THE HYDROLYSIS OF PHYTIN BY THE ENZYME PHYTASE CONTAINED IN WHEAT BRAN.

TWELFTH PAPER ON PHYTIN.

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INTRODUCTION.

It has been shown by the investigations of Suzuki, Yoshimura, and Takaishi[1] that rice bran contains an enzyme which rapidly hydrolyses phytin with formation of inosite and inorganic phosphoric acid. These authors concluded that wheat bran likewise contained a similar enzyme, because the inorganic phosphorus increased in wheat bran extracts on standing.

Plimmer[2] examined a large number of extracts prepared from the intestines, liver, pancreas, castor beans, etc., as to their action on organic phosphorus compounds. While some of these showed a slight cleavage action on phytin, none could be compared in activity to an aqueous extract of wheat bran. The hydrolytic action of these extracts was determined by estimating from time to time the amount of inorganic phosphorus split off from phytin solutions of known concentration.

Since the above experiments clearly demonstrated that large quantities of inorganic phosphate were liberated from phytin by wheat bran extracts, it appeared of interest to determine what products, in addition to inosite and inorganic phosphoric acid, were formed under these conditions. For this purpose wheat bran extract was allowed to act upon a dilute solution of phytin

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[1] U. Suzuki, K. Yoshimura, and M. Takaishi: *Bulletins of the College of Agriculture*, Tokyo, vii, pp. 503–512, 1907.

[2] R. H. A. Plimmer: *Biochem. Jour.*, vii, p. 43, 1913.

at a temperature of 37°. Inorganic phosphoric acid was determined in the solution from time to time.

It was found that about two-thirds of the total phosphorus was split off during the first sixteen days. Afterwards there was no appreciable change even on standing for about two years.

The solution had been prepared and the original determinations made by Dr. Plimmer. At his suggestion the writer undertook to examine the final reaction mixture for such products as had been formed.

These products were separated into two portions by precipitating the original solution with barium hydroxide. The precipitate contained inorganic barium phosphate and also those barium salts of organic phosphoric acids that were insoluble in the dilute alkaline solution. The filtrate, on the other hand, was found to contain inosite monophosphate, and free inosite.

The inorganic phosphate and other impurities were removed from the crude barium hydroxide precipitate, as will be described in the experimental part. The organic phosphoric acids which remained were obtained as amorphous barium salts. It was impossible to isolate any unchanged barium phytate. It is evident then that all the phytin had been partially hydrolyzed.

The above amorphous substance appeared to consist mainly of barium inosite triphosphate, but probably mixed with some barium inosite diphosphate. Owing to the difficulty of separating these compounds their isolation was not attempted.

Among the soluble substances which had been formed we were able to isolate and identify inosite monophosphate, a substance which we have previously isolated from wheat bran.[3] In addition to this, the solution also contained some free inosite which was isolated by means of its lead compound.

The action of this enzyme, phytase, upon phytin appears to proceed in several stages. Only a portion of the phytin is completely decomposed into inosite and phosphoric acid, but all the phytin is partially hydrolyzed with formation of certain lower phosphoric acid esters of inosite; *viz.*, inosite tri-, di-, and monophosphate, and inorganic phosphoric acid. The formation of these intermediate products is only possible through the destruc-

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[3] R. J. Anderson: this *Journal*, xviii, p. 441, 1914; and *New York Agricultural Experiment Station Technical Bulletins*, No. 36, 1914.

tion or inhibition of the enzyme before the hydrolysis is complete. The reason for this inhibition is not clear, but it may be due to the excess of phosphoric acid which is liberated.

It is interesting to note, and we call particular attention to the fact, that the organic phosphoric acids which remain as intermediate products of the action of the enzyme upon phytin, *viz.*, inosite triphosphate and inosite monophosphate, are identical with the substances which we have isolated previously from wheat bran after it has been digested in 0.2 per cent hydrochloric acid.

EXPERIMENTAL PART.

Commercial phytin, 100 grams, was dissolved in 500 cc. of water and filtered from the insoluble matter which weighed 3.5 grams when dried at 100°. The pale yellow solution was treated with 38 grams of oxalic acid dissolved in about 250 cc. of water. The calcium oxalate was filtered off, washed, and dried. It weighed 48 grams. The solution was diluted to 6000 cc. with water and was then found to contain 40 grams of phosphorus pentoxide. To it were added 800 cc. of an aqueous extract of wheat bran which contained 2.2 grams of P_2O_5. The solution was kept under toluene at a temperature of 37°. No hydrolysis occurred in a week. This was evidently due to the strongly acid reaction of the solution. It was nearly neutralized with ammonia, and 735 cc. of bran extract containing 1.53 grams of P_2O_5 were added. It was again kept at a temperature of 37° under toluene. In nine days one-half of the total phosphorus was hydrolyzed; in sixteen days two-thirds was hydrolyzed. In thirty-five days the amount of hydrolysis had not altered, and after about two years it was again the same. The total and inorganic phosphorus was determined as described by Plimmer and Page.[4]

The dark colored solution was filtered and barium hydroxide (Kahlbaum) added in slight excess. After standing over night the precipitate was filtered and washed in water. The filtrate and washings were evaporated on the water bath and the residue was examined, as will be described later.

The barium precipitate was dissolved in about 2.5 per cent hydrochloric acid, filtered, and precipitated by adding about an

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[4] R. H. A. Plimmer and H. J. Page: *Biochem. Jour.*, vii, p. 162, 1913.

equal volume of alcohol. The precipitate was filtered and washed in dilute alcohol. The substance was again precipitated four times in the same way. It was then precipitated by barium hydroxide three times from about 2 per cent hydrochloric acid, and finally twice more with alcohol from the same strength hydrochloric acid. After finally filtering it was washed free of chlorides in dilute alcohol and then in alcohol and ether and dried in vacuum over sulphuric acid. The substance was then a snow-white amorphous powder. It weighed 28.4 grams. It was free from chlorides and inorganic phosphate, and bases other than barium could not be detected.

The substance was then rubbed up in a mortar with 300 cc. of cold water and allowed to stand with occasional shaking for a few hours. It was then filtered and washed in water, alcohol, and ether, and dried in vacuum over sulphuric acid. The dry water-insoluble portion weighed 5.4 grams.

The filtrate from above was neutralized to litmus with barium hydroxide. The precipitate was filtered and washed in water, alcohol, and ether, and dried in vacuum over sulphuric acid. It weighed 23.6 grams.

These precipitates were analyzed after drying at 105° in vacuum over phosphorus pentoxide.

The first, water-insoluble portion gave the following result:

Found: C = 11.46; H = 1.93; P = 11.59; Ba = 39.94 per cent.

This substance is apparently largely composed of the dibarium inosite triphosphate.

Calculated for this: $C_6H_{11}O_{15}P_3Ba_2 = 690$.
C = 10.43; H = 1.59; P = 13.47; Ba = 39.71 per cent.

It is, however, not pure, but apparently contains some barium inosite diphosphate; because the carbon is high and the phosphorus is low.

The water-soluble substance which was precipitated with barium hydroxide gave the following:

Found: C = 9.63; H = 1.63; P = 10.91; Ba = 47.41 per cent.

This substance also appears to consist largely of the neutral barium salt of inosite triphosphate.

Calculated for the latter: $C_6H_9O_{15}P_3Ba_3 = 826$.
$C = 8.71$; $H = 1.08$; $P = 11.25$; $Ba = 49.88$ per cent.

The carbon, however, is high, and the phosphorus as well as the barium are low; which points to the presence of barium inosite diphosphate.

In the hope of approximately separating these barium inosite tri- and diphosphates, the substance, 23.6 grams, was digested in dilute acetic acid for several hours with occasional shaking. It was then filtered and washed in water, and the insoluble portion dried in vacuum over sulphuric acid. It weighed 10 grams.

The filtrate and washings containing the soluble portion of the substance were precipitated by adding lead acetate in excess. After standing over night the white amorphous precipitate was filtered and washed in water. It was suspended in water and decomposed by hydrogen sulphide, filtered, and the excess of hydrogen sulphide boiled off. It was again precipitated in the same manner with lead acetate and decomposed with hydrogen sulphide. The solution still contained a considerable quantity of barium. The barium was therefore removed with a slight excess of dilute sulphuric acid. After filtration of the barium sulphate the solution was precipitated by adding copper acetate in excess. The copper precipitate was filtered, washed, suspended in water, and decomposed with hydrogen sulphide. After removing the copper sulphide, the filtrate was evaporated in vacuum to small bulk, and finally dried in vacuum over sulphuric acid. There remained a thick, nearly colorless syrup. It was readily soluble in alcohol. The addition of chloroform to this solution caused the substance to separate in small oily drops; the addition of ether produced a cloudiness, and on standing a flocculent amorphous precipitate separated. These solutions could not be brought to crystallize. The acid preparation itself was kept for several weeks in the desiccator over sulphuric acid. It became a hard, sticky mass, but showed absolutely no tendency to crystallize. The color of the preparation, kept in this manner, gradually darkened.

Since the acid would not crystallize, the syrupy substance was analyzed after drying at 105° in vacuum over phosphorus pentoxide.

Found: C = 18.58; H = 3.82; P = 20.38 per cent.
Calculated for inosite triphosphoric acid: $C_6H_{15}O_{15}P_3 = 420$.
C = 17.14; H = 3.57; P = 22.14 per cent.
Calculated for inosite diphosphoric acid: $C_6H_{14}O_{12}P_2 = 340$.
C = 21.17; H = 4.11; P = 18.23 per cent.

This acid preparation is evidently also a mixture of the inosite tri- and diphosphoric acids.

EXAMINATION OF THE FILTRATE AFTER THE WATER-INSOLUBLE BARIUM SALTS HAD BEEN PRECIPITATED.

The filtrate was evaporated, as mentioned on page 477, and the residue taken up in hot water. It was decolorized with animal charcoal. The solution was neutral in reaction. It strongly reduced Fehling's solution on boiling, possibly due to sugars introduced with the bran extract. The solution was found to contain barium and also phosphorus in organic combination, evidently inosite monophosphate. The aqueous solution was precipitated by adding about an equal volume of alcohol and the white amorphous precipitate filtered off, the filtrate being reserved for further examination.

Isolation of inosite monophosphate. The above precipitate, which formed on the addition of alcohol, was dissolved in water, slightly acidified with acetic acid, and then precipitated with lead acetate in excess. After settling, this was filtered, washed in water, suspended in hot water, and decomposed with hydrogen sulphide. It was then filtered, and the filtrate boiled to expel excess of hydrogen sulphide. It was reprecipitated several times with lead acetate in the same manner until a white lead precipitate was obtained. This was finally decomposed with hydrogen sulphide, filtered, and evaporated to small bulk in vacuum and then dried in vacuum over sulphuric acid until a thick syrup remained. On scratching with a glass rod, this crystallized to a white solid mass. It was digested in alcohol and filtered, washed in alcohol and ether, and dried in the air. It weighed 1.6 grams. It had all the properties of inosite monophosphate. For further purification it was dissolved in a few cubic centimeters of water and filtered. Alcohol was then added until the solution turned cloudy; it was heated until it cleared up, and more alcohol was added until a faint permanent cloudiness remained. It was allowed to stand for about forty-eight hours at room temperature, when the substance had separated in massive, practically colorless crystals. After filtering, washing in alcohol and ether, and drying in the air, 1 gram of substance was obtained. When heated in a capillary tube it began to soften at 188°–189° and melted with decomposition and effervescence at 190°C. (uncorrected). The appearance and properties of the substance corresponded exactly with those described for inosite monophosphate and the analysis was therefore omitted.

Isolation of inosite. The filtrate, after precipitation of the above barium salt of inosite monophosphate with alcohol, was evaporated on the water bath until the alcohol was removed. It still contained barium, chlorides, etc. The barium was quantitatively precipitated with dilute sulphuric acid and the solution again concentrated on the water bath.

The addition of lead acetate caused no precipitate. Basic lead acetate was then added as long as any precipitate formed. This precipitate was filtered off and discarded. The solution was then heated to boiling, more basic lead acetate added, and the solution was finally made strongly alkaline with ammonia and allowed to stand over night. This precipitate was filtered and washed in water, and then decomposed in aqueous suspension with hydrogen sulphide. The filtrate was concentrated on the water bath and the inosite brought to crystallization by the addition of alcohol. After recrystallizing several times 0.5 of a gram of pure inosite was obtained in the characteristic needle-shaped crystals. It gave the reaction of Scherer and melted at 218°C. (uncorrected).

SUMMARY.

The chief products of the hydrolysis of phytin by the phytase in wheat bran are inorganic phosphoric acid and certain intermediate compounds apparently consisting of inosite tri-, di-, and monophosphates. These intermediate substances are identical with the compounds which we have previously isolated from 0.2 per cent hydrochloric acid extracts of wheat bran.

A portion of the phytin was completely hydrolyzed by the action of the enzyme into phosphoric acid and inosite; because the solution was found to contain some free inosite.

All the phytin was at least partially hydrolyzed; since the final reaction mixture did not contain any unchanged inosite hexaphosphate.