way are also accessible to acetylation when the fibroin is treated with boiling acetic anhydride.

3. The existence is thus established of two categories of serine residue in silk fibroin, differing in their accessibility to etherification by dimethyl sulphate.

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REFERENCES

- Bell, D. J. [1937]. *Biochem. J.* **31**, 1683.
 Bergmann, M. & Niemann, C. [1937]. *J. biol. Chem.* **122**, 577.
 Blackburn, S., Carter, E. G. H. & Phillips, H. [1941]. *Biochem. J.* **35**, 627.
 — Middlebrook, W. R. & Phillips, H. [1942]. *Nature, Lond.*, **150**, 57.
 Block, R. J. [1938]. *The Determination of the Amino-acids*. Minneapolis: Burgess Publishing Co.
 Campbell, W. R. & Hanna, M. I. [1937]. *J. biol. Chem.* **119**, 1.
 Carr, E. L. [1938*a*]. *Text. Research*, **8**, 125.
 — [1938*b*]. *Text. Research*, **8**, 399.
 Chibnall, A. C. [1942]. *Proc. Roy. Soc. B*, **131**, 136.
 Conway, E. J. & O'Malley, E. [1942]. *Biochem. J.* **36**, 655.
 Gleysteen, L. F. & Harris, M. [1940]. *Text. Research*, **11**, 117.
 Gordon, A. H., Martin, A. J. P. & Synge, R. L. M. [1943*a*]. *Biochem. J.* **37**, 79.
 — — — [1943*b*]. *Biochem. J.* **37**, 86.
 Haurowitz, F. [1938]. *Hoppe-Seyl. Z.* **256**, 28.
 Hirst, E. L. & Young, G. T. [1939]. *J. chem. Soc.* p. 1471.
 Huggins, M. L. [1943]. *Chem. Rev.* **32**, 195.
 Kise, M. A. & Carr, E. L. [1936]. *Text. Research*, **7**, 103.
 Linderström-Lang, K. & Jacobsen, C. F. [1941]. *J. biol. Chem.* **137**, 443.
 Martin, A. J. P. & Synge, R. L. M. [1941]. *Biochem. J.* **35**, 1358.
 Meyer, K. H., Fuld, M. & Klemm, O. [1940]. *Helv. chim. Acta*, **23**, 1441.
 Nicolet, B. H. & Saidel, L. J. [1941]. *J. biol. Chem.* **139**, 477.
 — & Shinn, L. A. [1941*a*]. *J. Amer. chem. Soc.* **63**, 2284.
 — — [1941*b*]. *J. biol. Chem.* **140**, 685.
 Rutherford, H. A., Patterson, W. I. & Harris, M. [1940]. *Text. Research*, **11**, 15.
 Skraup, Z. H. & Krause, E. [1909]. *Mh. Chem.* **30**, 447.
 Synge, R. L. M. [1939]. *Biochem. J.* **33**, 1931.
 — [1943]. *Chem. Rev.* **32**, 135.
 Van Slyke, D. D., Hiller, A. & MacFadyen, D. A. [1941]. *J. biol. Chem.* **141**, 681.
 du Vigneaud, V. & Meyer, C. E. [1932]. *J. biol. Chem.* **98**, 295.

The Relative Efficacy of Calcium Carbonate and Calcium Phosphate in Preventing Rickets in Rats

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Present knowledge of the metabolism of calcium and phosphorus suggests that calcium phosphate is likely to be more effective as a calcifying agent than the carbonate. Since the question of dietary supplies of calcium became topical with the proposal to add carbonate to bread, some experiments were begun to compare the power of the carbonate and the phosphate to prevent rickets in rats. The applicability of the findings to human rickets will be discussed after the presentation of the results.

EXPERIMENTAL

General plan. The general plan of the experiments was to place young rats on diets comprising mostly flour to which were added varying amounts of CaCO_3 or CaHPO_4 ; after 5 weeks on these diets the ash content of both femurs of each animal was determined. The rats at the beginning of the experiment were about 6 weeks old and weighed about

50 g.; they were kept in separate cages throughout the experiment and weighed weekly. Each group receiving a given level of Ca contained five animals. CaCO_3 was given as the preparation used to fortify bread, i.e. *creta preparata* B.P. The highest amount of each salt added to the diet was equivalent to 3% of Ca, i.e. 7.5% CaCO_3 or 10.1% CaHPO_4 .

Diets. In most of the experiments the basal diet consisted of flour to which was added 1% NaCl. The flour was either white (70% extraction) or National Wheatmeal (85% extraction). When white flour was used the rats were given supplements of aneurin and riboflavin equivalent to 10 μg . daily of the former and 4 μg . of the latter. No supplement was given with the wheatmeal flour. For comparative purposes one experiment was carried out with a maize diet similar to that commonly used for producing rickets in rats. This basal diet contained maize meal 76 parts, gluten 20 parts.

To the basal flour or maize diets were added 1% NaCl and varying amounts of the calcium salts. No further additions were made. With three parts CaCO_3 , the maize meal

diet is the rachitogenic diet 2965 of Steenbock & Black [1925] and is that normally used in this laboratory for producing rickets.

Examination of bones. The knee joints of the rats were X-rayed at the end of each experiment and in some instances during the course of the experiment. The femurs were removed on the day the rats were killed. They were cleaned by hand and extracted with ethanol in a Soxhlet apparatus, dried, weighed and ashed at dull-red heat. The results are expressed as the percentage of ash in the dried bones.

RESULTS

Effect of calcium carbonate and of calcium phosphate on the ash content of bones

(a) *White flour.* With diets containing no added Ca, the ash content of the bones was, in three experiments, between 32 and 35% (Fig. 1). With added CaHPO_4 , the maximal ash content of about 50% was reached with approximately 0.4% Ca

Fig. 1. White-flour diet: effect of CaCO_3 (continuous line) and CaHPO_4 (dotted line) on bone ash.

Fig. 2. Wheatmeal diet: effect of CaCO_3 and CaHPO_4 on bone ash.

and little difference was produced with an increase of Ca up to 3%. With added CaCO_3 , however, the ash content never exceeded 42%, a level reached with 0.2% Ca; higher levels of CaCO_3 reduced the ash content, which eventually fell to 28%, i.e. below that of the animals receiving no added Ca. In a preliminary experiment the rats received a supple-

ment of aneurin only and no riboflavin; although this influenced their growth rate (see below), it did not affect the ash content of the bones.

(b) *Wheatmeal flour.* In many respects, the experiments with wheatmeal flour (Fig. 2) gave results similar to those with white flour. The bones of animals on the basal diet with no added Ca had an ash content of about 32%. The addition of CaHPO_4 to the diet produced an ash content of about 50% with some 0.4% Ca, and there was no change with an increasing amount of Ca. Addition of CaCO_3 resulted in a maximal ash content at about 0.2% Ca, which rapidly fell with increasing amounts of dietary Ca to a value slightly below that of the animals on the basal diet. The only difference between the results with the white and with the wheatmeal flours appeared to be that the maximal calcification produced by the addition of CaCO_3 to wheatmeal flour was greater (about 50%) than that produced with white flour (about 42%); that is, with the former the maximal ash content was the same whether CaCO_3 or CaHPO_4 was added. Since, however, a slight difference in the amount of dietary CaCO_3 in the region of 0.2% Ca produced such a great effect in the bones it is possible that an addition to white flour slightly higher or lower than this might also have resulted in an ash content approaching 50%, and the apparent difference may be fortuitous.

(c) *Maize diet.* The results with the maize-gluten mixture were similar to those with flour (Fig. 3).

Fig. 3. Maize-gluten diet: effect of CaCO_3 and CaHPO_4 on bone ash.

The addition of CaHPO_4 in any concentration above 0.4% Ca produced a high degree of calcification. Lower concentrations (0.2% Ca) of CaCO_3 produced an improvement in calcification, but this deteriorated with higher concentrations. This diminution in effect was not so rapid as with the flour diets; for example, the ash content of the flour diets had reached its minimal level with 1.2% Ca, whereas with the maize diet there was a further decrease from 37% with 1.2% Ca, to 32% with 3% Ca.

Effect of calcium carbonate and calcium phosphate on growth

It is known that, other things being equal, the degree of rickets which an animal develops is proportional to its rate of growth. It might have been

Fig. 4. White-flour diet: effect of CaCO_3 and CaHPO_4 on growth.

Fig. 5. Wheatmeal diet: effect of CaCO_3 and CaHPO_4 on growth.

Fig. 6. Maize-gluten diet: effect of CaCO_3 and CaHPO_4 on growth.

thought that the differences in the calcification noted above were due to differences in rates of growth; the higher levels of dietary CaCO_3 , for example, might be producing their rachitogenic

effect by increasing the rate of growth. Such an explanation, however, must be discounted. A study of the growth curves shows that the rate of growth was lowered in each instance by the higher amounts of CaCO_3 in the diet (Figs. 4-6). Also the rate of growth on the flour diets was less with CaCO_3 than with the CaHPO_4 , whilst the reverse was true with the maize-gluten diet; as we have seen, in all of these diets the phosphate at high levels of intake produced better calcification than the carbonate.

There are other interesting features of the growth curves which are worth noting. Growth on the flour diets to some extent paralleled the ash content of the bones; this was especially noticeable with the white-flour diets containing CaCO_3 . It was less true with those containing CaHPO_4 , where the higher levels of this salt diminished growth, although they did not decrease the ash content of the bones. With the maize-gluten diet there was less parallelism; for example, with higher amounts of Ca, the CaHPO_4 resulted in a lower growth rate than CaCO_3 , although it produced better calcification. In all instances growth was better on the wheatmeal-flour diet than on the white-flour diet, even though the latter contained added aneurin and riboflavin [cf. Chick, 1940]. In the preliminary experiments, to which reference has already been made, in which aneurin only was given, there was, in the absence of added Ca, a slight average loss of weight (0.7 g./week) compared with a gain of 1.7 g. when both vitamins were given. When 0.2% Ca or more was added, either as CaHPO_4 or as CaCO_3 , the growth-promoting effect of riboflavin was not observed.

DISCUSSION

It is known that the calcification of bone, especially in the absence of vitamin D, is to a large extent dependent on the Ca/P ratio in the diets. The different effects of the addition of CaCO_3 and CaHPO_4 to the diet on the bones of the animals appear to be due to this cause. In the absence of added Ca the ratio Ca/P is always low; 0.16 with white flour (70% extraction), 0.10 with wheatmeal flour (unfortified; 85% extraction) and 0.10 with the maize-gluten diet (Tables 1, 2). Addition of CaHPO_4 increases the ratio, which reaches a value of one, or slightly more, for all the diets, with an addition of 1.2% Ca as CaHPO_4 , and increases only slightly with further additions. With additions of CaCO_3 the ratio increases to very much higher levels. With 0.2% Ca as CaCO_3 , the ratio is 2.05 for white flour, 1.10 for wheatmeal flour and 1.00 for the maize-gluten diet; with higher levels of CaCO_3 the ratio rises rapidly, so that with 1.2% Ca it is between 5 and 10 and with 3% Ca it is between 13 and 28. In all instances, therefore, maximal calcification occurred with a ratio between 1:1 and 2:1.

Table 1. *Ca and P content of flours and maize-gluten mixture*

	Ca (mg./100 g.)	P (mg./100 mg.)	Ca/P
White flour	16.7	106	0.16
Wheatmeal flour	20	200	0.10
Maize-gluten mixture	22.3	221	0.10

These values have been very kindly supplied by Miss E. M. Widdowson. The values for the white flour and the maize-gluten mixture were from actual analyses of the samples used in these experiments; the values for the wheatmeal flour were average values obtained from the analysis of a large number of samples, in which it was found that the range of variation was fairly small.

Table 2. *Ca/P ratios of flours and maize-gluten mixture with various additions of CaCO₃ and CaHPO₄*

Addition to diet (g./100 g.)	Ca/P		
	White flour	Wheatmeal flour	Maize-gluten mixture
0	0.16	0.10	0.10
CaCO ₃ 0.05	0.64	0.35	0.33
0.2	2.05	1.10	1.00
0.5	4.87	2.60	2.36
1.2	11.5	6.10	5.54
3.0	28.5	15.1	13.7
CaHPO ₄ 0.05	0.46	0.29	0.28
0.2	0.83	0.62	0.59
1.2	1.17	1.08	1.06
3.0	1.29	1.20	1.18

Adequate calcification depends not only on the ratio Ca/P but also on the absolute amounts of Ca and P; if both are low, the bones will not be well calcified. It may be that the poor calcification which occurred on the basal diets without added Ca was due to this fact and not to the very low Ca/P ratios. The amounts of P in the wheatmeal bread and maize-gluten diets are, however, fairly high. It seems likely therefore that the low ash content produced by the basal diets represents rickets of the 'low-calcium' type, whilst the low ash content produced by the higher levels of CaCO₃ represents rickets of the 'low-phosphorus' type. The mechanism of the production of 'low-phosphorus' rickets with high Ca/P ratios seems to be a reduction of the absorption of P by the excess of Ca.

It is recognized that the metabolism of Ca and P in human beings is appreciably different from that in rats. For example, rats usually absorb nearly 100% of their dietary Ca, a proportion much higher than that ever reached in human subjects. Nevertheless, a comparison of rickets in human beings and in rats is not without some value. In both there is excessive faecal loss of PO₄⁼ and sometimes of Ca, as

well as a low blood PO₄⁼. Administration of vitamin D in both cases restores these values to normal.

That CaCO₃ might be rachitogenic in human beings as well as in rats is suggested by experimental observations. McCance & Widdowson [1942] have demonstrated that absorption of PO₄⁼ in experimental subjects is lowered by the inclusion of CaCO₃ in bread. In experiments on babies and children, Stearns & Jeans [1934] showed that Ca and P were adequately retained in the body when given as milk or as CaHPO₄ but less was retained when Ca was given as carbonate, gluconate or lactate. Only one study, however, was made with CaCO₃.

The quantity of CaCO₃ at present added to wheatmeal bread is 65 mg. Ca to 100 g. flour (i.e. 0.065%). In our experiments with rats these small amounts always result in improved calcification. It would, however, be unwise to transfer these quantitative results directly to the question of human rickets. Moreover, the rats were receiving diets restricted to a degree which would certainly not obtain in human subjects. The work here reported may best be considered as tests of the relative value of CaCO₃ and CaHPO₄ in favouring bone formation, using rats as a 'biological indicator'. The results suggest that at high levels of dosage the carbonate is inferior to the phosphate. At lower levels, comparable with the quantities added to bread, CaCO₃ is as efficacious as CaHPO₄ in improving the calcification of the bone of the rat. Whether the carbonate is as good as the phosphate for children, as obtains with growing rats, in the quantities likely to be ingested, can be determined only by trial.

SUMMARY

1. The effect has been studied in rats of the addition of calcium carbonate or calcium monohydrogen phosphate to basal diets consisting of white flour, wheatmeal flour or a maize-gluten mixture.

2. In all instances the addition to the diet of carbonate increased the ash content of the bones when it was added in small amounts in the proportion present in National Wheatmeal bread; larger amounts, however, led to a reduction in the ash content. Phosphate, on the other hand, produced adequate calcification, not only in these small amounts, but also in amounts up to 3% Ca.

3. These differences were not due to the known aggravation of rickets which results from an acceleration of growth. With the white-flour diet, for example, the gain in weight was less with added carbonate than with phosphate.

4. The applicability of these results to human dietaries is discussed.

REFERENCES

- Chick, H. [1940]. *Lancet*, 2, 511.
 McCance, R. A. & Widdowson, E. M. [1942]. *J. Physiol.* 101, 44.
 Stearns, G. & Jeans, P. C. [1934]. *Proc. Soc. exp. Biol.*, N.Y., 32, 428.
 Steenbock, H. & Black, A. [1925]. *J. biol. Chem.* 64, 263.