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The chemical nature of enzymes

Nobel Lecture, December 12, 1946

Professor Westgren, Ladies and Gentlemen.

Why was it difficult to isolate an enzyme? Here I employ the word "isolate" as meaning preparing in pure condition. It does not seem difficult to isolate and crystallize an enzyme now, but it was difficult 20 years ago. The reasons were several. One was the inertia of men's ideas. Another reason was the influence exerted by Willstätter and his school, who held that enzymes were neither lipids, carbohydrates nor proteins and who believed enzymes to exist in excessively low concentrations in plants and animals. The misconception that colloidal substances had a chemistry different from that of crystalloids was another stumbling block. A yet more important reason why enzymes could not be isolated readily was because work with these substances requires a special technique. The enzyme is present in relatively low concentration in the raw material. It is often highly unstable. The raw material nearly always contains natural protectors which are left behind during the purification processes. In the absence of these natural protectors the enzyme may become destroyed by traces of heavy metals, by oxidation, by unfavorable pH, or even by autolysis.

I wish to tell next why I decided in 1917 to attempt to isolate an enzyme. At that time I had little time for research, not much apparatus, research money, or assistance. I desired to accomplish something of real importance. In other words, I decided to take a "long shot". A number of persons advised me that my attempt to isolate an enzyme was foolish, but this advice made me feel all the more certain that if successful the quest would be worthwile.

The reasons why I chose to work with urease were several. I had been working with urease as a reagent for the estimation of urea in muscle, blood and urine. This urease was prepared from soybean meal. In 1916 Mateer and Marshall found that the jack bean, *Canavalia ensiformis,* contained about 16-fold more urease than the soy bean. The jack bean appeared to me to be extraordinarily rich in urease and I could see no reason why this enzyme could not be isolated in pure form and characterized chemically. Claude Bernard has said that success or failure may depend upon the lucky choice of

some reagent or raw material. Willstätter was unfortunate in his choice of saccharase as an enzyme to isolate. I was fortunate in choosing urease.

I hoped that urease would turn out to be a globulin, since globulins are usually, if not always, present in beans and seeds, and since globulins are easy to precipitate by dialysis. Other reasons for choosing urease as the enzyme to isolate were because this enzyme can be estimated quantitatively very accurately and readily and because urease could be expected to be one enzyme, rather than a mixture of enzymes, acting as it does on such a simple substrate as urea.

I started trying to isolate urease in the fall of 1917, having been occupied previously with analytical methods. At this time our laboratory contained no adequate apparatus for grinding jack beans. We first used a coffee mill and then ground the coarse material with a mortar and pestle. Years later we constructed a mill which was run by an electric motor and which employed a bolting device. However, in the meanwhile we usually used commercial jack bean meal. This commercial meal was not always satisfactory.

The jack bean is a miniature world in itself and contains all of the elements required for life, growth and reproduction. I decided to isolate and characterize as many as possible of the chemical compounds present in the bean. I found various minerals, proteins, carbohydrates, lipids, extractives, pigments and enzymes to be present. Particular attention was paid to the proteins of the jack bean, since I expected to find that urease was one of these. I isolated two globulins in crystalline form and named these concanavalin A and concanavalin B. A third globulin which I called "canavalin" separated as spheroids upon dialysis. Years later Howell and I were able to crystallize this, after a preliminary digestion with trypsin. Many years later we discovered that concanavalin A is a hemagglutinin for the red cells of certain animal species as well as an excellent precipitant for certain polysaccharides. A lipid fraction of the jack bean was observed to function as a thromboplastic agent.

In attempting to concentrate and purify urease I employed fractional precipitation with alcohol, acetone and other organic solvents. Fractional precipitation with ammonium sulfate, magnesium sulfate and other neutral salts was tried. I tested a large number of salts of heavy metals as precipitants. I employed a very large number of reagents as adsorbents. This work covered many years. At times I grew discouraged and temporarily abandoned the quest, but always returned to it again.

At first we used to extract urease from jack bean meal with water. These aqueous extracts were viscous and therefore very difficult to filter. Glycerol

extracts were even more bothersome. I learned that Folin used 30 per cent alcoholic extracts of jack bean meal as a source of urease for analytical purposes. It was found that extraction with 30 per cent alcohol was of distinct advantage, inasmuch as this solvent dissolved most of the urease but failed to dissolve a rather large quantity of the other proteins. Hence a considerable purification was achieved through the use of this solvent. The alcoholic extracts filtered very rapidly, leaving the undissolved material behind on the filter paper. The only disadvantage of 30 per cent alcohol lay in the slow inactivating action of this solvent upon the urease. However, if kept at low temperatures there was no inactivation of the enzyme.

When kept at low temperatures 30 per cent alcoholic extracts of jack bean meal formed precipitates. These precipitates contained practically all of the urease, together with concanavalin A, concanavalin B and other proteins. At this time we had no ice chest in our laboratory and we used to place cylinders of 30 per cent alcoholic extracts on our window ledges and pray for cold weather.

It seemed to me of interest to employ dilute acetone instead of 30 per cent alcohol and to see whether this substitution would result in any improvement in the method of purification. Accordingly I diluted 316 ml of pure acetone to 1000 ml and used this as the means of extracting the urease. I

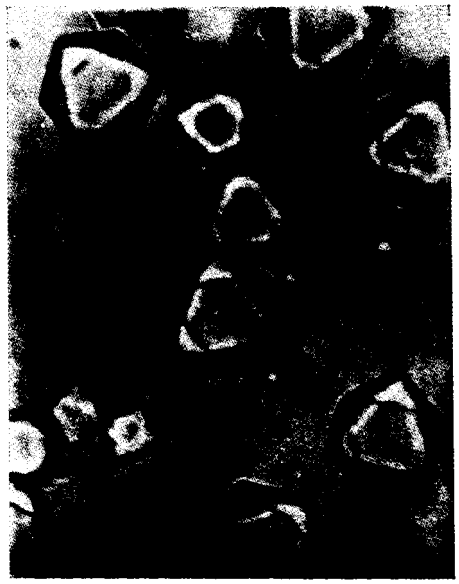

Fig. 1. Urease crystals.

routinely employed this dilution of acetone, since I had been preparing 30 per cent alcohol through diluting 95 per cent alcohol in this manner. The acetone extract was chilled in our newly acquired ice chest overnight. The next morning I examined the filtrate. It contained practically no precipitate, thus differing from alcoholic filtrates. However, upon observing a drop of the liquid under the microscope it was seen to contain many tiny crystals. These were of a shape that I had never observed previously. I centrifuged off some of the crystals and observed that they dissolved readily in water. I then tested this water solution. It gave tests for protein and possessed a very high urease activity. I then telephoned to my wife, "I have crystallized the first enzyme".

Now, I should like to tell this audience what enzyme crystals look like. (See Figs. 1 and 2.) This description applies also to proteins, since enzymes are proteins. Enzyme crystals are highly refractive and nearly always microscopic, since these compounds, being of high molecular weight, diffuse relatively slowly and therefore crystallize slowly. Enzyme crystals belong nearly always to the isometric or hexagonal systems. Enzymes may separate from solution as spheroids. This indicates a tendency to crystallize, as Dr. Northrop can tell you from his experience. Spheroids are nearer the crystalline state than purely amorphous material, such for example, as casein which has been

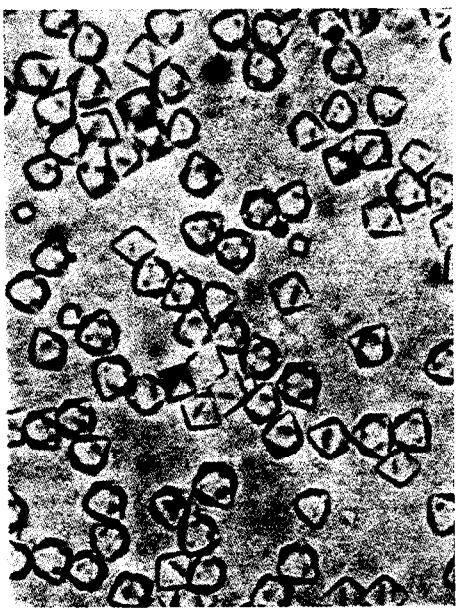

Fig. 2. Urease crystals.

precipitated from milk by the addition of acetic acid. Spheroids have sometimes been found by us to be aggregations of many needles either in parallel or concentrically arranged.

Having convinced myself that I had really isolated urease in crystalline form, I read a paper on this matter at Clifton Springs, New York and published an article in the August number of the Journal of Biological Chemistry for the year 1926. But now a difficulty arose. The commercial jack bean meal which we had been using suffered a decline in quality and we could obtain no urease crystals from it unless we added a small amount of acetic acid to the alcoholic filtrates. Even then, the yield of crystals was low. Analyses showed that the recent samples of jack bean meal contained only about one-half as much urease as the earlier samples. Accordingly attempts were made to obtain satisfactory meal, or satisfactory jack beans. We grew jack beans in one of the Cornell greenhouses. The beans grew poorly and the yield was less than the number of beans planted. We obtained jack beans from Texas, Guatemala and Honduras, but these were low in urease. I happened to meet a plant physiologist, Dr. Albert Muller, who said that he would grow me some jack beans at Mayaguez, Porto Rico. I gave him about a kilo of jack beans rich in urease, the last I had left. Some seven months later about a bushel of beans arrived from Porto Rico. These beans were rich in urease. The finely ground meal gave a high yield of urease crystals. Later on, we were able to obtain excellent jack beans from an Arkansas farmer. He has supplied us with jack beans ever since.

I wish to speak now about proofs of the identity of the crystals with urease. In cases of this sort one piece of experimental evidence is not sufficient to constitute a valid and satisfactory proof; one must submit many pieces of evidence. At this time I had no access to the ultra-centrifuge of Svedberg nor to the electrophoresis apparatus of Tiselius. However, I was able to offer evidence of another sort, as is shown below:

1. When the crystals separated there occurred a great increase in purity, namely from 700 to 1400-fold. Using other methods the increase in purity observed was very much less than this and at times there even occurred a decrease in purity.

2. When the distribution of urease was followed quantitatively it was found that of the urease passing into the filtrate as much as 40 per cent or more separated with the crystals.

3. Recrystallization increased the purity of the urease.

4. Adding traces of poisons, such as mercuric chloride or formaldehyde, to

jack-bean meal inactivated the urease and, at the same time, prevented the appearance of the crystals.

In obtaining the crystals I felt much the same as a person does who is trying vainly to place in position a piece of a machine. Suddenly the piece slides in as if covered with butter. One knows that it is now where it belongs.

During later work in crystalline urease I was fortunate to have in our laboratory a number of excellent men. These were Doctors Hand, Howell, Kirk, Poland and Dounce. We found that trypsin neither digested nor inactivated urease. Since trypsin is a proteolytic enzyme of second attack, it does not digest certain native proteins readily. Some proteins that are digested with great difficulty are hemoglobin, ovalbumin and the serum proteins. After urease had been denatured by acid or by heating it was found to be very readily digested by trypsin.

Pepsin acts best in a strongly acid medium and strong acid rapidly destroys urease. However, at pH 4.3 we found that urease was so slowly destroyed that it was possible to demonstrate a parallel digestion and inactivation by pepsin. In place of pepsin it was possible to use papain-cysteine.

In our laboratory Dr. J. Stanley Kirk was able to immunize rabbits to crystalline urease. Kirk started by giving rabbits as little as 0.03 mg of crystalline urease intraperitoneally. This dose was given twice weekly and was finally increased to 1000 lethal doses. The immunized rabbits contained antiurease in their blood serum. This antiurease could be purified by precipitating it by adding urease, washing the urease-antiurease precipitate, decomposing the complex with 0.05 N hydrochloric acid, bringing to pH 5.0 and centrifuging down the denatured urease. The antiurease was not harmed by this treatment and could be employed as an excellent precipitant in testing for urease. It gave a visible precipitate with solutions of urease diluted 1 to 500,000. However, urease allowed to stand for a few seconds with 0.05 *N* hydrochloric acid and then neutralized gave no precipitate with antiurease; neither did it possess any urease activity.

Northrop has made good use of physical methods to demonstrate the identity of pepsin with his pepsin crystals. While we have not employed such methods, Kubowitz and Haas, working in Warburg's laboratory have demonstrated that ultraviolet light is absorbed by highly dilute solutions of crystalline urease and that exactly the same wavelengths that are absorbed are those which destroy urease.

In 1930 Northrop of the Rockefeller Institute obtained pepsin in crystalline form. A short time later Northrop and Kunitz obtained crystalline tryp-

sin, crystalline chymotrypsin and also the zymogens of these enzymes in crystalline form. This monumental work was of very great help in bringing the scientific world to admit that enzymes can be isolated in pure and crystalline condition. In this connection I wish to note that Professor von Euler aided me greatly when I worked on urease in his laboratory at Stockholm's Högskola in 1929 and that I received valuable help in 1937 while working in the laboratory of Professor The Svedberg at the University of Uppsala.

The announcement of the crystallization of urease and pepsin was not accepted by some biological chemists. In Germany students of Willstätter attempted to show that our crystalline proteins were merely carriers of the enzymes. It suffices to say that these attempts to disprove our work failed, as they were bound to fail, since Northrop and I were right and since our evidence was unassailable.

To date about thirty enzymes have been obtained in crystalline and in presumably pure condition. Certain enzymes are colored, due to being combinations of specific proteins with such prosthetic groups as riboflavin phosphate, or hematin. Theorell has crystallized the yellow enzyme of Warburg and Christian and, remarkably enough, has been able to split the protein from the riboflavin phosphate and later to reunite these two components. He has shown conclusively that the union of these components proceeds stoichiometrically.

The enzymes catalase and peroxidase are compounds of protein with hematin and these two enzymes possess characteristic absorption bands. This property has greatly facilitated the crystallization of these two enzymes. In addition, it should be noted that these two enzymes have been shown to form compounds with hydrogen sulfide, sodium fluoride, sodium azide, etc.

All enzymes are proteins but not all proteins are enzymes. Many, if not all enzymes can be crystallized. The oxidizing enzymes all appear to be conjugated, or compound, proteins, while the hydrolytic enzymes, are, as far as we can tell, without prosthetic groups. However even hydrolytic enzymes must have reactive groups. Every enzyme requires a specific method for its purification. Our present methods of purifying both enzymes and proteins are crude and unsatisfactory for the most part. It is fairly certain that better methods will be discovered in the near future. One can purify an enzyme either by precipitating the impurities or else by precipitating the enzyme. The latter procedure is to be preferred, but the former procedure is often necessary at the beginning. When I speak of precipitating the enzyme I mean, of course, a more or less specific precipitation and not a general

precipitation of everything, such as occurs on adding a great excess of alcohol or acetone.

Some day every enzyme in living matter will have been discovered and described. Every chemical reaction which goes on will have been recorded. We can probably expect to find some enzymes which are glycoproteins, others which are lipoproteins and others which are nucleoproteins.

In 1917 the role played by enzymes was only partially understood. Digestive enzymes were well known, autolytic and oxidative enzymes were somewhat known, but not well understood. At present we realize the tremendous complexity of the cell. In muscle alone some sixty enzymes are known to occur. Thanks to relatively recent investigation, practically all of the complicated reactions involved in the breaking down of glycogen to carbon dioxide and water have been made clear.

The organic chemist has never been able to synthesize cane sugar, but by using enzymes, the biological chemist can synthesize not only cane sugar but also gum dextran, gum levan, starch and glycogen.

We know now of the existence of enzymes which employ phosphoric acid instead of water and which might be given the general term "phosphorases". There are the phosphorylases, transphosphorylases, phosphoisomerases, phosphomutases and phosphodismutases.

From the work of Cori and his associates we now have evidence that hormones function through their effect on enzymes. Thus, glucose is transformed into glucose-6-phosphate when it reacts with ATP in the presence of the enzyme known as hexokinase. This reaction, essential for utilization of glucose, is inhibited by the diabetogenic hormone coming from the anterior pituitary. This inhibiting action is abolished by insulin.

We can sum up by saying that as the result of discoveries in the field or enzyme chemistry some questions have been answered and many new questions have arisen. We live in an expanding universe in more senses than that of the astronomers.

In conclusion I wish to pay tribute to my former teacher, Otto Folin, who emigrated as a boy of seventeen from Småland to America and who, as Professor of Biochemistry at Harvard, inspired me as he did many other young men.