XL. THE DETERMINATION OF PHYTIC ACID.[1]

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THE method generally accepted for the determination of phytic acid (*i.e.* inositol-hexaphosphoric acid) is that of Heubner and Stadler [1914]. It was subsequently applied to plant products and foodstuffs by other workers [Rather, 1917; Arbenz, 1922; Averill and King, 1926] and Rather [1917] found that its accuracy is not affected by non-phytic acid substances in plant extracts. The method involves the titration of phytic acid in 0·6 % HCl solution with standard $FeCl_3$ (0·5–2·0 mg. Fe per ml.) in 0·6 % HCl, ammonium thiocyanate being used as internal indicator; Heubner and Stadler considered that the end-point is reached when a flesh pink colour is obtained which persists for 5 min.

A disadvantage of this method is that the end-point is not sharp owing to the presence of the colloidal precipitate of ferric phytate which forms during titration. Various attempts have been made to circumvent this difficulty [Knowles and Watkin, 1932; Andrews and Bailey, 1933], the most satisfactory method being that of Harris and Mosher [1934] who titrated past the end-point, filtered the precipitate and determined the excess $FeCl_3$ colorimetrically.

Phytic acid is determined in biological material by extraction with dilute HCl, the titration being performed directly on the extract. This procedure gives rise to a serious difficulty as it cannot be applied to materials which yield markedly coloured extracts.

Experiments by the author showed that phytic acid is partially adsorbed on substances such as norite charcoal, fuller's earth and alumina and therefore decoloration of the extracts by such adsorbents cannot be used as a preliminary to titration. It was found that solutions of $FeCl_3$ in $N/6$ HCl did not yield precipitates at room temperature with very dilute solutions of phytic acid in $N/6$ HCl. When heated at 100° however coagulated precipitates formed which were easily separable either by centrifuging or filtration. Under suitable conditions precipitation was complete and furthermore it was possible to decompose the precipitates quantitatively with NaOH into $Fe(OH)_3$ and sodium phytate. By using centrifuging for separating the ferric phytate precipitate it became possible to deal with small amounts of phytic acid and in order to take advantage of this fact a micro-method for the determination of phytic acid was developed. It was designed to determine up to 2 mg. (expressed as P) of phytic acid.

I. THE COLORIMETRIC DETERMINATION OF PHYTIC ACID.

This method differs from previous methods mainly in that (1) a fixed amount of standard $FeCl_3$ is added to the solution being analysed, (2) the ferric phytate is precipitated at 100°, (3) the precipitation is performed in $N/6$ HCl and (4) the excess Fe^{+++} after precipitation of the ferric phytate is determined by the thiocyanate method using a colorimeter.

Conditions of precipitation. The precipitations are done in boiling-tubes heated in the water-bath in racks. Experiments showed that a solution of 2·5 mg.

[1] A preliminary account of the work described in this paper was the subject of a paper read at the Biochemical Society meeting held on March 15th, 1935 [Young, 1935].

of Fe^{+++} in 5 ml. of *N* HCl was suitable for the precipitation of quantities of phytic acid up to 2 mg. (expressed as P) in 25 ml. of neutral or slightly acid solution. Under these conditions the required final concentration of *N*/6 HCl was obtained. After the other details of the method had been established a series of time experiments was performed in order to find the effect of the period of heating on the precipitation process. The following results were obtained:

mg. of phytic acid P taken	mg. of Fe^{+++} precipitated after a heating period of			
	5 min.	10 min.	15 min.	30 min.
0·20	0·14	0·17	0·20	0·19
0·50	0·54	0·53	0·53	0·53
1·00	1·07	1·08	1·07	1·06
1·50	1·57	1·57	1·57	1·58
2·00	2·04	2·03	2·03	2·03

Phosphoric acid is only liberated with difficulty when phytic acid is subjected to hydrolysis and the fact that decreasing values were not obtained with prolongation of the time of heating in the above experiments shows that no significant amount of hydrolysis occurs during the precipitation process. In the case of 0·2 mg. of phytic acid-P marked coagulation of the precipitate did not occur in less than 15 min. heating and since this period also represented the maximum precipitation of Fe^{+++} for this amount of phytic acid it was adopted for use in the method.

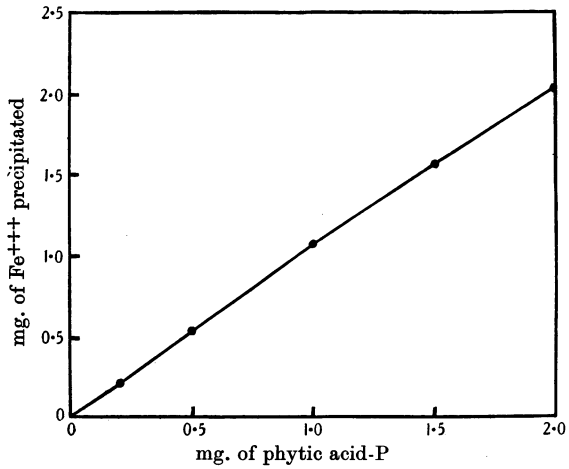
The determination of excess Fe^{+++}. The colorimetric thiocyanate method appeared to be the most suitable means of determining the excess Fe^{+++}, and Brouckère and Gillet [1933] have shown that this procedure can be applied with accuracy to solutions containing HCl up to *N* concentration.

The ferric phytate precipitation is performed in *N*/6 HCl solution (30 ml.), and after the precipitation the volume of the reaction mixture is made up to 50 ml. with *N*/6 HCl and then filtered. If 20 ml. of this filtrate are taken the maximum amount of the excess Fe^{+++} can be 1·0 mg. An investigation was therefore made of the determination of Fe^{+++} in quantities up to 1·0 mg. Using a standard containing 2·0% KCNS and 0·5 mg. of Fe^{+++} in 50 ml. of *N*/6 HCl it was possible to determine accurately amounts of Fe^{+++} between the limits 0·25–1·0 mg.

Quantitative results. Sodium phytate was prepared from a sample of commercial calcium magnesium inositolhexaphosphate ("Phytin" S.C.I., Basle) by the method of Posternak [1921]. The product was recrystallised twice and air-dried. It contained no detectable amounts of inorganic P and gave the following analyses: Na, 17·11%; total P, 11·41%; phytic acid-P (by the method of Heubner and Stadler), 11·48%; H_2O lost at 105–110°, 39·28%; $Na_{12}C_6H_6O_{24}P_6.3H_2O+35H_2O$ requires Na, 17·16%; total P, 11·57%; H_2O (calculated for $35H_2O$), 39·18%. Standard solutions were prepared from the crystalline sodium phytate. They were made just acid to litmus with HCl and were used for determining the amounts of Fe^{+++} precipitated in the colorimetric method by varying amounts of phytic acid. Blank experiments using water were also performed. The mean results obtained were as follows:

mg. of phytic acid P taken	mg. of Fe^{+++} found	mg. of Fe^{+++} precipitated
None	2·50	None
0·20	2·30	0·20
0·50	1·97	0·53
1·00	1·43	1·07
1·50	0·93	1·57
2·00	0·47	2·03

The graphical representation of these results shows that a relationship closely approaching a linear form exists between the varying amounts of phytic acid and the corresponding amounts of Fe^{+++} precipitated.



Graph showing ferric iron-phytic acid-P relationships in the colorimetric method.

Reagents. The following reagents are required for the estimation:

Standard $FeCl_3$ in *N* HCl solution. A solution of A.R. $FeCl_3$ in *N* HCl is made up and Fe^{+++} determined gravimetrically. By suitable dilution with *N* HCl a solution containing 0·5 mg. of Fe^{+++} per ml. is prepared.

N/2 and *N*/6 HCl.

A 10 % solution of A.R. KCNS in water.

Procedure. Into a dry pyrex boiling-tube are pipetted 25 ml. of the solution for analysis (neutral or just acid to litmus), and 5 ml. of the standard $FeCl_3$-HCl solution are added. The tube is covered by a glass bulb and heated in a rack in a boiling water-bath for 15 min. with the level of the water above that of the contents of the tube. The ferric phytate separates as an ivory coloured flocculent precipitate. After cooling for 15 min. in a bath of cold water the contents of the tube are made up to 50 ml. with *N*/6 HCl. The contents of the flask are filtered into a dry boiling-tube through a dry 9·0 cm. no. 31 Whatman filter. 20 ml. of the filtrate are pipetted into a 50 ml. flask (in cases where the amount of phytic acid approaches the upper limit determinable by the method the small excess of iron makes it preferable to use 30 ml. instead of 20 ml. of this filtrate), 5 ml. of *N*/2 HCl and 10 ml. of 10 % KCNS solution are added, the solution is made up to 50 ml. with *N*/6 HCl, mixed and compared without delay in a colorimeter with the standard. The standard is prepared as follows: 5 ml. of the standard $FeCl_3$-HCl solution in a 50 ml. flask are diluted with 25 ml. of water and made up to 50 ml. with *N*/6 HCl. 10 ml. of this solution are pipetted into a 50 ml. flask, 5 ml. of *N*/2 HCl and 10 ml. of 10 % KCNS solution are added and the volume is made up to 50 ml. with *N*/6 HCl. The most convenient colorimeter reading for the standard is 20 (2·0 cm.), and by using this reading and 20 ml. of the filtrate for the determination of excess Fe^{+++}, the mg. of Fe^{+++} precipitated by the phytic acid can be calculated from the expression $2{\cdot}50-\frac{25}{\text{Reading of unknown}}$.

From the amount of Fe^{+++} precipitated the quantity of phytic acid P is obtained by reference to the graph. For conversion of the results for phytic acid-P into phytic acid ($C_6H_{18}O_{24}P_6$) the factor is 3·55.

II. The determination of phytic acid in faeces.

In studying the metabolism of phytic acid in rabbits [Young *et al.*, 1935] it became necessary to determine phytic acid in faeces. Since this material yields coloured extracts, the following separation procedure was evolved. The phytic acid is precipitated from the extract with $FeCl_3$ in dilute HCl solution, the precipitate is separated by centrifuging, decomposed with NaOH, the $Fe(OH)_3$ removed by filtration and the filtrate (containing sodium phytate) used for the determination by the colorimetric method. The various stages of this procedure are considered in detail below.

Extraction of the phytic acid. Most recent workers have used 2% HCl for extracting phytic acid from biological material, *e.g.* Harris and Mosher [1934] used 25 ml. of 2% HCl for each g. of material and extracted for 3 hours with occasional shaking. In the present work it was found more convenient to use *N*/2 HCl for extraction. Repeated extraction showed that a single extraction under conditions to be described is sufficient.

Precipitation of ferric phytate. Previous workers have reduced the acidity of the extract to 0·6% HCl by dilution and then proceeded to titrate the phytic acid in this solution. In the present method the extract is first neutralised, made just acid to litmus with HCl and then filtered. To a measured volume of the filtrate is added one-fifth of its volume of a $FeCl_3$ solution in *N* HCl and the required acidity is thus obtained. This procedure has the advantage that it avoids a large increase in the volume of the extract and also brings about the precipitation and removal of much unwanted material from the extract preliminary to the ferric phytate precipitation. With 20 ml. of slightly acid extract (containing not more than 4 mg. of phytic acid-P) it was found necessary to use 4 ml. of a *N* HCl solution of $FeCl_3$ containing 1·25 mg. Fe per ml.

Determination of the phytic acid of the precipitate. The precipitate is suspended in about 10 ml. of hot water in the centrifuge-tube and then converted into sodium phytate and ferric hydroxide by the action of sodium hydroxide. By calculation and experiment 2 ml. of *N*/2 NaOH were found to be adequate for the treatment of precipitates containing up to 4 mg. of phytic acid-P. The ferric hydroxide coagulates and is removed by filtration and washed with hot water. The filtrate and washings are made just acid to litmus with HCl. In the case of a faeces determination the solution obtained at this point is colourless and also gives no colour with KCNS. The phytic acid content of this final solution is determined by the colorimetric method described above (I).

Quantitative results. After the above conditions had been established, the efficiency of the separation procedure was tested using slightly acid solutions containing 1, 2, 3 and 4 mg. of phytic acid-P. The following results were obtained:

mg. of phytic acid-P taken	mg. of phytic acid-P found	
1·00	0·96	0·98
2·00	1·98	1·96
3·00	2·94	2·94
4·00	3·92	3·92

The average recovery in these 8 experiments was 97·9%.

The method has the advantage that it eliminates interference by inorganic phosphate, *e.g.* a solution containing 1·25 mg. phytic acid-P gave a recovery of 1·20 mg. whereas a similar solution containing in addition 15 mg. of inorganic P (present as Na_2HPO_4) gave a recovery of 1·21 mg.

The method was tested with extracts of rabbit faeces. In each experiment two equal volumes (A and B) of the *N*/2 HCl extract were taken and a known amount of phytic acid added to B. The determinations were then performed as described in detail below. The results were as follows:

mg. of phytic acid-P			
Added to B	Found in A	Found in B	Difference
0·50	0·58	1·07	0·49
0·50	0·50	1·00	0·50
1·00	0·72	1·68	0·96
1·00	0·78	1·72	0·94
1·00	0·68	1·63	0·95
1·00	0·68	1·64	0·96
1·00	0·84	1·83	0·99
1·00	1·31	2·24	0·93
1·00	1·28	2·25	0·97
1·00	1·07	2·03	0·96

The average recovery of added phytic acid in this series of experiments was 96·4%.

No phytic acid was found when the method was applied to the faeces of rabbits fed for some days on a diet (cabbage) free from phytic acid thus showing that under these conditions the faeces contained no interfering substances.

Reagents. The following reagents are required.

$FeCl_3$ in *N* HCl solution. A solution of A.R. $FeCl_3$ in *N* HCl is prepared containing 1·25 mg. Fe^{+++} per ml.

N/2 HCl.

5*N* and *N*/2 NaOH solution.

Procedure. The faeces are dried, sieved and extracted by mechanical shaking for 2 hours in a glass-stoppered bottle with 25 ml. of *N*/2 HCl per g. of material. The extract is filtered on a Büchner funnel and 40 ml. of the filtrate are transferred to a 50 ml. flask, neutralised with 5*N* NaOH, made just acid to litmus with HCl, made up to 50 ml. with water and filtered. A measured volume of the filtrate (containing not more than 4 mg. of phytic acid-P) is transferred to a centrifuge-tube and one-fifth of its volume of the $FeCl_3$-HCl solution is added. The tube and its contents are heated in a boiling water-bath for 15 min., cooled in a bath of cold water for 15 min. and centrifuged for 10 min. at 3000 r.p.m. The supernatant fluid is decanted and the tube is allowed to drain. The precipitate is suspended in 10 ml. of hot water in the centrifuge-tube and heated in a boiling water-bath for 2 min. To the hot suspension 2 ml. of *N*/2 NaOH are added with stirring, and heating in the water-bath is continued for 15 min. with occasional stirring. The precipitate of $Fe(OH)_3$ is filtered on a 7 cm. no. 31 Whatman filter and washed with hot water. The filtrate and washings are allowed to cool, made just acid to litmus with HCl and the volume made up to 50 ml. with water. 25 ml. of this solution are used for the determination of the phytic acid by the colorimetric method.

Conclusions.

Although a detailed study of this procedure has only been made in the case of faeces it seems probable that it may prove of value in determining phytic acid in other biological materials. It minimises the action of interfering substances

and can be applied to materials yielding coloured extracts, which is not possible with the direct titration methods previously used. Furthermore the colorimetric method permits the accurate determination of smaller amounts of phytic acid than has been hitherto possible.

SUMMARY.

A description is given of a colorimetric method for determining phytic acid in amounts up to 2 mg. (expressed as P). A procedure is also described which has been developed to permit the determination of phytic acid in biological materials which yield coloured extracts and an account is given of its application to faeces.

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