Studies on the Skeletal Tissues

5. THE INFLUENCE OF AGE UPON THE DEGREE OF CALCIFICATION AND THE INCORPORATION OF ^{32}P IN BONE*

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Earlier work has established that as animals grow the cortical part of the long bones becomes more calcified (for example, Strobino & Farr, 1949; Rogers, Weidmann & Parkinson, 1952; Tarján & Szöke, 1957) and the bones incorporate decreasing amounts of administered isotopic tracers (for example, Falkenheim, 1942–3; Bonner, 1948). It has also been made apparent that the examination of samples of powdered whole cortical bone from young or adult animals gives only an average value for the tissue which is made up of parts with widely differing properties (Amprino & Engström, 1952; Engfeldt, Engström & Zetterström, 1952; Rogers, Weidmann & Jones, 1953; Tomlin, Henry & Kon, 1953; Weidmann, 1956). Since these differences are expressed at a microscopic level, i.e. each Haversian system seems likely to have its own degree of calcification and rate of incorporation of tracer, logically each should be examined separately. Radioautographic and X-ray studies such as those by Leblond, Wilkinson, Bélanger & Robichon (1950), Engfeldt *et al.* (1952) and Tomlin *et al.* (1953) have provided a semi-quantitative picture of the variations at the microscopic level in calcification and incorporation of ^{32}P in both adult and growing animals. In the present work an attempt has been made to obtain precise measurements on restricted regions and types of bone and thus to compromise between methods which have exactly measured the properties of whole bone samples and those which have assessed incorporation of ^{32}P at the microscopic level by radioautographic techniques. The two species of animals, cats and rabbits, were chosen because earlier work (Rogers *et al.* 1953) had shown certain differences existed between them and because the young animals of both species were large enough to allow the proper handling and cleaning of the bones.

METHODS

Owing to the large number of experiments involved and to local conditions, animals of various litters and breeds had to be used. In order to minimize complications due to variations between the growth rate of different breeds, each age group was constituted of animals which had shown similar rates of growth. The groups of adult animals of both species were selected over a considerable period of time and consisted of apparently healthy animals of weight greater than 2 kg. The ages within this group are consequently likely to vary rather widely. However, as will be seen from the recorded results, this does not seem to lead to greater variability in the bone characteristics than within the more exactly defined groups. The standard of error of the means of the results from the adult animals are no greater than those from the other groups.

The methods of dissecting and cleaning bones have been previously described (Weidmann & Rogers, 1950) and have proved satisfactory for the removal of bone marrow from the small bones of young animals. The central parts of the shaft cortex of the femoral and tibial bones were used for the investigation of cortical bone, and the metaphyseal trabeculae from both bones for cancellous tissue. In one-day-old animals the entire shaft is still highly vascularized and has a trabecular appearance, so that no complete distinction is possible between cortical and cancellous bones. From the age of 5 days onwards the metaphysis and diaphysis could be distinguished and were separated. In very young animals, however, particularly in young cats, the centre part of the medullary shaft was still covered with trabecular spicules, which could not be removed and so were included along with the samples of cortical bone. In order to obtain adequate amounts for the various estimations, bone-powder samples from femur and tibia had to be pooled. Calcium, phosphorus and nitrogen estimations, together with radioactivity measurements, were made as previously described (Weidmann & Rogers, 1950; Rogers *et al.* 1953). The activities of the subperiostal and subendosteal layers were estimated by the paraffin-protection method described by Weidmann (1956). The administration of ^{32}P was adjusted to body weight and 33 μc/kg. was injected intravenously in rabbits or intraperitoneally in cats. The animals were always killed for the measurement of radioactivity in the bones 4 hr. after injection of the isotope. The specific activity was expressed, as in previous work (Rogers *et al.* 1953), by the fraction

$$\frac{(\text{Counts/min./mg. of P}) \times 10^{8}}{\text{Administered counts/min./kg. body wt.}}$$

* Part 4: Weidmann, S. M. (1956).

Table 1. *Chemical composition of cortical bones in growing rabbits*

Results are mean values ±S.E.M. and are given as a percentage of dry, defatted bone. The Ca/P ratios are means of ratios and not ratios of means.

Age (days)	Wt. (g.) Mean	Range	No. of animals	Ca (%)	P (%)	Ca/P	N (%)	Ca/N
Embryo	—	—	2	25·4±0·28	12·0±0·14	2·10±0·05	—	—
1	54	31–77	26	25·2±0·14	11·6±0·08	2·17±0·01	3·93±0·04	6·36±0·09
5	89	61–123	16	24·9±0·21	11·2±0·05	2·22±0·02	4·09±0·06	5·96±0·08
9	145	135–175	12	25·4±0·21	11·6±0·19	2·19±0·04	4·12±0·09	6·20±0·16
14	245	210–290	8	25·7±0·30	12·2±0·13	2·12±0·13	4·17±0·06	6·21±0·13
28	447	342–700	11	26·5±0·02	12·4±0·08	2·12±0·02	3·65±0·06	7·28±0·16
84	794	700–1040	14	27·8±0·23	12·8±0·11	2·18±0·02	3·36±0·05	8·25±0·16
Adults	3000	2250–4000	46	28·6±0·15	12·7±0·06	2·27±0·01	3·21±0·03	8·87±0·10

Table 2. *Chemical composition of cortical bones in growing cats*

Results represent mean values ±S.E.M. and are given as a percentage of dry, defatted bone. The Ca/P and Ca/N ratios are means of ratios and not ratios of means.

Age (days)	Wt. (g.) Mean	Range	No. of animals	Ca (%)	P (%)	Ca/P	N (%)	Ca/N
1	90	55–114	8	25·0±0·32	11·4±0·22	2·20±0·03	4·40±0·10	5·70±0·11
5–6	115	95–145	13	24·5±0·24	11·4±0·14	2·17±0·02	4·62±0·02	5·35±0·07
7–9	200	97–236	14	24·5±0·10	11·5±0·09	2·15±0·02	4·87±0·08	5·06±0·09
21	246	207–290	6	24·1±0·24	11·5±0·14	2·10±0·02	4·87±0·08	4·95±0·12
42	450	290–598	13	25·2±0·22	11·4±0·11	2·22±0·02	4·60±0·05	5·49±0·08
84	690	585–780	6	25·2±0·39	11·4±0·08	2·18±0·03	4·66±0·06	5·35±0·17
180	1450	1150–2000	9	26·3±0·13	11·8±0·08	2·22±0·01	4·34±0·07	6·01±0·15
Adults	3000	2100–3600	27	27·4±0·14	12·2±0·06	2·24±0·02	4·01±0·06	6·97±0·12

RESULTS

Variations in chemical composition

Cortical bone. The calcium content of the cortical bone of both the rabbit and cat increases during growth, but, since the phosphate concentration also increases, the calcium/phosphorus ratio varies only slightly. The maximum limits of variation are from 2·10 to 2·27 (Tables 1 and 2). The total increases in both calcium and phosphorus concentrations are greater in the rabbit than in the cat. These increases, however, do not start in the rabbit until it is 1 week old and in the cat until it is 3 weeks old. Until this time, in the cat, the calcium concentration decreases slightly, whereas the phosphorus remains constant and the nitrogen increases slightly; consequently the calcium/nitrogen ratio decreases considerably, and the calcium/phosphorus ratio also decreases slightly. There is possibly a similar initial decrease in the calcification of the cortical bones of young rabbits, but it is much smaller.

Cancellous bone. Much less change is observed in the calcium and nitrogen concentrations of the cancellous bones of the rabbit (Table 3). Thus the degree of calcification of this type of bone, as measured by the calcium/nitrogen ratio, does not change significantly throughout the life of the animals. In the cat (Table 4) the degree of calcification falls quite markedly throughout growth.

Variations in the incorporation of ^{32}P *during growth*

The specific activities of samples of whole cortical and cancellous bones show the expected marked decrease during growth in both the rabbit and the cat, but there are differences between the two species of animal (Tables 5 and 6).

Cortical bone. The specific activity of phosphorus in the cortical bone of the rabbit (Table 5) is greatest in newly born animals and falls continuously during growth. In the cat (Table 6) the specific activity is highest in the 7- to 9-day-old group of animals, exceeding that of newly born kittens. After this maximum has been reached, a fall is observed with increasing age; the gradient of decline is not initially as steep as in the rabbit.

Cancellous bone. Specific activity in the cancellous bones of both species is greater throughout life than in the cortex. It rises in the cancellous bones of the cat in the first weeks of life, as in the cortical bones, but the increase is somewhat greater. This phenomenon of a maximum in the degree of incorporation into both types of bone during life seems to be characteristic of the cat.

Table 3. *Chemical composition of cancellous bones in growing rabbits*

Results represent mean values ±S.E.M. and are given as a percentage of dry defatted bone. The Ca/N ratios are means of ratios and not ratios of means.

Age (days)	Wt. (g.) Mean	Wt. (g.) Range	No. of animals	Ca (%)	P (%)	Ca/P	N (%)	Ca/N
1	54	31–77	26	—	—	—	—	—
5	89	61–123	16	24·8±0·25	11·6±0·08	2·13±0·02	4·34±0·15	6·00±0·22
9	145	135–175	12	25·2±0·18	11·7±0·19	2·19±0·12	4·06±0·13	6·31±0·17
14	245	210–290	8	25·0±0·29	11·9±0·11	2·08±0·05	4·50±0·11	5·58±0·18
28	447	342–700	11	25·4±0·30	11·8±0·10	2·12±0·02	3·87±0·10	6·63±0·23
84	794	700–1040	14	25·5±0·17	11·6±0·10	2·19±0·02	4·41±0·08	5·75±0·13
Adults	3000	2250–4000	46	26·2±0·15	11·4±0·06	2·29±0·01	4·37±0·05	6·00±0·09

Table 4. *Chemical composition of cancellous bones in growing cats*

Results represent mean values ±S.E.M. The Ca/P and Ca/N results are means of ratios and not ratios of means. The results are given in terms of dry, defatted bone.

Age (days)	Wt. (g.) Mean	Wt. (g.) Range	No. of animals	Ca (%)	P (%)	Ca/P	N (%)	Ca/N
1	90	55–114	8	26·8±0·11	12·0±0·03	2·23±0·03	4·09±0·05	6·56±0·06
5–6	115	95–145	13	25·0±0·30	11·8±0·18	2·12±0·03	4·05±0·10	6·40±0·13
7–9	200	97–236	14	25·6±0·26	11·9±0·07	2·16±0·02	4·32±0·06	6·12±0·08
21	246	207–290	6	25·0±0·31	11·8±0·22	2·12±0·22	4·44±0·06	5·65±0·14
42	450	280–598	13	24·2±0·16	11·1±0·09	2·19±0·02	4·71±0·05	5·13±0·04
84	690	585–780	6	24·5±0·33	11·3±0·08	2·17±0·02	4·93±0·10	4·97±0·05
180	1450	1150–2000	9	24·6±0·27	11·3±0·23	2·17±0·01	4·94±0·09	4·91±0·11
Adults	3000	2100–3600	27	25·2±0·15	11·2±0·08	2·27±0·02	4·91±0·06	5·31±0·09

Table 5. *Specific activity of inorganic phosphorus in bones of growing rabbits 4 hr. after the injection of 33 μc/kg. body wt. of carrier-free* ^{32}P *as* $H_3{}^{32}PO_4$

Results represent mean values ±S.E.M. For definition of specific activity, see Methods.

Age (days)	Wt. (g.) Mean	Wt. (g.) Range	No. of animals	Specific activity: Cortical bone	Specific activity: Cancellous bone
1	54	31–77	22	682±46	—
5	97	72–123	11	602±50	1620±139
9	147	135–175	12	525±43	1044±95
14	239	210–290	10	484±34	1020±96
28	447	342–700	10	216±43	854±121
84	794	700–1040	9	115±19	735±101
Adults	2960	2250–4200	28	48±5	332±37

Table 6. *Specific activity of inorganic phosphorus in bones of growing cats 4 hr. after the injection of 33 μc/kg. body wt. of carrier-free* ^{32}P *as* $H_3{}^{32}PO_4$

Results represent mean values ±S.E.M. For definition of specific activity, see Methods.

Age (days)	Wt. (g.) Mean	Wt. (g.) Range	No. of animals	Specific activity: Cortical bone	Specific activity: Cancellous bone
1	90	55–114	8	396±44	660±57
5–6	115	95–145	10	389±46	507±71
7–9	194	97–236	17	618±42	1204±101
21	247	228–268	4	553±58	1403±245
42	458	280–598	17	330±23	1390±117
84	692	585–780	9	206±25	886±112
180	1420	1150–1700	11	81±9	627±121
Adults	2940	2100–3500	15	19±3	273±142

Incorporation of ^{32}P in various layers of cortical bone during growth

The salient points of the work of Tomlin *et al.* (1953) are confirmed by the present results, but some findings are different, as might be expected from the different experimental approach. A single administration of ^{32}P was used in this work and the animals were killed 4 hr. later, as compared with the prolonged intake of ^{45}Ca used by Tomlin *et al.* (1953); hence the present results are likely to stress rapid exchange of the phosphate instead of reflecting deposition over a long period of time, which was the interest of the other authors. Shaft bones from at least three animals of each age group were examined by the paraffin-protection method (Weidmann, 1956) and the specific activities of phosphorus determined in the subperiosteal and subendosteal layers, together with the average activity of the shaft.

In younger rabbits (Table 7) the activity of the phosphorus in the subperiosteal layer is greater than in the subendosteal layer. The subperiosteal layer remains comparatively active in all periods of growth and decreases only when the animal reaches maturity. In the subendosteal layer, however, the activity increases gradually during growth. When the animal reaches about one-third of its full development (84 days) tracer incorporation occurs at about equal rates on both surfaces of the tubular shaft; in maturity the incorporation is some five to six times as great on the surface next to the marrow. A comparison of the specific activities of the two surface layers with those of the average activities in growing and mature shafts shows that the whole bone is relatively active during growth, but that, in the fully formed shaft, the activity is almost completely restricted to the subperiosteal and subendosteal layers of the bone, and particularly to the latter.

In the growing cat examination of the regional activities of the developing diaphysis shows a similar picture to that in the rabbit (Table 8). During the first weeks of post-natal life the specific activity in the periosteal region is much higher than that next to the endosteum. The subperiosteal activity decreases rapidly and the difference between the developing and mature shaft is very large in this region. There is a gradual increase in the specific activity of the endosteal region during growth, reaching that of the periosteal region at the age of 84 days and surpassing it in the later phases of life. The overall picture presented by both species of animal is the same.

DISCUSSION

One aspect of this work is to emphasize species difference and localized effects in the biochemical behaviour of the skeleton during growth. For

Table 7. *Specific activity of inorganic phosphorus in various layers of cortical bone of growing rabbits 4 hr. after the injection of ^{32}P*

Results represent mean values ±S.E.M. For the definition of specific activity, see Methods.

Age (days)	Wt. (g.) Mean	Wt. (g.) Range	No. of animals	Specific activity: Cortical bone	Specific activity: Subperiosteal layer	Specific activity: Subendosteal layer
5	97	84–110	3	829±14	1580±127	565±108
9	156	135–175	3	538±72	2248±371	520±41
14	229	210–263	4	438±63	1357±422	942±252
28	398	334–472	3	335±29	2262±182	771±6
84	889	819–939	3	118±32	1257±135	1557±376
Adults	2732	2450–3140	9	46±3	654±133	3910±666

Table 8. *Specific activity of inorganic phosphorus in various layers of cortical bone of growing cats 4 hr. after the injection of ^{32}P*

Results represent mean values ±S.E.M. For the definition of specific activity, see Methods.

Age (days)	Wt. (g.) Mean	Wt. (g.) Range	No. of animals	Specific activity: Cortical bone	Specific activity: Subperiosteal layers	Specific activity: Endosteal layers
9	191	174–213	4	738±103	3598±890	990±205
21	245	228–260	4	553±58	1849±413	683±52
42	535	445–598	7	412±34	2597±290	1138±145
84	668	585–780	6	233±60	1654±273	1308±137
180	1407	1170–1625	6	80±11	1900±202	3472±549
Adults	2813	2345–3470	6	29±6	477±70	2180±313

example, the cancellous trabeculae from the metaphyseal regions of cat bones, far from becoming more calcified during growth, actually show a marked decrease in the ratio of calcium to total nitrogen content as the animals get older, a change which continues almost throughout life. The cortex, too, of the long bones in these animals starts to increase in degree of calcification only after the animals have reached about 10 % of their adult weight. During the first two- or three-fold weight increase the degree of calcification declines, as indicated by a reduction of the calcium/nitrogen ratio from 5·70 to 4·95. This conclusion is unlikely to be upset by the inclusion of some trabeculae into the cortical samples from the very young cats since the amount of trabecular bone is decreasing rapidly during the first fortnight of life. This should mean that there is a decreasing contamination of the cortical bone, which would tend to emphasize an increase rather than a decrease in degree of calcification. In the rabbit, on the other hand, the cancellous bones remain approximately constant in composition throughout growth, whereas the cortex starts to increase in calcification as soon as the newly born animals' weight has increased rather more than 50 %, i.e. when it has achieved only 3 % of its adult weight. The division of bones into cortex and trabeculae, which has allowed adequate samples to be obtained throughout growth, is still very crude, and no doubt much more striking differences could be shown by examining more closely localized samples. Even from this work, however, it appears that during growth of the skeleton local species-specific alterations occur in the relative rates of deposition of salts and collagen, over 95 % of the nitrogen of adult bone being due to collagen (Rogers *et al.* 1952; Eastoe & Eastoe, 1954; Tarján & Szöke, 1957) in all species of animal so far examined. Tarján & Szöke (1957) have found that there is also a much larger proportion of the total nitrogen due to water-soluble compounds in bones of young rats. It still seems likely, however, that most of the alteration in total nitrogen of bones is due to changes in collagen-like substances, since the water-soluble substances of Tarján & Szöke (1957) have a large content of hydroxyproline (private communication) and may represent some form of soluble collagen.

Burns & Henderson (1935) drew attention to the decrease in calcium content of the cortical and trabecular bones which takes place during the growth of young kittens and suggested (Burns & Henderson, 1936), as had Bauer, Aub & Albright (1929), that the calcified trabeculae may form a reserve to be drawn upon during subsequent growth. If indeed the decrease both in quantity of trabecular bone and in the degree of calcification of those trabeculae that remain indicates that their salts are being used as an endogenous source for the growing skeleton (i.e. as a reserve), then the increase in incorporation of ^{32}P which occurs in week-old kittens in both cortical and trabecular bone may be a further reflexion of the mobilization and translocation of phosphate. It would be of great interest to study the influence of nutritional and hormonal status on the composition and metabolism of the cancellous bones of young kittens.

In an attempt to obtain more information about the localized metabolic differences the rapid uptake of ^{32}P has been examined in the surface layers under the periosteum and endosteum from the bones of the growing animals. The specific activities in these two layers show changes which are large when compared with those of the whole bone. In both species the subperiosteal layer declines in activity, whereas the subendosteal layer reaches its greatest activity in the mature animal. It seemed possible that the declining activity of the periosteal layer was reflecting the reduction in rate of deposition of bone salts which necessarily occurs as the circumferential growth rate of bone declines. This interpretation, however, cannot be accepted without reserve since Leblond *et al.* (1950) also found a periosteal zone of active ^{32}P deposition in the shaft of long bones by radioautographic technique at as short a time interval as 5 min. after intravenous injection of the isotope into rats. It would seem that deposition is not likely to play a significant part in defining this layer in a time interval of 5 min. and may not do so in the longer time of 4 hr. used here. Possibly some such factor as better blood supply or the easier penetration of ions into freshly deposited bone-salt crystals in very young animals may be involved (Neuman & Neuman, 1953). Leblond *et al.* (1950) also found that there is an endosteal layer of bone salt, which rapidly incorporates isotope, at the end regions of the shaft where it is expanding into the epiphysis. They refer to these regions as the funnels. This observation, however, is unlikely to help in interpreting the present results since only the middle region of the shaft has been used and the regions of the funnels are excluded. It is conceivable that the high value for the incorporation of phosphate into the endosteal layer of adult bone is due to a good regional blood supply; but, if so, it is also true, as Weidmann (1956) points out, that some other factor must intervene to prevent rapid equilibration of this layer with the blood phosphate in the cat. In this animal he showed that the maximum value for the endosteal layer is reached only 4 hr. after the injection of ^{32}P, whereas it is reached within 30 min. in the rabbit. In both species the specific activity of the serum phosphate reached a maximum in 30 min. despite the different routes of administration of the isotope, which was given intravenously in the rabbit and intraperitoneally in the cat.

SUMMARY

1. The degree of calcification (calcium/nitrogen ratio) of cortical bones in rabbits increases from about the fifth day of life and in the cat from about the ninth day of life. From the birth of kittens until they are 9 days old there is a continuous reduction in the degree of calcification.

2. The calcification of the cancellous bone in rabbits remains approximately constant during the whole of growth, whereas in cats this bone becomes less calcified during growth.

3. The amount of ^{32}P incorporated during the 4 hr. following administration declines throughout the growth of rabbits in both cancellous and cortical bone. In cats there is a maximum incorporation in both types of bone at an age of about 14 days. This maximum is more marked in the cancellous bone.

4. In both cats and rabbits there is a very marked change during growth in the incorporation of ^{32}P in the subperiosteal and endosteal layers of bone. The incorporation of ^{32}P in the former layers declines throughout growth, whereas that in the latter layers reaches its maximum in the adult.

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Enzyme Systems in Marine Algae

2. TRANS-α-GLUCOSYLATION BY EXTRACTS OF *CLADOPHORA RUPESTRIS**

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Duncan, Manners & Ross (1956) reported that unfractionated extracts of four species of marine algae (*Cladophora rupestris*, *Laminaria digitata*, *Rhodymenia palmata* and *Ulva lactuca*) showed hydrolytic activity towards a number of carbohydrates, including maltose. In dilute aqueous solution (0·04 %) this sugar was completely hydrolysed to glucose, whereas in concentrated solution (7–17 %) the synthesis of oligosaccharides was observed. By contrast, incubation of an algal extract with a concentrated solution of glucose did not result in oligosaccharide synthesis. The enzymic reaction therefore involves the transfer of α-glucosyl residues to glucosaccharides and not the enzymic polymerization of glucose; in this respect, the algal enzyme systems differ from that in *Aspergillus niger* (strain NRRL 330), which can synthesize disaccharides from glucose (Peat, Whelan & Hinson, 1955).

In the present paper the characterization of the oligosaccharides synthesized from maltose by an extract of *Cladophora rupestris* is described, and the acceptor specificity of the trans-α-glucosylase system is discussed.

* The paper by Duncan, Manners & Ross (1956) is regarded as Part 1.

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