orbitals of the terminal atoms. This suggests that the wave function of the unpaired electron has a node at the central atom and belongs to either the Σ_u^+ representation of $D_{\infty h}$ (linear species) or the B_2 representation of C_{2v} (nonlinear species). The occupation of such a nonbonding (or weakly antibonding) MO is predicted for three electrons placed in a simple Hückel MO scheme derived from linear combinations of three s atomic orbitals. The small *s* spin density at the central nucleus must then be due to spin polarization effects and is presumably negative.

The isotropic *g* shift of −0.04 for Ag_3 is surprisingly large, and it is difficult to reconcile such a shift with the established lack of silver hyperfine anisotropy in the spectrum. The sign of the *g* shift is consistent with matrix contributions to the spin–orbit interaction, as suggested for Na_3,[12] but the magnitude of the shift seems to be excessive for such a mechanism. It appears more likely to us that the *g* shifts in these triatomic clusters are due to intramolecular spin–orbit interactions. Because of the small anisotropic one-electron parameters expected for $^{107}Ag(5p)$,[11] considerable spin density present in such contributing atomic orbitals in Ag_3 would not give rise to resolved anisotropic hyperfine structure. It is possible, in principle, that the silver atoms contribute as much as 10% 5p character to the SOMO of Ag_3. Spin–orbit interaction between the ground state and very low-lying excited states having considerable Ag(5p) character would then give rise to negative *g* shifts.

Our analysis of the spectrum of Ag_3 in terms of an orthorhombic *g* tensor having all three principal values appreciably less than the free-spin value suggests that Ag_3 is probably bent, with a $^2B_2$ (C_v) ground state. A linear $^2\Sigma_u^+$ species would have an axial **g** tensor with $g_\parallel$ quite close to 2.0023. This conclusion does of course conflict with a recent laser Raman spectroscopic study of small silver clusters in Kr, from which it was concluded that Ag_3 is linear.[14]

In conclusion, we might add that all the $Ag–C_6D_6$ species formed in this experiment have not yet been positively identified, but it does appear that a $Ag_3\cdots C_6D_6$ pseudocomplex is formed.

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Enzyme-Catalyzed Organic Synthesis: NAD(P)H Regeneration Using Dihydrogen and the Hydrogenase from *Methanobacterium thermoautotrophicum*

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This paper describes several practical systems for the in situ regeneration of NAD(P)H from NAD(P), using dihydrogen as the ultimate reducing agent, in reactions catalyzed by the hydrogenase (H_2ase, EC 1.12.1.2) from *Methanobacterium thermoautotrophicum* (Scheme I). The development of simple and economical methods for regenerating the reduced nicotinamide cofactors represents an important intermediate step in the adaptation of enzymic catalysis to problems in practical organic synthesis.[1-5] Dihydrogen has the advantages as a reactant that it is inexpensive and a strong reducing agent and that its consumption leaves no byproducts. Previous hydrogenase-catalyzed reductions have been carried out on a small scale and have not provided the information concerning the stability and ease of manipulation of the enzymes involved that is required to judge the usefulness of these schemes for organic synthetic applications.[6-8]

Scheme I

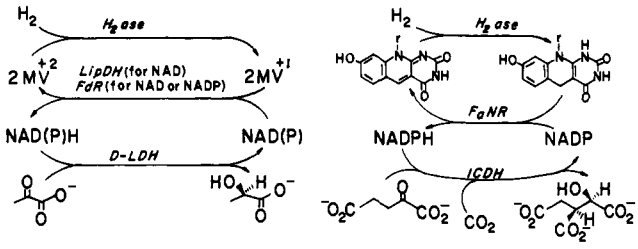

Table I. Synthesis of D-Lactate and Isocitric Acid

enzyme or cofactor	MV; lactate TN[a]	MV; lactate recovery, %	F_0; isocitrate TN[a]	F_0; isocitrate recovery, %
H_2ase	1.5×10^7[b]	78	6×10^5	76
LipDH	6×10^5	35[c]		
D-LDH	2×10^7	81		
F_0NR			1.5×10^7[b]	62
ICDH			3×10^5	78
NAD(P)(H)	1700[d]	68	1000[b]	40

[a] TN ≡ moles of product isolated per mole of enzyme or cofactor. [b] These turnover numbers are calculated by assuming the crude protein mixture used contained ~10% each of H_2ase and F_0NR by weight. [c] Calculated on the basis of the *total* LipDH added. [d] These numbers are calculated based on *isolated* product.

We have explored two redox cycles based on H_2ase. In one, H_2ase catalyzes the reduction of MV^{2+} to MV^+ (MV = methyl viologen), and MV^+ is used to reduce NAD(P) to NAD(P)H in reactions catalyzed by the flavoenzymes lipoamide dehydrogenase (LipDH, EC 1.6.4.3) or ferredoxin reductase (FdR, EC 1.6.99.4).[7-9] In the second, H_2ase catalyzes the reduction of cofactor F_0 to F_0H_2, and this soluble flavin analogue is used to reduce NADP to NADPH in a reaction catalyzed by F_0-NADP reductase (F_0NR, EC not assigned).[10,11] In the first cycle, FdR can accept either NAD or NADP as substrate; LipDH is specific for NAD.

The H_2ase and F_0NR required are present in quantity in the same preparation and are used in crude form. *M. thermoautotrophicum* was grown as described previously[12] and harvested, and the cells were broken in a French press (4-g wet cells, 0 °C, in 25 mL of 50 mM Tris, pH 7.5, 19000 psi). The resulting suspension was centrifuged at 14000*g* for 25 min and the supernatant passed through a DEAE column (2.2 × 3.5 cm). The resulting crude mixture of proteins (3.3 mg of protein[12] per mL of Tris buffer, ~38 mL) was immobilized in PAN gel[14] (20 g of polymer)

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(12) *M. thermoautotrophicum* was grown following the procedure of: Balch, W. E.; Wolfe, R. S. *Appl. Environ. Microbiol.* **1976**, *32*, 781-791. A 25-L fermentation generated ~90 g of cells; this cell mass yielded ~15000 U of H_2ase and ~2000 U of F_0NR. Details of this fermentation are outlined in supplementary material to this article.
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(14) Pollak, A.; Blumenfeld, H.; Wax, M.; Baughn, R. L.; Whitesides, G. M. *J. Am. Chem. Soc.* **1980**, *102*, 6324-6336: 1 g of PAN-1000 per 0.5-1 mL of enzyme solution was used in MV-mediated reactions; 30 mM MV^{2+} was present during immobilization to protect the H_2ase active site; for F_0-mediated reactions, the immobilizations were carried out in the presence of NADPH (1 mM) and FAD (5 mM).

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without further purification. The activity measured for H_2ase depended on the assay method used[15] but was approximately 5-6 U mg^{-1} (1 U = 1 μmol of F_0 or 2 μmol of MV^{2+} reduced/min) before immobilization; the immobilization yield was ~40% and the activity of the resulting swollen gel ~3-10 U mL^{-1} for H_2ase and ~1 U mL^{-1} for F_0NR. The gel-immobilized H_2ase (suspended in 50 mM Tris, pH 7.5) showed no loss in activity over 2 weeks when stored under H_2 at 25 °C in the presence of 2 mM MV and 2-mercaptoethanol or under Ar at 5 °C.

A preparation of D-lactate illustrates the operation of the MV-mediated redox cycle. In a 2-L, three-necked, round-bottomed flask was placed 1 L of solution containing pyruvate (400 mmol), NAD (0.2 mmol), MV^{2+} (2 mmol), DTT (3 mmol), and 300 U each of immobilized H_2ase (40 mL of gel, assay based on 2 mM MV^{2+}, pH 8.0), LipDH (8 mL of gel), and D-LDH (0.5 mL of gel).[16] The suspension was deoxygenated with a stream of Ar for 30 min and evacuated to 0.01 torr (*boiling*). Dihydrogen (1.2 atm) was introduced and the pH controlled at 7.8 using a pH-stat by adding oxygen-free 2 N KOH solution. An additional 500 U of LipDH was added every 2 days.[17] The reaction was completed in 12 days. The suspension was flushed with Ar to remove H_2, the gel was allowed to settle, and the solution was decanted and treated with 20 g of activated charcoal to remove MV and NAD(H). D-Lactate was isolated from this solution as its zinc salt (340 mmol of D-lactate, 10 mmol of L-lactate, 85% yield, 94% ee) as described previously.[5] Turnover numbers (TN) and quantities of enzymes recovered are summarized in Table I.

The operation of the redox system mediated by F_0 is illustrated by a preparation of *threo*-D_s(+)-isocitrate. In the same apparatus was placed 1 L of deoxygenated solution (0.1 M Tris, pH 7.8) containing α-ketoglutarate and $NaHCO_3$ (200 mmol each), $MnCl_2$ (5 mmol), DTT (3 mmol), NADP (0.1 mmol), and F_0 (0.08 mmol). PAN-immobilized H_2ase (500 U based on F_0, pH 7.5), F_0NR (~100 U),[15] and ICDH (100 U) were added, and the mixture was maintained at pH 7.8 under 1.2 atm of 4:1 mixture of H_2 and CO_2 for 12 days. Unreacted α-ketoglutarate was destroyed by using NH_4Cl (100 mmol) and glutamate dehydrogenase (100 U) and *threo*-D_s(+)-isocitric acid isolated as its barium salt (43 g, 96% purity, 100 mmol, 50% yield) as described previously.[5]

These procedures demonstrate the usefulness of the H_2ase from *M. thermoautotrophicum* as the basis for catalytic procedures for reducing nicotinamide cofactors by H_2 in situ. The thermodynamics of the overall reactions strongly favor reduction (for NAD + H_2 → NADH + H^+, $\Delta E_0' = 0.1$ V, $\Delta G_0' = -4.6$ kcal/mol, $K_{eq}' = 2400$, pH 7.0, 1 atm of H_2).[18] This H_2ase has a high specific activity;[9] it (and F_0NR if required) can be obtained in large quantities from a nonpathogenic organism using a simple isolation and can be used in crude form; it is stable and is not irreversibly inactivated by O_2; it accepts as substrates a number of cofactors and redox dyes (F_0, MV^{2+}, benzyl viologen, diquat, FAD, FMN, others) and can thus be utilized in a variety of ways. In addition, the other coupling enzymes required are either commercially available or readily prepared. The disadvantages of these systems are that the *M. thermoautotrophicum* fermentation is not trivial, yeast LipDH is unstable under the reaction conditions, H_2ase, FdR, F_0NR, and F_0 must be prepared, F_0NR is specific for NADP, and LipDH is specific for NAD.

In summary, this work demonstrates the practicality of organic synthetic procedures based on NAD(P)H-requiring enzymes, in which H_2/H_2ase is the ultimate reducing agent. The H_2ase used here seems the most attractive presently available for H_2 activation.[19] Of the two configurations tested for NAD → NADH, the most practical seems to be H_2/H_2ase/MV^{n+}/LipDH/NAD, although the problem of the instability of LipDH remains to be solved. Evaluation of the merits of this system for reduced nicotinamide cofactor regeneration, relative to others presently available or being developed (formate/formate dehydrogenase,[3,4] glucose 6-phosphate/G-6-P dehydrogenase,[5] various electrochemical procedures[2,20]) will almost certainly vary with the characteristics of the contemplated synthesis, and especially with its scale. H_2ase-based systems are of greatest interest in large-scale work, where the cost of the reagents is critical. In laboratory-scale work, where convenience is more important, the most attractive procedures are (in our experience) those based on glucose-6-PDH or formate dehydrogenase; the procedure described here is too complex for small-scale work.

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Supplementary Material Available: Details of the *M. thermoautotrophicum* fermentation (2 pages). Ordering information is given on any masthead page.

(15) Activities and assay conditions (32 °C, 1 atm H_2): 68.2 U mg^{-1} (pH 9.0, 2 mM MV^{2+}), 5.5 U mg^{-1} (pH 8.0, 0.1 mM MV^{2+}, and 6.0 U mg^{-1} (pH 7.5, 50 μM F_0). Reactions were followed by using the absorbance of the coupling agents: ε (F_0, at the isobectic point at 400 nm, pH 7.5) = 25 mM^{-1} cm^{-1}; ε [NAD(P)H, 340 nm] = 6.22 mM^{-1} cm^{-1}; ε (MV^+, 560 nm, pH 8.0) = 8.0 mM^{-1} cm^{-1}.

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Augmented and Diminished Spherands and Scales of Binding[1]

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We report the syntheses of three new spherands, the crystal structures of lithium salt complexes of two highly strained, bridged spherands, and a comparison of the binding abilities toward Li^+ and Na^+ of spherands **1-5** (Chart I), standard cryptands and crowns, and an open-chain model compound.

Treatment of 2,6-bis(3-bromo-2-hydroxy-5-methylphenyl)-4-methylanisole[2] with $(TsOCH_2CH_2)_2O$-KOH[3] gave **6**[4,5] (74%).

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