CLXI. CEREALS AND RICKETS.

VII. THE ROLE OF INORGANIC PHOSPHORUS IN CALCIFICATION ON CEREAL DIETS.[1]

BY JAMES TEMPLETON LOWE AND HARRY STEENBOCK.

From the Laboratory of Agricultural Chemistry, University of Wisconsin, Madison.

(*Received April 30th, 1936.*)

THE possibility that a particular agent is responsible for the poor calcifying properties of cereal grains has been a focal point of interest to students of nutrition for some years. It has long been recognised that grains are a poor source of Ca, yet this deficiency has failed to provide an adequate explanation.

E. Mellanby [1921; 1922, 1, 2; 1924; 1925; 1926, 1, 2; 1930] observed that a high cereal diet invariably had a deleterious effect upon the calcification of bones and teeth in dogs and rats. Oatmeal appeared unique in producing a greater severity of rickets than other cereal grains. Mellanby [1926, 2] proposed the idea that cereals contain some distinct anticalcifying substance which he provisionally called a toxamin. This anticalcifying action was found by Green and Mellanby [1928] to be neutralised by simultaneous administration of vitamin D, or by exposure of the cereal to ultraviolet light, according to the findings of Steenbock [1924] and Steenbock and Black [1924].

M. Mellanby [1928; 1929] emphasised the anticalcifying effect of cereals on teeth. Consideration of the chemical analyses of the cereals revealed that those which had the worst effect on teeth frequently contained the most Ca and P. Furthermore, the Ca/P ratio was not found responsible.

Green and Mellanby [1928] showed that oatmeal could be boiled with water or heated in the dry state at 120° for 18 hours without correction of its anti-calcifying properties. If, however, it were boiled with 1 % HCl for 1·5 hours and then neutralised, the product was found to have lost its anticalcifying effect.

Holst [1927] reported that oats contained a rickets-producing factor which could be extracted with 0·5 % HCl. Mirvish [1929; 1930] reported that when a dilute HCl extract of oats was injected into animals it produced a marked fall of blood Ca. Later Mirvish and Bosman [1929] suggested the identity of the blood Ca-reducing principle with the anticalcifying factor. Christiansen [1934] extracted oat flour with 0·5 % HCl and then dialysed the extract against distilled water. The dialysate when injected into rabbits produced a marked fall in serum Ca. However, similar effects on the serum Ca were obtained by injecting substances which had no anticalcifying properties.

Fine [1930] corroborated Mellanby's work with respect to the differences in the calcifying properties of cereals, but he ascribed the difference to variations in the vitamin D content. Harris and Bunker [1931] investigated the irregularity in the production of rickets on a rachitogenic diet and traced the difficulty to the yellow corn component in Ration 2965. They reported that the trouble could be

[1] Published with the permission of the Director of the Wisconsin Agricultural Experiment Station, Madison.

corrected by storing the ground corn meal for a few months before using. In a later paper [1934], these workers emphasised the variations in the P content of samples of fresh yellow corn obtained from different localities and showed that the Ca/P ratio in various rachitogenic rations may vary widely. Holmes and Tripp [1933] and Davies [1934] also reported widely different Ca/P ratios on various samples of Ration 2965, due chiefly to differences in P content.

Steenbock *et al.* [1927] have been unable to demonstrate that rolled oats are especially rickets-producing. They [1930] found that the cereals ranked in decreasing order of their anticalcifying effect thus: corn, rolled oats, wheat, regardless of whether they were fed alone or supplemented with $CaCO_3$ and with H_3PO_4. Of particular significance is their observation "that equalisation of the phosphorus content of the cereal rations did not make them equally effective in bone formation" and their suggestion that "phosphoric acid may not be equivalent in physiological properties to the organic phosphorus compounds in the cereals". More recent work by Templin and Steenbock [1933, 1] demonstrated that superior calcification was produced by immature yellow dent field maize as contrasted with the corresponding mature maize of the same variety and grown under identical conditions. In another paper by the same authors [1933, 2] the striking antirachitic effect of autolysed germinated maize was pointed out as compared with both mature maize and germinated maize, and attention was again directed to a consideration of the phosphorus component.

Bruce and Callow [1934] reported that "the apparent rachitogenic effect of cereals when compared with other material of the same phosphorus content is due to the fact that cereal phosphorus is not in an available form" and that "the differences between oatmeal, maize and white flour can be completely accounted for by differences in the total phosphorus content and in the proportion of inositolhexaphosphoric acid". Treatment of oatmeal with 1 % HCl was found to destroy its anticalcifying effect very strikingly as the inositolhexaphosphoric acid was hydrolysed. McCance and Widdowson [1935] found, after feeding phytin to 3 adults and 1 child, that 20–60 % of the phytin was excreted unchanged in the faeces.

Eddy *et al.* [1922] had reported earlier than when the inorganic P of the diets of rats was replaced by phytin, the rats were not protected against rickets although the food intake remained the same. Harris and Bunker [1935] recently studied the phytin content of different samples of mature corn, but reported no correlation between the phytin content of the corn and the severity of rickets produced by it.

Experimental.

The striking effect of immature yellow dent maize and of germinated, autolysed maize in promoting calcification when contrasted with the mature seeds suggested the advisability of studying these materials for their organic-inorganic P relations. The experiments of Templin and Steenbock [1933, 1, 2] were, therefore, repeated with this objective.

Mature yellow dent field maize on the cob obtained from the University Farm was dried with forced air circulation at a temperature of 42° for 36 hours, after which it was shelled and stored in a dry place. It was ground just before using. The manner of preparing the maize for germination and autolysis has been described in an earlier paper [1933, 2]. In the present work, "germinated maize" refers to that which has been germinated for a period of 96 hours, "germinated autolysed maize" to that which has been germinated for the same time and then autolysed for 10 days.

The method of assay employed in our experiments was unvaried. Six groups of rats between 3 and 4 weeks of age and weighing 50–60 g. with six rats in each group were used to test each of six rations. Litter-mate rats were uniformly distributed among the groups. Food consumption was equalised, the daily food intake of each rat being restricted to the amount taken by the rat with the lowest food consumption provided that it was not obviously abnormal. In addition to the modified Ration 2965 and distilled water, each rat received supplementary carotene. This was administered by dropper in oil[1] solution, each rat being given 5γ in one drop on alternate days. All the rations were finely ground, with the $CaCO_3$ thoroughly incorporated to prevent its settling out. All rations represented modifications of Ration 2965 in which the yellow maize was wholly or partially replaced by yellow maize of special selection or treatment. The rats were weighed weekly. After 5 weeks they were killed with ether, the costochondral junctions examined and the femora analysed for ash after alcohol extraction.

In Series I, germinated and germinated-autolysed maize was used to replace partially the mature maize component of Ration 2965. Preliminary experiments had shown that partial replacement avoided poor food consumption and provided better growth than complete replacement. As an added precaution, 2 % of yeast was included in all the rations.

Determinations of inorganic P and total P were made according to an adaptation of the Fiske and Subbarow method [1925]. Use of 0·8 % HCl for the extraction of inorganic P completely prevented the action of phytase. Determinations of phytin-P were made according to Harris and Mosher's [1934] modification of the Heubner-Stadler method [1914]. We were unable to avail ourselves of the recently reported modifications of this method by McCance and Widdowson [1935] and Young [1936], since their papers did not appear until after completion of our work.

The results of the experiments of Series I are presented in Table I. The importance of the inorganic P relations in these rations is clearly evident. Germinated autolysed maize showed an antirachitic superiority over mature maize

Table I. Series I. *Relation of inorganic P content to the rachitogenic properties of germinated and germinated autolysed maize.*

	Ration 2965 Maize component					Animals Each figure is average of 6 rats				
No.	Modification	Inorganic P %	Total P %	Inorganic % of total	Ca/P ratio	Gain in wt. g.	Daily food consumed g.	Femora g.	Ash g.	Ash %
1	Mature maize (basal)	0·02	0·33	6	4/1	45	7·7	0·103	0·030	28·8
2	Germinated autolysed maize, 50 %	0·14	0·33	42	4/1	34	7·7	0·124	0·055	46·2
3	Germinated autolysed maize, 25 %	0·07	0·33	21	4/1	45	7·6	0·115	0·043	38·5
4	Germinated maize, 50 %	0·06	0·33	18	4/1	29	7·1	0·093	0·022	24·2

as well as germinated maize. Determinations of the phytin-P of these rations revealed an inverse relation between the phytin-P content and the antirachitic effectiveness. Bruce and Callow's conclusions are thus generally confirmed.

[1] A vegetable oil sold under the trade name of Wesson Oil by Wesson Oil and Snowdrift Sales Company, New York.

Series II was designed to determine the effects of adding varying amounts of H_3PO_4 to the basal diet. H_3PO_4 was added to the rachitogenic ration in sufficient quantities to establish various arbitrary Ca/P ratios ranging from 4/1 to 1/1. The work of Shohl *et al.* [1932] was used as a guide. They stressed the importance of the Ca/P ratio in the study of rickets, but likewise showed the necessity of employing a basal rachitogenic ration of low P content. Our Ration 2965 was shown to have a Ca content of 1·2% and a P content of 0·3%, giving a Ca/P ratio of 4/1. The modifications of this ratio together with the results obtained are presented in Table II.

Table II. Series II. *Effect of various additions of H_3PO_4 to Ration 2965 on its rachitogenic properties.*

		Ration 2965 Maize component				Animals Each figure is average of 6 rats				
No.	Modification	Inorganic P %	Total P %	Inorganic % of total	Ca/P ratio	Gain in wt. g.	Daily food consumed g.	Femora g.	Ash g.	Ash %
7	Mature maize (basal)	0·02	0·33	6	4/1	35	6·9	0·088	0·026	29·9
8	Basal + H_3PO_4	0·06	0·37	16	3·5/1	36	7·0	0·098	0·031	31·2
9	Basal + H_3PO_4	0·12	0·43	28	3·1/1	39	6·9	0·105	0·037	35·4
10	Basal + H_3PO_4	0·20	0·51	39	2·6/1	39	7·1	0·120	0·053	43·8
11	Basal + H_3PO_4	0·32	0·63	51	2·1/1	43	7·1	0·151	0·073	48·2
12	Basal + H_3PO_4	0·92	1·23	75	1·1/1	35	6·3	0·139	0·069	48·7

The ash analyses in Series II present a well-defined illustration of the effect of varying the supplements of H_3PO_4 in a rachitogenic diet. The progressive improvement in calcification up to and including a Ca/P ratio of 2·1/1 runs exactly parallel with the rise in P content. The failure of improved calcification with a Ca/P ratio of 1·1/1 as compared with 2·1/1 becomes clear when we examine the Ca/P relation from a stoichiometrical viewpoint. The theoretical amounts of Ca and P required to produce $Ca_3(PO_4)_2$ are 2 parts of Ca to 1 of P. The critical Ca/P ratio appears to be between 2·6/1 and 2·1/1. In later work, therefore, it was attempted to confine Ca/P ratios to the zone where the greatest change in calcification took place, *viz.* between 3·1/1 and 2·6/1.

Since phytin is by far the principal source of P in cereal grains, Series III was set up primarily to compare the availability of this form of P with other forms. According to early workers, phytin is absorbed and the P is excreted as inorganic *phosphorus* in the urine. Scofone [1905], Giascosa [1905], Mendel and Underhill [1906], Cook [1909] reported that organic phosphorus compounds could be assimilated as such and that they were preferable to inorganic phosphate as a source of P for animals. Forbes and Keith [1914] reported no fundamental difference in nutritive values of phosphorus compounds and therefore no basis for a differentiation between their nutritive values.

Rogozinski [1910] showed that in dogs the unabsorbed phytin was present as such in the faeces, whereas in man it was hydrolysed by the bacteria in the large intestine. Rather [1918] found that after feeding large amounts of phytin in the natural state to pigs almost all the P was excreted in the form of orthophosphoric acid. He concluded that the pig has the power to hydrolyse phytin completely.

A few contemporary workers are inclined to believe that phytin is actually toxic to the animal organism [*cf.* Stockman, 1934]. Stockman and Johnston [1933] reported the production of symptoms of nervous degeneration in monkeys

by using a cereal extract presumably containing inositolhexaphosphoric acid. These observations have not been confirmed by Bruce and Callow [1934].

To determine the effect of phytin as such, we prepared phytin according to Boutwell's [1917] modification of the method of Clarke [1914]. From 15 pounds of wheat bran a net yield of 160 g. of phytin was obtained—a white amorphous powder, insoluble in water, but readily soluble in dilute mineral acids. It contained less than 0·01 % inorganic P, 14·0 % total P, and 1·8 % Ca.

In Series III phosphoric acid was added to the basal Ration 2965 in amounts necessary to establish Ca/P ratios of 2·9/1 and 2·3/1 respectively. Other rations were supplemented with phytin to provide equivalent amounts of P, and in one case Na glycerophosphate was added instead.

The results of the ash analyses in this series (Table III) reveal that phytin as a source of P was without significant value. Equivalent amounts of P given as

Table III. Series III. *Availability of phytin-P as compared with inorganic P.*

		Ration 2965 Maize component				Animals Each figure is average of 6 rats				
No.	Modification	Inorganic P %	Total P %	Inorganic % of total	Ca/P ratio	Gain in wt. g.	Daily food consumed g.	Femora g.	Ash g.	Ash %
13	Mature maize (basal)	0·02	0·33	6	4/1	44	8·4	0·110	0·030	27·8
14	Basal + H_3PO_4	0·15	0·46	33	2·9/1	50	8·5	0·128	0·046	36·2
15	Basal + phytin	0·02	0·46	4	2·9/1	39	8·2	0·106	0·030	27·8
16	Basal + H_3PO_4	0·26	0·57	45	2·3/1	43	8·5	0·139	0·062	44·5
17	Basal + phytin	0·02	0·57	3	2·3/1	42	8·5	0·108	0·032	29·4
18	Basal + Na glycerophosphate	0·02	0·57	3	2·3/1	46	8·5	0·167	0·088	52·8

phosphoric acid, however, resulted in a pronounced improvement in calcification. Na glycerophosphate was still more beneficial.

The limitations in the general applicability of these results, however, must be recognised, because Ration 2965 is not a balanced ration. Its high content of calcium carbonate is well known to produce a chemically unique intestinal environment which undoubtedly has some effect upon the character of the intestinal flora and the action of their phytases as well.

In view of the observations of Templin and Steenbock [1933, 1] that immature field maize was definitely less rachitogenic than the seed from which it was produced, P relations in immature maize were compared in Series IV. Large-sized ears of Golden Glow, a yellow dent maize, still in the milk stage and carrying a brown silk on well enclosed cobs, were husked in the laboratory and the kernels cut from the cob by hand. The maize was carefully dried and ground, the consistency of the final product approaching that of flour. It had a sweet agreeable odour. Portions of the original fresh immature maize were suspended in water, preserved with chloroform and toluene, and autolysed for 10 days. Comparisons were made with mature maize obtained from the same plot later. The rations were compounded so as to contain inorganic P comparable in amount with that contained in the immature maize ration. Immature autolysed maize was used in one ration, mature maize supplemented with the proper amount of H_3PO_4 in another. In still another ration phytin was substituted for H_3PO_4 on the basis of equivalent amounts of P.

The results of this series (Table IV) confirm the earlier reports from this laboratory that immature maize is definitely less rachitogenic than mature maize.

Table IV. Series IV. *Relation of inorganic P content to the rachitogenic properties of immature and immature autolysed maize.*

		Ration 2965 Maize component				Animals Each figure is average of 6 rats				
No.	Modification	Inorganic P %	Total P %	Inorganic % of total	Ca/P ratio	Gain in wt. g.	Daily food consumed g.	Femora g.	Ash g.	Ash %
20	Immature maize	0·24	0·39	61	3·4/1	49*	8·5	0·134	0·060	43·5
21	Immature autolysed maize	0·24	0·37	65	3·6/1	50·6	8·5	0·137	0·069	45·2
22	Mature maize	0·02	0·33	6	4/1	44†	8·5	0·101	0·031	31·2
23	Mature maize + H_3PO_4	0·24	0·55	44	2·4/1	48	8·5	0·147	0·070	47·4
24	Mature maize + phytin	0·02	0·55	4	2·4/1	44	8·5	0·111	0·039	34·5

* 1 rat died. † 2 rats died.

They also demonstrate that this difference can be correlated with a simultaneous rise in inorganic P. Immature maize and immature autolysed maize, both containing the same amounts of inorganic P, show comparable antirachitic effectiveness. Determinations of phytin-P on these samples showed that the increase in inorganic P could be almost entirely accounted for by the hydrolysis of phytin.

In an effort to answer the question whether the unavailability of phytin-P wholly explains the anomalous behaviour of cereal grains or whether there might be other anticalcifying factors present as claimed by Mellanby, the experiments of Series V were devised. Phytin was added to maize in the amount in which it naturally occurs, and then, by acid hydrolysis of the maize in one case and of phytin added to maize in another case, rations with the same inorganic P content were produced. If the hydrolysis of phytin with the corresponding liberation of inorganic P were alone responsible for improved calcification, comparable calcification should be obtained in the two rations. If factors other than phytin were involved and if these yielded to acid treatment along the lines suggested by Green and Mellanby [1928], Holst [1927] and others, it was to be expected that there would be a discrepancy in the results.

In preparing this series of rations, allowance was not made for the hydrolysis of other organic P compounds besides phytin. Consequently, the acid treatment of maize gave rise to a greater amount of H_3PO_4 than had been expected. In order to compensate for this, the treated maize was diluted with mature untreated maize to give a corresponding inorganic P content (Ration 28). To preclude any possibility of a deficiency of vitamin B, incurred by the drying of the maize, 4 % of yeast was added to each of the rations in this series, at the expense of the wheat gluten component.

Hydrolysis of the phytin was effected by heating the phytin in 0·05 % HCl solution in an autoclave at 15 pounds pressure for 6 hours. The solution was then evaporated at room temperature on the yellow maize component of Ration 2965. This treatment was found sufficient to convert all the phosphoric acid of phytin into the inorganic form.

The maize itself was hydrolysed in essentially the same manner. Sufficient 0·05 % HCl was added to produce a thick mash at p_H 4·4. This was heated in an autoclave at 15 pounds pressure for 6 hours. Practically quantitative hydrolysis of the organically bound phosphoric acid was effected. Sodium carbonate in amount calculated to neutralise the HCl was then added and the mixture dried at 43°. The amount of NaCl added later in compounding Ration 2965 was reduced to allow for the amount formed by neutralising the acid mixture.

The results of the ash analyses in this series (Table V) unquestionably confirm the importance of the inorganic P content and point to it as the prime factor in determining the antirachitic properties. The striking similarity in the extent of calcification on Rations 27 and 28 and again on Rations 29 and 30 where the

Table V. Series V. *Effect of acid hydrolysis of maize on its rachitogenic properties.*

	Ration 2965 Maize component					Animals Each figure is average of 6 rats				
No.	Modification	Inorganic P %	Total P %	Inorganic % of total	Ca/P ratio	Gain in wt. g.	Daily food consumed g.	Femora g.	Ash g.	Ash %
25	Mature maize (basal)	0·02	0·33	6	4/1	49	8·8	0·125	0·045	36·3
26	Untreated maize + untreated phytin	0·02	0·46	4	2·9/1	51	8·8	0·129	0·053	41·2
27	Untreated maize + hydrolysed phytin	0·16	0·46	35	2·9/1	46	8·8	0·150	0·072	48·4
28	Hydrolysed maize diluted	0·16	0·33	48	2·7/1	38	8·8	0·143	0·070	49·0
29	Hydrolysed maize + untreated phytin	0·25	0·46	54	2·9/1	49	8·8	0·173	0·095	55·2
30	Untreated maize + untreated phytin + H_3PO_4	0·25	0·76	33	4/1	52	8·8	0·182	0·099	54·5

inorganic P content was kept constant bear out this contention. The origin of the phosphoric acid appeared to be of no importance. No positive evidence suggesting the existence of unidentified factors involved in the anticalcifying effect of cereals was obtained. We desire, however, to call attention to the slight improvement in calcification observed in Ration 26 with the addition of phytin. This result is not in accord with our earlier findings, where it was entirely without effect. Apparently, a partial utilisation of phytin can occur under certain conditions.

In Series VI, mature yellow maize was hydrolysed in one case by heating with 0·05 % HCl for 2 hours in order to effect only a slight increase in inorganic P, and in another case for 8 hours to effect rather complete hydrolysis with the immediate objective of securing possible differential hydrolysis and destruction of the phytin and the hypothetical toxamin of Mellanby.

The "low inorganic P maize" produced by mild hydrolysis was used as the basis in compounding one ration. Assuming all the inorganic P to have arisen from hydrolysis of phytin, a corresponding amount of phytin was restored to the ration. For comparison, a ration using mature yellow maize to which H_3PO_4 had been added in amount exactly equal to that which had arisen from acid hydrolysis was used. Two other rations were prepared at a higher level of inorganic P, one using the maize which had been hydrolysed for 8 hours, diluted with equal parts of untreated maize, and the other using untreated maize and H_3PO_4 sufficient to equalise the inorganic P content. Phytin was restored to the acid-treated ration in an amount equal to that which had been hydrolysed. Two pairs of rations were compounded in this series, each pair containing identical amounts of both total as well as inorganic P. An odd group using yeast as a source of P was inserted to complete the series. It was fed at a level equivalent in P to a control ration to which H_3PO_4 had been added.

Data obtained from this experiment were expected to afford an insight into the possibility of the existence of the afore-mentioned other factors. Assuming

the phytin-P and inorganic P relations to be the complete explanation, comparable degrees of calcification should be obtained on these rations.

The results of this series (Table VI) on the whole reaffirm our previous conclusions. However, the fact that there occurred a consistent though slight

Table VI. Series VI. *Comparison of the rachitogenic properties of acid-hydrolysed maize with control maize containing same amounts of inorganic and total P.*

		Ration 2965 Maize component				Animals Each figure is average of 6 rats				
No.	Modification	Inorganic P %	Total P %	Inorganic % of total	Ca/P ratio	Gain in wt. g.	Daily food consumed g.	Femora g.	Ash g.	Ash %
31	Mature maize (basal)	0·02	0·33	6	4/1	54	8·4	0·118	0·038	32·1
32	Hydrolysed maize + phytin	0·09	0·40	22	3·3/1	59	8·4	0·151	0·067	44·5
33	Basal + H_3PO_4	0·09	0·40	22	3·3/1	60	8·4	0·138	0·060	43·0
34	Hydrolysed maize + phytin	0·16	0·47	34	2·8/1	52	8·4	0·155	0·074	47·3
35	Basal + H_3PO_4	0·16	0·47	34	2·8/1	62	8·4	0·158	0·071	44·5
36	Basal + yeast	0·04	0·40	10	3·3/1	65	8·4	0·137	0·052	43·0

superiority in calcification on the rations which contained acid-treated maize suggested that the state of the phosphorus compounds did not provide the complete answer. Whether the improvement in calcification was due to the destruction of Mellanby's hypothetical toxamin, the hydrolysis of fibre or other changes which can affect calcification, our data do not reveal.

The observed unavailability of phytin P to the rat suggested a study of the action of various enzymes on phytin. Plimmer [1913] found no evidence for the absorption of phytic acid, or for its hydrolysis by enzymes of the intestinal mucosa from a number of animals. Phytic acid was attacked readily by only one enzyme, phytase, which is found principally in bran and the castor bean. Hart *et al.* [1909] concluded that in the pig "when the food supply of P was entirely organic and 80 % of it consisted of phytin, the form of the excreted P was almost wholly inorganic".

We made some observations on the hydrolytic effect of extracts of the intestine from the rat and the chick on phytin as a substrate. No evidence for the enzymic hydrolysis of phytin was obtained even after 9 days' incubation. This suggests that future studies should concern themselves with the phytase activity of the intestinal flora and the influence that dietary changes have thereon.

Summary.

1. Germinated autolysed maize, immature maize, and HCl-treated maize, which had been previously demonstrated to be less rachitogenic than mature maize, were shown to owe this property primarily to an increased content of inorganic P.

2. Treatment of maize with HCl improved its antirachitic properties in proportion to the extent that its phytin was hydrolysed.

3. The inorganic P content of variously treated samples of maize bore a direct relation to the antirachitic effectiveness of the ration and an inverse relation to the phytin content.

4. Phytin proved itself to be a poorly available source of P when fed to the rat in Ration 2965, in contrast with phosphoric acid and sodium glycerophosphate.

5. Acid-treated maize was found to produce slightly better calcification than untreated maize, beyond that which could be accounted for by the increase in inorganic P content. The possible existence of other factors must still be given consideration.

REFERENCES.

Boutwell (1917). *J. Amer. chem. Soc.* **39**, 493.
Bruce and Callow (1934). *Biochem. J.* **28**, 517.
Christiansen (1934). *Biochem. Z.* **271**, 246.
Clarke (1914). *J. chem. Soc.* **105**, 535.
Cook (1909). *Bull. U.S. Bur. Chem.* No. 123.
Davies (1934). *Analyst*, **59**, 340.
Eddy, Miller and Heft (1922). *J. biol. Chem.* **50**, xix.
Fine (1930). *Cereal Chem.* **7**, 456.
Fiske and Subbarow (1925). *J. biol. Chem.* **66**, 374.
Forbes and Keith (1914). *Bull. Ohio agric. Exp. Sta. Tech. Series*, No. 5.
Giascosa (1905). *Biochem. Zbl.* **4**, 572.
Green and Mellanby (1928). *Biochem. J.* **22**, 102.
Harris and Bunker (1931). *Science*, **73**, 95.
—— —— (1934). *J. Lab. clin. Med.* **19**, 390.
—— —— (1935). *J. Nutrition*, **9**, 301.
—— and Mosher (1934). *J. industr. Engng Chem.*, Anal. Ed., **6**, 320.
Hart, McCollum and Fuller (1909). *Res. Bull. Wis. agric. Exp. Sta.* No. 1; *Amer. J. Physiol.* **23**, 246.
Heubner and Stadler (1914). *Biochem. Z.* **64**, 422.
Holmes and Tripp (1933). *Cereal Chem.* **10**, 313.
Holst (1927). *J. Hyg., Camb.*, **26**, 437.
McCance and Widdowson (1935). *Biochem. J.* **29**, 2694.
Mellanby, E. (1921). *Spec. Rep. Ser. med. Res. Coun., Lond.*, No. 61.
—— (1922, 1). *Brit. med. J.* **1**, 832.
—— (1922, 2). *Brit. med. J.* **2**, 849.
—— (1924). *Brit. med. J.* **1**, 895.
—— (1925). *Spec. Rep. Ser. med. Res. Coun., Lond.*, No. 93.
—— (1926, 1). *Brit. med. J.* **1**, 515.
—— (1926, 2). *J. Physiol.* **61**, *Proc.* xxiv.
—— (1930). *Brit. med. J.* **1**, 677.
Mellanby, M. (1928). *Physiol. Rev.* **8**, 545.
—— (1929). Diet and Teeth. London: H.M. Stationery Office.
Mendel and Underhill (1906). *Amer. J. Physiol.* **17**, 75.
Mirvish (1929). *Nature, Lond.*, **124**, 410.
—— (1930). *Biochem. J.* **24**, 233.
—— and Bosman (1929). *Brit. J. exp. Biol.* **6**, 350.
Plimmer (1913). *Biochem. J.* **7**, 72.
Rather (1918). *Bull. Ark. agric. Exp. Sta.* No. 147.
Rogozinski (1910). *Chem. Zbl.* **81**, II, 1549.
Scofone (1905). *Biochem. Zbl.* **3**, 606.
Shohl, Brown, Chapman, Rose and Saurwein (1932). *J. biol. Chem.* **98**, 215.
Steenbock (1924). *Science*, **60**, 224.
—— and Black (1924). *J. biol. Chem.* **61**, 405.
—— —— and Thomas (1927). *J. industr. Engng Chem.* **19**, 906.
—— —— —— (1930). *J. biol. Chem.* **85**, 585.
Stockman (1934). *J. Hyg., Camb.*, **34**, 10.
—— and Johnston (1933). *J. Hyg., Camb.*, **33**, 204.
Templin and Steenbock (1933, 1). *Biochem. J.* **27**, 2055.
—— —— (1933, 2). *Biochem. J.* **27**, 2061.
Young (1936). *Biochem. J.* **30**, 252.