Jack Bean Urease (EC 3.5.1.5). A New Purification and Reliable Rate Assay*

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ABSTRACT: Jack bean urease (EC 3.5.1.5) has been reproducibly purified to a hitherto unobtained and constant specific activity. The procedure is capable of handling "poor" jack bean meal. A chloroform–acetone powder of jack bean meal is extracted with 30% acetone containing 1% mercaptoethanol at 39° for 5 min.

The filtrate from this step is allowed to stand for 48 hr at 4°, and the crystals of the enzyme are harvested by centrifugation. After recycling gel filtration in the presence of 1 mM mercaptoethanol and 1 mM EDTA (Sephadex G-200), and concentration by ammonium sulfate precipitation, the enzyme has reached maximum specific activity. A reliable pH-Stat assay in the presence of 2 μM dithiothreitol has been devised, which should allow detailed investigations of the mechanism of action of this enzyme to be carried out with good precision.

This is the first of a series of papers describing the preparation, properties, and work on the mechanism of action of jack bean urease (urea amidohydrolase, EC 3.5.1.5).

Many hundreds of papers have been published on urease since Sumner (1926) first produced the classical octahedral crystals and showed that the enzyme was a protein. Sumner extracted jack bean meal with 31.6% (v/v) acetone at 22° for 4–10 min, and the enzyme crystallized from the filtrate at 2–2.5°. Dounce (1941) established the first reliable recrystallization procedure from aqueous citrate–acetone. The next significant improvement on the above basic procedure was made by Gorin *et al.* (1962). Reithel *et al.* (1964) and Mamiya and Gorin (1965) included mercaptoethanol in an aqueous acetone extraction at 28°. Gorin's best enzyme had a specific activity comparable with that of Sumner's.

However, Lynn (1967)[1] has recently reported the isolation of urease whose specific activity is approximately three times that of any previously reported preparation. Moreover, no crystallization was attempted, a procedure which has been used by other workers to obtain maximum (and constant) specific activity.

All workers have stressed that the yield and specific activity of the isolated urease depend markedly upon the quality of the jack bean meal. In our hands, the procedure of Mamiya and Gorin (1965) worked reasonably well on some batches of meal, but gave miserably low yields on others, so that we were forced to develop a reliable method of purification.

The activity of urease has been measured by the release of ammonia and carbon dioxide from urea over fixed intervals of time. The techniques include Nesslerization (Sumner, 1926; Kistiakowsky *et al.*, 1952), titration of the ammonia (Van Slyke and Cullen, 1914; Sumner and Hand, 1928; Gorin *et al.*, 1962; Gorin and Chin, 1966), and manometric determination of carbon dioxide (Van Slyke, 1927; Van Slyke and Archibald, 1944). In addition, color reactions are available, at least in principle (Momose *et al.*, 1965).

Andrews (1965), in a different connection, has reported briefly on a pH-Stat assay for urease, but he obtained nonlinear traces over the first 2–3 min. In this laboratory, a similar (but quite distinct) procedure has been independently developed which provides a reliable rate assay for the enzyme.

A new purification and rate assay are the subjects of the present paper.

Experimental Section

Extraction, Purification, and Crystallization of the Enzyme. Jack bean meal (~1700 g, Mann Research Laboratories) was homogenized with 4 l. of redistilled chloroform (−20°) in a Waring Blendor. The chloroform was removed by filtration at 10°. The filter cake was homogenized with 3 l. of redistilled acetone (−20°) in an *explosion-proof* blender and filtered. The resulting filter cake was washed with 2 l. of acetone (−20°) and filtered to semidryness using a rubber dam. The cake was then dried for 1 day *in vacuo* at 10° to remove acetone, using traps at −80°. The resulting powder was finely divided, dried *in vacuo* for a further 25 hr over concentrated H_2SO_4, and finally stored in well-sealed jars at 4°. The over-all loss in weight was about 5%.

A solution of 1725 ml of distilled water and 25 ml of mercaptoethanol (Eastman, White Label) was warmed in a 5-l. beaker to about 39°. The solution was removed from heat and stirred with a Teflon-covered paddle

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[1] The paper actually states 5300 Sumner units/mg. This figure is stated to be too high by a factor of 10 (personal communication to B. Z. from K. R. Lynn, 1968).

driven by an overhead motor. When the temperature had fallen to 38.0°, redistilled acetone (750 ml, ~25°) was added;[2] the temperature rose to ~41°. Jack bean meal, prepared as previously described (500 g, ~25°), was added with vigorous stirring, causing the temperature to drop to ~39.5°. The suspension was stirred for a total of 5 min, during which the temperature fell about 1°. The suspension was filtered into ice-cold receivers through two previously prepared 25-cm Büchner funnels, each funnel containing two layers of Whatman No. 1 paper supported on a fiberglass screen. The filtration process took about 20 min, starting with a slight vacuum and ending with full water-pump vacuum. Again, the final stages were assisted by the application of rubber dams. Some frothing of the filtrate was not harmful.

The filtrate (1.8–2.0 l., pH 6.2–6.5), which is pale yellow and faintly turbid, was assayed and allowed to stand at 4° for 48 hr. After 12 hr, the solution was nearly opaque and a small amount of white solid (a mixture of amorphous and crystalline urease) had settled out. Large crystals of urease were seen upon microscopic examination after 48 hr.

Three such extractions were carried out in sequence, and after 48 hr, the product was collected by serial centrifugation at 3000*g* for 30 min at 0° in Teflon-clad stoppered cups. The turbid supernatant was assayed and discarded, leaving a predominantly white pellet. The pellets were serially suspended at 0° in a total of about 20 ml of buffer A (0.02 M phosphate, pH 7.1, 1 mM in EDTA and 1 mM in mercaptoethanol) and centrifuged for 30 min at 35,000*g*. The resulting pellet was reextracted with 10 ml of buffer A and recentrifuged. The combined supernatants from these last two steps were dialyzed at 4° against three changes of the same buffer (for a total dilution factor of 10^6), centrifuged, and applied to a column of Sephadex G-200.

An LKB 4900A ReCyChrom system, monitored by an LKB 8301A Uvicord detector (280 mμ) and an LKB 6520A recorder, was used for upward-flow gel filtration on Sephadex G-200. After the Sephadex had been equilibrated with buffer and packed in a 3.2 × 90 cm bed, the column was cooled to 4° by circulating water. The column was washed with buffer at least 1 day. Dialyzed urease from the previous step was applied and eluted at 25–35 ml/hr. The large urease peak was recycled two or three times, and on each cycle, all other absorbing material was short circuited to waste. The peak was finally collected in fractions at 4°. Fractions with an A_{280}/A_{260}[3] greater than 1.75 (Shimadzu QV-50 spectrophotometer) and specific activity greater than 4000 (see below) were pooled and centrifuged to remove traces of Sephadex particles.

The enzyme at this stage was either dialyzed in preparation for ion-exchange chromatography or electrophoresis or concentrated by the addition of 2.3 volumes of saturated ammonium sulfate in buffer A, followed after several hours at 4° by centrifugation.

Spectra were taken on a Cary 14 spectrophotometer using buffer A as solvent. After addition of an aliquot of urease to the sample cell, the absorbance of impure samples at 360 mμ was generally 2–4% of that at 280 mμ because of light scattering due to colloidal particles not readily removed by centrifugation. The base line between 360 and 320 mμ in the presence and absence of enzyme was extrapolated to shorter wavelengths (Beaven and Holiday, 1952), giving a correction of 5–10% of the net absorbance at 280 mμ. With purer samples of the enzyme, while the absorbance at 360 mμ was still of the order 2–3% of that at 280 mμ, the 360–320 mμ base-line correction amounted to only some 3–4% of the net absorbance at 280 mμ.

The sedimentation coefficient of urease in buffer A was determined in a Beckman-Spinco Model E analytical ultracentrifuge at 20°. The temperature was controlled with a Spinco RTIC temperature control unit.

Urease of specific activity 4490 was dialyzed exhaustively against boiled-out distilled water under nitrogen and then centrifuged. Aliquots were submitted to spectrophotometric measurement and to drying under high vacuum (10^{-5} mm) at 120–140° for 4 hr, in order to set up a weight standard for the spectral measurements.

Urease was crystallized by the procedure of Dounce (Dounce, 1941; Mamiya and Gorin, 1965). The initial presence of buffer A did not interfere with the crystallization.

Hydrolyzed starch (Connaught Research Laboratories) was used in the preparation of starch gels according to the method of Smithies (1959). The discontinuous buffer system consisted of 0.1 M phosphate (pH 7.1), 1 mM in EDTA and 1 mM in mercaptoethanol, in the buffer compartments and 0.01 M phosphate (pH 7.1), 1 mM in EDTA and 1 mM in mercaptoethanol, in the 13% starch gel. Electrophoresis was carried out at 4° for 11.5 hr with a field of 6 V/cm. After electrophoresis, the gel was split and stained for protein with Amido Black 10B (G. T. Gurr, London) in methanol–acetic acid–water (4:1:5, v/v) (Giri, 1956, 1957).

The Assay of Urease. Enzyme activity was measured with a recording pH-Stat (Radiometer TTT1c, SBR2c, and SBU1a, Copenhagen) equipped with a 0.5-ml Agla syringe buret (Burroughs Wellcome & Co., England). The pH-Stat was standardized frequently at the temperature of the assay with 1:1 0.05 *m* phosphate buffer (Bates, 1964). For routine assays, 10-ml aliquots of 0.05 M recrystallized urea in boiled-out distilled water were thermostated at 38 ± 0.1° for at least 15 min in 20-ml glass vessels whose rubber stoppers were boiled in distilled water before each use. Into the titration vessel were placed a combination electrode (GK 2026C) used only for urease, a glass stirrer, and the buret tip. These were rinsed in buffer A between assays.

An aliquot of 1 mM dithiothreitol (20 μl) was added just prior to the addition of urease.[4] The rate of uptake

[2] Reagent grade acetone also gave satisfactory results.

[3] Abbreviations used: A_{280}, etc., absorbance at 280 mμ, etc. A. S., concentration by ammonium sulfate, followed by dialysis.

[4] The dithiothreitol solution is stabilized with 0.5 mM EDTA.

of 0.00971 N HCl at pH 7.0 was recorded; 1 IU of enzyme activity gave a convenient rate in the assay system and highly concentrated solutions of the enzyme were diluted before assay as much as 800-fold in buffer A.

All glassware used in the handling of urease was cleaned with chromic acid, rinsed thoroughly with distilled water, and boiled in distilled water or steamed.

Results and Discussion

The Assay of Urease. One unit (IU) of activity of urease is defined as that amount of enzyme which causes the decomposition of 1 μmole of urea/min, under the standard assay conditions (38°, pH 7.0, 0.05 M urea, 2 μM dithiothreitol). Specific activity is defined as (activity/ml)/A_{280}, *i.e.*, (IU/ml)/A_{280}.

The reaction catalyzed by urease is $H_2NC(=O)\text{-}NH_2 + 2H_2O \rightarrow 2NH_3 + H_2CO_3$. At pH 7.0, acid must be added to maintain constant pH. Although ammonium carbamate is formed under the conditions of the assay (*cf.* Sumner *et al.*, 1931), its decomposition is not rate limiting (Roughton, 1941; Jensen, 1959). This is attested to by the fact that the rate is linear in enzyme concentration (see below). Thus carbamate must be present in steady-state concentration, since it is clearly not affecting the velocity measurements (Wong, 1965).

The carbonic acid and ammonia produced have acidity constants in the pH range of interest, so that the observed uptake of acid at pH 7.0 must be corrected for the ionization of the products. The appropriate acidity constants are given in Table I. It may readily be shown that $-\mathrm{d[urea]}/\mathrm{d}t = (\mathrm{d[H^+]}/\mathrm{d}t)/(2f_N - f_C)$, where $f_C = (1 + 2K_2/[H^+])/(1 + [H^+]/K_1 + K_2/[H^+])$, $f_N = [H^+]/(K_3 + [H^+])$, K_1 and K_2 are the first and second apparent dissociation constants of carbonic acid, and K_3, is the dissociation constant of ammonium ion. The rate, v, is defined as a fraction of the chart width per min, where one chart width is equivalent to 0.50 ml of 0.00971 N HCl. It follows that: activity $= 4.855v/(2f_N - f_C) = 4.264v$, since $1/(2f_N - f_C) = 0.8782$ at pH 7.0 and 38°.

The reaction is zero order to within at least 5%, throughout the hydrolysis of 1% of the urea. The initial rate of hydrolysis of urea is linear in enzyme concentration between 0.0027 and 0.52 IU per ml in the 10-ml assay system (Table II). Therefore, the popularly held conception that protein solutions of this dilution are heavily adsorbed to clean glassware seems to find little support in the present work.

The assay procedure is rapid and readily reproducible to within ±1%. Albeit the concentration of dithiothreitol in the assay system is low (2 μM), it was used routinely and is essential if the urease had not been stored in the presence of mercaptoethanol. Dithiothreitol could be replaced by mercaptoethanol, but higher levels of mercaptoethanol were necessary (10^{-4} M). Urease which had been stored in the presence of 1 mM mercaptoethanol had activity unaffected by dithiothreitol. In the absence of any SH compound, the observed rate decreased slowly and continuously throughout the first 1% of hydrolysis. This effect could be reversed by the addition of 1 μM dithiothreitol.

TABLE I: Acidity Constants and the pH Function at pH 7.0.

	H_2CO_3[a,b]		$^+NH_4$[c]	
Temp (°C)	10^7K_1	$10^{11}K_2$	$10^{10}K_3$	$1/(2f_N - f_C)$
20	4.147	4.20	3.972	0.8432
25	4.452	4.69	5.676	0.8546
30	4.710	5.13	8.072	0.8631
38	5.010	5.86	13.74	0.8782[d]
50	5.161	6.73	28.84	0.9045

[a] Apparent constants. [b] Harned and Davis (1943); Harned and Scholes (1941). [c] Bates and Pinching (1950). [d] At pH 6.9, this term is 0.8483.

TABLE II: Variation of Rate with the Concentration of Urease.[a]

10^{10} × [Urease] (M)	Rate[b] (μmoles of Urea Hydrolyzed/min)	Relative Normalized Rate
3.84	5.18[c]	1.029
0.768	1.006	1.00
0.0753	0.0983	0.997
0.0191	0.0268	1.072

[a] Specific activity = 4580; molecular weight taken as 483,000; a solution of urease containing 1 mg/ml has $A_{280} = 0.589$. [b] Initial rate in 0.05 M urea at pH 7.0 and 38°, in the 10-ml assay system. [c] Titrated with 0.097 N HCl.

The relative activities of urease (in micromoles of 0.05 M urea hydrolyzed per minute, at pH 7.0) at 50, 38, 30, 25, and 20° are 1.40, 1.00, 0.743, 0.605, and 0.487, respectively. The effects of other components are generally small; for example, the relative activities at pH 7.0 are 0.55 in 1 M sodium chloride and 0.43 in 40% ethanol.

The specific activity of urease was also determined under the conditions of the most recent assay (Gorin and Chin, 1966) at pH 9.0 (0.25 M urea, 0.05 M Tris, 0.01 M phosphate, and 1 mM EDTA, at 25°). Urease with a specific activity of 4580 (present assay) gave a specific activity of 1700 ((micromoles of NH_3 released per minute, at pH 9.0 and 25°) per milligram of urease).

Finally, it should be noted that there is absolutely no hydrolysis of urea under the conditions of the present assay in the absence of enzyme.

Extraction and Purification of Urease. While the procedure of Mamiya and Gorin (1965) worked reasonably on some batches of jack bean meal (Mann lot no. M2523 and N1002), the yields of purified enzyme obtained were markedly lower (~20%). With other meals (Mann lot no. S2318 and S3318), it failed abjectly.

TABLE III: Extraction of Chloroform–Acetone Powder of Jack Bean Meal at 39° with 30% Acetone Containing 1% Mercaptoethanol.

Prepn	Yield in 30% Acetone Extract[a] (mg of Urease/100 g of Jack Bean Meal)	Recovery in Crystals[b] (%)
44[c]	63	67
45[c]	48	72
46[d]	53	61
48[d]	54	83
49[d]	48	90
50[d]	61	68

[a] Based on an A_{280} of 0.589 for a solution containing 1 mg/ml of urease, and on a specific activity of 4600 for pure urease. [b] Percentage of the initially extracted activity which crystallized in 48 hr at 4°, measured after dialysis against 0.02 M phosphate (pH 7.1), containing 1 mM EDTA and 0.1 or 1 mM mercaptoethanol. [c] Lot S2318. [d] Lot S3318.

Since chloroform–acetone powders had proved successful in the preparation of pig liver carboxylesterase (Horgan *et al.*, 1969), this technique was applied to jack bean meal. The yield of urease in aqueous buffer extracts of two lots (S2318 and S3318) was nearly doubled by the chloroform–acetone treatment, giving ~100 mg of urease/100 g of meal. Both Gorin and Sumner report similar figures. The centrifuged aqueous extract had a specific activity of 16 and was difficult to purify, as Gorin *et al.* (1962) have pointed out. This led us to try modifications of the original acetone extraction, in order to find a dependable procedure.

A successful procedure involves extraction of jack bean meal at 39° with 30% aqueous acetone containing 1% mercaptoethanol. More than half of the available activity of the meal is found in the acetone solution, and an average of 73% of this is recovered as crystals after 48 hr (Table III). The specific activity at this stage is 3000–3500. Other experiments have shown that the primary factors controlling the amount of activity extracted into aqueous acetone containing 1% mercaptoethanol are the concentration and temperature

TABLE IV: Some Properties of Urease after Gel Filtration on Sephadex G-200, Followed by Precipitation with Ammonium Sulfate.

	Gel Filtration[a]			Ammonium Sulfate Precipitation[b]		
Prepn	Yield[c] (mg of Urease/100 g of Jack Bean Meal)	Sp Act. [(IU/ml)/A_{280}]	A_{280}/A_{260}	Yield[c] (mg of Urease/100 g of Jack Bean Meal)	Sp Act. [(IU/ml)/A_{280}]	A_{280}/A_{260}
44	33	4300[d]	1.8[d]	32[e]	4480	1.84
45	20[f]	4300[d]	1.8[d]			
46	25	4300	1.75	Applied to DEAE-cellulose		
48	28	4350	1.77	25[g]	4310	1.81
49	31	4480	1.89	Applied to Porath column		
50	20	4350[d]	1.85[d]	18[h]	4580	1.76
					↓	
					4610[i]	1.88
					→ 4490[j]	1.98
					↓	
					4860[k]	1.95
					→ 4520[l]	1.95

[a] Buffer A, except for preparations 44, 45, 46, and 48, for which the buffer differed in being only 0.1 mM in mercaptoethanol. The yield and properties were determined after dialysis against the buffer used for gel filtration. [b] The precipitation was carried out in the buffer used for gel filtration. [c] The yield is based on an A_{280} of 0.589 for a solution containing 1 mg/ml of urease and on a specific activity of 4600. [d] Not dialyzed. The absorbance was determined without extrapolation of the base lines. [e] The enzyme was precipitated by the addition of solid ammonium sulfate to 70% saturation and then dialyzed against 0.02 M phosphate (pH 5.9), 1 mM in EDTA, and 0.1 mM in mercaptoethanol. [f] Seven cycles on the ReCyChrom. [g] The enzyme was precipitated by the addition of solid ammonium sulfate to 70% saturation and then dialyzed against 0.02 M phosphate (pH 7.1), 1 mM in EDTA, and 0.1 mM in mercaptoethanol. [h] The enzyme was precipitated in the presence of 5 mM mercaptoethanol by the addition of saturated ammonium sulfate in buffer A. It was dialyzed against 5 mM mercaptoethanol in buffer A. [i] After dialysis under nitrogen against 0.02 M phosphate (pH 7.1), containing 1 mM EDTA and *no mercaptoethanol* prepared in boiled-out distilled water. [j] After dialysis against buffer A. [k] After dialysis against boiled-out distilled water in a glass-stoppered vessel under oxygen-free nitrogen. [l] After crystallization of the material with specific activity 4580 followed by dialysis against buffer A.

TABLE V: Sedimentation Coefficients of Different Preparations of Urease.

Prepn No. and Prior Treatments[a]	s_{20}[b] (S)	Species Concn[c] (%)	Mercaptoethanol[d] (mM)
46 (DEAE-cellulose, A. S.) (Figure 1B)	18.4	70	0.1
	27.5	23	
	35	5	
	~37	2	
48 (G-200)	18.4	97	0.1
	27.7	3	
48 (G-200, dialyzed)	18.1	93	0.1
	27.0	7	
48 (G-200, A. S.)	18.8	87	0.1
	28.4	13	
49 (G-200, dialyzed)	17.6	>99	1
49 (Porath column, A. S.)	18.0	99	1
	26.1	1	
50 (G-200, A. S., 5 mM in mercaptoethanol) (Figure 1A)	18.5	>99	2.3

[a] Abbreviation: A. S., concentration by ammonium sulfate, followed by dialysis. [b] Bar angle, 70°, except for preparation 46 (65°). [Urease] = 3–12 mg/ml. [c] Based on relative areas of the various peaks observed with schlieren optics. [d] Buffer for ultracentrifugation: 0.02 M phosphate, pH 7.1, containing 1 mM EDTA and mercaptoethanol as listed. The prior treatment involved the same buffer except for preparation 50.

of the aqueous acetone rather than the length of time of the extraction. The present procedure ensures exact reproducibility of conditions by minimizing the opportunity for evaporation of acetone at the elevated temperature. The factors controlling the crystallization of urease from aqueous acetone extracts of jack bean meal are not understood, but the present procedure is adequately reproducible in this regard.

Urease was separated from nucleic acids and other impurities by recycling gel filtration on Sephadex G-200. A characteristic small peak of absorbance (280 mμ) was eluted slightly before the enzyme. Urease was collected in fractions after two or three passes through the gel bed, and those fractions with the highest specific activity and A_{280}/A_{260} ratio were pooled and dialyzed or else concentrated with ammonium sulfate. The results of several preparations are listed in Table IV.

An average of 25 mg of urease/100 g of jack bean meal was recovered in the peak fractions from the gel filtration, accounting for about 50% of the enzyme initially extracted into 30% acetone. The enzyme after gel filtration and dialysis had a specific activity of at least 4300 and had an A_{280}/A_{260} ratio of ~1.8.

The urease was concentrated by precipitation with ammonium sulfate at 70% saturation, followed by dialysis against buffer A (preparations 49 and 50), or the corresponding buffer containing only 0.1 mM mercaptoethanol (preparations 44–48). The specific activity and the A_{280}/A_{260} ratio either were not affected or were slightly increased in this procedure (Table IV).

Electrophoresis of urease on cellulose using a Porath column (LKB Model 5801A, size 60) gave no change in specific activity or in the A_{280}/A_{260} ratio (preparation 49). Ion-exchange chromatography on XE-64 resin, in maleate buffers, has been reported to be useful in purifying urease (Lynn, 1967), but when 8 mg of urease of specific activity 4480 was dialyzed for 36 hr at 4° against a maleate buffer at pH 6.57 (0.046 M disodium maleate, 0.004 M sodium bimaleate, 0.055 M Na_2SO_4, 1 mM EDTA, and 1 mM mercaptoethanol; $\mu = 0.3$), the specific activity dropped to 3620. When this material was applied to, and eluted from, a column of DEAE-Sephadex in the same maleate buffer, only 58% of the original activity was recovered and the specific activity of the peak enzyme was 3240. It seems likely that maleate is inactivating urease by reaction with the sulfhydryl groups (Morgan and Friedman, 1938).

The gel filtrate of preparation 46 was submitted to ion-exchange chromatography on DEAE-cellulose at pH 6.6 (0.05 M phosphate, 1 mM in EDTA, and 0.1 mM in mercaptoethanol), using a linear salt gradient, 0–0.4 M NaCl in 2 l. of starting buffer, to elute the bound protein. The peak contained 30% and the tailing fractions another 20% of the applied activity. After concentration by ammonium sulfate precipitation and dialysis (in the presence of 0.1 mM mercaptoethanol), the effluent enzyme had a specific activity at 4670 and an A_{280}/A_{260} ratio of 1.84. While the specific activity was improved 9% by the ion-exchange procedure, it is still not much higher than that often attained without this extra purification step.

Urease of specific activity 4490 was crystallized and then dialyzed exhaustively against buffer A, giving a specific activity of 4520. This sample gave a single

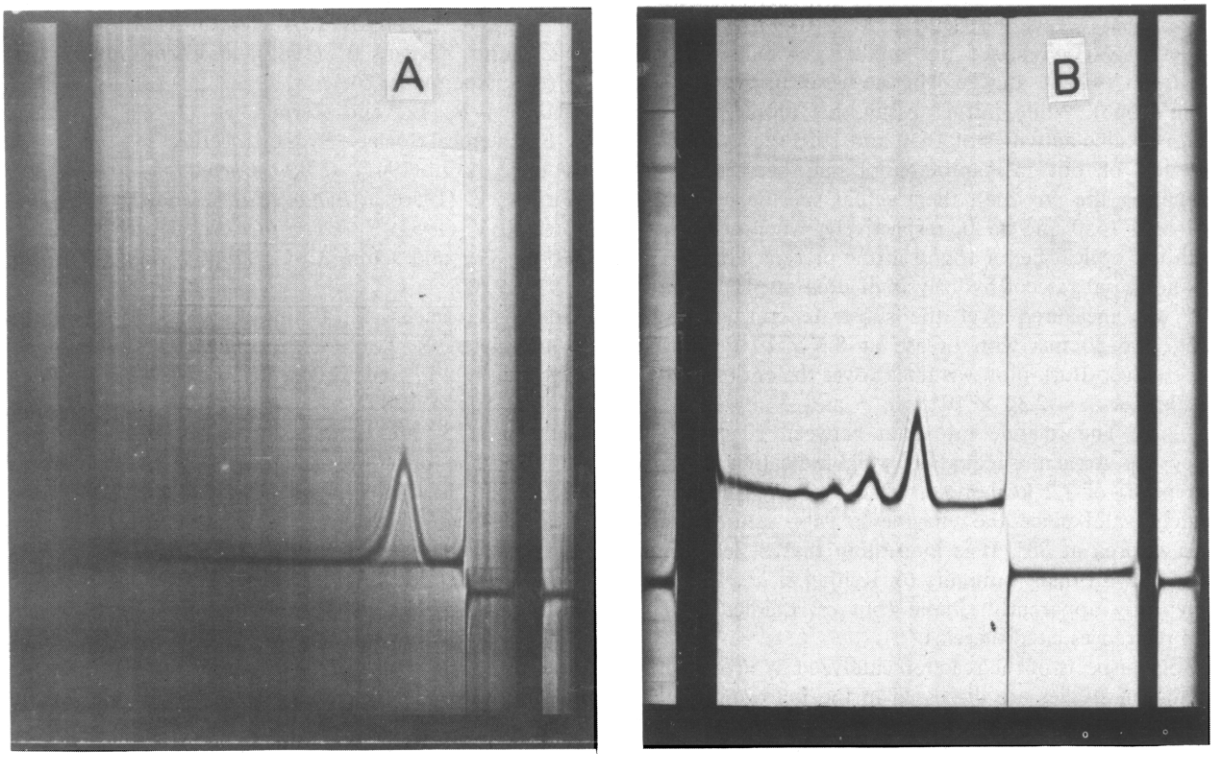

FIGURE 1: Sedimentation velocity experiments at 20°. (A) Urease: preparation 50, after Sephadex G-200, concentration by ammonium sulfate, and dialysis against buffer A (containing 5 mM mercaptoethanol). The actual run is in buffer A containing 2.3 mM mercaptoethanol, with urease of specific activity 4580 at 4.3 mg/ml. s_{20} = 18.5 S. The photograph was taken 6 min after reaching speed (42,040 rpm) in a double-sector cell and using a bar angle of 70°. (B) Urease: preparation 46, after DEAE-cellulose, concentration by ammonium sulfate, and dialysis against buffer A (containing 0.1 mM mercaptoethanol). The run is in the same buffer, with urease of specific activity 4670 at 3.5 mg/ml. The peaks have s_{20} values of 18.4, 27.5, 35, and ~37 S, contributing 70, 23, 5, and 2%, respectively, to the total area. The photograph was taken 4 min after reaching speed (59,780 rpm) in a single-sector cell and using a bar angle of 65°.

protein band on starch gel electrophoresis and consisted of an 18.5S species in the ultracentrifuge.

While a specific activity of 4500–4600 and an A_{280}/A_{260} ratio of 1.8–1.9 for urease are readily reproduced by these procedures, the product was frequently not homogeneous in the ultracentrifuge (Table V). In preparation 48, for example, the percentage of urease in the 18S form drops from 97 to 87 without any change in specific activity. Similarly, the effluent from the DEAE-cellulose column in preparation 46 shows that the specific activity can be maximal even though 30% of the urease has sedimentation coefficients greater than 18S (Figure 1). Since the 18S form has a molecular weight of 483,000 (Sumner *et al.*, 1938), this establishes that the aggregation of urease observed in this work does not interfere with the catalytic efficiency of the enzyme.

Urease prepared in the presence of 1 or 5 mM mercaptoethanol had full specific activity and existed at least 99% in the 18S form (Table V and Figure 1). No trace of forms of lower molecular weight were seen in the ultracentrifuge. The patterns of sedimentation coefficients in the presence of lower amounts of mercaptoethanol are paralleled by the appearance of a fine colloidal precipitate in concentrated solutions of urease; this largely redissolves upon the addition of sufficient mercaptoethanol. A clean, sealed solution stored in the presence of 5 mM mercaptoethanol remains optically clear. The turbidity and the species of higher molecular weight observed in the ultracentrifuge presumably reflect the same reversible aggregation which Hellerman *et al.* (1933, 1934) demonstrated under mild oxidizing and reducing conditions. The present results are consistent with the proposition that urease is fully stable as the 18S species under all the preparative conditions, *so long as sufficient mercaptoethanol is present.* All deviations from this proposition may be readily understood in terms of the slow decomposition of mercaptoethanol in the presence of air.

Urease of specific activity 4490 was dialyzed free of all other reagents with the exclusion of oxygen. The urease did not lose specific activity and had a spectrum in buffer A unchanged from that before removal of the protective agents. The wavelength of maximum absorbance is 278.5 mμ. From the dry weight of an aliquot, it is found that a solution containing 1 mg/ml of urease has an $A_{278.5}$ of 0.601 and an A_{280} of 0.589. These figures agree well with data of Reithel and Robbins (1967), but are appreciably smaller than those found by Gorin and Chin (1966).

The maximum specific activity reproducibly obtained was about 4600. This corresponds to 2709 IU/mg, and all yields of urease have been calculated using this factor. Urease with a specific activity of 4580 in the

present assay gave a specific activity of 1700 (micromoles of NH_3 released per minute per milligram of urease at pH 9.0 and 25°) in the assay of Gorin and Chin (1966). While, in their assay, they reported maximum specific activities of 1650 and 1920 (according to whether or not mercaptoethanol had been used in preparing the enzyme), their actual specific activities are only 1550 and 1530, respectively, when corrected with the optical factor found in the present work.

The initial rate of hydrolysis of urea at pH 7.0 and 38° was measured over the substrate concentration range 0.5–50 mM with urease at 8.3×10^{-11} M. A Lineweaver–Burk plot was linear over the entire range, yielding $k_{cat} = 2.34 \times 10^4$ sec^{-1} and $K_m = 3.28 \times 10^{-3}$ M. The relative rate data between 20 and 38° obey the Arrhenius equation well, indicating an apparent E_a of 7.2 kcal/mole. This is at least approximately valid since the enzyme is 94% saturated in 0.05 M urea at 38°. It is interesting that at least one enzyme with many subunits (6, Reithel *et al.*, 1964) is kinetically apparently simple (but *cf.* Laidler (1958) and references cited therein).

Some aspects of the reaction catalyzed by urease and inhibition studies are discussed in the following paper (Blakeley *et al.*, 1969).

Added in Proof

Professor Gorin has advised (personal communication to R. L. B., 1969) that the concentration of acetone used by Mamiya and Gorin (1965) was actually 32% and not 36% as published.

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