Table I. Assays of Partially Inhibited Urease

Residual specific enzymatic activity[a] (%)	Protein concentration (mg/ml)	Radioactivity (nCi/ml)	[Protein-bound inhibitor]/[protein][b] (μmol/g)
Urease I, [^{14}C]Acetohydroxamic Acid			
1.27[c]	2.35_1	0.365_7	8.78_8
18.16[d]	0.97_6	0.126_4	7.31_7
33.76[e]	1.02_7	0.106_6	5.86_4
93.5[f]	2.35	$<0.001_3$	<0.03
Urease II, Ammonium [^{32}P]Phosphoramidate			
5.07[c]	0.93_4	1.41_3	8.53_3
36.19[d]	0.41_4	0.427	5.81_7
51.83[g]	0.43_0	0.327	4.28_9
99.2[h]	0.65_5	<0.006	<0.05

[a] Expressed as a percentage of the original specific enzymatic activity. [b] [Protein-bound inhibitor] in units of μmol/ml; [protein] in units of g/ml. [c] Maximally inhibited enzyme. [d] Reactivated for 5 min at 38°. [e] Reactivated for 15 min at 38°. [f] Maximally inhibited enzyme, reactivated for 2 hr at 38° and dialyzed at 4° in the phosphate buffer (four cycles). [g] Reactivated for 10 min at 38°. [h] Maximally inhibited enzyme, dialyzed for 18 hr at 38° against the *N*-ethylmorpholine buffer, and then against the same buffer at 4° (6 × 250 vol).

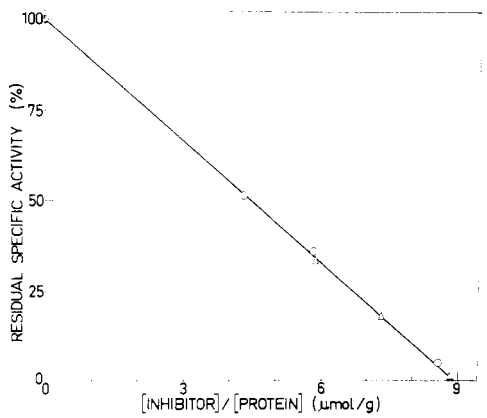

Figure 1. Correlation of residual specific enzymatic activity with incorporation of radioactively labeled inhibitor into urease: [inhibitor] bound to protein, in units of μmol/ml; [protein] in units of g/ml; ○, [^{32}P]phosphoramidate; △, [^{14}C]acetohydroxamic acid.

to yield an equivalent weight of 111,800 daltons. The maximum specific activity of urease that we have observed repeatedly is 90,000 (μkat/l.)/A_{280}, and therefore correction of the measured value gives a best estimate of 105,000 ± 1000 daltons for the equivalent weight.[18] This equivalent weight implies a molecular weight of 420,000 or 525,000 for the commonly observed species of urease (483,000),[19] and we have therefore begun a reinvestigation of the molecular weight and subunit structure of this enzyme. It should be noted, however, that our measurements are all based on $A_{1cm}^{1\%}$ = 5.89 at 280 nm for the pure enzyme,[16,17] a result which is complicated by our discovery that the enzyme is a metalloenzyme and contains 2 ± 0.3 g-atom of nickel/105,000 g of protein.[7] If a pure sample of the apoenzyme of appropriate size can be produced,[20] some refinement (possibly as much as 5%) of this equivalent weight may be possible.

The presence of two nickel atoms per 105,000 daltons poses such problems as: what is the ultimate subunit structure of the enzyme; are the inhibitors "seeing" only one of the nickel atoms; are the environments of the nickel atoms different? Answers to these questions must await further work.

Finally, it should be noted that other metalloenzymes also form complexes with hydroxamic acids[21] and hydroxyurea.[22] The present work indicates that phosphoramidate may also be a useful reversible probe of the sites of metalloenzymes.

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Jack Bean Urease (EC 3.5.1.5). A Metalloenzyme. A Simple Biological Role for Nickel?

Sir:

In 1926, Sumner isolated from jack beans *(Canavalia ensiformis)* the first crystalline enzyme, urease, and defined the proposition that enzymes could be proteins devoid of organic coenzymes and metal ions.[1] It is therefore with some sadness that in this communication we adduce evidence which strongly indicates that urease is a nickel metalloenzyme.

The availability of highly purified urease in large quantities[2,3] has allowed us to determine its absorption spectrum at high concentrations. The native enzyme exhibits electronic and/or vibrational transitions in the region 320–1150 nm, part of which are shown in Figure 1 (A, A′). This spectrum, together with the inhibition of the enzyme by hydroxamic acids and phosphoramidate,[6] led us to reexamine the

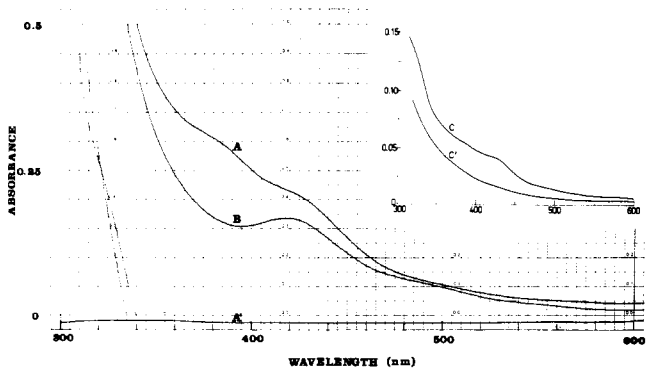

Figure 1. Absorption spectra at 25°: (A) urease at 43.3 mg/ml in O_2-free 0.029 *M* phosphate buffer, pH 7.0 (1 m*M* each in EDTA and β-mercaptoethanol); (A′) buffer base-line; (B) urease at 42.2 mg/ml, 10 min after the solution in A was made 10 m*M* in acetohydroxamic acid. (Similar spectra of hydroxamate-inhibited urease are obtained in buffers containing 0 and 5 m*M* β-mercaptoethanol.) Note that the spectra continue on the 0.5–1.0 absorbance range at low wavelength. Inset: (C) spectrum (taken between 1 and 12 min after acidification, and corrected for base-line variation) of urease at 11.9 mg/ml in O_2-free 0.1 *M* acetate, pH 3.8 (1 m*M* in β-mercaptoethanol); (C′) spectrum of urease (corrected for base-line variation) at 11.6 mg/ml, 2.0 hr after solution in C was made 1 m*M* in EDTA. The enzyme retained 6.1% of its original activity (assayed at pH 3.5).

metal ion content. We found 2.0 ± 0.3 g-atom of nickel[7,9] per 105,000 g of enzyme.[6]

The absorption spectrum of native urease (in the presence of 1 m*M* β-mercaptoethanol), which has shoulders at ~316, ~376, and ~425 nm, and broad maxima at 725 and 1060 nm, is similar to those of Ni(II) phosphoglucomutase (410, 760, and 1300 nm)[10] and Ni(II) carboxypeptidase A (412, 685, and 1060 nm).[11] We find that the spectrum of urease is reversibly modified by β-mercaptoethanol,[12] and inhibition by acetohydroxamic acid[6,13] produces marked spectral changes (Figure 1; B, A′).[14] It is relevant that large spectral changes also occur when Ni(II) carboxypeptidase A, in which Ni^{2+} is relatively weakly bound, is inhibited by β-phenylpropionate.[11]

Gorin reported that urease was rapidly dissociated from an 18*S* species to an ~10*S* species at pH 3.5 and 25°, with only slow loss of activity, and that EDTA in the assay solution was inhibitory.[15] Building on these observations, we found that EDTA increased approximately threefold the rate of inactivation of urease at pH 3.8 and 0°.[16] To comment on whether or not the metal ion in urease was essential for activity, we measured the specific activity and metal content of urease which had been denatured for limited periods at pH ~3.7 in the presence of EDTA.[17] The residual specific activity was a linear function of the nickel content of the partially denatured enzyme within the limits of accuracy of atomic absorption spectrometry.

The effect of low pH and EDTA on the spectrum of urease is also shown in Figure 1 (C, C′). An appreciable absorbance change occurs on the time scale of loss of activity, consistent with the loss of a metal ion under these conditions.[18]

Thus far we have been unable to remove the nickel ion and to replace it with reconstitution of enzymatic activity.[19] None the less, the weight of evidence indicates that jack bean urease is a metalloenzyme, and the fact that sword bean urease[20] and certain bacterial ureases[21] are also inhibited by hydroxamic acids suggests that nickel may not be artifactual (i.e., replacing another accepted "biological" metal ion) in jack bean urease.[22] Urease, therefore, appears to be the first example of a nickel metalloenzyme, and nickel may well be an essential trace element in jack beans. Heretofore, nickel was the only element between vanadium and zinc in the fourth row of the periodic table which had not been recognized as essential, or as having some biological function.[23] Yet the discovery of a serum globulin which appears to bind nickel very tightly,[24] together with documentation of alternations of serum nickel concentrations in certain diseases,[25] suggests that nickel may have a biological role in animals. There now exists a precedent that such role may be as part of a metalloenzyme system. This enzyme and related systems continue under active investigation in this laboratory.[26]

Acknowledgments. We are indebted to Professor Bert Vallee for the initial metal ion analysis and to the Australian Research Grants Committee for support.

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(18) Activity was measured by pH-stat at pH 3.5 and 38°, under which conditions the native enzyme shows 36% of the activity at pH 7.0 and 38° (*cf.* ref 15). We have not shown calculated molar absorption coefficients for the transitions associated with the metal ion because they are complicated by Rayleigh scattering at both pH's, the dissociation of urease to a smaller species at pH 3.5, as well as our inability to produce at this stage a clearly defined apoenzyme under mild conditions.
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A Model for the Carbonyl Adduct of Ferrous Cytochrome P_{450}

Sir:

Cytochrome P_{450}[1] is the name given to the active site in a widespread class of hemoproteins which activate molecular oxygen during the hydroxylation of C–H bonds in metabolism, hormone regulation, and drug detoxification. This cytochrome derives its name from the unusual 450-nm Soret band exhibited by its ferrous carbonyl derivative. The position of this 450-nm peak is different from all other known iron(II) porphyrin carbonyl derivatives. Recent spectral studies of model ferric porphyrin complexes clearly indicate that both the resting, low-spin and substrate-bonded high-spin ferric forms of P_{450} have in common an axial *thiolate* ($R-S^-$) ligand.[2] In the interim, Stern and Peisach[3] reported that the combination of a ferrous heme, CO, excess thiol, and excess KOH in DMSO–EtOH[4] produces a 450-nm absorbance characteristic of ferrous–carbonyl P_{450} along with another species present in ~50% abundance. Their work implies that thiolate is required to produce the characteristic 450 peak.

We have found conditions which afford the 450-nm peak in ~100% abundance. Addition of a benzene solution of $\overset{\oplus}{Na}SCH_3$ solubilized by dibenzo-18-crown-6 macrocyclic ether to Fe(TpivPP)[4,5] or Fe(PPIXDEE) followed by exposure of this solution to CO results in a Soret band at 449 nm (Figure 1). This absorbance is quite sensitive to the polarity of the solvent and of the porphyrin. For example, in DMSO the Soret band for both TpivPP and PPIXDEE is found at 462 nm,[6] and the combination Fe(TPP), CO, and $\overset{\oplus}{Na}SCH_3$ in benzene exhibits the corresponding band several nanometers lower.[7] The species giving rise to the 449 absorption is extremely sensitive to O_2 which discharges the 449 peak. Undoubtedly because of this air sensitivity at low porphyrin concentrations (5×10^{-6} *M*), a 50–100-fold excess of $\overset{\oplus}{Na}SCH_3$ is required to produce the 449 chromophore;[8] whereas at higher porphyrin concentration (10^{-3} *M*) a one- or twofold excess of $\overset{\oplus}{Na}SCH_3$ is sufficient to afford >95% of the 449 peak. It is significant that with *mercaptan* rather than *mercaptide* as the axial base the Soret band for the ferrous carbonyl TpivPP or PPIXDEE derivative is found at 422 nm[6] (Table I).

The other characteristic feature of the absorption spectrum of cytochrome P_{450} is the broad featureless band centered at ~550 nm. Unlike other hemoproteins, the α and β bands of the porphyrin ligand are not clearly separated. The extinction coefficients for both the visible and the Soret band compare very well with those of the natural enzyme, at least in the case of Fe(PPIXDEE).[9]

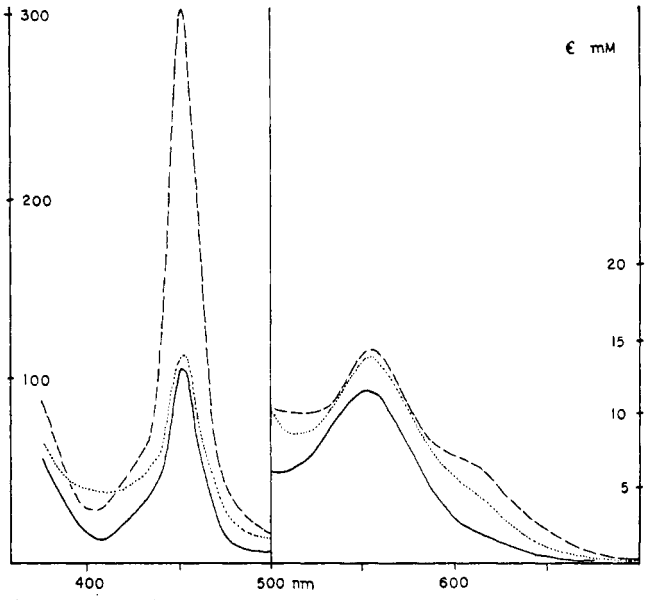

Figure 1. Plots of wavelength (nm) vs. millimolar extinction coefficient for: (—) reduced P_{450} cam + CO at 5° in 50 m*M* potassium phosphate buffer (ref 1), (- - -) Fe(TpivPP) and $\overset{\oplus}{Na}SCH_3$ + CO in benzene at 25°, (···) Fe(PPIXDEE) and $\overset{\oplus}{Na}SCH_3$ + CO in benzene at 25°.

Table I. Spectral Data for Fe(TpivPP) + B + CO

B	ν_{CO}[a,b]	λ_{max}[a,c]
N-MeIm[f]	1964 (1965)	427[e]
THF[f]	1961[d] (1955)	417[d,e]
THT[f]	1970 (1972)	428
n-C_3H_7SH	1970	422
$\overset{\oplus}{Na}SCH_3$	1945 1902[g]	449

[a] Benzene solution; numbers in parentheses refer to spectra obtained in KBr pellets. [b] In cm^{-1}. [c] In nm. [d] THF as solvent. [e] Reference 5. [f] These complexes have been isolated and fully characterized. [g] Spectrum with ^{13}CO.

The prior spectral studies of ferric porphyrins[2] and the present work reproducing the P_{450} ferrous Soret strongly imply that an axial *mercaptide* ligand is present throughout the P_{450} catalytic cycle—encompassing two ferric and two ferrous stages.[1,2a]

The ν_{CO} infrared bands for the carbonyl mercaptide and complexes having other axial bases are listed in Table I. Although the differences are not large, the ν_{CO} value for the complex having an axial mercaptide ligand is the lowest ν_{CO} frequency for a ferrous carbonyl porphyrin of which we are aware. Since ν_{CO} for the enzyme is unknown, comparison cannot be made with the natural system. However, determination of this frequency does set a target for studies with the P_{450} ferrous carbonyl complex. Comparison of ν_{CO} values in Table I supports the intuitively reasonable hypothesis that mercaptan is a better π-acid ligand than mercaptide (which may, in fact, be a π-donor[10]). It seems likely that the mercaptide axial base may have an important electronic effect necessary to activate dioxygen for the subsequent hydroxylation event. This point must remain a speculation until a model dioxygen complex has been prepared and characterized.