Jack bean urease (EC 3.5.1.5). II. The relationship between nickel, enzymatic activity, and the "abnormal" ultraviolet spectrum. The nickel content of jack beans[1]

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At low pH, EDTA promotes the loss of the tightly bound nickel ions from jack bean urease. The specific activity of soluble enzyme after partial EDTA-promoted inactivation is a linear function of the nickel content. The results are consistent with the presence of 2.0 nickel ions per 97 000-dalton subunit in pure urease. The time scale for loss of enzymatic activity and nickel under these conditions is similar to that for loss of the "abnormal" tail absorption in the ultraviolet and visible absorption spectrum of urease (including the shoulder at ~420 nm). This indicates that nickel in urease is essential for enzymatic activity and establishes that the metal ions are in part responsible for the tail absorption in the ultraviolet spectrum of urease. After partial inactivation in the presence of EDTA either at low pH or in 2.5 *M* guanidinium chloride at neutral pH, urease did not regain activity in the presence of Ni^{2+}. As yet apourease has not been produced reversibly. Jack bean seeds grown hydroponically without added nickel were low in both urease activity and nickel (10 and 6%, respectively, of parent seeds). Several other metal ions were readily available. This result suggests that metal ions other than nickel cannot substitute for nickel in the formation of normally active urease.

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A faible pH, l'EDTA favorise la perte des ions nickel étroitement liés à l'uréase de la fève Jack. L'activité spécifique de l'enzyme soluble après inactivation partielle produite par l'EDTA est une fonction linéaire de la teneur en nickel. Les résultats confirment la présence de 2.0 ions nickel par sous-unités de 97 000 daltons dans l'uréase pure. Dans ces conditions la perte de l'activité enzymatique et du nickel en fonction du temps est semblable à celle de la perte de l'absorption "anormale" (*tail absorption*) dans le spectre ultraviolet et visible de l'uréase (incluant l'épaulement à ~420 nm). Ceci indique que le nickel de l'uréase est essentiel à l'activité enzymatique et montre que les ions métalliques sont partiellement responsable de l'absorption anormale (*tail absorption*) dans le spectre ultraviolet de l'uréase. Après inactivation partielle en présence d'EDTA, soit à faible pH soit dans le chlorure de guanidinium 2.5 *M* à pH neutre, l'uréase ne recouvre pas son activité en présence de Ni^{2+}. Jusqu'à présent, l'apouréase n'a pas été produite de façon réversible. Les graines de fève Jack croissant dans des conditions hydroponiques, sans addition de nickel, ont une activité uréasique faible (10% des graines parentes) et contiennent peu de nickel (6% des graines parentes). Plusieurs autres ions métalliques sont facilement disponibles. Ce résultat suggère que les ions métalliques autres que le nickel ne peuvent se substituer au nickel dans la formation de l'uréase normalement active.

[Traduit par le journal]

ABBREVIATIONS: EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; kat, katal; A_{280}, etc., absorbance at 280 nm, etc., using a cuvet of 1-cm path length.

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Introduction

The discovery of stoichiometric amounts of tightly bound nickel in jack bean urease (1, 2) raises many interlocking questions regarding the role of nickel in the growth and development of the jack bean and in the structure and mechanism of action of urease. In this paper, we describe the effect of EDTA at low pH in promoting the irreversible loss of both enzymatic activity and nickel, accompanied by alterations in the ultraviolet and visible absorption spectra of the enzyme. Some attempts at reversible removal of nickel from urease are described, and the metal ion and urease contents are reported for jack beans grown in a medium low in nickel. Results of these experiments establish firmly that urease, as it finds itself in the jack bean, is a nickel metalloenzyme.

Materials and methods

Materials

Distilled water was deionized immediately before use to give a conductivity of $<2 \times 10^{-6}\ \Omega^{-1}\ cm^{-1}$. For preparation of solutions free of oxygen or carbon dioxide (saturated with nitrogen), this water was boiled for ~20 min, then cooled and manipulated under nitrogen. All glassware was cleaned as previously described (3). All plastic or rubber material was cleaned with detergent, boiled in the presence of EDTA, and then boiled in water. Dialysis tubing was washed thoroughly (internally and externally) with distilled water and buffers containing EDTA and β-mercaptoethanol. *N*-Ethylmorpholine (bp 137.5–138°C) was distilled from KOH and collected under N_2 and stored at 4°C in the dark. Buffers were otherwise prepared from reagent grade components. All measurements of pH were made at 25°C using a Radiometer PHM4c pH meter fitted with combination electrode GK2401C and standardized to three decimal places according to Bates (4).

Urease was extracted and crystallized from six 500-g portions of defatted jack bean meal exactly as described (3). The yellow-green urease crystals were dialyzed (normally to give a specific activity of 68 to 70 (mkat/L)/A_{280}) and applied to recycling gel chromatography (3) on Sephadex G-200 (three cycles) in the presence of 5 m*M* β-mercaptoethanol. The specific activity after this step was normally 85 to a maximum of 93 (mkat/L)/A_{280} (cf. Ref. 5). Some preparations have been further chromatographed on DEAE-cellulose.[6] After this step, concentration by ultrafiltration and dialysis into a storage buffer (0.02 *M* phosphate buffer, pH 7.14, 1 m*M* in EDTA and 20 m*M* in β-mercaptoethanol), the specific activity of freshly prepared urease was normally 86 to a maximum of 93 (mkat/L)/A_{280} (6).

Urease was diluted into a neutral buffer (usually 5 m*M* in β-mercaptoethanol) prior to assay at pH 7.0 and 38°C with a pH-stat exactly as described (3), unless otherwise specified. Spectra were recorded using a Cary 14 or 17 spectrophotometer at 25.0 ± 0.1°C. Specific activity is expressed in terms of (mkat/L)/A_{280} (7), where A_{280} refers to the net absorbance after correction as described elsewhere (footnote 6 and Refs. 3, 6, 7). Concentrations of urease (protein) were evaluated spectrophotometrically at pH 7 (3, 7) using 6.20 as the value of the net $A^{1\%}_{1\,cm}$ at 280 nm (6).[6] The concentration of active sites (normality, *N*) is based on an equivalent weight (5, 6) of 96 600 for enzyme of maximal specific activity (93 (mkat/L)/A_{280}).[7] The specific activity of highly purified samples of urease always fell slowly over several months to 60–80 (mkat/L)/A_{280}.

In one instance, the normal purification procedure (3) was modified to include 1 m*M* dimethylglyoxime in the initial extraction of commercial jack bean meal and 1 m*M* dimethylglyoxime plus 10% (v/v) ethanol in all subsequent steps. After gel chromatography on Sephadex G-200, the enzyme was dialyzed into oxygen-free distilled water prior to analysis.

Inactivation of urease by EDTA at low pH

Stock urease in oxygen-free 5 m*M* HEPES buffer, pH 7.00, had a specific activity of 81.1 (mkat/L)/A_{280}. The effect of EDTA on the rate of inactivation of urease at $\sim 3 \times 10^{-7}\ N$ was studied in oxygen-free 0.1 *M* acetate buffers at 0°C. Samples were periodically assayed using a pH-stat (3) with 50 m*M* urea at pH 3.50 and 38°C. Since urease is slowly inactivated during the assays, initial gradients were used (6).

For preparative-scale experiments, stock urease was prepared by exhaustive dialysis at 4°C against oxygen-free 0.05 *M* *N*-ethylmorpholine buffer (pH 7.1, 1 m*M* in β-mercaptoethanol) and then against oxygen-free distilled water. Samples of stock urease were treated at 0°C with aliquots of concentrated acetate buffer. Inactivation was halted by the addition of sufficient *N*-ethylmorpholine to produce a pH of ~7. The solutions were immediately dialyzed at 4°C against the *N*-ethylmorpholine buffer and then clarified by centrifugation (6). Samples for metal ion analysis (atomic absorption) were dialyzed against oxygen-free distilled water.

To investigate the possibility that EDTA-inactivated enzyme may have been capable of reactivation, aliquots were diluted (to a concentration of 0.12 μ*M* with respect to the 97 000-dalton subunit) into oxygen-free 0.05 *M* *N*-ethylmorpholine–HCl buffer, pH 7.16 (5 m*M* in β-mercaptoethanol). To each sample was added a small volume of $NiCl_2$ solution, and enzymatic activity was measured (3) periodically during equilibration at 4°C. A similar experiment was carried out at 0°C in 0.1 *M* acetate buffer, pH 3.50, in the presence of 7×10^{-5} *M* β-mercaptoethanol.

A sample of stock urease in oxygen-free distilled water was treated at 25°C with an aliquot of concentrated acetate buffer in the presence of 1 m*M* β-mercaptoethanol and an absorption spectrum was recorded. At 21 min after acidification, EDTA was added. The absorbance at 385 nm was monitored until a second spectrum was recorded at 100 min after addition of EDTA. In independent experiments, the rate of loss of enzymatic activity was measured under conditions identical to those of the spectral study. Periodically, aliquots were diluted into the acetate buffer containing no EDTA and assayed immediately at pH 3.50 and 38°C as described above (6).

Inactivation of urease by guanidinium chloride and by urea

Native urease at 4.8 mg/mL was dialyzed against oxygen-free 0.1 *M* Tris–HCl buffer (10 m*M* in EDTA, 2.5 *M* in

[6] Hinds, J. A., Gazzola, C., Dixon, N. E., Fihelly, A. K., Winzor, D. J., Blakeley, R. L. & Zerner, B., manuscript submitted.

[7] Dixon, N. E., Gazzola, C., Blakeley, R. L. & Zerner, B., manuscript submitted.

guanidinium chloride (Schwarz-Mann, Ultrapure), pH 7.6) at 4°C (two changes, 24 h) and then against a similar buffer containing no EDTA (four changes, 16 h). An aliquot was dry ashed and assayed spectrophotometrically for nickel (2). After dilution into 0.1 *M* Tris–HCl buffer, pH 7.6, containing 10^{-5} *M* Ni^{2+}, or 10^{-4} *M* Ni^{2+}, or 10^{-3} *M* Ni^{2+} plus ~15 m*M* β-mercaptoethanol at 0°C, followed by periodic assay (3) over 18 h, no reactivation was detected. On dialysis at 4°C against 0.1 *M* Tris–HCl buffer, pH 7.6, containing 10^{-5} *M* Ni^{2+}, or 10^{-4} *M* Ni^{2+}, or 10^{-4} *M* Ni^{2+} plus 1 m*M* β-mercaptoethanol (two changes, 24 h), a heavy precipitate of protein formed in each case. After further dialysis into the same buffer containing 1 m*M* EDTA and 5 m*M* β-mercaptoethanol, the turbid samples had no enzymatic activity.

Urease (18 μg/mL) was equilibrated at 38°C with oxygen-free 2.0 *M* guanidinium chloride in 0.1 *M* Tris–HCl buffer (pH 7.60, 12 μ*M* in β-mercaptoethanol and either 2.5 μ*M* or 10 m*M* in EDTA). Aliquots were assayed by the normal procedure (3) except that 2.0 *M* guanidinium chloride was present in the assay system. For native urease, the rate of uptake of acid under these conditions was 37% of normal; the enzyme appeared to be slowly inactivated during the assay.

Urease (4 mg/mL) was dialyzed at 25°C against two changes (24 h) of 0.05 *M* Tris–HCl buffer, 10 m*M* in EDTA and initially 9.0 *M* in recrystallized urea, and then into 0.10 *M* Tris–acetic acid buffer, pH 7.4 (first with 10^{-5} *M* Ni^{2+} for 48 h, then with 1 m*M* EDTA and 5 m*M* β-mercaptoethanol for 24 h). Because of the urease-catalyzed hydrolysis of urea, the pH of the buffer containing urea rose rapidly to pH ~9. The remaining soluble protein was assayed.

Jack bean seeds

Jack bean seeds were germinated on moist paper towels and grown to maturity in Hoagland's No. 1 solution (8) which had been modified by reducing the levels of phosphate and manganese to 500 and 25 μ*M*, respectively, and substituting Sequestrene 138 (Geigy Chemical Co., New York, NY) for ferric tartrate as the iron source. Molybdate was added at 1.8 μ*M*. Sodium silicate was added to the solution as a precaution against the possible development of manganese toxicity (9). The plants were supplied with combined nitrogen (15 m*M* nitrate), but no cobalt was added to the nutrient solution. Analytical grade reagents and deionized water were used. At all stages, plant growth and seed development were apparently normal.

Four washed beans from each source were transferred to two 500-mL conical flasks. To each vessel were added 5 mL of redistilled 70.5% nitric acid and 1 mL of 30% hydrogen peroxide. The vessels were heated at ~100°C on a hot plate and further similar quantities of these reagents were added after ~1 h. After the initial reaction had subsided (~3 h), 40 mL of nitric acid was added to each flask and the temperature was increased over 8 h to ~160°C. More nitric acid (50 mL) was added and evaporated over 24 h at ~160°C. The residues were dry ashed at 450°C for ~16 h. Two more cycles of wet ashing with 4- to 5-mL portions of nitric acid at ~160°C followed by dry ashing at 450°C overnight gave a white ash which was solubilized by treatment with 10-mL portions of nitric acid (evaporated off at 160°C) and distilled ~5.8 *M* HCl (evaporated off at 100–120°C). The residues were completely dissolved at ~100°C in ~0.1 *M* HCl containing 10^{-4} *M* EDTA and transferred quantitatively to 10-mL volumetric flasks (6).

For measurements of the total urease activity, beans were crushed between brass plates and chloroform–acetone powders were prepared on a small scale (3). Weighed quantities (5–7 g) of the powder were subjected to four cycles of extraction at 25°C into 25 mL of 0.1 *M* citrate buffer (pH 6.26, 1 m*M* in EDTA and 5 m*M* in β-mercaptoethanol) for 1 h, followed by centrifugation (14 000 × *g*, 4°C, 30 min) and assay (3). The percentages of total extractable urease recovered in each successive buffer extraction were as follows: parent seeds, 71, 21, 6, and 2%; low-nickel daughter seeds, 72, 20, 6, and 2%. A portion of the buffer extract of the low-nickel seeds was made 10^{-4} *M* in Ni^{2+} and equilibrated at 38°C for 14 h, during which time the urease activity did not change significantly in comparison with a control.

Results

EDTA-promoted inactivation of urease at low pH

The loss of activity of urease at 0°C at low pH followed a first-order rate law. At pH values of 3.57 and 3.75, k_{obs} in the presence of 1 m*M* EDTA was 2.1×10^{-3} s^{-1} and 1.0×10^{-3} s^{-1}, respectively, while at pH 4.02 in the presence of 2.4 m*M* EDTA, k_{obs} was 4.4×10^{-4} s^{-1}. Each of these values was about 10 times the corresponding value in the absence of EDTA and, when the EDTA concentration was doubled, k_{obs} was almost doubled. These results establish that EDTA promotes the inactivation of urease at low pH.

When urease was treated with EDTA at low pH on a preparative scale for different periods of time at 0°C, the residual soluble enzyme was deficient in both nickel and specific enzymatic activity, as shown in Fig. 1. The line drawn describes a theoretical relationship between nickel content and urease activity, based on the assumptions that these two properties are linearly correlated and that fully active urease (specific activity,[6] 93 (mkat/L)/A_{280}) contains 2.0 g-at. Ni/96 600 g of protein (2). The presence of β-mercaptoethanol did not prevent the EDTA-promoted loss of activity and nickel at low pH (points *3*, *4*, and *5* in Fig. 1).

Urease, whose residual specific activity after treatment with EDTA at low pH was 18% of the maximum value, displayed, on analytical ultracentrifugation at pH 7.0, two well-resolved symmetrical peaks (Fig. 2). The two peaks have $s_{20,w}$ values of 11.5 and 19.4 S, with areas in the ratio 47:53, respectively. Fully active urease displayed a single peak, $s_{20,w}$ 18.8 S, under the same conditions (Fig. 2).

In an attempt to bring about reactivation, EDTA-inactivated urease (Fig. 1, point *2*) was equilibrated at pH 7.16 and 4°C in an oxygen-free *N*-ethylmorpholine buffer in the presence of Ni^{2+} (0, 0.20, 1.0, or 10 μ*M*). A 10–15% loss of activity occurred over about 25 h, independently of the concentration of Ni^{2+}. In analogous reactivation attempts at pH 3.50, the same enzyme lost ~75% of its residual specific activity over 1 h, independently of the absence or presence (17 μ*M*) of

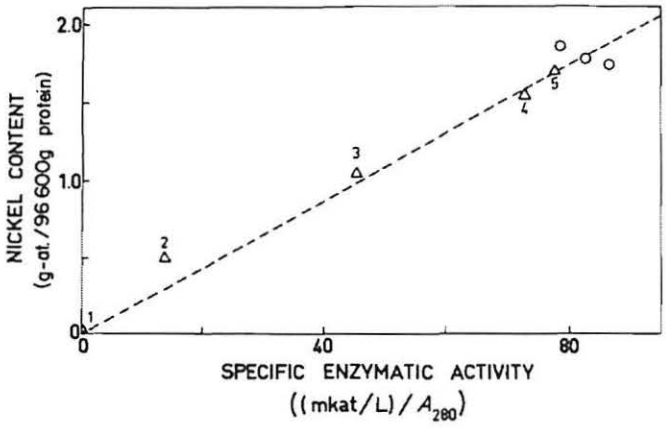

FIG. 1. Correlation of the residual specific enzymatic activity (3) with nickel content for partially inactivated (△) and native (○) urease samples. The line corresponds to 2.0 g-at. of Ni/mol of 96 600-dalton subunits in fully active urease. Prior to neutralization and dialysis, urease was equilibrated in oxygen-free 0.1 *M* acetate buffers under various conditions: (*1*) 11.3 mg/mL, pH 3.8, 1 m*M* EDTA, 1 m*M* β-mercaptoethanol, 2.5 h at 25°C and 20 h at 4°C; (*2*) 4.0 mg/mL, pH 3.75, 5 m*M* EDTA, 17 min, 0°C; (*3*) 6.6 mg/mL, pH 3.7, 1 m*M* EDTA, 5 m*M* β-mercaptoethanol, 20 min, 0°C; (*4*) as in (*3*), but for 5 min; (*5*) as in (*3*), but for 100 s.

Ni^{2+}. No evidence has been obtained for reactivation of EDTA-inactivated urease by low concentrations of Ni^{2+}.

The absorbance of urease at 385 nm (10) started to decrease with time immediately upon acidification to pH 3.8 at 25°C. Upon subsequent addition of EDTA (to a final concentration of 1 m*M*), the rate of decrease in A_{385} was accelerated (data not shown). A graph of A_{385} as a function of time after addition of EDTA is shown in Fig. 3. Progress curves for the decrease in enzymatic activity with and without EDTA under these conditions are also shown in Fig. 3. Again inactivation is promoted by EDTA. The time course for loss of activity cannot be simply correlated with the change in A_{385} in Fig. 3 because nearly half the overall total loss of A_{385} had taken place while the initial spectrum was recorded at low pH prior to addition of EDTA. Further, the tail absorption is partly due to Rayleigh scattering associated with the large size of the urease hexamer (molecular weight, 590 000)[6] and the dissociation to subunits which occurs after acidification (11) must cause an absorbance decrease due to decreased Rayleigh scattering, independently of absorbance changes associated with the nickel ions.

Effects of dimethylglyoxime, guanidinium chloride, and 9 M urea on urease

When urease was prepared by the normal procedure (3), modified to include 1 m*M* dimethylglyoxime (plus 10% ethanol where necessary for solubility) throughout, the yield of urease activity was normal at all stages.

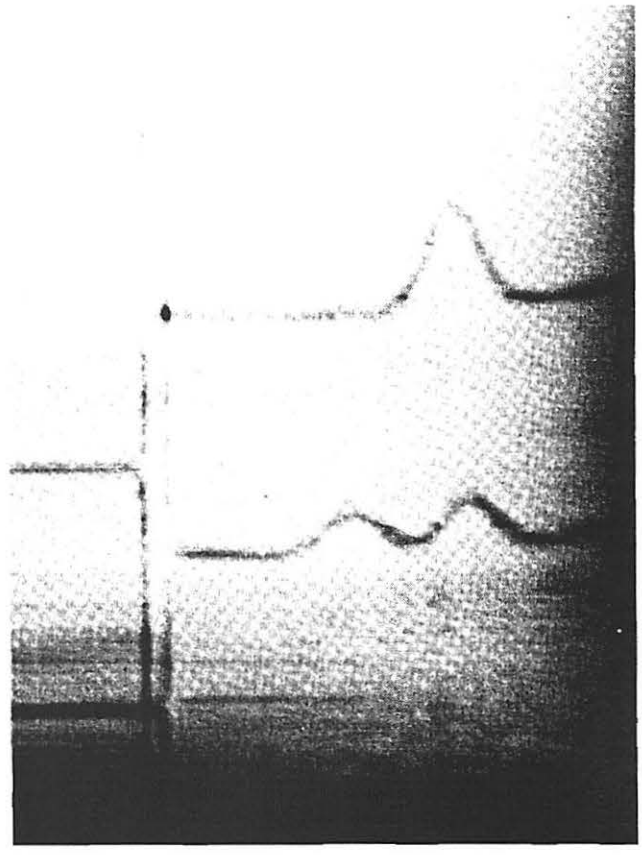

FIG. 2. Sedimentation velocity experiments at 20°C in 0.029 *M* phosphate buffer (pH 7.00, 1 m*M* each in EDTA and β-mercaptoethanol). Above: native urease, 0.87 mg/mL, 92.1 (mkat/L)/A_{280}. Below: EDTA-treated urease, 1.21 mg/mL, 17 (mkat/L)/A_{280}, from Fig. 1 (point 2). A Spinco model E ultracentrifuge equipped with Schlieren optics and two 12-mm single-sector cells was used at 40 000 rpm. Sedimentation coefficients in water ($s_{20,w}$) were calculated from the values determined in buffer, using the specific viscosity (1.016) and density (1.0018 g cm^{-3}) of the buffer at 20°C, and assuming a $\bar{v}$ of 0.734 cm^3 g^{-1} for both samples of urease (see footnote 6 in text).

After gel chromatography on Sephadex G-200, this urease had a specific activity of 78.7 (mkat/L)/A_{280} and a nickel content of 0.99 μg Ni/mg urease (atomic absorption spectrometry). These are both somewhat less than the values for pure urease (93 (mkat/L)/A_{280} and 1.21$_6$ μg Ni/mg urease, respectively (footnote 6 and Ref. 2)) and it is likely that the enzyme prepared in the presence of dimethylglyoxime was only about 81–86% pure, possibly because of an altered fractionation on gel chromatography in the presence of 10% ethanol. Manganese was undetectable (less than 0.2 g-at./mol of subunits).

After dialysis in 2.5 *M* guanidinium chloride at pH 7.6 for 40 h in the presence of 10 m*M* EDTA, urease contained less than 0.2 g-at. Ni/mol of 97 000-dalton subunits, was completely inactive, and was not reactivated in the presence of Ni^{2+} under various conditions

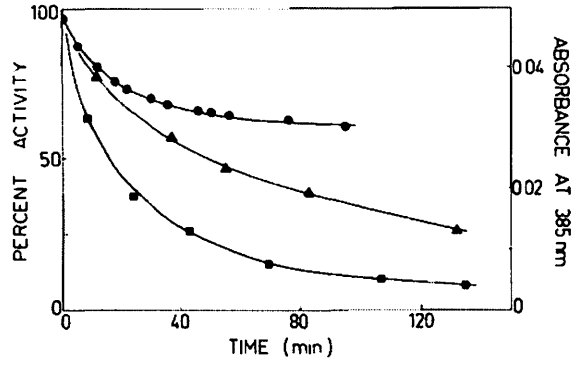

FIG. 3. Inactivation of urease (11.0 mg/mL) in oxygen-free 0.1 *M* acetate buffer (pH 3.8, 1 m*M* in β-mercaptoethanol) at 25.0°C. Progress curves for the loss of enzymatic activity in the presence (■) and absence (▲) of 1 m*M* EDTA. Progress curve for the loss of absorbance at 385 nm (●) in the presence of 1 m*M* EDTA; in this experiment, the enzyme had undergone substantial loss of tail absorption at pH 3.8 prior to addition of EDTA and initiation of the absorbance trace (see Materials and methods).

in Tris buffers. Nickel ion complexes to urease (12) much more tightly than it does to Tris (13), so that if simple equilibria are involved, urease should have been reactivated.

In 2.0 *M* guanidinium chloride at pH 7.6 and 38°C, urease lost 50% of its activity gradually over 2.0 h in the presence of 2.5 μ*M* EDTA. The initial rate of loss of enzymatic activity was only half as great in the presence of 10 m*M* EDTA. Evidently EDTA is stabilizing the enzyme in 2.0 *M* guanidinium chloride, presumably by removing traces of adventitious metal ions or by binding to the enzyme-bound nickel ions or by complexing the liberated nickel ions.

After treatment of urease with 9 *M* urea at pH ~ 9 and 25°C for 24 h, precipitation of about 75% of the protein occurred on removal of the urea (and ammonium carbonate) by dialysis in the presence or absence of Ni^{2+}. The residual soluble urease had specific activities of 88.9 (mkat/L)/A_{280} in the control and 83.3 (mkat/L)/A_{280} in the nickel-treated sample. These experiments provide no evidence for the reversible formation of apourease in the presence of high concentrations of urea.

Metal ion and urease content of jack bean seeds

Table 1 shows the metal ion and urease content of normal jack bean seeds (parent seeds) and of beans grown hydroponically from these in the absence of added nickel. The low-nickel seeds had only 6% of the parental level of nickel, while the levels of manganese, iron, and cobalt were 70, 128, and 29%, respectively, of those in the parent seeds. The amount of extractable urease in the parent seeds corresponded to 0.11% of the weight of the seeds, in excellent agreement with the value of 0.10% calculated from Sumner's data (14). The low-nickel seeds had only 10% of the normal urease activity, which reflects closely the decreased nickel content. Equilibration of the extracted proteins with nickel ion did not produce any increase in enzymatic activity.

TABLE 1. Metal ion and urease content of jack bean seeds

Parameter	Value	
	Parent seeds[a]	Low-nickel daughter seeds[b]
Metal ions (μg/g of seeds)[c]		
Ni	8.0	0.48
Mn	22.6	15.9
Co	0.56	0.16
Fe	15.1	19.3
Urease (μkat/g of seeds)[d]		
Powder[e]	72.9	7.1
Seeds[f]	~66	~6.4
Urease/nickel (kat/g of nickel)[g]	~8.2	~13.3
Proportion of total Ni in active urease (%)[h]	~17	~28

[a]Sigma Chemical Co., lot No. 17B-0920.
[b]From a crop of jack beans raised hydroponically with no added nickel.
[c]Assessed by atomic absorption spectrometry following wet and dry ashing. The reagent blank contained no Ni, Mn, or Co. The reported values for Fe have been corrected for a reagent blank corresponding to 1.5 μg/g of seeds.
[d]Assessed by pH-stat assay of serial buffer extracts.
[e]Chloroform–acetone powder of crushed beans.
[f]A weight loss of ~10% occurs during the preparation of the chloroform–acetone powder of jack beans.
[g]This ratio for pure urease (specific activity, 93 (mkat/L)/A_{280}; 2.00 g-at. Ni 96 600 g protein) is 47.44 kat/g Ni.
[h]Calculated from the ratio of urease activity to nickel for the beans and for pure urease.

Discussion

Urease contains 2 (2.00 ± 0.12) g-at. of Ni/mol of 96 600-dalton subunits (1, 2) and this nickel is retained in the presence of EDTA at neutral pH even when the enzyme has lost up to 30% of its original specific activity (2). Building on work of Gorin (11), we found that under acid conditions (pH 3.5–4.0), EDTA promotes the inactivation of urease. The residual specific activity is a linear function of the nickel content of partially inactivated enzyme (Fig. 1) and is completely consistent with the presence of 2.0 Ni^{2+} ions/96 600-dalton subunit in fully active urease. Further, the data in Fig. 1 suggest that the inactive subunits have lost both nickel ions.

After partial inactivation in the presence of EDTA at low pH, followed by neutralization, the residual soluble urease displayed roughly equal amounts of a 19S species (same as for the native enzyme) and an 11S species (Fig. 2). No other species could be detected. Since the residual soluble urease retained ~18% of the maximal specific activity of the enzyme and

~25% of the normal nickel content of the enzyme, nickel-deficient enzymatically inactive urease with a molecular weight near that of the native enzyme must be capable of existence. This is the first indication that some of the nickel is not essential for the maintenance of quaternary structure.

The ultraviolet spectrum of urease has an "abnormal" tail absorption which extends into the visible region (footnote 6 and Refs. 1, 10, 12). A characteristic feature of this spectrum at pH 7.0 in the presence and absence of β-mercaptoethanol (1, 10) and also at pH 3.8 (1) is a shoulder near 420 nm. This shoulder is apparently associated with Ni^{2+} ion in an octahedral environment (10, 15); the nickel (Fig. 1), the shoulder (1), and much of the tail absorption (Fig. 3) are lost on the time scale of loss of enzymatic activity. Further, reversible inhibition by acetohydroxamic acid, phosphoramidate, fluoride ion, and β-mercaptoethanol correlates with their reversible effects on this region of the urease spectrum, strongly indicating that these inhibitors bind to the nickel ions (footnote 7 and Refs. 1, 5, 10). It follows that the nickel in jack bean urease is part of the active site, and a detailed catalytic role for nickel ion in the enzymatic hydrolysis of urea and other substrates has been postulated.[8]

Attempts to reactivate the soluble EDTA-inactivated urease by addition of Ni^{2+} at pH 3.50 and at pH 7.16 were not successful, despite the fact that the partially inactivated enzyme was not substantially denatured (judging from the velocity sedimentation results). Since urease is reported to be fully active after treatment with 2.0 *M* guanidinium chloride in the presence of EDTA (16), but to lose activity rapidly and synchronously with the loss of nickel in 3 *M* guanidinium chloride at pH 7 (17), and since the manganese in superoxide dismutase from *Escherichia coli* is reversibly removed in 2.5 *M* guanidinium chloride (18), a variety of attempts was made to use this denaturant to form an apourease capable of reactivation. Our failure reversibly to form apourease in the various experiments may reflect complications associated with the sulfhydryl groups, the large polypeptides, the quaternary structure (19), or the possible role of nickel in the correct refolding of urease (20).

Urease which was prepared at all stages in the presence of dimethylglyoxime had a specific activity and a nickel content which were essentially normal. Dimethylglyoxime is a good chelating agent for Ni^{2+} ions and its lack of effect renders very unlikely the possibility that nickel in purified urease is an artifact due to the replacement of some other metal ion by adventitious Ni^{2+} during purification.

Seeds from jack beans grown hydroponically in the absence of added nickel had a normal size and appearance and therefore presumably contained the normal amounts of most proteins and carbohydrates. However, these seeds were low both in urease and in nickel (10 and 6%, respectively, with respect to the parent seeds). If an apourease is present in buffer extracts of the low-nickel beans, it, too, is not activated by addition of Ni^{2+}. These results would be partially explained if (*a*) nickel is required to bring about synthesis of apourease in the developing seed, and (or) if (*b*) no other metal ion will substitute for nickel to produce a *normally active* holoenzyme. In consideration of the latter possibility, the growth medium contained Mg^{2+}, Ca^{2+}, Fe^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+}, and MoO_4^{2-}, and the daughter seeds which were low in both nickel and urease, contained manganese and iron in nearly normal amounts. The total nickel in the low-nickel seeds is only ~35% of that present as urease-bound nickel in the parent seeds. The fact that depressed urease activity parallels depressed nickel content in the presence of all of these other metal ions is consistent with the proposition that of these, only nickel can produce fully active urease.

Urease in the seeds of legumes is involved in the mobilization (upon germination) of nitrogen stored as arginine or canavanine (12). Purified soybean urease has recently been reported to contain nickel (21) and Polacco has suggested that nickel may be a universal requirement for plant ureases (22).

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