SECTION C, PHYSICAL SCIENCES

Isolation of Crystalline Urease[1]

G. GORIN, M. F. BUTLER, J. M. KATYAL, and J. E. BUCKLEY

Department of Chemistry, Oklahoma State University, Stillwater

The enzyme urease was first obtained in crystalline form by Sumner (1926), and the method of isolation was a remarkably simple one; jack-bean meal was extracted with 32% aqueous acetone, the extract was filtered, and crystals of the enzyme, which formed in the filtrate on standing in the cold, were separated by centrifugation. These operations, when successful, accomplish the separation of fairly pure enzyme from about 500 parts by weight of inactive material, and a fortunate combination of factors in delicate balance must operate to make this possible; therefore, careful control of manipulative details is necessary to achieve the desired results.

Sumner repeatedly insisted that the preparation of crystalline urease required neither special skill nor a unique grade of meal (Sumner *et al.*, 1931; Sumner and Sisler, 1944). However, jack-beans grown in different localities and/or in different conditions may be expected to differ in gross composition as well as in urease content (Sumner and Hand, 1928; Sumner and Holloway, 1928; Sumner, 1937) and they may, accordingly, give different results. Sumner himself relates that, in and about the year 1928, he was unable to obtain jack-bean meal that would give crystalline urease by the procedure originally devised by him, although appropriate modification of the procedure gave a low yield of crystals having lower specific activity (Sumner and Holloway, 1928). Clearly, then, the procedure described in the standard reference works (Sumner, 1951; Sumner, 1955) is not uniformly successful.

The procedure has unquestionably been successfully applied by many other investigators, and some (Landen, 1940; Hofstee, 1948-9) reported activities approximately equal to that found by Sumner for its purest preparations, 130 units/mg. (Sumner *et al.*, 1938; Sumner, 1951). On the other hand, many other investigators have reported lower activities (Hellerman *et al.*, 1943; Desnuelle and Rovery, 1949; Ambrose *et al.*, 1951; Kistiakowski *et al.*, 1952), and many others have not reported the activity at all. Urease is very sensitive to a variety of factors that are not yet fully understood, and the absolute values of the activity determined at different times and places may not be strictly comparable (Hofstee, 1948-9); furthermore, in most cases no deliberate attempt may have been made to attain the highest activity. Therefore, definite conclusions about the absolute activity of "pure" enzyme cannot yet be made; but it is fair to deduce from the evidence cited that the preparation of (nearly) pure urease entails considerable difficulty.

For this reason, it was thought appropriate to present, in the present paper, an account of some experiences encountered in this Laboratory in the course of preparing crystalline urease. After many unsuccessful and partially successful attempts several preparations of high activity (89-106

---

[1] This work was performed under Contract AF 18(603)-135, Air Force Office of Scientific Research, Aeromedical Division, administered by the Research Foundation, Oklahoma State University.

S.U./mg.) were obtained, and it is believed that a detailed description of the successful procedure, and a briefer discussion of related matters, should prove of value to other investigators interested in the preparation of the crystalline enzyme.

General Precautions

Urease is extraordinarily sensitive to inactivation . Ordinary distilled water was found to contain sufficient metal ions to cause substantial inactivation (Sumner and Hand, 1928). Certain metal ions are absorbed on glassware so strongly that they are not removed by repeated washings. In all the work to be described, the water used was obtained by redistilling ion-exchange purified water through an all-glass apparatus. Whenever possible, the glassware employed was taken new, and not used for any other purposes.

Assay for Urease Activity

Sumner and Hand (1928) defined a unit of activity (now generally known as a Sumner unit, S.U.) as the amount of enzyme which, when added in 2% gum arabic solution to 3% urea in 9.8% neutral phosphate buffer would produce 1 mg. of ammonia nitrogen in 5 minutes at 20.0°. Samples of fairly high activity can be assayed conveniently by mixing Nessler's reagent directly with the acidified reaction mixture (Sumner, 1951), but if much inert proteinaceous material is present the ammonia must first be aerated off. Also, acetone interferes with the determination.

The ammonia can also be determined by straightforward acidimetric titration after removing it from the reaction mixture by aeration (Van Slyke and Cullen, 1914; Van Slyke and Archibald, 1944). It was ascertained in this work that the ammonia can be determined without removal from the buffer medium by differential titration with hydrochloric acid using "Alka-Ver" indicator (Hach Chemical Co., Ames, Iowa). Neither acetone nor the components of the meal interfere with this method of assay, which is therefore quite convenient and which has been used in most of the work.

Solutions.—(1) Potassium dihydrogen phosphate, 28.0 g., and disodium hydrogen phosphate, 68.0 g. in 1 l. of solution; (2) urea, 3.00 g., in phosphate buffer to make 100 ml.; (3) bovine serum albumin (35% sterile solution, Nutritional Biochemicals Corp., Cleveland, Ohio) diluted to 2%; (4) (UPA Solution) 39.0 ml. of solution (2) and 1.00 ml. of solution (3); this solution is best mixed freshly before use, although it may be stored for a day or two in the refrigerator.

Procedure.—One milliliter of UPA solution was diluted with 1.00 ml. of water, two drops of "Alka-Ver" indicator were added, and the mixture was titrated with 0.1 *M* hydrochloric acid to the first purple color; V = volume of acid used in ml. One milliliter of UPA solution was diluted with enough water so that the volume after addition of the urease solution would be 2.00 ml., the urease was added, and the mixture allowed to stand exactly 5 minutes; the assay was conducted at 20.0 ± 0.5°. At the end of the 5-minute period, V ml. of 0.1 *M* acid was added *at once*, and the titration was then continued to the purple end point; V' = volume of acid used in ml. If $(V' - V)$ exceeded 3 ml., the assay was repeated with a more dilute urease sample, although approximate estimates of activity could be made with up to 5.00 ml.

The activity A was calculated from the expression:

$$A = (V' - V) \times M \times 14.0$$

where *M* is the molarity of the acid. The results may not correspond exactly to those of Sumner because of the uncertainties, already alluded to, concerning the repeatability of activity determinations, and also because serum albumin was used as a protective colloid instead of gum arabic. However, the results obtained by the above assay were reproducible within 5%, and correspond at least approximately to the activity in Sumner units (S.U.).

Characterization of Jack-Bean Meal

It is clear from the introductory discussion that the nature of the jack-bean meal used in the preparation of urease may have a profound effect on the course and the results of the isolation procedure. Hence, some effort has been made carefully to identify and characterize the meal used. The beans were grown by Mr. Ernest Nelson, Route 1, Waldron, Arkansas, in 1958; the grinding was carried out in a special stainless-steel mill at the U. S. Soil, Plant, and Nutritional Laboratory, Ithaca, N. Y., by Dr. Walter L. Nelson. The specific activity was determined by stirring 1.00 g. of meal in 100 ml. of water for three minutes, filtering, and assaying the filtrate; the value found was 230 S.U./g.

In order to get some measure of the total amount of material soluble in water, 10 g. of meal were suspended in 100 ml. of water at 25 ± 0.05°, stirred mechanically for one hour, and filtered with suction. An aliquot portion of the filtrate (which was cloudy) was evaporated to dryness with careful heating, and the residue per ml. was 30 mg.

Extraction Procedure

The extraction mixture was prepared from 160 ml. of acetone and enough water to make 500 ml.; its temperature was adjusted to 28°, and it was added to 100 g. of jack-bean meal in a 1-l. beaker. The mixture was stirred continuously for 5 minutes and immediately filtered with gentle suction through a sheet of Whatman #41 filter paper on a 27.5 cm. Buchner funnel. Filtration was continued for 10 minutes at room temperature and the filtrate transferred to a 500-ml. graduated cylinder (the filtrate should not be kept under vacuum any longer than necessary); then the apparatus was put in a cold room at 4° and filtration was continued for 15-20 minutes longer. The total amount of filtrate was 350-400 ml. Three other 100-g. samples of meal were treated in the same way.

After standing 24 hours at 4°, the filtrates were cloudy and in some cases a small amount of white sediment was present in the bottom of the cylinders. The filtrates were stirred, transferred to 250-ml. polyethylene centrifuge bottles with plastic caps, and centrifuged at 7000 r.p.m. and -10° for 1.5 hours (Lourdes centrifuge, Model LR). The clear centrifugate was decanted, and the bottles containing residue were inverted on paper towels and allowed to drain in the cold room until the smell of acetone was no longer evident. The residue was not allowed to dry out; it should be noted that the residue itself has a penetrating odor, not to be confused with that of acetone. It was found helpful to examine the residue microscopically, and this part of the procedure will be discussed in the subsequent section.

The residues were combined, *thoroughly* mixed with 10-12 ml. of water, transferred to two 10-ml. polyethylene centrifuge tubes, and allowed to stand 0.5 hours in the cold room. Then the suspension was centrifuged at 10,000 r.p.m. and 0° for 0.5 hours, and, after decanting from the residue, the centrifugation was repeated once more. The centrifugate finally obtained *(Solution A)* was clear or only faintly opalescent.

To Solution A was added citrate buffer (1 *M* trisodium citrate and

1 *M* citric acid, about 25:1, pH 6) in the ratio of 0.05 ml. per ml. of solution, and ice-cold acetone was then added, drop by drop, with constant stirring, until the solution *just* became cloudy. The cloudy solution was allowed to stand overnight, and was then centrifuged at 10,000 r.p.m. and 0° for 0.5 hours. A homogeneous or nearly homogeneous precipitate of urease crystals was obtained at this time. The precipitate was taken up in 0.02 *M* phosphate buffer (0.02 *M* potassium dihydrogen phosphate and 0.02 *M* disodium hydrogen phosphate, about 2:3, pH 7.0) or in water, and separated from insoluble material, if any, by centrifugation at 10,000 r.p.m. and 0°; the centrifugate will be designated as *Solution B*.

If a second crystallization was desired, Solution B was treated with citrate buffer and acetone and the precipitate separated as already described. The residue from centrifugation was taken up in 0.02 *M* phosphate buffer *(Solution C)*.

Since the enzyme may deteriorate on standing, the solutions finally obtained were used as soon as practicable, and in no case allowed to stand more than four days; in this period, little or no decrease in activity was noted. Some experiments indicate that solutions of enzyme in buffer are stable for much longer periods of time, but this matter has not been fully investigated.

Microscopic Examination

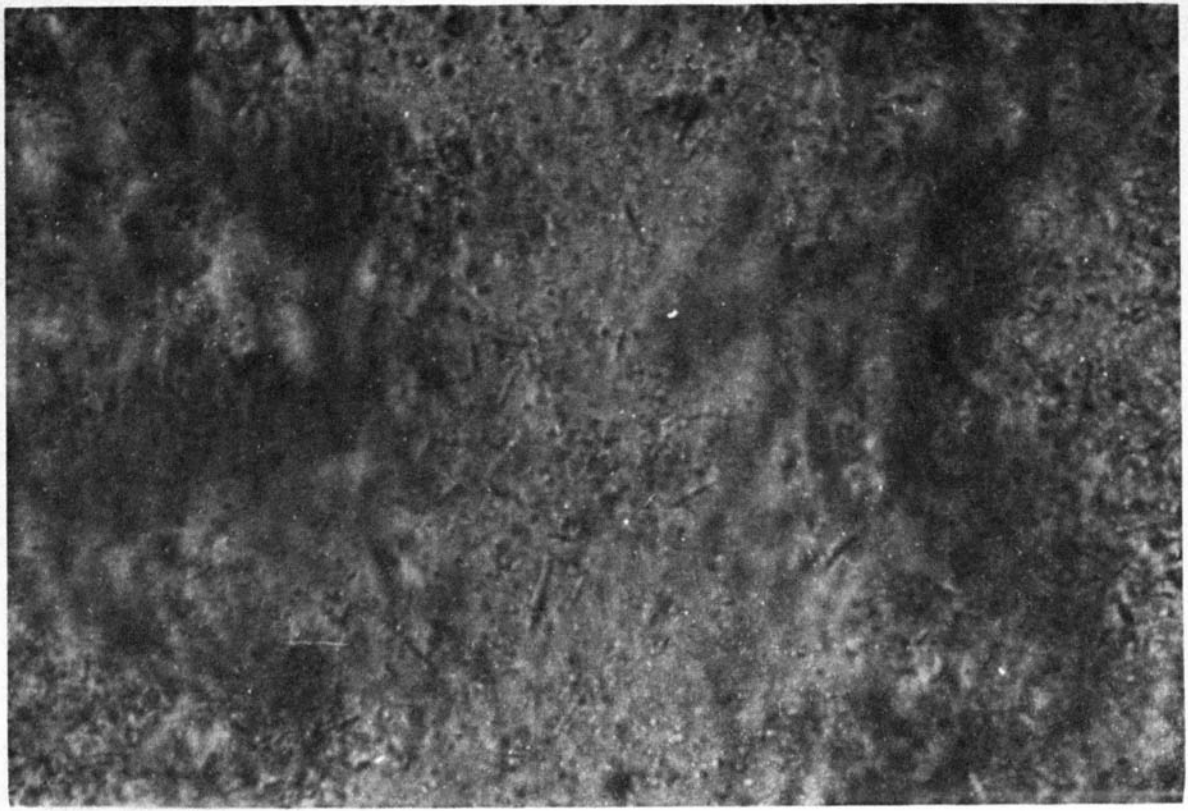
Crystalline urease was first discovered by microscopic examination of the aqueous acetone extract (Sumner, 1926), and such examination is helpful in developing the isolation procedure and following its course. A beautiful picture of large and well-defined crystals has been published (Sumner, 1951). However, the crystals of urease usually obtained are smaller and harder to see; indeed, they may be missed by the inexperienced observer. A discussion of the applicability and limitations of the technique may therefore be helpful.

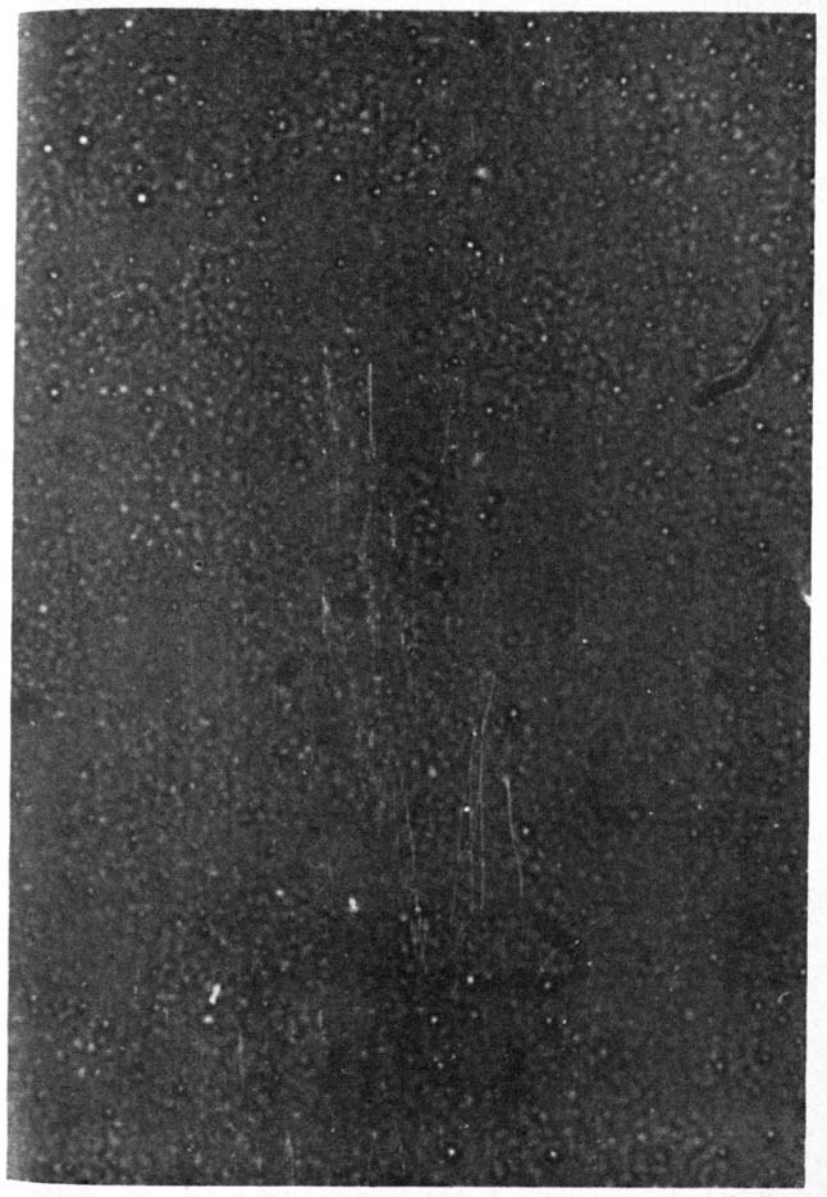
A good microscope is an absolute necessity. To view the crystals in the aqueous acetone filtrate, high power was used, and the illumination reduced so the background was rather dark. Against this background, the crystals stood out as shiny particles; at first sight they appeared quite round, and only by careful focusing the octahegral edges could be made out.

The size and number of crystals varied considerably, and it was quite difficult to estimate, even very roughly, how much crystalline enzyme was present in any preparation. For this reason, no systematic microscopic examination was made at this stage in the present work.

After the filtrate had been centrifuged, examination of the residue was more informative. For this purpose, a little material was suspended in centrifugate to give a thin suspension, and a droplet of this was examined. Plate I shows a representative view, obtained under high power. It should be noted that the field is full of small crystals; these are crystalline urease. Most of the crystals are not in sharp focus, because of the thickness of the sample; when the sample is being viewed, one can focus on individual crystals and better discern their geometrical shape. In addition, some amorphous material and some needle-like crystals can be seen. In some preparations, the amount of needle-like material was much greater than this.

When the residue is treated with water, the urease crystals dissolve more easily than the needle-like crystals and the amorphous material, and the proper amount of water to use in this step is that sufficient to effect this separation. Microscopic examination of the suspension being





extracted is helpful in determining how much water should be added. In the experiments being described, the amount of water was kept to a minimum in order to obtain as concentrated a solution of urease as possible, and several urease crystals could still be seen in the residue from the high-speed centrifugation. However, the amount of urease should now be small relative to the amorphous and needle-like material.

Plate II shows a representative microphotograph, taken under oil immersion, of a suspension of the precipitate obtained after adding buffer and acetone to Solution A and centrifuging. In this photograph, only one type of crystals can be seen. This preparation, when dissolved in buffer, had an activity of about 90 S.U./mg.

Results and Discussion of Procedure

The results of six preparations are summarized in Table I.

In each preparation, 400 g. of meal was used, which contained 92,000 S.U. Of this, it can be seen that only about one third was recovered in the filtrate. Although this is a large loss, it seemed more expedient to take this loss without trying to modify the procedure, inasmuch as a plentiful supply of meal was available. While it is easily possible to extract a greater amount of enzyme, e.g. by using a smaller proportion of acetone, this is accompanied by a greater amount of inactive material, and the subsequent separation is correspondingly more difficult.

In each extraction, 2000 ml. of aqueous acetone was used, and only 1400-1600 ml. were recovered; the remainder was absorbed on the meal. The amount of absorbed liquid is even greater when gravity filtration is used, as in Sumner's suggested procedure. Since the specific activity of the extracting liquid is presumably uniform, it is desirable to recover as much filtrate as possible, and this is the reason for utilizing suction filtration.

The filtrates contained 19-22 S.U./ml., and the specific activity is of greater importance than the total amount of activity recovered, since an appropriate level of activity at this point appears to be a necessary, though not a sufficient, condition for successful crystallization of the enzyme. In many of the early attempted preparations carried out in this work, the activity of the filtrate was much less than this, and no crystalline urease was indeed obtained (Sumner and Holloway, 1928).

In the extraction with aqueous acetone, considerable activity is lost, i.e. the remaining meal and absorbed extractant do not contain the difference between the initial total activity and that found in the filtrate. It appears, therefore, that the treatment causes considerable inactivation of the enzyme; since the activity of the aqueous acetone filtrate decreases very slowly in the cold, it is believed that inactivation occurs mainly while the extracting liquid is warm. In some of the early, unsuccessful experiments, exposure to aqueous acetone at room temperature was unduly prolonged, and this may have been the main, or a contributing, cause for obtaining filtrates of low specific activity. It should not be concluded, however, that the operation would be more successful if conducted at low temperature throughout; cold aqueous acetone does not extract the enzyme well.

The separation of crystals by centrifugation may give rise to some difficulty, since crystals as small as those obtained in this work and shown in Plate I are precipitated slowly. There are indications that some of the early failures may have been due, in part, to the fact that the crystals were not precipitated; a good refrigerated centrifuge was not available at that time. Even in the conditions described in the recommended procedure, the centrifugates still contained some urease crystals.

TABLE I
SUMMARY OF UREASE PREPARATIONS

	Filtrate	Centrifugate	Soln. A	Soln. B	Soln. C
No. 1					
Volume, ml.	1465	—	10	4	—
Activity, S.U./ml.	21.6	8.5	930	1010	—
No. 2					
Volume	1450	—	10	—	—
Activity	19.9	9.0	815	—	—
No. 3					
Volume	1560	—	10	4.5	—
Activity, S.U./ml.	19.7	6.6	1090	1010	—
Activity, S.U./mg. protein	—	—	—	96	—
No. 4					
Volume	1565	—	10	7	4.6
Activity, S.U./ml.	22.5	5.2	995	646	928
Activity, S.U./mg. protein	—	—	—	—	102
No. 5					
Volume	1569	—	10	5	5.5
Activity, S.U./ml.	20.7	4.9	822	948	841
Activity, S.U./mg. protein	—	—	—	—	96
No. 6					
Volume	—	—	10	4	—
Activity, S.U./ml.	20	5.1	819	1090	—
Activity, S.U./mg. protein	—	—	—	87	—

It should be noted that a variable, but in all cases quite appreciable, amount of enzyme activity remained in the centrifugate; this, of course, may be due mainly to urease that had not yet crystallized.

In some cases (not those described in detail above) a substantial "second crop" of crystals could be obtained on letting the centrifugate stand for an additional 24 hours. However, it did not prove worthwhile to carry out an additional centrifugation, and hence only the first crop of crystals was harvested routinely. The residues were taken up in about 12 ml. of water, and about 10 ml. of clear solution was obtained after high-speed centrifugation. In the early unsuccessful attempts, much larger volumes of water were used in the mistaken belief that the entire residue should be dissolved. Actually it is desirable at this point to separate the urease from the less soluble needle-like and amorphous materials, as has already been discussed. In the experiments being described, the amount of water used was, as a matter of fact, probably somewhat less than that which could be used to advantage; the volume of solution was kept at a minimum to make the subsequent crystallization as efficient as possible. A considerable amount of activity was found in the residue from the high-speed centrifugation, indicating that some urease had not been dissolved.

As can be seen, the first crystallization was attended by a considerable reduction of activity. On the other hand, a second crystallization could be carried out without much loss.

In some of the solutions finally obtained, the protein concentration was determined by Kjeldahl determination, using the factor 15.8% for the

nitrogen content. The activity could then be calculated in S.U. per mg. of protein, and the values are reported in Table I.

Acknowledgment

The authors are deeply indebted to Dr. W. L. Nelson, Department of Biochemistry, Cornell University, for gifts of urease meal and for much help and advice concerning all phases of the work described.

LITERATURE CITED

Ambrose, J. F., G. B. Kistiakowsky, and A. G. Kridl. 1951. Inhibition of urease by silver ions. J. Am. Chem. Soc., 73: 1232-1236.

Desnuelle, P., and M. Rovery. 1949. Sur l'inactivation de l'urease par l'isocyanate de phenyle. Biochim. Biophys. Acta, 3: 26-33.

Hellerman, L. F., F. P. Chinard, and V. R. Deitz. 1943. Protein sulfhydryl groups and the reversible inactivation of the enzyme urease. J. Biol. Chem., 147: 443-462.

Hofstee, B. H. J. 1948-9. The activation of urease. J. Gen. Physiol., 32: 339-349.

Kistiakowsky, G. B., P. C. Mangelsdorf, Jr., A. J. Rosenberg, and W. H. R. Shaw. 1952. The effects of electrolytes on urease activity. J. Am. Chem. Soc., 74: 5015-5020.

Landen, E. W. 1940. Quantum yield as a function of wavelength for the inactivation of urease. J. Am. Chem. Soc., 62: 2465-2468.

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

Sumner, J. B., and D. B. Hand. 1928. Crystalline urease. II. J. Biol. Chem., 76: 149-162.

Sumner, J. B., and R. G. Holloway. 1928. Variations in jack bean meal as affecting the yield of crystals. J. Biol. Chem., 79: 489-492.

Sumner, J. B., D. B. Hand, and R. G. Holloway. 1931. Studies of the intermediate products formed during the hydrolysis of urea by urease. J. Biol. Chem., 91: 333-341.

Sumner, J. B. 1937. The story of urease. J. Chem. Ed., 14: 255-259.

Sumner, J. B., N. Gralén, and I. Eriksson-Quensel. 1938. The molecular weight of urease. J. Biol. Chem., 125: 37-44.

Sumner, J. B., and E. B. Sisler. 1944. Crystalline urease vs. urease preparations for urea determinations. Arch. Biochem., 4: 207-210.

Sumner, J. B. 1951. Urease, in J. B. Sumner and Karl Myrback, "The Enzymes," Vol. I, New York, Academic Press, pp. 873-892.

Sumner, J. B. 1955. Urease, in S. P. Colowick and N. O. Kaplan, "Methods in Enzymology," Vol. II, New York, Academic Press, pp. 378-9.

Van Slyke, D. D., and G. E. Cullen. 1914. A permanent preparation of urease, and its use in the determination of urea. J. Biol. Chem., 19: 211-28.

Van Slyke, D. D., and R. M. Archibald. 1944. Manometric, titrimetric, and colorimetric methods for measurement of urease activity. J. Biol. Chem., 154: 623-642.