Subsequent reduction of the mixture of methyl 7-*cis*,9-*cis*- *and* 7-*cis*,9-*cis*,13-*cis*-retinoates with $LiAlH_4$ (room temperature, 2 hr) followed by MnO_2 oxidation (CH_2Cl_2, 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 chromatography (Biosil A, 25% $CHCl_3$–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 complicated by the accidental equivalence of H-11 and H-12; however, with $Pr(fod)_3$ 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).

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(1) New Geometric Isomers of Vitamin A and Other Carotenoids. II. For paper I, see ref 2.
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(3) In 7 ft × 0.25 in.-Corasil-II or 1 ft × 0.25 in. μ-Porasil columns.
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(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 (60%) with **2** being formed in 30–35% yield. Diacetoxylation 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 Horner reaction exhibited decreased selectivity. Thus, in THF or 25% HMPA–THF, the ratio of *tt*-**3** to *tc*-**3** was 5:1 and 7:3, respectively. Similar stereoselectivity has been reported for other systems: G. R. Pettit, C. L. Herland, and J. P. Yardley, *J. Org. Chem.*, **35**, 1389 (1970), and ref 8 therein.
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(15) Fellow of the John Simon Guggenheim Foundation, 1974–1975.

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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 urease,[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 reversible 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 weight[4] of urease, measured by correlation of specific enzymatic activity with the incorporation of the reversibly bound inhibitors, [^{14}C]acetohydroxamic acid[5,6] and [^{32}P]phosphoramidate. Further, it led us to reexamine the metal ion content of urease which is reported in the following communication.[7]

[U-^{14}C]Acetohydroxamic acid was prepared from [U-^{14}C]acetamide[8] by treatment with hydroxylammonium 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°. [^{14}C]Acetohydroxamic acid was assayed spectrophotometrically,[10] using acetohydroxamic acid as standard.

Ammonium [^{32}P]phosphoramidate was prepared essentially as described by Sheridan et al.[11] The recrystallized product had constant specific radioactivity (177.3 μCi/mmol), and was free of contaminating radioactivity,[14] and of material which did not hydrolyze in acid to give inorganic phosphate.[15]

Scintillation counting of aqueous samples was carried out in Instagel (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 Beckman LS 250 liquid scintillation system. Counting efficiencies were measured using an internal standard of [^{14}C]toluene (New England Nuclear) or ammonium [^{32}P]phosphoramidate (25 μl of a 3.442 m*M* aqueous solution) dispensed with a Grunbaum pipet (Labindustries). All dilutions and measurements were made in duplicate.

Urease was prepared as previously described,[16] except that the storage buffer was 5 m*M* in β-mercaptoethanol. Two totally independently prepared samples (I and II) of the enzyme were used. Ureases I and II had specific activities[17] of 84,620 and 84,610 $(\mu kat/l.)/A_{280}$, respectively. Assays of inhibited enzyme samples and of control samples of native urease were performed at 10°, at which temperature 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 m*M* each in EDTA and β-mercaptoethanol, pH 7.0) was equilibrated at 38° with 4.9 m*M* [U-^{14}C]acetohydroxamic acid for 10 min. Urease II (5.30 ml, 2.46 mg/ml, in 0.05 *M* *N*-ethylmorpholine buffer, 1 m*M* in EDTA and 5 m*M* in β-mercaptoethanol, pH 7.12) was equilibrated at 38° with 23.2 m*M* 2-(*N*-morpholino)ethanesulfonic acid (to produce pH ~6.0) and 11.9 m*M* ammonium [^{32}P]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 × 35 cm) of Sephadex G-50 preequilibrated with the appropriate oxygen-free buffer. As expected from the slow reactivation at 4° of the enzyme-inhibitor complexes,[6] appropriate 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 the maximally inhibited enzyme were equilibrated at 38° for varying lengths of time, cooled to 0°, passed through columns (2.2 × 14.5 cm) of Sephadex G-25 at 4°, and similarly assayed. The results of these assays are given in Table I.

A plot of the residual specific enzymatic activity of the effluent protein (expressed as a percentage of its specific activity before treatment with radioactive inhibitor) vs. the ratio [protein-bound inhibitor]/[protein] is strictly linear (Figure 1). The least-squares line so obtained extrapolates

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).

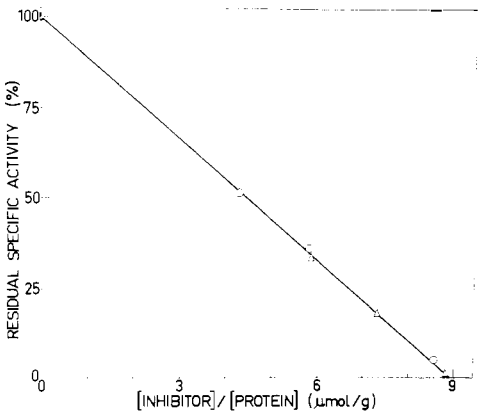

Figure 1. Correlation of residual specific enzymatic activity with incorporation of radioactively labeled inhibitor into urease: [inhibitor] bound to protein, in units of μmol/ml; [protein] in units of g/ml; ○, [^{32}P]phosphoramidate; △, [^{14}C]acetohydroxamic acid.

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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(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