Subsequent reduction of the mixture of methyl 7-cis,9-
cis- and 7-cis,9-cis-13-cis-retinoates with LiAlH₄ (room
temperature, 2 hr) followed by MnO₂ oxidation (CH₂Cl₂,
room temperature, 0.75 hr) gave the corresponding mixture
of retinal isomers. Separation of 7-cis,9-cis- and 7-cis,9-
cis,13-cis-retinal was readily effected by column chroma-
tography (Biosil A, 25% CHCl₃–hexane) with the latter
eluting first.¹⁴ The NMR spectra of the pure isomers are
presented in Figures 1 and 2. The spectrum of 7-cis,9-cis-
retinal is identical with that of HPLC purified sample from
the mixture prepared previously. The olefinic region is well
resolved and all vinylic hydrogens can be unambiguously
assigned. The spectrum of 7-cis,9-cis,13-cis-retinal is com-
plicated by the accidental equivalence of H-11 and H-12;
however, with Pr(tod)ζ shift reagent, the familiar first order
d and d of d signals for H-12 and H-11, respectively, are
again present (see A in Figure 2).

We are currently examining alternate approaches to stereoselective 15 + 5 condensations of 7,9-cc-1 as well as new
routes to pure 7-c-1.

Acknowledgment. This work was supported by grants
from the Public Health Services (EY00918, AM17806).

References and Notes

(1) New Geometric Isomers of Vitamin A and Other Carotenoids. II. For paper I, see ref 2.
(3) In 7 ft X 0.25 in.-Coralil or 1 ft X 0.25 in. — v-mosil columns.
(4) For a summary of synthesis of 7-trans of retinal and carotenoids, see O.
(5) When β-ionone was treated with 1 equiv of Pb(OAc)₄, considerable
amounts of 10,10-diacetate were formed (10%). With 2 equiv of
Pb(OAc)₄, the diacetate became the major product (80%) with 2 being
formed in 30–35% yield. Diacetylation was effectively suppressed by
using a 1.5-fold excess of β-ionone. See J. W. Ellis, J. Org. Chem.,
34, 1154 (1969), for analogous preparations.
(6) Compounds 2 to 7 show expected spectroscopic properties which will
be disclosed in a full paper in the future.
(7) With more polar solvents the homolytic reaction exhibited decreased selectivity. Thus, in THF or 25% HMPA-THF, the ratio of t-3 to t-5 was
5:1 and 7:3, respectively. Similar stereoselectivity has been reported for
Chem., 38, 1389 (1970), and ref 8 therein.
(8) Prolonged irradiation of t-3 greatly decreased the overall yield of both
cis-3 and cis-5. Careful monitoring of the photoreaction by NMR spec-
trscopy circumvented this problem.
(10) A lower yield (86–70%) of 8 was obtained when DMDO was used as
solvent. This was presumably due to a slow interaction of NaBH₄ with this
solvent. On the other hand, see H. M. Bell, C. W. Vanderslice, and
(11) The use of more reactive reducing reagents (e.g., LAH) in an attempt to
convert 5 directly to 7 only resulted in the formation of allylic rearranged
isomer.
(12) V. Ramamurthy, G. Tustin, C. C. Yau, and R. H. Liu, Tetrahedron, 31,
193 (1975).
(1966).
(14) This elution pattern is consistent with 7-cis isomers of retinal (ref 2).
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Inhibition of Jack Bean Urease (EC 3.5.1.5) by
Acetohydroxamic Acid and by Phosphoramidate. An
Equivalent Weight for Urease

Sir:

On the basis of earlier reports of new substrates for ure-
ase,¹,² and our finding that semicarbazide was a substrate,³
we quite wrongly predicted that phosphoramidate would
also be a substrate. Instead we found that it produced re-
versible inhibition with kinetic characteristics surprisingly
similar to those of acetohydroxamic acid. This finding has
enabled us to obtain two totally independent assessments of
the operational equivalent weight⁴ of urease, measured by
correlation of specific enzymatic activity with the incorpo-
ration of the reversibly bound inhibitors, [14C]acetohydro-
oxamic acid⁵,⁶ and [32P]phosphoramidate. Further, it led us to
reexamine the metal ion content of urease which is reported
in the following communication.⁷

[U-14C]Acetohydroxamic acid was prepared from [U-
14C]acetamide⁸ by treatment with hydroxyammonium
chloride for 1 hr at 86–96° with the exclusion of moisture.
The recrystallized product had constant specific radioactivity
(17.70 μCi/mmol) and mp 88.7–89.1°, lit.⁹ mp 89°.
[14C]Acetohydroxamic acid was assayed spectrophotomet-
rically,¹⁰ using acetohydroxamic acid as standard.

Ammonium [32P]phosphoramidate was prepared essen-
tially as described by Sheridan et al.¹¹ The recrystallized
product had constant specific radioactivity (177.5 μCi/
mmol), and was free of contaminating radioactivity,¹⁴ and
of material which did not hydrolyze in acid to give inorgan-
ic phosphate.¹⁵

Scintillation counting of aqueous samples was carried out
in a liquid scintillation counter (Packard Instrument Co., Inc.)
or in a medium prepared from toluene (Mallinckrodt, scintillation grade; 48
vol), Triton X-100 (35 vol), and Liquifluor (New England
Nuclear; 2 vol) using a Nuclear Chicago Mark I or a Beck-
man LS 250 liquid scintillation system. Counting efficien-
cies were measured using an internal standard of [14C]to-
luene (New England Nuclear) or ammonium [32P]phos-
phoramidate (25 μl of a 3.442 mM aqueous solution) dispersed with a Grubbaum pipet (Labindustries). All dilutions and
measurements were made in duplicate.

Urease was prepared as previously described,¹⁶ except
that the storage buffer was 5 mM in β-mercaptoethanol.
Two totally independently prepared samples (I and II) of the enzyme were used. Ureases I and II had specific activi-
ties¹⁷ of 84,620 and 84,610 (μkat/l.)/A₂₈₀, respectively.
Assays of inhibited enzyme samples and of control samples
of native urease were performed at 10°, at which tempera-
ture the reactivation of inhibited enzyme during assay is
negligible.

Urease I (5.10 ml, 5.62 mg/ml, in oxygen-free 0.02 M
phosphate buffer, 1 mM each in EDTA and β-mercapto-
thanol, pH 7.0) was equilibrated at 38° with 38° with 4.9 mM
[U-14C]Acetohydroxamic acid for 10 min. Urease II (3.30
ml, 2.46 mg/ml, in 0.05 M N-ethylmorpholine buffer, 1
mM in EDTA and 5 mM in β-mercaptoethanol, pH 7.12)
was equilibrated at 38° with 23.2 mM 2-(N-morpholino)e-
thanolsulfonic acid (to produce pH ~6.0) and 11.9 mM am-
monium [32P]phosphoramidate for 10 min.
In each experiment, the sample of inhibited enzyme was
cooled rapidly to 0°, and passed at 4° through a column
(3.0 X 35 cm) of Sephadex G-50 pre-equilibrated with the
appropriate oxygen-free buffer. As expected from the slow
reactivation at 4° of the enzyme-inhibitor complexes,⁶ ap-
propriate assays showed that the protein-inhibitor peaks
were completely separated from the unbound radioactive
inhibitors. The peak protein fraction ("maximally inhibited
enzyme") was assayed immediately for enzymatic activity,
protein concentration, and radioactivity. Aliquots (3.0 ml
of material which did not hydrolyze in acid to give inorgan-

icer phosphate.¹³

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The nickel atoms; are the environments of the nickel atoms...22 The present work indicates that phosphoramidate may also be a useful reversible probe of the sites of metalloenzymes.

Table 1. Assays of Partially Inhibited Urease

<table>
<thead>
<tr>
<th>Residual specific enzymatic activity (%)</th>
<th>Protein concentration (mg/ml)</th>
<th>Radioactivity (nCi/ml)</th>
<th>[Protein-bound inhibitor] (^b) (µmol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urease I, (^{14}C)Acetoxyhydroxamic Acid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.27(^c)</td>
<td>2.351</td>
<td>0.365</td>
<td>8.785</td>
</tr>
<tr>
<td>18.16(^d)</td>
<td>0.976</td>
<td>0.1264</td>
<td>7.317</td>
</tr>
<tr>
<td>33.76(^e)</td>
<td>1.027</td>
<td>0.1066</td>
<td>5.864</td>
</tr>
<tr>
<td>93.5(^f)</td>
<td>2.35</td>
<td>&lt;0.001</td>
<td>3 &lt;0.03</td>
</tr>
<tr>
<td>Urease II, Ammonium (^{32}P)Phosphoramidate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.07(^g)</td>
<td>0.934</td>
<td>1.413</td>
<td>8.533</td>
</tr>
<tr>
<td>36.19(^h)</td>
<td>0.414</td>
<td>0.427</td>
<td>5.817</td>
</tr>
<tr>
<td>51.83(^i)</td>
<td>0.430</td>
<td>0.327</td>
<td>4.289</td>
</tr>
<tr>
<td>99.2(^j)</td>
<td>0.655</td>
<td>&lt;0.006</td>
<td>0.005</td>
</tr>
</tbody>
</table>

\(^a\)Expressed as a percentage of the original specific enzymatic activity. \(^b\) [Protein-bound inhibitor] in units of umol/ml; [protein] in units of g/ml. \(^c\)Maximally inhibited enzyme. \(^d\)Reactivated for 5 min at 38\(^\circ\). \(^e\)Reactivated for 15 min at 38\(^\circ\). \(^f\)Maximally inhibited enzyme, reactivated for 2 hr at 38\(^\circ\) and dialyzed at 4\(^\circ\) in the phosphate buffer (four cycles). \(^g\)Reactivated for 10 min at 38\(^\circ\). \(^h\)Maximally inhibited enzyme, dialyzed for 18 hr at 38\(^\circ\) against the N-ethylmorpholine buffer, and then against the same buffer at 4\(^\circ\) (6 x 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 umol/ml; [protein] in units of g/ml; G, \(^{125}P\)phosphoramidate; A, \(^{14}C\)acetoxyhydroxamic 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 re-investigation of the molecular weight and subunit structure of this enzyme. It should be noted, however, that our measurements are all based on \(A_{280}\) = 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 apo-enzyme 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 acids21 and hydroxurates.20 The present work indicates that phosphoramidate may also be a useful reversible probe of the sites of metalloenzymes.

References and Notes

11. 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.14,21 However, the compound did not melt below 300\(^\circ\). Sheridan has acknowledged that their reported mp 233-234\(^\circ\) is in error (personal communication from R. C. Sheridan to N.E.D., 1974).
18. An equivalent weight of 207,000-317,000 daltons has been reported for sword bean urease inhibited by \([^{14}C]\)caproxyhydroxamic acid: K. Kobashi, J. Hase, and T. Komai, Biochem. Biophys. Res. Commun., 23, 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 Summer units/mg.
20. See footnotes 18 and 19 in ref 7.
24. This work was supported by the Australian Research Grants Committee.

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Communications to the Editor

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 quantities2-3 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