CLXX. THE MINERAL CONSTITUENTS OF BONE.

II. THE INFLUENCE OF AGE ON THE MINERAL CONSTITUENTS OF BONES FROM KITTENS AND PUPS.

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METHODS of bone analysis and sampling have been described in a previous paper [Burns & Henderson, 1935]. In this investigation changes in calcium, phosphate and carbonate of bone from kittens and pups were studied in relation to age. Dry fat-free calcified cartilage, cancellous bone and samples of cortex from the middle of the shaft and from the growing ends of the diaphyses were used.

Table I. *Calcium and carbonate in the cortex from the middle of the shaft (cats).*

Age (days) ...	0–6	7–13	14–20	21–48	49–84	160	Adult*	Adult*
No. of samples...	9	8	6	12	9	1	1	1
Ca %:								
Range	17·8–22·9	16·3–20·6	19·0–20·6	18·7–22·2	20·7–22·9	—	—	—
Mean	20·9	18·5	19·8	20·7	21·9	23·3	24·0	25·5
Standard deviation σ	1·5	1·37	0·60	—	—	—	—	—
CO_2 as % Ca:								
Range	9·4–12·3	9·5–11·0	8·6–9·6	9·0–10·7	9·5–10·7	—	—	—
Mean	10·6	10·0	9·1	10·0	10·0	10·9	11·5	12·9
σ	0·92	0·53	0·12	—	—	—	—	—

* Unknown breed.

From Table I it may be seen that the mean value for the calcium content of the dry fat-free cortex of the middle of the shaft of the femur fell slightly between the first and second weeks of post-natal life, and then rose very slowly over several months. When the carbonate content is expressed as that percentage of the calcium with which it can combine, it is evident that the carbonate also fell and then rose over the same periods, but that the carbonate fell more markedly than the calcium, so that its proportion to the latter also decreased. This decrease in the proportion of carbonate to calcium was moreover continued into the third week, and although the difference between the figures for the first and second weeks is of doubtful significance, there seems to be probably a real difference between the first and third weeks. After 21 days the proportion of carbonate to calcium slowly rose to reach its highest level in the adult animals. The change in calcium content and in the carbonate content relative to the calcium was also found in the pups (Table III).

The standard deviations for the calcium values for the groups 0–6 days, 7–13 days (Table I), are such that the difference between the means is not

statistically significant, but if the bones are re-grouped according to the texture of the cortex rather than age [Burns & Henderson, 1936], then the 3-day bone (Ca 17·8 %) falls into the second group and the 8-day bone (Ca 20·6 %) falls into the first group, and the means become 21·2 and 18·1 %, with standard deviations 1·07 and 1·12 respectively. Three pairs of samples of bone from litter-mates of the same age (two at 23 days, and one at 50 days) had calcium contents which agreed within 2 %, but a pair from day-old animals and another at 3 days gave figures which differed by 5–6 %. In these two pairs however the difference in calcium content was again accompanied by differences in size of bone and texture of cortex, the harder cortex or smaller bone (i.e. the bone in which post-natal change was least advanced) being in each case associated with the higher calcium content.

In view of the following facts it seems probable that the rapid post-natal growth and remodelling of the bone results in a decrease of the calcium content even of the cortex in the middle of the shaft: (1) In litter 1 when the change in bone texture was marked between birth and 3 days the Ca content fell from 20·7 to 17·8 % in this period. (2) In litter 7, between 3 and 10 days the content fell from 22·9 to 19·8 %. (3) In litter 4 and the pups the changes in bone texture between 1 and 8 and 1 and 24 days respectively were accompanied by a drop in Ca of 4 and 3 % (changes not significant in themselves but consistent in direction). (4) The difference in the Ca of the first two groups is just significant when they are classed by texture and not by age.

In nine animals femur and humerus were both examined, and differences in calcium content ranged from −5 to +8 % with an average of +2·2 % (the humerus being to this extent more calcified than the femur). In seven pairs of femora and tibiae the femora were more calcified than the tibiae by an average of 2·5 % (range of difference −8 to +6·5 %). There was thus no evidence of significant differences in the rates of calcification of the different bones of kittens and pups as found by Hammett [1925] for rats.

From the calcium, carbonate and phosphate of the bone was calculated the ratio of calcium combined with phosphate, called by Howland et al. [1926] the residual Ca/P (here called R (Ca)/P).

For forty-six samples of cortex from the middle of the shaft this ratio did not vary with age; twenty-three figures lay between 1·88 and 1·93, fifteen between 1·82 and 1·88, and eight between 1·74 and 1·82. No figure below 1·84 was found in animals over 45 days old, and the adults showed ratios of 1·88–1·91. For the dogs, the ratio ranged from 1·81 to 1·92, the sample from the adult animal and two samples from 60-day pups showing 1·88 and 1·90.

Table II. *Calcium and carbonate in the cortex from the ends of the diaphyses (dogs).*

Age (days) ...	0–6	7–13	14–20	21–49	160	Adult	Adult
No. of samples ...	2	6	6	8	2	2	2
Ca %:							
Range	16·8 and 17·8	16·0–17·8	17·8–19·6	17·2–19·0	18·6 and 19·4	20·7 and 21·7	23·1 and 24·9
Mean	17·3	16·9	18·5	18·1	19·0	21·2	24·0
σ	—	0·58	0·57	—	—	—	—
CO_2 as % Ca:							
Range	9·6 and 10·1	8·2–9·0	7·7–8·9	7·7–9·3	9·1 and 9·3	11·3 and 11·6	13·4 and 13·2
Mean	9·8	8·6	8·5	8·4	9·2	11·4	13·3

From Table II it may be concluded that the calcium content of the cortex from the growing ends of the diaphysis remained low throughout the growth period and then slowly increased approximately to that of the cortex from the

middle of the shaft in the adult animals. There was a slight increase just significant (i.e. difference exceeding 2σ) after the period of rapid post-natal growth. Throughout the growth period, the proportion of calcium combined with carbonate was always less in the cortex from the diaphyseal ends than in that from the middle of the shaft. The early period of growth was again accompanied by a drop in the carbonate relative to the calcium. In the adult the ratio of carbonate to calcium also approximated to that found in the cortex from the middle. In six animals in which femur, humerus and tibia were examined, the calcium of the cortex from the ends of the femur exceeded that of the humerus by an average of 2 % and that of the tibia by an average of 5 %, but differences equalling these were found between the two ends of the same bone, and no significant difference between the bones could be demonstrated.

Table III. *Calcium and carbonate in the cortex of pups' bones.*

	Middle					Embryos		Ends				
Age (days) ...	<1	24	60	160*	Adult†	Middle	Ends	<1	24	60	160*	Adult†
Ca %	21·7	20·9	22·4	24·5	25·8	16·9	12·8	17·2	18·4	19·6	17·7	23·3
CO_2 as % Ca	12·7	11·2	11·4	13·0	13·9	6·1	4·7	11·1	9·8	10·8	9·7	12·3

* 2nd litter. † Unknown breed.

The changes in the cortex from the ends of the diaphyses in the pup (Table III) were similar to the changes in the kitten, except that at 160 days both calcium and carbonate were relatively low. This was possibly a sampling error, since there was such a marked difference in shape between the long shaft and relatively small epiphysis of the 160-day bone and the much shorter bone at 60 days, that it was difficult to determine the proportion of the cortex to take for analysis.

R (Ca)/P was determined for twenty-eight samples of this tissue from cats and eight samples from dogs. Four figures from two adult cats ranged from 1·87 to 1·91. Of the remaining twenty-four, three lay between 1·88 and 1·95, fifteen between 1·82 and 1·88 and six between 1·74 and 1·82. These in no way varied with age. The figure for the adult dog was 1·87 and for the seven growing animals ranged from 1·81 to 1·91 without relation to age.

In the neo-natal period the cancellous tissue showed more marked changes in calcium content with age than did the cortex (Tables IV and V).

Table IV. *Calcium and carbonate contents of cancellous bone in cats.*

Age (days) ...	0–6	7–13	13–20	21–49	50–84	160	Adult	Adult
No. of samples ...	11	6	9	16	18	1	1	1
Ca %:								
Range	17·8–23·63	13·8–17·9	14·7–16·9	12·5–17·6	12·0–16·8	—	—	—
Mean	20·96	15·8	16·1	14·7	14·4	14·5	16·1	17·4
σ	1·5	1·2	0·71	—	—	—	—	—
CO_2 as % Ca:								
Range	9·3–11·7	8·2–10·2	7·5–9·4	6·7–9·6	7·7–10·0	—	—	—
Mean	10·3	9·1	8·4	8·4	8·9	8·3	10·3	12·6

The mean value for the calcium content of this tissue in the kittens fell between the first and second weeks from 20·9 to 15·8 %, a quite significant drop, the lowest value in the first week being found in the brittle 3-day bone, whilst the highest value in the second group was found in the hard 8-day bone. From the second week there was a further slight drop followed later by a rise, but the

figure for the adult animals did not exceed some figures found at all stages of the growth period. On the other hand whilst the initial drop was also found in the pups (Table V), the 160-day-old pup and the adult dog both showed significant increases in calcium content. It is not possible to decide whether this is a species difference, or whether the 160-day-old pup was better able to utilize the bones in the diet than the 160-day-old kitten.

Table V. *Calcium and carbonate content of cancellous bone and calcified cartilage in dogs.*

	Cancellous bone					Embryos		Calcified cartilage			
Age (days) ...	<1	24	60	160	Adult	Cancellous	Calcified cartilage	<1	24	60	160
Mean Ca %	22·2	18·7	17·3	22·1	22·1	15·7	8·5	20·3	17·4	19·7	19·2
CO_2 as % Ca	11·4	10·5	9·3	10·2	14·0	4·4	3·6	10·4	8·4	9·2	9·0

In both dogs and cats, the initial fall in the calcium of the cancellous bone was accompanied by an even greater fall in the carbonate content, and the carbonate remained low relative to the calcium until growth ceased, when it again rose towards the figure for adult bone as found in the cortex of the fully grown animal.

Differences up to 15 % were found between the calcium contents of the cancellous bone from the two ends of the same bone, and in the seven kittens in which femur, humerus and tibia were examined no differences exceeding this were found between the different bones, the average difference being of the same order as for the cortex.

Sixty-two determinations of phosphate were made on the tissue from cats and R (Ca)/P for the two adults was 1·87. Nine figures ranging from 1·88 to 1·94 were found at ages varying from 3 to 50 days. The other figures ranged from 1·68 to 1·87 and averaged 1·81, thirteen falling below 1·80. Out of the nine figures for dogs, four fell between 1·88 and 1·92, and others ranged from 1·81 to 1·87. The 160-day-old animal gave 1·87 and the adult 1·92.

The calcium and carbonate contents of the calcified cartilage from kittens of different ages are shown in Table VI and of pups in Table V.

Table VI. *Calcium and carbonate contents of the calcified cartilage of kittens.*

Age (days) ...	0–6	7–13	14–20	20–48	49–84	160
No. of samples ...	8	5	6	14	17	1
Ca %:						
Range	15·6–20·2	17·1–18·6	16·0–18·4	17·1–19·8	14·6–18·7	—
Mean	18·1	17·9	17·5	17·9	17·2	17·2
CO_2 as % Ca:						
Range	6·5–9·9	6·7–7·5	6·1–7·0	6·2–8·6	7·2–9·5	—
Mean	8·5	7·1	6·6	7·4	8·1	7·3

The figures for the one litter of pups (Table V) suggested that the post-natal drop in calcium was also to be found in the calcified cartilage but the more numerous figures for the kittens did not support this. In these animals the mean calcium remained roughly constant throughout the growth period. The post-natal drop in the proportion of carbonate to calcium was however found. In the pups this proportion was much the same as in the other rapidly growing tissues, i.e. the cancellous bone and the cortex from the ends of the diaphyses, but in the kittens it was consistently lower. In the kittens 50 estimations of R (Ca)/P were

made and 14 as high as 1·88 occurred at 1, 8, 11, 50 and 84 days. The other thirty-six figures lay between 1·71 and 1·87 and averaged 1·82. In the pups the figures ranged from 1·84 to 1·88 and averaged 1·85.

In the embryos, the calcium content and the proportion of carbonate to calcium in all the tissues were lower than the lowest figures found after birth, but again the proportion of carbonate was highest in the cortex from the middle of the shaft and lowest in the calcified cartilage. The figures for R (Ca)/P however, 1·76, 1·82, 1·85 and 1·88, were similar to figures from post-natal samples.

As was found previously for water, the results of the analyses of bones of kittens for calcium failed to indicate the systemic differences noted by Hammett [1925] for rats, so a femur and humerus from each of two animals were examined for water and ash by his methods, whilst the other femur and humerus were examined for Ca by the methods used in this investigation.

Table VII.

Kitten 1.

	Wt. g	Water %	Ash %	Ca % Cortex	Ca % Cancellous
F.	1·250	65·8	14·08	20·15	16·83
H.	1·388	65·3	14·91	21·07	17·63

Kitten 2.

	Wt. g	Water %	Ash %	Ca % Cortex	Ca % Cancellous
F.	1·402	65·9	13·89	20·6	16·85
H.	1·468	66·1	14·57	20·8	16·64

Water differences (Table VII) lay within the accuracy of the estimation (an accuracy determined mainly by the speed with which the bones were cleaned and weighed). The ash of the humerus exceeded that of the femur by 6 and 4 %, and the calcium of the dry cortex from the middle of the shaft of the humerus exceeded that of the femur by 4 and 1 % respectively.

It seems possible that the difference in ash content between the two bones of the rat may be associated with the different rates of growth of the bones in that animal. In the more slowly growing humerus, the more heavily calcified portions of the bone would form a greater proportion of the whole than in the more rapidly growing femur. In kittens this difference in the rate of growth is negligible, and differences in rate of ash deposition are also small.

Bauer et al. [1929] noticed a diminution in the number of trabeculae in kitten bones shortly after birth. They concluded from this that calcium is stored in the trabeculae and that this calcium store is drawn on during the period of rapid post-natal growth. From the change in calcium content in the different parts of the bone, and the complete destruction and rebuilding of the cortex which takes place during this period, it seems however that the trabeculae do not differ from cortical bone in this respect, at this stage. The proportion of cancellous bone to total bone in the young kittens decreased from 17 % in the new-born to 9 % at 16 days, but in view of the difficulty of securing all the cancellous bone the significance of these figures is uncertain. Any theory explaining the processes of ossification must take into account bone destruction as well as bone growth. Since both appear to take place side by side in the same bone, it seems that bone growth cannot be wholly controlled by changes in the systemic blood supply, but must be largely determined by purely local factors and that, at any rate as long as growth continues, bone is in dynamic equilibrium with its tissue fluids.

Consideration of the values for R (Ca)/P shows that out of the 186 samples from the bones of kittens, 138 (i.e. 76 %) gave ratios ranging from 1·82 to 1·95,

and averaged 1·87. Hammett [1925] found an amount of magnesium in the bones of rats equivalent to about 2–3 % of the calcium. A few estimations of magnesium were made in the course of this investigation, by colorimetric determination as phosphate after precipitation of calcium. Magnesium figures varied from 0·35 to 0·49 % with corresponding calcium 16·3 to 24·4 %. It was found that figures for R (Ca)/P 1·91, 1·78, 1·77, 1·84 and 1·86, became when corrected for magnesium 1·97, 1·85, 1·84, 1·90 and 1·92 and a total Ca/P (CO_2 not determined) was increased from 1·91 to 1·97. If therefore an average of 0·06 is added to R (Ca)/P as found to correct for magnesium, it is found that 138 out of the 186 lie between 1·88 and 2·01, and of these 87 lie between 1·91 and 1·97. Only 13 lie between 1·98 and 2·01, whilst 38 are between 1·88 and 1·91. This is roughly the distribution to be expected if the true figure is 1·94, the theoretical figure for tertiary calcium phosphate. There are however 48 figures (26 % of the total) with ratios below 1·88, which seem to fall outside the range of experimental error. Shear & Washburn [1932–33] have criticized the use of micro-methods in the analysis of bone and have developed a much more accurate technique, but for this are needed amounts of material much in excess of those available in a detailed study of young growing bone. They point out that with examinations made by the less accurate methods "deviations from the theoretical composition (i.e. $3Ca_3(PO_4)_2CaCO_3$) have usually been ascribed either to shortcomings of the analytical methods employed, or to experimental error. It is not impossible, however, that these discrepancies may have been due to actual variations in the composition of bone". The forty-eight samples in this study with corrected R (Ca)/P of less than 1·88 all occurred in bones from animals less than 60 days old, and they occurred most frequently in the most actively growing tissues; e.g. of the total samples they formed 35 % in the cancellous bone, 25 % in the calcified cartilage, 21 % in the cortex from the ends of the shaft and 17 % in the cortex from the middle of the shaft. This suggests that the actual period of rapid growth or destruction of bone may be marked by the presence in the bone of phosphate other than the tertiary calcium or magnesium salt. Shear & Kramer [1928] have adduced evidence to show that in ossification the secondary salt $CaHPO_4$ may be first deposited. The corrected ratios 1·74–1·87 found in the forty-eight samples would be compatible with a mixture of 10–20 % of the secondary salt and 80–90 % of the tertiary salt. Roseberry et al. [1931] showed (1) that there were none of the characteristic lines of crystalline $CaHPO_4$ in the X-ray spectrogram of fresh young bone, and (2) that a mixture of such bone and crystalline $CaHPO_4$ in the ratio 10:1 (i.e. bone salt:$CaHPO_4$ approximately 3:1) gave such lines quite clearly. They considered that if the secondary salt occurs, it is present in smaller proportion than this. It is not clear however whether their method would detect $CaHPO_4$ if it formed less than 25 % of the total salt. Since only a few samples of growing bone could contain as much as this (from R (Ca)/P) and samples from different parts of the same bone may differ considerably, it is clear that the detection of such small quantities of the secondary salt would be difficult.

The small amounts of non-tertiary phosphate indicated may moreover only be soluble phosphates accumulated locally during bone growth or destruction and would not therefore be detected by the physical methods. If such phosphate were not combined with calcium, it would only constitute from 2 to 6 % of the total, and its presence could only be finally demonstrated by its isolation. Almost all the samples examined for phosphate were analysed for both total and inorganic phosphate. No definite evidence of the presence of organically combined phosphate was found in the trichloroacetic acid filtrate.

Roseberry et al. [1931] also showed that (1) the main crystalline compound in the bone salt belonged to the apatite group and was very similar to dahlite ($2Ca_3(PO_4)_2CaCO_3$); (2) that bone salt contained no calcite (crystalline $CaCO_3$); (3) that tertiary calcium phosphate is a crystalline member of the apatite series. Kramer & Shear [1928] have already shown that old bone (diaphysis) contains more carbonate in proportion to calcium than does young bone (metaphysis). The results presented here show that the carbonate content of bone is roughly proportional to the rate at which that particular portion of bone is being laid down and removed. The relative carbonate content of calcified cartilage is low at all ages, that of the cancellous tissue and the rapidly growing cortex slightly higher, while the highest figure is found in the more slowly growing cortex of the middle of the shaft. When growth at the ends of the shaft has ceased, the carbonate content of the whole cortex and of the cancellous bone, in dogs and cats, tends to approximate to the figure found in dahlite (i.e. 14·2 % of the calcium combined with carbonate). Sendroy & Hastings [1926–27] showed that when serum and certain salt solutions were shaken with $CaCO_3$, no calcium was precipitated, but when shaken with $Ca_3(PO_4)_2$ calcium carbonate was precipitated. If at some stage in the growth cycle of bone tertiary calcium phosphate were in contact with fluids containing carbonate it seems probable that the salt would react to form a carbonato-phosphate complex, and that, up to the point at which all the salt was converted into a dahlite-like compound, the longer the phosphate was in contact with the fluid, the more compound salt would be formed. In the cortex of the middle of the shaft, where, after the post-natal spurt in growth, growth and destruction take place relatively slowly, the phosphate remains in contact with the tissue fluids of the bone long enough to maintain a high and increasing proportion of carbonate. In the rapidly removed calcified cartilage, on the other hand, the carbonate remains relatively low.

The very low carbonate contents of the bone tissues from the embryo pups suggest that the insoluble bone salt may be originally deposited as phosphate without the presence of any carbonato-complex. Further work is being carried out on embryos to test this point. But, within limits, the carbonate content of a bony tissue does seem to be a measure of the age of the tissue, and therefore a measure of bone growth in the region of the bone from which the tissue was taken.

SUMMARY.

1. Immediately after birth in pups and kittens there is a decrease in salt content of the diaphysis. This decrease is most marked in the cancellous tissue. This period must be avoided in any experiments designed to test a decalcifying or recalcifying agent.

2. Later the calcium content of the cortex rises slowly, but in the cancellous bone it displays irregularity and remains low in the kitten, while rising slowly in the pup.

3. In kittens, the difference between femur and humerus is very slight, and can probably be explained by the very slightly slower rate of growth in the latter.

4. In temporary tissues such as calcified cartilage, the carbonate is always low relative to the calcium, but its proportion increases in the tissues which are destroyed more slowly.

5. In the bone of the adult cat and dog, the proportion of carbonate to calcium approaches that of dahlite.

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