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# Effect of chelating agents on hydrogenase in *Azotobacter chroococcum*

## Evidence that nickel is required for hydrogenase synthesis

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The chelating agents EDTA, *o*-phenanthroline, nitrilotriacetic acid (NTA), ethylenediamine-bis(*o*-hydroxyphenylacetic acid) (EDDA) or dimethylglyoxime prevented the expression of hydrogenase activity in batch cultures of nitrogen-fixing *Azotobacter chroococcum*, but did not inhibit preformed enzyme. The inhibition was reversed either by adding a mixture of trace elements ($Cu^{2+}$, $Mn^{2+}$, $Zn^{2+}$, $Co^{2+}$) or $Ni^{2+}$ or, to a lesser degree, $Co^{2+}$ alone. $Ni^{2+}$ or $Ni^{2+} + Fe^{2+}$ also enhanced the rate of hydrogenase derepression in *A. chroococcum* in the absence of any added chelator, if the medium was first extracted with 8-hydroxyquinoline. *A. chroococcum* accumulated $^{63}Ni^{2+}$ by an energy-independent mechanism. Both $Ni^{2+}$ uptake and hydrogenase synthesis were equally inhibited by either NTA, EDTA, EDDA or dimethylglyoxime. The evidence suggests a role for $Ni^{2+}$ in hydrogenase synthesis.

Hydrogen-uptake hydrogenase activity is widespread among bacteria (Adams *et al.*, 1981). Its physiological importance is to provide energy for $CO_2$ reduction in autotrophs (Schlegel, 1976) and to recycle hydrogen produced by nitrogenase in nitrogen-fixing aerobes (Evans *et al.*, 1981). Factors affecting hydrogenase synthesis by various types of bacteria include $H_2$ and $CO_2$, $O_2$, organic carbon substrates (Schlegel, 1976; Aragno & Schlegel, 1978; Maier *et al.*, 1978, 1979; Walker & Yates, 1978; Simpson *et al.*, 1979; Lepo *et al.*, 1980; Pedrosa *et al.*, 1980; Friedrich *et al.*, 1981*b*) and, in *Azotobacter*, nitrogen sources (Lee & Wilson, 1943; Partridge *et al.*, 1980).

Autotrophic growth in *Alcaligenes eutrophus*, *Xanthobacter autotrophicum*, *Pseudomonas flavum* and *Arthrobacter sp. IIX* is nickel-dependent (Thauer *et al.*, 1980); chelating agents inhibited this growth by preventing nickel uptake (Tabillion *et al.*, 1980). Chelating agents prevented the synthesis of both the membrane-bound and soluble hydrogenases in *A. eutrophus* H16, rather than any other enzyme directly involved in autotrophy (Friedrich *et al.*, 1981*a*). In contrast, autotrophic growth by *Nocardia opaca* is not nickel-dependent (Thauer *et al.*, 1980) but the single, soluble, hydrogenase is activated and stabilized by $Ni^{2+} + Mg^{2+}$ ions after purification (Aggag & Schlegel, 1974).

Abbreviations used: NTA, nitrilotriacetic acid; EDDA, ethylenediamine-bis(*o*-hydroxyphenylacetic acid).

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This present report describes the inhibition of hydrogenase synthesis in *A. chroococcum* by chelating agents and presents evidence that nickel is required for development of hydrogen-uptake activity in this organism.

## Materials and methods

### *Materials*

*o*-Phenanthroline, EDDA and dimethylglyoxime were purchased from Sigma; all other chelators and salts were from BDH. Radionuclides were obtained from The Radiochemical Centre, Amersham, and Lumax was from LKB. $H^3H$ was purchased as tritium gas and diluted with $H_2$ to $5 \times 10^8$ c.p.m./ml to store as stock. This was diluted further (100-fold) with $H_2$ before use.

### *Organisms and growth*

*Azotobacter chroococcum* (NCIB 8003) strain MCC1, *str*$^r$,*nal*$^r$, was grown in batch culture on Burk's sucrose medium (Newton *et al.*, 1953) or in sulphate-limited chemostat culture (Baker, 1968) on $B_6$ medium (Dalton & Postgate, 1969) with sucrose (20 g/l), sulphate (50 $\mu$M) and NTA (50 $\mu$M). Batch and chemostat cultures were grown under air at 30°C; in the latter the culture volume was 600 ml, air flow 320 ml/min, dilution rate 0.1 $h^{-1}$, and the pH was maintained at 7.1 ± 0.1. Sulphate limitation was established as described by Partridge *et al.* (1980).



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The above level of NTA was only one-tenth that normally employed in $B_6$ medium. Therefore, to avoid precipitation of metal phosphates and ferric hydroxide, the medium was supplied to the chemostat through a dual-feed system from two 10 litre vessels. $CaCl_2,2H_2O$, NTA and $FeSO_4,7H_2O$ (at pH 2) were each autoclaved separately and added aseptically to sterilized trace elements. The second 10 litre vessel contained sucrose, $K_2HPO_4$ and $KH_2PO_4$. Both solutions were added simultaneously to the culture vessel by a Watson–Marlow Flow Inducer.

*Extraction of media*

Metal contaminants were removed from Burk's medium by three overnight extractions with 1% 8-hydroxyquinoline (Gentry & Sherrington, 1950; Umland, 1962) in 1,2-dichloroethane at room temperature. $NaMoO_4$ and $CaCl_2,2H_2O$ solutions were extracted at pH 8.0; sucrose solution and the remaining salts except $FeSO_4,7H_2O$ were treated separately at pH 7.0. The concentrated salts were combined and diluted with double-distilled water. All glassware was soaked for 2 days in 40% (v/v) $HNO_3$ and rinsed in deionized and distilled water. *Azotobacter* grown in such extracted media required added trace elements ($B_6$ medium, Table 2) and $Fe^{2+}$ for maximum growth rates, whereas it grew well in untreated media without added trace elements.

*Hydrogenase assay*

Hydrogenase activity was measured anaerobically by $H^3H$ uptake with Methylene Blue as the electron acceptor, essentially as described by Pedrosa *et al.* (1982). $H^3H$ in $H_2$ (1 ml) was injected into 8 ml serum bottles containing cells (0.5 ml; absorbance 15–30 at 540 nm in an EEL spectrophotometer), 0.2 M-sodium phosphate buffer, pH 8.0 (0.5 ml) and Methylene Blue (7.5 mM) under argon. After incubation at 30°C in a shaking water bath (120 strokes/min, amplitude 5 cm), for 45 min samples were diluted 10-fold in distilled water. $H^3H$ uptake was determined in 0.2 ml aliquots by using a Beckman LS7500, programmed for automatic quench compensation, with NE250 or Lumax (20% in toluene) (10 ml) as scintillant.

*Hydrogenase derepression*

Bacteria, grown in sulphate-limited $N_2$-fixing continuous culture with NTA (50 $\mu$M) to an absorbance of 25–27 EEL units were washed aseptically in Burk's carbon-free medium and resuspended in one-half of the initial volume of Burk's medium with sucrose (15 mM) but without added NTA. Derepression of hydrogenase was followed at 30°C under air in a shaking water bath; activity was measured at intervals of 2–4 h for up to 24 h.

*Nickel uptake*

Nickel accumulation was estimated in sulphate-limited *A. chroococcum* grown with NTA (50 $\mu$M). The cells were washed and resuspended in extracted Burk's medium, without added NTA but with sucrose (15 mM) and $FeSO_4,7H_2O$ (18 $\mu$M). Radioactive nickel (3.5 or 7 nmol of $^{63}NiCl_2,6H_2O$, sp. radioactivity 0.77 mCi/$\mu$mol) was added to 10 ml of cells (absorbance 25–30 units) in 25 ml conical flasks, incubated at 30°C with shaking at 60 strokes/min. Duplicate samples (1 ml) were withdrawn at intervals, centrifuged for 1 min in an Eppendorf centrifuge and washed in 0.1 M-phosphate buffer, pH 7.0, containing $^{58}NiCl_2$ (0.5 mM) before resuspending in the same buffer at 4°C. Radioactivity was estimated in 0.2 ml aliquots by using a Beckman LS7500 with 5 ml of Lumax (20% in toluene) as scintillant. Alternatively, the cells were filtered through cellulose nitrate micro filters (Sartorius) and washed twice with 5 ml of cold 0.1 M-phosphate buffer, pH 7.0. The cellulose nitrate filters were dissolved in NE250 (5 ml) and counted directly.

*Protein estimation*

Protein was measured by the method of Lowry *et al.* (1951) with bovine serum albumin (Sigma) as the standard.

## Results

*Effect of chelating agents on hydrogenase*

A range of chelating agents added to the growth media resulted in depressed hydrogenase activity in *A. chroococcum* grown in batch culture (Table 1). Their relative inhibiting efficiency was in the order: *o*-phenanthroline > NTA $\gg$ EDTA > EDDA $\simeq$ dimethylglyoxime. The effect was not due to inhibition of preformed enzyme: hydrogenase activity assayed *in vitro*, either in whole cells or in crude extracts was not inhibited by any of these chelators at ten times the minimum concentration which caused substantial depletion (>90%) of enzyme activity during growth.

Cells grown in the presence of NTA (50 $\mu$M) developed hydrogenase activity after resuspension in NTA-free medium (Fig. 1). Activity did not increase if either chloramphenicol (75 $\mu$g/ml) was added or carbon substrate was omitted, indicating that protein synthesis was involved. An increase in cell density occurred during derepression.

*Effect of trace elements on hydrogenase*

The low levels of chelating agents (e.g. 50 $\mu$M NTA) needed to depress hydrogenase activity suggested that these agents prevented trace metal ion uptake. Inclusion of additional trace elements (in the proportions normal for continuous culture), together with NTA in the growth media of batch

Table 1. *Effect of chelating agents on hydrogenase in batch-grown cultures of Azotobacter chroococcum*
Batch cultures (50ml) were set up with 1% inocula from a sulphate-limited continuous culture of *A. chroococcum* growing without chelators. After 24h growth with the chelators listed, hydrogenase activity was measured by $H^3H$ uptake with Methylene Blue (see the Materials and methods section). The data are representative of six experiments in which activity was measured in duplicate cultures at equivalent absorbance values.

| Chelator | Concentration ($\mu$M) | $H_2$ uptake (nmol·min$^{-1}$·mg of protein$^{-1}$) | Inhibition (%) |
|---|---|---|---|
| *o*-Phenanthroline | 50 | 20 | 97 |
| | 20 | 65 | 94 |
| | 2 | 440 | 56 |
| | 0.5 | 570 | 44 |
| NTA | 200 | 20 | 97 |
| | 50 | 38 | 96 |
| | 20 | 100 | 90 |
| | 2 | 238 | 76 |
| | 0.4 | 366 | 63 |
| EDTA | 50 | 111 | 86 |
| | 10 | 570 | 29 |
| | 1 | 712 | 11 |
| EDDA | 1000 | 108 | 89 |
| | 500 | 121 | 87 |
| | 50 | 734 | 25 |
| | 10 | 794 | 17 |
| Dimethylglyoxime | 500 | 96 | 90 |
| | 50 | 762 | 22 |
| | 10 | 878 | 12 |
| Control (without added chelating agent) | | 907 ± 106 | |

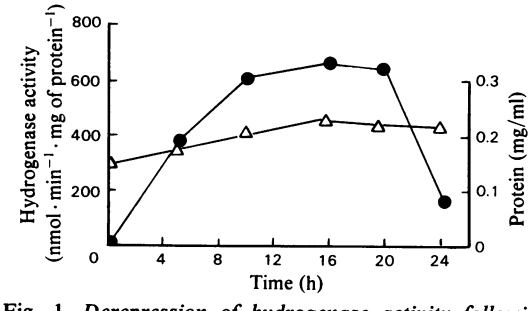


Fig. 1. *Derepression of hydrogenase activity following the removal of NTA*
Cells grown in batch culture with NTA (50$\mu$M) were harvested aseptically during the logarithmic phase, washed twice by centrifugation and resuspended in Burk's medium with sucrose (15mM). Suspensions (12 × 5ml) in 25ml conical flasks were incubated aerobically at 30°C and duplicates were frozen at intervals in liquid $N_2$. Hydrogenase activity and protein concentration were determined as described in the Materials and methods section. ●, Hydrogenase; △, protein.

cultures, caused hydrogenase activity to increase *pro rata* (Table 2). However, addition of trace elements neither inhibited nor stimulated hydrogen uptake assayed *in vitro* and attempts to reactivate apoprotein, if present, by incubation of NTA-grown cells and crude extracts with iron and trace elements were unsuccessful. This again suggests that the effect of trace elements is on synthesis of hydrogenase rather than activity of preformed enzyme. When trace elements were tested individually, only nickel or cobalt resulted in significantly higher activity during growth on NTA (Table 2). Nickel was more effective than cobalt: 4$\mu$M-$Ni^{2+}$ or 32$\mu$M-$Co^{2+}$ caused an approx. 5-fold increase in hydrogenase activity.

The effects of iron and trace elements on hydrogenase synthesis were examined further by resuspending NTA-grown cells in media from which contaminant trace metals had been extracted with 8-hydroxyquinoline. Additional trace elements caused a 75% increase in the growth rate over medium supplemented with iron alone. In contrast, growth with unextracted media did not benefit from addition of trace elements.

Some hydrogenase activity developed in the washed cells even in the absence of added iron or trace elements. Absorbance measurements confirmed that growth occurred, an effect probably due to carry-over of iron and endogenous trace elements. Addition of nickel (2$\mu$M) caused a consistent enhancement of hydrogenase activity by up to 80%, whereas cobalt, copper, manganese or zinc did not affect derepression in a reproducible manner. Addition of iron, either alone or with the other metals ($Co^{2+}$, $Mn^{2+}$, $Zn^{2+}$) individually, also stimulated the development of hydrogenase activity slightly. The maximum rate of increase occurred in the presence of $Fe^{2+}$ + $Ni^{2+}$ (Fig. 2).

Table 2. *Effect of trace elements on hydrogenase activity in A. chroococcum grown in the presence of NTA*
Batch cultures (50 ml) were grown as described previously with NTA (50 $\mu$M) and trace elements as listed.

| Metal ion supplement | Concentration ($\mu$M) | Protein concentration after 24 h (mg/ml) | Hydrogenase activity after 24 h (nmol·min$^{-1}$·mg of protein$^{-1}$) |
|---|---|---|---|
| None | — | 0.22 | 44 |
| | — | 0.31 | 34 |
| $B_6$* | ×1* | 0.33 | 71 |
| | ×2 | 0.30 | 109 |
| | ×3 | 0.31 | 122 |
| | ×4 | 0.28 | 201 |
| $CuCl_2$ | 0.8 | 0.31 | 27 |
| $MnCl_2,4H_2O$ | 1.0 | 0.21 | 45 |
| $ZnCl_2$ | 22.0 | 0.22 | 62 |
| $CoCl_2,6H_2O$ | 4.0 | 0.21 | 155 |
| | 8.0 | 0.25 | 199 |
| | 32.0 | 0.16 | 283 |
| $NiCl_2,6H_2O$ | 1.0 | 0.26 | 101 |
| | 2.0 | 0.19 | 167 |
| | 4.0 | 0.21 | 279 |

* Addition of $B_6$ (×1) gave the following metal concentrations ($\mu$M): $CuCl_2$, 0.4; $MnCl_2,4H_2O$, 0.5; $ZnCl_2$, 11.0; $CoCl_2,6H_2O$, 4.0.

*Nickel uptake*

*A. chroococcum* accumulated $^{63}Ni^{2+}$ under the same conditions in which hydrogenase derepressed (Fig. 3). Uptake was biphasic: a rapid binding, which was completed within approx. 10 min, and which was not reversed by washing with $^{58}Ni^{2+}$, and a slower, linear increase which was partly reversed by the addition of a 100-fold excess of $^{58}NiCl_2$ or toluene. Bacteria exposed to $^{63}Ni^{2+}$ for 2 h, then washed and suspended in $^{63}Ni$-free medium lost 15 and 10% radioactivity with toluene (1%) or $^{58}Ni^{2+}$ (100 $\mu$M) respectively in 15 min. The rate of $^{63}Ni^{2+}$ uptake was temperature-, but, apparently, not energy-dependent: it occurred in cells which had been starved of carbon for 2 h or under strictly anaerobic conditions. Juglone (1 mM) or 2,4-dinitrophenol (1 mM) completely inhibited respiration by *A. chroococcum* but enhanced the rate of nickel uptake. In contrast, NaCN (1 mM), which also inhibited respiration, completely inhibited $^{63}Ni^{2+}$ binding, possibly because of nickel–cyano complex formation in the external medium (Tabillion & Kaltwasser, 1977). $NaN_3$ (1 mM), on the other hand, affected neither respiration nor nickel uptake.

*Effect of chelating agents*

Chelating agents which inhibited hydrogenase synthesis were tested for their ability to inhibit nickel uptake by *Azotobacter*. With the exception of *o*-phenanthroline, they all inhibited nickel assimilation but differed in their effectiveness: NTA $\gg$ EDTA > EDDA $\simeq$ dimethylglyoxime. Fig. 4 shows that there is a close correlation between the inhibition of nickel binding and hydrogenase synthesis by these four chelating agents. Presumably, *o*-phenanthroline,

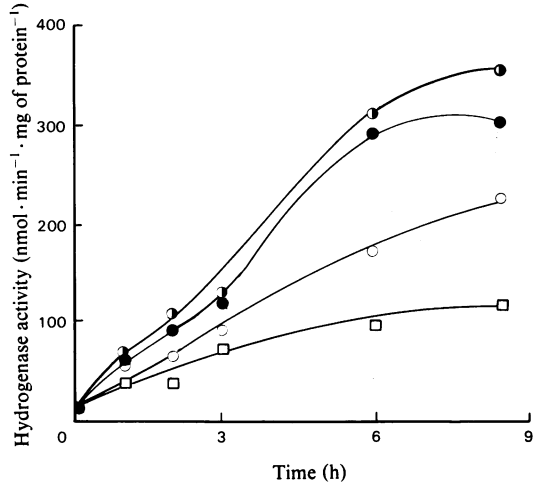


Fig. 2. *Effect of iron and nickel on the derepression of hydrogenase in NTA-grown cells*
Cells (400 ml) from a sulphate-limited continuous culture of *A. chroococcum* growing with NTA (50 $\mu$M) were washed twice by centrifugation and resuspended in 8-hydroxyquinoline-extracted Burk's medium (200 ml) containing sucrose (15 mM). Derepression of hydrogenase was followed in 35 ml suspensions in 100 ml conical flasks shaken in air at 30°C. Samples (5 ml) were withdrawn at different times and immediately frozen in liquid $N_2$. Hydrogenase activity was measured as described in the Materials and methods section. The data are representative of three separate experiments. The following were present throughout derepression: ◑, $FeSO_4,7H_2O$ (7 $\mu$M) + $NiCl_2$ (2 $\mu$M); ●, $NiCl_2$ (2 $\mu$M); ○, $FeSO_4,7H_2O$ (7 $\mu$M); □, no added trace metals.

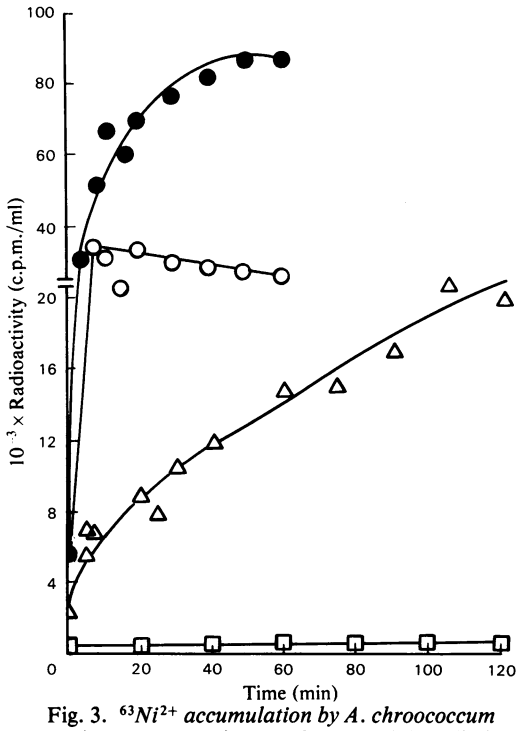


Fig. 3. *$^{63}Ni^{2+}$ accumulation by A. chroococcum*
Cells (absorbance 22) taken from a sulphate-limited continuous culture containing 50$\mu$M-NTA were resuspended to absorbance 27 in 8-hydroxyquinoline-extracted Burk's medum. $^{63}NiCl_2$ (0.7$\mu$M) was added after 2h incubation at 30°C. At intervals duplicate samples (0.5ml) were pipetted into 2ml of 0.1M-sodium phosphate buffer, pH7.0, in ice and collected on cellulose nitrate filters (0.45$\mu$M). Filters were washed twice with cold buffer (5ml) and dissolved in NE250 (5ml) to estimate $^{63}$Ni incorporation. △, $^{63}$Ni uptake without respiratory inhibitors; □, $^{63}$Ni uptake with NaCN (1mM); ○, $^{63}$Ni uptake with 2,4-dinitrophenol (1mM); ●, $^{63}$Ni uptake with juglone (1mM).

which inhibited hydrogenase synthesis but not nickel uptake, blocked synthesis by a different mechanism. Alternatively, the cells may have been permeable to *o*-phenanthroline but $Ni^{2+}$ was not released for hydrogenase synthesis.

## Discussion

Several observations in this study suggest a role for nickel ions in the synthesis of the hydrogen-uptake hydrogenase of *A. chroococcum*. Firstly, several chelating agents inhibited the development of hydrogenase activity (presumed synthesis) *in vivo* but did not affect hydrogenase activity *in vitro*. The concentrations (2–5$\mu$M) required for substantial inhibition by some of these agents were much lower than the concentration of $Fe^{2+}$ (18$\mu$M), which is a known constituent of *A. chroococcum* hydrogenase (Van der Werf & Yates, 1978) and hydrogenases generally (Adams *et al.*, 1981). Secondly, the addition of $Ni^{2+}$ overcame the effect of NTA more effectively than did other metal ions (although $Co^{2+}$ also prevented inhibition), and optimal hydrogenase activity developed in the presence of $Ni^{2+} + Fe^{2+}$. Thirdly, the extremely close correlation between the abilities of several chelators to inhibit both hydrogenase synthesis and $^{63}Ni^{2+}$ uptake, in view of the widely different stability constants of metal ion-chelator complexes (Sillen & Martell, 1971), clearly indicates a role for $Ni^{2+}$ in hydrogenase synthesis.

It was expected that $Fe^{2+}$ would stimulate hydrogenase synthesis, since all hydrogenases so far isolated are iron–sulphur proteins (Adams *et al.*, 1981). The stimulation by $Co^{2+}$ is less easy to explain, but since the log(stability constants) of NTA–$Ni^{2+}$ (11.5) and NTA–$Co^{2+}$ (10.8) complexes are similar (Sillen & Martell, 1971), $Co^{2+}$ may have had a sparing effect on $Ni^{2+}$ present as a contaminant in the medium. $Ni^{2+}$ commonly contaminates mineral salts at levels sufficient for growth of $Ni^{2+}$-dependent autotrophic bacteria (Thauer *et al.*, 1980). $Co^{2+}$, unlike $Ni^{2+}$, failed to enhance hydrogenase derepression by *A. chroococcum* in media freed from contaminant trace metals, which suggests that $Co^{2+}$ has no direct role in hydrogenase synthesis.

Friedrich *et al.* (1981*b*) showed that EDTA was more effective than NTA as an inhibitor of hydrogenase synthesis in *Alcaligenes eutrophus*, whereas the reverse is true in *A. chroococcum*. The pH was similar in both investigations, but the autotrophic growth media for *Alcaligenes* spp. (Schlegel *et al.*, 1961) is relatively high in phosphate and low in calcium compared with $B_6$. It is not clear whether these differences in growth media or other factors determined the relative effects on these two chelating agents. Possibly nickel-binding sites within or on the cell walls are not equally accessible to the chelating agents. In *A. eutrophus* uptake is active (Tabillion & Kaltwasser, 1977) whereas in *A. chroococcum* accumulation appears to occur passively by diffusion. The two systems may therefore differ both in components and characteristics.

Both toluene (1%) and $^{58}Ni^{2+}$ (100-fold excess) released $^{63}Ni^{2+}$ from *Alcaligenes eutrophus* in an apparently biphasic manner; first rapidly and then slowly, suggesting the presence of free and bound $^{63}Ni^{2+}$ in the cell. $^{58}$Ni removed 25%, and toluene 35%, of the radioactivity in 15min. In *Azotobacter* the loss rate with either toluene or $^{58}Ni^{2+}$ was slower than from *A. eutrophus*. This could reflect either a slower rate of $^{58}Ni^{2+}$ incorporation (one is active and rapid, the other passive and slow), a less rapid reaction with toluene, or alternatively a greater proportion of absorbed $Ni^{2+}$ is bound in *Azotobacter* than in *A. eutrophus*. However it appears that toluene reacts rapidly with *Azotobacter*: after 15min the

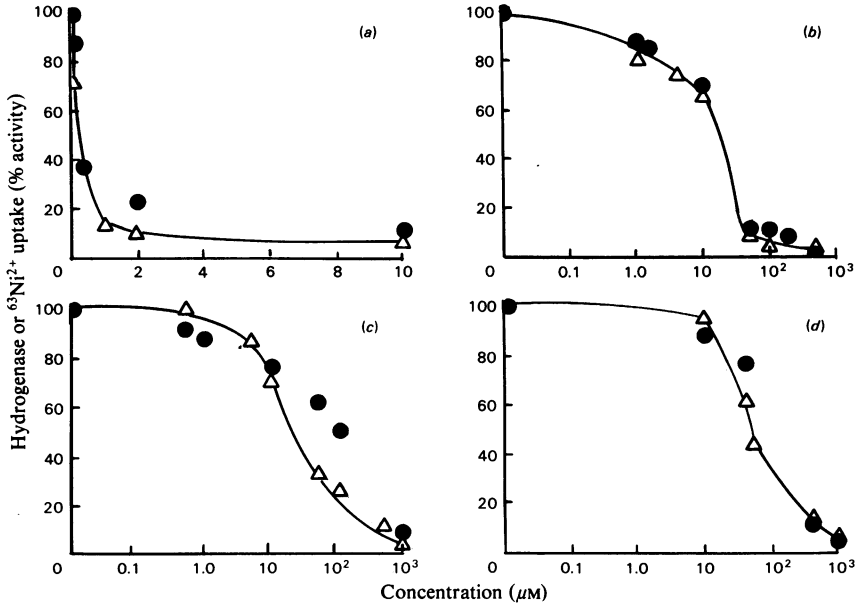


Fig. 4. *Effect of chelating agents on nickel binding and hydrogenase in A. chroococcum*
Bacteria were grown in batch cultures with chelating agents as described in Table 1. Hydrogenase activity and nickel binding by cells were estimated as described in the Materials and methods section; duplicate samples were taken to measure $^{63}Ni$ accumulated after 3h. Control suspensions (absorbance 30–35) without chelating agents bound 4300 ± 750c.p.m. of $^{63}Ni^{2+}$. (*a*) NTA; (*b*) EDTA; (*c*) EDDA; (*d*) dimethylglyoxime. ●, Hydrogenase activity; △, $^{63}Ni$ uptake.

cells were clearly disrupted and the results ($Ni^{2+}$ loss) unreliable. The results suggest that $Ni^{2+}$ may be more strongly bound in *Azotobacter* than in *A. eutrophus*.

## References


Adams, M. W. W., Mortenson, L. E. & Chen, J. S. (1981) *Biochim. Biophys. Acta* **594**, 105–176

Aggag, M. & Schlegel, H. G. (1974) *Arch. Microbiol.* **100**, 25–39

Aragno, M. & Schlegel, H. G. (1978) *Arch. Microbiol.* **116**, 221–229

Baker, K. (1968) *Lab. Pract.* **17**, 817–824

Dalton, H. & Postgate, J. R. (1969) *J. Gen. Microbiol.* **56**, 307–319

Evans, H. J., Purohit, K., Cantrell, M. A., Eisbrenner, G., Russell, S. A., Hanus, F. J. & Lepo, J. E. (1981) *Proc. Int. Symp. Nitrogen Fixation 4th*, 84–96

Friedrich, B., Heine, E., Finck, A. & Friedrich, C. G. (1981*a*) *J. Bacteriol.* **145**, 1144–1149

Friedrich, C. G., Friedrich, B. & Bowien, B. (1981*b*) *J. Gen. Microbiol.* **122**, 69–78

Gentry, C. H. R. & Sherrington, L. G. (1950) *Analyst* **75**, 17–21

Lee, S. B. & Wilson, P. W. (1943) *J. Biol. Chem.* **151**, 377–385

Lepo, J. E., Hanus, F. J. & Evans, H. J. (1980) *J. Bacteriol.* **141**, 664–670

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

Maier, R. J., Campbell, N. E. R., Hanus, F. J., Simpson, F. B., Russell, S. A. & Evans, H. J. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 3258–3262

Maier, R. J., Hanus, F. J. & Evans, H. J. (1979) *J. Bacteriol.* **137**, 824–829

Newton, J. W., Wilson, P. W. & Burris, R. H. (1953) *J. Biol. Chem.* **204**, 445–451

Partridge, C. D. P., Walker, C. C., Yates, M. G. & Postgate, J. R. (1980) *J. Gen. Microbiol.* **119**, 313–319

Pedrosa, F. O., Dobereiner, J. & Yates, M. G. (1980) *J. Gen. Microbiol.* **119**, 547–551

Pedrosa, F. O., Stephan, M., Dobereiner, J. & Yates, M. G. (1982) *J. Gen. Microbiol.* **128** in the press

Schlegel, H. G. (1976) *A. van Leeuwenhoek J. Microbiol. Serol.* **42**, 181–201

Schlegel, H. G., Kaltwasser, J. & Gottschalk, G. (1961) *Arch. Mikrobiol.* **38**, 209–222

Sillen, L. G. & Martell, A. E. (1971) *Chem. Soc. Spec. Publ.* **25**

Simpson, F. B., Maier, R. J. & Evans, H. J. (1979) *Arch. Microbiol.* **123**, 1–8

Tabillion, R. & Kaltwasser, H. (1977) *Arch. Microbiol.* **113**, 145–151

Tabillion, R., Weber, F. & Kaltwasser, H. (1980) *Arch. Microbiol.* **124**, 131–136

Thauer, R. K., Diekert, G. & Schönheit, P. (1980) *Trends Biochem. Sci.* **5**, 304–306

Umland, F. (1962) *Z. Anal. Chem.* **190**, 186–208

Van der Werf, A. N. & Yates, M. G. (1979) in *Hydrogenases: their Catalytic Activity, Structure and Function* (Schlegel, H. G. & Schneider, R., eds.), pp. 307–326, Verlag E. Goltze KG, Göttingen

Walker, C. C. & Yates, M. G. (1978) *Biochimie* **60**, 225–231