THE ISOLATION AND CRYSTALLIZATION OF THE ENZYME UREASE.

PRELIMINARY PAPER.

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After work both by myself and in collaboration with Dr. V. A. Graham and Dr. C. V. Noback that extends over a period of a little less than 9 years, I discovered on the 29th of April a means of obtaining from the jack bean a new protein which crystallizes beautifully and whose solutions possess to an extraordinary degree the ability to decompose urea into ammonium carbonate. The protein crystals, which are shown in Fig. 1, have been examined through the kindness of Dr. A. C. Gill, who reports them to be sharply crystallized, colorless octahedra, belonging by this definition to the isometric system. They show no double refraction and are from 4 to 5μ in diameter.

While the most active solutions of urease prepared in this laboratory by Sumner, Graham, and Noback[1] and by Sumner and Graham[2] possessed an activity of about 30,000 units per gm. of protein present, the octahedra, after washing away the mother liquor, have an activity of 100,000 units per gm. of dry material. In other words, 1 gm. of the material will produce 100,000 mg. of ammonia nitrogen from a urea-phosphate solution in 5 minutes at 20°C. At this temperature the material requires 1.4 seconds to decompose its own weight of urea.

The crystals, when freshly formed, dissolve fairly rapidly in distilled water, giving a water-clear solution after centrifuging from the slight amount of insoluble matter that is present. The solution coagulates upon heating and gives strongly the biuret,

[1] Sumner, J. B., Graham, V. A., and Noback, C. V., *Proc. Soc. Exp. Biol. and Med.*, 1924, xxi, 551.

xanthoproteic, Millon, Hopkins and Cole, ninhydrin, and unoxidized sulfur tests. The phenol reagent of Folin and Denis gives a strong color, while the uric acid reagent gives none. The material can be entirely precipitated by saturating with ammonium sulfate. The Molisch test is negative and Bial's test is negative also. The absence of pentose carbohydrate, as shown by Bial's

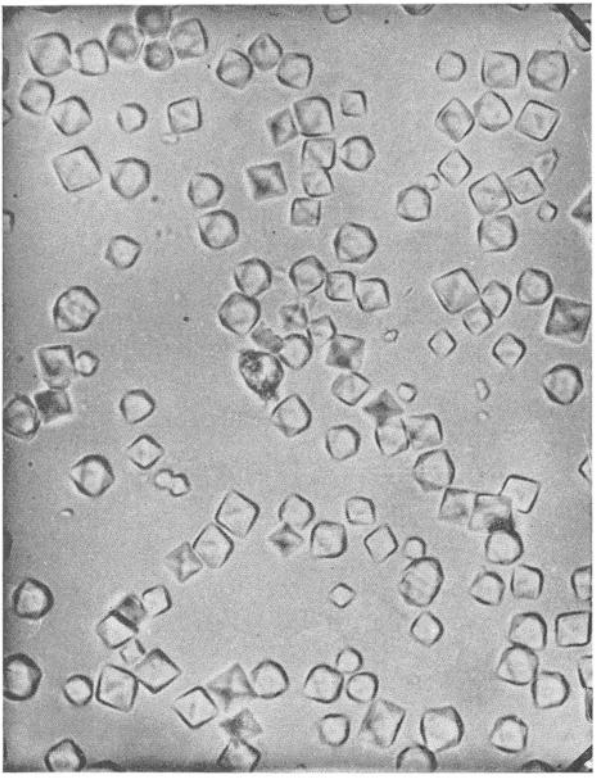

FIG. 1. Photomicrograph of urease crystals magnified 728 diameters.

test is especially pleasing as we have experienced a great deal of trouble in the past in freeing jack bean proteins from this substance.

The octahedral crystals, when freshly prepared, are very soluble in dilute alkali or dilute ammonia, and are either dissolved or coagulated by dilute mineral and organic acids, depending upon

the concentration of acid. Even so weakly acid a substance as primary potassium phosphate is capable of causing an irreversible coagulation. Although the crystals dissolve in distilled water I am inclined to regard the material as globulin inasmuch as a precipitate is formed when carbon dioxide is passed into its solution and this precipitate immediately redissolves upon the addition of a drop of neutral phosphate solution.

Owing to the fact that I have not had large enough amounts of the material to work with I am unable to give accurate figures for its nitrogen content at the present time, but this can be stated to be not far from 17 per cent, as shown by micro-Kjeldahl determinations made on several preparations. The content of ash is certainly low, so low that a considerable amount of material will have to be used to obtain this figure. Determination of the enzyme activity of the crystals has been somewhat interfered with, owing to the fact that dilute solutions of the crystals produce less ammonia from urea than one would calculate from results obtained from more concentrated solutions. If this effect is real, rather than apparent, it may be due to the instability of the enzyme at great dilutions. When in concentrated solution the activity is not lost very rapidly, provided the material is kept in the ice chest.

When old the crystals are entirely insoluble in distilled water, salt solutions, and dilute ammonia. In this condition the enzyme activity is almost nil. I have made several attempts to purify the fresh crystals by a second crystallization but have never succeeded in obtaining more than traces of crystals and these have been insoluble in water and inactive.

It may be worth noting that practically all of our previous ideas concerning the nature of urease appear to be confirmed by the discovery of the octahedral crystals and by study of their properties. I undertook the task of isolating urease in the fall of 1917 with the idea that it might be found to be a crystallizable globulin, in which case the proof of its isolation would be greatly simplified. Other reasons for choosing urease were that the quantitative estimation of urease is both rapid and accurate, that urease can be reasonably expected to be an individual enzyme, rather than a mixture of enzymes, and that the jack bean appears to contain a very large amount of urease, if it is permissible to draw a parallelism between the urease content of the jack bean and the

amounts of other enzymes found in other plant and animal materials.

In previous work in collaboration with Graham and Noback[1,2] and in unpublished work of my own it has been found that urease is very completely precipitated, together with the jack bean globulins, by cooling its 35 per cent alcoholic solution to −5 to −10°C., provided the reaction is sufficiently acid. We have found that urease can be precipitated by neutral lead acetate and neutralized cadmium chloride and that most of the urease can be reextracted by decomposing the precipitate with potassium oxalate; that urease can be precipitated by tannic acid without very much inactivation and that urease can be rendered insoluble, with loss of a part of its activity, by the action of dilute alcohol or very dilute acid.

Although the literature contains numerous references to a coenzyme of urease, I believe that no specific coenzyme exists. My evidence rests upon the fact that the loss of activity that occurs when the octahedral crystals are separated from a jack bean extract is almost exactly equal to the activity obtained when these crystals are washed with dilute acetone and then dissolved in water. If anything could separate an enzyme from its coenzyme crystallization might be expected to do so. The proteins in impure urease solutions doubtless exert a protective action as buffers and both proteins and polysaccharides may exert protective colloidal action.

I present below a list of reasons why I believe the octahedral crystals to be identical with the enzyme urease.

1. The fact that the crystals can be seen by the microscope to be practically uncontaminated by any other material.

2. The great activity of solutions of the crystals.

3. The fact that solvents which do not dissolve the crystals extract little or no urease and that to obtain solutions of urease one must dissolve the crystals.

4. The fact that the other crystallizable jack bean globulins, concanavalin A and B, carry with them very little urease when they are formed from solutions that are comparatively rich in urease.

[2] Sumner, J. B., and Graham, V. A., *Proc. Soc. Exp. Biol. and Med.*, 1925, xxii, 504.

5. The unique crystalline habit of the octahedra and their ready denaturation by acid.

6. The fact that the crystals are purely protein in so far as can be determined by chemical tests, combined with evidence from previous work to the effect that urease behaves like a protein in its reactions towards heavy metals, alkaloid reagents, alcohol, and acids.

7. The fact that the crystals are nearly free from ash and the fact that we have previously prepared solutions of urease that contained neither iron, manganese, nor phosphorus.

The method which I have used to obtain the crystals is extremely simple. It consists in extracting finely powdered, fat-free jack bean meal with 31.6 per cent acetone and allowing the material to filter by gravity in an ice chest. After standing overnight the filtrate is centrifuged and the precipitate of crystalline urease is stirred with cold 31.6 per cent acetone and centrifuged again. The crystals can be now dissolved in distilled water and centrifuged free from insoluble and inactive matter that has passed through the filter during the filtration. Of the urease extracted from the meal as much as 47 per cent may be present in the crystals. If one uses coarsely ground jack bean meal that has not been freed from fat the crystals are still obtained, but in traces only. I have carried out the process described above about fifteen times since first discovering the crystals and have always had success. The method is described in the experimental part of this paper in detail. It is probable that not all of the precautions that I have taken are necessary, but I think it best to give a description of the method exactly as it has been used by me.

Preparation of Urease Crystals.

Distill commercial acetone from a mixture of fused calcium chloride and a little soda-lime to remove water and acids. Place 158 cc. of the distillate in a 500 cc. graduate. Dilute with distilled water to the 500 cc. mark, mix, and cool to 22°C. Dilute exactly to mark and pour upon 100 gm. of "Arlco" jack bean meal which has been placed in a beaker. Stir for 3 or 4 minutes to break up the lumps. Now pour the material upon a 28 cm. Schleicher and Schüll filter, number 595. It is best to pour the mixture on the filter where there are four thicknesses and to add more only when

the paper has become moistened higher up, as in preparing the Folin-Wu blood filtrate, but even if this is done the filtrate may come through not quite clear at first and in this case the first 50 cc. should be refiltered. When all, or nearly all of the material is on the filter place in an ice chest at 2–2.5°C. and allow to remain overnight. The next morning centrifuge off the crystals that are present in the filtrate, using cold 50 cc. centrifuge tubes and cold holders for the tubes. (I have centrifuged for 7 minutes at a moderate speed and then cooled the tubes and holders in the ice chest for 10 minutes before centrifuging more of the filtrate.)

When all of the crystals have been centrifuged off drain the tubes against clean filter papers to remove the last drops of the mother liquor. The crystals can now be stirred up with 5 to 10 cc. of ice-cold 31.6 per cent acetone and centrifuged again and the tubes again drained against filter papers. The crystals are now dissolved in from 15 to 40 cc. of distilled water at room temperature by stirring, and the solution is centrifuged free from insoluble matter. For examination under the microscope it is best not to separate the crystals from all of the original mother liquor as they are insoluble in this.

The Arlco jack bean meal referred to above is an extremely finely ground, fat-free meal obtained from The Arlington Chemical Company, Yonkers, N. Y.

Estimation of Urease Action.

Make a solution containing 3 per cent of urea, 5.4 per cent of K_2HPO_4, and 4.25 per cent of KH_2PO_4; preserve with toluene and place in a bath of 20.0°C. Pipette 1 cc. of the diluted enzyme solution into a test-tube that contains no adsorbed mercury and allow to remain in bath to reach its temperature. Now rapidly add to the enzyme 1 cc. of the urea-phosphate solution and mix. Allow the action to proceed for 5 minutes. At the end of this interval blow into the solution 1 cc. of normal hydrochloric acid to inactivate the enzyme and mix immediately. Wash the material into a 100 cc. volumetric flask, dilute, Nesslerize, dilute to mark, mix, and compare with a standard containing 1 mg. of ammonium nitrogen that has been prepared at the same time. If the color of the unknown is much lighter or darker than that of the standard the test must be repeated, using a more con-

centrated, or a more dilute enzyme solution, as the case may be.

This procedure can be used only with solutions of urease that are relatively pure. For example, urease prepared by extracting jack bean meal with 30 per cent alcohol gives results by this method that are about 3 per cent too low on account of the interfering action of the protein that is present. The protein can be removed by precipitation with potassiomercuric iodide containing hydrochloric acid and an aliquot of the filtrate can be Nesslerized in cases of this sort.[3]

Determination of Dry Weight of Urease in Solutions.

As the addition of absolute alcohol to solutions of the crystals did not precipitate all of the protein, which was probably on account of the lack of electrolytes, I was obliged to pipette the solution into a weighed glass dish and evaporate to dryness in a boiling water oven. The temperature of the oven was about 94°C. The dish and contents were then dried to constant weight in a desiccator over sulfuric acid.

Determination of Total Nitrogen.

As I have not yet prepared the material on a large scale the macro-Kjeldahl method was not used. The material was digested in a large Kjeldahl flask with a small amount of sulfuric acid and copper sulfate. After digestion the material was washed into a large glass tube and the ammonia aerated off and Nesslerized. It was found impossible to digest the material in a glass tube, such as is ordinarily used, as it foamed very badly.

CONCLUSION.

A new crystallizable globulin has been isolated from the jack bean, *Canavalia ensiformis*. From the reasons given elsewhere in this paper I am compelled to believe that this globulin is identical with the enzyme urease.

I wish to thank Professor A. C. Gill for examining the crystals and Professor S. H. Gage and Professor B. F. Kingsbury for photographing them.

[3] Sumner, J. B., *J. Biol. Chem.*, 1919, xxxviii, 59.