XXVI. THE COUNTERACTION BY FAT OF THE ANTICALCIFYING ACTION OF CEREALS

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MELLANBY [1921; 1925] showed that rickets was produced in young puppies when large amounts of cereals were added to diets relatively poor in calcium and vitamin D. The greater the proportion of cereals in the diet, the greater was the degree of rickets produced. Some cereals appeared to cause worse rickets than others, and these differences bore no relation to the calcium or phosphorus content of the cereals. In all cases, the rachitogenic action could be counteracted by the addition to the diet of more calcium salts or vitamin D. Furthermore the so-called "anticalcifying" or rachitogenic action of the cereals could be destroyed by acid hydrolysis. He therefore postulated the presence of some toxic substance in cereals, which he termed a "toxamin", and which interfered with calcification and could be destroyed by hydrolysis.

Holst [1927] found that he could extract (with 0.5% HCl) from oatmeal a substance which caused rickets in rats when added to a diet of starch which would otherwise give osteoporosis but no rickets. He noted that the ash of oatmeal did not produce rickets when added to such a starch diet, and therefore concluded that the rachitogenic factor was an organic substance destroyed by ashing.

Green & Mellanby [1928] confirmed on rats the previous experiments of the latter on dogs, and Thomas & Steenbock [1936] obtained similar results, although they did not find the same differences in the rachitogenic actions of the various cereals.

Bruce & Callow [1934] suggested that the rachitogenic action of whole cereals might be due to the relatively high proportion (50–80% of the total phosphorus) of phytin-phosphorus which they contain. They found that phytin-P was not well absorbed by the rat, and claimed that the antirachitic potency of various cereals, in a low P diet, was inversely proportional to the percentage of P present as phytin.

They also found that the addition of Na phytate to rachitogenic diets of the type used by Mellanby (i.e. low Ca-high P cereal diets) increased the degree of rickets produced, while a corresponding amount of Na phosphate did not. They concluded that phytin-P has an "anticalcifying" action, and write that "it is possible to assume that this action is the result of lowering of the availability of calcium by binding it as a calcium salt which is at least as insoluble as calcium phosphate".

McCance & Widdowson [1935] found that phytin was not well absorbed from the human intestine, 36–63% being excreted unchanged in the faeces.

Lowe & Steenbock [1936] supported the claim of Bruce & Callow that the phytin-P content of the diet showed an inverse relation to the antirachitic potency, and that phytin was a poor source of P when added to a high Ca-low P diet. They also showed that acid hydrolysis of maize improved its antirachitic properties in proportion to the extent to which its phytin-P was hydrolysed.

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Recently Mottram & Palmer [1937] made experiments in which they counteracted the rachitogenic action of a pure cereal diet by adding sufficient calcium to give a favourable Ca : P ratio. When excess of Ca was added, the diet again produced rickets. They concluded that the rachitogenic action of cereals was not specific, but was due simply to their unbalanced Ca : P ratio. The more extensive researches of Lowe & Steenbock [1936] showed, however, that not only the ratio of Ca to P, but also the percentage of P present as phytin-P in the cereal, determined its rachitogenic potency.

It should, however, be noted that Mellanby [1937] has recently announced that he has made further experiments on the subject and has come to the conclusion that phytin itself cannot account for the rachitogenic (or, as he terms it, "anticalcifying") action of cereals, but that some other organic P compound in cereals probably causes this action.

Furthermore, Bruin & Boumann [1937], unlike Bruce & Callow [1934], found that the giving of sodium phytate instead of sodium phosphate in a high cereal diet (Ca : P = 4 : 1) did not increase the rachitogenic action of the rations, and they concluded that the phytin content is not the cause of the rachitogenic action of cereals. They also found that an aqueous extract of oats promoted rickets when added to a rice diet, the active principle involved being soluble in alcohol.

These experiments are not directly comparable with those of Mellanby and of Bruce & Callow, since the Ca : P ratio was different, and the relatively large amounts of calcium carbonate added to the cereal diets might be expected to create very different conditions in the intestine.

There therefore seems some reason to believe that the rachitogenic action of cereals, in diets which are relatively, although not absolutely, deficient in calcium and vitamin D, is due to the relatively high proportion of phytin (or some other organic P compound) in cereals. This P compound may decrease the availability of the calcium of the diet by forming a calcium salt which is not readily absorbed. The diet would thus be changed by the presence of phytin from one with just sufficient available calcium to allow normal calcification, to a rachitogenic one with too little available calcium.

Recent work on the subject seems to indicate that the interference with calcification is due to a disturbance of the absorption from the intestine of Ca and P, rather than to the presence of a toxic substance. It is therefore necessary to consider what is known concerning the conditions required for intestinal absorption of Ca.

Ca can of course only be absorbed if it is present in the intestine in a water-soluble form. The reaction of the intestinal contents is such that CaCl_2 cannot exist, and since there are always phosphates in the intestine, there is a likelihood of insoluble calcium phosphate being formed which is not absorbed. There is, however, a possible way in which a soluble Ca compound can be formed, and that is by combination with fatty acids (which are formed in large quantities during the digestion of neutral fats) to form soluble Ca soaps which are absorbed in conjunction with the bile acids.

The experimental evidence for the influence of fatty acids on calcium absorption was pointed out by Stolte [1912], who spoke of a competition in the intestine between phosphates and carbonates on the one hand and fatty acids on the other for combination with the Ca. When there is too much phosphate present in relation to the fatty acids, insoluble calcium phosphate is formed and the Ca involved is lost to the body.

Klinke [1928] suggested that fatty acids combine in the intestine with Ca to

form Ca soaps which are dissolved by the bile acids to water-soluble and diffusible complexes. He found that by shaking 0.01 *M* deoxycholic acid with CaCO_3 , 2.6 mg./100 ml. of Ca was dissolved, with $\text{Ca}_3(\text{PO}_4)_2$ 4.6 mg. %, and with Ca oleate 13.0 mg./100 ml. of Ca was dissolved.

Boyd *et al.* [1932] found that moderate amounts of fat (10%) increase the intestinal absorption of Ca and P, probably by making an acid reaction in the intestine so that Ca soaps are formed, which are well utilized. Hickmans [1924] stated that the absorption of Ca in children was optimal if the food contained a proportion of 0.04–0.08 g. Ca to 1 g. fat.

From the study of the literature cited above, the rachitogenic action of cereal diets appears to be due to interference with the absorption of Ca from the intestine. Now, it was noted that the experimental diets used by Mellanby and others to demonstrate this rachitogenic action of cereals, contained little fat. Since it is known that fats have a great influence on the intestinal absorption of Ca, it seemed probable that the addition of fat to such cereal diets would counteract their rachitogenic tendencies by creating more favourable conditions in the intestine for Ca absorption.

Rat-feeding experiments were therefore made, using the high cereal diets originally described by Green & Mellanby [1928] to produce rickets in young rats. Fat was added to some of the diets and a calorically equivalent amount of starch to others, to test whether the addition of fat alone would prevent the rickets produced by these cereal diets. The quantity of fat added was about 11% of the total weight of food, a proportion which is quite usual in human diets.

METHODS

Young albino rats, of the laboratory's own stock, about 4 weeks old, weighing 50–60 g. were used. They were kept in separate glass cages, with metal screen bottoms, and were given water and their appropriate ration *ad lib.* They were weighed weekly, and killed after 33 days when it was seen by roentgenographs that rickets had developed.

The basal diet used, and the proportion of flour to basal diet, were the same as those used by Green & Mellanby [1928] to show the rachitogenic action of cereals in rats. The diet was made up freshly every 4 or 5 days as described by these authors. It consisted of

24 g. extracted casein (Hoffmann-La Roche),
32 g. marmite (yeast extract),
32 g. lemon juice (fresh),
25 g. dried cabbage,
12 g. NaCl.

The dried cabbage was made by shredding fresh cabbages, from which the outer green leaves were removed, and drying it at 55° for 24–36 hr. The dried shreds were then ground to powder and mixed with the other ingredients of the basal diet. Fresh lemons were used for each occasion.

Portions of the freshly made basal diet were then mixed with the various flours plus either starch or fat for the experimental rations, and the cakes so formed were kept in a refrigerator. About 20 g. were given to each animal daily, this quantity being more than any one animal ate per day.

The ratio of basal diet to flour was usually 1 : 3, as in the experiments of Green & Mellanby. Where fat was added, it was added in the proportion of 0.5 part of fat to 1 part of basal diet and 3 parts of flour. The diet therefore

contained 11 % fat. In the control diets, fat was replaced by twice the weight of starch (1 part basal, 3 flour, 1 starch) since 1 part of fat was taken to be about equal in energy value to 2 parts of starch.

The results of the feeding tests were estimated by three different methods. Firstly roentgenographs of the hind limbs of anaesthetized animals were made in all cases. Since this technique is so highly developed, it seemed the best method of determining the degree of rickets.

As a second method, the femora were dissected out immediately after death and cut and stained by the line test method as described by Dyer [1931]. The stained bones were then photographed.

As a third method, the ash percentage of the femora was estimated in the first series. The results of this method, however, were less satisfactory than those of the other two. The ash contents are given in Table I as percentage of the fat-extracted dry weight of the bones. The method used to obtain the fat-extracted dry weight of the bones was that described by Chick *et al.* [1926].

The rats were weighed weekly, and the averages of the gains in weight during the experimental period are given in Table I.

Table I

Series	Group	Diet	No. of rats	Gain in wt. g.	End wt. g.	Degree of rickets by		Ash content of femora %
						Roentgenographs	Line test method	
I	I	Basal + brown flour	5	35	91	1(+ +), 4(+ + +)	—	40.1
	II	Brown flour + starch	5	26	81	1(0), 4(+ + +)	—	43.2
	III	Brown flour + lard	5	45	109	5(0)	—	44.4
	IV	White flour	5	23	70	1(±), 1(+ +), 3(+ + +)	—	40.5
	V	White flour + starch	5	37	92	5(+ + +)	—	41.4
	VI	White flour + lard	5	36	89	5(0)	—	44.4
II	I	White flour + starch	5	29	85	2(+), 1(+ +), 2(+ + +)	2(+), 1(+ +), 2(+ + +)	—
	II	White flour + lard	5	52	108	1(+), 4(0)	1(+), 4(0)	—
	III	White flour + olive oil	5	31	89	1(+ +), 1(+), 3(0)	2(±), 3(0)	—
III	I	2 parts wholemeal + starch	5	33	93	2(+), 3(+ +)	1(+), 4(+ +)	—
	II	2 parts wholemeal + lard	5	45	102	1(+), 1(±), 3(0)	1(+), 3(0), 1 missing	—
IV	I	White bread	5	42	99	1(0), 1(±), 1(+), 1(+ +), 1(+ + +)	2(0), 2(+ +), 1(+ + +)	—
	II	White bread + lard	5	54	109	5(0)	5(0)	—

Roentgenographs of all animals were made, but since it is not possible to reproduce them all owing to lack of space, the degree of rickets is recorded in Table I.

Experimental procedure. Since the different feeding tests were spread over the period from February to August, they will be considered in separate series. The experimental technique used was the same in all cases, and the feeding period was always 32–34 days.

The series were as follows:

Series I. White and brown flours with (1) starch and (2) lard.

Series II. White flour with (1) starch, (2) lard, and (3) olive oil.

Series III. Wholemeal flour (smaller quantity) with (1) starch, (2) lard.

Series IV. White bread (1) alone, (2) with lard.

The flours used were the following:

(I) "White flour"—wheaten flour of 70–71 % extraction from English and Swiss mills.

(II) "Brown flour"—wheaten flour of 85 % extraction with 20 % rye flour as commonly used in Switzerland.

(III) "Wholemeal"—wheaten flour of 99 % extraction from an English mill.

The lard used was good quality pig's lard as sold for human consumption. Lard, olive oil and coconut oil have been shown by Steenbock & Black [1925] to contain no vitamin D.¹

The results of all the series are given in Table I. The gains in weight and end weights, and in the first series the ash content of the femora, are given as average values of the 5 rats in each group. The degree of rickets as seen by the roentgen and line test methods is given for each rat. The following signs are used to express the degree of rickets: 0=no rickets, $\pm$ =doubtful rickets; + =slight rickets; ++ =medium rickets; and +++ =severe rickets.

The ash content of the femora is given as percentage of the fat-extracted dry weight.

Series I

In the first series 6 groups of 5 rats each were fed with the following rations:

Group I. Brown flour (3 parts) + basal diet (1 part).

Group II. Brown flour (3 parts) + basal diet (1 part) + starch (1 part).

Group III. Brown flour (3 parts) + basal diet (1 part) + lard (0.5 part).

Group IV. White flour (3 parts) + basal diet (1 part).

Group V. White flour (3 parts), basal (1 part) + starch (1 part).

Group VI. White flour (3 parts), basal (1 part) + lard (0.5 part).

On the twenty-fourth day, 2 rats of each group were X-rayed, and it was seen that rickets had developed in the rats with no fat in the diet, i.e. in groups I, II, IV and V. On the thirty-third and thirty-fourth days all the rats were killed (with coal gas) and X-rayed immediately after death.

It will be seen from Figs. 1–12 that the rats of groups I, II, IV and V, whose diets contained brown or white flour with or without starch, all had marked rickets, while those of groups III and VI, whose diets contained lard, had no rickets.

It is not possible to reproduce here the roentgenographs of all the rats in the experiment; but those not shown here were essentially similar in each group to the representative cases reproduced in Figs. 1–12, which were chosen for their approximately equal growth curves.

There were only two exceptions to this uniformity in the degree of rickets in each group, namely rats No. 6 in group II and No. 20 in group IV, both of which showed very mild or no rickets in contrast to the marked rickets of the other rats in these groups. This is probably to be explained by the fact that these two rats gained only 12 and 11 g. respectively during the experimental period of 33 days, while the majority of the rats gained from 20 to 50 g. It is of course well known that rickets is more likely to develop during periods of vigorous growth, and it was the experience of previous authors (e.g. Green & Mellanby) that rats on such a diet only develop rickets when actively growing.

As can be seen from Table I, the rats of groups III and VI, with lard in the diet, grew better on the whole than those of groups I, II, IV and V without fat.

¹ Daily administration of the non-saponifiable fraction of 30 g. of the lard used in these experiments did not cure rickets in rats on a vitamin D-free diet, whilst 0.1 ml. cod liver oil cured them in 10 days.

Fig. 1.

Fig. 2.

Fig. 5.

Fig. 6.

Fig. 3.

Fig. 4.

Fig. 7.

Fig. 8.

Fig. 9.

Fig. 10.

Fig. 11.

Fig. 12.

This was true for the groups as a whole, as can be seen from Table I. It is therefore clear that the presence of lard afforded a real protection from rickets which was not due to an inhibition of growth.

Table I also shows the ash content of the bones of the various groups of rats. It will be seen that the average values are slightly higher in the two groups which received fat and developed no rickets. While this is in agreement with the roentgenographs, the latter were taken to be a more reliable index of the degree of rickets.

Series II

The second series of experiments was made partly to confirm the results of the first series, and partly to test whether olive oil would have the same action as lard in preventing rickets. Three groups of 5 rats each were therefore fed on the following diets:

Group I. White flour (3 parts), basal diet (1 part), starch (1 part).

Group II. White flour (3 parts), basal diet (1 part), lard (0.5 part).

Group III. White flour (3 parts), basal diet (1 part), olive oil (0.5 part).

After 34 days on these diets, the rats were killed and the hind limbs X-rayed. The tibiae and femora were then dissected out and preserved in formalin for investigation by the line test method.

The results are shown in Table I. It was found that the rats of group I, without fat in the diet, all developed rickets. In group II with lard, 4 rats had no rickets and the remaining one had only very slight rickets. The addition of lard had therefore prevented rickets in 4 out of 5 animals.

Group III, with olive oil, showed no rickets in 3 rats and only slight rickets in the remaining two. The addition of olive oil afforded also, therefore, protection against rickets, although perhaps less complete protection than lard. It may be noted that the addition of lard improved the growth of the animals, as in the first series, while that of olive oil did not.

Series III

A third series of experiments was made in which the proportion of flour in the diet was reduced from 3 parts to 2 parts per 1 part of basal diet. This gave the diet slightly more resemblance to a human diet, although even such a proportion of cereal is much higher than is usually eaten in British diets.

The diets used were as follows:

Group I. Wholemeal (2 parts), basal diet (1 part), starch (1 part).

Group II. Wholemeal (2 parts), basal diet (1 part), lard (0.5 part).

Each group contained 5 rats as usual and all the animals were killed after 32 days. They were then X-rayed, and the femora and tibiae dissected out and preserved in formalin for the line test.

The results are shown in Table I. It will be seen that all the rats in group I, with no fat in the diet, developed rickets, while in group II with lard, 3 rats had no rickets and 2 very slight rickets. As in previous series, the rats receiving lard grew better than those on the fat-free diet; in spite of this more vigorous growth, which would otherwise tend to cause more severe rickets, the protection against rickets was complete in 3 rats and partial in the remaining 2.

Series IV

A fourth series of experiments was made to test whether white bread (which is the form in which cereals are chiefly eaten in British diets) would exert a similar rachitogenic action in these diets, and whether such an action could be prevented by the addition of lard. Fresh white bread, made of wheat flour of about 70% extraction, was bought every 4 or 5 days, and made up into the following diets:

Group I. Bread (4 parts), basal diet (1 part).

Group II. Bread (4 parts), basal diet (1 part), lard (0.5 part).

Five rats were put on each diet, and killed after 32 days. They were then X-rayed, and the tibiae and femora dissected out and preserved in formalin for the line test.

The results are shown in Table I. It will be seen that of the rats in group I without fat, one had bad rickets (+++), two had slight rickets (+) and two had none (0). In group II with lard, all five rats were without rickets.

It seems then that white bread has a similar rachitogenic action to white flour in these diets, but that this action is prevented by the presence of lard, as in the case of flour.

A fifth series of experiments was made in which coconut oil was used as the source of fat, but since the rats did not eat this diet well, they grew less well than the control groups on the same diet without fat. The fact that the rats on the coconut oil diet developed less severe rickets than the others is therefore of uncertain significance.

DISCUSSION

In the introduction, experiments of Mellanby and others were quoted showing that a diet which contains only just enough calcium and vitamin D to allow normal calcification, becomes rachitogenic when it includes a high proportion of cereals. The present experiments, using the same diets which Green & Mellanby used for rats, fully confirm this observation.

It seemed probable from the further researches of Bruce and Callow, and Thomas & Steenbock, that the rachitogenic or so-called "anticalcifying" action of cereals in such diets was due to a disturbance of the intestinal absorption of Ca. Bruce & Callow concluded that the high proportion of phytin in cereals caused this interference with Ca absorption by forming an insoluble and therefore non-absorbable Ca compound in the intestine. It is now probable that this substance is not Ca phytate but a Ca phosphate, since Patwardhan [1937] has shown that there is an enzyme in the rat's intestine which splits phytin. Laskowski [1937], in this laboratory, has confirmed this fact and has demonstrated, by direct absorption tests with rats, that the rate of absorption of P from Na-phytate is in proportion to the rate at which the phytate is split by the enzyme into soluble (and therefore absorbable) Na phosphate. In the presence of Ca, however, an insoluble Ca phosphate may be formed which is not absorbed. This is probably the explanation of the observation of Lowe & Steenbock [1936] that phytin is only assimilated in rats when the diet is low in calcium, since a high calcium content would precipitate all the P as insoluble Ca phosphate in the intestine.

To counteract the loss of Ca by precipitation in the intestine, it was necessary to add to the diet some substance which created favourable conditions for the absorption of Ca. Fatty acids are known to exert such an effect by acidifying

the intestinal contents and by combining with Ca to form Ca soaps, which are dissolved by the action of the bile acids and then readily absorbed. Fat was therefore added to the diets to ensure a constant supply of fatty acids in the intestine during the absorption of the food products.

The experimental results justified these expectations, since the addition of 11 % fat (free from vitamin D) prevented the rachitogenic action of the cereal diets of Mellanby. Of three fats tested, lard, olive oil and coconut oil, lard gave the best protection against rickets and also the best growth curves; olive oil was rather less favourable to growth and less certain in its prevention of rickets. Coconut oil gave very incomplete protection against rickets, but it also interfered with the appetite and growth of the animals, so that its purely protective action cannot be judged from these experiments.

It seems clear from these results that the rachitogenic action of cereals in such diets is due to the incomplete nature of the diet, namely to deficiency of fat. A normal amount of fat in the diet seems to be important for the solubility in the intestine (and hence for the absorption and utilization) of Ca and P.

This of course does not mean that Ca and P cannot be absorbed in the absence of fatty acids if the conditions in the intestine are favourable. For instance Mellanby and others have shown that normal calcification can be obtained from these cereal diets if the cereal is hydrolysed, if vitamin D is added or if the Ca : P ratio is made optimal by the addition of a Ca salt [cf. Mottram & Palmer, 1937]. In these cases the conditions in the intestine presumably become changed so as to make the absorption of sufficient Ca and P possible.

From a practical point of view, the important fact is that even in a diet containing a high proportion of cereals and relatively little Ca and vitamin D, as in the experimental diets studied, the so-called anticalcifying tendency of the cereals is counteracted by the presence of about 10 % fat.

SUMMARY

1. Rickets produced in young rats by a diet containing a high proportion of wheat flour or bread and low Ca and vitamin D contents, is prevented by the addition of 11 % lard or olive oil.

2. The explanation is offered that the absorption of Ca from these diets is promoted by the formation of Ca soaps with the fatty acids, while in the absence of fat the Ca combines with the phytin (or its split-product) to form an insoluble compound which cannot be absorbed.

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DESCRIPTION OF PLATE III

- Figs. 1—12. Roentgenographs of knee-joints of rats.
Figs. 1 and 2. Rats Nos. 3 and 4, of group I (brown flour).
Figs. 3 and 4. Rats Nos. 8 and 9, of group II (brown flour + starch).
Figs. 5 and 6. Rats Nos. 17 and 19, of group IV (white flour).
Figs. 7 and 8. Rats Nos. 23 and 25, of group V (white flour + starch).
Figs. 9 and 10. Rats Nos. 12 and 15, of group III (brown flour + lard).
Figs. 11 and 12. Rats Nos. 26 and 29, of group VI (white flour + lard).