XV. AN INVESTIGATION OF PHYTIN.

BY ROBERT HENRY ADERS PLIMMER AND
HAROLD JAMES PAGE.

*From the Ludwig Mond Research Laboratory for Biological Chemistry,
Institute of Physiology, University College, London.*

(Received January 11th, 1913.)

The chemistry of the substance phytin has attracted the attention of workers in all parts of the world. It is generally regarded as the calcium magnesium salt of inositol phosphoric acid (phytic acid), but the presence of organic constituents other than inositol has been described.

OCCURRENCE.

Phytin occurs in the seeds of a large number of plants and was first extracted from them by Palladin [1895], who distinguished it from coagulable protein. Schulze and Winterstein [1896] prepared it by the same method and showed that it contained phosphoric acid, calcium and magnesium. They supposed it to be identical with the aleurone or globoid bodies described by Pfeffer [1872]. A better method of preparation was discovered by Posternak [1903] and he extracted the substance from various seeds. Hart and Andrews [1903] found that the phosphorus contained in the bran of cereals was chiefly in an organic form of combination, and Patten and Hart [1904] isolated a substance from wheat bran which they regarded as identical with phytin; the same substance was isolated from other cereals by Hart and Tottingham [1909] and Anderson [1912, 4] has prepared it from cotton seed meal. The distribution of phytin in various plants has been investigated by Suzuki and Yoshimura [1907], Staniszkis [1909], and Geys [1910].

PREPARATION.

Palladin and Schulze and Winterstein prepared phytin by extracting the fat-free seeds of *Sinapis nigra* with 10 per cent. sodium chloride solution, boiling to remove coagulable proteins and filtering when the solution had

cooled. On again boiling the solution the phytin was precipitated; it was filtered off, washed with boiling water and dried.

The method of preparation used by Posternak has been patented and phytin is now a commercial article (manufactured by the Gesellschaft für chemische Industrie in Basle). The preparation is as follows.

Oil-cake is extracted with dilute hydrochloric acid (0.5–1 per cent.) and the extract is freed from protein by one of the usual methods. Sufficient sodium acetate is added to remove free hydrochloric acid and then the necessary amount of a soluble calcium salt is added to bring the calcium content of the solution to a fixed amount. The phytin is completely precipitated with copper acetate as a complex copper, calcium and magnesium salt. The precipitate is washed with distilled water and decomposed with hydrogen sulphide. The filtrate from the copper sulphide is evaporated *in vacuo* to a syrup, treated with alcohol and the solid mass dried and powdered.

The extract may be freed from impurities by oxidation, and organic acids such as formic may be used instead of hydrochloric acid.

Phytic acid may be prepared by extracting acidified solutions of phytin with a mixture of one volume of alcohol and three volumes of ether. A syrup is obtained on evaporation which may be precipitated as copper salt as before.

Acid salts of phytic acid may be prepared by dissolving phytin in dilute mineral acids and precipitating with alcohol; with just sufficient acid to dissolve the phytin the precipitate is insoluble in water, but if more acid be used a precipitate of the soluble acid salt of the type CaH_2X is obtained; with still more acid the precipitate is the acid salt of the type CaH_4X .

COMPOSITION OF PHYTIN.

The method of preparation of phytin from extracts of seeds and bran does not exclude the presence of calcium and magnesium phosphate in the preparations which have been made.

There are many conflicting statements concerning the precipitation of phytin by ammonium molybdate; some authors have stated that it is precipitated, some that it is not precipitated; some have said that it gives a white precipitate, others a yellow precipitate. The yellow precipitate has been attributed by many observers to impurities in the phytin. Starkenstein [1910] claimed to have shown that phytin contained inorganic phosphate and that it was decomposed by drying at 100° , but Jegoroff [1912] showed the contrary. Jegoroff pointed out that there is no satisfactory method for

estimating inorganic phosphate in the presence of phytic acid though in some cases that of Schulze and Castoro had been of use. Fingerling and Hecking [1911] have also complained of the absence of a suitable method for the estimation of inorganic phosphate in extracts of fodders containing phytic acid.

None of the investigators mention the methods by which they have ascertained the calcium, the magnesium and the phosphoric acid content of phytin. Most of the methods for the estimation of calcium and magnesium in the presence of phosphoric acid are long and involve possibilities of error. This may account for the very different values which have been found, e.g.:

		Cu	Mg	P	Na	K	Observer
CaMg salt	...	—	—	15.13	—	—	Schulze and Winterstein.
Mg salt	...	—	7.78	18.44	—	—	Winterstein.
CaMg salt	...	13.42	8.05	22.30	—	—	Posternak [1900]*.
CaNa salt	...	8.41	—	19.42	18.79	—	„ [1903].
„	...	8.16	—	19.13	19.02	—	„ „
CaMgK salt	(wheat)	1.13	5.80	16.38	—	2.60	Patten and Hart.
„	(maize)	0.48	6.70	14.17	—	5.60	Hart and Tottingham.
„	(oats)	8.60	4.70	16.70	—	1.50	„ „
„	(barley)	0.00	7.90	14.46	—	11.20	„ „
„	(rice)	5.18	17.48	23.48	—	trace	Suzuki and Yoshimura.
CaMg salt	(commercial)	11.96	1.45	20.32	—	—	Horner.
CaMgK salt	(from phytic acid)	13.03	4.29	19.09	—	6.42	Anderson [1912, 2].
CaMgK salt	(wheat bran)	2.82	4.72	14.42	—	—	„ [1912, 3].

The carbon, hydrogen and phosphorus content of phytin and of phytic acid as given by various workers are also very different:

		C	H	P	Observer
Phytic acid	...	9.87	3.70	25.89	Posternak.
„	...	9.97	3.66	26.00	„
„	(wheat)	10.63	3.38	25.98	Patten and Hart.
„	(maize)	10.30	—	26.07	Hart and Tottingham.
„	(oats)	10.22	—	25.97	„ „
„	(barley)	10.54	—	25.88	„ „
„	(cotton seed)	11.71	3.07	26.35	Anderson [1912, 4].
„	„	10.89	3.25	27.11	„ „
„	„	11.02	3.30	26.36	„ „
Calculated for $C_6H_{24}O_{27}P_6$		10.08	3.36	26.05	—
CaMg salt	...	9.65	2.88	15.13	Schulze and Winterstein.
CaNa salt	...	7.25	1.34	19.42	Posternak.
„	...	7.43	1.49	19.13	„
CaMgK salt	(wheat bran)	17.30	3.63	16.38	Patten and Hart.
„	„	21.47	3.69	14.42	Anderson [1912, 3].

* Cited in Jegoroff's paper.

The difficulty of burning all the carbon in the presence of phosphoric acid has never been mentioned and the different values may be assigned to this cause.

THE PREPARATION OF PHYTIC ACID.

Phytic acid was prepared by Posternak, who described it as a thick transparent brownish syrup. Patten and Hart again prepared phytic acid from the phytin of the bran of wheat. They pointed out the great difficulty in obtaining it free from calcium (compare the precipitation of phytin with copper acetate) and they adopted the following procedure. The copper compound which is precipitated from the extract is decomposed by hydrogen sulphide. The filtered solution is made alkaline with sodium hydroxide and precipitated with barium chloride. The barium salt is washed until free from alkali, suspended in water and decomposed with the exact amount of sulphuric acid to combine with the barium. The solution is again precipitated with barium chloride in alkaline solution and treated as before. The process is once more repeated and then copper acetate is added. The copper compound which is precipitated is washed, suspended in water and decomposed with hydrogen sulphide. The filtrate from the copper sulphide is evaporated to a syrup and the residue is dried at 110°.

CONSTITUTION OF PHYTIC ACID.

The evidence for the constitution at present accepted for phytic acid is based upon the work of Winterstein and of Posternak. Winterstein [1897] prepared a magnesium salt of phytic acid and obtained on hydrolysis with concentrated hydrochloric acid at 130° a 60 per cent. yield of inositol. Posternak analysed the acid and determined its molecular weight by the freezing point method. He gave it the formula $C_2H_8O_9P_2$ and represented it as anhydro-oxymethylene diphosphoric acid,

He confirmed the observation of Winterstein that it yielded inositol on hydrolysis (98 per cent. yield) and explained the formation of this product by the polymerisation of the formaldehyde which is first formed.

This constitution of phytic acid was challenged by Neuberg [1908, 1] who quoted Suzuki and Yoshimura's result that inositol is obtained by hydrolysis with the enzyme, phytase. Winterstein [1908] was also of opinion that the inositol nucleus existed in phytic acid. Neuberg and Suzuki and Yoshimura

[1907] put forward formulae representing phytic acid as inositol hexaphosphoric acid:

Contardi [1909] contributed further evidence to show that phytic acid is a phosphoric acid ester of inositol: he obtained 18 g. of inositol from 100 g. of phytin by acid hydrolysis.

Starkenstein [1910] from titrations of phytic acid supposed that phytic acid was derived from pyrophosphoric acid and Vorbrodt [1910], who analysed a "sphero" crystalline barium salt, believed that the phytic acid molecule contained two inositol nuclei.

The synthesis of esters of phosphoric acid and inositol has been effected by Contardi [1910] and by Anderson [1912, 1]. Contardi heated together inositol and phosphoric acid and obtained inositol hexaphosphoric acid together with a quantity of inositol diphosphoric acid. The inositol hexaphosphoric acid agreed very closely in its properties with natural phytic acid. By the same method Anderson obtained inositol tetraphosphoric acid as the chief product of the reaction; inositol hexaphosphoric acid was not formed. Anderson [1912, 2] has also prepared an ester of inositol and pyrophosphoric acid. Carré [1911] has maintained that the substances prepared by Contardi are mixtures of inositol and phosphoric acid, but Contardi [1912] refuted these statements.

Most observers seem to agree that phytic acid is composed of only inositol and phosphoric acid, but the data concerning the yield of inositol obtained on hydrolysis do not support this conclusion. Posternak alone obtained a nearly theoretical yield; other workers have obtained yields varying from 20 to 75 per cent. and in the majority of cases the yield has not been given. Levene [1909] claimed to have separated phytin into two constituents; one of them yielded inositol on hydrolysis; the other a carbohydrate which gave pentose reactions and furfural on distillation with hydrochloric acid. Neuberg [1909] severely criticised the claims of Levene and showed that inositol gave small quantities of furfural when distilled with concentrated hydrochloric acid. Considering the scanty data of the yield of

inositol on hydrolysis, Levene's claim that another organic constituent is present in phytin must be considered. Anderson [1912, 3] finds that the phytin of bran does not consist of one substance, but is a mixture.

The above summary of the work on phytin shows that there are several lines of investigation which require attention:

- (1) The presence of inorganic phosphates in phytin.
- (2) The analysis of the calcium and magnesium content of phytin.
- (3) The removal of calcium in the preparation of phytic acid.
- (4) The yield of inositol by the hydrolysis of phytin or phytic acid.
- (5) The presence of other organic compounds besides inositol in phytic acid.

Our investigations are not complete but we believe we have sufficient data to justify their publication, especially as our joint work has ceased for some time, although we hope to continue it at some future date.

EXPERIMENTAL.

Commercial phytin has been employed almost entirely in these investigations, but for comparison phytin (neutral calcium phytate) was prepared from bran. 200 g. of wheat-bran were extracted with successive quantities of N/5 hydrochloric acid until no appreciable amount of phosphorus was contained in the extract. The combined extracts were made alkaline with ammonia; this caused the precipitation as neutral calcium phytate of the whole of the calcium in the extract. The residual phytic acid in the solution was precipitated by adding calcium chloride solution until no further precipitation ensued. The whole was then filtered and the precipitate thoroughly washed with water and dried. The product was dissolved in dilute hydrochloric acid and again thrown down by adding ammonia. The precipitate was re-dissolved in hydrochloric acid and the solution decolourised by shaking with charcoal. After filtering, the phytin was precipitated with ammonia and filtered off. It was washed four times by removing from the filter, triturating with distilled water and filtering, and then dried at 100°. Yield = 6 g. or 3 per cent.

ANALYSIS OF PHYTIN.

(i) *Estimation of the Total Phosphorus.* The total phosphorus content of phytin was estimated by the method of Neumann as modified by Plimmer and Bayliss [1906]. Several samples were analysed. The analytical data are all given on p. 168.

(ii) *Estimation of Inorganic Phosphorus.* Inorganic phosphate in the presence of organic phosphates can be readily estimated by precipitation with ammonium magnesium citrate as ammonium magnesium phosphate and conversion into magnesium pyrophosphate as was shown by Plimmer and Bayliss in the case of caseinogen. The same method can be applied to the estimation of inorganic phosphate in the presence of glycerophosphate and ethyl phosphate, as was shown by one of us in a previous communication, but in the presence of phytic acid this method cannot be employed. The solution of phytic acid obtained after removal of the calcium by oxalic acid or an oxalate not only inhibits the precipitation of small amounts of inorganic phosphate as ammonium magnesium phosphate but also, when larger amounts are present, renders the precipitation slow and the precipitate consists of ammonium magnesium phosphate and ammonium magnesium phytate. This is shown by the following experiments.

Various amounts of a solution of sodium phosphate were added to a solution of phytic acid prepared by adding oxalic acid to 10 g. of phytin dissolved in water, filtering and diluting to one litre.

A. 10 c.c. phytic acid sol. + 1 c.c. Na_2HPO_4 sol. gave no ppt.					
,, ,, + another 1 c.c. Na_2HPO_4 sol. gave no ppt.					
,, ,, + yet another 1 c.c. Na_2HPO_4 gave ppt. = 0.0692 g. P_2O_5 .					
					Hence ppt. from phytic acid
B. 10 c.c. phytic acid sol. + 1 c.c. Na_2HPO_4 sol. gave ppt. = 0.0313 g. P_2O_5 .					= 0.0072
,, ,, + 2 c.c. ,, ,, = 0.0629 ,, = 0.0147					
,, ,, + 3 c.c. ,, ,, = 0.0925 ,, = 0.0202					
,, ,, 1 c.c. ,, ,, = 0.0241 ,,					
C. 10 c.c. phytic acid sol. + 5 c.c. Na_2HPO_4 sol. gave ppt. = 0.1187 g. P_2O_5 .					= 0.0075
,, ,, + 5 c.c. ,, ,, = 0.1350 ,, = 0.0238					
,, ,, 5 c.c. ,, ,, = 0.1112 ,,					

The estimation of inorganic phosphate in the presence of phytic acid was carried out by Hart and Andrews [1903] by precipitation with ammonium molybdate at 65° in the presence of very dilute nitric acid. This method was used by McCollum and Hart in their phytase experiments. Under these conditions there is a possibility that phytic acid is hydrolysed with the liberation of inorganic phosphate. Experiments were therefore made to ascertain if inorganic phosphate was completely precipitated by ammonium molybdate in the presence of seminormal to normal nitric acid at room temperature. The yellow solution which is first formed on adding ammonium molybdate to a solution of sodium phosphate in the presence of dilute nitric acid gradually deposits ammonium phosphomolybdate in a crystalline state and the solution becomes colourless in 24 to 48 hours. On determining the

amount of P_2O_5 in this precipitate by the Neumann method, by dissolving it, after filtration and washing, in semi-normal sodium hydrate, boiling off the ammonia and titrating with semi-normal sulphuric acid it was found to be quantitative:

1 c.c. Na_2HPO_4 sol. gave $Mg_2P_2O_7 = 0.0241$ g. P_2O_5 .
 1 c.c. " " + 25 c.c. H_2O + 25 c.c. 2N. HNO_3 } gave { 0.0226 g. P_2O_5 .
 + 25 c.c. Am. molybdate sol. } { 0.0219 " "

Applying this method to the solution of phytic acid the following amounts were obtained:

10 c.c. phytic acid sol. + 25 c.c. 2N. HNO_3 + 25 c.c. H_2O } gave { 0.0043 g. P_2O_5 .
 + 10 c.c. Am. molybdate sol. } { 0.0042 " "
 { 0.0044 " "

Total P_2O_5 in 10 c.c. phytic acid sol. by Neumann's method = 0.0871 gm.

Further proof that the estimation of inorganic phosphate in the presence of phytic acid can thus be effected is given by the following experiments which were carried out in the same way:

(a) 1 c.c. Na_2HPO_4	(b) 10 c.c. phytic acid sol.	(c) 1 c.c. Na_2HPO_4 + 10 c.c. phytic acid sol.	Sum of (a) and (b)
0.0223 g. P_2O_5	0.0044 g. P_2O_5	0.0264 g. P_2O_5	0.0267
0.0226 "	0.0054 "	0.0270 "	0.0281
0.0222 "	0.0051 "	0.0270 "	0.0273

Hydrolysis of the phytic acid by dilute nitric acid at room temperature therefore does not occur. Employing this method of estimation it has been shown that hydrolysis does not occur at 37° but at 65° and at 75° phytic acid is slowly hydrolysed [Plimmer, 1913, 2, p. 79].

If proteins be present in solution they are precipitated by phosphomolybdic acid with the ammonium phosphomolybdate. The estimation can be carried out by filtering the yellow precipitate, washing free from acid, dissolving in sodium hydrate and then precipitating in the usual way with magnesium citrate mixture and ammonia and finally converting into magnesium pyrophosphate.

The above three solutions after the estimation by Neumann's method were filtered and the inorganic phosphate estimated as magnesium pyrophosphate:

(a)	(b)	(c)	(a) + (b)
0.0239 g. P_2O_5	0.0042 g. P_2O_5	0.0281 g. P_2O_5	0.0281
0.0240 "	—	0.0290 "	—
0.0239 "	0.0050 "	0.0289 "	0.0289

The estimation of inorganic phosphate in phytin can be readily effected by this method; the presence of calcium does not interfere with the

precipitation. A known weight of phytin is dissolved in normal nitric acid, excess of ammonium molybdate is added and the solution is allowed to stand. When the solution is colourless the crystalline precipitate is filtered off and estimated by the Neumann method, or by filtering, washing free from acid, dissolving in sodium hydrate and precipitating as ammonium magnesium phosphate.

The results of the estimations in different samples of phytin are given on p. 168.

(iii) *Estimation of Calcium and Magnesium.* The previous investigators have not stated the method which they employed for the estimation of these elements in phytin. They have in all probability precipitated the calcium as oxalate and converted it into calcium oxide, either before or after oxidation of the organic matter, and estimated the magnesium in the filtrate as magnesium pyrophosphate. This method involves several possibilities of error; if phosphoric acid be not removed it may be thrown down with the calcium oxalate and some of the magnesium may also be precipitated as magnesium oxalate. The method of separation of these constituents in a mixture is again a long and tedious process.

If oxalic acid or an oxalate be added to a solution of phytin a precipitate is formed, but this precipitate does not consist of pure calcium oxalate. This is shown by the following experiment:

10 g. of commercial phytin were dissolved in decinormal acetic acid and filtered from a small quantity of insoluble matter. This was dried at 110° and analysed: it contained 13.74 per cent. of calcium (estimated as CaSO_4 , see later) and 17.4 per cent. of phosphorus (by Neumann's method). The filtrate was precipitated with 4.1 g. of oxalic acid dissolved in 50 c.c. of water. The precipitate was washed and dried at 110° ; it weighed 5 g. and was analysed:

0.2188 g. heated to redness to constant weight gave 0.0956 g. residue. Calculated amount for pure calcium oxalate is 0.0957 g.

0.3364 g. gave 0.2782 g. CaSO_4 (see later) and 0.0031 g. P by Neumann's method.

0.3030 g. gave 0.0028 g. P.

$\text{Ca} = 24.31$ per cent. $(\text{COO})_2\text{Ca}$ requires $\text{Ca} = 31.25$ per cent.

$\text{P} = 0.92$ per cent.

$\text{P} = 0.93$ per cent.

Another preparation of calcium oxalate from phytin contained 0.77 per cent. total P and 0.27 per cent. inorganic P.

Calcium can therefore not be estimated by precipitating with oxalic acid.

Aron [1907] showed that the calcium in milk, urine, etc., could be estimated as calcium sulphate after oxidation of the organic matter by the

Neumann method. When the oxidation is completed the nitric acid is removed as far as possible by diluting with water and again evaporating. The sulphuric acid which remains is then diluted and four volumes of alcohol are added. The calcium sulphate is precipitated and is filtered off, dried and weighed.

Beyond these few estimations of calcium Aron made no other analyses, but he states that other elements might be estimated in the filtrate.

This method therefore required verification and extension for the estimation of magnesium. Analyses of mixtures of calcium chloride, magnesium sulphate and sodium phosphate were consequently made to test the method. The mixtures were evaporated down in a small conical flask with 10 c.c. of sulphuric acid; the acid was diluted with water and precipitated with four to five volumes of alcohol. The calcium sulphate was filtered off on a Gooch crucible, and dried at a red heat. The magnesium was estimated in the filtrate as pyrophosphate after evaporating off the alcohol, oxidising the remainder of the alcohol with nitric acid, diluting with water and making alkaline with ammonia. Excess of phosphoric acid was present in the mixtures to precipitate the whole of the magnesium. The results were:

Calcium		Magnesium	
Taken	Found	Taken	Found
0.1736	0.2540	0.1484	0.1490
0.1736	0.2066	0.0297	0.0310
0.0347	0.0523	0.1484	0.1493
0.1736	0.1885	0.1484	0.1495
0.0264	0.0569	0.0337	0.0332
0.0264	0.0328	0.0337	0.0332
0.0264	0.0588	0.0337	0.0329

Magnesium can thus be correctly estimated but the values for calcium are too high. The high values are due to the presence of sodium in the solution as is shown by experiments with mixtures of calcium chloride and sodium phosphate and calcium chloride and phosphoric acid in various proportions:

Calcium		In presence of sodium phosphate	Calcium		In presence of phosphoric acid
Taken	Found		Taken	Found	
0.0233	0.0285	5 c.c.	0.0233	0.0233	10 c.c.
	0.1038	10 c.c.		0.0233	15 c.c.
	0.1944	15 c.c.		0.0232	20 c.c.
	0.2740	20 c.c.		0.0231	25 c.c.

The presence of sodium salts in the solution thus causes a serious error in the estimation of calcium as calcium sulphate.

If the amount of alcohol used in the precipitation be reduced and if the amount of sodium or potassium in the solution be not greater than 0.1 g. the

estimation of calcium can be accurately effected, especially if not more than one volume of alcohol be used in the precipitation:

3 volumes alcohol		2 volumes alcohol		1 volume alcohol	
Calcium found	Amount of Na present, in g.	Calcium found	Amount of K present, in g.	Calcium found	Amount of K present, in g.
0·0231	0·0	0·0232	0·0	0·0231	0·0
0·0231	0·0	0·0245	0·0394	0·0228	0·0
0·0284	0·1228	0·0281	0·0591	0·0230	0·0197
0·0268	0·0614	0·0383	0·0788	0·0230	0·0394
0·0258	0·0307	0·0237	0·0197	0·0228	0·0591
0·0238	0·0184	0·0238	0·0158	0·0230	0·0788
0·0233	0·0123	0·0237	0·0118	0·0227	0·0788

The amount of calcium taken in all these experiments was 0·0233 g.

Unless the amount of potassium and sodium (if present) in phytin exceed 20 per cent. (0·1 g. in 0·5 g. required for an analysis) the calcium can be accurately estimated by precipitation with one volume of alcohol as calcium sulphate and the magnesium can be estimated in the filtrate as magnesium pyrophosphate. The calcium and magnesium content of various samples of phytin have been estimated by oxidising the organic matter with 10 c.c. sulphuric acid and nitric acid, removing the nitric acid by diluting and evaporating, adding 10–20 c.c. of water and then one volume of alcohol (20–60 c.c.) to precipitate the calcium sulphate. Alcohol was removed from the filtrate by evaporation. On oxidation with nitric acid and on making alkaline with ammonia the magnesium was precipitated as ammonium magnesium phosphate and estimated as pyrophosphate. Excess of P_2O_5 is always present in phytin: the remainder can be estimated by adding magnesia mixture and determining it as pyrophosphate. The sum of the figures obtained then gives the total P_2O_5 content. It has been found to be the same as by the Neumann method. For the data see p. 168.

(iv) *Loss of Weight on Heating and in vacuo over Sulphuric Acid.* Phytin loses weight at 110° and *in vacuo* over sulphuric acid. The greater part of the loss takes place during the first heating; the weight continues to decrease very slowly and loss of weight seems to go on indefinitely. One specimen was heated at 110° for 54 hours. The loss in weight under both conditions is about the same, but it takes place more slowly *in vacuo*.

Part of the loss in weight may be due to alcohol; one of the commercial specimens examined gave the iodoform reaction.

The gradual decrease in weight on heating is probably due to a slow conversion into pyrophosphate.

(v) *Estimation of Carbon and Hydrogen.* In the course of many combustions of organic phosphorus compounds the difficulty of burning all the

carbon has been noticed. Page [1912] has shown that the values for carbon in the case of the hydroxyphosphinic acids were 1–2 per cent. below the true value even after mixing the substance intimately with lead chromate. The values were still lower by the Fritsch-Messinger wet method of combustion.

Previous observers have never mentioned any difficulty in ascertaining the carbon content of phytin or other organic phosphorus compounds nor have they mentioned any modification of the ordinary method which they used.

One of the specimens of phytin was analysed by mixing intimately with the finest copper oxide and it seems improbable that all the carbon was not burnt.

Results of Analyses.

Total Phosphorus.

Commercial phytin :

Sample 1	0.1120 g. : 37.8 c.c. $\frac{N}{2}$ NaOH.	18.68 per cent.
2	0.0682 g. : 24.7 c.c. „	20.04 „
3		17.74 „
4	0.0579 g. : 18.7 c.c. „	18.57 „
5	0.0694 g. : 24.1 c.c. „	19.22 „
6	0.0614 g. : 21.2 c.c. „	19.11 „
7	0.0544 g. : 18.7 c.c. „	19.76 „
8	0.0550 g. : 18.5 c.c. „	18.62 „
Bran (1)	0.6704 g. : 15.8 c.c. „	1.30 „
Phytin from bran	0.1326 g. : 0.0714 g. $Mg_2P_2O_7^*$	15.00 „

* Estimated by Mg method after ppt. of $CaSO_4$.

Inorganic Phosphorus.

Commercial phytin :

Sample 2	0.2664 g. : 4.3 c.c. $\frac{N}{2}$ NaOH.	0.89 per cent.
4	0.6152 g. : 10.8 c.c. „	0.97 „
5	0.9642 g. : 17.1 c.c. „	0.98 „
6	0.6626 g. : 11.7 c.c. „	0.98 „
7	0.5350 g. : 9.5 c.c. „	0.98 „
8	0.8726 g. : 14.8 c.c. „	0.94 „
Phytin from bran	0.3524 g. : 16.7 c.c. „	2.62 „

Calcium and Magnesium.

Commercial phytin :

		Per cent.	
		Ca	Mg
Sample 1	0.4552 g. : 0.1984 g. $CaSO_4$: 0.0092 g. $Mg_2P_2O_7$.	12.82	0.44
2	0.5630 g. : 0.2340 g. „ : 0.0194 g. „	12.2	0.75
3	0.4090 g. : 0.1828 g. „ : 0.0104 g. „	13.15	0.56
4	0.5235 g. : 0.2207 g. „ : 0.0195 g. „	12.40	0.81
5	0.4245 g. : 0.1744 g. „ : 0.0155 g. „	12.10	0.78
6	0.4224 g. : 0.1775 g. „ : 0.0151 g. „	12.36	0.78
7	0.4052 g. : 0.1681 g. „ : 0.0151 g. „	12.20	0.81
8	0.4466 g. : 0.1873 g. „ : 0.0155 g. „	12.34	0.76
Phytin from bran	0.1326 g. : 0.0962 g. „ : 0.0000 g. „	21.12	0.0
Bran (1)	3.3430 g. : 0.0150 g. „ : 0.0786 g. „	0.13	0.51
(2)	4.4868 g. : 0.0176 g. „ : 0.1116 g. „	0.11	0.54
(3)	4.2028 g. : 0.0228 g. „ : 0.1058 g. „	0.15	0.55

Loss of Weight (a) at 110° and (b) in vacuo over H₂SO₄.

Commercial phytin :

Sample 3 (a)	5.0229 g. : 0.3425 g. = 6.82 per cent.
3 (b)	5.6868 g. : 0.3874 g. = 6.81 ,,
4 (a)	0.7376 g. : 0.0544 g. = 7.37 ,,
5 (a)	1.7712 g. : 0.1510 g. = 8.52 ,,
6 (a)	1.5502 g. : 0.1164 g. = 7.51 ,,
7 (a)	0.8472 g. : 0.0628 g. = 7.41 ,,
8 (a)	2.1870 g. : 0.1532 g. = 7.00 ,,

Carbon and Hydrogen.

Commercial phytin :

Sample 2	0.3052 g. : 0.0815 g. CO ₂ : 0.0917 g. H ₂ O. C = 7.28%, H = 3.33%.
	0.2392 g. : 0.0655 g. ,, : 0.0740 g. ,, C = 7.47%, H = 3.44%.

If these figures be compared with those of previous workers (p. 159) considerable differences will be noticed. These figures agree most closely with those of Horner for commercial phytin and of Anderson for the preparation made from phytic acid. The total phosphorus content is generally higher and so is the calcium content, but the magnesium content is much lower; in all the specimens examined magnesium was less than one per cent. Commercial phytin seems to be the calcium salt and the magnesium may be present as impurity.

The carbon content is also lower, but the hydrogen content agrees with the analyses of other workers.

The commercial preparations are very constant in their phosphorus, calcium and magnesium content, but they differ from the preparation from wheat bran, which was the neutral calcium salt of phytic acid and hence contained less phosphorus and more calcium; the bran preparation contained no magnesium.

Analyses of the phosphorus, calcium and magnesium in wheat bran were made for comparison. It will be observed that bran contains more magnesium than calcium, which is the reverse of phytin.

PREPARATION OF PHYTIC ACID.

Phytic acid was prepared from commercial phytin and from the phytin from bran by the method of Patten and Hart and also by the removal of the calcium as oxalate. Neither method sufficed to remove the calcium entirely, and it was at first thought that some hitherto undescribed product was present.

Preparation 1. 50 g. of commercial phytin (sample 3 above) were

dissolved in a minimum quantity of dilute hydrochloric acid and just sufficient sodium acetate solution was added to render the solution neutral to Congo-red paper. Copper acetate solution was then added until no further precipitation occurred, and the copper salt filtered off by suction. Since it was impossible to wash the precipitate thoroughly on the paper it was removed, made into a uniform cream with distilled water and passed through muslin to ensure the complete breaking up of all lumps. The mass was filtered and the washing procedure repeated twice. (All subsequent washings were effected in this way.) The washed copper phytate precipitate was suspended in water and the mixture treated with hydrogen sulphide until saturated and set aside for twelve hours. The copper sulphide was filtered off and washed. The filtrate and washings were exactly neutralised with sodium hydroxide and excess of barium chloride solution added. The precipitate of barium salt was filtered off and washed twice as described above. It was then suspended in water and the exact quantity of sulphuric acid necessary to combine with the whole of the barium was added, and the barium sulphate filtered off and washed. The solution was neutralised with sodium hydrate and the whole procedure of precipitation with barium chloride and decomposition with sulphuric acid repeated twice. The solution after removal of the barium for the third time was neutralised with caustic soda and then acidified with acetic acid. Copper acetate solution was added and the copper salt so obtained filtered off, washed and decomposed as described above. The filtrate from the copper sulphide was evaporated to a syrup at 100°.

Preparation 2. A specimen of phytic acid was prepared in the same manner from 5 g. of the phytin from bran.

Preparation 3. 100 g. of commercial phytin (sample 4 above) were dissolved as far as possible in decinormal acetic acid. The insoluble residue (amounting to 4 g.) was filtered off and washed. To the filtrate was added the calculated quantity of oxalic acid (37.5 g. allowing for the residue, which contained 11.81 per cent. of calcium) dissolved in water. The calcium oxalate was filtered off and a portion of the filtrate tested with a little more oxalic acid to ascertain if the calcium had been completely removed; no further precipitate was produced. Since calcium oxalate is known to occlude magnesium oxalate to a considerable degree it was thought that the precipitated calcium oxalate would remove at the same time the small quantities of magnesium present. The filtrate was therefore evaporated to a small volume *in vacuo*; a small amount of calcium oxalate separated out and was filtered off. The solution was then concentrated to a syrup at 100°.

The presence of calcium in the syrups of phytic acid.

In the case of all three preparations of phytic acid it was noticed that after drying at 100° an appreciable scum or skin formed on the surface and that when small samples of each specimen were treated with alcohol a small white flocculent precipitate was formed. Each of the preparations was therefore dissolved in a small quantity of water and sufficient alcohol added to precipitate completely the insoluble substance. This was filtered off, washed thoroughly with alcohol, re-dissolved in water and re-precipitated with alcohol. It separated as a white flocculent precipitate which rapidly became stringy and formed a sticky mass on the bottom of the vessel; on standing under alcohol for some time it became quite hard and apparently crystalline. The yields were: (1) 3 g., (2) 1.3 g., (3) 12 g.

On analysis when dried at 110° these precipitates were found to contain phosphorus and calcium, and the third preparation magnesium as well:

Prep. 1	0.2162 g. : 0.0883 g. CaSO_4 : Ca=12.01 per cent.
2	0.2197 g. : 0.1004 g. ,, : Ca=13.45 ,,
3	0.2586 g. : 0.0654 g. ,, : Ca= 7.46 ,,
	0.0357 g. $\text{Mg}_3\text{P}_2\text{O}_7$: Mg= 3.12 ,,
Prep. 1	0.0527 g. : 21.10 c.c. $\frac{\text{N}}{2}$ NaOH : P=20.79 per cent.
2	0.0227 g. : 8.95 c.c. ,, : P=21.80 ,,
3	0.0304 g. : 11.80 c.c. ,, : P=21.50 ,,

Another preparation by the oxalic acid method was found to contain 21.1 per cent. of phosphorus, 11.10 per cent. of carbon and 2.98 per cent. of hydrogen.

The alcoholic filtrates were evaporated to syrups at 100° . They agreed in most of their properties with the descriptions of other workers.

When dried to constant weight at 100° and *in vacuo* over sulphuric acid for three months the syrup from Preparation 1 was analysed:

Prep. 1	0.3444 g. : 0.1498 g. CO_2 : 0.1108 g. H_2O . C=11.86, H=3.57 per cent.
	0.0986 g. : 38.0 c.c. $\frac{\text{N}}{2}$ NaOH. P=21.34 per cent.

$(\text{C}_2\text{H}_5\text{O}_6\text{P}_2)_3$ requires C=10.09 and H=3.36 per cent. P=26.07 per cent.

The carbon and hydrogen figures are higher than those required for phytic acid whilst the phosphorus figure is much lower. It is very probable that some alcohol is retained in the syrup and is not removed by drying; it is also possible that some esterification took place between the alcohol and the phytic acid.

FORMATION OF INOSITOL BY HYDROLYSIS OF PHYTIC ACID.

The hydrolysis of phytin by dilute nitric acid at the atmospheric pressure and at various temperatures has been investigated by one of us [Plimmer, 1913, 2]; even after seventeen days the separation of the phosphoric acid was not quite complete.

64 g. of phytic acid (the syrup after removal of the crystalline calcium compound in Preparation 3, prepared from 100 g. phytin by treatment with oxalic acid) were dissolved in 250 c.c. of twice normal sulphuric acid and heated in a corked flask in a boiling water bath for 36 hours; about 55 per cent. of the phosphorus was then present as inorganic phosphate; the heating was continued at 75° in a thermostat for 164 hours; the solution now contained 79 per cent. of the phosphorus in an inorganic form. The hydrolysis was proceeded with for another 118 hours after diluting with four volumes of twice normal sulphuric acid. 84 per cent. of the total phosphorus was now present as phosphoric acid. Since the hydrolysis proceeded so slowly it was discontinued. The solution was evaporated to 200 c.c. and an equal volume of alcohol was added, followed by ether until a permanent cloudiness was produced. Inositol crystallised out when the mixture was set aside at 0° for twenty-four hours and was removed by filtration. Two further quantities were obtained on adding ether to the filtrate and keeping at 0°. The total yield amounted to 4.5 g. The alcohol and ether were distilled from the solution and the volume made up to 500 c.c. This solution contained 10.38 g. total phosphorus and 9.11 inorganic phosphorus, i.e. 1.27 g. organic phosphorus or 5.08 g. of phytic acid (25 per cent P). Allowing for the samples removed during hydrolysis the liquid from which the inositol was isolated contained 48 g. of phytic acid, and allowing for the organic phosphorus still present the amount of phytic acid hydrolysed is 42 g. This quantity should yield 10.06 g. of inositol. Since the amount actually obtained was only 4.5 g. it represents less than 50 per cent. of the theoretical.

The inositol was recrystallised from water, alcohol and ether; it melted at 220–222°. Analysis:

0.2140 g. :	0.2550 g. CO ₂ :	0.1230 g. H ₂ O :	C = 32.5 per cent.,	H = 6.4 per cent.
C ₆ H ₁₂ O ₆ requires	...	...	C = 40.0 per cent.,	H = 6.7 per cent.

The figure for carbon is too low. A residue was left in the boat and amounted to 7 per cent.; it contained particles of carbon which it was impossible to oxidise completely after many hours in a current of oxygen.

This combustion again shows the difficulty of burning carbon in the presence of phosphoric acid. The ash was found to contain 52.14 per cent. of P_2O_5 .

SUMMARY.

1. Inorganic phosphates in phytin can be readily estimated by precipitation with ammonium molybdate in semi-normal nitric acid at room temperature.

2. The calcium content of phytin cannot be estimated by precipitation as calcium oxalate, but is easily ascertained by precipitation as calcium sulphate.

The magnesium content of phytin can then be estimated as magnesium pyrophosphate.

3. There is great difficulty in removing the calcium from phytin in the preparation of phytic acid.

4. The yield of inositol obtained on the hydrolysis of phytic acid by acids is not quantitative; we are inclined to believe that another organic constituent is present in phytin, and hope to carry out further experiments on the production of inositol by the hydrolysis of phytic acid at some future date.

The expenses of this work were partly defrayed by a grant from the Government Grant Committee of the Royal Society.

REFERENCES.

- Anderson (1912, 1), *J. Biol. Chem.* **11**, 471.
— (1912, 2), *J. Biol. Chem.* **12**, 97.
— (1912, 3), *J. Biol. Chem.* **12**, 447.
— (1912, 4), *J. Biol. Chem.* **13**, 311.
Aron (1907), *Biochem. Zeitsch.* **4**, 268.
Carré (1911), *Bull. Soc. Chim.* iv, **9**, 195; *Chem. Zentr.* 1911, **1**, 1196.
Collison (1912), *J. Biol. Chem.* **12**, 65.
Contardi (1909), *Atti. R. Accad. Lincei*, v, **18**, **1**, 64; *Chem. Zentr.* 1909, **1**, 1102.
— (1910), *Atti. R. Accad. Lincei*, v, **19**, **1**, 23; *Chem. Zentr.* 1910, **1**, 1032.
— (1912), *Gazz. chim. Ital.* **42**, **1**, 408; *Chem. Zentr.* 1912, **11**, 186.
Fingerling and Hecking (1911), *Biochem. Zeitsch.* **37**, 452.
Geys (1910), *Zeitsch. ges. Brauwesen*, **33**, 347–349; *Chem. Zentr.* 1910, **11**, 982.
Hart and Andrews (1903), *Amer. Chem. J.* **30**, 470.
Hart and Tottingham (1909), *J. Biol. Chem.* **6**, 431.
Horner (1907), *Biochem. Zeitsch.* **2**, 428.
Jegoroff (1912), *Biochem. Zeitsch.* **42**, 432.
Levene (1909), *Biochem. Zeitsch.* **16**, 399.
Neuberg (1908, 1), *Biochem. Zeitsch.* **9**, 557.
— (1908, 2), *Biochem. Zeitsch.* **9**, 551.
— (1909), *Biochem. Zeitsch.* **16**, 406.

- Page (1912), *J. Chem. Soc.* **101**, 423.
Palladin (1895), *Zeitsch. Biol.* **31**, 199.
Patten and Hart (1904), *Amer. Chem. J.* **31**, 564.
Pfeffer (1872), *Jahrb. wiss. Bot.* **8**, 465.
Plimmer (1913, 1), *Biochem. J.* **7**, 43.
— (1913, 2), *Biochem. J.* **7**, 72.
Plimmer and Bayliss (1906), *J. Physiol.* **33**, 439.
Posternak (1900), *Rev. gen. Bot.* **12**, 5, 65.
— (1903), *Compt. rend.* **137**, 202, 337, 439; *Compt. rend. Soc. Biol.* **55**, 1190.
Schulze and Winterstein (1896), *Zeitsch. physiol. Chem.* **22**, 90.
— (1903), *Zeitsch. physiol. Chem.* **40**, 120.
Staniszkiš (1909), *Anzeiger Akad. Wiss. Krakau*, 95; *Chem. Zentr.* 1909, II, 918.
Starkenstein (1910), *Biochem. Zeitsch.* **30**, 56.
Suzuki and Yoshimura (1907), *Bull. Coll. Agric. Tokyo*, **7**, 495.
Vorbodt (1910), *Anzeiger Akad. Wiss. Krakau*, A, 414; *Chem. Zentr.* 1911, I, 895.
Winterstein (1897), *Ber.* **30**, 2299.
— (1908), *Zeitsch. physiol. Chem.* **58**, 118.