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_{250}, 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 re-investigation of the molecular weight and subunit structure of this enzyme. It should be noted, however, that our measurements are all based on A_{250} = 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\(^{22}\) and hydroxylurea.\(^{22}\) The present work indicates that phosphoramidate may also be a useful reversible probe of the sites of metalloenzymes.

### References and Notes

10. R. C. Sheridan, J. F. McCullough, and Z. T. Wakefield, Inorg. Synth., 13, 23 (1971). The infrared spectrum (KBr) of the pure material agreed completely with that of ammonium phosphoramidate prepared independently.\(^{14}\) However, the compound did not melt below 300°. Sheridan has acknowledged that their reported mp 233-234° is in error (personal communication from R. C. Sheridan to N.E.D., 1974).
17. An equivalent weight of 207,000-317,000 daltons has been reported for sword bean urease inhibited by [3H]caprylohydroxamic acid: K. Kobashi, J. Hase, and T. Komai, Biochem. Biophys. Res. Commun., 33, 34 (1966). In this work, no attempt was made to demonstrate, by then existing reliable chromatographic procedures, the complete separation of the enzyme-inhibitor complex from unbound hydroxamic acid. The authors did not examine the problems of reactivation, and finally their calculations relate to a maximum specific activity for the sword bean enzyme of 126 Sumner units/mg.
19. See footnotes 18 and 19 in ref 7.
23. This work was supported by the Australian Research Grants Committee.

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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
metal ion content. We found 2.0 ± 0.3 g-atom of nickel per 105,000 g of enzyme.

The absorption spectrum of native urease (in the presence of 1 mM 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) and Ni(II) carboxypeptidase A (412, 685, and 1060 nm). We find that the spectrum of urease is reversibly modified by β-mercaptoethanol, and inhibition by hydroxymethylene acid produces marked spectral changes (Figure 1; B, A). It is relevant that large spectral changes also occur when Ni(II) carboxypeptidase A, in which Ni²⁺ is relatively weakly bound, is inhibited by β-phenylpropionate.

Gorin reported that urease was rapidly dissociated from an 18S species to an ~10S species at pH 3.5 and 25°C, with only slow loss of activity, and that EDTA in the assay solution was inhibitory. Building on these observations, we found that EDTA increased approximately threefold the rate of inactivation of urease at pH 3.8 and 0°C. 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. The residual specific activity was a linear function 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.

Thus far we have been unable to remove the nickel ion and to replace it with reconstitution of enzymatic activity. None the less, the weight of evidence indicates that the metal ion in urease is a metalloenzyme, and the fact that sword bean urease and certain bacterial ureases 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. 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. Yet the discovery of a serum globulin which appears to bind nickel very tightly, together with documentation of alternations of serum nickel concentrations in certain diseases, suggests that nickel may have a biological role in animals. There now exists a precedent that such a role may be as part of a metalloenzyme system. This enzyme and related systems continue under active investigation in this laboratory.

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

References and Notes

3. Sumner reported a maximum specific activity of 133 units per milligram of crystalline urease. Our highest specific activity is 5400 (U/mg)/A₅₈₀, which is equivalent to 182 units/mg in Sumner's terms.
7. Analyses carried out by atomic absorption spectrometry through the courtesy of the Queensland Government Analyst, and totally independently by the Department of Mining and Metallurgical Engineering at the University of Queensland. At the same time, analyses for Ca, Mn, and Co were negative, as was chemical analysis for Fe. The molar ratio of Fe/Ni in urease is <0.02 as determined by atomic absorption spectrometry; clearly the presence of any adventitious iron would be of significance in a spectral examination of the reversible binding of hydroxamic acid to urease. The nickel analysis has been confirmed independently using dimethylglyoxime.
9. A preliminary analysis in 1969 on our then purest sample of urease, dihydrated into O₂-free distilled water and then lyophilized, gave as the only metals present in significant amount, Ca > Al > Ni > Fe > Zn. Since Ca and Al are common contaminants of many proteins, since the amounts showed no simple relationship to Sumner's molecular weight (483,000 daltons), and since the analysis for Ni was low, we erroneously concluded that urease was not a metalloenzyme. The properties of urease have not changed during the period of our investigations nor, indeed, have they altered appreciably since Sumner's time.
12. The calculated difference spectra of urease (0.02 M phosphate, pH 7.0, 1 mM EDTA) with varying [β-mercaptoethanol] vs. urease shows saturation effects with absorption maxima at ~363 and ~412 nm and a shoulder at ~316 nm. A value of ~1.4 × 10⁻⁵ M is inferred from these spectra for the dissociation constant of the β-mercaptoethanol-urease complex. In the absence of β-mercaptoethanol, the spectrum of the native enzyme does not show shoulders at ~316 and ~376 nm.
14. The spectral change and inhibition by hydroxymethylene acid are fully reversible. The spectral change occurs on a time scale consistent with the relative slow rate of inhibition by urease of hydroxymethylene acid.
16. EDTA produces its effect both in the presence and absence of β-mercaptoethanol, and regardless of whether residual activity is assayed at pH 7.0 or pH 3.5. However, the detailed role of EDTA is not yet fully understood.
17. After dialysis into O₂-free distilled water, urease at 4–7 mg/ml was equilibrated with 0.1 M acetate buffer (either 5 mM in β-mercaptoethanol and 1 mM EDTA or 5 mM in EDTA alone) at pH 3.7 and 0°C. The pH was adjusted to ph ~7 by addition of a calculated amount of Na₂CO₃. After centrifugation and dialysis against O₂-free 0.05 M Na₂HPO₄-NaCl solution, the samples were assayed for enzymatic activity, for protein, for nickel, and other metal ion content.
18. Activity was measured by pH-stat at pH 3.5 and 38°C, under which conditions the native enzyme shows 38% of the activity at pH 7.0 and 38°C (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 3.5 and 38°C.
19. The Chicken liver mitochondrial pyruvate carboxylase from which the manganese ion has not yet been reversibly removed: M. C. Scrutton, M. F. Utter, and A. S. Midyan, J. Biol. Chem., 241, 3480 (1966).
A Model for the Carbonyl Adduct of Ferrous Cytochrome P450

Sir:

Cytochrome P450 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(III) porphyrin carbonyl derivatives. Recent spectral studies of model ferric porphyrin complexes clearly indicate that both the resting, low-spin and substrate-bound high-spin ferric forms of P450 have in common an axial thiolate (R-S-) ligand. In the interim, Stein and Peisach reported that both the resting, low-spin and substrate-bonded high-spin ferric forms of P450 have in common an axial thiolate (R-S-) ligand.5 In the interim, Stein and Peisach5 reported that the combination of a ferrous heme, CO, excess thiol, and excess KOH in DMSO-EtOH produces a 450-nm absorption characteristic of ferrous-carbonyl P450 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 NaSCH3 solubilized by dibenzo-18-crown-6 macrocyclic ether to Fe(TppPP) 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 TppPP and PPIXDEE is found at 462 nm, and the combination Fe(TPP), CO, and NaSCH3 in benzene exhibits the corresponding band several nanometers lower.6 The species giving rise to the 449 absorption is extremely sensitive to O2 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 NaSCH3 is required to produce the 449 chromophore, whereas at higher porphyrin concentration (10^{-3} M) a one- or two-fold excess of NaSCH3 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 TppPP or PPIXDEE derivative is found at 422 nm (Table 1).

The other characteristic feature of the absorption spectrum of cytochrome P450 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).7

The prior spectral studies of ferric porphyrins8 and the present work reproducing the P450 ferrous Soret strongly imply that an axial mercaptide ligand is present throughout the P450 catalytic cycle—encompassing two ferric and two ferrous stages.9

The pCO infrared bands for the carbonyl mercaptide and complexes having other axial bases are listed in Table 1. Although the differences are not large, the pCO value for the complex having an axial mercaptide ligand is the lowest pCO frequency for a ferrous carbonyl porphyrin of which we are aware. Since pCO 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 P450 ferrous carbonyl complex. Comparison of pCO values in Table 1 supports the intuitively reasonable hypothesis that mercaptan is a better σ-acid ligand than mercaptide (which may, in fact, be a σ-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.

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Table 1. Spectral Data for Fe(TppPP) + B + CO

<table>
<thead>
<tr>
<th>B</th>
<th>( \lambda_{CO}^{a,b} )</th>
<th>( \lambda_{max}^{a,c} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Mel/m</td>
<td>1964</td>
<td>427^e</td>
</tr>
<tr>
<td>THF/f</td>
<td>1965</td>
<td>417^d,e</td>
</tr>
<tr>
<td>n-C3H7SH</td>
<td>1970</td>
<td>428</td>
</tr>
<tr>
<td>NaSCH3</td>
<td>1945</td>
<td>422</td>
</tr>
<tr>
<td></td>
<td>1902^a</td>
<td>449</td>
</tr>
</tbody>
</table>

^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 pCO.

Communications to the Editor