LXXVIII. THE OCCURRENCE OF A PHYTIN-SPLITTING ENZYME IN THE INTESTINES OF ALBINO RATS¹

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A LARGE proportion of the total phosphorus of the cereals and other vegetable foodstuffs has been shown to exist in the form of phytin, the calcium magnesium salt of inositolhexaphosphoric acid. McCance & Widdowson [1935] have shown that the amount of phytin-P may vary between 41 and 68 % of the total phosphorus of the cereals. According to Bruce & Callow [1934] the availability of this form of phosphorus to the mammalian organism is very low. A summary of work dealing with this aspect may be found in the paper by McCance & Widdowson. That phytin does not undergo alteration in the digestive tract of animals was first shown by Scofone [1905]. Plimmer [1913] found that extracts of the intestines of the dog, cat, sheep, ox and rabbit failed to hydrolyse the sodium salt of phytic acid. McCollum & Hart [1908], however, found in calf liver a phytin-splitting enzyme. Lowe & Steenbock [1936, 1] failed to notice any hydrolytic action of intestinal extracts of rats and chicks on phytin, even though they observed a certain amount of hydrolysis of phytin in the intestines when it was fed to the rats. They are of the opinion [Lowe & Steenbock, 1936, 2] that any hydrolysis of phytin which takes place in the intestines is due to the activity of the intestinal flora or to the phytase of the vegetable part of the diet.

A search by the author for a phytin-splitting enzyme in the intestines of the albino rats has proved successful and the experimental evidence is presented in the following pages.

EXPERIMENTAL

Preparation of the enzyme. The method of extraction of the enzyme from the intestines was similar to that used by Martland & Robison [1929] for the extraction of phosphatase from bones. Albino rats (4 to 6 at a time) were killed with chloroform, their intestines were removed, slit open and washed carefully in running water to free them of all extraneous matter. They were then ground with 5 to 10 ml. of chloroform and left in contact with about 50 ml. distilled water at room temperature for 4 days. An active extract was thus obtained on filtration, but it contained appreciable quantities of inorganic phosphate. Attempts to remove this by dialysis sometimes resulted in a loss of activity of the enzyme. The addition of five volumes of 95 % alcohol to the extract gave a precipitate which was separated by centrifuging, washed with absolute alcohol and dried *in vacuo* over sulphuric acid. It was then suspended in a small volume of water or in Michaelis's [1931] veronal-acetate-HCl buffer solution of pH 7.6, allowed to stand for 2 hours, centrifuged and the centrifugate dialysed in cellophane bags against distilled water or buffer solution of pH 7.6 for 4-6 hours. This procedure gave an active enzyme preparation containing only small

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amounts of inorganic phosphate. Dialysis for longer periods was not tried for fear of a loss of activity.

Preparation of the barium salt of inositolhexaphosphoric acid. Commercial phytin was dissolved in dilute hydrochloric acid and the solution filtered. The filtrate was adjusted to pH 4.0 with baryta and to it were added two volumes of 95 % alcohol. A white precipitate was formed, which was removed by filtration, washed with alcohol and dried *in vacuo* over sulphuric acid. The precipitation at pH 4.0 was repeated twice; finally the acid barium salt was dissolved in dilute hydrochloric acid and the solution was adjusted to pH 8.4 with baryta. The insoluble barium salt separated as an amorphous precipitate. One volume of 95 % alcohol was added and the precipitate was separated by filtration, washed with absolute alcohol and dried *in vacuo* over sulphuric acid. Found: P, 12.17 %; inorganic P, 0.20 %. $C_6H_6(PO_4Ba)_6$ requires P, 12.64 %.

For hydrolytic experiments the barium was removed by precipitating with the requisite amount of *N* sulphuric acid, and *N* NaOH was added to pH 8.0 to give the sodium salt. A determination of the total and inorganic P in the final solution gave the amount of phytin-P in solution.

All the following experiments have been carried out at 37° in presence of chloroform. Some experiments have been made with sodium glycerophosphate as substrate mainly for purposes of comparison. Phosphorus was determined by the Bell & Doisy [1920] modification of Briggs's method.

Hydrolysis of sodium phytate and sodium glycerophosphate by the intestinal enzyme preparations. To 0.50 ml. of the substrate were added 0.50 ml. enzyme preparation and 0.40 ml. of 0.012 *M* $MgSO_4$ or water. Total and inorganic P were determined in an aliquot portion. The results are given in Table I.

Table I

	pH	Inorganic P liber- ated in 43 hours mg. per ml.	Total P mg. per ml.	% hydro- lysis
A.† In unbuffered solutions:				
Sodium phytate + enzyme preparation 2	8.4	0.228	0.567	40.2
„ + boiled enzyme preparation 2	8.4	0.00	0.567	Nil
„ + enzyme preparation 3	8.6	0.138	1.256	11.0
„ + boiled enzyme preparation 3	8.6	0.03	1.256	Trace
„ + enzyme preparation 3 + $MgSO_4$	8.6	0.316	1.20	26.3
„ + boiled enzyme preparation 3 + $MgSO_4$	8.6	0.04	1.20	Trace
Sodium glycerophosphate + enzyme preparation 3 + $MgSO_4$	8.4	1.51	1.622	93.1
„ + boiled enzyme preparation 3 + $MgSO_4$	8.4	0.02	1.622	Trace
B.*† In buffered solutions:				
Sodium phytate + enzyme preparation 13 + $MgSO_4$	7.4	0.215 in 24 hr.	0.514	41.7
Sodium glycerophosphate + enzyme preparation 13	7.4	0.387 in 24 hr.	0.424	91.3

* 1 ml. substrate + 2 ml. enzyme preparation + 1 ml. buffer mixture.

† In A the concentration of $MgSO_4$ was 0.0034 *M* and in B 0.00187 *M*.

The effect of pH on the hydrolysis of sodium phytate by the intestinal enzyme preparations. To 1.0 ml. of the substrate were added 2.0 ml. buffer solution (Michaelis) of the desired pH and 1.0 ml. of the enzyme preparation. Total and inorganic P were determined at 0 hour and after 24 hours. The results in % phytin-P hydrolysed are given in Table II.

Table II

pH	8.6	8.2	7.8	7.4	7.2	7.0	6.8	6.6	6.1
% hydrolysis	9.9	12.0	12.7	11.2	10.9	7.9	7.6	7.7	6.0

The hydrolysis obtained in the above experiment is of a low order. Owing to the generally low activity of the enzyme, dilution with buffer solution resulted in further lowering of the activity. More active enzyme preparations caused greater hydrolysis but they also caused a shift in the pH of the reaction mixture. The above experiment has been given because no change in pH was observed in this case, and although the figures for hydrolysis are low they represent the effect of the change of pH on the rate of hydrolysis of sodium phytate by the intestinal enzyme preparations.

The influence of Mg^{++} ions on the hydrolysis of sodium phytate. To 1.0 ml. of the substrate were added 2 ml. enzyme preparation and varying amounts of 0.015 *M* $MgSO_4$ solution. The volume was made up to 4 ml. by the addition of buffer mixture of pH 7.4. The tubes were incubated for 24 hours. The results are given in Table III.

Table III

Particulars	$MgSO_4$ <i>M</i>	P liberated in 24 hr. mg. per ml.	Total phytate-P mg. per ml.	% hydro- lysis
With enzyme preparation 13	Nil	0.146	0.512	28.5
	0.00093	0.197	0.514	38.5
	0.00187	0.215	0.514	41.7
	0.00375	0.204	0.516	39.5
With enzyme preparation 12	Nil	0.123	0.221	55.7
	0.00093	0.187	0.224	83.9
	0.00187	0.178	0.222	80.3

The optimum seems to lie between 0.00093 and 0.00187 *M* concentration of Mg^{++} ions.

Experiments with enzyme preparations from the intestines of the guinea-pig and the rabbit and the bones of the rat and the rabbit. The methods of extraction of

Table IV

	Inorg. P		Inorg. P released mg. per ml.	Total P mg. per ml.	% hydro- lysis
	At 0 hr. mg. per ml.	At 24 hr. mg. per ml.			
A. Intestinal enzymes:					
Sodium phytate + guinea-pig enzyme prep.	0.014	0.052	0.038	0.518	7.2
Sodium phytate + guinea-pig enzyme prep. + MgSO ₄	0.012	0.056	0.044	0.515	8.6
Sodium glycerophosphate + guinea-pig enzyme prep.	0.01	0.046	0.036	0.452	7.9
Sodium glycerophosphate + guinea-pig enzyme prep. boiled	0.018	0.021	—	—	Nil
Sodium phytate + rabbit enzyme prep.	0.041	0.054	0.013	0.577	Trace
Sodium phytate + rabbit enzyme prep.	0.041	0.052	0.011	0.577	Trace
Sodium glycerophosphate + rabbit enzyme prep.	0.036	0.376	0.340	0.500	71.7
Sodium glycerophosphate + rabbit enzyme prep. boiled	0.034	0.040	0.006	0.500	Nil
B. Bone enzymes:					
Sodium phytate + rabbit bone enzyme + MgSO ₄	0.016	0.018	Nil	0.533	Nil
Sodium phytate + rabbit bone enzyme + MgSO ₄	0.016	0.016	Nil	0.533	Nil
Sodium glycerophosphate + rabbit bone enzyme + MgSO ₄	0.020	0.424	0.404	0.452	89.4
Sodium glycerophosphate + rabbit bone enzyme boiled + MgSO ₄	0.016	0.016	Nil	0.533	Nil
Sodium phytate + rat jaw-bone enzyme	0.025	0.025	Nil	0.701	Nil
Sodium glycerophosphate + rat jaw-bone enzyme	0.00	0.920	0.920	2.498	36.8

N.B. All reactions were carried out at pH 7.4 except in the last two experiments where pH values were 8.7 and 9.4 respectively.

the respective enzymes were similar to that used in the case of rats' intestines. The above results (Table IV) show that the rabbit and guinea-pig intestinal enzymes exert only a feeble action, if any, on sodium phytate.

DISCUSSION

The foregoing evidence shows that sodium inositolhexaphosphate is hydrolysed by enzyme preparations made from the intestines of albino rats and that in the process orthophosphoric acid is set free. Such enzyme preparations also contain a glycerophosphatase as shown by their action on the solutions of sodium glycerophosphate. The enzyme preparations were made from rats of all ages. In one case (enzyme preparation 2, Table I) it was made from young rats hardly 3 weeks old and not yet weaned. They were suddenly and unexpectedly killed by their mother. Their intestines were treated as usual and yielded an active enzyme. Since these rats fed almost exclusively on milk, the origin of the intestinal phytase from vegetable foodstuffs can be ruled out. Sodium phytate is not hydrolysed by the bone enzyme, as is shown in Table IV, and is hydrolysed very feebly, if at all, by preparations from the intestines of guinea-pigs and rabbits.

The optimum *pH* for the intestinal phytase is about 7·8 while the optimum for plant phytase is *pH* 5·6 [Adler, 1915]; this can be considered as additional evidence against the former enzyme being of plant origin.

No attempt has been made by the author to determine whether the preparations contained one enzyme capable of acting on glycerophosphate as well as on phytate of sodium or two independent enzymes. The intestinal phytase resembles phosphatase in that the action of both is accelerated by the presence of Mg^{++} ions.

The evidence leaves hardly any doubt about the fact that the hydrolysis of sodium phytate caused by the intestinal preparations is truly enzymic in nature. What part this enzyme plays in the hydrolysis of phytin in the intestines and whether the intestines of other animals contain phytase is yet unknown. Partial hydrolysis of phytin observed by McCance & Widdowson and Lowe & Steenbock may possibly be ascribed to the rather slow action of the intestinal phytase.

SUMMARY

1. An enzyme capable of hydrolysing phytase is shown to exist in the intestines of albino rats.
2. This intestinal phytase exerts its optimum activity at about *pH* 7·8 and its action is accelerated by Mg^{++} ions.
3. It is suggested that a partial hydrolysis of phytin takes place in the intestines of albino rats.
4. Intestines of guinea-pigs and rabbits yielded inactive or feebly active extracts.

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