LXXV. CEREALS AND RICKETS. THE RÔLE OF INOSITOLHEXAPHOSPHORIC ACID.

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As the result of a long series of investigations, E. Mellanby [1921; 1922; 1924; 1925; 1926; 1930; 1931; *cf.* Green and Mellanby, 1928] and M. Mellanby [1929] have demonstrated that certain cereals contain an anticalcifying factor having the effect of producing or intensifying rickets in dogs or rats and of retarding calcification of the teeth in dogs. Oatmeal, for instance, was found to have a marked anticalcifying effect in comparison with white flour or rice when the diet contained a large proportion of cereal. M. Mellanby [1929] summarised the evidence as to the nature of this factor as follows: "It has been impossible to obtain evidence to show that the anticalcifying action of cereals on the teeth is due to any known constituent. Neither the fat, carbohydrate, calcium and phosphorus (absolute and relative amounts) nor the acid-base ratio of the mineral constituent explains this action of cereals. There is some evidence that it is associated with protein and liberated from it by proteolytic digestion."

Certain points with regard to these investigations should be noted. The great majority of the experiments were carried out with dogs, which, unlike rats, have a low natural ability to make use of dietary calcium and phosphorus in the absence of vitamin D. It is possible, however, to assume that, with a low level of vitamin D, the Ca/P ratio will have a similar effect in controlling absorption of these elements in dogs and rats. Further, since the effect of adding calcium carbonate was to improve calcification, though only slightly, it seems justifiable to regard the diets as being deficient in calcium. The Ca/P ratio of the cereal has however been over-emphasised in relation to that of the diet as a whole. The cereals used all have calcium contents which are small in comparison with those of the other constituents of the diet, but the phosphorus contents are considerable. Using the figures given by E. and M. Mellanby, it will be found that a diet containing oatmeal (P, 0·392 %) will have a lower Ca/P ratio than a diet containing rice (P, 0·096 %), although the cereals alone have Ca/P ratios of 1 : 5·7 and 1 : 10·7, respectively.

Steenbock *et al.* [1930] compared the calcifying powers of diets containing different cereals with supplements of calcium carbonate and phosphoric acid, using the percentages of ash in the femurs of rats as the basis of comparison. With 1, 2 or 3 % of calcium carbonate in the diet, the calcifying power was in the order: whole wheat > rolled oats > yellow maize, which was the same order as that of the phosphorus contents. Without calcium carbonate, whole wheat showed slightly less calcifying power than the other two cereals. When the phosphorus content was equalised by addition of phosphoric acid, the order of calcifying power of the diets was practically reversed, although the differences were smaller. They say, "Equalisation of the phosphorus content of the cereal rations did not make them equally effective in bone formation, but it did make

the corn (maize) more effective with its $CaCO_3$ supplement. It must however be considered that inorganic phosphorus added as phosphoric acid may not be equivalent in physiological properties to the organic phosphorus compounds in the cereals. Our experiments certainly demonstrate the fact that inequality of phosphorus intake is not the sole reason for the difference in calcifying action of the various cereals."

Fine [1930] also confirmed the existence of differences in the calcifying properties of cereals, but was inclined to ascribe such differences to variations in vitamin D content rather than to variations in content of an anticalcifying factor.

Quite recently György *et al.* [1933] concluded that the existence of a rachitogenic factor in cereals, although lacking material proof, was supported strongly by their results, although they differed from Mellanby in finding that maize meal was most highly rachitogenic. They also confirmed the reduction of the rachitogenic effect by treatment with acid, but showed that the high proportion of sodium chloride resulting from subsequent neutralisation was alone capable of exerting a slight antirachitic effect.

It seemed to us that the influence of absolute and relative amounts of calcium and phosphorus on calcification was worthy of further study. Although there were no grounds for supposing that the elusive anticalcifying factor was entirely a matter of calcium and phosphorus content, a quantitative analysis of the cereal effect from that point of view was a necessary preliminary to further investigation. The suggestion of Steenbock *et al.* that the phosphorus compounds of cereals differed in their biological availability, might, moreover, contain an important clue to the solution of the problem, and attention should be paid to the nature of the phosphorus compounds.

The fact that in the work of Steenbock *et al.* the cereal with the highest phosphorus content had the lowest calcifying power when the phosphorus contents of the diets had been equalised strongly suggests that cereals contain a form of phosphorus less available than phosphoric acid. Independent evidence does, in fact, exist that this is the case.

The mineral components of a diet are too frequently expressed as a total percentage only, with little or no reference to the actual form in which those elements are present. Most cereals are relatively rich in phosphorus, but from 50–80 % of this is present in the form of inositolphosphoric acids, "phytin-phosphorus" [for literature, *cf.* Koehler, 1926], and it is a debatable question whether phosphorus in this form is absorbed at all from the digestive tract. The earlier literature is reviewed by Schaumann [1910]. Starkenstein [1910] concluded from his own investigations on organic phosphates in human urine that only a small proportion of orally administered inositolhexaphosphate was absorbed unchanged, the great part being hydrolysed by bacteria in the intestine. Plimmer [1913, 1] demonstrated that inositolhexaphosphoric acid diffused only slowly through parchment membranes, and that the acid was not hydrolysed by intestinal extracts from carnivora or herbivora. He concluded that there was no evidence of direct absorption, but that after hydrolysis by an enzyme in the foodstuff or by bacterial action it would enter the circulation as inositol and phosphoric acid.

As regards the availability of phytin-phosphorus in rickets, the evidence in the literature is conflicting. Eddy *et al.* [1922] demonstrated that supplements of phytin were ineffective in protecting rats from rickets when added to Sherman-Pappenheimer's diet No. 84. More recently there has been a controversy about the antirachitic activity of "Vitaphos," a preparation of calcium magnesium

inositolhexaphosphate. Von Wendt [1930] claimed that "Vitaphos" contained a form of vitamin D which was not toxic in excessive doses. Vonder Mühll [1931] contested this and demonstrated that the curative effect on rickets in rats could be explained as a result of the addition of phosphorus compounds. A more detailed investigation was recorded by Bleyer and Fischler [1931, 1, 2], who claimed that "Vitaphos" restored the composition of rats' bones to normal when added to McCollum's diet No. 3143 in the proportion of from 3 to 7 %. Their experiments are however open to several objections.

In the present communication there is described an investigation of the differences in calcifying power between cereals and of the availability of phytin-phosphorus, using the method of measuring the calcifying powers of diets which has been described in the preceding paper.

The series of diets compared were all of the high calcium-low phosphorus type, made up from 32 parts of a basal mixture and 68 parts of cereal. In certain cases a source of phosphorus was added. All contained 2 % of calcium. One diet, the "routine rachitogenic diet," had a mean Ca/P ratio of 10·3/1; the others had a mean Ca/P ratio of 5·5/1. In this way a comparison has been made of the effects due to the addition to rachitogenic diets of phosphorus in various forms,

(*a*) as a component of the cereal itself,

(*b*) as disodium hydrogen phosphate, and

(*c*) as sodium or calcium magnesium inositolhexaphosphates.

Further, the effects on oatmeal of cooking and of treatment with dilute acid have been investigated.

It has not been found possible to apply the same method to diets of the low calcium-high phosphorus type, but we have included here an account of some preliminary qualitative comparisons of such diets.

Experimental.

Analytical methods.

The determinations of calcium and phosphorus in dietary constituents and in diets were made by Neumann's method as described by Aron [1912]. Air-dry samples were taken, as variation in the content of moisture affects the absolute figures only slightly, without altering the relative proportions of calcium and phosphorus found.

Phytin-phosphorus (as inositolhexaphosphoric acid) was determined by the method of Heubner and Stadler [1914], with certain of the modifications introduced by Andrews and Bailey [1932].

Diets and materials. The "basal" portion of the diets was composed of:

Wheat gluten	20
Meat powder	6
Calcium carbonate	5
Sodium chloride	1
	32

The components were drawn from the same stocks throughout the investigation. In the "routine rachitogenic diet" (R.R.), and in some of the other experimental diets, the "cereal" portion consisted of a mixture of yellow maize meal (20 parts) and white flour (48 parts); in other diets it consisted of oatmeal or of maize.

The following values were found for certain of the constituents.

Meat powder, Ca, 0·045%; P, 0·75%. Wheat gluten, P, 0·148%. Maize meal, Ca, 0·04%; P, 0·37% (P soluble in HCl, 0·35%; phytin-P, 0·34%; phytin, as $C_6H_{18}O_{24}P_6$, 1·2%.) White flour,

Ca, 0·018%; P, 0·078% (P soluble in HCl, 0·026%; phytin-P, 0·014%; phytin, as $C_6H_{18}O_{24}P_6$, 0·05%). The oatmeal was "Ingles Midlothian Scotch, fine," and contained Ca, 0·06%; P, 0·406% (P soluble in HCl, 0·35%; phytin-P, 0·32%; phytin, as $C_6H_{18}O_{24}P_6$, 1·1%). Disodium hydrogen phosphate was used as the anhydrous salt, dried at 120° and ground to pass 80 mesh. The calcium magnesium inositolhexaphosphate used was a commercial brand of "phytin." Found: Loss at 100°/15 mm., 13·2%, and, referred to anhydrous material, Ca, 11·8%; P, 22·0% (phytin-P, 22·0%; phytin, as $C_6H_{18}O_{24}P_6$, 78·1%). The sodium salt was prepared from this by the method of Posternak [1921], recrystallised from water, dried partially *in vacuo* and ground to pass 80 mesh. (Found: P, 19·2%; phytin-P, 18·5%; inorganic P, 0·7%.) The mixing of small amounts of these addenda with the diets was carried out in batches of 2–4 kg. in a large ball mill for a period of 2·5–3 hours.

We have met with some difficulty in reproducing the exact composition of a diet as regards the total calcium and phosphorus content when the diets have been mixed in bulk. For this reason, where it was necessary to make up more than one batch of any given diet, each batch has been analysed separately. Thus the figures given represent the actual diets which were employed in feeding.

Biological methods.

The practical details of the rat tests were essentially as described by Bourdillon *et al.* [1931]. Rats weaned at approximately 4 weeks of age (50 g. weight) were put on the R.R. diet for 2 weeks, and then the knee-joints were radiographed under anaesthesia to confirm the satisfactory development of rickets. The rats in each litter were then sorted into pairs comparable in weight and sex, as far as was possible, and transferred to the diets to be tested, the two members of a pair being put on different diets. Daily doses of a standardised solution of irradiated ergosterol in olive oil were given in most cases. In general it was arranged that the two members of a pair should have either equal doses or doses which, taking into consideration the differences between the two diets, would produce comparable degrees of healing. 12 doses were given in 14 days. At the end of 14 days of this regime, the rats were killed by coal-gas and the knee-joints again radiographed. The degree of healing in each case was then estimated by comparison with the standard scale.

The general theory of the method used, and its relationship to the method of estimation of vitamin D by radiography has been discussed in the preceding paper [Bruce and Callow, 1934]. For a pair of rats on different diets, A and B, showing healing scale numbers n_2 and n_1, the apparent dose ratio is given by:

$$\frac{a_A}{a_B} = \text{antilog}\,[(n_2 - n_1) \times 0{\cdot}1505].$$

If ${}_A\phi_B$ is the multiplicative factor for diet A with respect to diet B, *i.e.* the influence of diet A in multiplying the apparent magnitude of a dose of vitamin D, the effect of which has been determined for diet B, and if the ratio of the doses actually administered is d_A/d_B, then

$$\frac{a_A}{a_B} = {}_A\phi_B \times \frac{d_A}{d_B},$$

and

$${}_A\phi_B = \frac{a_A}{a_B} \times \frac{d_B}{d_A}.$$

Comparison of high calcium-low phosphorus diets.

In the following section the protocols are given in full in one case, in order to illustrate the method of calculation, but in the other cases only in summary form.

(i) *Comparison of maize + white flour mixture with oatmeal.* Diet R.R. (Ca, 1·97%; P, 0·227%; Ca/P = 8·7) was compared with diet No. 10 (Ca, 2·0%; P, 0·406%; Ca/P = 4·9), in which the cereal portion consisted entirely of oatmeal.

(*a*) In the first experiment, with two litters divided into 8 pairs, the doses of vitamin D in International Units, and the degrees of healing obtained were as given in Table I.

Table I.

	Diet No. 10		Diet R.R.		
Litter No.	Dose (International Units)	Healing. (Scale Units)	Dose. (International Units)	Healing. (Scale Units)	Difference in healing. (No. 10 – R.R.) Δ
384	Nil	2	Nil	1	1
	0·5	4·5	0·5	1·5	3
	1	10	1	5	5
	2	11	2	4	7
1776	Nil	1	Nil	0	1
	0·5	9	0·5	4·5	4·5
	1	10·5	1	7	3·5
	2	11	2	6	5

The mean difference in degrees of healing between rats receiving equal doses of vitamin D on diets No. 10 and R.R. was 3·75, and, from this:

Apparent dose ratio $a_{10}/a_{\text{R.R.}} = \text{antilog}\,(\Delta \times 0{\cdot}1505) = 3{\cdot}7$.

Since the actual dose ratio, $d_{10}/d_{\text{R.R.}}$, is unity, this apparent dose ratio is equal to ${}_{10}\phi_{\text{R.R.}}$, the ratio of the antirachitic potencies of diets No. 10 and R.R.

(*b*) In the second experiment (Table II) two litters were divided into 5 pairs in which the dose ratio $d_{10}/d_{\text{R.R.}}$ was 1 : 4.

Table II.

	Diet No. 10		Diet R.R.		
Litter No.	Dose. (International Units)	Healing. (Scale Units)	Dose. (International Units)	Healing. (Scale Units)	Difference in healing. (No. 10 – R.R.) Δ
405	0·125	2	0·5	3	–1
	0·25	5	1	6	–1
	0·5	5·5	2	6·5	–1
1822	0·125	1	0·5	4·5	–3·5
	0·25	5	1	6	–1

Mean value of $\Delta = -1{\cdot}5$.

$$ {}_{10}\phi_{\text{R.R.}} = \frac{d_{\text{R.R.}}}{d_{10}} \times \frac{a_{10}}{a_{\text{R.R.}}} = \frac{4}{1} \times \text{antilog}\,(-1{\cdot}5 \times 0{\cdot}1505) = 2{\cdot}4. $$

The mean value of ${}_{10}\phi_{\text{R.R.}}$ is 3·2.

(ii) *Addition of calcium magnesium inositolhexaphosphate to maize + white flour mixture.* (*a*) Diet R.R. (Ca, 1·98%; P, 0·195%; Ca/P = 11·5): 8 rats, dose 1 unit; 2 rats, dose 0·5 unit.

Diet No. 18 was made up with the same cereal and base, except that the calcium carbonate was reduced from 5% to 4·77%, and calcium magnesium inositolhexaphosphate was added in the proportion of 27·6 g. to 2993 g. of diet. (Ca, 1·93%; P, 0·364%; Ca/P = 5·3): 10 rats, dose 0·5 unit.

8 pairs, $d_A/d_B = 0{\cdot}5$, mean $\Delta = -1{\cdot}75$, ${}_{18}\phi_{\text{R.R.}} = 1{\cdot}09$.

2 pairs, $d_A/d_B = 1$, mean $\Delta = +0{\cdot}75$, ${}_{18}\phi_{\text{R.R.}} = 1{\cdot}30$.

${}_{18}\phi_{\text{R.R.}}$ (mean, 10 pairs) = 1·13.

(*b*) Diet R.R. (Ca, 1·90; P, 0·203%; Ca/P = 9·4): 12 rats, dose 1 unit.

Diet No. 24 (equivalent to No. 18) (Ca, 1·94%; P, 0·348%; Ca/P = 5·6): 10 rats, dose, 1 unit.

10 pairs, $d_A/d_B = 1$, mean $\Delta = 0{\cdot}81$, ${}_{24}\phi_{\text{R.R.}} = 1{\cdot}32$.

For 20 pairs the mean factor for the phytin diet is 1·23.

(iii) *Addition of disodium hydrogen phosphate to maize + white flour mixture.* (*a*) Diet R.R. (as above): 2 rats, dose 0·5 unit; 2 rats, dose 1 unit; 1 rat, dose 2 units.

Diet No. 15, consisting of diet R.R. with the addition of 8·11 g. of Na_2HPO_4 anhydr. per kg. (Ca, 1·90%; P, 0·392%; Ca/P = 4·9): 2 rats, dose 0·125 unit; 2 rats, dose 0·25 unit; 1 rat, dose 0·5 unit.

5 pairs, $d_A/d_B = 0{\cdot}25$, mean $\Delta = 0{\cdot}80$, ${}_{15}\phi_{R.R.} = 5{\cdot}27$.

(*b*) Diet R.R. (Ca, 1·98%; P, 0·195%; Ca/P = 10·2): 7 rats, dose 1 unit.

Diet No. 19 (as No. 15, above) (Ca, 1·98%; P, 0·368%; Ca/P = 5·4): 7 rats, dose 0·25 unit.

7 pairs, $d_A/d_B = 0{\cdot}25$, mean $\Delta = 2{\cdot}36$, ${}_{19}\phi_{R.R.} = 9{\cdot}06$.

(*c*) Diet R.R. (Ca, 1·74%; P, 0·186%; Ca/P = 9·4): 6 rats, dose 1 unit.

Diet No. 21 (as Nos. 15 and 19, above) (Ca, 1·91%; P, 0·359%; Ca/P = 5·3): 6 rats, dose 0·25 unit.

6 pairs, $d_A/d_B = 0{\cdot}25$, mean $\Delta = 2{\cdot}67$, ${}_{21}\phi_{R.R.} = 10{\cdot}08$.

Mean, for all groups, 18 pairs, ${}_{PO_4}\phi_{R.R.} = 8{\cdot}35$.

(iv) *Comparison of calcium magnesium inositolhexaphosphate with disodium hydrogen phosphate.* Diet No. 18, containing added calcium magnesium inositolhexaphosphate, *cf.* (ii, *a*), above: 12 rats, dose 0·5 unit.

Diet No. 19, containing added Na_2HPO_4, *cf.* (iii, *b*), above: 12 rats, dose 0·25 unit.

12 pairs, $d_A/d_B = 2$, mean $\Delta = 3{\cdot}5$, ${}_{18}\phi_{19} = 0{\cdot}15$.

(v) *Comparison of sodium inositolhexaphosphate with disodium hydrogen phosphate.* Diet No. 48 consisting of diet R.R. with the addition of 14 g. of dry sodium inositolhexaphosphate per kg. (Ca, 2·06%; P, 0·420%; Ca/P = 4·9): 4 rats, dose nil; 6 rats, dose 0·25 unit; 6 rats, dose 0·5 unit.

Diet No. 49, consisting of diet R.R. with the addition of 11 g. of anhydrous Na_2HPO_4 per kg. (Ca, 2·12%; P, 0·418%; Ca/P = 5·1): 4 rats, dose nil; 6 rats, dose 0·125 unit; 6 rats, dose 0·25 unit.

4 pairs, dose nil, mean $\Delta = -4{\cdot}25$, ${}_{48}\phi_{49} = 0{\cdot}23$.

12 pairs, $d_A/d_B = 2$, mean $\Delta = -2{\cdot}29$, ${}_{48}\phi_{49} = 0{\cdot}23$.

Mean, for 16 pairs, ${}_{48}\phi_{49} = 0{\cdot}23$.

(vi) *Comparison of oatmeal with maize + white flour mixture containing added disodium hydrogen phosphate.* (*a*) Diet No. 13, consisting of diet R.R. with the addition of 8·66 g. of anhydrous Na_2HPO_4 per kg. (Ca, 2·00%; P, 0·433%; Ca/P = 4·6): 6 rats, dose nil.

Diet No. 10, containing oatmeal, *cf.* (i, *a*), above: 6 rats, dose nil.

6 pairs, dose nil, mean $\Delta = 2{\cdot}92$, ${}_{13}\phi_{10} = 2{\cdot}75$.

(*b*) Diet No. 15, *cf.* (iii, *a*): 2 rats, dose nil; 2 rats, dose 0·125 unit; 2 rats, dose 0·25 unit; 2 rats, dose 0·5 unit.

Diet No. 10: 2 rats, dose nil; 2 rats, dose 0·125 unit; 2 rats, dose 0·25 unit; 2 rats, dose 0·5 unit.

8 pairs, $d_A/d_B = 1$, mean $\Delta = 2{\cdot}5$, ${}_{15}\phi_{10} = 2{\cdot}38$.

(*c*) Diet No. 21, *cf.* (iii, *c*): 4 rats, dose 0·25 unit; 6 rats, dose 0·5 unit; 4 rats, dose 1 unit.

Diet No. 20 (as No. 10) (Ca, 1·97%; P, 0·377%; Ca/P = 5·2): 4 rats, dose 0·25 unit; 6 rats, dose 0·5 unit; 4 rats, dose 1 unit.

14 pairs, $d_A/d_B = 1$, mean $\Delta = 3{\cdot}89$, ${}_{21}\phi_{20} = 3{\cdot}85$.

Mean, for all groups, 28 pairs, ${}_{PO_4}\phi_{OATMEAL} = 3{\cdot}2$.

(*d*) A repetition of (*c*), but with each diet cooked by stirring into four parts of boiling water and heating for half an hour.

Diet No. 21 *c* (phosphate): 4 rats, dose 0·125 unit; 4 rats, dose 0·25 unit; 4 rats, dose 0·5 unit.

Diet No. 20 *c* (oatmeal): 4 rats, dose 0·25 unit; 4 rats, dose 0·5 unit; 4 rats, dose 1 unit.

12 pairs $d_A/d_B = 0{\cdot}5$, mean $\Delta = 2{\cdot}04$, ${}_{21C}\phi_{20C} = 4{\cdot}06$.

(vii) *Comparison of oatmeal with maize + white flour mixture containing added sodium inositol-hexaphosphate.* Diet No. 28, consisting of diet R.R. with the addition of 8·5 g. of dry sodium inositolhexaphosphate per kg. (Ca, 1·90%, P, 0·362%; Ca/P = 5·2): 15 rats, dose 0·5 unit.

Diet No. 20, containing oatmeal, *cf.* (vi, *c*), above: 15 rats, dose 0·5 unit.

15 pairs, $d_A/d_B = 1$, mean $\Delta = -0{\cdot}50$, ${}_{28}\phi_{20} = 0{\cdot}84$.

(viii) *Comparison of oatmeal with maize.* Diet No. 37, with maize as sole cereal (Ca, 2·00%; P, 0·353%; Ca/P = 5·7) : 6 rats, dose 0·5 unit.

Diet No. 38, with oatmeal as sole cereal (Ca, 2·07%; P, 0·379%; Ca/P = 5·5) : 6 rats, dose 0·5 unit.

6 pairs, $d_A/d_B = 1$, mean $\Delta = -2{\cdot}83$, ${}_{37}\phi_{38} = 0{\cdot}38$.

(ix) *Comparison of oatmeal with maize and added disodium hydrogen phosphate.* Diet No. 39, with maize as the sole cereal and 2·46 g. of anhydrous Na_2HPO_4 added per kg. (Ca, 2·16%; P, 0·365%; Ca/P = 5·9) : 13 rats, dose 0·5 unit; 7 rats, dose 1 unit.

Diet No. 40, with oatmeal as sole cereal (Ca, 2·11%; P, 0·365%; Ca/P = 5·8) : 13 rats, dose 0·5 unit; 7 rats, dose 1 unit.

20 pairs, $d_A/d_B = 1$, mean $\Delta = 0{\cdot}20$, ${}_{39}\phi_{40} = 1{\cdot}07$.

(x) *Comparison of cooked, acid-treated and raw oatmeal.* Diet No. 42 was prepared by boiling 4080 g. of oatmeal for four hours with a mixture of 23 litres of water and 1 litre of concentrated hydrochloric acid. After 1·5 hours the product no longer gave a blue colour with iodine. It was then neutralised with anhydrous sodium carbonate (548 g.), the basal mixture (1920 g.) was stirred in, and cooking continued for a further hour. The moisture content of the product was 66%, and analysis, calculated on the oven-dried diet, gave: Ca, 2·25%; P, 0·364%, Ca/P = 6·2; phytin, as $C_3H_{18}O_{24}P_6$, about 0·007%.

Diet No. 41 was prepared by boiling 4080 g. of oatmeal for 4 hours with 24 litres of water then stirring in the basal mixture and an additional 390 g. of NaCl, and cooking for a further hour. The moisture content of the product was 70%, and analysis, calculated on the oven-dried diet gave: Ca, 2·19%; P, 0·364%; Ca/P = 6·0; phytin, as $C_6H_{18}O_{24}P_6$, 0·64%.

Diet No. 43 was the usual dry mixture, with oatmeal as the sole cereal, but with an additional 56·7 g. of NaCl per kg. (Ca, 1·96%; P, 0·324%; Ca/P = 6·1; phytin, as $C_6H_{18}O_{24}P_6$, 0·66%).

A simultaneous comparison was made on triads of litter-mates, without doses, with 21 rats on diet No. 41, 21 rats on diet No. 42, and 20 rats on diet No. 43.

Diets Nos. 41 and 42, mean $\Delta = 3{\cdot}46$, ${}_{42}\phi_{41} = 3{\cdot}66$.
Diets Nos. 42 and 43, mean $\Delta = 3{\cdot}32$, ${}_{42}\phi_{43} = 3{\cdot}32$.
Diets Nos. 41 and 43, mean $\Delta = 0{\cdot}28$, ${}_{41}\phi_{43} = 0{\cdot}91$.

Comparison of low calcium-high phosphorus diets.

The results obtained from the feeding of low calcium-high phosphorus diets have not been very satisfactory. It was first attempted to produce rickets, suitable for radiographic comparison, either by diets which consisted essentially of those described above, but with the omission of calcium carbonate and with added phosphorus compounds. Only a mild type of rickets was obtained, and the radiographic pictures more nearly resembled those found in prophylactic tests. It was found difficult to secure satisfactory growth on low calcium diets, and a slight tendency towards spontaneous healing was often found from the third week onwards. Three classes of diet were employed, one containing oatmeal and the other two containing maize + white flour mixture with the addition of sodium inositolhexaphosphate or sodium phosphate to bring the total phosphorus content up to the same level. Such slight differences as were observed in the rickets resulting after 14–28 days on these diets indicated that the rachitogenic power of the oatmeal diet was slightly less than that of the other two diets, but differences were so small and variable that no real significance could be attached to the result. Using the type of diet described by Green and Mellanby [1928] we were again only able to produce a mild type of rickets in spite of improved growth, but could not detect a difference between the effect of oatmeal and of maize-white flour mixture.

In another experiment the A/R ratio was used as a measure of calcification. Rats weaned at about 4 weeks old were fed one of three diets containing in common the basal mixture: wheat gluten, 20 parts; meat powder, 6 parts; dried liver, 3 parts; sodium chloride, 1 part; No. 44 contained oatmeal (70 parts),

No. 45 contained white flour (70 parts), and sodium inositolhexaphosphate (1·3 parts), and No. 46 contained white flour (70 parts) and disodium hydrogen phosphate (1·1 parts). The phosphorus contents were practically equal, *viz.* 0·38, 0·36 and 0·38 %, respectively. Rats on each of the diets were killed at intervals of a week, from 4 to 8 weeks after the beginning of the experiment. In Table III are shown the time interval, number of rats killed, and average A/R value.

Table III. *Values of* A/R (*no. of rats in parentheses*).

Time (weeks)	4	5	6	7	8
Diet No. 44 (oatmeal)	0·374 (1)	0·433 (2)	0·374 (2)	0·347 (2)	0·391 (2)
Diet No. 45 (inositol-hexaphosphate)	0·545 (1)	0·384 (2)	0·361 (2)	0·428 (2)	0·495 (2)
Diet No. 46 (phosphate)	0·472 (1)	0·468 (2)	0·458 (2)	0·545 (3)	0·502 (1)

It will be seen that the rats on the phosphate diet, after the fourth week, have a consistently higher value of A/R than the rats on the inositolhexaphosphate diet. The rats on the oatmeal diet have a value of A/R which is generally below the value for the rats on the other diets; this is largely to be accounted for by the fact that the rate of growth was very much higher for these rats than for those on either the phosphate or inositolhexaphosphate diets, and an assessment of the anticalcifying power of the oatmeal diet relative to the other two is not possible. In the two latter cases the rates of growth fell off after the third week. With all three diets the growth was unsatisfactory after the sixth week, and an increase in the A/R ratio occurred.

Discussion.

Results with high calcium diets.

The results of the direct comparisons of high calcium diets (i) with diet R.R., (ii) with diet R.R. containing added phosphate and (iii) with the oatmeal diet are summarised in Table IV, in which the diets in the first column are arranged in order of decreasing antirachitic potency.

Table IV.

Characteristic of Diet A	Ca/P (mean)	${}_A\phi_B$: Diet B; R.R.	${}_A\phi_B$: Diet B; R.R. + Na_2HPO_4	${}_A\phi_B$: Diet B; oatmeal
Acid-treated oatmeal	6·2	—	—	3·3
R.R. + Na_2HPO_4	5·2	8·35	(1)	3·2
Maize + Na_2HPO_4	5·4	—	—	1·1
Oatmeal	5·4	3·2	0·32	(1)
Cooked oatmeal	5·9	—	—	0·91
R.R. + Na inositolhexaphosphate	5·1	—	0·23	0·84
Maize	5·7	—	—	0·38
R.R. + Ca Mg inositolhexaphosphate	5·4	1·2	0·15	—
R.R.	10·3	(1)	0·16	0·32

Considering first the diets which have been directly compared with diet R.R., it is seen that the addition of calcium magnesium inositolhexaphosphate causes quite a small increase in the antirachitic potency of the diet, whilst addition of disodium hydrogen phosphate to give the same content of phosphorus causes a very considerable increase. The oatmeal diet, again with the same total phosphorus content, is in an intermediate position. The comparisons with diet R.R. containing added phosphate confirm these relationships, but the diet with added calcium magnesium inositolhexaphosphate is here slightly less antirachitic than diet R.R., and it is evident that the effect of the addition in these amounts is negligible. Sodium inositolhexaphosphate, on the other hand,

appears to cause a definite increase in antirachitic potency, but it should be noted that the specimen used contained a certain amount of inorganic phosphate.

The oatmeal diet has been compared with five other diets containing the same proportion of cereal and of practically the same composition with respect to calcium and phosphorus. The value of ϕ for acid-treated oatmeal diet is 3·3 when compared with the untreated oatmeal diet. This might equally well be explained either by the assumption that the treatment has rendered the phosphorus more readily available, or by the assumption that treatment with acid has destroyed a rachitogenic factor of some other nature. In support of the first assumption are the facts that inositolphosphates were hydrolysed to phosphates by this treatment and that the availability of the phosphorus was consequently greatly increased. Moreover, the addition of phosphate to diet R.R. yields a diet of antirachitic potency practically equal to that of the acid-treated oatmeal diet with the same phosphorus content.

Confirmation of this view is afforded by comparison of the oatmeal diet with two others which may be regarded as "artificial oatmeal" diets. In the first of these the cereal was maize, with nearly the same proportion of inositolhexaphosphate but with less total phosphorus, and this was brought up to the same level as in oatmeal by the addition of disodium hydrogen phosphate. In the second diet the low phosphorus content of the maize+white flour mixture (estimated at total P, 0·17 %; phytin-P, 0·11 %; other P, 0·06 %) was raised by the addition of sufficient sodium inositolhexaphosphate (containing a little phosphate) to give a mixture (total P, 0·41 %; phytin-P, 0·32 %; other P, 0·09 %) comparable with the oatmeal (total P, 0·41 %; phytin-P, 0·32 %; other P, 0·09 %). The values of ϕ for these two diets, relative to the oatmeal diet, are 1·1 and 0·84 respectively. They are in fact equal to that of the oatmeal diet within the limits of experimental error. It is therefore concluded that, under these conditions, the differences in antirachitic potency between oatmeal and either maize or a maize+white flour mixture (20 : 48) can be practically entirely accounted for by the differences in content of phosphorus in available forms and in the non-available form of inositolhexaphosphate. E. Mellanby [1930], referring to the cereal effect in rickets, said "a greater deficiency of calcium and phosphorus in the body can be brought about by diets which actually contain more of these substances, so that an analysis of mineral intake may be quite misleading as to the efficacy of a diet in adequately supplying these substances to the tissues." The work now described in this paper is in full accordance with this view, in that it has shown that in high calcium diets cereals are apparently more rachitogenic than other material of the same mineral composition, just because their phosphorus content is illusory—the diet is deficient in available phosphorus in spite of being rich in total phosphorus.

Results with low calcium diets.

It is to be regretted that the above experiments are necessarily confined to rats on a high calcium-low phosphorus type of diet, the only conditions under which methods of quantitative comparison of calcifying or anticalcifying action have been worked out. However, the results of the preliminary experiments with rats on low calcium-high phosphorus diets described in this paper, although by no means definitive, give an indication that inositolphosphoric acid has, under such conditions, an anticalcifying action comparable with that of phosphoric acid for the same amount of phosphorus. It is possible to assume that this action is the result of lowering the availability of the calcium by binding it as a calcium salt which is at least as insoluble as calcium phosphate.

Inositolhexaphosphoric acid forms a calcium salt which is sparingly soluble in water, but exact data of its solubility relations are lacking. Under physiological conditions, the experiments of Starkenstein [1914] indicate that the toxicities of orthophosphates and inositolphosphates, which are attributed to their powers of precipitating calcium, are equal in solutions of equal normality ($M/3$ and $M/12$, respectively), when they are administered by mouth or subcutaneously, but that inositolhexaphosphate is three times as toxic as orthophosphate when injected intravenously. Starkenstein suggests that conversion of calcium into a non-ionised form is a factor in this toxic action. In low calcium diets there is thus, in addition to the factor of the low availability of the phosphorus, this fact that inositolhexaphosphoric acid is a precipitant of calcium and tends to reduce the latter to a deficiency level. The exact extent of this tendency is not susceptible of measurement by the methods we have been able to employ, but evidence from other sources shows conclusively that an effect of this kind exists.

The cereal factor in calcification.

It is of particular interest to discuss further the experimental results of M. Mellanby [1929]. The evidence presented with respect to the modifying influence of certain forms of treatment on cereals shows a remarkable parallelism with the known effect of similar treatment on inositolphosphates. The destruction of the anticalcifying factor by boiling oatmeal with 1 % hydrochloric acid, first demonstrated by Green and Mellanby [1928], and its relative stability to sodium hydroxide are comparable with the relative rates of hydrolysis of inositolphosphate by acid and alkali [Plimmer, 1913, 2]. The conclusion that the destruction of the anticalcifying factor during germination and malting may be enzymic is in accordance with the known occurrence of phytase in malt [Adler, 1916]. Further, the possibility of association of the anticalcifying factor with cereal proteins, indicated by digestion experiments on the proteins of oatmeal, recalls the combination of inositolphosphate with protein substances in oatmeal demonstrated by Lindenbaum [1926; *cf.* Koehler, 1926; Mnich, 1931]. It would be of interest to extend experimentally the correlation of anticalcifying properties with the relative proportions of inositolhexaphosphate and phosphate, already demonstrated in this paper in the case of acid treatment of oatmeal.

Vitamin D testing.

The distinction between phytin-phosphorus and other phosphorus compounds in cereals with respect to availability in rachitogenic diets is of significance in connection with certain aspects of vitamin D testing. It has been a common experience in this and other laboratories that unexplained variations in the severity of rickets produced by a rachitogenic diet occur from time to time in spite of a reasonably careful control of the diet [*cf.* Bourdillon *et al.* 1931]. One possible factor in this variation may be variations in the proportions of phytin and other phosphorus in samples of maize of similar total phosphorus content. Moreover, since phytase is present in the grain, the proportion of inorganic phosphate may increase on prolonged storage [*cf.* Minkovska, 1926].

Nervous degeneration.

The word "toxamin" was applied by E. Mellanby to "substances [in cereals] of unknown composition which have harmful effects in the body and can be antagonised by specific vitamins" [E. Mellanby, 1930]. As well as an anticalcifying toxamin, toxamins producing nervous symptoms are believed to be present

in cereals. It seemed worth while investigating whether inositolhexaphosphoric acid, which in these experiments accounted for the anticalcifying toxamin, also played some part in the production of nervous degeneration. This idea was supported, during the course of this work, by the appearance of a paper by Stockman and Johnston [1933], in which they described the extraction from cereals of an acid material which produced symptoms of nervous degeneration in monkeys. It seemed likely, considering their methods, that the material contained inositolhexaphosphoric acid, and this led us to try the effect of sodium inositolhexaphosphate on monkeys. The results obtained have so far been negative and are given in summary form only. Three female *Rhesus* monkeys were used, weighing 2·7–3·1 kg. They were kept under observation for a preliminary period of 3 months and were in good condition when the experiment was started. The monkeys were then dosed by stomach tube. One monkey, which served as control, was given a daily dose of 1 g. of sodium chloride in 100 ml. of water. Doses of 5–10 g. of sodium inositolhexaphosphate in 100 ml. of water daily given to the other two produced only severe diarrhoea and sometimes vomiting. After 2 weeks, the dose was reduced to 1·25 g. and 1·5 g. in 100 ml. daily. At this level no toxic symptoms were observed even after 3 months. For the first 2 weeks of this period, normal diet was continued, after this the intake of calcium and of vitamin A was restricted, and the proportion of cereal (wheat, white maize and rice) was greatly increased.

At no stage in the experiment was there any significant difference in behaviour or growth between the control monkey and the experimental animals. It seems evident that sodium inositolhexaphosphate is not the primary cause of the nervous degeneration and other toxic effects observed by Mellanby and by Stockman and Johnston.

Summary and conclusions.

A method of quantitative comparison of the influence of different cereal diets of the high calcium-low phosphorus type has been applied to the comparison of oatmeal, a maize + white flour mixture and maize.

In the type of diet used, containing a great excess of calcium, the replacement of maize + white flour by oatmeal causes a slight healing of rickets. The concomitant increase in phosphorus content might be assumed to account for this, but when the total phosphorus contents of the two diets are equalised by the addition of disodium hydrogen phosphate to the maize + white flour diet, the healing effect of the latter is considerably greater than that of the oatmeal diet.

This can be accounted for by the fact that a large proportion of the phosphorus present in oatmeal is actually in the form of inositolhexaphosphoric acid, which has been shown to be a poorly available form.

After treatment with 1 % hydrochloric acid the oatmeal diet has an antirachitic potency equal to that of the maize + white flour + phosphate diet, and the hydrolysis of inositolhexaphosphoric acid which takes place in this process affords sufficient explanation of the increased potency.

A series of quantitative comparisons has shown that the differences between oatmeal, maize + white flour, and maize can be completely accounted for by differences in the total phosphorus content and in the proportion of inositolhexaphosphoric acid.

In diets of high calcium content the apparent rachitogenic effect of cereals when compared with other material of the same phosphorus content is due to the fact that the cereal-phosphorus is not in an available form.

These experiments are, it should be emphasised, concerned only with high calcium-low phosphorus diets. Where defect in calcium is the factor limiting calcification, there are grounds for believing that excess of inositolhexaphosphoric acid will interfere with the absorption of calcium in the same way as excess of phosphoric acid. The same quantitative methods used with the high calcium diets cannot be applied here, but preliminary experiments have shown that diets containing excess of phosphate or of phytate are about equal in anti-calcifying power.

The relative proportions of phytin-phosphorus and other phosphorus in cereals is of significance in diets used for vitamin D testing.

Administration of sodium inositolhexaphosphate by mouth to monkeys produced no specific nervous symptoms. Thus it appears that this substance is not related to the toxamins of cereals which produce nervous degeneration.

A preliminary note on the subject of these investigations has been published previously [Bruce and Callow, 1932].

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