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Purification of the Membrane-Bound Hydrogenase of *Escherichia coli*

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The membrane-bound hydrogenase (EC class 1.12) of aerobically grown *Escherichia coli* cells was solubilized by treatment with deoxycholate and pancreatin. The enzyme was further purified to electrophoretic homogeneity by chromatographic methods, including hydrophobic-interaction chromatography, with a yield of 10% as judged by activity and an overall purification of 2140-fold. The hydrogenase was a dimer of identical subunits with a mol.wt. of 113000 and contained 12 iron and 12 acid-labile sulphur atoms per molecule. The ε_{400} was $49000\,M^{-1}\cdot cm^{-1}$. The hydrogenase catalysed both H_2 evolution and H_2 uptake with a variety of artificial electron carriers, but would not interact with flavodoxin, ferredoxin or nicotinamide and flavin nucleotides. We were unable to identify any physiological electron carrier for the hydrogenase. With Methyl Viologen as the electron carrier, the pH optimum for H_2 evolution and H_2 uptake was 6.5 and 8.5 respectively. The enzyme was stable for long periods at neutral pH, low temperatures and under anaerobic conditions. The half-life of the hydrogenase under air at room temperature was about 12h, but it could be stabilized by Methyl Viologen and Benzyl Viologen, both of which are electron carriers for the enzyme, and by bovine serum albumin. The hydrogenase was strongly inhibited by carbon monoxide ($K_i = 1870$ Pa), heavy-metal salts and high concentrations of buffers, but was resistant to inhibition by thiol-blocking and metal-complexing reagents. These aerobically grown *E. coli* cells lacked formate hydrogenlyase activity and cytochrome c_{552}.

The enzyme hydrogenase (EC class 1.12) was first discovered by Stephenson & Stickland (1931), who observed that *Escherichia coli* could use H_2 to reduce a variety of substrates. The enzyme, which catalyses the reversible activation of molecular H_2, was later shown to be widespread in both bacteria and algae (see Mortenson & Chen, 1974; Schlegel & Schneider, 1978) and is now known to be involved in all physiological reactions in which H_2 is evolved or consumed. In recent years the enzyme has been the subject of much research, but hydrogenase purification has often been impeded by the sensitivity of the enzyme to O_2 and its association with membranes in some organisms. However, the enzyme has been isolated and purified to homogeneity from anaerobic bacteria (Chen & Mortenson, 1974; van der Westen *et al.*, 1978; Hatchikian *et al.*, 1978), from photosynthetic bacteria (Gitlitz & Krasna, 1975; Gogotov *et al.*, 1976; Adams & Hall, 1977) and from hydrogen bacteria (Schneider & Schlegel, 1976). The data reported have established that hydrogenases are all iron–sulphur proteins, but they differ markedly in their physical and catalytic properties, e.g. iron–sulphur content, O_2- and temperature-sensitivity, electron-carrier specificity and specific activity.

Abbreviation used: SDS, sodium dodecyl sulphate.

To date, hydrogenase has not been purified from the facultative-anaerobic Enterobacteriaceae. After the initial work of Stephenson & Stickland (1931), there have been several attempts to characterize and purify the hydrogenase of *Escherichia coli* in cell-free extracts (Bovarnick, 1941; Joklick, 1950*a*,*b*; Gest, 1952), but this work was hampered by the particulate nature of the enzyme preparations used. We present here, almost 50 years after the discovery of the enzyme, details of the solubilization and purification of the hydrogenase of *E. coli*. The enzyme was isolated from aerobically grown cells and, after solubilization with deoxycholate and pancreatin, was further purified by chromatographic methods to electrophoretic homogeneity. It possesses many properties in common with the hydrogenases isolated from strictly anaerobic bacteria (*Clostridium* and *Desulfovibrio*).

We have also considered the possible role of hydrogenase in the aerobically grown cells with respect to the activities of physiologically related enzyme systems and potential electron carriers for the enzyme. Formate hydrogenlyase is a multienzyme system that catalyses the oxidation of formate to H_2 and CO_2, an efficient way of removing formate during the anaerobic metabolism of carbohydrates. This involves the function of formate dehydrogenase 'H'

(after H_2 formation) (EC class 1.2) hydrogenase and unidentified intermediate electron carrier(s) (Peck & Gest, 1957; Gray & Gest, 1965; Ruiz-Herrera & Alvarez, 1972). The discovery of a soluble low-redox-potential *c*-type cytochrome (c_{552}) in *E. coli* (Gray *et al.*, 1963) led to the proposal that this pigment was a cofactor for the hydrogenase in formate metabolism (Gray & Gest, 1965; Cole & Wimpenny, 1966). Formate may also be oxidized with O_2 (under aerobic conditions) or nitrate (under anaerobiosis) as the terminal electron acceptor. This involves a second formate dehydrogenase, 'N' (membrane-bound form; after nitrate reduction), which is linked to the aerobic respiratory chain or to nitrate reductase (EC 1.7.99.4) (see Gray *et al.*, 1966; Ruiz-Herrera & DeMoss, 1969; Enoch & Lester, 1975). However, the interrelationship between hydrogenase and these various enzymes under different growth conditions is still not clear. We therefore decided to investigate this and identify any physiological electron carrier(s) for the hydrogenase in the aerobically grown cells.

Materials and Methods

All chemicals and reagents were of the highest available purity. Deoxycholate, pancreatin (P-1750), chymotrypsinogen, 3-lactoglobulin, polymyxin B, lysozyme, proteinase (type VI), NAD^+, NADH, $NADP^+$, FMN and FAD were obtained from Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K.; chymotrypsin A_4, catalase, aldolase, ovalbumin and bovine serum albumin from Boehringer Corp. (London) Ltd., Lewes, East Sussex, U.K.; Whatman DE52 from Whatman Biochemicals, Maidstone, Kent, U.K.; octyl-Sepharose CL-4B, Sephacryl S-200 and DEAE-Sephacel from Pharmacia (G.B.) Ltd., London W.5, U.K. Hydroxyapatite (spheroidal) and all other chemicals were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K. The bipyridylium salts (Methyl Viologen analogues I, II and III, see below) were donated by Dr. J. R. Bolton, University of Western Ontario, London, Ont., Canada. *Megasphaera elsdenii* flavodoxin was a gift of Dr. S. G. Mayhew, University College, Dublin, Ireland. *Desulfovibrio desulfuricans* (Norway strain) cytochrome c_3 was donated by Dr. C. Hatchikian, CNRS/LCB, Marseille, France. The ferredoxins of *Spirulina maxima*, *Chromatium* and *Clostridium pasteurianum* were obtained from Dr. K. K. Rao of this Department.

Organism

Escherichia coli cells (strain MRE 600) were obtained as a frozen paste from the Microbiological Research Establishment, Porton Down, Wilts., U.K. The cells were grown aerobically by continuous-flow culture at 37°C under glycerol limitation at a dilution rate of $0.78\,h^{-1}$ in a medium containing glycerol, yeast extract and mineral salts as described by Elsworth *et al.* (1968).

H_2-evolution assay

The hydrogenase was routinely assayed by the evolution of H_2 from Methyl Viologen reduced by sodium dithionite. In 15ml sealed vials, shaken at 30°C, the 2ml reaction mixture contained 20mM-potassium phosphate buffer, pH7.0, Methyl Viologen (2.5mM) and sodium dithionite (10mM) under a N_2 atmosphere. Hydrogenase was added by syringe to start the reaction and the H_2 evolved was determined as described by Rao *et al.* (1976). A unit of enzyme catalysed the evolution of 1μmol of H_2/min under these conditions. Rates of 30nmol of H_2 evolved/min were routinely measured.

H_2-uptake assay

H_2 uptake was measured at 30°C on a Unicam SP.800 spectrophotometer by using anaerobic cuvettes (no. 26; Starna Ltd., London E.13, U.K.). The 3ml reaction mixture contained 20mM-potassium phosphate buffer, pH7.0, with the electron acceptor in the cuvette and the hydrogenase in the side arm. After repeated degassing with a vacuum pump and flushing with H_2, the reaction was started by mixing the components under a H_2 atmosphere and was measured by the reduction of the acceptor at the appropriate wavelength. The electron acceptors used and their absorption coefficients (ε) were: Methyl Viologen, $12000\,M^{-1}\cdot cm^{-1}$ at 600nm; Benzyl Viologen, $8100\,M^{-1}\cdot cm^{-1}$ at 555nm; Methylene Blue, $7000\,M^{-1}\cdot cm^{-1}$ at 601nm; Neutral Red, $7600\,M^{-1}\cdot cm^{-1}$ at 450nm; Safranine T, $9450\,M^{-1}\cdot cm^{-1}$ at 550nm; potassium ferricyanide, $13200\,M^{-1}\cdot cm^{-1}$ at 405nm; and phenosafranin, $1150\,M^{-1}\cdot cm^{-1}$ at 400nm. All electron acceptors were reduced at a constant rate, but only after an initial lag period of up to 5min (see Adams & Hall, 1979).

Other enzyme assays

Formate oxidase activity was determined by the rate of formate-dependent O_2 uptake at 25°C by using an oxygen electrode (Hansatech Ltd., King's Lynn, Norfolk, U.K.) with formate (10mM) in 50mM-potassium phosphate buffer, pH7.4 (Gray *et al.*, 1966). Formate hydrogenlyase activity was measured at 30°C by the rate of H_2 evolution (see above) with formate as the electron donor by using the reaction mixtures described by Peck & Gest (1957) and Azoulay & Marty (1970). Formate dehydrogenase activities were determined at 30°C spectrophotometrically by using anaerobic cuvettes (see above) with either Methylene Blue, Benzyl Viologen (Peck & Gest, 1957) or phenazine methosulphate plus 2,6-dichlorophenol-indophenol (Lester & DeMoss, 1971) as the electron acceptors under conditions as de-

scribed in the references. 2,6-Dichlorophenol-indophenol reduction was measured at 600nm (ε 20600$M^{-1}\cdot cm^{-1}$): Formate dehydrogenase 'H' will only reduce low-redox-potential electron acceptors that are reduced by one electron, e.g. Benzyl Viologen, whereas formate dehydrogenase 'N' is specific to Methylene Blue or phenazine methosulphate. For the above assays, crude cell extracts were prepared as for the hydrogenase with the omission of the deoxycholate.

Polyacrylamide-gel electrophoresis

The method of Davis (1964) was used for disc electrophoresis in 10% (w/v) polyacrylamide. Gels were stained with Coomasie Brilliant Blue and destained electrophoretically. Hydrogenase activity was located by Methyl Viologen reduction as described previously (Ackrell *et al.*, 1966), with Methyl Viologen (0.25% w/v) in 20mM-potassium phosphate buffer, pH7.0, at 30°C. The blue band of reduced Methyl Viologen, which is rapidly oxidized by air, was further made to react with 2,3,5-triphenyltetrazolium chloride (2.5% w/v) to give a bright-red permanent band of the reduced formazan. Controls were run in a H_2-free system and without Methyl Viologen. R_F values were calculated by comparing the migration of the hydrogenase with that of the dye Bromophenol Blue.

Isoelectric focusing

The isoelectric point of the hydrogenase was determined in polyacrylamide gel by the method of Gainer (1973), with pH3–5 Ampholines (LKB Produkter).

Molecular-weight determination

The molecular weight of the hydrogenase was determined by gel filtration on a column (2.2cm×90cm) of Sephacryl S-200 equilibrated with 50mM-potassium phosphate, pH7.0, containing 50mM-KCl, as described by Andrews (1964), with bovine serum albumin, ovalbumin, aldolase and β-lactoglobulin as molecular-weight markers. Polyacrylamide-gel electrophoresis in the presence of SDS was performed by the method of Weber *et al.* (1972). Bovine serum albumin, catalase, ovalbumin, aldolase, β-lactoglobulin and chymotrypsinogen were used as molecular-weight references.

Iron and acid-labile-sulphide determination

Total iron was determined by the bathophenanthroline method (Brumby & Massey, 1967). Acid-labile sulphide was determined by the method of Fogo & Popowsky (1949) as modified by Suhara *et al.* (1975). Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as the standard.

Purification of hydrogenase

Unless otherwise stated, all steps were performed at 4°C under an atmosphere of O_2-free N_2, and all buffers were flushed with N_2 before use. All centrifugation steps were carried out in the 6×100ml rotor of an MSE 18 refrigerated centrifuge at 10°C.

(1) *Crude sonicated material.* Frozen cells (120g) were thawed in 3vol. of 50mM-Tris/HCl buffer, pH8.0, containing deoxycholate (3%, w/v), and were disrupted by sonication with a Dawe Soniprobe at full power (about 4A) for 20min. The temperature was maintained below 15°C by cooling in an ice bath.

(2) *Supernatant.* The sonicated material was centrifuged at 40000*g* for 2h, and the sediment was discarded.

(3) *$(NH_4)_2SO_4$ fractionation and pancreatin treatment.* Solid $(NH_4)_2SO_4$ was added to the supernatant to give 15% saturation and the mixture was centrifuged at 40000*g* for 20min. The supernatant was brought to 45% saturation with respect to $(NH_4)_2SO_4$ and then centrifuged at 40000*g* for 1h. The sediment was suspended in 50mM-potassium phosphate buffer, pH7.0, and dialysed overnight against the same buffer. Pancreatin (10mg/g of cells) was added to the dialysate, and the suspension was incubated at 35°C for 1h in a shaking water-bath. After cooling, the mixture was clarified by centrifugation (40000*g* for 1h).

(4) *DEAE-cellulose chromatography.* The supernatant was applied directly to a column (3.4cm×44cm) of DEAE-cellulose DE52 equilibrated with 50mM-potassium phosphate buffer, pH7.0, at 80ml/h. The column was then washed with 400ml of the equilibration buffer, which eluted a considerable quantity of protein. The hydrogenase was eluted with a linear gradient (1200ml) of 50–250mM-potassium phosphate, pH7.0, collected in 7ml fractions. The activity was eluted at approx. 90mM-phosphate. Those fractions containing activity greater than 0.6 unit/ml were combined and used directly for the next step.

(5) *Octyl-Sepharose CL-4B chromatography.* The pooled DEAE-cellulose DE52 fractions were applied directly to a column (1.8cm×30cm) of octyl-Sepharose equilibrated with 100mM-$(NH_4)_2SO_4$ in 10mM-potassium phosphate buffer, pH7.0, at 30ml/h. The liquid that came out of the column retained the yellowish-green appearance of the DEAE-cellulose DE52 fractions, but contained no hydrogenase activity. The column was washed with 100ml of the equilibration buffer and the activity was eluted with a 400ml linear gradient containing, initially, the equilibration buffer, and finally, deoxycholate (0.50%, w/v) and Triton X-100 (0.05%, v/v) in 1mM-potassium phosphate buffer, pH7.0. Fractions of 2ml were collected at 20ml/h. The enzyme was eluted as a sharp peak at approx. 55mM-$(NH_4)_2SO_4$. Fractions with

activity greater than 0.6 unit/ml were combined and dialysed against 10 litres of 25mM-Tris/HCl buffer, pH7.5.

(6) *DEAE-Sephacel chromatography*. The dialysed fractions were passed through a column (1.8cm × 44cm) of DEAE-Sephacel equilibrated with 25mM-Tris/HCl buffer, pH7.5, at 15ml/h. The column was washed with the equilibration buffer containing 100mM-NaCl (100ml), and the activity was eluted with a 800ml linear gradient of 100–250mM-NaCl in 3ml fractions. The activity was displaced from the gel at 190–200mM-NaCl, and all active fractions containing more than 30% of the activity found in the peak fraction were pooled and dialysed against 1mM-potassium phosphate buffer, pH7.0.

(7) *Hydroxyapatite chromatography*. The dialysed fractions were loaded on to a column (2.2cm × 14cm) of hydroxyapatite equilibrated with 1mM-potassium phosphate buffer, pH7.0, at 20ml/h. After washing the column with the equilibration buffer (50ml), the enzyme activity was eluted with a 800ml linear gradient of 1–150mM-potassium phosphate, pH7.0. Fractions of 3ml were collected. All the hydrogenase activity eluted between 18 and 22mM-phosphate. Those fractions containing pure hydrogenase as judged by polyacrylamide-gel electrophoresis were combined, concentrated by ultrafiltration (Amicon ultrafilter, PM-10 membrane), and dialysed against 20mM-potassium phosphate buffer, pH7.0.

Results

Cellular location of the hydrogenase

The hydrogenase was not associated with the outer cell membrane or located within the periplasmic space, since extensive washing of whole cells with lysozyme (Neu & Heppel, 1965), EDTA at high pH values (van der Westen *et al.*, 1978) or polymyxin B (Cerny & Teuber, 1971) released less than 1% of the total activity. After sonication of a cell suspension followed by centrifugation (45000*g* for 90min), less than 30% of the total activity was in the supernatant. This suggested that the enzyme was firmly associated to the inner cell membrane.

Solubilization and purification procedure

Polyacrylamide-gel electrophoresis and the elution pattern from a column of Sephacryl S-200 were used to assess the molecular weight and thus probable degree of membrane association of the hydrogenase (see Adams & Hall, 1978). After sonication of a cell extract, there was no migration of the activity on the gels [Fig. 1, gel (*a*)], and the activity was eluted with the void volume from the Sephacryl S-200 column (mol.wt. >200000). After treatment of whole cells in the presence of deoxycholate and then incubation in the presence of pancreatin, increasing amounts of the

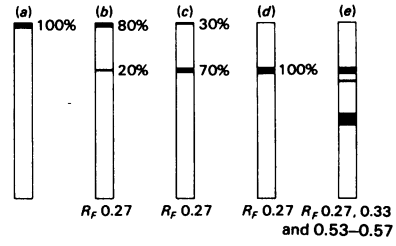


Fig. 1. *Electrophoretograms of E. coli hydrogenase activity during solubilization procedure*

The gels were run and stained for hydrogenase activity as described in the Materials and Methods section. The percentage values refer to estimated stain intensities. (*a*) After sonication (mol.wt. >250000); (*b*) After sonication in the presence of deoxycholate. (*c*) After sonication in the presence of deoxycholate followed by incubation at 35°C for 1h. (*d*) After sonication in the presence of deoxycholate followed by incubation at 35°C for 1h in the presence of pancreatin (see the text) (mol.wt. ≃110000). (*e*) After prolonged staining (30min) of the gel obtained in (*d*).

enzyme migrated on the gels to the same R_F value of 0.27 [Fig. 1, gels (*b*)–(*d*)]. The activity shown in gel (*d*) eluted as a single peak from a Sephacryl S-200 column corresponding to a mol.wt. of about 110000. Also, when subjected to DEAE-cellulose chromatography, the activity was eluted as a single peak, showing homogeneity of the hydrogenase activity. Attempts to further 'solubilize' the activity shown in gel (*d*) by incubation (2h at 30°C) of the preparation with urea, proteinase, trypsin, chymotrypsin and pancreatin were unsuccessful, as judged by migration of the activity on the gels and elution from a Sephacryl S-200 column. This preparation thus constituted the membrane-free enzyme.

The activity bands on the polyacrylamide gels [Fig. 1, gels (*a*)–(*d*)] appeared after less than a 10min incubation with Methyl Viologen (see the Materials and Methods section). However, after prolonged incubation (over 30min) of the membrane-free preparation (purification 8-fold, see Table 1) with Methyl Viologen, additional bands formed [gel (*e*)]. The band at R_F 0.53–0.57 was very diffuse. Thus this preparation appeared to contain different 'states' of the enzyme, which differed in electrophoretic mobility and reactivity towards Methyl Viologen reduction, although by chromatographic methods the preparation was homogeneous with respect to hydrogenase activity. If a membrane-free preparation (purification 8-fold, or, after DEAE-cellulose chromatography, purification 160-fold) was incubated (40°C for 1h) with urea (1.5M) and then passed down a Sephacryl S-200 column equilibrated with urea (2.0M), the activity still eluted as a single peak with, as before, a mol.wt. of about 110000, and still gave the same

Table 1. *Purification of E. coli hydrogenase*

H_2-uptake activity is expressed as μmol of H_2 consumed/min per mg of protein. The reaction was performed as described in the Materials and Methods section, with Methyl Viologen (10mM) as the electron acceptor. The Methyl Viologen concentrations used in both assays are approx. 2.5 times the apparent K_m values (see the text).

Purification step	Volume (ml)	Total activity (units)	Recovery (%)	Total protein (mg)	Specific activity (units/mg)	Purification (fold)	H_2-uptake activity
Crude sonicated material	475	1370	100	29200	0.047	1.0	0.057
Supernatant	400	959	70	13700	0.070	1.5	0.078
$(NH_4)_2SO_4$/pancreatin	510	932	68	2450	0.38	8.1	0.32
DEAE-cellulose	190	480	35	65.7	7.3	156	7.2
Octyl-Sepharose CL-4B	52	288	21	8.44	34	726	30
DEAE-Sephacel	60	165	12	2.55	65	1380	59
Hydroxyapatite	42	137	10	1.37	100	2140	89

pattern of activity bands [gel (*e*)]. However, after chromatography on octyl-Sepharose CL-4B (purification 730-fold, see Table 1), only one activity band could be detected on the gels, at an R_F of 0.27, even after prolonged incubation with Methyl Viologen. It was thus concluded that the multiple bands on the gels obtained with less pure enzyme preparations were an artifact of electrophoresis caused presumably by the binding of the enzyme to other proteins, which also affected the enzyme activity.

The octyl-Sepharose CL-4B column was eluted with $(NH_4)_2SO_4$ plus phosphate. Phosphate could be used alone, but the activity peak was very broad, although there was little difference in the purification achieved. Although this column material could be recycled after use, repeated recycling led to compression of the column when the detergent and deoxycholate were used for elution of the activity.

Typical results of the purification procedure are summarized in Table 1. The hydrogenase was purified about 2100-fold to a specific activity of 100 units/mg of protein, with a yield of 10% as judged by activity. The enzyme from the hydroxyapatite column gave a single band of protein after electrophoresis in polyacrylamide gel (R_F 0.27) coincidental with a single band of hydrogenase activity. The preparation was thus judged to be pure. The results given in Table 1 indicate that the hydrogenase represents about 0.05% of the total cellular protein of *E. coli* grown under these conditions. The ratio of H_2-evolution to H_2-uptake activity increases slightly, with purification remaining constant at about 1.1 after chromatography on octyl-Sepharose CL-4B. This suggests that the same enzyme is responsible for catalysing both reactions. The binding of the hydrogenase to other cellular proteins, the presence of small amounts of other hydrogenase types and/or modifiers of hydrogenase activity could account for the discrepancy observed with the enzyme preparations before the octyl-Sepharose CL-4B chromatography step.

Physical properties of the hydrogenase

The molecular weight of the hydrogenase was estimated to be 113000 (±4000) after gel filtration on a calibrated column of Sephacryl S-200. SDS/polyacrylamide-gel electrophoresis of the purified enzyme (10–50μg) revealed only one protein band, which corresponded to a mol.wt. of 56000 (±2000). Isoelectric focusing of the pure enzyme on polyacrylamide gel also gave a single protein band, with a pI value of 4.2 (±0.15). These results indicated that the native enzyme exists as a dimer with subunits of the same molecular weight. These experiments also confirmed the homogeneity of the enzyme preparation. Analysis of the iron and acid-labile-sulphide content of the hydrogenase showed them to be present in approximately equimolar amounts, values of 11.8 ± 0.8 and 10.9 ± 1.4 g-atoms/113000g of protein were obtained respectively (values from three determinations). The enzyme preparation as isolated was brown, absorbed light throughout the visible region of the spectrum and had an absorption maxima at 280nm and a shoulder around 400nm. The ratio A_{400}/A_{280} was 0.35 and ε_{400} was $48800 \pm 3000\,M^{-1} \cdot cm^{-1}$, corresponding to about $4000\,M^{-1} \cdot cm^{-1}$ per g-atom of Fe. This value compares favourably with those obtained for other 12Fe–12S hydrogenases (Hatchikian *et al.*, 1978; van der Westen *et al.*, 1978).

The purified hydrogenase was sensitive to inactivation by oxygen. The half-life of the enzyme (10–100μg/ml in 20mM-phosphate buffer, pH 7.0) was about 12h at room temperature under air, compared with 11 days when the preparation was stored under N_2 at the same temperature. These values were the same when the residual activity was determined by the H_2-evolution or the H_2-uptake assay. Methyl Viologen and Benzyl Viologen, which will act as electron carriers for the enzyme, and bovine serum albumin, protected the enzyme from O_2. The effect of increasing concentrations of Benzyl Viologen on enzyme stability is shown in Fig. 2. Activity could not

be restored to the O_2-inactivated hydrogenase by flushing with H_2 or by the addition of thiol-containing reagents, i.e. mercaptoethanol, dithiothreitol or glutathione. The effect of H^+ concentration on the stability of the hydrogenase on storage (Fig. 3) showed the enzyme to be fairly labile, except at neutral pH. The enzyme was thus routinely stored in neutral buffer (20mM-potassium phosphate, pH7.0) at 4°C under N_2. Over prolonged periods the hydrogenase was stored in liquid N_2.

The effect of temperature on the activity and the stability of the purified hydrogenase under N_2 is shown in Fig. 4. The rate of H_2 evolution from dithionite-reduced Methyl Viologen over a 1h period was maximum at 35°C. Almost total inactivation of the enzyme occurred after a 15min incubation at 70°C.

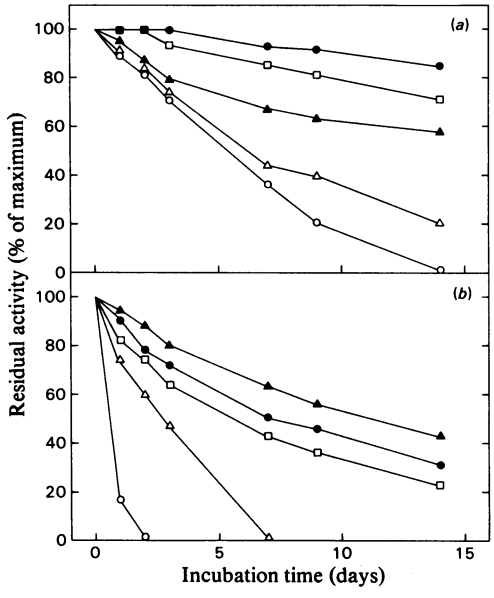


Fig. 2. *Effect of Benzyl Viologen on the stability of the hydrogenase*

The hydrogenase (20μg/ml in 20mM-potassium phosphate buffer, pH7.0) was incubated with various concentrations of Benzyl Viologen under air in sealed vials at (*a*) 4°C, or (*b*) 20°C. At intervals, duplicate samples (50μl) were taken and assayed by H_2 evolution from reduced Methyl Viologen under standard conditions (see the Materials and Methods section). The Benzyl Viologen concentrations used were: ○, none; △, 0.07mM; □, 0.7mM; ●, 7.0mM; ▲, none (incubation carried out under N_2). Control experiments with heat-treated samples (80°C for 15min) of hydrogenase showed that the measured activity was not due to bacterial contamination. The results are the average values for two separate experiments.

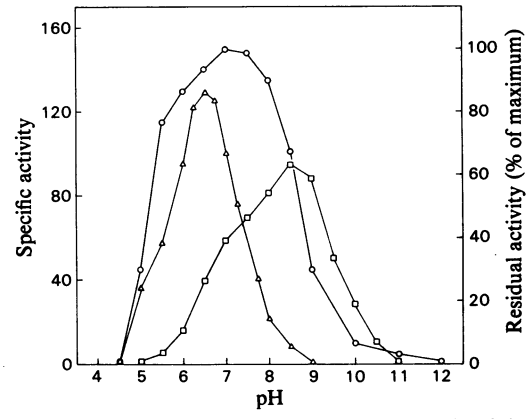


Fig. 3. *Effect of pH on hydrogenase activity and stability*

The effect of pH on hydrogenase activity was measured by the H_2-evolution (△) and H_2-uptake (□) assays as described in the Materials and Methods section with Methyl Viologen (2.5mM) as the electron carrier in both assay systems. The results are expressed as μmol of H_2 evolved or consumed/min per mg (specific activity), and each point represents the average for three determinations. The effect of pH on enzyme stability (○) was measured by incubating the enzyme (20μg/ml) under different pH conditions under an atmosphere of N_2 in sealed vials at 20°C in a shaking water-bath. After a 20h incubation, duplicate samples (50μl) were removed and assayed by H_2 evolution from reduced Methyl Viologen under standard conditions at pH7.0 (see the Materials and Methods section). The results are expressed as a percentage of the maximum activity remaining and are the average values from two separate experiments. The buffers used for all the above experiments were: citric acid/sodium phosphate (pH4.0–6.5) (McIlvaine, 1921); 20mM-potassium phosphate (pH6.7–7.3); 20mM-Tris/HCl (pH7.5–8.5); glycine/NaOH (pH9.0–10.0) (Gomori, 1955); sodium phosphate (pH10.5–11.0); and KCl/NaOH (pH12.0) (Bates & Bower, 1956).

Catalytic properties of the hydrogenase

The ability of the enzyme to both evolve and consume H_2 in the presence of various electron carriers is shown in Table 2. There was no correlation between the ratios of activities and the redox potential of the electron mediator. Methyl Viologen was the most efficient electron donor for H_2 evolution. Structural analogues of Methyl Viologen (I, II and III; see below) with more negative redox potentials were much less active, owing probably to steric hindrance of electron transfer. The computer-calculated V and K_m values (see Wilkinson, 1961) for Methyl Viologen at pH7.0 were 136±3μmol of H_2/min per mg of protein and 0.92±0.01mM respectively in the H_2-evolution assay, and 137±9μmol of H_2/min per mg of protein and 3.48±0.34mM respectively in the H_2-uptake assay.

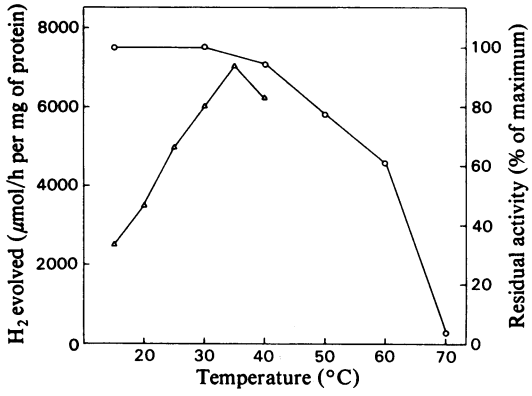
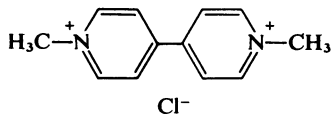


Fig. 4. *Effect of temperature on the stability and the activity of the hydrogenase*

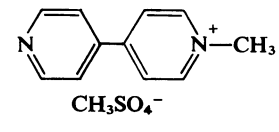
The effect of temperature on enzyme activity (△) was measured by assaying the enzyme (0.5μg) at various temperatures by H_2 evolution from reduced Methyl Viologen as described in the Materials and Methods section. The amount of H_2 evolved after a 1h incubation was determined. The stability (○) was determined by incubation of the hydrogenase (20μg/ml in 20mM-potassium phosphate buffer, pH7.0) in sealed vials under a N_2 atmosphere at various temperatures for 15min. The residual activity was then determined by removing samples (50μl) and assaying by H_2 evolution from reduced Methyl Viologen under standard conditions. All points represent averages for three determinations.

shows a fairly sharp optimum for H_2 evolution in the neutral–acidic region with Methyl Viologen as the electron carrier. Similar curves were obtained with Benzyl Viologen, Safranin T and phenosafranin (pH optima at 5.5, 6.0 and 6.2 respectively). The electron donor, sodium dithionite, is unstable in acidic media (see Mayhew, 1978); however, the rates of H_2 evolution remained constant (for 30min) at all pH values tested. The pH optimum for H_2 uptake was in the alkaline region and one may speculate that the difference in pH optima between the two activities reflects the changing substrate/product (H^+) concentration, and the ionic nature of the active site of the enzyme. The K_m for Methyl Viologen in the H_2-evolution assay was not significantly different at pH6.0 or 8.0 from that calculated at pH7.0.

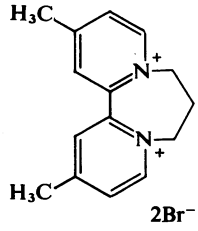
In the absence of an added electron carrier, the pure hydrogenase evolved H_2 from dithionite at about 0.3% of the rate obtained in the presence of Methyl Viologen (2.5mM). The same results were obtained by using the crude sonicated material (prepared also in the absence of deoxycholate; see the Materials and Methods section) or partially purified hydrogenase preparations. The pure enzyme was unable to evolve H_2 from FMN, FAD, NAD^+, $NADP^+$ (all at concentrations of up to 10mM), *M. elsdenii* flavodoxin or the ferredoxins of *Spirulina maxima* (2Fe), *Chromatium* (8Fe) or *Clostridium pasteurianum* (8Fe) (all at concentrations of up to 0.5mM), all reduced with dithionite; it was also unable to reduce any of the above with H_2. Also, the hydrogenase would not catalyse H_2 evolution from reduced synthetic iron–sulphur analogues (4Fe) of the active sites of ferredoxins (see Adams *et al.*, 1977). *Desulfovibrio desulfuricans*



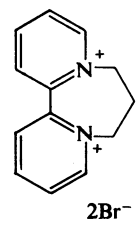
Methyl Viologen



Methyl Viologen analogue I



Methyl Viologen analogue II



Methyl Viologen analogue III

Benzyl Viologen had a much greater apparent affinity for the enzyme in the H_2-evolution assay (K_m 0.070±0.009mM), perhaps reflecting some hydrophobic interaction with the active site. The dependence of enzyme activity on H^+ concentration (Fig. 3)

Table 2. *Electron-carrier specificity in the H_2-evolution and H_2-uptake assays*

The activity of the hydrogenase with the various electron carriers (final concn. 1 mM) was measured as described in the Materials and Methods section. The results are the average values for three separate determinations (error ± 5%). H_2-evolution activity is expressed as μmol of H_2 evolved/min per mg, and all the carriers were reduced with sodium dithionite (10mM). H_2-uptake activity is expressed as μmol of H_2 consumed/min per mg. The E_0' value refers to pH 7.0.

Electron carrier	E_0' (mV)	H_2 evolution	H_2 uptake	$\frac{H_2 \text{ evolution}}{H_2 \text{ uptake}}$
Methyl Viologen analogues				
I	−730	16.6	—	—
II	−740	1.6	—	—
III	−560	0	—	—
Methyl Viologen	−440	72.3	35.5	2.0
Benzyl Viologen	−360	43.1	47.9	0.9
Neutral Red	−325	7.6	23.0	0.3
Safranine T	−289	8.4	6.3	1.3
Phenosafranine	−252	9.0	9.2	1.0
Methylene Blue	+11	0	123	—
Potassium ferricyanide	+360	0	153	—

(Norway strain) cytochrome c_3 (1–5 μM) could mediate electron transfer to the pure hydrogenase from dithionite with rates of H_2 evolution similar to those obtained with low concentrations of Methyl Viologen (1–5 μM). As with the pure enzyme, crude extracts of *E. coli* were also unable to evolve H_2 from the reduced nicotinamide or flavin nucleotides, flavodoxin or the ferredoxins.

Inhibition and inactivation of the hydrogenase

The hydrogenase-catalysed H_2 evolution from reduced Methyl Viologen was strongly inhibited by CO, as shown in Fig. 5. The inhibition was non-competitive with respect to the electron carrier, but could be completely reversed by flushing with N_2 for 5 min to remove the CO. Both the inhibition and the re-activation were independent of light. A linear reciprocal plot indicated that one molecule of CO was bound per molecule of enzyme, and from the slope the inhibitor constant (K_i) was calculated to be 1870 Pa. However, even under an atmosphere of pure CO, some hydrogenase activity was detected (3–4% of the control) and the rate of H_2 evolution was constant (for 1 h). Inhibition by CO has been reported for all hydrogenases so far tested (see Mortenson & Chen, 1974), except the enzyme from *Alcaligenes eutrophus* (Schneider *et al.*, 1979).

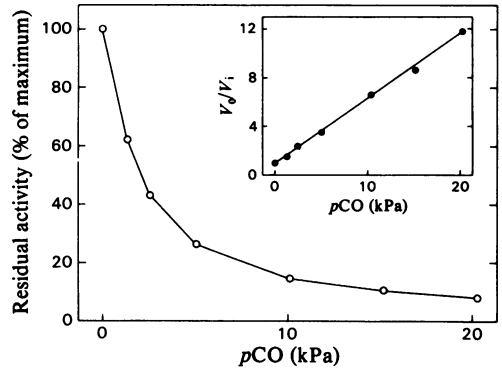


Fig. 5. *Inhibition of hydrogenase by CO*

The activity of the hydrogenase (1.0 μg) was determined by H_2 evolution from reduced Methyl Viologen under standard conditions as described in the Materials and Methods section, except that the N_2 atmosphere was replaced by N_2/CO mixtures. ○, Residual activity; ● (inset), reciprocal plot where V_i is the residual activity in the presence of CO and V_0 the activity in the absence of CO. All points represent averages for three determinations.

The effect of a number of inhibitors on H_2-evolution activity is shown in Table 3. The metal-complexing reagents azide, cyanide and EDTA were not inhibitory, suggesting that the iron was firmly bound in the active enzyme, although the iron-complexing reagent bathophenanthroline did cause some inhibition (as shown). The heavy metals mercury and copper caused severe inhibition. The enzyme (1.5 μg/ml) was completely inhibited by $HgCl_2$ at a concentration of only 10 μM. This could arise by interaction with amino, carboxy, imidazole or thiol groups. The inhibition was not relieved by increasing the electron carrier (Methyl Viologen) concentration. The lack of significant inhibition by arsenate, iodoacetate, phenylmethanesulphonyl fluoride, *p*-chloromercuribenzoate, sodium mersalyl and *N*-ethylmaleimide suggests that, if thiol groups are available, their modification does not abolish catalytic activity. Metabolites related to the proposed physiological role of hydrogenase (see the Discussion section), such as formate, nitrate, hydroxylamine,

glucose, lactate, pyruvate, succinate, fumarate, ATP or NADH (all up to 10mM final concn.) neither stimulated nor inhibited hydrogenase activity.

The hydrogenase was not inactivated by incubation (at 30°C for up to 2h) with the proteolytic enzymes chymotrypsin, trypsin or proteinase, but was susceptible to protein-denaturation reagents. Incubation (30°C for 1h) of the enzyme (35μg/ml) with urea (6M), guanidinium chloride (2M) or sodium perchlorate (0.5M) resulted in 50, 100 and 50% inactivation respectively when the enzyme (diluted 50-fold) was assayed by H_2 evolution. The hydrogenase was also inhibited by increasing concentrations of salts or buffers. When assayed in 0.5M-potassium phosphate, pH7.5, 0.5M-Mops (4-morpholinepropanesulphonic acid), pH7.5, or 0.5M-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], pH7.5, buffers, or 0.5M-KCl (in 20mM-potassium phosphate, pH7.5), the enzyme was inhibited by 50±8% relative to the control (buffer concentration of 20mM, or in the absence of KCl). With Tris/HCl buffer, pH7.5, increasing the buffer concentration from 20mM to 100mM resulted in a 60±3% decrease in hydrogenase activity. However, inhibition by all these buffers was totally reversible, as all the activity was recovered on dilution. The enzyme exhibited the same H_2 evolution activity (±8%) in all these buffers (pH7.5) at a buffer concentration of 20mM. Dimethyl sulphoxide (15%, v/v), an organic aprotic solvent important because of its use in the preparation of active-site analogues of ferredoxins (see Holm & Ibers, 1977), caused 50% inhibition when the enzyme was assayed in its presence. Again, the inhibition by dimethyl sulphoxide was reversible by dilution.

Table 3. *Inhibition of E. coli hydrogenase*

The hydrogenase (0.5μg/ml) was incubated in a shaking water-bath at 30°C for 15min with the inhibitor (0.1, 1.0 or 10.0mM) in 20mM-potassium phosphate buffer, pH7.0. Methyl Viologen and sodium dithionite were then added and the residual activity was determined by H_2 evolution as described in the Materials and Methods section. The results are average values for three separate experiments (error ± 5%).

Inhibitor	Residual activity (%)		
[Inhibitor] ...	0.1mM	1.0mM	10.0mM
N-Bromosuccinimide	86	5	0
Sodium arsenate	100	100	85
Potassium iodide	100	100	90
Bathophenanthroline	92	87	48
Potassium ferricyanide	100	100	72
Copper(II) sulphate	94	68	0
Phenylmethanesulphonyl fluoride	100	94	63
p-Chloromercuribenzoate	100	84	—
N-Ethylmaleimide	100	100	77
Sodium mersalyl	100	91	52

Activities of physiologically related enzymes

The aerobically grown *E. coli* cells, used as the source of hydrogenase, were assayed for the presence of physiologically related enzymes. The results, given in Table 4, show that formate hydrogenlyase and formate dehydrogenase 'H' (Benzyl Viologen-dependent) activities were not detectable in these cells. Also, there was no H_2 evolution with formate as the electron source when Methyl Viologen (2.5mM) was also present (as electron carrier to the hydrogenase). However, cell extracts would oxidize formate with O_2, Methylene Blue or phenazine methosulphate/2,6-dichlorophenol-indophenol as the electron acceptors. Fujita & Sato (1966) reported that cytochrome c_{552} is located in the periplasmic space and is quantitatively released into the surrounding medium when cells are converted into spheroplasts by lysozyme/EDTA treatment. We were unable to detect this cytochrome by difference-spectra (dithionite-reduced minus ferricyanide-oxidized) analysis of the spheroplast medium of the aerobically grown cells.

Table 4. *Enzyme activities in E. coli cells*

Activities were determined by using a crude cell extract as described in the Materials and Methods section. The results are expressed as μmol of formate oxidized/min per mg of cell protein and are the average values from three determinations.

Enzyme		Activity
Formate oxidase		0.008
Formate dehydrogenase	'H' (Benzyl Viologen)	0
	'N' (Methylene Blue)	0.140
	'N' (Phenazine methosulphate/2,6-dichlorophenol-indophenol)	0.064
Formate hydrogenlyase		0
Cytochrome c_{552}*		0

* This could not be detected (see the text).

Discussion

Aerobically grown *E. coli* cells have been reported to contain between 0 and 8% of the hydrogenase activity found in anaerobically grown cells, as determined by the H_2-uptake assay (Pichinoty, 1962; Cole & Wimpenny, 1966; Gray *et al.*, 1966). Owing to the different assay conditions used, a comparison with our results is difficult. However, from the results of Yamamoto & Ishimoto (1978), the H_2-evolution activities (determined with Methyl Viologen as the electron carrier) of cell extracts of *E. coli* grown anaerobically on glucose or fumarate plus H_2 were about 300 and 50% respectively of the activity determined by us with the extracts of aerobically grown cells.

The absence of formate hydrogenlyase activity in our cells would arise from the lack of the formate dehydrogenase 'H' and/or lack of intermediate electron carriers to the hydrogenase. The inability of formate to serve as an electron source for H_2 evolution in the presence of Methyl Viologen (as an artificial electron carrier) confirms that formate dehydrogenase 'H' is absent (see Gest & Peck, 1955). A deficiency of formate dehydrogenase 'H' in aerated cultures has been reported, although the activity of formate dehydrogenase 'N' increases under aerobic growth conditions (Peck & Gest, 1957; Gray *et al.*, 1966; Ruiz-Herrera & Alvarez, 1972). We found that the activities of this enzyme were similar to those given by Ruiz-Herrera & Alvarez (1972). Thus in these aerobically grown cells, formate oxidation would proceed via formate dehydrogenase 'N' and the aerobic respiratory chain with O_2 as the terminal electron acceptor.

Barrett & Sinclair (1967) found high concentrations of cytochrome c_{552} in aerobically grown *E. coli* cells, but in anaerobic cultures the cytochrome was not detectable. In contrast, and in accordance with our results, other workers have found cytochrome c_{552} synthesis to be repressed by O_2 such that it is almost or completely absent from aerobically grown cells (Wimpenny *et al.*, 1963; Gray *et al.*, 1966; Cole & Wimpenny, 1966; Ruiz-Herrera & Alvarez, 1972). The physiological role of this cytochrome is in doubt, since Cole & Wimpenny (1968) suggest that it functions in the nitrite reductase complex, and Ruiz-Herrera & Alvarez (1972) report that cytochromes are not involved in H_2 formation from formate. Similarly, Douglas *et al.* (1974) concluded that cytochrome c_{552} is not required for formate hydrogenlyase activity.

We were unable to find, in crude or purified extracts, a physiological electron carrier that would mediate electron transfer to the hydrogenase from dithionite or reduced nicotinamide or flavin nucleotides. Thus either such an electron carrier is not present in the cells, or it is labile and destroyed during the preparation of the extract. Although both ferredoxin (2Fe) and flavodoxin are present in aerobically grown *E. coli* cells (Vetter & Knappe, 1971; Knoell & Knappe, 1974), neither flavodoxin, ferredoxin (both 2Fe and 8Fe) nor active-site analogues (4Fe) of ferredoxins were able to interact with the hydrogenase. Interestingly, cytochrome c_3 of *Desulfovibrio*, a low-potential cytochrome analogous to cytochrome c_{552} of *E. coli*, would donate electrons to the purified hydrogenase from dithionite. Of the *c*-type cytochromes so far isolated from *E. coli* cells (see Fujita, 1966), only cytochrome c_{552} has a sufficiently low redox potential (E_0 −200mV) to donate electrons to hydrogenase. However, this problem has still to be resolved, and an as yet unidentified electron carrier for the hydrogenase is a distinct possibility.

In view of the absence of the formate hydrogenlyase pathway and physiological electron carriers in the aerobically grown cells which we used, it would seem that the hydrogenase, although present with a significant activity within the cell, has no apparent function. Presumably, within the intact membrane, the hydrogenase is not inactivated by the aerobic conditions.

We decided to study the effect of potential regulators on hydrogenase activity. Nitrate, an important regulator of the enzymes that function in formate oxidation, causes a decrease in the hydrogenase activity of anaerobically grown *E. coli* cells (Wimpenny & Cole, 1967; Cole & Wimpenny, 1968). Ruiz-Herrera & Alvarez (1972) reported that nitrate repressed the synthesis of the formate hydrogenlyase complex, whereas formate increased the activity of this multienzyme system. They also found that azide and cyanide completely inhibited formate hydrogenlyase activity. We found that these compounds neither stimulated nor inhibited the activity of the purified hydrogenase and so, presumably, the above observations were due to regulatory processes (nitrate and formate) or to the inhibition of other components of the formate hydrogenlyase system (cyanide and azide). Other electron donors for nitrate reduction (lactate, pyruvate and NADH), metabolites of nitrate (hydroxylamine, ammonia) and ATP also had no effect on the activity of the purified hydrogenase. The ability of *E. coli* to grow on malate/H_2 or fumarate/H_2 (Macy *et al.*, 1976) prompted the testing of these substrates on hydrogenase activity. However, malate, fumarate and succinate had no effect. We thus conclude that the activity of the hydrogenase, as isolated, is not subject to direct regulation by the concentrations of intermediary metabolites.

Yamamoto & Ishimoto (1978) measured the hydrogenase activity (both H_2 evolution and H_2 uptake) of *E. coli* cell extracts grown under different conditions (fumarate plus H_2 or glucose). They concluded, after analysis of the activities by polyacrylamide-gel electrophoresis, that multiple forms of hydrogenase (up to six types) were present and that

the relative amounts of each form depended on the growth conditions. In spite of the heterogeneous staining of hydrogenase activity on polyacrylamide gels, our results suggest that only one form of hydrogenase is present in the aerobic cells (or that other forms are present in amounts below detection) and that the anomalous behaviour we observed during electrophoresis probably arises from the association of the hydrogenase with other cell components. The hydrogenase-activity band we obtained (R_F 0.27 in 10%, w/v, acrylamide) was also present in all the preparations of Yamamoto & Ishimoto (1978). It should be mentioned here that Ackrell *et al.* (1966) reported a multiplicity of hydrogenase-activity bands after gel electrophoresis of *E. coli* cell extracts (cells grown both aerobically and semi-aerobically), although similar results that they obtained with *Clostridium pasteurianum* hydrogenase were later shown to be artifacts (Nakos & Mortenson, 1971). However, we must await the purification of the hydrogenase(s) of anaerobically grown *E. coli* before any conclusions can be made.

Results concerning the properties of purified hydrogenase from the Enterobacteriaceae have not been previously reported, so we decided to compare the *E. coli* enzyme with other purified hydrogenases. The enzymes of the strict anaerobes *D. vulgaris* (van der Westen *et al.*, 1978), *D. gigas* (Hatchikian *et al.*, 1978) and *C. pasteurianum* (Chen *et al.*, 1976) all contain 12 iron and 12 acid-labile-sulphide atoms per molecule, but are of lower molecular weight (50000, 89500 and 60500 respectively) than *E. coli* hydrogenase (113000), which also differs from the other enzymes in that it consists of a dimer of two equal subunits. *C. pasteurianum* hydrogenase is particularly O_2-sensitive (Nakos & Mortenson, 1971), much more so than *E. coli* hydrogenase, although the *D. vulgaris* enzyme is relatively O_2-insensitive, e.g. purification under aerobic conditions was possible. The specific activity of *E. coli* hydrogenase in the H_2-evolution assay is similar to that found with *D. gigas* hydrogenase, but the maximal activity (*V*, Methyl Viologen as electron carrier) is only about 2% of that observed with the *D. vulgaris* and *C. pasteurianum* enzymes. We were unable to identify a physiological electron carrier for *E. coli* hydrogenase. In contrast, the hydrogenases of *Desulfovibrio* are specific to cytochrome c_3 and *C. pasteurianum* hydrogenase to ferredoxin. However, the hydrogenases of the photosynthetic bacteria *Chromatium*, *Thiocapsa* and *Rhodospirillum* (Gitlitz & Krasna, 1975; Gogotov *et al.*, 1976; Adams & Hall, 1979) as yet lack a defined physiological electron carrier. The latter enzymes all contain four iron and four acid-labile-sulphur atoms per molecule, are relatively insensitive to O_2 and show much lower H_2-evolution activities compared with the other purified hydrogenases detailed above. The hydrogenase of the facultative anaerobe *E. coli* thus has more in common with the enzymes of the strict anaerobes rather than those of the photosynthetic bacteria. To date, the only hydrogenase purified from an aerobic bacterium is the enzyme from *Alcaligenes eutrophus* (Schneider & Schlegel, 1976; Schneider *et al.*, 1979). This hydrogenase is so far unique in that it possesses a bound flavin group, will reduce NAD^+ directly with H_2 and contains 2Fe–2S active centres.

The purification procedure reported here for *E. coli* hydrogenase gives a good yield of the pure enzyme (10%) in view of its cellular location within a membrane and presumed association with electron-transport-chain components. The availability of the pure enzyme will be of great significance to researchers attempting to elucidate the role of this hydrogenase and the associated electron-transport pathways in both aerobically and anaerobically grown *E. coli* cells.

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