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Studies on the Skeletal Tissues

4. THE RENEWAL OF INORGANIC PHOSPHATE IN BONES OF VARIOUS SPECIES OF SMALL MAMMAL AS A FUNCTION OF TIME*

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The uptake of ^{32}P and ^{45}Ca by bones varies considerably with type, area and age of the tissue. Differences according to bone type (diaphysis, metaphysis, alveolus) are well established (Rogers & Weidmann, 1951); the faster rate of phosphorus renewal in *spongiosa* as compared with *compacta* was shown to be statistically significant in various species of small mammals (Rogers, Weidmann & Jones, 1953). Variations in the absorption of isotopes in the different areas of bone shaft were clearly demonstrated by radioautography (Amprino, 1952; Engfeldt, Engström & Zetterström, 1952) and also by measurements of radioactivity (Rogers *et al.* 1953). The age of bone greatly influences the rate of isotope incorporation. The uptake of minerals is much quicker in the developing bones of the young than in the mature bones of the adult animal (Leblond, Wilkinson, Bélanger & Robichon, 1950).

These variations were attributed to local structural differences in the bone tissue, such as differences in micro-structure (Amprino, 1952), crystal size (Hevesy, Levy & Rebbe, 1940), vascularity (Neuman & Riley, 1947), but also to differences in the chemical composition, e.g. degree of calcification (Hevesy *et al.* 1940; Amprino, 1952).

Isotope incorporation is comparatively rapid immediately after the administration of the tracer, and slows down markedly within a few hours. Hevesy *et al.* (1940) explained the initial phase as an ion-exchange reaction and the subsequent slower phase as a biological recrystallization. It was thought that the rate of reaction in the initial

phase decreased with the exhaustion of the easily exchangeable fraction of bone-salt crystallites, and that the second phase was entirely governed by the rate of recrystallization. Neuman & Neuman (1953) defined recrystallization more explicitly as an ionic-diffusion through the solid crystal. One of the main reasons for regarding the initial uptake as an ion-exchange absorption was that the reaction was too fast to be accounted for by new bone formation and could, therefore, only indicate that an equilibrium was quickly established between the exchangeable ions of the surface area of the bone and the blood minerals. Bauer (1954), however, explained the decrease in turnover rates of bone salt by the rapid fall of isotope concentration in the blood. Since bone and blood minerals are in a dynamic equilibrium, bone deposits formed in the early phase of the reaction, when the tracer concentration of the blood is still high, contain more isotope than those formed in the later stages.

Radioautographic pictures showed heavy concentrations of ^{32}P and ^{45}Ca on the outside surfaces of the shaft, and fewer deposits in the Haversian systems. The areas of high tracer concentration coincided with areas of low mineral-salt content. It was suggested (Amprino, 1952; Engfeldt *et al.* 1952) that the turnover rate is proportional to the relative amount of recently formed but not yet fully calcified bone. The radioautographic findings were later confirmed by radioactivity measurements in bone-powder samples taken from the various regions of the shaft bone of the rabbit (Rogers *et al.* 1953). Significant differences were found in the turnover rates of phosphorus between subperiosteal, 'middle' and endosteal layers. These

* Part 3: Rogers, Weidmann & Jones (1953).

investigations are continued in the present work, in which a more efficient method for the extraction of newly labelled compounds from bone has allowed a more accurate measurement of the turn-over rates. The various layers of the shaft cortex of the rabbit and cat are compared, because the examination of their whole shaft bones showed a remarkable difference in the rate of tracer uptake (Rogers *et al.* 1953).

METHODS

All samples of bone were taken from adult, healthy specimens of rabbits, cats and rats.

Bone. The preparation of bone samples has been reported previously (Weidmann & Rogers, 1950; Rogers *et al.* 1953). Carrier-free $H_3^{32}PO_4$ was administered intraperitoneally to the cat and the rat, but intravenously to the rabbit. Comparative measurements showed that, provided that the ^{32}P entered the circulation system, the route of administration made no difference to the specific activities of serum and bone phosphorus. In early rabbit experiments an almost complete loss of tracer occurred occasionally after intraperitoneal injection; consequently intravenous injection was adopted for all subsequent rabbit experiments. Measurements of radioactivity were carried out as described earlier (Rogers *et al.* 1953). The specific activity was expressed by the usual formula

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

in which the theoretical count for $33\mu C = 7.35 \times 10^7$ counts/min. was taken as administered count. The activity of the isotope solution was always measured before the injection into the animal.

Extraction of bone samples

A method for the differential extraction of highly active bone-salt fractions from bone fragments (*a*) and bone-powder samples (*b*) has been devised, with saturated $(NH_4)_2SO_4$ solutions as solvents. For the differential extraction of bone fragments, tubular pieces of 1 in. length were selected from the central sections of femur and tibia. The technique of bone-powder sampling from the endosteal, subperiosteal and 'middle' layers of the shaft bone has been described in a previous paper (Rogers *et al.* 1953).

(*a*) **Differential extraction of bone fragments (paraffin-protection method).** All bone surfaces not to be extracted were covered with paraffin wax. For instance, when the outside layer was extracted the transverse section was

covered with a thin layer of melted paraffin wax and the endosteal layer was protected by filling the marrow cavity entirely with wax; when the endosteal layer was examined, the outside surface and the transverse section were protected with a thin layer of wax. For the investigation of the 'middle' layer, shaft tubes of 1 in. length which were entirely covered with paraffin wax, were cut transversely into five smaller fractions to supply a sufficiently large transverse surface for the extraction. The middle layer includes, therefore, the entire cross-section of the shaft.

The bone fragments were shaken with 11 ml. of 90% saturated ammonium sulphate soln. for 1 hr. at room temperature, and samples of the solution were used for the estimations.

(*b*) **Differential extraction of bone powder.** Approximately 0.1 g. of bone powder was stirred vigorously with 3 ml. of saturated $(NH_4)_2SO_4$ soln. for 15 min. in the cold, and filtered through a small Büchner funnel. It was then washed twice with 3 ml. of saturated $(NH_4)_2SO_4$ soln. and three times with 3 ml. of distilled water. The solutions were combined and made up to 25 ml., and samples were taken for the phosphorus and radioactivity estimations. Phosphorus was estimated by a modification (Martland & Robison, 1926) of the Briggs method.

(*c*) **Repeated differential extraction of bone fragments.** In an attempt to determine the total uptake of ^{32}P in the two outside layers of cortical bone, the bone fragments were shaken four times as described above (*a*). As seen from Fig. 1, the specific activities decrease rapidly with each subsequent extraction, particularly in the short-term experiments. Hence, four successive extractions appear to be adequate to estimate all the labelled compounds in bone. By plotting the specific activities against the number of extractions, and extrapolating to an infinite number of extractions, an approximate value of the total ^{32}P content can be obtained through graphical estimation. The total ^{32}P uptake was used as an indicator of the amount of exchangeable phosphorus. Non-labelled phosphates, which were recently formed but not fully calcified, are dissolved simultaneously with the labelled compounds in saturated $(NH_4)_2SO_4$ solns. (Engfeldt *et al.* 1952). Phosphorus estimations showed that the amount of soluble phosphate decreases gradually in the four successive extractions. If the soluble phosphates represent the active fraction of bone minerals then the percentage exchangeable phosphorus can be calculated from the relationship:

$$\frac{\text{Total exchangeable P}}{\text{Extracted P}} = \frac{\text{Total } ^{32}P}{\text{Extracted } ^{32}P}$$

Exchangeable P (g.) is expressed as a percentage of total P in the bone.

Table 1. Comparison of specific activities in bones and bone parts of two groups of rabbits in different stages of development, measured 4 hr. after the injection of ^{32}P

Results represent mean values $\pm$ S.E.M. Figures in parentheses are the number of animals used. For definition of specific activities see Methods.

Phase of development	Range of weights (g.)	Average sample of whole bone		Differential extraction with paraffin wax-protection method from shaft bone (outer layer)
		Cortical	Cancellous	
Mature	1900-4200	46 $\pm$ 3 (29)	324 $\pm$ 33 (21)	565 $\pm$ 133 (9)
Growing	2000-3400	99 $\pm$ 8 (8)	821 $\pm$ 65 (8)	1844 $\pm$ 267 (6)

RESULTS

Turnover rate of phosphorus in bones of different species of animal

In a recent comparative study of fully grown rabbits, rats and cats, a relatively slow turnover rate of phosphorus was observed in the cortical bone of the cat (Rogers *et al.* 1953). It was stated then that the number of animals and the periods of observation were inadequate to define the species differences. In the present work more animals were used and the experimental period was extended to 30 days. The number of animals used to determine an experimental point varied between 3 and 44, and most results are based on at least six animals. Owing to the large variations in the mineral metabolism of bones between individual animals, representative mean values are difficult to establish. The specific activity of cortical and cancellous bones in some rabbits of normal adult weight was significantly above the average. In those cases the uptake of ^{32}P of the periosteal layer of the diaphysis was also rather high. The latter phenomenon is a characteristic of animals still in the process of growth (Weidmann & Rogers, unpublished work.) By comparing the figures obtained for immature with those for mature animals (Table 1) it will be seen that the probability that the two groups of animals belong to the same population is very small ($P < 0.01$). Such animals were, therefore, not included in the assessment of means for normal adults.

Table 2 shows that the uptake of phosphorus by bones of the rabbit and rat is very fast, which is in good agreement with many previous findings, and that uptake of phosphorus is comparatively slow in the cat. This is particularly evident during the first hours following the injection of the tracer. After 0.5 hr., about half of the ultimate total of tracer has been incorporated in the cortical bone of the rabbit and the rat, whereas the cat contains only about one-sixth of the maximum amount. As the tracer concentrations increase with time, the species differences diminish. It takes, however, several days in the cat to reach levels similar to those found in the rabbit and rat 4 hr. after the injection. The scatter of results is very large in long-term experiments; the high ^{32}P concentrations in the cortical bones of the rabbit and rat at the 30 days' interval are, therefore, difficult to appraise, and it is consequently uncertain whether the total uptake of ^{32}P in these species is in fact larger than in the cat.

The cancellous bones of all three species contain six to seven times more ^{32}P than the cortical bones; the rate of tracer incorporation is also faster. Species differences are similar to those found in the cortical bones. The initial reaction in cancellous

Table 2. *Specific activity of inorganic phosphorus in bones and serum of various animals, measured at different time intervals after the injection of $33 \mu\text{C/kg. of body wt. of carrier-free } ^{32}\text{P as H}_3^{32}\text{PO}_4$*

Results represent mean values $\pm$ s.e.m. Figures in parentheses are the number of animals used. For definition of specific activity see Methods.

Time	Cortical bone			Cancellous bone			Serum*		
	Rabbit	Cat	Rat	Rabbit	Cat	Rat	Rabbit	Cat	Rat
10 min.	6 $\pm$ 1 (3)	—	19 $\pm$ 1 (5)	—	—	—	134 200	127 000	100 000
0.5 hr.	34 $\pm$ 4 (11)	7 $\pm$ 1 (6)	28 $\pm$ 3 (6)	306 $\pm$ 61 (7)	64 $\pm$ 6 (5)	—	149 000	151 000	82 000
4 hr.	46 $\pm$ 3 (29)	29 $\pm$ 4 (18)	65 $\pm$ 3 (44)	324 $\pm$ 34 (21)	286 $\pm$ 47 (15)	373 $\pm$ 18 (42)	21 900	33 600	10 600
1 day	45 $\pm$ 12 (4)	—	43 $\pm$ 4 (3)	315 $\pm$ 100 (3)	—	—	7 840	12 600	2 300
9 days	61 $\pm$ 10 (13)	50 $\pm$ 12 (7)	45 $\pm$ 4 (5)	534 $\pm$ 113 (8)	466 $\pm$ 91 (5)	—	1 980	2 800	1 100
30 days	85 $\pm$ 12 (10)	50 $\pm$ 14 (8)	94 $\pm$ 14 (8)	469 $\pm$ 140 (11)	488 $\pm$ 105 (7)	204 $\pm$ 47 (7)	982	800	2 550

* Variation of s.e.m. $\approx$ 11 %.

bones is again comparatively slow in the cat, proving that the slow rate of mineral-salt renewal is a general characteristic of cat bones.

The specific activity of the cortical bones, after reaching a maximum, does not decrease in the course of 30 days in any of the three species investigated. No losses of isotope were observed either in the cancellous bones of the rabbit or of the cat. The only decrease of isotope concentration occurred in the trabeculae of the rat, which lost almost half their original uptake. This is most likely due to an extensive resorption of these bones, whose mineral salt is utilized for skeletal growth. In contrast to the cat and the rabbit skeletal growth does not cease in the adult rat (Singer, Armstrong & Premer, 1952). The present results confirm, therefore, the irreversible incorporation of ^{32}P , and this is true for the cancellous bones, which are most active biologically, as well as for the cortical bones. According to Neuman & Mulryan (1952) the apparent lack of equilibrium between bone and plasma minerals is due to a recrystallization of bone-salt crystallites, whereby the isotope is rendered unavailable for exchange reactions.

The specific activity of serum decreases rapidly in all three species, the rate being greatest in the rat and least in the cat, whereas the rabbit occupies a middle position. There appears to be a direct connexion between the decrease of tracer concentration in the serum and the isotope incorporation into the bones, particularly so in the period between 0 and 4 hr. In the cat, where the tracer disappears most slowly from the serum, the uptake of ^{32}P by the bones is also slowest, whereas both processes are quickest in the rat. A comparatively high specific activity was observed in the serum of the rat at the 30 days' interval which is, presumably, due to the partial dissolution of the labelled trabeculae discussed above.

Uptake of phosphorus into different parts of long bones

The problem whether inorganic phosphorus of serum or other compounds, such as organic phos-

phate esters, react directly with bone phosphorus, is of considerable interest. Intermediate reactants should be recognizable by their comparatively high specific activities. In an attempt to obtain information about possible precursors, samples of bone powder were extracted with chemically inert salt solutions. Various salt solutions were tested, and sodium acetate buffers (pH 5-6) gave the best results. Since the completion of these experiments, Zetterström (1952), with saturated ammonium sulphate solns. as solvents, succeeded in extracting fairly active fractions from bones of rabbits and rats. Ammonium sulphate is a very good separating agent, probably because of its acid reaction (pH of saturated ammonium sulphate is 3.8), and the capacity of the sulphate ion to form a comparatively insoluble calcium salt, thereby replacing the phosphate. External conditions, such as time and intensity of agitation, or temperature of the solution, have, within reasonable limits, very little effect on the extraction. A modified version of the technique of Zetterström (1952) was applied in these experiments.

Differential extraction of bone fragments. In preliminary experiments the cortex of bone shaft was subdivided into outer, 'middle' and inner layers by the usual procedure (Rogers *et al.* 1953) and the bone-powder samples were subjected to differential extraction. The arbitrary separation of the shaft into three layers by means of a dental burr can never be wholly satisfactory, however thin the burred-off layer may be; some fraction of the less-active middle area will always be contained in the endosteal and periosteal samples. By using bone fragments for the differential extraction with the paraffin wax-protection method highly active fractions were obtained, particularly from the endosteal area, the specific activity of which was occasionally 200 times that of the bone average. The differential extraction by the paraffin wax-protection method seems to select the newly labelled compounds very efficiently.

Table 3 contains the specific activities of rabbit bones and bone parts. The three areas of the shaft

Table 3. *Comparison of the specific activities of inorganic phosphorus in bones of the rabbit obtained by various methods*

Results represent the mean values $\pm$ S.E.M. Figures in parentheses are the number of animals used. The figures for middle and inner layers are the same as for the outer layer. For definition of specific activities see Methods.

Time	TCA-extract of shaft (average) (1)	TCA-extract of bone powder from various layers of the shaft			Differential extract of the various layers of bone shaft			TCA-extract of metaphysis (average) (8)	Differential extract of bone powder from metaphysis (9)
		Outer (2)	Middle (3)	Inner (4)	Outer (5)	Middle (6)	Inner (7)		
0.5 hr.	34	41 $\pm$ 5 (7)	33 $\pm$ 5	182 $\pm$ 42	854 $\pm$ 178 (4)	141 $\pm$ 23	5050 $\pm$ 1030	306	2663 $\pm$ 689 (7)
4 hr.	46	43 $\pm$ 4 (8)	24 $\pm$ 3	141 $\pm$ 53	654 $\pm$ 133 (9)	149 $\pm$ 30	3910 $\pm$ 666	324	2100 $\pm$ 376 (7)
1 day	45	51 $\pm$ 7 (3)	26 $\pm$ 2	117 $\pm$ 18	504 $\pm$ 165 (3)	63 $\pm$ 7	1254 $\pm$ 281	315	855 $\pm$ 239 (3)
9 days	61	66 $\pm$ 17 (6)	29 $\pm$ 4	116 $\pm$ 14	242 $\pm$ 46 (3)	106 $\pm$ 21	803 $\pm$ 60	534	693 $\pm$ 104 (8)
30 days	85	103 $\pm$ 10 (10)	38 $\pm$ 4	123 $\pm$ 17	972 $\pm$ 111 (5)	148 $\pm$ 28	892 $\pm$ 127	469	610 $\pm$ 40 (11)

were examined by differential extraction of bone fragments, as well as by dissolving bone powder samples in 20% trichloroacetic acid (TCA). By presenting these two sets of results side by side the appraisal of turnover reactions is facilitated. A comparison of the specific activities in the differential extracts of the three layers with that of the shaft average (first column) shows that the latter conveys a wholly inadequate picture of the complexity of turnover reactions. The process of labelling varies distinctly with the area of bone, especially in the first hours after the injection; e.g. the ratio of specific activities at the 0.5 hr. interval varies between 6:1:36 for the outer:middle:inner layers (Table 3, columns 5-7). The endosteal area has a very fast rate of tracer uptake which was first demonstrated by the present technique. In the rabbit endosteum maximum uptake is reached almost immediately after the administration of the

total ^{32}P concentration. Hence, the fall in the specific activities of P in ammonium sulphate extracts is also due to decreased solubility of the newly labelled phosphates. The specific activities of the cancellous bones are relatively lower than those of the endosteal area of the shaft, which, considering the high biological activity of the cancellous bones, is rather surprising. The two sets of results are, however, not strictly comparable because the former were obtained by differential extraction of bone-powder samples and the latter from bone fragments. In powders there is an appreciable co-dissolution of non-labelled phosphates, which naturally reduces the specific activity. This does not occur to the same extent in bone fragments, where a solid surface is exposed to the solvent action.

The results of the examination of cat bones are contained in Table 4. A comparison with Table 3

Table 4. *Comparison of the specific activities of inorganic phosphorus in bones of the cat obtained by various methods*

Results represent mean values $\pm$ S.E.M. Figures in parentheses are the numbers of animals used. The figures for middle and inner layers are the same as for the outer layer. For definition of specific activities see Methods.

Time	TCA-extract of shaft (average)	TCA-extract of bone powder from various layers of the shaft			Differential extract of the various layers of bone shaft			TCA-extract of metaphysis (average)	Differential extract of bone powder from metaphysis
		Outer	Middle	Inner	Outer	Middle	Inner		
0.5 hr.	7	8 $\pm$ 1 (5)	8 $\pm$ 2	18 $\pm$ 3	179 $\pm$ 38 (5)	32 $\pm$ 5	778 $\pm$ 185	72	550 $\pm$ 96 (7)
4 hr.	24	25 $\pm$ 6 (7)	16 $\pm$ 5	48 $\pm$ 8	477 $\pm$ 70 (6)	80 $\pm$ 14	2180 $\pm$ 313	284	2160 $\pm$ 310 (10)
9 days	50	54 $\pm$ 18 (3)	32 $\pm$ 10	72 $\pm$ 20	—	—	—	466	803 $\pm$ 190 (3)
30 days	50	81 $\pm$ 23 (7)	43 $\pm$ 8	104 $\pm$ 35	186 $\pm$ 8 (3)	67 $\pm$ 13	268 $\pm$ 81	488	581 $\pm$ 100 (5)

tracer, followed by a sharp decrease of activity. It could be assumed that this decrease in activity is exclusively due to rapid bone resorption in the endosteal layer. This possibility is ruled out, however, by the fact that at the 30 days' interval the percentage decrease of specific activity in the differential extract is 82% (Table 3, column 7) as compared with the loss of only 32% (Table 3, column 4) in the total tracer concentration. The decrease in the specific activity in the differential extract is therefore mainly due to the reduced solubility of labelled phosphates.

Predominance of the endosteal activity is a characteristic feature in the process of bone renewal of the adult animal. In single-injection experiments the amount of tracer uptake is much bigger in the endosteum than in the periosteum. Another important difference between the two layers is that the tracer concentration falls in the inner area with time, whilst it increases in the outer layer.

The tracer-uptake in the metaphyseal trabeculae of the rabbit is as fast as in the endosteum of the diaphysis. The isotope concentration decreases sharply in the differential extracts after the 0.5 hr. interval, but no concomitant losses occur in the

shows that, generally, much more active fractions can be extracted from the rabbit than from the cat. The comparatively slow onset of bone-salt renewal in the cat is very clearly demonstrated with the paraffin wax-protection method. At the 0.5 hr. interval all three layers of the bone shaft and the cancellous bones of the cat contain only a fraction of the tracer amount present in the rabbit at the same time interval. Another interesting difference between the two species is the increase of the phosphorus uptake in the cat between 0.5 and 4 hr., as opposed to the decrease in the rabbit. A comparison of the TCA-extracts from bone-powder samples of the three layers of the two species also shows that in the cat the process of labelling progresses at a much slower rate than in the rabbit. The changes in specific activity can be explained in the same way as in the rabbit.

Influence of time upon the solubility of newly labelled compounds

The decrease of solubility of the labelled phosphates was explained by Amprino (1952) and Zetterström (1952) as a progressive mineralization of fresh bone-salt deposits. This process can be

conveniently measured by comparing specific activities in the differential extracts of bone-powder samples with those of the TCA-extracts of the residue.

Cancellous bone. Table 5 shows that the tracer distribution becomes rapidly uniform throughout the trabecular spicules of the rabbit. In the early phases of the reaction there are large differences between the specific activities of the easily soluble,

reaction affects the entire mass of trabecular spicules, and that these bones are renewed within the remarkably short time of 9 days.

The processes are largely the same in the cat (Table 5), but the rate of transformation of new mineral deposits into less-soluble compounds is markedly slower, uniformity being reached only after 30 days. This is further evidence for the comparatively slow bone-salt renewal in the cat.

Table 5. *Influence of time upon the solubility of labelled phosphorus compounds in the trabecular spicules of the metaphyses of rabbits and cats*

Results are expressed as specific activity and represent mean values $\pm$ S.E.M. For definition of specific activity see Methods. Figures in parentheses are the number of animals used. The figures for differential extraction are the same as for whole bone.

Time	Rabbit			Cat		
	TCA-extract of whole bone (average)	Differential extraction of bone-powder samples		TCA-extract of whole bone (average)	Differential extraction of bone-powder samples	
		(NH ₄) ₂ SO ₄ fraction	TCA-extract of residue		(NH ₄) ₂ SO ₄ fraction	TCA-extract of residue
0.5 hr.	306 (7)	2663 $\pm$ 689	135 $\pm$ 16	64 (5)	526 $\pm$ 96	56 $\pm$ 6
4 hr.	342 (9)	1104 $\pm$ 331	114 $\pm$ 15	226 (7)	2042 $\pm$ 317	197 $\pm$ 69
1 day	315 (3)	855 $\pm$ 239	257 $\pm$ 89	—	—	—
9 days	534 (8)	693 $\pm$ 104	607 $\pm$ 146	477 (3)	803 $\pm$ 190	470 $\pm$ 116
30 days	477 (11)	601 $\pm$ 40	501 $\pm$ 51	512 (5)	581 $\pm$ 100	465 $\pm$ 105

Table 6. *Influence of time upon the solubility of labelled phosphorus compounds in the various layers of cortical bone of adult rabbits*

Results are expressed as specific activity and represent mean values $\pm$ S.E.M. Figures in parentheses are the number of animals used. The figures for differential extraction are the same as for whole bone.

Time	Outer layer			Inner layer		
	TCA-extract of whole layer (average)	Differential extraction of bone-powder samples		TCA-extract of whole layer (average)	Differential extraction of bone-powder samples	
		(NH ₄) ₂ SO ₄ fraction	TCA-extract of residue		(NH ₄) ₂ SO ₄ fraction	TCA-extract of residue
0.5 hr.	41 (5)	159 $\pm$ 27	24 $\pm$ 3	152	678 $\pm$ 197	61 $\pm$ 7
4 hr.	54 (7)	172 $\pm$ 17	29 $\pm$ 5	160	572 $\pm$ 209	77 $\pm$ 18
1 day	51 (3)	248 $\pm$ 31	44 $\pm$ 7	117	381 $\pm$ 64	117 $\pm$ 32
9 days	122 (8)	278 $\pm$ 73	110 $\pm$ 38	160	327 $\pm$ 17	103 $\pm$ 21
30 days	163 (8)	285 $\pm$ 48	174 $\pm$ 51	164	301 $\pm$ 22	180 $\pm$ 23

recently labelled compounds in the differential extracts and the old-established bone-salts in the TCA-solute of the residue, but these differences have already decreased 24 hr. after the injection. The fall in the specific activities in the differential extracts is not due to an overall loss of radioactivity, as shown by the almost constant radioactivity of the TCA-extracts of whole bones, but is caused by a transformation of recent deposits into less-soluble compounds, as seen from the increasing specific activities in the residues. In the rabbit the differences are completely absent 9 days after the injection of the isotope, as indicated by the similar specific activities of total bone solute, ammonium sulphate fraction and residue. This implies that the

Cortical bone. Bone-powder samples of the two outside layers of rabbit shaft were examined by differential extraction, and a somewhat different picture from the cancellous bones was obtained (Table 6). The specific activity in the ammonium sulphate extracts of the endosteum decreases during the course of 30 days, but it always remains distinctly above that of the residue, which means that the process of renewal is not completed within 30 days in the endosteum. The explanation for the changes in solubility may be the same as those given for similar changes in the cancellous bones.

In contrast to the endosteum, the total isotope concentration increases in the periosteum. Since the specific activities of both fractions, ammonium

sulphate and residue, and the total ^{32}P content increase simultaneously, it is quite obvious that, apart from a gradually decreasing solubility of the first deposits, an additional incorporation of ^{32}P takes place in the periosteum in the later phases. Thus additional evidence was obtained for the interesting difference in the biological activity between the two surface areas of the shaft.

Total ^{32}P -uptake by the two outside layers of bone shaft and its relationship to the percentage of exchangeable phosphorus

As seen from Fig. 1, the incorporated ^{32}P can be extracted almost completely by repeated differential extractions, particularly if the deposits are of recent origin. From 0.5- and 4-hr.-old deposits the first elution removes approximately 75 % of the incorporated ^{32}P . The extraction is less effective as the labelled phosphates in bone become older; from 30-day-old deposits the first elution removes only

Fig. 1. Repeated differential extractions from the inner and outer layers of the cortical bone of the rabbit at different time intervals after the injection of radioactive phosphate. Shaded areas represent the average specific activity of the layer.

about 30 % of the total ^{32}P present in bone. When the ^{32}P -uptake is used as an indicator, it is possible to calculate the percentage of exchangeable phosphorus. The calculation is based on the fact that non-labelled phosphates are dissolved together with ^{32}P -compounds, and that these soluble phosphates are regarded as corresponding to the exchangeable part of bone salts (see Methods). The values for exchangeable phosphorus contained in Table 7 represent only part of the total active phosphorus in bone shaft because the 'middle' area is not included. Since the bulk of ^{32}P is concentrated in the two outside layers, the sum of exchangeable phosphorus in the two outside layers (columns 3 and 6) forms the major fraction of the active bone salts.

The percentage of exchangeable phosphorus varies between 3.29 and 4.73 in the rabbit, and from 2.24 to 4.41 in the cat when measured at different time intervals after the administration of the tracer. Since the active fraction of bone salt is a constant value, the true value lies, presumably, between the two figures. An assessment of the active phosphates is rendered difficult owing to the fact that the ammonium sulphate extract contains bone salts of different solubilities (Zetterström, 1952), and consequently of differing exchangeabilities. The ^{32}P reacts in the first place with the most reactive fraction of bone minerals, and thus, by calculating the exchangeable phosphorus in relation to the ^{32}P , present at the 0.5 hr. interval, the less-active fractions are not taken into consideration, i.e. the 0.5 hr. results are probably too low. On the other hand, the values calculated from the 30-day-old ^{32}P deposits are too large, because part of the labelled phosphates have already become insoluble and can no longer be accounted for as easily exchangeable phosphorus. In view of these limitations of the method, the results are in satisfactory agreement with the figure (5 %) quoted by Neuman & Weikel (1954) for the exchangeable pool.

The active fraction is higher in the endosteum than in the periosteum in both species. On both surfaces the rabbit shows a higher percentage of exchangeable phosphorus than the cat.

Table 7. *Percentage of exchangeable phosphorus in the two outside layers of cortical bone of the rabbit and cat at different time intervals after the injection of radioactive phosphate*

Results are expressed as percentage of total phosphorus in shaft bone.

Time	Rabbit			Cat		
	Subperiosteal layer	Endosteal layer	Sum in both layers	Subperiosteal layer	Endosteal layer	Sum in both layers
0.5 hr.	1.58	1.71	3.29	0.92	1.32	2.24
4 hr.	2.02	2.27	4.29	0.68	0.66	1.34
30 days	2.15	2.58	4.73	2.02	2.09	4.11

DISCUSSION

In mature animals, where no growth occurs, ionic-exchange, re-crystallization and Haversian replacement were suggested as the only mechanisms regulating the mineral-salt uptake (Neuman & Weikel, 1954). A rapid initial tracer uptake occurs in the diaphyseal and metaphyseal parts of the bone shaft, which confirms previous findings. This is, however, not a general phenomenon because the initial uptake in the cat is relatively slow. The main distinguishing feature between the cat and the two other species is not in the extent, but in the rate, of renewal. Hence, the differences cannot be explained by variations in the amount of exchangeable bone-salt crystallites. The slow incorporation of ^{32}P into the bones of the cat in the first phase of the reaction is not due to lack of available phosphate, because the specific activity of the serum inorganic phosphate is not lower than that of the two other species. Turnover rates of phosphorus were also compared in the bone marrow of the cat and the rabbit. The acid-soluble phosphate compounds of bone marrow are more slowly renewed in the cat, but the differences between the two species are too small to account for the large differences in bone metabolism (unpublished results). A slow phosphorus turnover is, however, not a general characteristic of the cat; the turnover rates of the phosphorus compounds in the liver, for instance, are above those of the rabbit (unpublished results). It appears, therefore, that a local factor at the site of ossification is responsible for the species differences in bone-salt renewal.

By applying an improved technique for the separation of labelled phosphates from old-established bone, much new information was obtained about the mode of bone-salt renewal in the adult animal. Turnover reactions are far better demonstrated when, instead of the TCA-extracts from average samples of whole bone, differential extracts of bone parts are used for the measurement of radioactivity. The species differences are biggest in the areas most active biologically: the endosteal surface of bone shaft and the trabeculae of the metaphysis. In the past, quantitative differences in the tracer incorporation between cancellous and cortical bones were explained, with some justification, by structural variations between the two types of bone. The same reasoning is, however, hardly applicable to the differences found in the corresponding bones of the two species. It is particularly difficult to see how surface factors could cause such large differences in the rate of renewal, as observed in corresponding bone areas of the cat and the rabbit.

Apart from the much bigger initial ^{32}P -uptake, the progress of labelling differs distinctly in the

rabbit and cat. In the cat the quickest uptake occurs several hours after the injection of the tracer, and is then four to five times larger than in the first 0.5 hr. This is not compatible with the view that ionic-exchange absorption in the easily exchangeable fraction accounts for the fast initial phase of the reaction. The delayed uptake in the cat could be due to a comparatively slow diffusion across membrane barriers, or to a low turnover rate of an organic-phosphate precursor, but there is no evidence for this.

When the rate of the initial reaction is high, the solubility of the labelled compounds also decreases more rapidly. Considering that subsequent ^{31}P -deposits render the labelled compounds more insoluble, there is no need to assume different reaction mechanisms for initial uptake and re-crystallization. The high turnover rate of the endosteal area of the shaft and the cancellous bones is due, not to a quicker re-crystallization, but to a more efficient biological functioning which regulates both the initial uptake and the decrease in solubility by the same mechanism. Accretion of bone salt (Haversian replacement) is in principle not different from these reactions; where the initial reaction is quicker more bone salt will be deposited eventually.

The increasing insolubility of labelled phosphates is best demonstrated by repeated differential extractions, carried out at different time intervals after the administration of the tracer. Two reasons could account for the change in solubility: (a) the isotopes migrate into the deeper strata of crystals; (b) a progressive mineralization of the newly deposited bone salt takes place, whereby the highly labelled compounds become buried under layers of non-labelled phosphates. The fact that the tracer distribution rapidly reaches uniformity in the cancellous bones favours the second explanation. It is not likely that the speed of any type of ion-migration is such as to advance throughout the whole thickness of a trabecular spicule within a few days, as was found in the rabbit. The rapid total renewal of the cancellous bones is, presumably, partly due to the specific lamellar arrangement in the microstructure (Amprino & Engström, 1952) which affords an easy access of the body fluids to the bone-salt crystallites within the bone. The surfaces of the crystal units are quickly labelled, followed by equally quick ^{31}P deposition, which decreases the solubility of the newly labelled compounds uniformly throughout the spicule. Owing to the dynamic equilibrium between bone and blood minerals, equal amounts of bone salt are dissolved as deposited, but losses of ^{32}P are not observed because of the large $^{31}\text{P}/^{32}\text{P}$ ratio. Favourable structural arrangements may explain the quicker rate of renewal and the deeper range of

reaction in cancellous as compared with cortical bones, where the reaction is restricted to a few surface layers, but it does not explain the difference in the rate of renewal between the cancellous bones of the rabbit and cat. There must be other factors, apart from structural ones, on which the rate of mineral salt turnover depends.

Bone-salt renewal occurs in mature shaft bones mainly on the marrow side, i.e. in the same area where resorption is known to take place. Whether the two processes are connected with each other, e.g. through strain and stress, cannot be decided from the present experiments. But the close vicinity of such opposite processes rather suggests a cellular regulation (Newton, 1939).

SUMMARY

1. The rate of tracer uptake in long bones varies according to species; the incorporation of ^{32}P is considerably slower in the cat than in the rabbit or rat.

2. No decrease of tracer concentration occurs in the cortical parts of the long bones of the rabbit, cat or rat, or in the cancellous parts of the two former species in the course of 30 days. The decrease of the specific activity of phosphorus in the cancellous bones of the rat is explained by the continued skeletal growth of this animal in adult age.

3. The species differences are much better demonstrated with a newly developed technique, which affords an almost complete separation of the newly labelled bone salts from old-established bone. Large differences were found in the function of various parts of the shaft.

4. The different aspects of bone renewal in the adult animal, such as initial uptake, recrystallization,

Haversian replacement, mobilization of fixed isotopes, equilibrium between bone and blood minerals, are discussed in the light of the new observations.

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Determination of Cocarboxylase in Blood

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Cocarboxylase estimation has received considerable attention from Ochoa & Peters (1938) and Westenbrink & Steyne-Parvé (1949), among others. Most of the accepted methods employ alkali-washed yeast apoenzymes activated by excess of thiamine, and some also include iodoacetate to prevent the phosphorylation of added thiamine (Blanchaer & Cohen, 1949). Goodhart & Sinclair (1939) developed a method for estimating cocarboxylase in heated blood, and Westenbrink & Steyne-Parvé

(1949) used acid to extract cocarboxylase from blood, before applying their 'two-stage' method for assay.

In a system such as heated blood, which contains diphosphopyridine nucleotide and adenosine triphosphate in sufficient quantity to allow cocarboxylase synthesis by yeast enzymes (Lipton & Elvehjem, 1939), it seems unwise to include excess of thiamine. Further, now that carboxylase is recognized as a sulphydryl enzyme, which is