On Phytic Acid, its Importance in Metabolism and its Enzymic Cleavage in Bread Supplemented with Calcium

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I. IMPORTANT PROPERTIES OF PHYTIC ACID

Phytic acid, a hexaphosphoric ester of inositol, is found in all plant seeds examined, in grains as well as in oilseeds, and undoubtedly represents a store of phosphoric acid that is hydrolyzed in sprouting. For this reason green plants never contain considerable amounts of phytic acid. The cleavage in the seed is evidently caused by a specific enzyme called phytase, but other phosphatases from plant tissue are able to split the substance to some degree.

In most cases no phytase activity is found in resting seeds. The enzyme seems to be formed during sprouting of the seeds. Wheat and rye, and to a certain extent barley and buckwheat, are, however, exceptions from this rule. If the whole flour of these grains is suspended in a buffer solution at pH 5 and left at a temperature of 40° for 2 hr. all the phytic acid in wheat and rye, and a considerable part of the acid in barley and buckwheat, is split into inositol and free phosphoric acid. In flours of any other seeds examined no cleavage of phytic acid takes place under these conditions. Experiments with pure phytase preparations have proved the conditions mentioned to be optimal, and confirm the conclusion that these grains do not contain phytase. Table 1 shows the results of investigations by Pedersen (1940) in Møllgaard's laboratory.

Total P was in the experiments of Pedersen and in other experiments referred to in this paper determined by destruction of the substance with concentrated sulphuric and nitric acids and precipitation as ammonium phosphomolybdate; this was heated at 550° to form phosphomolybdic acid (Hansen & Græsholm, 1935).

The phytic P was determined by extraction with 0·5N-HCl and precipitation as iron phytate; this was decomposed with NaOH, and the P in the dissolved sodium phytate was determined according to the method for total P (Pedersen, 1940).

Ca was determined by destruction with concentrated sulphuric acid, dissolution of the ash in diluted hydrochloric acid and precipitation as oxalate, which was titrated with permanganate (Hansen & Græsholm, 1935).

Table 1. *The phytic acid and phytase content of certain cereals and seeds*

	Total P (%)	Phytic P (%)	Phytic P (as % of total)	Phytic P split in 2 hr. by the naturally present phytase (%)
Wheat	0·319	0·273	86	100
Wheat bran	1·099	0·944	86	100
	1·120	0·965	86	100
Rye	0·342	0·250	73	100
Barley	0·281	0·182	65	69
	0·322	0·205	64	94
Buckwheat	0·304	0·192	63	55
Maize	0·255	0·205	80	0
	0·330	0·270	82	2
	0·248	0·199	80	4
Oats	0·345	0·228	66	8
Cotton-seed meal:				
Brazil	1·047	0·812	78	4
Siam	1·446	1·183	82	3
Linseed meal	0·989	0·772	78	2
Ground-nut meal	0·992	0·529	76	0
Coconut meal	0·505	0·258	51	0
Palm-kernel meal	0·576	0·481	84	0
Rape-seed meal	0·814	0·649	80	10
Sunflower-seed meal	0·944	0·803	85	0
Soya-bean meal	0·629	0·426	68	0

Table 1 shows that a very considerable part of the total P in seeds is present as phytic acid; in the grains used for human food this amounts to 75–85%. Maize and oats contain no phytase, but Pedersen (1940) has shown that a considerable part of their phytic acid can be split if the flours are suspended in a solution of phytase extracted from wheat bran, and kept under optimal conditions.

Phytic acid forms an interesting calcium salt. In Fig. 1 is shown a titration curve for calcium phytate. It was made by dissolving equivalent amounts of sodium phytate and calcium chloride in water at pH 2 and titrating with 0·1N-NaOH, the potentials being measured with the glass electrode. The molarity of the solution was 0·0067 at the beginning of the titration. The figure shows that the curve has two turning points. At pH 3·7 a precipitate of calcium phytate was formed, probably a basic salt. The analysis of the substance showed a P : Ca ratio of 1·25 : 1.

In his investigation of the solubility of calcium phytate Hoff-Jørgensen (1944) found that a salt of a constant composition was precipitated within the pH interval of 4·6–6·9, the P : Ca ratio being approximately 1·2 : 1. This seems to be in fair agreement with our result and corresponds to the empirical formula: $C_6H_2O_{24}P_6Ca_5$, which means, that the salt formed is pentacalcium phytate.

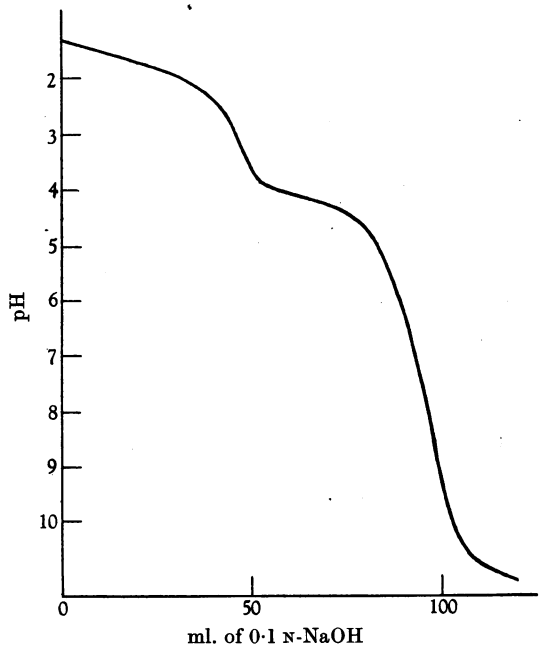

Fig. 1. Titration curve of calcium phytate.

It is important, that this salt is precipitated at a rather acid reaction, because it means that the presence of phytic acid in the intestines may seriously interfere with the absorption of calcium, even in the first parts of the small intestine, where the reaction corresponds to a pH of 4–5.

On the other hand we have found that certain organic oxy-acids forming complex calcium salts are able to shift the precipitation point of calcium phytate to a more alkaline reaction. Fig. 2 shows the effect of increasing concentrations of lactic, tartaric, citric and gluconic acids. It will be seen that tartaric and citric acids especially, in higher concentrations, effect a very large displacement of the pH of precipitation. Lactic acid also has a considerable influence. This is particularly remarkable, since considerable amounts of lactic acid are produced by fermentation in the intestines of many animals, and to some extent in those of man. These results have been confirmed by Christensen (1944), who in the laboratory of Møllgaard measured quantitatively the effect of oxy-acids on the solubility of pentacalcium phytate. An excess of this substance was shaken for 24 hr. at a temperature of 20° with solutions of sodium chloride, tartaric acid and citric acid. From the suspension 10 ml. were filtered and the contents of Ca and P

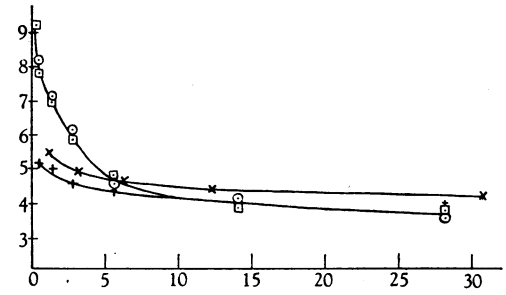

Fig. 2. The influence of oxy-acids upon the precipitation point of calcium phytate. The abscissa represents the quotient (m-equiv. phytate : m-equiv. oxy-acids) × 10, the ordinate the pH at which precipitation of calcium phytate takes place. × lactic acid, ⊙ tartaric acid, ⊡ citric acid, + gluconic acid.

determined. The results are given in Fig. 3. It is evident that the two acids increase enormously the solubility of the phytate. The same effect was found with $Ca(IO_3)_2.6H_2O$, so that it presumably takes place with any insoluble calcium salt, the

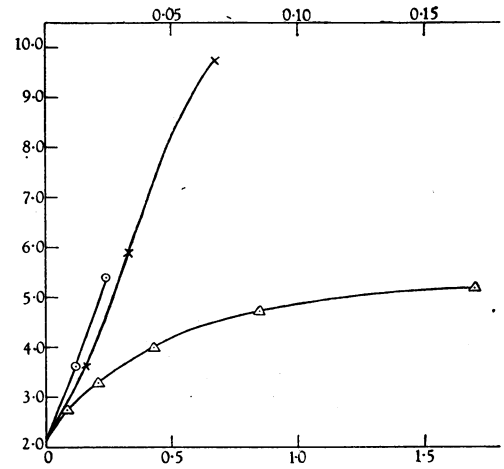

Fig. 3. The influence of oxy-acids on the solubility of calcium phytate. The abscissa represents the molar concentrations of sodium chloride and of tartaric and citric acid, the ordinate gives the concentration of calcium phytate in g./l. △ NaCl, × tartaric acid, ⊙ citric acid.

insoluble phosphates included. The influence of oxy-acids on calcium absorption may therefore be very far-reaching. The old question of the importance of fatty acids for this absorption may find its solution in that effect.

Phytic acid also forms insoluble iron salts. The exact composition of these salts is not known at present, but McCance, Edgecombe & Widdowson (1943) have demonstrated that the ferrous as well as the ferric ion is precipitated by sodium phytate at a pH of 6·5 and that the precipitate always contains more phosphorus than the equivalent of iron, being presumably a double phytate of sodium and iron. This means that both ferrous and ferric phytates are more insoluble at the pH obtaining in the largest part of the small intestine, than the corresponding phosphates or hydroxides. Phytic acid may therefore also interfere with the absorption of iron from the intestine.

II. THE REACTION IN THE INTESTINE

As far as we know an element must be in solution in order to be absorbed from the intestine. As the solubility of calcium and phosphate ions is strictly dependent on the reaction of the solvent, the pH in the contents of the different parts of the intestinal canal must be of decisive importance in the absorption of these elements when they are present together. The same holds presumably for iron, because it forms insoluble precipitates with phosphoric acid in neutral solution. It is therefore regrettable that the whole question of the reaction in the intestinal canal of animals and man has not yet been given the attention it deserves. It seems that up to now the only extensive investigation was that done by Hagens in the laboratory of Møllgaard. Hagens measured the reaction in the different parts of the intestine of pigs under varying conditions of feeding. Because of his unfortunate death during the German occupation, his work cannot yet be published in full. Only part of it has appeared (Hagens, 1943). In Tables 2–5 are given the results of his unpublished measurements on pigs under normal conditions of feeding. All the values were measured with the glass electrode on samples taken from tubes inserted in the stomach, the duodenum, the jejunum and the ileum. Full details of this technique will soon be published by Borch-Madsen (1946). In the jejunum the tubes were set 3–4 m., in the ileum 13–15 m., from the pylorus. The measuring took place immediately after the sample had been taken. In some of the animals the results were controlled by direct measurement with glass electrodes in the tubes. In these cases a calomel electrode was connected with the stomach and the rectum of the animal. The readings were about the same for both calomel electrodes and corresponded very closely with the results on the samples. In all cases the samples were taken at such time after feeding that the intestine in the section of the tube was known to be filled with food. Tables 2–5 give, therefore, values of pH during digestion.

Table 2. *pH in the stomach of the pig. Samples taken* 30 *min. after feeding*

	Samples from the outer liquid layers of food		Samples from the central mass of food	
Pig no.	No. of samples	pH range	No. of samples	pH range
T73	8	1·14–2·79		
T73	13	1·02–3·78		
T73	11	1·06–2·21	11	1·66–4·59
T73	16	1·35–3·48	16	1·79–5·29
T43	6	1·43–2·40	7	1·76–4·48
T43	5	1·81–3·66	4	3·61–4·19
T43	5	1·91–1·78	5	1·30–5·08
T43	5	1·34–3·03	5	1·34–3·99
T15	9	1·64–3·27		
T43	6	0·95–1·95	6	1·54–4·26

From Table 2 it appears that the pH in the central mass generally has a higher value than in the outer layers of the food. This was to be expected because the hydrochloric acid diffuses from outside into the food mass. In the liquid contents of the stomach the pH varies between 1 and 3.

Table 3. *pH in the duodenum of the pig. Samples taken* 45 *min. after feeding*

Pig no.	No. of samples	pH range
T73	11	2·46–6·15
T73	9	3·07–6·04
T73	11	3·30–6·10
T73	12	2·34–6·29
T43	6	4·03–5·04
T43	5	4·29–5·37
T43	6	3·97–5·97
T43	5	3·93–5·93
T15	14	4·04–5·66
T43	9	2·65–5·60
T33	6	3·91–6·12
T33	5	4·19–5·12
T33	7	4·31–5·36
T33	7	3·17–4·78
T6	8	3·60–5·60
T6	9	3·47–4·97

The values of pH in the duodenum vary widely. This would be expected because the stomach is emptied periodically. If the sample is taken immediately after the opening of the pylorus, the

Table 4. *pH in the jejunum of the pig. Samples taken* 3 *hr. after feeding*

Pig no.	No. of samples	pH range
T73	7	5·14–7·68
T73	5	5·30–5·61
T73	10	5·37–6·54
T73	8	5·66–6·03
T43	6	5·73–6·23
T43	5	5·92–6·38
T43	5	5·80–6·25
T43	6	5·98–6·52
T15	16	5·64–6·75
T43	7	5·59–6·35

pH will be considerably lower than later, when the contents of the duodenum have been partly neutralized by the pancreatic juice. The reaction, however, always remains acid, and corresponds generally to pH values between 3 and 6.

The variations of pH in the jejunum are much smaller than in the duodenum. It is further remarkable that the reaction still remains acid. On average the pH is between 5 and 6·5.

Table 5. *pH in the ileum of the pig. Samples taken 5 hr. after feeding*

Pig no.	No. of samples	pH range
T73	5	6·68–7·73
T73	2	7·25–7·51
T73	2	6·71–6·89
T43	6	6·72–7·39
T43	5	7·04–7·49
T43	4	7·35–7·57
T43	6	6·53–7·28
T15	6	6·75–7·33
T43	5	7·38–7·60
T33	4	7·20–7·36
T33	4	7·30–7·35
T33	4	7·10–7·46
T33	2	6·70–7·98

Table 5 shows that the reaction in the lower parts of the small intestine varies about the point of neutrality.

As a general result of these measurements, the following ranges for pH in the different parts of the intestine of pigs can be quoted:

Duodenum	3 –6
Middle of jejunum	5 –6·5
Lower part of ileum	6·5–7·5

This means that the reaction is *acid* in the largest part of the small intestine. Not until its end does the reaction become alkaline. Presumably the same holds for other mammals with a relatively short intestinal canal, man included.

III. ABSORPTION OF CALCIUM, PHOSPHATE AND IRON

The conditions of reaction mentioned are very important for the absorption of some of the most valuable foodstuffs. As considerable amounts of $HPO_4^=$ are not formed until the pH reaches the value of 6 and the quotient $\frac{H_2PO_4^-}{HPO_4^=}$ does not amount to 1 until the pH is 7, the calcium and the phosphate ions can remain in solution in the contents of a large part of the small intestine, and in these upper sections absorption of both ions can take place. In the lower parts of the small intestine these ions may be precipitated as $CaHPO_4$ and therefore withdrawn from absorption. Probably the same conditions are decisive for the absorption of the ferrous ion.

If, however, the intestine contains considerable amounts of phytic acid, Ca^{++} may be precipitated even at a pH of 3·7 and absorption very probably prevented within the whole range of the small intestine. To a certain extent the same may happen to the ferrous ion, which is precipitated as a double phytate at pH 6·5.

On the other hand, if the phytic acid is split to inositol and free phosphoric acid, the conditions are of course entirely altered. As far as we know the gastric and intestinal juices of the higher mammals including man contain no phosphatase that could effect the cleavage of phytic acid. The substance therefore cannot be digested unless the food itself contains a specific phytase. This may happen very frequently with domestic animals because some grains ordinarily used for feeding stuffs (wheat, rye, barley), and young green plants often contain considerable amounts of this enzyme. There is no doubt that an enzymic cleavage of phytic acid by means of food phytase takes place to a large extent in the stomach of pigs in the preliminary period of the digestion, where the pH amounts to 4·5. That this cleavage is of the greatest importance to the well-being of the animals will be shown later in this paper. In man, such cleavage of phytic acid by means of food phytase is not likely ever to take place, because he ordinarily eats food that in some way or another has been heated to a temperature that destroys any enzymic activity.

The precipitation of the calcium and ferrous ions, however, can be prevented if the intestinal food masses contain considerable amounts of oxy-acids, forming soluble complexes with these ions. In domestic animals this may be the case because they normally produce some lactic acid by fermentation in the stomach and the upper parts of the intestine, and lactic acid is known to shift the precipitation point of calcium phytate. If the lactic acid fermentation is sufficiently extensive it may reduce to a minimum the precipitation of phytates as well as phosphates of calcium and iron. Absorption of calcium ions would still take place because the equilibrium of calcium complex, calcium ion, phytate and phosphate ions is continuously readjusted as Ca^{++}, $CH_3CHOHCOO^-$ and $H_2PO_4^-$ are absorbed. The same holds for the ferrous ion. Under these conditions the harmful effect of phytic acid may be eliminated. Such conditions cannot be supposed to exist in man because the lactic acid fermentation in his intestinal canal is very slight so long as the secretion of hydrochloric acid in his stomach is normal. Unless he directly consumes oxy-acids, he cannot take advantage of their complex-forming capacity. In man, therefore, phytic acid may presumably unfold its whole harmful effect on the absorption of calcium and iron.

If no cleavage of phytic acid takes place, the phosphoric acid in it cannot be absorbed because, except for glucose- and glycerol-phosphoric esters, we know of no organic compound of phosphoric acid that is absorbed as such. In this case phytic acid presumably has another harmful effect, namely that its phosphoric acid is withdrawn from metabolism. If the food consists largely of grain products, a dangerous decrease of the phosphorus supply may result. The risk of this happening is definitely greater in the pig and in man than in most other mammals.

Further, there is very good reason to believe that the precipitation of calcium phytate in the small intestine may depress not only the absorption of calcium but also of phosphorus, by making the phytic acid inaccessible to the action of phytase, if this enzyme is present in the food. Under such conditions oxy-acids may promote absorption of phosphorus by bringing calcium phytate and, in the lower parts of the intestine, calcium phosphate into solution.

IV. EXPERIMENTAL EVIDENCE OF THE HARMFUL EFFECT OF PHYTIC ACID ON THE ABSORPTION OF CALCIUM AND IRON

A. *Review of literature.* Owing to the length of time during which British and Danish investigations on these problems have been out of touch the references in this paper to British work cannot be complete. If some important British work on this matter is not mentioned, it is earnestly hoped that the authors will understand that this omission was caused by wartime circumstances.

Harrison & Mellanby (1939) expressed the opinion that the pronounced rachitogenic effect of oatmeal was a consequence of the fact that phytic acid formed insoluble calcium salts, and that in this way the absorption of phosphoric acid as well as of calcium was seriously reduced. An experimental proof that phytic acid in the food really diminished the absorption of calcium was not published by these or any other authors writing on the subject at that time. Pedersen (1940), however, brought forward some evidence. In the course of extensive investigations of the rachitogenic effect of phytic acid, he found that the calcium content of the faeces of pigs fed a ration containing phytic acid was strictly correlated with the content of phosphoric acid. As the amount of undigested phosphoric acid in these experiments was shown to be approximately equivalent to the amount of phytic acid in the food, the amount of unabsorbed calcium was directly correlated with the content of phytic acid in the food. This finding afforded evidence that the amount of phytic acid in the food limits quantitatively the absorption of calcium by pigs.

In the same year Krieger & Steenbock (1940) published investigations on the availability of phytic acid phosphorus to rats. It is known that these animals produce some kind of 'phytase' in their intestinal canal, and therefore are able to split phytic acid to a certain extent. Using the percentage of bone ash as criterion, the two authors found that the availability of phytic phosphorus decreased markedly as the calcium content of the food increased, a fact that can hardly be explained otherwise than by assuming that calcium in the food prevents the splitting of phytic acid by precipitating it as an insoluble salt.

Two years later McCance & Widdowson (1942–3*a*) reported investigations on the absorption of calcium from bread. Their experiments were of particular interest because they were performed on human beings. They found that the absorption of calcium and magnesium decreased markedly when bread made from flour of 69% extraction was replaced by bread made from flour of 92% extraction, that is, when the content of phytic acid in the food was increased. They further found that an addition of phytic acid to white bread (69% extraction) caused a fall in the absorption of calcium that was so serious that the calcium balance in some subjects became negative. Finally it was shown that addition of $CaCO_3$ or $CaHPO_4$ improved the absorption of calcium considerably. In further experiments it was found that with diets containing respectively white bread, brown bread with partially split phytic acid and brown bread with the phytic acid intact the absorption of calcium decreased progressively in the order given.

It appears from all these researches that there is very good evidence that phytic acid in the food markedly prevents the absorption of calcium from the intestine of pigs and human beings, and that probably the same holds for magnesium.

Mellanby (1921, 1925) demonstrated that oatmeal has a marked rachitogenic effect on young dogs. Templin & Steenbock (1933) showed that the disappearance of this effect after boiling with acid was accompanied by a change of organic P to inorganic P in the cereals, and suggested that the organic P might not be as available as the inorganic. Bruce & Callow (1934) considered that the rachitogenic effect of cereals was due to their high content of phytic acid with phosphorus in a comparatively unavailable form. Harrison & Mellanby (1939) accepted the idea of the rachitogenic effect of phytic acid but laid stress on the calcium precipitating effect of this acid.

The influence of phytic acid upon the absorption of iron has been investigated by Widdowson & McCance (1942) in human beings. Brown bread with a high content of phytic acid seemed to depress the absorption of iron. As iron balances present

considerable technical difficulties, the two authors rightly regarded their results as suggestive rather than conclusive, and tried to obtain further information on the matter by new experiments in which the rise in the concentration of serum iron following a large dose of a soluble iron salt was taken as criterion of the absorption (McCance & Widdowson (1942–3*b*). In fourteen out of sixteen observations the addition of phytic acid prevented the full rise of the serum iron following the intake of the soluble iron salt. Taken together, these experiments support the opinion that phytic acid in the food has a depressing action on the absorption of iron from the intestine of man.

B. *New experiments.* In the course of investigations on rickets in pigs, performed in the laboratory of Møllgaard, it was established beyond all doubt that oats and maize have a very marked rachitogenic effect, whereas barley, wheat and wheat bran could be incorporated in the diet in large amounts without any such effect. As the phytic acid content of these grains is approximately the same (Table 1), it is evident that the suggestion of Bruce & Callow (1934) could not contain the whole truth. There must be some other difference in the composition of these grains responsible for their very unequal effect. As it was shown by Pedersen (1940) that the rachitogenic grains, oats and maize contained no phytase whereas this enzyme was found in varying amounts in the non-rachitogenic grains (Table 1), it was very obvious that this difference might provide the key to the understanding of the whole question of the rachitogenic effect of cereals. To investigate this possibility Pedersen made very extensive researches on pigs in the laboratory of Møllgaard. Having proved that the phytic acid in oats and maize could be split when the ground cereal was suspended under optimal conditions in a solution of phytase prepared by extracting wheat bran with saturated lime water, he proceeded to prepare such an extract on a scale sufficient for the treatment of the

Table 6. *Main results of experiments by Pedersen* (1940) *on the effect of phytic acid and phytase in the fodder on the development of rickets in groups of two pigs*

Group no.	State of phytic acid in fodder	State of phytase in fodder	Phytic P added as sodium phytate (g.)	Blood P (mg. %)	Degree of rickets
RI	Intact	Active	0	—	0
				—	0
RII	Intact	Inactive	0	—	+ +
				—	(+)
RIII	Split 50%	Inactive	0	—	+
				—	+
RVII	Intact	Inactive	2·5	3·47	+ +
				4·23	+ +
RVIII	Split	Inactive	2·5	7·88	0
				7·82	0
RIX	Intact	Active	2·5	5·18	0
				5·66	0
RX	Intact	Inactive	1·0	3·84	+ + +
				3·33	+ + +
RXI	Split	Inactive	1·0	7·40	(+)
				5·42	(+)
RXII	Intact	Inactive	0	4·55	+ +
				5·58	+
RXIII	Intact	Active	0	6·91	0
				7·12	0
RXV	Intact	Inactive	0*	5·27	(+)
				5·35	+ +
RXVI	Intact	Inactive	0†	7·25	0
				7·54	0

In the last column the degree of rickets is indicated as follows:

0 No macro- or microscopical rickets.
(+) Uncertain macro- or microscopical rickets.
\+ Slight macro- and microscopical rickets.
\+ + Severe macro- and microscopical rickets.
\+ + + Florid macro- and microscopical rickets.

* 15 g. $CaCO_3$ and 7000 i.u. vitamin D added daily.
† 12·5 g. $CaHPO_4.2H_2O$ and 7·5 g. $CaCO_3$ added daily.

whole ration of growing pigs. He prepared further pure crystalline sodium phytate in sufficient amounts to supply it to pigs as a source of phosphoric acid.

Groups of young pigs of 15–20 kg. live weight were then formed, each containing two animals. They received diets composed of different cereals with blood meal as a principal source of protein. Vitamins were partly provided by direct addition of concentrates (4000 i.u. of vitamin A, 200 i.u. vitamin B_1, 30 Sherman units of vitamin B_2 and 20 mg. ascorbic acid daily) and partly by 0·5 l. skim milk/kg. of dry food. The Ca content of the diet was in all experiments approximately 8 g./kg. of dry matter and the content of NaCl *c.* 6 g. The content of available phosphorus varied according to the amount of phytic acid in the food. For some groups the phytic acid was split before feeding by means of the treatment with phytase solution described above (indicated in Table 6 by 'split'). In some cases the whole food was heated before feeding to destroy the phytase (indicated by 'inactive'); in some, the phytase in the food was left intact and further increased by the addition of an extract of wheat bran (indicated by 'active'). Finally, in some groups, sodium phytate was directly added to the food. Each experiment lasted 6 days with a preliminary period of 8 days. The main results of these investigations are given in Table 6.

It will be seen from Table 6 that signs of rickets and a low phosphorus content of the blood are associated with the presence in the intestine of intact phytic acid. Rickets was absent or only very slight in the groups where the phytic acid had been split before feeding, or the animals received active phytase.

The results for groups RXV and RXVI show that even the addition of vitamin D cannot suppress entirely the rachitogenic effect of phytic acid but that the addition of $CaHPO_4$ prevents it completely.

It follows from these experiments that the phosphorus of phytic acid cannot be absorbed and participate in metabolism unless it is in some way split into free phosphoric acid and inositol, and this cannot take place in the intestine of the higher animals unless the food itself contains a certain amount of active phytase.

This conclusion was further substantiated by Pedersen's observation that, when the phytic acid was not split before feeding or the food contained no active phytase, the faecal phosphorus was very nearly equal to the phytic phosphorus in the food. If, on the other hand, the phytic acid was split, or the animal was fed active phytase, the faecal phosphorus was always less than the phytic phosphorus in the food.

Man commonly eats food with the phytase activity entirely destroyed by heating, and there seems to be no possibility of any cleavage of phytic acid in his intestinal canal. The rachitogenic effect of phytic acid must therefore be more serious for man than for the pig unless human food is, in some way or another, supplied with inorganic phosphate in sufficient amounts to prevent the fall in absorption.

V. EXPERIMENTAL EVIDENCE OF THE BENEFICIAL EFFECT OF ORGANIC OXÝ-ACIDS ON THE ABSORPTION OF CALCIUM AND PHOSPHORUS FROM RATIONS CONTAINING PHYTIC ACID

It has been mentioned that an addition of oxy-acids to the food or a considerable production of lactic acid in the intestinal canal of mammals might prevent the harmful effect of phytic acid on the absorption of calcium and phosphorus.

Evidence of such effect is found in the investigations of Møllgaard (1945) on the effects of oxy-acids on pigs. Four pigs, all females, weighing 20–23 kg., were used for these experiments. Their food was made up of feeding stuffs commonly used for raising young pigs in Denmark, namely barley, wheat, wheat bran, blood meal and lucerne meal. Sufficient $CaHPO_4$ and $CaCO_3$ were added to provide, together with the calcium and phosphorus already present, a total supply of 9 g. Ca and 6 g. P/kg. dry matter; the animals were given to drink, with each kg. dry matter, 500 g. of skim-milk. About 60 % of the total phosphorus was present as phytic acid. The phytase activity of the grains and bran was not destroyed before feeding, so that the animals had the chance to split the phytic acid during digestion. The effect of oxy-acids on the absorption of calcium and phosphorus was studied under such relatively favourable conditions. Tartaric and lactic acids were used and the pigs were divided into two groups. In the first period of the experiment with tartaric acid pigs 43 and 45 received daily 13·5 g. of the acid and pigs 47 and 48 none. In the second period pigs 47 and 48 received 16·5 g. of the acid, and pigs 43 and 45 served as controls. The experiment with lactic acid was similarly arranged. Each experimental period lasted 6 days with a preliminary period of 7–8 days. The results are given in Table 7.

The results show that the two oxy-acids caused a considerable increase in the absorption of phosphorus and particularly of calcium.

Experiments were also performed with diets containing only 4 g. Ca/kg. dry matter (Table 8).

This time the percentage absorption was naturally somewhat higher in all groups, but the effect of the oxy-acid was again obvious.

Table 7. *Effect of oxy-acids on the absorption of* P *and* Ca *by the pig*

No. of animal and period	Intake (g./day) P	Intake (g./day) Ca	Percentage absorption P	Percentage absorption Ca	Oxy-acids added to food (g./day)
43_{I}	5·62	7·64	42·3	43·2	Tartaric acid 13·5
45_{I}	5·62	7·64	42·3	46·3	„ „ 13·5
47_{II}	6·90	9·29	43·6	35·6	„ „ 16·5
48_{II}	6·90	9·29	44·1	38·6	„ „ 16·5
43_{III}	8·16	10·97	41·2	42·3	Lactic acid 8·0
45_{III}	8·16	10·97	37·5	39·7	„ „ 8·0
47_{IV}	9·41	12·66	43·1	41·9	„ „ 8·8
48_{IV}	9·41	12·66	37·1	39·3	„ „ 8·8
47_{VI}	10·81	14·31	38·4	37·3	Lactic acid 20·0
48_{VI}	10·81	14·31	39·3	40·3	„ „ 20·0
47_{I}	5·62	7·64	28·3	30·8	Controls for tartaric acid
48_{I}	5·62	7·64	34·7	38·5	
43_{II}	6·90	9·29	35·2	34·2	
45_{II}	6·90	9·29	36·4	26·4	
47_{III}	8·16	10·97	37·7	36·2	Controls for lactic acid
48_{III}	8·16	10·97	33·0	34·6	
45_{IV}	9·14	12·66	36·1	35·8	
47_{V}	10·21	13·49	39·2	34·7	Controls for lactic acid
48_{V}	10·21	13·49	37·2	32·5	
45_{VI}	10·81	14·31	36·0	32·9	
Mean of all experiments with oxy-acids in the food			40·6	40·2	
Mean of all experiments without oxy-acids in food			37·3	33·8	

Table 8. *Effect of tartaric acid on the absorption of* P *and* Ca *by the pig from a diet relatively low in calcium*

No. of animal and period	Intake (g./day) P	Intake (g./day) Ca	Percentage absorption P	Percentage absorption Ca	Oxy-acid added to food (g./day)
45_{VII}	9·05	5·94	56·1	48·0	25·5 g. tartaric acid
47_{VIII}	9·05	5·94	54·5	51·5	
48_{VIII}	9·05	5·94	46·3	42·9	
Mean			52·3	47·5	
47_{VII}	9·05	5·94	45·2	38·2	No oxy-acid
48_{VII}	9·05	5·94	41·9	34·7	
45_{VIII}	9·05	5·94	46·5	45·3	
Mean			44·5	39·4	

In the experiments described so far no vitamin D was given to the pigs. Table 9 contains results of tests done at the same time with two other pigs which received 1200 i.u. daily of vitamin D but no oxy-acids.

It will be seen that the effect of vitamin D was somewhat greater than that of oxy-acids in the experiments where the calcium content of the food was high, but was of the same order of magnitude in the experiments with food lower in calcium, a fact that underlines the importance of these acids in the absorption of calcium and phosphorus.

Table 9. *Effect of vitamin D added to the food on the absorption of* P *and* Ca *by the pig*

No. of animal and period	Intake (g./day) P	Intake (g./day) Ca	Percentage absorption P	Percentage absorption Ca	Vitamin D (i.u./day)
86_{I}	5·47	6·71	45·5	39·8	1200
87_{I}	5·47	6·71	44·6	41·1	1200
86_{II}	6·36	7·79	49·8	47·4	1200
87_{II}	6·36	7·79	41·5	41·5	1200
86_{III}	7·59	10·14	50·1	50·0	1200
87_{III}	7·59	10·14	43·1	43·1	1200
86_{IV}	8·50	10·37	55·8	56·6	1200
87_{IV}	8·50	10·37	47·5	47·8	1200
Mean			47·2	45·9	

At the conclusion of these experiments tubes were inserted in the jejunum of pigs 45 and 48 and in the ileum of pig 47 for the collection of intestinal contents from which it was proposed to cultivate lactic acid bacilli in order to get a culture that would multiply in the intestine of the pig. By adding such culture to the food it should be possible to increase the concentration of lactic acid in the intestinal contents. Success was achieved with pig no. 48. The isolated bacillus formed, when grown on sterilized skim milk, large amounts of lactic acid, but at temperatures between 30 and 40° only minute amounts of volatile fatty acids. The three pigs were fed ordinary skim milk in a preliminary period and after that they received the same amount of skim milk inoculated with the culture and left at 39° for 24 hr. In both periods samples were taken from intestinal tubes 3 hr. after feeding, and the lactic acid content was determined by a modified method of Flieg (1937). The results are seen in Table 10.

Table 10. *Effect of acidified skim milk on the concentration of lactic acid in the contents of the small intestine of pigs*

Pig no.	(m-equiv. lactic acid/kg. contents) Preliminary period	Experimental period	Percentage increase in lactic acid	Skim milk fed (kg.)
45	75	119	58·7	0·5
47	134	168	25·4	0·5
48_{I}	90	104	15·6	0·5
48_{II}	90	131	45·5	0·5

As the culture increased the production of lactic acid in the pigs' intestine, a large-scale experiment with pigs was set up in order to investigate whether these animals would grow better when fed skim milk inoculated with this culture in addition to an ordinary ration containing phytic acid phosphorus in amounts of 60 to 66% of the total phosphorus. The outcome of this first experiment was that the

growth of the group which received the inoculated milk was distinctly superior to the growth of the control group. Because of the importance of the problem in practical feeding the experiment will be repeated, but the preliminary results already suggest that lactic acid fermentation is of advantage to growth and thereby support the view that the elimination of the harmful effects of phytic acid is of real importance to the metabolism of the pig.

VI. THE NEED FOR INCREASING THE ABSORPTION OF CALCIUM, PHOSPHORUS AND IRON FROM THE ORDINARY FOODSTUFFS USED FOR HUMAN CONSUMPTION

The experiments described in §§ IV and V afford good reason for the conclusion that the theoretical assumptions discussed in § III were correct. The presence of considerable amounts of phytic acid in the food must be considered as harmful to the absorption of calcium, phosphorus and iron. The harmful effect can be prevented either by splitting the phytic acid in the food or by adding sufficient amounts of calcium phosphate and of iron salts to the food.

In animals the cleavage of phytic acid during digestion may to some extent be brought about by the phytase present in some of the feeding stuffs, and it can be further enhanced by an increase in the production of lactic acid in the intestine. In human beings this is seldom possible because their food is mostly heated to such temperature that any enzymic activity is destroyed. There are, therefore, only two possibilities of preventing the harmful effect of phytic acid in human food: the cleavage of the acid before consumption, and the addition to the food of suitable amounts of calcium phosphate and of iron salts. This can, of course, be done by incorporating in the ration other foodstuffs rich in calcium, phosphorus and iron, but containing little or no phytic acid. In modern industrial society, however, the bulk of the population of any country has not access to such foodstuffs. It consumes mostly foods which are produced in large amounts and at relatively cheap prices by the food industry. It is therefore of decisive importance for the well-being and ability to work of these communities that foodstuffs should reach consumption in such a state that they supply the consumers with the amino-acids, vitamins and minerals necessary for nutritional welfare and do not contain any substance that in some way or another may be harmful to metabolism.

With the purpose of improving in this respect the daily bread of the Danish population, work was done in the Physiological Laboratory of the Agricultural Experimental Station and the Central Laboratory of the Co-operative Bakeries in Denmark. It concerns for the present the possibility of increasing the calcium content and of splitting the phytic acid of rye bread so extensively consumed by the Danish population. Further work on the supply of iron and vitamins will, however, follow.

VII. THE ADDITION OF CALCIUM TO BREAD

The necessity to increase the calcium content of the Danish common rye bread is made obvious by Table 11 which lists Danish foodstuffs containing appreciable quantities of calcium. Out of seventy-four Danish foodstuffs analyzed we found only fourteen which could serve as a source of calcium and of these only nine contained considerable quantities.

Table 11. *The calcium content of some Danish foodstuffs*

1% and more		0·5–1·0%		0·1–0·5%	
Spinach	2	Cucumber	0·8	Carrots*	0·3
Lettuce	1	Green beans	0·6	Leeks	0·4
Kale*	2	White cabbage*	0·9	Cauliflower	0·2
Milk	1	Brussels-sprouts*	0·5	Tomatoes	0·15
Cheese	1·5			Fish*	0·1–0·17

* Generally accessible.

Of these foodstuffs only five, marked with an asterisk in the table, are available at prices within the reach of the general population in the towns of Denmark. The table shows that the diet of the ordinary town population in Denmark must be exceedingly poor in calcium. The prevalence of dental diseases in this country bears witness to the harmful effect of this condition.

It was therefore agreed that approximately 0·5% of calcium should be added to the rye bread produced by all the forty-one bakeries belonging to the Workers' Co-operative in Denmark. This was

Table 12. *Calcium content of bread from* 30 *bakeries*

Bakery	Ca in dry matter (%)	Bakery	Ca in dry matter (%)
Aalborg	0·54–0·40	Nyborg	0·37
Aarhus	0·54–0·33	Nykøbing F	0·60
Assens	0·46	Nykøbing M	0·56
Esbjerg	0·30–0·36	Nærum	0·36–0·53
Fredericia	0·24–0·20	Randers	0·41
Frederikshavn	0·49	Ringsted	0·45–0·47
Grenaa	0·57–0·51	Roskilde	0·40–0·60
Glostrup	0·48–0·44	Rønne	0·30–0·46
Haderslev	0·53	Skive	0·47
Helsingør	0·45–0·51	Slagelse	0·41–0·57
Holbæk	0·46	Svendborg	0·48–0·50
Kalundborg	0·46–0·43	Thisted	0·54
Køge	0·41–0·42	Vejle	0·51
København	0·47–0·60	Viborg	0·38–0·58
Nakskov	0·42	Vordingborg	0·46–0·70

done by adding 1 % of $CaCO_3$ to the flour. The calcium content of the bread manufactured by the Co-operative in September 1945 is given in Table 12.

It appears from the table, that the bread from the great majority of bakeries contained 0·4 to 0·6 % of calcium, which should be satisfactory.

VIII. THE SPLITTING OF PHYTIC ACID IN THE DOUGH

The splitting of the phytic acid in the dough is a much more difficult undertaking than the addition of calcium, particularly because this addition very naturally delays the splitting by precipitating the acid as insoluble calcium phytate.

In our work we have, however, taken advantage of the fact that oxy-acids are able to keep the calcium and the phytate ions in solution. It is shown in Fig. 2 that tartaric acid is able to dissolve the ions even in alkaline solution. Lactic acid, of course, is not so effective in concentrations which are possible in the dough, but it can work within the pH interval 4·5–5·5 and this is quite sufficient, because the optimal pH for the action of phytase is approximately 5. We used tartaric acid for the study of the fundamental conditions of cleavage, but in the practical application of the method in the bakeries we have made use of the lactic acid produced in the dough by fermentation. In all experiments in the laboratory and in bakeries the rye flour was of 98 % extraction.

A. *The effect of pH on the cleavage of phytic acid in the dough.* To 100 g. of rye flour containing 0·5 % Ca was added 1 g. of tartaric acid, and the pH was brought to the values quoted in Table 13 by adding water and suitable amounts of HCl and NaOH, the total amount of fluid added being 80 ml. The dough was left at a temperature of 28–30° for 4 hr. The results are given in Table 13.

Table 13. *The effect of pH on the cleavage of phytic acid in rye dough during incubation in the presence of tartaric acid*

pH	Moisture in dough (%)	Total P in dry matter (%)	Phytic P in dry matter: Initial (%)	Phytic P in dry matter: After 4 hr. (%)	Extent of hydrolysis (%)
4·60	47·4	0·510	0·383	0·167	56·4
4·95	47·7			0·109	71·5
5·25	48·2			0·065	83·0
5·47	48·7			0·082	78·6
5·70	48·2			0·071	81·5
5·93	48·1			0·081	78·9

It appears from the table that there is an optimum at pH 5·3, but after this the curve flattens out, so that the difference in action between pH 5·3 and 5·9 is not marked.

B. *The effect of the water content on the cleavage of phytic acid in the dough.* To 100 g. of rye flour containing 0·5 % Ca was added 1 g. of tartaric acid. The pH was brought to 5·5–5·6 and varying amounts of water were added to the dough which was left at a temperature of 28–30° for 4 hr. The results appear in Table 14.

Table 14. *The effect of the water content on the cleavage of phytic acid in rye dough during incubation in the presence of tartaric acid*

Moisture content (%)	pH	Total P in dry matter (%)	Phytic P in dry matter: Initial (%)	Phytic P in dry matter: After 4 hr. (%)	Extent of hydrolysis (%)
49·1	5·5	0·510	0·383	0·043	88·8
46·9	5·5			0·064	83·3
44·5	5·6			0·090	76·5
41·3	5·5			0·111	71·0

The table shows that the water content of the dough has a considerable effect on the velocity of cleavage. To reach a maximum in 4 hr. the water content should be increased to 48–49 %.

C. *The effect of temperature on the cleavage of phytic acid in the dough.* To 100 g. of rye flour with 0·5 % Ca were added 1 g. of tartaric acid and 82 ml. water. The pH was stabilized at 5·3 and the dough left at the temperatures indicated in Table 15 for 4 hr.

The table shows that the rate of hydrolysis increases with increasing temperature, but that it seems not to have reached maximum even at 39°. Table 16 shows that it is a question of the concentration of the oxy-acid.

D. *The effect of the concentration of the oxy-acid on the cleavage of phytic acid in the dough.* To 100 g. of rye flour with 0·5 % Ca were added the amounts of

Table 15. *The effect of temperature on the cleavage of phytic acid in rye dough during incubation in the presence of tartaric acid*

Temperature (°)	pH	Moisture content (%)	Total P in dry matter (%)	Phytic P in dry matter: Initial (%)	Phytic P in dry matter: After 4 hr. (%)	Extent of hydrolysis (%)
10–12	5·3	50·7	0·536	0·409	0·264	35·5
18–20		50·4			0·180	56·0
25–27		50·2			0·120	70·7
39		50·1			0·066	83·7

Table 16. *Effect of concentration of tartaric acid on the cleavage of phytic acid in rye dough during incubation at* 28–30°

Tartaric acid (g./100 g. flour)	pH	Moisture content (%)	Total P in dry matter (%)	Phytic P in dry matter: Initial (%)	Phytic P in dry matter: After 4 hr. (%)	Extent of hydrolysis (%)
1·6	5·7	48·5			0·011	96·9
0·8	6·0	49·0	0·489	0·361	0·083	77·0
0·4	6·15	48·5			0·129	64·3
1·5*	5·3	50·0	0·536	0·409	0·082	80·0

* Temperature of incubation 25–27°.

tartaric acid shown in Table 16. The pH was brought to 5·7–6·1 and 80 ml. water added. The dough was kept at 28–30° for 4 hr.

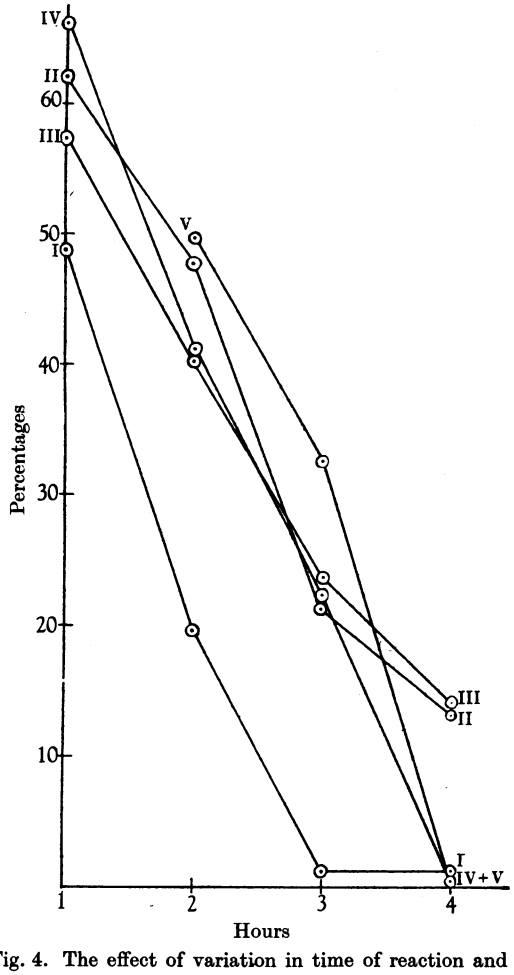

Fig. 4. The effect of variation in time of reaction and in concentration of Ca and of tartaric acid on the cleavage of phytic acid. Curve I. 1% tartaric acid + 0·1% Ca. Curve II. 1% tartaric acid + 0·17% Ca. Curve III. 1% tartaric acid + 0·5% Ca. Curve IV. 1·5% tartaric acid + 0·5% Ca. Curve V. 1·6% tartaric acid + 0·5% Ca. The abscissa indicates the reaction time in hours, the ordinate represents phytic P in percentages of phytic P at the beginning of experiment.

It appears from the table that the rate of hydrolysis increases with the concentration of the oxy-acid until maximum is reached at 1·6%. It is further shown that, with this concentration, phytic acid is largely split even at a temperature of 25–27°.

E. *The effect of variation in time of reaction and concentration of* Ca *and of oxy-acid.* Fig. 4 shows the influence of variation in the time of reaction and in concentration of Ca and of tartaric acid.

From the curves in Fig. 4 the following facts are evident.

(1) The velocity of cleavage of phytic acid is clearly dependent on the quantitative ratio of Ca to tartaric acid.

(2) The degree of splitting depends on the time of reaction.

F. *Summary of results.* From all these experiments the optimal condition for the enzymic cleavage of phytic acid in the rye dough can be stated. They are as follows:

Water content	48–49%
pH	5·1–5·3
Tartaric acid content	1·5%
Temperature of reaction	28–30°
Time of reaction	4 hr.

Further experiments have shown that these conditions are applicable only to fresh flour. If the flour has been kept for a long time at room temperature (e.g. 1 month), the phytase content evidently decreases and the degree of splitting after 4 hr. is diminished.

IX. INFLUENCE OF THE BAKING PROCESS ON THE SPLITTING OF PHYTIC ACID

The investigations just described have established the optimal conditions for the splitting of phytic acid in the dough. They have not taken account, however, of the possible effect of the baking process itself. This was done in the experiments summarized in Table 17.

In all these experiments the dough was acidified by the culture of the lactic acid bacillus mentioned on p. 596. In some of the experiments 1·5% tartaric acid was also added to the dough.

Table 17. *Effect of baking on the phytic acid content of rye bread*

(Fermentation time, i.e. time interval between final preparation of dough and placing in oven, 4 hr. Fermentation temperature 28–30°. Ca content of flour 0·5%.)

	Addition to dough	pH	Moisture (%)	Total P in dry matter (%)	Phytic P in dry matter (%)	Phytic P (as % of total P)
Dough	Tartaric acid + culture of	4·61	44·2	0·523	0·125	23·90
Bread	lactic acid bacillus	4·75	38·5	0·523	0·055	10·52
Dough	Tartaric acid + culture of	4·82	44·82	0·507	0·105	20·71
Bread	lactic acid bacillus	5·20	39·19	0·507	0·035	6·90
Dough	Culture of lactic acid	4·98	45·83	0·517	0·120	23·21
Bread	bacillus alone	5·20	38·76	0·517	0·061	11·80
Dough	Tartaric acid + culture of	4·86	46·56	0·509	0·086	16·89
Bread	lactic acid bacillus	5·16	40·22	0·509	0·035	6·88
Dough	Culture of lactic acid	4·92	47·25	0·509	0·089	17·48
Bread	bacillus alone	5·08	40·44	0·509	0·054	10·61

It will be seen that the baking process invariably causes a considerable decrease in phytic acid P. This means that the splitting is continued and probably even accelerated by the baking process. This result is very naturally explained by the fact that the temperature in the bread increases very slowly up to about 100° during baking. As the phytase is not destroyed until the temperature reaches about 70°, there is always time for it to act.

The table shows further that the cleavage of phytic acid in the dough prepared with the culture alone was only slightly less than that obtained with added tartaric acid. This indicates that under practical conditions of baking the splitting of phytic acid might probably be effected by a strong lactic acid fermentation alone. That this can be done is proved by the experiments described in Table 18.

The acidifier in these experiments was the lactobacillus mentioned on p. 596. The flour contained in all cases 0·5 % Ca.

To understand the results it must be kept in mind that the oven in the Central Laboratory is of relatively small size compared with the big ovens in the bakeries. In a small electrically heated oven the temperature rises rapidly to maximum which means that the phytase is destroyed within a short time after the breads are put in the oven. In the big ovens, on the contrary, the temperature increases slowly and the phytase has a considerable time to do its work.

For this reason the influence of the time of holding the dough before baking must be more pronounced with the small oven. The table shows clearly that this is the case. In the big bakeries there was little difference in the extent to which the phytic acid was split after holding times of 1 and

Table 18. *The phytic acid content of rye bread baked after lactic acid fermentation of the dough*

(Fermentation temperature 28–31°. Ca content of flour 0·5%.)

Bakery	Date	Fermentation time* (hr.)	pH	Moisture (%)	Total P in dry matter (%)	Phytic P (as % of total P)
Central Laboratory	8. xi	1	5·70	37·0	0·449	30·51
Central Laboratory	8. xi	1	5·63	39·1	0·475	28·42
Glostrup	8. xi	1	5·21	37·2	0·404	19·18
Central Laboratory	9. xi	2	5·22	36·4	0·493	16·84
Central Laboratory	9. xi	2	5·22	39·7	0·524	16·79
Central Laboratory	10. xi	3	5·17	37·2	0·511	10·96
Central Laboratory	10. xi	3	5·19	37·8	0·528	8·14
Slagelse	8. xi	2	4·81	36·2	0·475	9·47
Slagelse	9. xii	1·75	5·96	37·8	0·375	17·07
Helsingør	15. xi	1·5	5·32	36·5	0·406	17·98
Helsingør	15. xi	1·5	5·26	37·2	0·404	18·07
Aarhus	15. xi	2	—	35·5	0·456	15·35
Glostrup	20. xi	1	5·11	39·3	0·422	18·72
Glostrup	20. xi	1	5·17	38·1	0·425	18·59
Viborg	6. xii	2	4·90	—	0·484	18·80
Roskilde	19. xii	1·75	4·86	36·9	0·421	15·68
Rønne	19. xii	2·5	4·90	40·4	0·502	17·93

* The fermentation time indicates the time interval between final preparation of dough and placing in oven.

2 hr. and the variation in moisture content did not greatly affect the results. This meant that big bakeries should have no difficulty in splitting phytic acid to such an extent that bread would contain 82 to 85 % of its total phosphoric acid in a state available for absorption.

After these results had been obtained it was decided that all the bakeries belonging to the Co-operative should as soon as possible change their method of baking according to the following rules:

(1) The dough should be acidified with the lactobacillus mentioned on p. 596. The cultures should be delivered from the Central Laboratory in order to ensure uniformity.

(2) The pH in the bread should be kept at 5·0–5·3.

(3) The moisture content of the dough should be increased to 46–48 %.

(4) The fermentation time should be about 2 hr.

After one year's trial the results from thirty Co-operative bakeries were as set out in Table 19.

Table 19. *Available phosphorus in rye bread baked by Co-operative bakeries according to the rules mentioned above*

Bakery	Non-phytic P (as % of total P)			Bakery	Non-phytic P (as % of total P)		
Aalborg			84	Nyborg			83
Aarhus		81	85	Nykøbing F			80
Assens			90	Nykøbing M			79
Esbjerg		87	91	Nærum		81	68
Fredericia		82	80	Randers			81
Frederikshavn			79	Ringsted		84	84
Grenaa			79	Roskilde	83	84	83
Glostrup	79	81	81	Rønne		84	82
Haderslev			81	Skive			77
Helsingør	83	82	96	Slagelse	84	84	79
Holbæk			87	Svendborg		83	83
Kalundborg		84	85	Thisted			82
Køge		82	88	Vejle			80
København	75	77	67	Viborg		87	81
Nakskov			73	Vordingborg		82	90

The table shows that the percentage of non-phytic P in the bread from twenty-three of the thirty bakeries was 80–85. For six of them the percentage lay between 75 and 80; one only had a percentage under 75.

These results seem to be satisfactory from a nutritional point of view. Of course the phytic acid ought to be split entirely, but this goal is probably unattainable as long as $CaCO_3$ must be used for the enrichment of the bread with Ca, because calcium lactate itself delays the cleavage to a certain degree. The conditions will be much more favourable as soon as it is possible to import $CaHPO_4$ in sufficient amounts to supply our bread with 0·5 % of Ca, because this salt has only a very slight influence on the splitting process. This is seen from Table 20.

It appears that the delaying effect of $CaHPO_4$ is very small whether lactic or tartaric acid is used. It is therefore the intention of the Central Laboratory to supply the bakeries with $CaHPO_4$ instead of $CaCO_3$, as soon as this salt is available.

Table 20. *The effect of* $CaHPO_4$ *on the cleavage of phytic acid in rye dough*

(Temperature of fermentation 27–29°. Fermentation time 4 hr.)

pH	Lactic acid (%)	$CaHPO_4$ added (%)	Non-phytic P (as % of phytic P in the flour)
5·3	*c.* 1·0	0	80·13
5·1	*c.* 1·0	1·7	80·76
	Tartaric acid (%)		
5·0	*c.* 1·0	0	89·79
5·1	*c.* 1·0	1·7	85·13

X. THE STATE OF PHOSPHORIC ACID IN THE BREAD AFTER THE CLEAVAGE OF PHYTIC ACID

So far the question of the state of the phosphoric acid after cleavage had not been taken into consideration. To find out whether it is all present as phosphoric ions or partly in organic combination, the analyses reported in Table 21 have been carried out. The table shows the analytical distribution of the phosphoric acid after almost complete cleavage of the phytic acid in the dough. The first half of the table contains the analyses of the flour used for making the dough and the second half the analyses of the dough after splitting. The term inorganic P means the amount of P directly precipitated by ammonium molybdate in an extract of the dough with 0·5N-HCl. The term residual P means the difference between the total P and the sum of inorganic P and phytic P.

Table 21. *The distribution of various forms of phosphorus in rye flour and in dough prepared from the flour*

(Ca-content in Exp. 1, 0·1 %; in Exps. 2 and 3, 0·5 %.)

Exp.	Phytic P (as % of total P)	Inorganic P (as % of total P)	Residual P (as % of total P)	Tartaric acid added to flour (%)
		Rye flour		
1	74·7	14·3	11·0	1·0
2	75·9	11·1	13·0	1·5
3	75·1	10·0	14·9	1·6
		Dough		
1	0·8	69·7	29·5	
2	0·4	63·9	35·7	
3	0·4	62·0	37·6	

It appears from the table that the residual P increases during the cleavage of the phytic acid. This means that the total phosphoric acid even after complete cleavage is not present as phosphoric ions, but that some of it is in fact bound to organic substances.

Table 22. *Distribution of various forms of phosphorus in rye bread*

Bread no.	pH	Moisture (%)	Total P in dry matter (%)	Phytic P (as % of total P)	Inorganic P (as % of total P)	Residual P: Extractable* (as % of total P)	Residual P: Non-extractable* (as % of total P)
94	5·17	37·18	0·511	10·96	65·36	18·79	4·89
95	5·19	39·79	0·538	8·14	64·77	19·89	7·20
97	5·10	35·47	0·456	15·35	62·06	16·67	5·92
100	5·32	36·50	0·406	17·98	59·11	14·54	8·37
102	5·10	39·33	0·422	18·72	61·61	15·64	4·03

* With 0·5N-HCl.

In order to elucidate the nature of these phosphoric compounds we have further determined how much of the residual P is soluble in 0·5N-hydrochloric acid. This is seen in Table 22, which presents the analytical distribution of the phosphorus after cleavage in the bakeries.

The table shows that a certain fraction of the residual P cannot be dissolved in 0·5N-HCl. This is probably identical with the phosphorus contained in the proteins of the flour and is, according to present knowledge, set free during digestion in the intestinal canal. The extractable residual P, however, may be, at least partly, made up of mono- and diphosphoric esters of inositol, which are not measured in the analytical determination of phytic acid, because they are not precipitated by iron. It is more probable that this fraction results from phosphorylation during the fermentation of the dough in rising. It must be kept in mind that under practical conditions a certain amount of yeast is added to the dough to effect rising, and in the fermentation with yeast processes of phosphorylation of carbohydrates play, as is well known, a considerable role. If that is true, the extractable residual P would also be available for absorption in the intestines. The question has not yet been definitely settled, but it seems reasonable to assume that, at all events, a large part of the extractable residual P is available in digestion.

SUMMARY

1. In grains used for human consumption and for feeding farm animals 75 to 85% of the total phosphorus is found in phytic acid.

2. The rachitogenic effect of cereals depends on the presence in them of phytic acid. Experimental evidence is given to show that when this is hydrolyzed the rachitogenic effect disappears.

3. The rachitogenic grains, oats and maize, contain no phytase whereas considerable amounts of this enzyme are present in the non-rachitogenic grains, wheat, rye and barley. With these grains an enzymic cleavage of phytic acid may take place in the intestine of animals.

4. Phytic acid forms a calcium salt insoluble at a pH of 3–4, which means that phytic acid may precipitate Ca even in the first parts of the small intestine, thus causing a serious fall in its absorption. Experiments, which prove this harmful effect, are described.

5. Oxy-acids, such as tartaric, citric and lactic acids, are shown to shift the precipitation point of calcium phytate to a more alkaline reaction. It is demonstrated that these oxy-acids considerably improve the absorption of Ca and P from the intestine of pigs.

6. It is shown that the feeding to pigs of a culture of a lactobacillus which can grow in their intestine increases the amount of lactic acid formed during digestion. Pigs fed such cultures grow better than controls not receiving the culture.

7. Rye bread in Denmark is improved by the addition of 0·5% of Ca and hydrolysis of 80 to 85% of the phytic acid by enzymic fermentation during the preparation of the dough and the first stages of baking. Analyses of the bread from 30 bakeries belonging to Co-operative Societies in Denmark are published.

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Ox-spleen β-Glucuronidase; its Purification and a Study of some Factors Involved in Assaying its Activity

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Masamune (1934) showed that aqueous extracts of ox kidney contained an enzyme which appeared to be specific in catalyzing the hydrolysis of β-glucuronides at the glucosidic link. The method of purification developed by Masamune for this 'β-glucuronidase' involved autolysis of the kidney with water and precipitation of the enzyme with ethanol. Oshima (1936) carried the purification a stage further than Masamune by adsorbing the enzyme on kaolin and subsequently eluting it with sodium phosphate. In repeating the work of the Japanese investigators Fishman (1939*a*, *b*) found difficulty in obtaining active enzyme preparations and devised an entirely new method for its purification from ox spleen. In this method acetone precipitation of the enzyme from the aqueous spleen extract was followed by an isoelectric precipitation of inactive protein. The enzyme preparation was further purified by fractionation with various concentrations of ammonium sulphate.

In view of the suggestion by Fishman (1940) that β-glucuronidase might catalyze the synthesis of conjugated glucuronides in the animal body, its chemical behaviour merits further study. The present research was therefore initiated with the ultimate intention of carrying out a systematic physicochemical examination of β-glucuronidase action. During the preliminary work Fishman's method (1939*a*) of purifying the enzyme from ox spleen was repeated. Since it was found that a large loss in activity occurred as a result of the acetone precipitation a different method was devised and is described below. Some factors involved in the estimation of β-glucuronidase activity in spleen extracts were investigated and are also described.

EXPERIMENTAL

The estimation of β-glucuronidase activity

Bio-synthetic *l*-menthol glucuronide prepared by the method of Williams (1938) was crystallized three times from water. The substrate solution was prepared by suspending 500 mg. *l*-menthol glucuronide ($C_{16}H_{28}O_7.1{\cdot}5H_2O$) in about 10 ml. water. A few drops of N-NaOH were added and the mixture was warmed to effect solution. After cooling it was titrated electrometrically to pH 5·0 with N-NaOH and diluted to 25 ml.

Enzyme (0·50 ml.) was added to acetate buffer (0·10M, 1·0 ml., pH 5·0) and substrate (0·50 ml.) in a 15 ml. pyrex centrifuge tube which was stoppered with a rubber bung and incubated for 2 hr. at 25°. The protein was then precipitated, centrifuged down, and the reducing power of a suitable measured sample of supernatant liquid was determined by Levvy's modification (1946) of the cerimetric method. A control tube in which enzyme solution, buffer and water were placed in the same proportion as above was treated in an identical manner. Since the ceric sulphate was standardized against glucurone the amount of reducing material found in each tube was calculated in terms of glucuronic acid by applying a correction factor. From the difference between the two estimations the amount of glucuronic acid liberated by 1 ml. of enzyme solution was calculated.

Under the conditions of the activity measurement it was found that the time-action curve was of zero order. If the concentration of the enzyme solution used for assay was adjusted so that the amount of substrate hydrolyzed was less than 10 %, the rate of hydrolysis was directly proportional to the enzyme concentration. It is therefore proposed to define one β-glucuronidase unit (G.U.) as that amount of enzyme which will liberate 0·100 mg. of glucuronic acid from the substrate *l*-menthol glucuronide under the conditions specified.