Effect of level of dietary calcium on the skeleton of the rat

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1. The total body calcium (Expt 1) of litter-mate male rats given a diet adequate in phosphorus and high (0·74 %) or low (0·13 %) in Ca from the age of 3 weeks was determined after 21, 48 and 60 weeks on the diet. In Expt 2, the ash content of ten groups of bones from 10-week-old rats given these same diets for 7 weeks was studied to ascertain whether all groups were equally affected by the difference in dietary Ca level. Four groups of bones, i.e. skull and mandibles, vertebras, the shafts and the ends of long bones, were consequently chosen for examination in Expt 3 where their growth and composition were studied in rats given the Ca diets for 24, 48 or 60 weeks from the age of 3 weeks.

2. Total body Ca (Expt 1), expressed as g Ca or as a percentage of the net body-weight or of net dry, fat-free carcass weight, was always significantly higher in rats given the higher level of Ca. The dietary effect was greatest after 21 weeks.

3. The higher level of dietary Ca led to a highly significant increase in the weight of the dry, fat-free bone and in the percentage ash content of all ten bone groups after 7 weeks (Expt 2). The greater treatment differences in ash content occurred with the less well mineralized bones.

4. In Expt 3, irrespective of diet, the four groups of bones developed at different rates. In rats given the diets for 24 weeks or longer, the vertebras and the shafts of long bones showed the greatest proportional increase in weight of dry, fat-free bone and ash relative to the corresponding values for 3-week-old rats; the greatest change in percentage ash content occurred in the ends of the long bones.

5. Increasing the level of Ca in the diet increased the weight of the dry, fat-free bone and its ash content. The effect of diet decreased with increasing time on the diets, in general persisting most strongly in the skull and mandibles and declining most rapidly in the ends of the long bones. The percentage Ca and P content of the bone ash was only slightly affected by diet and, except for the Ca in the ash of vertebras, significant differences were only found in the composition of the ash of bones from 27-week-old rats.

The effect of various levels of dietary calcium on the total body Ca of rats has been studied in several long-term experiments by Sherman and co-workers (Sherman & MacLeod, 1925; Sherman & Booher, 1931; Whitcher, Booher & Sherman, 1936; Toepfer & Sherman, 1936; Lanford, Campbell & Sherman, 1941). Lanford & Sherman (1938) extended the work of Toepfer & Sherman (1936) and determined the total body Ca content of rats given diets containing adequate phosphorus and 0·20, 0·64 or 0·80 % Ca until 12 months old, each of these experimental rats receiving the same Ca diet as had been given to its mother since infancy. Most Ca was found in the rats given the high-Ca diets and, although the difference between the high- and low-Ca groups decreased with increasing age, it was still evident in 1-year-old animals. On the other hand, Henry & Kon (1953) used offspring from rats that had always received a diet containing about 0·46 % Ca and, in conjunction with a metabolic study, determined the ash and Ca content of the humerus and femur in rats on either a low-Ca diet (0·13 %) or a high-Ca diet (0·77 %) with short periods on the low-Ca diet. These workers concluded that, although calcification was initially better on the high-Ca diet, the difference had disappeared by the time the rats were 9–12 months old. As at least

99 % of the body Ca is in the skeleton (Bessey, King, Quinn & Sherman, 1935) the observations of Lanford & Sherman (1938) and Henry & Kon (1953) appear to be at variance. This apparent disagreement could possibly have arisen from the difference in the dietary regimen of the two breeding stocks, for the use by Lanford & Sherman of rats bred from animals already receiving the high- or low-Ca diets would tend to intensify any dietary effects (Toepfer & Sherman, 1936). It is also possible that the discontinuity in the feeding of the high-Ca diet by Henry & Kon may have limited the response and that the bones examined by them were not as susceptible as other parts of the skeleton to differences in dietary Ca level.

Studies of the effect of different levels of dietary Ca on bones have generally been of relatively short duration and limited to single bones, but in an experiment lasting 320 days Menczel, Schraer, Pakis, Posner & Likins (1963) found an increase in the percentage ash content of the ends and shafts of the tibia but not of the whole femur of rats, when the level of dietary Ca was raised from 0·1 to 0·8 %. Similarly Williams, Mason & McDonald (1964), using an X-ray technique, found differences in the density of the ninth caudal vertebra of rats given diets containing 0·1 or 0·5 % Ca and 0·39 % P from 74 days of age to death. Schraer, Siar & Schraer (1963), also using X-rays, found in a 59-day experiment that the seventh caudal vertebra and the femur diaphysis responded differently in rats given diets containing 0·025–1·49 % Ca and 0·29–0·89 % P.

In the present work the total body Ca and the weight and mineral content of several groups of bones have been studied in rats that from the age of 3 weeks were given, for periods of up to 60 weeks, a low-Ca diet containing 0·131 % Ca and 0·355 % P, or a high-Ca diet containing 0·744 % Ca and 0·795 % P. The levels of P in these diets were considered sufficient for the formation of the soft tissues of the rats during the period of most rapid growth, and also for maximum retention of the dietary Ca (Henry, Kon, Todd, Toothill & Tomlin, 1960). In order to select bone groups for examination in the long-term experiment, the degree of calcification of several different bones was first determined in a preliminary experiment lasting for 7 weeks. The rats used in the present experiments, like those used by Henry & Kon (1953), were from the stock colony at this Institute.

EXPERIMENTAL

Diets and general technique

The composition and analyses of the experimental diets and details concerning the vitamin supplement are given in Table 1. These diets contained the same basic ingredients, i.e. whole ground wheat and dried whole milk, as those used by Lanford & Sherman (1938) and Henry & Kon (1953). Several batches of each diet were used and the values given for Ca, P and moisture are means. Male hooded Norwegian rats were removed, when 3 weeks old, from mothers on the stock diet (McKinlay, 1951) and housed in twos or threes in stock-colony cages where they had unrestricted access to the experimental diet and distilled water. All animals were given a weekly supplement of fat-soluble vitamins (Table 1) throughout the experiments.

Analytical methods

Diets were mixed with 10% (w/v) sodium acetate solution and dried before being ashed at 550°. The ash was then digested with hot HNO_3 (approx. 8N) for the determination of Ca and P. Moisture was determined by drying the diet samples at 102–103°.

Carcasses of 51- and 63-week-old rats were extracted with a boiling chloroform–methanol mixture (2:1, v/v) and the dry, fat-free carcasses weighed before being ashed at 550° in silica dishes. Carcasses of younger rats were ashed without prior treatment. The ash was dissolved in hot HCl (approx. 5·5N) for the determination of Ca.

Table 1. *Components and composition (%) of the experimental diets and their mean content of calcium, phosphorus and moisture*

	Diet	
Component	Low-Ca	High-Ca
Wheat, whole ground	86	83·9
Milk, dried whole	10	9·7
Ca- and P-free salts*	4	3·9
$CaHPO_4.2H_2O$	—	2·5
Composition		
Ca	0·131	0·744
P	0·355	0·795
Moisture	12·7	12·5

* Salt mixture no. 6 of de Loureiro (1931) with calcium phosphate omitted.

A weekly supplement of 1·4 mg DL-α-tocopherol, 360 i.u. vitamin A and 26 i.u. vitamin D_3 in arachis oil was given by pipette for the whole of the experimental period.

Bones were fat-extracted with a boiling ethanol–benzene mixture (2:1, v/v) in a Soxhlet apparatus (Taylor & Moore, 1954) and dried to constant weight at 102–103°. Ash content was determined by heating to constant weight at 600° in silica crucibles. For the determination of Ca and P the ash was dissolved in HNO_3 (approx. 8N), transferred to Pyrex dishes and boiled.

Ca was determined by McCrudden's (1911) volumetric method and P by the molybdivanadate method (Kitson & Mellon, 1944; Hanson, 1950).

Plan of experiments and preparation of carcasses and bones

Expt 1. *Total body Ca of rats given a high- or low-Ca diet for periods up to* 60 *weeks.* Eight litters of seven 3-week-old male rats were allocated so as to give seven treatment groups of similar body-weight. From each litter one animal was killed immediately, three were given the high- and three the low-Ca diet (Table 1). Two rats from each litter, one on each diet, were killed after 21, 48 and 60 weeks. Each rat was weighed before it was killed and, immediately after death, the alimentary tract was removed and its contents were weighed. The tract and contents were then discarded. The fur and paws of the rats were brushed to remove any adherent diet and the carcasses of the 3- and 24-week-old rats were then ashed without further treatment. Fat was extracted from the carcasses of older rats before they were ashed, so as to avoid any effect on body-weight of varying amounts of body fat.

Expt 2. Bone calcification in rats given a high- or low-Ca diet for 7 weeks. Six pairs of 3-week-old litter-mate male rats of similar body-weight were given the high- or low-Ca diet (Table 1) for 7 weeks and then killed and eviscerated. The long bones, i.e. each humerus, radius, ulna, femur, tibia and fibula, were dissected out from the freshly killed animal and the flesh was removed. The shaft of each of these bones was then cut at the proximal and distal ends with an extremely fine coping saw; transverse cuts were made unless otherwise stated and the bone dust was discarded. The humerus was cut at the proximal end at about 2 mm from the epiphysial line on the anterior surface and, at the distal end, across the supratrochlear fossa. Cuts were made across both ends of the radius at about 1 mm from the epiphysial lines. The ulna was cut through the semilunar notch and at about 1 mm from the distal epiphysial line. The distal end of the femur was removed at about 1 mm from the epiphysial line on the anterior surface, and the proximal end by a slanting cut near to the greater and lesser trochanters. The fibula shaft was detached from its proximal epiphysis and from the distal end of the tibia shaft which was then cut at the proximal end at about 2 mm from the epiphysial line on the anterior surface and, at the distal end, at the point of attachment of the transverse crural ligament adjacent to the medial malleolus. The bones were then split longitudinally with a scalpel, and marrow was washed away with a fine jet of distilled water. Although this method was satisfactory for the shafts, it did not completely remove the marrow from the proximal and distal ends. The remainder of the carcass was skinned and heated in liquid paraffin on a water-bath (Taylor & Moore, 1954) for just sufficient time to enable the flesh to be easily detached from the skeleton. The following bones were then removed and cleared of soft tissue: all the cervical, thoracic, lumbar and sacral, and first ten caudal vertebras, the ribs, scapulas and pelvic girdle. The skull and mandibles were prepared by removing the brain, teeth, periotic capsules, tympanic bullae, zygomatics, the ethmoid and the cartilagenous bones of the nasal area. The bones from each rat were then divided into ten groups (Table 3) and fat was extracted before they were dried and ashed. All the material in each bone group from each rat was used for a single determination of ash content.

Expt 3. Bone calcification in rats given a high- or low-Ca diet for periods up to 60 weeks. Six litters of nine 3-week-old male rats were used and litter-mates were allocated at random to the treatments. Three rats from each litter were killed immediately and eviscerated, three were given the high- and three the low-Ca diet (Table 1). Two rats from each litter, one on each diet, were killed after 24, 48 and 60 weeks and eviscerated. Four bone groups that represented the range of percentage ash values and dietary responses obtained in Expt 2 (Table 3) were chosen for study. The bone groups were (1) skull and mandibles, (2) all the cervical, thoracic, lumbar and sacral, and first eight caudal vertebras, (3) shafts of long bones, (4) proximal and distal ends of long bones; the long bones were as specified in Expt 2.

The long bones of 3-week-old rats, and the skull, mandibles and vertebras of all rats were removed, cut and prepared for extraction of fat as described in Expt 2. The long bones of older rats were removed after the carcass had been heated in liquid paraffin and, after extraction of fat, cuts were made across the ends of the shafts as in

Expt 2. Similar groups of bones from 3-week-old litter-mates were pooled so as to provide sufficient material for analysis, but with older rats each group of bones examined consisted of material from one animal only. After the groups of bones had been dried to constant weight they were ground in a ball mill (Analytical Micropulverizer; Glen Creston Ltd) and then with a pestle and mortar until all the material passed a 100-mesh sieve. Attempts to remove the marrow from small samples of the powdered bones by centrifuging them in an ethanol–carbon tetrachloride mixture of suitable density were not successful as some marrow always adhered to the calcified fraction; consequently, the duplicate determinations of ash, Ca and P content were made on samples of the untreated fat-free powdered bone.

RESULTS

Expt 1. *Total body Ca of rats given a high- or low-Ca diet for periods up to* 60 *weeks*

The mean values relating to body-weight and Ca determinations for seven litters of rats are given in Table 2. Values for the eighth litter were discarded as two rats in this litter died before the end of the experiment. Net body-weight was calculated by subtracting the weight of the contents of the alimentary tract from the live weight of the

Table 2. *Expt* 1. *Mean values for body-weight, total body calcium and percentage body calcium of seven litters of rats that from the age of* 3 *weeks were given a high- or low-calcium diet for* 0, 21, 48 *or* 60 *weeks*

	Age at autopsy (weeks)	Experimental diets: High-Ca (H)	Low-Ca (L)	Difference (H − L)† with SE (df)
Net body-weight (g)‡	3	41·5 ± 1·58 (6 df)		
	24	298	270	28* ± 11·5 (18)
	51	367	375	−8NS ± 11·5 (18)
	63	407	399	8NS ± 11·5 (18)
Net dry, fat-free carcass weight (g)§	51	78·1	77·7	0·4NS ± 2·23 (12)
	63	84·4	80·9	3·5NS ± 2·23 (12)
Total Ca in body (g)	3	0·30 ± 0·015 (6 df)		
	24	3·05	2·12	0·93*** ± 0·100 (18)
	51	3·75	3·40	0·35** ± 0·100 (18)
	63	4·02	3·60	0·42*** ± 0·100 (18)
Total Ca as % net body-weight	3	0·73 ± 0·019 (6 df)		
	24	1·03	0·79	0·24*** ± 0·024 (18)
	51	1·02	0·91	0·11*** ± 0·024 (18)
	63	0·99	0·91	0·08** ± 0·024 (18)
Total Ca as % net dry, fat-free carcass weight	51	4·80	4·38	0·42*** ± 0·066 (12)
	63	4·77	4·45	0·32*** ± 0·066 (12)

† Significance of difference between diets (H − L) is denoted as follows: NS, $P > 0{\cdot}1$. * $P < 0{\cdot}05$. ** $P < 0{\cdot}01$. *** $P < 0{\cdot}001$.

‡ Live weight minus weight of contents of alimentary tract.

§ Weight of dried, fat-free carcass without the alimentary tract and its contents.

rat (Sherman & MacLeod, 1925). The net dry, fat-free carcass weight is the weight of the dried fat-extracted carcass without the alimentary tract and its contents.

After 21 weeks on the diets, i.e. at 24 weeks of age, rats given the high-Ca diet had a greater ($P < 0.05$) net body-weight than those on the low-Ca diet, but after 48 and 60 weeks there was no significant effect of diet on net body-weight or net dry, fat-free carcass weight.

The total body Ca of rats on the high-Ca diet for 21 weeks was very significantly greater ($P < 0.001$) than that of litter-mates given the low-Ca diet for the same period and although the effect of diet on body Ca became less marked with time, it was still significant ($P < 0.001$) after 60 weeks. Both groups of rats made large gains in total body Ca during the first 21 weeks of the experiment, but thereafter the succeeding increments, as shown by the 51- and 63-week-old rats, became noticeably smaller. Linear regressions relating weight (g) of Ca in the body (y) and the logarithm of the age of the rat in weeks ($\log x$) were calculated from values for 24-, 51-, and 63-week-old rats and, over this limited period of time, represented the relationships satisfactorily. The following equations were obtained

$$\text{High-Ca diet} \quad y = -0.09 + 2.27\,(\pm 0.27)\log x,$$
$$\text{Low-Ca diet} \quad y = -2.85 + 3.62\,(\pm 0.30)\log x,$$

and show that the increase in total body Ca with the logarithm of age was highly significant ($P < 0.001$) for both dietary groups; the figures in parentheses are the respective standard errors of the regression coefficients with 13 degrees of freedom. The rate of gain in total body Ca during this period was, however, significantly less ($P < 0.01$) for the rats given the high-Ca diet than for those given the lower level of dietary Ca.

When the total body Ca was expressed as a percentage of the net body-weight, Table 2 shows that the mean value for the 24-week-old rats on the low-Ca diet, in contrast to that for rats of the same age on the high-Ca diet, was only slightly higher than the mean for the 3-week-old animals. The dietary effect was very marked after 21 weeks ($P < 0.001$) with the higher value for rats on the high-Ca diet and, although the difference in the percentage total body Ca due to diet decreased from 0·24 after 21 weeks to 0·11 and 0·08 after 48 and 60 weeks respectively, it remained significant.

When the total body Ca was expressed as a percentage of the net dry, fat-free carcass weight, as for 51- and 63-week old rats, significantly greater values ($P < 0.001$) were again obtained at each age for rats given the higher level of Ca (Table 2).

Expt 2. Bone calcification in rats given a high- or low-Ca diet for 7 weeks

The mean live weight of rats at 3 weeks of age was 52.4 ± 0.08 g, there being no difference between groups. At 10 weeks of age the mean live weights for the high- and low-Ca diets were 113·3 and 95·5 g respectively with a standard error of the difference of 4·57 g (5 df). This difference was significant at between probability levels of 1–5 %. The results in Table 3 show that the rats given the high-Ca diet had heavier ($P < 0.001$) and more highly ($P < 0.001$) mineralized bones than those given the lower level of Ca.

The different bones differed in their degree of mineralization, and the mean values for percentage ash content (Table 3) ranged from 58·3 to 72·0% in rats given the high-Ca diet and from 47·7 to 67·6% in those given the low-Ca diet. In each dietary group, the extreme values were those for the shafts and ends of the long bones but, in contrast, the differences between the four groups of vertebras were very small. The treatment response of the various bones differed and was much more marked in the ends of the long bones than in the shafts, whereas the treatment responses obtained for the vertebras were all consistently large and very similar to each other.

Table 3. *Expt 2. Mean values for weight and ash content of dry, fat-free bones from six pairs of 10-week-old litter-mate rats given a high- or low-calcium diet from the age of 3 weeks*

	Weight (mg)			Ash (%)		
Bone group	High-Ca diet (H)	Low-Ca diet (L)	Difference (H − L)† with SE (5 df)	High-Ca diet (H)	Low-Ca diet (L)	Difference (H − L)† with SE (5 df)
Skull and mandibles	944	525	419 ± 6·5	69·9	63·0	6·9 ± 0·16
Vertebras‡: cervical	206	121	85 ± 4·8	66·8	57·0	9·8 ± 0·31
thoracic	347	202	145 ± 9·4	65·6	55·3	10·3 ± 0·35
lumbar and sacral	607	328	279 ± 12·8	64·7	53·7	11·0 ± 0·46
caudal	394	215	179 ± 10·6	64·9	54·8	10·1 ± 0·97
Ribs	314	188	126 ± 8·0	68·5	60·6	7·9 ± 0·42
Pelvic girdle	414	242	172 ± 9·8	66·2	56·7	9·5 ± 0·37
Scapulas	142	87	55 ± 3·9	68·4	61·2	7·2 ± 0·49
Long bones§: shafts	716	415	301 ± 13·1	72·0	67·6	4·4 ± 0·27
proximal and distal ends	802	508	294 ± 16·0	58·3	47·7	10·6 ± 0·44
Total: all bone groups	4886	2831	2055 ± 83·0	66·4	57·7	8·7 ± 0·31

† All dietary differences (H − L) were significant at $P < 0{\cdot}001$.
‡ All cervical, thoracic, lumbar, sacral and first ten caudal.
§ Humeri, radii, ulnas, femurs, tibiae, fibulae.

Expt 3. Bone calcification in rats given a high- or low-Ca diet for periods up to 60 weeks

One rat allocated to the low-Ca diet for 24 weeks and one allocated to the high-Ca diet for 48 weeks died shortly after the beginning of the experiment and estimates of the missing values have been used in the calculations.

Live weight. The mean live-weight gains were 275, 360 and 397 g over 24, 48 and 60 weeks respectively of the experiment. Differences between the two diets were very small, the largest being 4 ± 8·6 g after 24 weeks.

Bone weight. Irrespective of diet, the weights of the four groups of dry, fat-free bones increased at considerably different rates and, compared with values at 3 weeks of age, the vertebras and shafts from 63-week-old rats were about fourteen to fifteen times heavier, whereas the weights of the skull and mandibles and the ends of the long bones increased only about six to eight times (Table 4).

The higher level of Ca in the diet led to an increase in the weight of the dry, fat-free bones and after 24 weeks the effect was highly significant for skull and mandibles

Table 4. *Expt 3. Mean values for weight, percentage ash content and total weight of ash of dry, fat-free bones from six litters of rats that from the age of 3 weeks were given a high- or low-calcium diet for 0, 24, 48 or 60 weeks*

Bone group	Age at autopsy (weeks)	Weight of bone (g)			Ash content (%)			Weight of ash (g)‡		
		High-Ca diet (H)	Low-Ca diet (L)	Difference (H − L)§ with SE (13 df)	High-Ca diet (H)	Low-Ca diet (L)	Difference (H − L)§ with SE (13 df)	High-Ca diet (H)	Low-Ca diet (L)	Difference (H − L)§ with SE (13 df)
Skull and mandibles	3	0·297 ± 0·0052 (5 df)‖			61·2 ± 0·13 (5 df)‖			0·182 ± 0·0035 (5 df)‖		
	27	1·87	1·47	0·40*** ± 0·071	72·2	70·5	1·7*** ± 0·21	1·35	1·04	0·31*** ± 0·052
	51	2·24	1·98	0·26** ± 0·071	72·9	72·1	0·8** ± 0·21	1·64	1·43	0·21** ± 0·052
	63	2·42	2·10	0·32*** ± 0·064	73·2	72·4	0·8** ± 0·19	1·77	1·52	0·25*** ± 0·048
Vertebras¶	3	0·270 ± 0·0078 (5 df)‖			53·0 ± 0·14 (5 df)‖			0·143 ± 0·0097 (5 df)‖		
	27	3·16	2·58	0·58** ± 0·139	69·1	66·8	2·3*** ± 0·27	2·19	1·72	0·47*** ± 0·098
	51	3·82	3·48	0·34* ± 0·139	69·6	68·4	1·2*** ± 0·27	2·66	2·38	0·28* ± 0·098
	63	4·07	3·71	0·36* ± 0·127	69·3	68·7	0·6* ± 0·25	2·82	2·55	0·27* ± 0·090
Long bones‡‡: shafts	3	0·136 ± 0·0040 (5 df)‖			62·5 ± 0·14 (5 df)‖			0·085 ± 0·0055 (5 df)‖		
	27	1·54	1·27	0·27** ± 0·071	73·9	72·3	1·6*** ± 0·20	1·14	0·92	0·22** ± 0·053
	51	1·88	1·72	0·16* ± 0·071	74·3	73·3	1·0*** ± 0·20	1·39	1·26	0·13* ± 0·053
	63	2·01	1·89	0·12† ± 0·065	75·1	74·7	0·4* ± 0·18	1·51	1·41	0·10† ± 0·048
Long bones‡‡: proximal and distal ends	3	0·201 ± 0·0069 (5 df)‖			44·8 ± 0·22 (5 df)‖			0·090 ± 0·0073 (5 df)‖		
	27	1·17	1·02	0·15** ± 0·036	67·2	65·3	1·9*** ± 0·29	0·78	0·67	0·11*** ± 0·026
	51	1·29	1·19	0·10* ± 0·036	68·2	67·0	1·2** ± 0·29	0·88	0·80	0·08** ± 0·026
	63	1·26	1·24	0·02 NS ± 0·033	67·7	67·9	−0·2 NS ± 0·26	0·85	0·84	0·01 NS ± 0·024

‡ Calculated from values for each rat for weight of dry, fat-free bone and mean percentage ash content of two samples of the powdered bone material.
§ Significance of difference between diets (H − L) is denoted as follows: NS, $P > 0·1$. † $P < 0·1$. *$P < 0·05$. **$P < 0·01$. ***$P < 0·001$.
‖ Bones from three litter-mates pooled to form composite litter sample for examination.
¶ All cervical, thoracic, lumbar, sacral and first eight caudal.
‡‡ Humeri, radii, ulnas, femurs, tibiae, fibulae.

($P < 0{\cdot}001$) and for vertebras, shafts and ends of long bones ($P < 0{\cdot}01$). Progressively smaller treatment effects were observed when the rats were given the diets for longer periods and by 60 weeks the dietary differences for the shafts and the ends of long bones failed to reach the 5 % level of significance.

Percentage ash content. In all bone groups there was an increase in percentage ash content with increasing age, the greater part of this increase occurring between 3 and 27 weeks of age (Table 4). Irrespective of diet, large increases occurred in the ends of the long bones, whereas in the vertebras, and particularly in the skull and mandibles and the shafts of long bones, gains were smaller.

The higher level of Ca in the diet led to an increase in the percentage ash content of the bones, and after the rats had been on the diets for 24 weeks, the treatment differences were highly significant ($P < 0{\cdot}001$) for all four bone groups (Table 4). After 48 weeks the treatment differences had decreased appreciably and by 60 weeks the differences, though even smaller, reached at least the 5 % level of significance with the exception of the ends of the long bones.

Weight of ash. The ash weight of each bone group was calculated for each rat from the corresponding weight of the dry, fat-free bone and the mean value for percentage ash content of two samples of the powdered bone material. The mean values of these calculated weights of ash are given in Table 4.

The weights of bone ash increased with increasing age in all bone groups, but at the end of each treatment period and irrespective of diet, the vertebras had increased most both in absolute amounts and in proportion to the corresponding value for 3-week-old rats. As a result of these different rates of development, the relative contributions of the different bone groups to the total weight of bone ash changed during the 60 weeks of the experiment. Thus in the 3-week-old bones, the skull and mandibles accounted for about 36 % of the total weight of bone ash, the vertebras 29 % and the shafts and ends of the long bones each 17–18 %, but the corresponding values for the bones of 63-week-old rats were about 25, 41, 22 and 12 % respectively.

The higher level of Ca in the diet led to an increase in the weight of bone ash, and after the rats had been on the diets for 24 weeks the effect was highly significant ($P < 0{\cdot}01$) for all groups of bones (Table 4). After 48 weeks the treatment effect was less but was still significant for all bone groups. By 60 weeks, however, the differences reached the 5 % level of significance, or higher, only for the skull and mandibles and the vertebras.

To illustrate the changes with time in the weight of ash of different bones from rats on each diet, the mean values for the weight of bone ash (Table 4) in relation to the logarithm of the age of the rats are shown on Fig. 1. These relationships have been further examined by regression analysis. The regression lines are inserted on Fig. 1, and the increase in weight of bone ash with proportional increase in age was highly significant ($P < 0{\cdot}001$) in all instances except for the ends of long bones from rats on the high-Ca diet ($P < 0{\cdot}05$). Between the ages of 27 and 63 weeks, the rate of increase in weight of bone ash was always slightly less for rats given the high-Ca than for those given the low-Ca diet and, except for skull and mandibles, the difference within each bone group was significant ($P < 0{\cdot}05$).

Ca and P contents. The percentage Ca (Table 5) and P (Table 6) in the dry, fat-free bones increased with increasing age. The effect of the level of Ca in the diet on both percentage Ca and P content of the bones decreased with increasing time, but differences in the Ca and P values, except for the ends of the long bones after 60 weeks, were significant at the 10% level or higher.

The percentage Ca and percentage P contents of the bone ash were only slightly affected by the level of dietary Ca and, except for the Ca content of the ash of vertebras, significant differences ($P < 0{\cdot}1$) were only found in the ash of bones from 27-week-old rats.

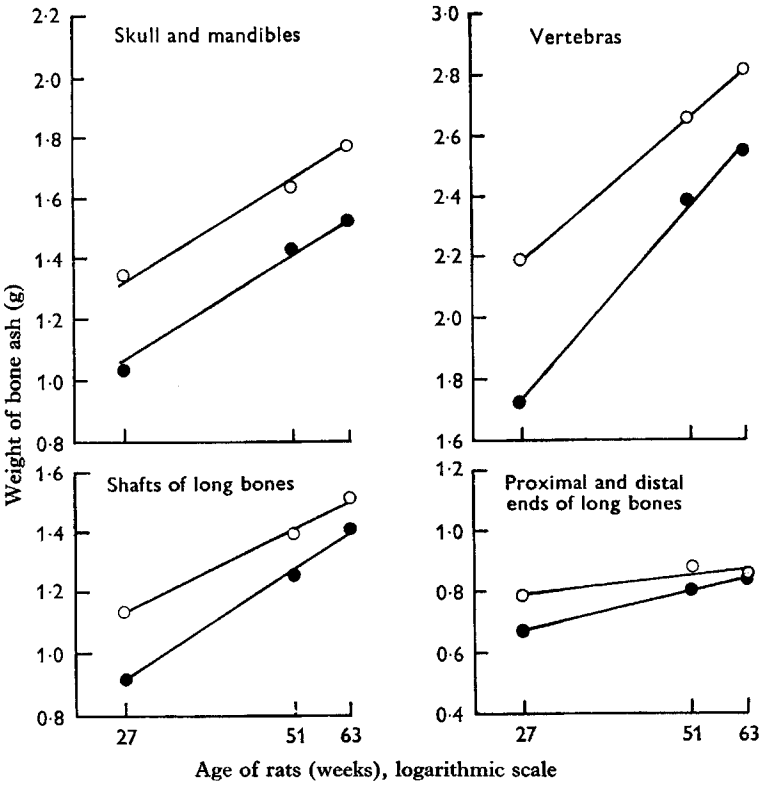

Fig. 1. Weight of ash of four groups of bones (see p. 90) from rats on the high-calcium (○) or low-calcium (●) diet from the age of 3 weeks.

DISCUSSION

In Expt 1, the rats given either level of dietary Ca for 21–60 weeks appeared healthy and grew satisfactorily. Although the rats on the low-Ca diet for 21 weeks grew at a slightly slower rate than litter-mates on the high-Ca diet, the level of Ca had no effect on net body-weight or net dry, fat-free carcass weight when the diets were given for 48 or 60 weeks.

During the first 21 weeks of Expt 1, the rats grew rapidly but, whereas the increase in percentage net body Ca for rats on the high-Ca diet was 0·30, the amount of Ca

Table 5. *Expt* 3. *Mean values for calcium content of bones and ash of bones from six litters of rats that from the age of* 3 *weeks were given a high- or low-calcium diet for* 0, 24, 48 *or* 60 *weeks*

Bone group	Age at autopsy (weeks)	Ca in dry, fat-free bone (%) High-Ca diet (H)	Low-Ca diet (L)	Difference (H − L)‡ with SE (13 df)	Ca in ash (%) High-Ca diet (H)	Low-Ca diet (L)	Difference (H − L)‡ with SE (13 df)
Skull and mandibles	3	22·01 ± 0·040 (5 df)§			35·95 ± 0·080 (5 df)§		
	27	26·62	25·91	0·71*** ± 0·099	36·87	36·73	0·14† ± 0·075
	51	26·74	26·41	0·33** ± 0·099	36·65	36·63	0·02 NS ± 0·075
	63	27·06	26·73	0·33** ± 0·091	36·99	36·89	0·10 NS ± 0·068
Vertebras‖	3	18·80 ± 0·067 (5 df)§			35·47 ± 0·069 (5 df)§		
	27	25·24	24·26	0·98*** ± 0·115	36·50	36·30	0·20** ± 0·050
	51	25·45	24·92	0·53*** ± 0·115	36·59	36·45	0·14* ± 0·050
	63	25·40	25·07	0·33** ± 0·105	36·68	36·48	0·20*** ± 0·046
Long bones¶: shafts	3	23·06 ± 0·049 (5 df)§			36·92 ± 0·054 (5 df)§		
	27	27·18	26·56	0·62*** ± 0·122	36·80	36·72	0·08 NS ± 0·123
	51	27·29	27·02	0·27* ± 0·122	36·76	36·83	−0·07 NS ± 0·123
	63	27·70	27·49	0·21† ± 0·111	36·86	36·82	0·04 NS ± 0·113
Long bones¶: proximal and distal ends	3	15·90 ± 0·083 (5 df)§			35·49 ± 0·063 (5 df)§		
	27	24·74	23·86	0·88*** ± 0·118	36·82	36·55	0·27** ± 0·064
	51	25·08	24·59	0·49** ± 0·118	36·79	36·72	0·07 NS ± 0·064
	63	24·82	24·81	0·01 NS ± 0·108	36·64	36·55	0·09 NS ± 0·058

‡ Significance of difference between diets (H − L) is denoted as follows: NS, $P > 0{\cdot}1$. † $P < 0{\cdot}1$. * $P < 0{\cdot}05$. ** $P < 0{\cdot}01$. *** $P < 0{\cdot}001$.

§ Bones from three litter-mates pooled to form composite litter sample for examination.

‖ All cervical, thoracic, lumbar, sacral and first eight caudal.

¶ Humeri, radii, ulnas, femurs, tibiae, fibulae.

Table 6. *Expt* 3. *Mean values for phosphorus content of bones and ash of bones from six litters of rats that from the age of* 3 *weeks were given a high- or low-calcium diet for* 0, 24, 48 *or* 60 *weeks*

Bone group	Age at autopsy (weeks)	P in dry, fat-free bone (%) High-Ca diet (H)	Low-Ca diet (L)	Difference (H − L)‡ with SE (13 df)	P in ash (%) High-Ca diet (H)	Low-Ca diet (L)	Difference (H − L)‡ with SE (13 df)
Skull and mandibles	3	11·47 ± 0·013 (5 df)§			18·74 ± 0·033 (5 df)§		
	27	12·96	12·76	0·20*** ± 0·043	17·96	18·09	−0·13** ± 0·038
	51	12·90	12·76	0·14** ± 0·043	17·68	17·71	−0·03 NS ± 0·038
	63	13·01	12·90	0·11* ± 0·039	17·79	17·81	−0·02 NS ± 0·035
Vertebras‖	3	10·04 ± 0·018 (5 df)§			18·95 ± 0·029 (5 df)§		
	27	12·37	11·99	0·38*** ± 0·050	17·89	17·95	−0·06 NS ± 0·054
	51	12·49	12·23	0·26*** ± 0·050	17·96	17·90	0·06 NS ± 0·054
	63	12·21	12·12	0·09† ± 0·046	17·63	17·64	−0·01 NS ± 0·049
Long bones¶: shafts	3	11·45 ± 0·030 (5 df)§			18·32 ± 0·033 (5 df)§		
	27	13·35	13·17	0·18*** ± 0·034	18·08	18·21	−0·13*** ± 0·022
	51	13·24	13·10	0·14** ± 0·034	17·83	17·86	−0·03 NS ± 0·022
	63	13·46	13·35	0·11** ± 0·031	17·90	17·87	0·03 NS ± 0·020
Long bones¶: proximal and distal ends	3	8·11 ± 0·042 (5 df)§			18·09 ± 0·041 (5 df)§		
	27	12·06	11·74	0·32*** ± 0·046	17·95	17·99	−0·04 NS ± 0·048
	51	12·08	11·91	0·17** ± 0·046	17·72	17·78	−0·06 NS ± 0·048
	63	11·94	11·95	−0·01 NS ± 0·042	17·63	17·60	0·03 NS ± 0·044

‡ Significance of difference between diets (H − L) is denoted as follows: NS, $P > 0{\cdot}1$. †$P < 0{\cdot}1$. *$P < 0{\cdot}05$. **$P < 0{\cdot}01$. ***$P < 0{\cdot}001$.

§ Bones from three litter-mates pooled to form composite litter sample for examination.

‖ All cervical, thoracic, lumbar, sacral and first eight caudal.

¶ Humeri, radii, ulnas, femurs, tibiae, fibulae.

supplied by the low-Ca diet was only sufficient to allow a corresponding increase of 0·06, and it is possible that the percentage net body Ca of the rats on the low-Ca diet may in fact have been less at some time between 3 and 24 weeks of age than it was at 3 weeks of age, as found by Lanford & Sherman (1938) for rats given 0·20 % Ca in the diet. In the present experiment the percentage net body Ca was higher at all times for rats given the higher level of dietary Ca and the increase for 51-week-old rats was 0·11, which is slightly higher than the corresponding value of 0·073 found by Lanford & Sherman (1938) for rats given diets containing 0·20 or 0·80 % Ca.

Since the total body Ca of rats given the high-Ca diet in Expt 1 was always significantly higher than that of litter-mates given the low level of Ca, in general agreement with Lanford & Sherman (1938), it was evident that there was a difference in the degree of mineralization and possibly also in the size of the skeletons of the two groups of rats that, at least in terms of total Ca, could be detected even in the 63-week-old animals.

The examination, in Expt 2, of various parts of the skeleton showed that, as has been found for other mammals (Mitchell, Hamilton, Steggarda & Bean, 1945; Benzie, Boyne, Dalgarno, Duckworth, Hill & Walker, 1955; Blair, Diack & MacPherson, 1963), different groups of bones in the rat may not be alike in their degree of mineralization. Thus the mean values for the percentage ash content of the 10-week-old bones ranged from 58·3 to 72·0 in rats given the high-Ca diet and from 47·7 to 67·6 in those given the low-Ca diet, the extreme values for both diets being those for the ends and shafts of long bones respectively. Increasing the level of Ca in the diet caused highly significant increases in the weights and percentage ash contents of the dry, fat-free bones, and the larger treatment responses, in terms of percentage ash content, occurred in the less well mineralized bones such as vertebras, pelvic girdle and the ends of long bones.

The rats in Expt 3 appeared healthy and grew satisfactorily, but here there was no significant effect of diet on live-weight gain, possibly because the rats were first examined after 24 weeks on the diets rather than after 21 weeks as in Expt 1. In the four groups of bones studied, both the weights of the dry, fat-free bones and their weights of ash increased with increasing age of the rats and, irrespective of diet, the largest increases in relation to the corresponding values for 3-week-old rats were consistently found for the vertebras and shafts of long bones. The percentage ash content of the dry, fat-free bones also increased with increasing age, but in this instance the increase was most marked for the ends of the long bones. The values for percentage ash content of the dry, fat-free shafts and ends of long bones of the 27- to 63-week-old rats were similar to those reported by Ellinger, Duckworth, Dalgarno & Quenouille (1952) for adult rats.

Increasing the level of dietary Ca increased the weight of the dry, fat-free bones and their ash content. Although the effect of diet in terms of bone weight and ash values progressively decreased as the length of time the rats were on the diet increased, it remained significant ($P < 0·1$) for all bone groups excepting the ends of long bones at 60 weeks and persisted most strongly in the skull and mandibles. The percentage Ca and P content of the bone ash was only very slightly affected by the level of Ca in the diet as was found by Blair & Benzie (1964) for pigs.

In order to compare the present results with those of Henry & Kon (1953), the percentage ash and Ca contents were first calculated for the whole, dry, fat-free long bones of the individual 51-week-old rats from the values already determined for the ends and the shafts of the bones. The mean differences between responses to high and low levels of dietary Ca were $1{\cdot}06 \pm 0{\cdot}183$ (4 df) and $0{\cdot}37 \pm 0{\cdot}061$ (4 df) for percentage ash and percentage Ca content respectively, and both were significant at the 1% level. The corresponding values found by Henry & Kon (1953) for the whole dry, fat-free right humerus and femur of 12-month-old rats were 0·8% ash and 0·43% Ca; these estimates were obtained for three pairs of litter-mates basically given high- or low-Ca diets that were almost identical with the corresponding diets used in the present experiment. The significant treatment differences now found in long bones of 51-week-old rats appear to dispute the observation of Henry & Kon (1953) that calcification was similar in the bones of 1-year-old rats, but it must be noted that the differences recorded in the two experiments are of the same order and that the estimates given by Henry & Kon derive from fewer rats, which may explain why these relatively small differences were not established as significant.

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