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Some Aspects of Hydrogenase Activity and Nitrogen Fixation in *Azotobacter* spp and in *Clostridium pasteurianum*

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SUMMARY: Hydrogenases from *Azotobacter vinelandii* and *Clostridium pasteurianum* reduced methylene blue, ferricyanide, benzyl- and methyl-viologens when hydrogen was the donor. Methylene blue was the most effective acceptor. Hughes press preparations of either organism in tris (2-amino-2-hydroxymethyl propane-1:3-diol) buffer (pH 8.0) resulted in the best extraction of the enzyme. Hydrogenase from *C. pasteurianum* was readily inactivated by traces of oxygen but this could be prevented by sodium dithionite. Pyridine nucleotides and cytochrome *c* are reduced by hydrogenase, but in extracts of *Azotobacter*, however, the addition of iron was required to couple DPNH to cytochrome *c*. The mechanism appears to involve the reduction of Fe^{3+} to Fe^{2+} enzymically and the Fe^{2+} is oxidized by cytochrome *c* non-enzymically. The effect of pH value and nature of buffer on this system was examined. Mo^{5+} did not reduce cytochrome *c*, with or without hydrogenase.

Metal-deficiency experiments, inhibitor studies, activation of dialysed preparations, and the results of radioactive tracer assays of purified protein fractions, showed that iron is the main metal constituent of hydrogenase. Molybdenum, however, is required for the fixation of nitrogen.

Since the work of Bortels (1930) it has been known that molybdenum is required for nitrogen fixation in *Azotobacter* species, and that vanadium can partially replace it in this process. Horner, Burk, Allison & Sherman (1942), Esposito & Wilson (1955), Nicholas (1957) and Bové, Bové & Arnon (1957) confirmed that molybdenum is essential when *Azotobacter* is utilizing atmospheric nitrogen, although its replacement by vanadium may vary with species. Takahashi & Nason (1957) and Keeler & Varner (1957) showed that tungstate is a competitive inhibitor of molybdate in nitrogen fixation, and Keeler & Varner (1957*a*) demonstrated the incorporation of tungstate into the same protein fraction as molybdenum. Esposito & Wilson (1955) showed that calcium and iron were also required for nitrogen fixation in *Azotobacter vinelandii* and Nicholas (1957, 1958*a, b*) showed that a deficiency of iron or molybdenum restricted the fixation of nitrogen in *Azotobacter* and in *Clostridium*.

The enzyme hydrogenase of a range of micro-organisms, which reversibly activates hydrogen gas (Stephenson & Stickland, 1933) was shown by Lee & Wilson (1948) to be correlated with nitrogen-fixing capacity in *Azotobacter*; organisms grown with combined nitrogen contained significantly less hydrogenase than those which were utilizing gaseous nitrogen only. Hoch, Little & Burris (1957) identified a hydrogen exchange reaction in root-nodule bacteria

(*Rhizobium*). Hydrogenase is, however, present in bacteria which do not fix nitrogen, e.g. *Escherichia coli*. Hydrogenase is also associated with the terminal component of the system responsible for the photochemical formation of hydrogen and for the evolution of this gas in the fermentation of carbohydrates, amino acids, purines and related substances. Shug, Wilson, Green & Mahler (1954) and Shug, Hamilton & Wilson (1956) suggested that hydrogenase from *Clostridium pasteurianum* is a flavoprotein enzyme dependent on molybdenum, whereas Peck & Gest (1957) showed that in *Clostridium butylicum* the enzyme is primarily an iron-containing flavoprotein. In the present paper some effects of trace metals on nitrogen fixation and hydrogenase activity in various *Azotobacter* species and in *Clostridium pasteurianum* are presented.

METHODS

Organisms. *Azotobacter vinelandii* (O) and *Clostridium pasteurianum* W-S were kindly supplied by Dr P. W. Wilson, Wisconsin University, Madison, U.S.A. *A. chroococcum* 8008 was obtained from the National Collection of Industrial Bacteria, Teddington, London.

Culture media. The bacteria were grown in the following media (g./l. deionized water): for *Azotobacter chroococcum*; sucrose, 10; KH_2PO_4 , 1; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2; Na acetate, 0.2; Ca citrate, 0.2; NaCl, 0.1; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.001; adjusted to pH 7.5; for *Azotobacter vinelandii* (O); sucrose, 50; KH_2PO_4 , 0.1; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.4; Na citrate, 0.2; Ca acetate, 0.2; NaCl, 0.1; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.001; for *Clostridium pasteurianum*; sucrose, 20; malic acid, 0.5; KH_2PO_4 , 0.5; K_2HPO_4 , 0.5; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2; NaCl, 0.1; CaCO_3 , 5; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.01; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.001. The water used for solution was deionized in a Permutit Deminrolit Mark IV plant.

Removal of trace metals from culture media. Methods described previously (Nicholas, 1952) were used to remove Mo and Fe from a solution of macro-nutrient constituents of the media. The phosphates were purified and autoclaved separately and when cold were added to the rest of the sterilized medium. This procedure prevented the precipitation which usually occurs when phosphates are autoclaved with the other constituents of the medium. The trace metals, spectrographically tested for purity, were supplied by Johnson Matthey (Hatton Garden, London, E.C.1.) The purified culture solutions were adjusted to pH 7.8 with 5% (w/v) sodium hydroxide which had been passed through a well-washed Amberlite (IRA-400) anion exchange column (20 in. $\times$ 3 in.) to remove molybdate. The Mo content of the solution after passage through the resin was $< 0.001 \mu\text{g./ml.}$ as determined by bioassay (Nicholas, 1952).

Growth of organisms. All strains of *Azotobacter*, maintained on nutrient agar slopes at 25°, were subcultured every 6 days. The media for slopes consisted of (g./l. deionized water): 1, yeast extract; 2, peptone; 20, sucrose; 1, KH_2PO_4 ; 0.2, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 0.2, CaAc ; 0.008, $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$; 0.005, $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$; 25, agar; adjusted to pH 7.5. Inocula for large-scale experi-

ments were prepared in 1 l. of appropriate culture medium. Cultures of *Azotobacter vinelandii* (O) were well aerated with sterile air dispersed in the media by passage through sintered-glass disks.

Inoculum of *Clostridium pasteurianum* for large-scale experiments was grown under semi-anaerobic conditions in 4 oz. medicine bottles fitted with screw caps containing 100 ml. of the following medium (g./l. deionized water): 20, glucose; 1 tryptone (Difco); 1, agar, 1, CaCO_3 .

For large-scale experiments the bacteria were grown in 10 l. Pyrex jars each containing 8 l. medium. The *Azotobacter* spp. were grown for 48 hr. at 30° and *Clostridium pasteurianum* for 48 hr. at 37° . Cultures of *A. vinelandii* (O) were well aerated with sterile air. Nitrogen gas was passed through cultures of *C. pasteurianum* to maintain anaerobic conditions. The bacteria, collected in the cold in a Sharples centrifuge at 30,000 g, were washed twice in 0.85 % (w/v) NaCl solution and stored in a deep-freeze cabinet at -17° .

Preparation of cell-free extracts. Washed organisms were disrupted in a Hughes press (1951) at -80° . *Azotobacter vinelandii* (O) organisms, suspended in 0.2 M-tris (2-amino-2 hydroxymethyl-propane-1: 3-diol) buffer (pH 8.0) at 8 g. wet wt. organism/24 ml. buffer, were put in the press precooled to -20° with solid carbon dioxide. The frozen suspension of organisms was disrupted by dropping a steel weight (112 lb.) on to the press plunger in a special pile-driver. *Clostridium pasteurianum* was suspended in 0.1 M-phosphate buffer (pH 7.5) and treated in the same way. The crushed material was collected in a plastic tube and after thawing was centrifuged at 20,000 g for 30 min. (unless otherwise stated). The supernatant solution, which was used in some experiments as a source of hydrogenase, was also fractionated by conventional methods.

Assay of hydrogenase. Hydrogen uptake by whole organisms and their extracts was measured in a Warburg apparatus, with a variety of H-acceptors including benzyl and methyl-viologen, methylene blue, and potassium ferri-cyanide. The routine assay with methylene blue was as follows. *Main compartment:* 1 ml. 0.2 M-phosphate buffer (pH 7.5); 0.5 ml. 10^{-2} M- $\text{Na}_2\text{S}_2\text{O}_4$ in 0.1 M-phosphate (pH 7.5); 0.5 ml. 8 % (w/v) gelatin; 0.5 ml. enzyme preparation. *Side-arm:* 0.5 ml. 3×10^{-2} M-methylene blue. *Centre well:* 0.2 ml. 20 % (w/v) KOH. Gas phase hydrogen; bath temperature 33° . The addition of 15 μ mole sodium dithionite stabilized the hydrogenase and gelatin protected it from inhibition by the various dye acceptors. The specific activity of the enzyme is expressed in μ l. H_2 /mg. N or protein/hr. i.e. QH_2 (acceptor).

Total nitrogen and protein determinations. Total nitrogen was determined in whole organisms and in extracts by the micro-Kjeldahl method. Protein was determined in extracts by the Folin method of Lowry, Rosebrough, Farr & Randall (1951). Enzyme extracts prepared in tris buffer and those fractionated with ammonium sulphate were thoroughly dialysed against running tap water for 24 hr. before protein or nitrogen determinations. Growth determinations were based on turbidity measurements and the total N content of centrifuged deposits from cultures.

Cofactors and other compounds. Diphosphopyridine nucleotide (DPN) and

triphosphopyridine nucleotide (TPN), both 98 % pure, and cytochrome *c* (95 % pure) were obtained from Sigma Chemical Company, U.S.A. Reduced DPN was enzymically prepared by the alcohol dehydrogenase method of Pullman, Colowick & Kaplan (1954). Crystalline alcohol dehydrogenase was prepared by Racker's method (1955) from Acorn yeast (Distillers Company, Ltd., Bristol). TPN was reduced by the isocitric acid dehydrogenase enzyme prepared from acetone powder of pig heart.

RESULTS

Hydrogenase in whole organisms and in extracts

The effects of various acceptors on hydrogenase activity in whole cells of *Azotobacter vinelandii* (O) and *Clostridium pasteurianum* are shown in Table 1. The concentration of each acceptor was that required for maximum hydrogen uptake. The specific activity of the enzyme in *C. pasteurianum* was considerably greater than in *A. vinelandii* (O). Methylene blue was the most effective acceptor, especially in the *Clostridium*. Enzyme activity in both organisms was

Table 1. *Effect of various H-acceptors on hydrogenase activity in whole Azotobacter vinelandii* (O) *and Clostridium pasteurianum*

Warburg assay. Main compartment: 1 ml. 0.2 M-phosphate (pH 7.5); 0.5 ml. 10^{-3} M- $\text{Na}_2\text{S}_2\text{O}_4$ in 0.1 M-phosphate (pH 7.5); 0.5 ml. 8 % (w/v) gelatin; 0.5 ml. suspension washed organism (*Azotobacter* equiv. 1.96 mg. N/ml.; *Clostridium* equiv. 2.08 mg. N/ml.). Sidearm: 0.5 ml. acceptor at concentration indicated. Centre well: 0.2 ml. 20 % KOH. Gas phase hydrogen; bath temperature 38°.

Acceptor	Molar concentration	<i>A. vinelandii</i>		<i>C. pasteurianum</i>	
		Without dithionite	With dithionite	Without dithionite	With dithionite
		H ₂ uptake (μl./mg. N/hr.)			
Methylene blue	3 × 10 ⁻²	6,700	9,400	36,600	58,600
Potassium ferricyanide	3 × 10 ⁻¹	4,750	5,350	9,100	22,400
Benzyl viologen	3 × 10 ⁻³	50	50	3,000	4,200
Methyl viologen	3 × 10 ⁻³	10	10	1,700	2,000

much decreased when benzyl- or methyl-viologen replaced methylene blue. Traces of oxygen readily inactivated the enzyme in the *Clostridium* and sodium dithionite stabilized it when methylene blue or ferricyanide were the acceptors.

The amount of enzyme extracted from whole organisms by various methods was similar (as %) in the two organisms: Hughes press, 98; Mickle disintegrator, 75; alumina grinding or ultrasonic treatment, 60. The effect of suspending the bacteria in buffers before the Hughes press treatment is shown in Table 2. Maximum extraction of the hydrogenase after centrifuging at 23,000 *g* was achieved in both organisms with 0.2 M-tris buffer (pH 8.0). The relative effectiveness of four acceptors for measuring enzyme activity in extracts was similar to that obtained with whole organisms.

Although it was not possible to detect an uptake of hydrogen in a Warburg apparatus with extracts of either organism when using cytochrome *c* or

Fig. 1

Fig. 2

Fig. 1. Reduction of DPN and TPN by hydrogenase extracted in 0.1 M-phosphate (pH 7.5) from *Clostridium pasteurianum* W-S measured at 340 m μ . Extracts prepared in a Hughes press and centrifuged at 20,000 g and supernatant solution used. Reaction mixture (8 ml.) in a special Thunberg tube with a silica cuvette attachment, contained 0.2 mmole phosphate buffer (pH 7.5), 0.1 ml. extract. Sidearm: 4 μ mole DPN or TPN. Thunberg tube evacuated and filled with H₂ (free from O₂). Δ — Δ = DPN + enzyme; $\circ$ — $\circ$ = DPN + boiled enzyme; $\times$ — $\times$ = TPN + enzyme.

Fig. 2. Enzymic reduction of cytochrome *c* by hydrogenase in a phosphate extract of *Clostridium pasteurianum* W-S measured at 550 m μ . Extracts prepared in a Hughes press and centrifuged at 20,000 g and supernatant solution used. Reaction mixture (8 ml.) in 1 cm. cuvette fitted to a Thunberg tube contained 0.2 mmole phosphate (pH 7.5), 0.1 ml. extract, 8 μ mole KCN. Sidearm: 0.1 μ mole cytochrome *c*. Thunberg tube evacuated and filled with H₂ (free from O₂) or He. $\square$ — $\square$ = enzyme + KCN (H₂); Δ — Δ = enzyme (H₂); $\bullet$ — $\bullet$ = enzyme (He); $\circ$ — $\circ$ = enzyme + KCN (He); $\times$ — $\times$ = boiled enzyme (H₂ or He).

Table 2. *Hydrogenase activity of whole organisms and Hughes press extracts of Azotobacter vinelandii* (O) and of *Clostridium pasteurianum*

Warburg assay. Main compartment: 1 ml. 0.2 M-phosphate (pH 7.5); 0.5 ml. 10⁻² M-Na₂S₂O₄ in 0.1 M-phosphate (pH 7.5); 0.5 ml. 8% (w/v) gelatin; 0.5 ml. suspension of washed organisms, homogenate, particles or supernatant solution. (*Azotobacter*, washed suspension, equiv. 1.9 mg. N/ml.; supernatant solution, equiv. 2 mg. N/ml. *Clostridium*, washed suspension equiv. 2.08 mg. N/ml.; supernatant solution, equiv. 1 mg. N/ml.) Sidearm: 0.5 ml. 8 $\times$ 10⁻⁴ M-methylene blue. Centre well: 0.2 ml. 20% w/v KOH. Gas phase hydrogen; bath temperature 33°.

QH₂(MB)/ml./hr.

Extractant	Whole organisms	Homogenate	Supernatant solution after centrifuging at 23,000 g for 1 hr.	Particles after centrifuging at 23,000 g	% extracted in supernatant solution
<i>A. vinelandii</i> (O)					
0.1 M-phosphate (pH 7.5)	35,900	10,900	3,000	22,000	28
0.2 M-tris (pH 8.0)	35,900	16,200	10,400	1,000	63
<i>C. pasteurianum</i> W-S					
0.1 M-phosphate (pH 7.5)	278,000	480,000	288,000	210,000	60
0.2 M-tris (pH 8.0)	276,000	652,000	669,000	8,300	100

pyridine nucleotides as acceptors, the results in Figs. 1 and 2 show that extracts of the *Clostridium* reduced cytochrome *c* or DPN (but not TPN) in the presence of hydrogen. The reduction of cytochrome *c* by hydrogenase from *Azotobacter* has already been reported (Hyndman, Burris & Wilson, 1953). Extracts of *Azotobacter* reduced cytochrome *c* when DPNH was the electron-donor, provided that ferric iron was added (Fig. 3). In this system DPNH reduced Fe^{3+} to Fe^{2+} enzymically as measured by the formation of the ferrous *o*-phenanthroline complex (Fig. 4) and Fe^{2+} is known to reduce cytochrome *c* non-enzymically (Webber, Lenhoff & Kaplan, 1956). The latter reaction, however, is markedly affected by the pH value and the composition of buffer

Fig. 3

Fig. 4

Fig. 3. Enzymic reduction of cytochrome *c* by DPNH measured at 550 $m\mu$ in 1 cm. optical cells, in a crude extract of *Azotobacter vinelandii* (O) in the presence of ferric chloride. Cuvette contained: 0.2 mmole phosphate (pH 6.8), 0.1 μ mole cytochrome *c*; 0.1 ml. extract, 0.1 μ mole DPNH. This gave endogenous rate; then 1 μ mole ferric chloride added. O—O = enzyme; ●—● = boiled enzyme.

Fig. 4. Enzymic reduction of ferric chloride by DPNH in a crude extract of *Azotobacter vinelandii* (O) in presence of *o*-phenanthroline. Ferrous *o*-phenanthroline complex measured at 550 $m\mu$ in 1 cm. optical cell. Cuvette contained: 0.20 mmole phosphate buffer (pH 6.5); 0.1 ml. enzyme; 0.1 μ mole DPNH; 0.2 μ mole *o*-phenanthroline; no endogenous rate; then 0.1 μ mole ferric chloride added. O—O = enzyme; ●—● = boiled enzyme.

used (Figs. 5–7). The effect of different valency states of Mo on the reduction of cytochrome *c* was studied with and without hydrogenase; only Mo^{3+} reduced it chemically. Mo^{5+} was without effect, with or without the enzyme. Thus in *Azotobacter* hydrogen is linked enzymically to cytochrome *c*, and Fe but not Mo is required in this system.

Fig. 5

Fig. 6

Fig. 5. Chemical reduction of cytochrome *c* by ferrous sulphate in phosphate buffer measured at 550 mμ in 1 cm. optical cells at: pH 5, ●—●; pH 6, ×—×; pH 7, ○—○. Cuvette contained: 0.20 mmole phosphate; 0.1 μmole cytochrome *c*; 0.1 μmole ferrous sulphate.

Fig. 6. Chemical reduction of cytochrome *c* by ferrous sulphate in acetate buffer measured at 550 mμ in 1 cm. optical cell. At pH 3, ○—○; pH 4, ◻—◻; pH 5, ●—●; pH 6, ×—×; pH 7, ○—○; Cuvette contained: 0.20 mmole acetate; 0.1 μmole cytochrome *c*; 0.1 μmole ferrous sulphate.

Fig. 7. Chemical reduction of cytochrome *c* by ferrous sulphate in citrate buffer measured at 550 mμ in 1 cm. optical cell. At pH 3, ○—○; pH 4, ●—●; pH 5, ×—×; pH 6, ◻—◻; pH 7, ○—○. Cuvette contained: 0.20 mmole sodium citrate; 0.1 μmole cytochrome *c*; 0.1 μmole ferrous sulphate.

Effects of trace metals on nitrogen fixation and hydrogenase

A molybdenum requirement for growth of *Azotobacter chroococcum* 8003 is shown in Fig. 8, the optimum requirement being 0.01 $\mu\text{g. Mo/ml.}$ The organisms had a wide tolerance for the metal; it did not become toxic until 0.1 mg. Mo/ml. was reached. Iron was required in greater amounts than Mo as illustrated (Fig. 9). Sodium vanadate replaced molybdate in the growth of *A. chroococcum* to the extent of 70 % (Fig. 10).

The effects of deficiencies of Fe and Mo on nitrogen-fixation and hydro-

Fig. 8. Effect of Mo content of culture medium on the growth of *Azotobacter chroococcum* 8003. Abscissa: % growth based on turbidity measurements. Ordinate: $\mu\text{g.}$ or mg. Mo/ml. medium.

Fig. 9. Effect of Fe content of culture medium on the growth of *Azotobacter chroococcum* 8003. Abscissa: % growth based on turbidity measurements. Ordinate: $\mu\text{g.}$ Fe/ml. medium.

Fig. 10. Effect of sodium vanadate in replacing sodium molybdate for the growth of *Azotobacter chroococcum* 8003.

genase activity in *Azotobacter vinelandii* (O) and *Clostridium pasteurianum* are illustrated in Table 8. A deficiency of Fe in both organisms markedly decreased hydrogenase activity, but Mo deficiency had no effect on the *Azotobacter* enzyme and only a small effect on the *Clostridium* enzyme. Both metals, however, were required for nitrogen fixation; a shortage of Mo had the most profound effect since it was independent of yield of organism.

Table 8. *Hydrogenase activity and nitrogen fixation in Azotobacter vinelandii* (O) and *Clostridium pasteurianum* W-S

Methylene blue was used as H-acceptor for assay of hydrogenase.

Organism	...	<i>Azotobacter vinelandii</i>			<i>Clostridium pasteurianum</i>		
		Full culture	Fe omitted	Mo omitted	Full culture	Fe omitted	Mo omitted
Nutrient solution							
Hydrogenase Q_{H_2} (MB)/ mg. N/hr. in whole organism		4,620	1,150	4,150	58,400	16,200	48,100
Total N_2 fixed mg. N/ 8 l. culture		1,000	600	416	670	436	240
Dry weight yields of organism as % normal		100	35	70	100	45	75

Table 4 shows the effects of sodium tungstate and sodium vanadate on hydrogenase activity and nitrogen-fixation; 2×10^{-3} M-tungstate in the medium depressed nitrogen fixation but had no effect on hydrogenase activity. A similar result was obtained when molybdenum was omitted; the addition of

sodium tungstate to these cultures depressed further the nitrogen fixation but had no effect on hydrogenase. Sodium vanadate at equivalent concentrations to tungstate did not depress nitrogen fixation.

Table 4. *Hydrogenase activity and nitrogen fixation in Azotobacter vinelandii (O) and in Clostridium pasteurianum W-S*

Culture medium	Hydrogenase in whole organism QH_2 (MB)/mg N fixed		Total N fixed by organism/l. medium	
1. Full culture	8,600	108,000	590	68
2. Full culture + 10^{-3} M-sodium tungstate	8,000	124,000	500	56
3. Full culture + 2×10^{-3} M-sodium tungstate	7,700	84,000	250	18
4. Molybdenum omitted	8,600	87,000	220	24
5. Molybdenum omitted; sodium tungstate 10^{-3} M added	7,900	—	200	—
6. Molybdenum omitted; 2×10^{-3} M sodium vanadate added	8,200	—	210	—

The concentration of hydrogenase from Clostridium pasteurianum

The method described by Shug, Wilson, Green & Mahler (1954) for purifying hydrogenase from *Clostridium* was not successful in our hands since the enzyme is irreversibly inactivated by traces of oxygen. Ethanol inactivated the enzyme no matter what precautions were taken to keep the preparations in a reduced state, e.g. by adding sodium dithionite and storing in Thunberg tubes under hydrogen. Dr D. Westlake has recently experienced similar difficulties with this purification (Dr P. W. Wilson, private communication).

A fractionation of hydrogenase from *Clostridium pasteurianum* is shown in Table 5. The organism was grown in media freed from iron to which was returned 0.75 mg. ferric chloride labelled with $0.66 \mu\text{C } ^{59}\text{Fe}$ per l. The various protein fractions were assayed for radioactivity in a scintillation counter (Nicholas, Lloyd-Jones & Fisher 1957) and for hydrogenase activity. The results (Fig. 11) show that iron concentrated in the more purified fractions of the enzyme. Molybdenum did not accumulate in these fractions, and the addition of sodium molybdate did not increase the activity of the purified enzyme. Similar results were obtained with hydrogenase from *Azotobacter* although a good purification of this enzyme was not achieved.

The inhibition of the partially purified enzyme from *Azotobacter* was very pronounced when it was dialysed at 4° against 0.1 M-phosphate buffer (pH 7.5) + 0.01 M-sodium dithionite containing 5×10^{-3} M. *o*-phenanthroline. After dialysis for 6 hr. the enzyme preparation was transferred to a fresh solution of the same buffer + dithionite and dialysis continued for a further 6 hr. by which time excess *o*-phenanthroline was removed. At this stage reactivation of the enzyme was tried by returning metals (including Fe, Mo) to samples of the dialysed enzyme preparation. The results (Table 6) show that dialysis against

Table 5. *Concentration of hydrogenase from Clostridium pasteurianum W-S*

Fraction	Total N (mg.)	Total enzyme units QH ₂ (M.B.)	Enzyme specific activity (units/mg./protein)	Recovery of enzyme (%)	Total ⁵⁵ Fe (μ g.)	μ g. ⁵⁵ Fe/ mg. protein	Recovery of ⁵⁵ Fe (%)
1. Whole organisms	82	4,500,000	54,000	—	—	—	—
2. Crude extract from organisms crushed in a Hughes press in 0.1 M-phosphate (pH 7.5)	58	3,800,000	65,000	100	885	15.3	100
3. Supernatant solution after centrifuging (2) at 23,000 g for 1 hr.	25	4,100,000	165,000	100	497	20	56
4. Residue left after centrifuging (2) at 23,000 g for 1 hr.	35	800,000	22,000	21	397	12	45
5. Ribonuclease treatment of (3): 3 mg./100 ml./15 min. at 37°. Ppt. from 0.50 % ammonium sulphate fraction, centrifuged at 23,000 g, dissolved in 0.2 M-phosphate (pH 7.5)	3.2	55,100	18,360	1	113	35	11
6. Supernatant solution left after treatment (5)	4.6	3,180,000	690,000	84	304	66	9
7. Ppt. from 60-70 % saturation with ammonium sulphate of fraction (6). Ppt. dissolved in 0.1 M-phosphate (pH 7.5)	0.5	420,000	840,000	11	112	224	12

o-phenanthroline markedly decreased enzyme activity. Subsequent dialysis against dithionite in phosphate buffer, however, resulted in a recovery of enzymic activity, presumably because the *o*-phenanthroline was removed from the enzyme, thus indicating that Fe^{2+} was held more tightly by the protein than by the chelate. When no further recovery of enzyme activity was observed, and no *o*-phenanthroline could be detected in the preparation, the addition of Fe^{2+} reactivated the enzyme by 35–48 %, whereas the addition of molybdate produced little effect. The addition of Zn, which has a higher affinity for *o*-phenanthroline than has Fe^{2+} , did not stimulate the enzyme.

It was not possible to remove the iron from the *Clostridium* enzyme preparation by dialysis, although numerous methods were tried. This may mean that iron is very tightly bound in the enzyme.

Fig. 11. Relation between the iron content and hydrogenase activity of protein fractions prepared from a Hughes press extract of *Clostridium pasteurianum* W-S.

DISCUSSION

Hydrogenase reduces pyridine nucleotides in a number of micro-organisms when hydrogen is the donor (Korkes, 1955; Vishniac & Ochoa, 1952; Shug & Wilson, 1954; Peck & Gest, 1954). Cytochrome *c* is also a suitable acceptor (Hyndman *et al.* 1953); Shug, Hamilton & Wilson, 1956). In the present work these effects have been confirmed and it is also shown that iron is required to couple DPNH to cytochrome *c* in extracts of *Azotobacter vinelandii* (O). The mechanism involves the enzymic reduction of Fe^{3+} to Fe^{2+} by DPNH and then the reduction of the cytochrome by Fe^{2+} non-enzymically. The fact that the non-enzymic reduction to Fe^{2+} is fast at physiological pH values makes it a likely mechanism for cytochrome reduction *in vivo* in *Azotobacter*. Webber, Lenhoff & Kaplan (1956) showed that Fe^{3+} was reduced to Fe^{2+} by flavo-proteins in extracts of *Pseudomonas fluorescens* and that Fe^{2+} reduced cytochrome *c* chemically. Although extracts of *Clostridium pasteurianum* reduce cytochrome *c* *in vitro* as effectively as do those from *A. vinelandii* (O) this cannot be the natural system since *C. pasteurianum* is devoid of cytochrome. Thus here the cytochrome acts as would a suitable redox dye H-acceptor and it has

Table 6. *Dialysis of hydrogenase from Azotobacter vinelandii (O) and its reactivation by iron*

(1) Original enzyme in phosphate (pH 7.5)	(2) Dialysis against 10 ⁻³ M-Na ₂ S ₂ O ₄ in phosphate (pH 7.5)	(3) Dialysis for 6 hr. against 10 ⁻² M- Na ₂ S ₂ O ₄ + 5 × 10 ⁻³ M- o-phenanthroline in phosphate buffer (pH 7.5)	(4) (3) transferred after 6 hr. to 10 ⁻³ M-Na ₂ S ₂ O ₄ in phosphate buffer (pH 7.5) and dialysis continued for 8 hr.	(5) 20 µg. Fe added/ ml. enzyme from (4)	(6) 20 µg. Mo added/ ml. enzyme from (4)
Hydrogen uptake (µl./ml. enzyme preparation/hr.)					
Experiment 1					
1500	1620 (6 hr.)	360	960	1280	1040
	1200 (12 hr.)				
Experiment 2					
1740	1860 (6 hr.)	780	1050	1500	1120
	1740 (12 hr.)				

no physiological significance. Mo^{6+} or Mo^{5+} cannot replace Fe^{2+} in this reaction; only Mo^{3+} reduced cytochrome *c* non-enzymically. This is of interest since Shug *et al.* (1954) suggested that Mo is required to couple hydrogen to cytochrome *c* in hydrogenase from *C. pasteurianum*. Evidence presented in the present paper however suggests that Fe and not Mo is the main constituent of hydrogenase. This is in accord with the results of Waring & Werkman (1944) with *Aerobacter indologenes*, Hoberman & Rittenberg (1943) with *Proteus vulgaris*, Peck & Gest (1957) with *C. butylicum* and Postgate (1951) and Sadana & Jagannathan (1956) with *Desulfovibrio desulfuricans*.

Molybdenum is required for nitrogen fixation and only in *Azotobacter chroococcum* did vanadium replace it to the extent of 70 %. The precise role of Mo in the fixation process must await further work. The metal is probably associated with the enzyme system which activates nitrogen. It has already been shown that Mo is important for nitrogen metabolism since nitrate reduction from micro-organisms requires Mo for its functioning (Nicholas & Nason, 1954; Nicholas & Stevens, 1955). The inhibitor and metal deficiency studies reported here have shown hydrogenase and nitrogenase to be distinct enzyme systems.

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