The first and second peaks in the Fourier transform of the iron edge EXAFS (Figure 1) fit unequivocally to sulfur and iron (Table I). Unfortunately, the coordination number derived from this and other EXAFS analyses are quite sensitive to the Debye–Waller factor, which we cannot determine very precisely (Table I). The Fe–Fe coordination number may be less accurate than the Fe–S number since apparent differences in the relevant Debye–Waller factors between the enzyme and model are larger in the former case. We conclude that there are iron–sulfur clusters in this hydrogenase with Fe–S and Fe–Fe distances nearly identical with those found in well-characterized iron–sulfur proteins and model compounds.

The Fourier transform of the nickel EXAFS (Figure 2a) contains one major peak, which was isolated by Fourier filtering. The amplitude and phase functions of the peak could be fit to sulfur (Figure 2b) but not to nitrogen (or oxygen). An apparently reasonable fit to nitrogen could be constructed only by distorting the nitrogen phase function by setting ΔE_0 to 30; this behavior was also observed in attempts to fit Ni(toluene-3,4-dithiolato)$_2^{2-}$ to nitrogen. Thus we conclude that sulfur is the *dominant* scatterer in the nickel EXAFS in F_{420}-reducing hydrogenase. The Debye–Waller factors of the model and protein are again apparently somewhat different, diminishing our confidence in the accuracy of the Ni–S coordination number.

The distance of 2.19 Å obtained for Ni(toluene-3,4-dithiolato)$_2^{2-}$ is as would be expected for a square-planar Ni(II) complex[15] and is close to the average distance of 2.168 Å found in the equivalent square-planar cobalt species.[16] An estimate of ca. 1.3 Å for the covalent radius of low-spin Ni(III) was obtained from the crystal structure of $Ni^{III}[C_6H_3(CH_2NMe_2)_2\text{-}1,5]I_2$,[17] while a value of ca. 1.2 Å has been calculated from $Ni^{III}Br_3(PPhMe_2)_2$.[17,18] Using 1.04 Å as a sulfur covalent radius,[19] the distance of 2.25 Å observed in the enzyme is within the expected region.

The positions of the EPR *g* values in the enzyme suggest the nickel is low-spin Ni(III)[2] with the unpaired electron in an orbital of d_{z^2} symmetry[20–22] and it has been suggested on this basis that the nickel in a hydrogenase in crude membrane fractions from *Methanobacterium bryantii* is a rhombically distorted octahedron.[20] Similarly, square-pyramidal geometry is also possible. The crystal structure and EPR of the low-spin square-pyramidal nickel(III) complex $Ni^{III}[C_6H_3(CH_2NMe_2)_2\text{-}1,5]I_2$[17] provides solid evidence for this possibility.

In summary, the iron EXAFS indicates the presence of iron–sulfur clusters. The dominant scatterers in the nickel EXAFS comprised approximately three sulfur atoms at ca. 2.25 Å. Our inability to fit a nitrogen or oxygen scatterer at a distance of around 2.0 Å to the EXAFS suggests that none are present, though a minor contribution may have gone undetected. In addition, the distant nitrogen observed by electron spin echo spectroscopy[5] was not detected. As a working hypothesis, we suggest the nickel in F_{420}-reducing hydrogenase has equatorial sulfurs with either one or two loosely held axial ligands in roughly a tetragonally distorted octahedron or square pyramid. Further work aimed at identifing other ligand atoms is in progress.

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Nickel Coordination in Deazaflavin and Viologen-Reducing Hydrogenases from *Methanobacterium thermoautotrophicum*: Investigation by Electron Spin Echo Spectroscopy

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Two hydrogenases that differ in their in vitro activities[1] have recently been purified from *Methanobacterium thermoautotrophicum* (strain ΔH). In vitro, one hydrogenase (FH_2ase) catalyzes the reduction of the physiological two-electron acceptor 7,8-didemethyl-8-hydroxy-5-deazaflavin (F_{420}) by hydrogen, whereas the other has only been observed to reduce the artificial one-electron acceptor methylviologen. Both enzymes, on isolation, contain nickel and iron sulfur clusters and show an essentially superimposable CW EPR spectrum for the nickel paramagnet at 77 K.[1] The FH_2ase has 1 equiv of bound FAD, which seems to be absent from the viologen-reducing enzyme (MVH_2ase). Using electron spin echo spectroscopy, we demonstrate here that the environments of the nickel paramagnet in these two enzymes are in fact different.

The presence of a tightly bound paramagnetic nickel center in both hydrogenases has been confirmed by the hyperfine coupling in the CW EPR powder spectra of the ^{61}Ni enriched enzymes.[2] The powder spectra of the hydrogenases with natural abundance $^{58,60}Ni$ appear almost identical, but the features are broad enough that small hyperfine splittings are not resolved.[2] However, these splittings can be measured and the identification of nuclei that are more loosely coupled to the electron spin can be made by the technique of electron spin echo envelope modulation (ESEEM).[3]

ESEEM measurements do show a clear difference between the two hydrogenases: the F_{420}-reducing hydrogenase is found to have a nitrogen (^{14}N, $I = 1$) nucleus near the paramagnetic nickel center whereas this interaction is absent in the methylviologen-reducing hydrogenase.

It has been observed in three-pulse ESEEM experiments (Figure 1)[4] in solids that as the time between the second and third pulses (T) is increased, there is a decrease in the echo intensity due to electron spin relaxation processes. Often superimposed on this decay is a periodic modulation of echo intensity, due to nuclei coupled to the electron spin. The depth and frequencies of components of this modulation convey information as to the identity of nearby nuclei, their number, and the distance separating them from the electron spin. Consequently, a combination of EPR, ENDOR, and ESEEM studies probe directly the immediate magnetic environment of a paramagnet. Mims has shown how the echo modulation amplitude depends on nuclear spin eigenvalues and eigenvectors.[5]

The three-pulse ESEEM patterns for the F_{420}-reducing hydrogenase and the methylviologen-reducing hydrogenase are shown in Figure 1. The difference between the patterns can be explained by the presence of a nitrogen-14 nucleus near the paramagnetic center in FH_2ase. The Fourier transform of the time-domain pattern of FH_2ase has three sharp lines at 0.4, 1.2, and 1.6 MHz

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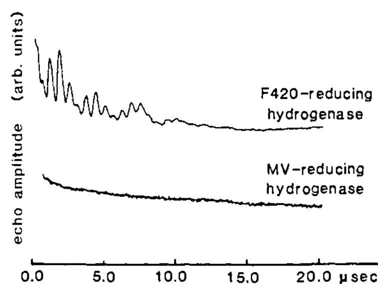

Figure 1. Hydrogenases were prepared as in ref 1. Concentrated enzyme (0.6 mM in Ni) was used in either 50 mM Tris at pH 7.5 or 50 mM phosphate buffer at pH 7.0 at room temperature. All ESEEM experiments were carried out at 10 K and at 9.0 GHz using the three-pulse or stimulated-echo sequence ($\pi/2 - \tau - \pi/2 - T - \pi/2 - \tau$...echo). T is always varied from 0 to 20 μs. τ was incremented in 30-ns steps. These experiments were repeated for different magnetic field values, from 2750 to 3220 G in 10-G steps. All spectra were added to obtain a composite spectrum such that a spherical average, performed in the computer simulation, is the appropriate comparison.

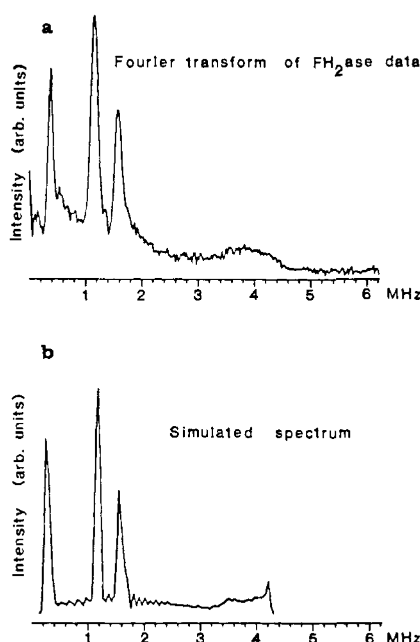

Figure 2. Parameters used in the simulations: Zeeman term $\gamma_N H_0 = 0.9$ MHz; quadrupolar term

$$\frac{eqQ_{zz}}{4h} = 0.42 \text{ MHz} \qquad \frac{Q_{xx} - Q_{yy}}{Q_{zz}} = 0.48$$

Euler angles connecting the quadrupolar and dipolar tensors are $\alpha = 18°$, $\beta = 2°$, and $\gamma = 0°$; isotropic hyperfine $|a| = 1.8$ MHz, Ni–N distance $R \geq 3.0$.

and a broad line at about 4 MHz (Figure 2a). Varying the static field leaves the FH_2ase modulation frequencies nearly unchanged, indicating that the sharp lines lie near the zero-field nuclear quadrupolar splittings, so that the relative orientation of the enzyme with respect to H_0 is not important. This is possible if the nuclear Zeeman splitting and the electron–nuclear splitting are similar over the width of the powder pattern and cancel each other out for one of the electron spin states. For the opposite electron spin state the Zeeman and hyperfine energies add, and the net field is approximately twice the applied field. The large anisotropy of these interactions and the electron–nuclear coupling explains the broad line at 4 MHz.[6]

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Table I. Summary of Hyperfine and Zero-Field Nuclear Quadrupolar Couplings for Nitrogen

compound (assignment)	isotropic hyperfine $\|a\|$, MHz	obsd zero-field quadrupolar splitting, MHz	ref
propylamine heme (propylamine)	2.0	0.45, 1.75, 2.2	6c
imidazole heme mercaptoethanol (imidazole N-3)	2.0	0.4, 1.7, 2.1	6c
myoglobin nitroxide (imidazole N-3)	2.5	0.35, 1.15, 1.5	6c
imidazole heme-NO (imidazole N-3)	1.8	0.7, 1.4, 4.0	6c
cytochrome P_{450} (?)	1.8	0.7, 1.5, 4.1	6c
Cu^{2+}(imidazole)$_4$	1.75	0.75, 1.5, 4.0	6a, 7
cysteine		0.4, 0.7, 1.1	7
L-serine		0.8, 0.9	7
L-asparagine		0.3, 0.8, 1.1	7
thioacetamide		0.4, 1.2, 1.6	7
FH_2ase	1.8	0.4, 1.2, 1.6	this work

The hyperfine tensor components A_x, A_y, and A_z are

$$A_x = a - \frac{1}{2}\frac{\gamma_N\gamma_e h}{r^3} \qquad A_y = a - \frac{1}{2}\frac{\gamma_N\gamma_e h}{r^3} \qquad A_z = a + \frac{\gamma_N\gamma_e h}{r^3}$$

where $|a|$ is the isotropic Fermi contact term. The electron–nucleus distance can be estimated by using the point dipole approximation and fitting the anisotropic term to the spectrum. The experimental data cannot be matched by simulations using a large anisotropic term ($r \sim 1.9$ Å): these simulations show distortions in the lines as well as the appearance of additional maxima. The data can be fit rather well with simulations employing an *isotropic* hyperfine term of 1.8 MHz (Figure 2b). The lines are sharper for the simulated spectrum when compared to the experimental one (Figure 2a). The broadening requires that there be *some* contribution from the anisotropic term; however, the isotropic hyperfine term is much greater than the dipolar contribution (anisotropic term). A total hyperfine interaction of about 1.8 MHz (the anisotropic term being small on this scale) makes it unlikely that the nitrogen observed in the ESEEM is directly coordinated to the nickel. From ENDOR studies of directly coordinated nitrogen, the hyperfine splittings have been shown to range from 5 to 100 MHz.[7] In ESEEM measurements porphyrin nitrogens show hyperfine splitting of 5 MHz.[6c]

Two derived quantities, the hyperfine coupling constant and the quadrupolar splitting, are to be examined for structural consequences: the total hyperfine coupling of greater than 1.8 MHz is large enough to suggest that there is a significant "overlap" between the electronic orbitals. (In contrast, the isotropic hyperfine coupling for protons in hydrated Cu^{2+} ions is 0.2 MHz.)[3b] We point out that there can be significant electron density at the nucleus if the nitrogen is, for example, part of an aromatic heterocyclic ring coordinated to the nickel or if the nitrogen we detect is strongly hydrogen bonded to a ligand of the nickel. This size nitrogen-14 hyperfine coupling constant is consistent with the magnitude of couplings obtained by Mims for the far, unliganded nitrogen in coordinated imidazole, in a series of metal–imidazole complexes as well as in proteins. Table I shows a summary of the coupling constants observed for various compounds. *Several* nitrogen-containing potential ligands are present in these hydrogenases. The F_{420} enzyme contains stoichiometric amounts of the nitrogenous coenzyme FAD while the viologen-reducing hydrogenase does not.[2] It is possible, therefore that the observed spectrum is due to nitrogen-14 hyperfine from FAD (e.g., N-1, N-10) coordinated to the nickel, perhaps related to the N-5,C-4

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biscoordination proposed for Cd(II) flavin complexes by Hemmerich et al.[8]

The quadrupolar splittings needed to simulate the FH_2ase modulation curve do not correspond well to the pure nuclear quadrupolar resonance splitting for histidine or imidazole or backbone nitrogens; however, Ashby et al.[9] have shown that these energy levels are sensitive to changes in environment. Table I shows some of the quadrupolar transitions obtained in other systems. Recent EXAFS data[11] suggest multiple sulfur coordination to nickel in this enzyme.

The ESEEM study described here thus suggests a nitrogen nucleus from the protein or possibly the FAD coenzyme in the FH_2ase is close to the nickel paramagnet. Taken with the EXAFS data, these results begin to define the likely ligands to the nickel. The striking distinction between the FH_2ase and MVH_2ase data indicates the discriminating power of the ESEEM approach.

Acknowledgment. We thank the National Institutes of Health, USPHS, for support of this project under GM31574, and we are grateful to Michael K. Bowman and John S. Waugh for many helpful discussions of the analysis of electron–nuclear interactions.

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Additions and Corrections

Electrocatalytic Oxidation of Carbon Monoxide in a CO/O_2 Fuel Cell [*J. Am. Chem. Soc.* **1983**, *105*, 7456]. JIANXIN WU and CLIFFORD P. KUBIAK*

Page 7457, second paragraph: The rate constant $k^h = 1.86 \times 10^2$ cm s^{-1} should be $k^h = 1.86 \times 10^{-2}$ cm s^{-1}.

Self-Diffusion of Water at the Protein Surface: A Measurement [*J. Am. Chem. Soc.* **1984**, *106*, 428–9]. C. F. POLNASZEK and R. G. BRYANT*

Page 429, lines 24–28: The sentence should read as follows—The neglect of these effects can be shown to result in a calculated diffusion constant that is somewhat *larger* than the correct value and a calculated distance between the centers of the interacting particles that is *smaller* than the correct value.[8]

Book Reviews *

Atmospheric Pollution, Its History, Origins and Prevention. 4th Edition. By A. R. Meetham, D. W. Bottom, S. Cayton, A. Henderson-Sellers, and D. Chambers. Pergamon Press, Oxford and New York. 1981. xi + 232 pp. $15.00.

This book covers a wide range of topics on air pollution, including the sources, measurement, effects, and control of pollution. It also provides a brief discussion on the air pollution laws administered in the Western European countries and the United States of America. While the initial chapters of the book appear remotely related to the atmospheric pollution, it is not until one reads past Chapter 10 that the usefulness of the book can be realized. While I trust the authors had some good purpose to scope the book which includes a lengthy discussion on the sources of pollution such as fuels, furnaces, and other industrial equipment, this discussion could have been brief without losing the purpose served. For example, the engineering drawings of the archaic boilers, such as Cochran and Lankashire types, occupy so many pages; the book could have been just as useful without them.

The chapter of measurement of air pollution is succinct, clear, and reflects the start-of-the-art, though it is limited to two pollutants, namely the particulates (smoke) and sulfur dioxide which are of major concern in England. In fact, the whole book places much emphasis on these two pollutants, only having a brief reference to others. The discussion on atmospheric dilution processes and their effect on spacial variability of pollution is given in simple terms requiring little special training for the reader to understand the subject. The discussion on the British approach of using tall chimneys as effective means of reducing ground-level concentrations of pollution is skillfully handled which leaves a novice to air pollution with the impression that the solution to pollution could be just as simple. The effect of using tall chimneys at large distances away from the sources is not explained well. The long-range transport of pollutants and their effect in acid participation is a major concern in North America and Scandinavian Countries where dilution with the use of tall chimneys is not considered as a solution to pollution. As such, the air pollution law in the United States limits the maximum height of the chimneys that industry can use while the British regulate the minimum height of the chimneys which places no limitation on how tall they can be.

All this and the discussion on air pollution laws and their administration in several countries provides a good start for the reader in learning about air pollution, its sources, effects, and control methods.

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*Unsigned book reviews are by the Book Review Editor.

Survey of Contemporary Toxicology. Volume I. Edited by Anthony T. Tu (Colorado State University). John Wiley and Sons, New York, NY. 1980. ix + 357 pp. $39.50.

It is difficult to review a book that is one volume in a series designed to survey the field of contemporary toxicology when the editor does not give a list of the topics that will be covered in other volumes. The reader is forced to place Volume I in a perspective about which he is uncertain.

The real strength of this book is the comprehensive manner in which it treats the toxicology of substances in foods or that have food related origins. Indeed six of the seven chapters (Chapters 2–7) deal with this