Purification and Properties of Urease Derived from Hydrated Seeds of Jack Bean, Canavalia ensiformis (L) DC.[1]

Prem P. Sehgal[2] **and Aubrey W. Naylor**

Department of Botany, Duke University, Durham, North Carolina

Received September 7, 1965.

Summary. Urease from jack bean meal and hydrated seeds has been obtained in 25 to 33 % yield with specific activity in the range of 1000 to 1070 units/mg protein. A purification of 100 to 130-fold was achieved from meal and fully soaked seeds. Use of β-mercaptoethanol and EDTA was found essential to obtain this high yield and purity. Amino acid analysis showed all 18 amino acids commonly found in proteins. Electrophoresis of urease from soaked seeds (specific activity: 1025 units/mg protein) on a starch-gel block showed 2 peaks. Upon ultracentrifugation of urease samples having a low specific activity (less than 25 % pure), the major portion of the urease was probably present in a peak having a sedimentation value of 11 to 12. With relatively pure samples (55–100 % pure), S values in the range of 18 to 20 and 24 to 26 were obtained. Usually the purest samples of urease tested without any prior storage lacked the 24 to 26 S peak or the higher polymeric forms. The percentage areas under none of the ultracentrifuge peaks corresponded to the percentage purity of the sample analyzed. It is argued that the physical state of urease in the cell when associated with other seed proteins is as yet uncertain. In crude extracts, a portion of urease exists in a 12 S form but so far data on its origin and specific activity in relation to other species of urease are not available.

Urease is of wide occurrence in bacteria, fungi and higher plants (2, 23, 30). Sumner (28) and Varner (31) have thoroughly reviewed the literature through 1960 in this field. Some recent work on urease has been on standardizing (6) or slightly modifying (24) the original method of Sumner (27) to get reproducible results. The latter procedure, or described modifications of it, are, however, applicable only to meal derived from a jack bean variety long cultivated in the United States because of its high urease content. Japanese workers (9–10) have successfully obtained crystalline urease from jack bean meals (*Canavalia ensiformis*, var. *gladiata*) possessing low initial quantities of urease. Gorin et al. (7) have made chemical studies on the number of methionyl, cystinyl and cysteinyl residues per mole of urease and attempted to dissociate the molecule into monomers by treating with guanidine hydrochloride (8, 19). Hydroxamic acids have recently been found to be specific inhibitors of urease and the inactive hydroxamic acid-urease complex has been isolated (13) and its properties studied (12).

So far, there has been no systematic attempt to isolate urease from soaked seeds and cotyledons of young seedlings. All methods referred to above were designed for and work only on dry meal of jack bean seeds. In this paper, procedures for the preparation of highly purified urease from hydrated jack bean seeds and commercial meal are reported.

In the hands of different workers, using a variety of techniques, urease has been shown to have S_{20w} values ranging from 4 to 7 to as high as 17 to 19, 25 to 27, 34 to 36 and 44 to 47 (4, 7, 14, 24, 25, 29). In crude extracts from jack bean meal (14) only half of the urease activity sedimented at the rate ascribed to crystalline urease (S_{20w} of 17–19). The remainder of the activity was associated with 3 discrete components of higher molecular weight. Most investigators, however, consider the 17 to 19 S component to be that of the active enzyme and believe that urease molecules exist in higher polymeric forms.

In the present study, sedimentation coefficient values for urease samples in different stages of purification will be reported. It is proposed that a portion of active urease may exist in a 12 S form.

Experimental Procedures

Determination of Urease Activity. Enzyme activity was determined in micro-Conway dishes (3). Treatment with 1 % siliclad solution (Clay-Adams,

New York) provided a water repellent layer that prevented the substrate and enzyme solution drops from spreading and mixing before it was desired that they do so.

A 0.5% boric acid in 20% alcohol solution containing an indicator was used to absorb the liberated ammonia (3). A saturated solution of K_2CO_3 was employed to stop the reaction after 5 minutes and to liberate ammonia from the reaction mixture. Diffusion was allowed to occur at room temperature for 2 to 3 hours. Fifty μl of 0.05 M urea in phosphate buffer, pH 7.0 (2.8% anhydrous KH_2PO_4 and 6.8% anhydrous Na_2HPO_4) and 25 μl of urease solution (properly diluted in 0.01 M phosphate buffer, pH 7.0 so that hydrolysis of the substrate does not exceed 20% level) were placed side by side on the distal end in the peripheral ring of the micro-Conway dish and the proximal side of it contained 1 ml of saturated K_2CO_3. The central well contained 2 ml of the boric acid solution with the indicator (3). The micro-Conway dishes were sealed air tight with plate glass covers which had been smeared on the edges with green surgical soap. Each unit was lifted and tapped lightly to mix only the test solution and substrate. After 5 minutes' incubation at room temperature (23 ± 1°), each dish was tilted to mix K_2CO_3 with the reaction mixture and swirled to get proper diffusion.

The amount of ammonia liberated was measured by titrating the solution in the central well with standard 0.004 N HCl. The end point was sharp and readily reproducible. Each determination was replicated 3 to 4 times.

Definition of the Urease Unit. In the present study, one urease unit is defined as the amount of enzyme which will hydrolyze 1 μmole of urea per minute at room temperature (23 ± 1°). One Sumner unit (28) is approximately 7 times larger than 1 International Enzyme unit (20).

Preparation of DEAE-Cellulose Column. Selectacel, DEAE-cellulose type 20 and 40, lot 1406 obtained from Carl Schleicher and Schuell Company, Keene, New Hampshire, was treated according to the method of Peterson and Sober (17). After the last traces of alkali had been removed by washing with water, the DEAE-cellulose was then suspended in 0.05 M phosphate buffer pH 5.8 containing 500 mg/liter of EDTA-disodium salt dihydrate, M.W. 372.25 (Matheson, Coleman and Bell) and 0.5 ml/liter of 2-mercaptoethanol (Eastman Kodak catalogue No. 4196, Eastman grade).

A column of treated DEAE-cellulose (2.8 × 2.5 cm) was prepared at room temperature and allowed to equilibrate for 12 to 24 hours at 4°. Generally a good equilibration of the column was obtained with 0.5 to 1.0 liter of the above buffer at a flow rate of 30 to 40 ml per hour.

Starch Gel Electrophoresis. Vertical electrophoresis on starch gel was carried out according to Smithies (26). Hydrolyzed starch manufactured by Connaught Medical Research Laboratories, Canada

and distributed in the United States by Fisher Scientific Company, was used in the experiments reported here. The buffer and bridge solution used were those of boric acid-sodium hydroxide (Smithies, 1959). A constant voltage of 6 volts per cm of gel was applied for 20 hours at 4°. Protein concentrations of about 10 mg/ml were used at the starting point.

Protein Determination. The method of Lowry et al. (15) was used for determination of protein. In the first 2 steps of the purification procedure, protein was precipitated from solution by adding an equal volume of freshly prepared 20% trichloroacetic acid at room temperature.

In the last stage of purification on DEAE-cellulose column, each 5 to 10 ml fraction of the eluate was read at 280 mμ on the Beckman DU spectrophotometer. The amount of protein was calculated from a standard curve prepared from Sigma urease powder V. The various tubes comprising the peak of urease activity were pooled and a small porton dialyzed for protein determination by Lowry's method (15).

Ultracentrifugation. A 0.8 to 1.2% solution of enzyme in pH 5.8 phosphate buffer (0.01–0.025 M) containing 0.5 ml/liter β-mercaptoethanol and 0.5 g/liter EDTA was analyzed on a Spinco Model E ultracentrifuge at 59,780 rpm (25°). The observed sedimentation coefficients were not corrected to standard conditions. Relative amounts of the different components were determined either by measuring the areas under the peaks enlarged from the photographic plates on graph paper or by weighing cut outs of the magnified peaks on a uniform heavy paper.

A period of a few hours to a maximum of about 16 to 20 hours usually elapsed between assay for urease activity and its run on the ultracentrifuge. There was, however, no storage involved before the enzymatic assay.

Amino Acid Analysis. Protein in the eluate (0.25 M phosphate buffer) from the DEAE-cellulose column in the last stage of purification was precipitated by adding solid ammonium sulfate to saturation. The precipitate was dialyzed until free of ammonium sulfate. The enzymatic protein used for the amino acid analyses was hydrolyzed in vacuo with 6.0 N HCl for 24 hours at 110 ± 1°.

The analyses were carried out according to the gradient elution method of Piez and Morris (18) on a Technicon Chromobead Type C resin column maintained at 60°.

Cystine in the protein was determined as cysteic acid according to Moore's (16) method. Trytophan was estimated spectrophotometrically by using the Beaven and Holiday technique (1).

Enzyme Preparation. Jack beans for the present investigations were obtained in the years 1961 to 1963 from Mr. Ernest Nelson, Route No. 1, Waldon, Arkansas. This is the source from which Sumner and others have obtained the material for their work.

Seeds obtained from the above source were soaked for 20 to 24 hours in water and only those fully swollen were selected, husked and frozen at −20°.

A loss of approximately 3 to 5 % in urease activity of the soaked and frozen seeds occurred during a storage period of 4 to 6 months. Seeds stored for more than 3 months were discarded.

Acetone used in the various steps was redistilled at 56.5° after treating with a small amount of potassium permanganate and anhydrous potassium carbonate.

The purification procedure employed for Sigma jack bean meal and soaked seeds is outlined below.

1 and 2a. Homogenization and Aqueous Extract. The stored cotyledons were homogenized in a Waring blendor with 1.5 ml of a water-mercaptoethanol mixture per g of cotyledons. Mercaptoethanol was used at the rate of 0.5 ml per liter of water. The homogenate was filtered through a double layer of cheesecloth and finally squeezed by hand. The residue was blended a second time with the same volume of water and processed as above. Both of the extracts were mixed and centrifuged for 30 minutes at $13,000 \times g$ in a Servall Model RC-2 centrifuge. The residue was discarded and the cold supernatant treated with 0.6 volume acetone. Precipitation with acetone was unnecessary when Sigma jack bean meal was employed as the starting material.

2b. Acetone Precipitation (0.6 vol). Acetone, chilled to −20°, was used in this step. The aqueous extract was brought to about 1° and kept in a salt-ice mixture bath maintained at about −10°. After about 10 % of acetone had been added, the temperature of the solution was maintained in the range of −4 to −2°. After an amount of acetone equivalent to 0.6 volume had been added, the mixture was allowed to stand in the bath for 30 minutes. The chilled mixture was then centrifuged at −5° for 30 minutes at $13,000 \times g$. The supernatant was discarded and the residue homogenized in batches in a Kontes glass homogenizer with water (0.5 ml mercaptoethanol/liter water). The homogenate was spun at $27,000 \times g$ for 30 minutes. The residue was washed a second time with water and centrifuged and the residue discarded.

3 and 4. Ammonium Sulfate Fractionations. Solid ammonium sulfate was added in batches with constant stirring to the clear extract obtained in step 2a from jack bean meal or the supernatant obtained after centrifugation in step 2b (if soaked seeds had been used as the starting material) to the point of 65 % saturation. The mixture was left at room temperature for 30 to 45 minutes and was then centrifuged at $13,000 \times g$ for 30 minutes. The supernatant was discarded and the precipitate was suspended in water and dialyzed at 4° for 12 hours against 14 liters of cold distilled water containing 1 ml/liter mercaptoethanol with 3 to 4 changes of water during this period. The dialyzates from the dialysis bags were pooled and centrifuged at $13,000 \times g$ for 30 minutes. The residue was discarded and

Table I. *Purification of Urease*

Stages	Total protein in g	Total units $\times 10^{-3}$	Yield	Jack bean meal urease Specific activity in units/mg protein	Soaked seeds urease specific activity in units/mg protein
1. Homogenate from starting material (500 g meal)	...	543.8	100.0	...	...
2. a. Aqueous extract*	63.78	536.2	98.6	8.4	12.3
b. 0.6 vol acetone precipitation	...	...	...	...	18.0
3. 65 % $(NH_4)_2SO_4$ fractionation	26.68	534.1	98.19	20.1	60.7
4. 55 % $(NH_4)_2SO_4$ fractionation	15.73	521.0	95.79	32.8	89.4
5. 0.6 vol acetone fractionation followed by solubilization	2.73	407.9	75.0	149.2	117.1
6. Crude crystals in 0.025 M citrate buffer, pH 6.0 with 0.3 vol acetone	0.35	187.2	34.43	541.1	681.2
7. Recrystallization 2 ×	0.25	165.4	30.32	661.2	719.6
8. Eluate (ii) from DEAE-cellulose column (0.25 M buffer)	0.14	164.8	30.31	1161.6	1189.9

* Extraction was done with distilled water containing 0.5 ml/liter mercaptoethanol.

this time solid ammonium sulfate was added to the clear supernatant at room temperature in batches to the point of 55 % saturation. Gentle stirring was maintained during the period ammonium sulfate was being added. After leaving for about 30 minutes at room temperature, the mixture was centrifuged at 13,000 × g for 30 minutes. The precipitate obtained on centrifugation was dissolved in water and dialyzed at 4° for 6 to 8 hours against 14 liters of water containing 0.1 % mercaptoethanol. During this period 2 to 3 changes of cold water were made. Solid KH_2PO_4 was added to the dialyzate to make a M/15 solution (9.073 g/liter) and cooled to about 1° before proceeding to the next step.

5. *Acetone Fractionation and Solubilization (0.6 vol).* This acetone fractionation was carried out in a manner similar to that in step 2b. If, however, soaked seeds were used as the starting material, solubilization by water containing mercaptoethanol did not suffice. Further successive treatments found essential for solubilizing more urease are shown in table II. Usually 75 to 80 % of the enzyme units were recovered. Since both the yield and specific activity data with 0.6 volume acetone were more consistent than with 0.3 volume, the larger amount of acetone was employed routinely and treatments i, ii and iii (in table II) were successively used for extracting the precipitate.

6 and 7. *Crude Crystals and Recrystallization.* Crude crystals were obtained by the method of Dounce (5), employing pH 6.0, 0.5 M citrate buffer (5 ml for every 100 ml of urease solution) and cold acetone. After adding the citrate buffer to the urease solution in the required amount, pH of the solution was again checked and adjusted to 6.0 with citric acid or sodium citrate. Generally the protein concentration at this stage was about 1.0 to 2.0 %. Cold (−20°) acetone (0.3 vol) was added dropwise and the solution was subsequently kept at 1 to 2° for 6 to 8 hours. Crude crystals were collected on centrifuging for 30 minutes at 27,000 × g, and dissolved in water (25–30 ml) containing 1 ml/liter of mercaptoethanol. The insoluble material separated on centrifugation was discarded and the supernatant was treated again as above to recrystallize urease at least twice.

8. *DEAE-Cellulose Column and Concentration of the Eluate.* The urease crystals (200–300 mg protein), dissolved in 0.05 M, pH 5.8 phosphate buffer, usually having an activity of 625 to 700 units/mg protein, were poured on a DEAE-cellulose column equilibrated as described above. It was washed with 0.05 M phosphate buffer (same as used for equilibrating the column) till all the unadsorbed protein was eluted. This fraction had no urease activity. Urease from the column was eluted by 0.25 M phosphate buffer, pH 5.8 containing both mercaptoethanol (0.5 ml/liter) and EDTA (500 mg/liter). Fractions of 3 to 5 ml were collected and those comprising the urease peak were tested individually for specific activity. Tubes showing the highest specific activity were pooled and tested again. Concentration was effected either by ammonium sulfate precipitation at full saturation or by dialyzing out the inner solution against solid sucrose. When ammonium sulfate was employed for concentration, about 10 % of the protein was lost and usually the specific activity also fell by about 10 to 15 % compared with the eluate. With commercial sugar (3–4 lbs/100 ml solution), a 10 to 15-fold concentration could easily be obtained in 7 to 10 hours at room temperature or in the cold. A maximum loss of only 1 to 2 % protein and specific activity was noted.

Results and Discussion

Enzyme Preparation. The procedure described can be used for both fully hydrated seeds and jack bean meal. The yields obtained were consistently in the range of 25 to 33 % and the specific activity usually 1025 units/mg protein. Use of 0.6 volume acetone was found to be essential for soaked seeds. It was unnecessary to heat the dialyzates at 50° after $(NH_4)_2SO_4$ fractionations (10).

Stability. It was found necessary to use EDTA (0.5 g/liter) and β-mercaptoethanol (0.5 ml/liter) to preserve urease activity throughout the purification procedure. Specific activity and yields were consistently low if either of the reagents was omitted. In the absence of mercaptoethanol, urease aggregated, became insoluble, and was lost during the stages of centrifugation where residues were routinely discarded and supernatants kept. This might also be the reason for the poor yields obtained (maximum of ca. 10 %) when Sumner's procedure (27) is followed.

Starch-gel Electrophoresis. Starch-gel electro-

Table II. *Successive Solubilization of Urease Fractionated by Acetone Second $(NH_4)_2SO_4$ Step*

Acetone	Specific activity in units/mg protein*			
	0.3 vol	0.4 vol	0.5 vol	0.6 vol
(i) Water	134.0	89.8	52.7	88.3
(ii) 0.005 M citrate pH 6.0	245.1	106.9	99.8	106.9
(iii) 0.025 M citrate pH 6.0 + 1 % mercaptoethanol	162.5	159.6	114.0	198.1
Avg specific activity**	153.1	104.3	64.1	114.7

* Starting with equal volumes of solution, urease was precipitated with 0.3, 0.4, 0.5 or 0.6 volumes of acetone. The precipitates obtained in each case were successively extracted with (i) water, (ii) citrate and (iii) citrate plus mercaptoethanol. Urease activity and protein concentration were determined in each of the extracts and specific activity was calculated for a total of 12 combinations.

** Average specific activity was calculated for the combined supernatants from steps i to iii for each acetone fractionation.

Table III. *Sedimentation Coefficient Values in 0.01 M–0.025 M Phosphate Buffer, pH 5.8 and Percentage Protein in Relation to Increasing Specific Activity of Urease*

Sample no.	Activity in units/mg protein	Percentage purity	Peak no. S values	Percentage areas under each peak			
				I (11–12)	II (19–20)	III (24–26)	IV (33–36)
1	29.3	2.8	99	traces of 19S	...	...	
2	64.3	6.2	48	52	...	...	
3	257.0	24.7	53	47	...	. .	
4a*	192.8	18.5	38	49	6	...	
4b**	564.1	54.1	6	82	7	3	
5a	642.6	61.7	12	85	3	...	
5b**	706.9	67.1	...	74	18	8	
6	1041.0	100.0***	5	95	...	...	

* 7 % of protein had an S value of approximately 4 in sample 4a and about 2 % in 4b.

** Samples 4b and 5b are the eluates from DEAE-cellulose column, whereas samples 4a and 5a refer to the material poured over such columns.

*** As this was the most purified preparation, percentage purity in this case is arbitrarily taken to be equal to 100. Other figures in this column are calculated on that basis.

phoresis of urease of the highest purity (145 Sumner units/mg protein) showed 2 peaks. Approximately 5 to 10 % of the protein moved faster than the rest. As the former peak could not be eluted quantitatively and without denaturation it has not been ascertained whether the fast-moving fraction was a form of urease or a contaminant. Creeth and Nichol (4) also concluded from the Rayleigh interferograms that not more than 10 % of another resolvable electrophoretic component was present in their preparations.

Amino Acid Analysis. In the present study, urease obtained from fully imbibed seeds (144 Sumner units/mg protein) was analyzed for amino acid composition. All 18 of the amino acids that commonly occur in proteins were found in urease. Hanabusa (11) had indicated that tryptophan was either absent or present in amounts too small to be detected by paper chromatography. We found tryptophan to be definitely present, though in small amounts. Our data showed that tryptophan and cysteine +1/2 cystine have the lowest number of residues per mole of urease. Aspartyl and glutamyl residues, on the other hand, are present in the largest proportions. This is in agreement with Hanabusa's (11) conclusions. Kinetic data for the destruction and release of each amino acid at 24, 48 and 72 hours of protein hydrolysis have to be obtained before the correct number of residues per mole for each amino acid can be accurately calculated.

Sedimentation Coefficient Values. From the data in table III it may be concluded that if urease was less than 25 % pure (specific activity below 260 units/mg protein, 38 to 99 % of the protein had S values ranging between 11 and 12, while the remainder of the protein had an S value between 19 and 20. Earlier we have argued (21) that urease may exist in 12 S form. With urease preparations having specific activities ranging from 570 to 1030 units/mg of protein (55–100 % purity), 75 to 95 % of the protein had S values in the range of 19 to 20. All

of the material in sample 1 under our experimental conditions moved with an apparent S value of 11 to 12. Also as the purification proceeds, the majority of the protein moves with an S value of 19 to 20 (sample 6). It is important to note, however, that the percentage areas under none of the peaks correspond with the percentage purity of the preparation.

Creeth and Nichol (4) who studied the chemical interaction of urease samples having a range of specific activity from 10 to 80 Sumner units/mg protein (70–570 International units) in buffer systems ranging in pH from 6.1 to 8.8, observed 3 major components in their preparations: S_{20w} 18.8 to 21 (55–90 %), S_{20w} 28 to 31 (13–28 %), and the heaviest fraction having a S_{20w} value of 36 to 40 (0–10 %). Besides these 3 major components, they observed a very slow moving boundary estimated to have an S value within the range of 4 to 6 and still another component at 12 S. In addition, they noted that the relative areas under separate peaks varied considerably. Their observations are thus extended further in the present study. It is quite probable that urease may have a 12 S form. Whether S values of lower than 12 and those higher than 36 reported in the literature represent active urease or urease as it exists in the cell is uncertain. Also, the presence of multiple peaks of ureease with S_{20w} values of 35 to 37 and 46 to 47 in crude extracts (14) remains to be confirmed.

S_{20w} values obtained in our laboratory by sucrose density gradient centrifugation with crude urease preparations confirm the presence of an active 12 S urease peak (22) in jack bean. Further work on its characterization and origin is in progress.

Literature Cited

1. BEAVEN, G. H. AND E. R. HOLIDAY. 1952. Ultraviolet absorption spectra of proteins and amino acids. In: Advances in Protein Chemistry. M. L.

Anson, K. Bailey, and J. T. Edsall, eds. Academic Press, New York. 7: 320–86.

2. BOLLARD, E. G. 1957. Translocation of organic nitrogen in the xylem Aust. J. Biol. Sci. 10: 292–301.

3. CONWAY, E. J. 1962. Microdiffusion Analysis and Volumetric Error, 5th Edition. Crosby Lockwood and Son Ltd., London. 467 p.

4. CREETH, J. M. AND L. W. NICHOL. 1960. Evidence for the chemical interaction of urease in solution. Biochem. J. 77: 230–39.

5. DOUNCE, A. L. 1941. An improved method for recrystallizing urease. J. Biol. Chem. 140: 307–08.

6. GORIN, G., M. F. BUTLER, J. M. KATYAL, AND J. E. BUCKLEY. 1960. Isolation of crystalline urease. Proc. Okla. Acad. Sci. 40: 62–70.

7. GORIN, G., E. FUCHS, L. G. BULTER, S. L. CHOPRA, AND R. T. HERSH. 1962. Some properties of urease. Biochemistry 1: 911–16.

8. GORIN, G., J. E. ROBBINS, AND F. J. REITHEL. 1964. Dissociation of urease by guanidine hydrochloride. Federation Proc. 23: 264.

9. HANABUSA, K. 1961. High-yield crystallization of urease from jack bean. Nature 189: 551–53.

10. HANABUSA, K. 1962a. Preparation of urease crystal from jack bean. Nature 193: 1078.

11. HANABUSA, K. 1962b. Qualitative studies on the amino acid composition of urease. Keio J. Med. Japan 11: 65–67.

12. HASE, J. AND K. KOBASHI. 1964. Properties of urease-hydroxamic acids complex. Proc. 6th Intern. Congr. Biochem. New York (Abstracts). Vol. IV, 312.

13. KOBASHI, K., J. HASE, AND K. UEHARA. 1962. Specific inhibition of urease by hydroxamic acids. Biochim. Biophys. Acta 65: 380–83.

14. KUFF, E. L., G. H. HOGEBOOM, AND M. J. STRIEBICH. 1955. The sedimentation behaviour of alcohol dehydrogenase and urease in crude solutions. J. Biol. Chem. 212: 439–48.

15. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265–75.

16. MOORE, S. 1963. On the determination of cystine as cysteic acid. J. Biol. Chem. 238: 235–37.

17. PETERSON, E. A. AND H. A. SOBER. 1962. Column chromatography of proteins: Substituted cellulose. In: Methods in Enzymology, Vol. V. S. P. Colowick and N. O. Kaplan, eds. Academic Press, New York. p 3–27.

18. PIEZ, K. A. AND L. MORRIS. 1960. A modified procedure for the automatic analysis of amino acids. Anal. Biochem. 1: 187–201.

19. REITHEL, F. J., J. E. ROBBINS, AND G. GORIN. 1964. A structural subunit molecular weight of urease. Arch. Biochem. Biophys. 108: 409–13.

20. REPORT OF THE COMMISSION ON ENZYMES OF THE INTERNATIONAL UNION OF BIOCHEMISTRY. 1961. Pergamon Press, New York. Vol. 20, Proc. 4th Intern. Congr. Biochem. Vienna, 1958.

21. SEHGAL, P. P. AND A. W. NAYLOR. 1964. Ontogentic, physical and chemical studies on urease in Canavalia ensiformis DC. Plant Physiol. 39: xx.

22. SEHGAL, P. P., R. J. TANIS, AND A. W. NAYLOR. 1965. A low molecular weight form of urease. Biochem. Biophys. Res. Commun. 20: 550–54.

23. SENECA, H., J. K. LATTIMER, M. S. ROBERTS, O. K. TROC, P. MILNER, AND P. PEER. 1960. Relationship of drug resistance to the production of urease and citrase by some coliform bacilli: A preliminary report: Antibiotics annual. Antibiotica Inc., New York. p 834–38.

24. SHADAKSHARASWAMY, M. AND R. M. HILL. 1962. Fractionation of urease on CM-cellulose. Arch. Biochem. Biophys. 97: 607–09.

25. SHEPPARD, E. 1951. Ultracentrifugal studies on ultraviolet light irradiated urease and ultracentrifugal studies on trypsin and the inhibitor-trypsin compound. Ph.D. Thesis, Polytechnic Inst. of Brooklyn, New York (Doctoral Dissertations 18: 11).

26. SMITHIES, O. 1959. An improved procedure for starch gel electrophoresis: Further variations in the serum proteins of normal individuals. Biochem. J. 71: 585–87.

27. SUMNER, J. B. 1926. The isolation and crystallization of the enzyme urease. Preliminary paper. J. Biol. Chem. 69: 435–41.

28. SUMNER, J. B. Urease. In: The Enzymes, Vol. I, part II. J. B. Sumner and K. Myrback, eds. Academic Press, New York. p 873–92.

29. SUMNER, J. B., N. GRALÉN, AND I. B. ERIKSSON-QUENSEL. 1938. The molecular weight of urease. J. Biol. Chem. 125: 37–44.

30. SUMNER, J. B. AND G. F. SOMERS. 1953. Chemistry and Methods of Enzymes, 3rd Edition. Academic Press, New York. 462 p.

31. VARNER, J. E. 1960. Urease. In: The Enzymes, 2nd Edition, Vol. IV. P. D Boyer, H. Lardy, and K. Myrback, eds. Academic Press, New York. p 247–51.