64. RATE OF REJUVENATION OF THE SKELETON

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THE first experiments in which labelled (radioactive) phosphorus ^{32}P was applied as an indicator [Chievitz & Hevesy, 1935], showed that some of the phosphorus atoms of the mineral constituents of the bone exchange rapidly with those present in the plasma. This result was corroborated and extended by later work on this subject [Hevesy *et al.* 1937; Cook *et al.* 1937; Dols *et al.* 1937; 1939; Artom *et al.* 1938; Cohn & Greenberg, 1939; Lefevre & Bale, 1939]. The question as to what extent the P contents of the mineral constituents of the bone are replaced in a given time by plasma P remained unanswered however. This is an important question, as the rate of this replacement is a measure of the rate of rejuvenation of bone tissue. The extent to which bone P is replaced by plasma P in the course of a given time can be determined by comparing the activity of 1 mg. of bone P with that of 1 mg. of inorganic plasma P. The activity of the plasma P, i.e. its ^{32}P content, changes appreciably, however, with time. The ^{32}P atoms, like all P atoms present at any moment in the plasma, exchange with the P atoms present in the various organs and in doing so are removed from the plasma and replaced by tissue P. The application of the above-mentioned consideration implies a constancy of the activity of the plasma inorganic P. To secure such a constancy, we administered labelled phosphate all through the experiment, instead of doing so at the start of the experiment, as in all investigations mentioned above. By taking blood samples at intervals, we ascertained that the activity of the inorganic P of the plasma remained constant. At the end of the experiment, the bone sample was purified from all non-mineral constituents and the radioactivity of 1 mg. of bone P compared with that of 1 mg. of plasma inorganic P.

Experiments with frogs

Experiments of 5 to 240 min. duration were carried out with frogs. As early as 5 min. after injecting 0.3 ml. physiological NaCl solution containing a negligible proportion of labelled sodium phosphate into the lymph sac, the mineral constituents of the tibia contained some labelled phosphate, as shown in Table 1.

Table 1. *Labelled P contents of plasma and tibia of a frog*

Wt. 45 g.; temperature 22°; determinations 5 min. after the start of the experiment.

Fraction	^{32}P content per mg. P (specific activity)
Plasma	100
Epiphysis	0.026
Diaphysis	0.013

With increasing time, the ^{32}P content of the mineral constituents of the tibia increases (Fig. 1). After the lapse of 1 hr., the specific activity of the epiphysis P amounts only to 1/600 of the corresponding magnitude of the inorganic P of the

plasma. Thus only 1/600 of the epiphysial P was replaced by plasma P within 1 hr. In the next 3 hr. a further 1/900 part of the epiphysis P exchanged. The first point in the curve was obtained by analysing the right, the second point by analysing the left tibia. For the diaphysis, the corresponding figures were found to be 1/900 and 1/1200, respectively. In the course of 4 hr., therefore, only a minute part of the tibia P is replaced by plasma P. A still smaller replacement is found when the frog is kept at 0°.

Fig. 1. Extent of replacement of frog's bone P by labelled P.

Experiments with rabbits

After the lapse of 2 hr., 1/530 and 1/1800, respectively, of the tibia epiphysis P and diaphysis P were found to be replaced by plasma P. With increasing time, an increase of the replacement of the bone P takes place, as shown in Fig. 2 and Table 2; this increase diminishes, however, with increasing time, as would be expected. The bone tissue contains numerous small crystals formed by mineralization of the matrix. The crystals are built up on similar lines to the mineral apatite.¹ While the atoms situated on the uppermost layer of the crystals [Paneth, 1922] exchange easily with those present in the surrounding liquid, those situated inside the crystal are prevented from doing so, except at very high temperatures. The exchange between bone phosphate and plasma phosphate which we observe in experiments of short duration is due to a replacement process between the phosphate ions situated on the surface of the apatite crystals and those of the plasma or lymph. Should the surface exchange be exhausted, an increase of the time of the experiment may at first have no effect at all on the extent of replacement. If, however, in the course of time a dissolution and reprecipitation of the apatite crystals takes place, new and far-reaching possibilities

¹ From X-ray measurements, it was concluded [Caglioti, 1936] that the inorganic part of the bone has the composition of about $3\text{Ca}_3(\text{PO}_4)_2 \cdot \text{CaCO}_3 \cdot x\text{H}_2\text{O}$ with the hexagonal structure of hydroxyapatite, the length of the axes being $a = 9.2 \times 10^{-8}$ cm. and $c = 6.9 \times 10^{-8}$ cm., the axis being oriented parallel to the length of the bone. The organic parts consist of polypeptide chains, supported and stretched.

of an exchange between plasma P and bone P will arise. From these considerations it follows that an exchange taking place within a long interval cannot be extrapolated from results obtained in experiments of short duration. We have, therefore, carried out experiments in which we kept the activity of the plasma inorganic P of rabbits at a constant level for several days or weeks.

Table 2. *Extent of replacement of the bone P of the rabbit by labelled P*

The labelled phosphate was injected intravenously during the experiment.

Fig. 2. Extent of replacement of rabbit's bone P by labelled P.

Experiment of long duration

To obtain a constant level of the plasma inorganic P, the first day every 30 min. and later twice every day, labelled sodium phosphate of negligible weight was administered by subcutaneous injection to rabbits. After removal of the marrow, the bone was first extracted for 12 hr. with hot ether-alcohol. The bone was then treated with hot alkaline glycerol solution for further 6 hr. The fractions obtained were dissolved in HNO_3 and their P contents precipitated as molybdate. The molybdate was dissolved in dilute NH_3 and precipitated as ammonium magnesium salt. An aliquot of the sample obtained was used in the colorimetric P determination, while another aliquot was reprecipitated as ammonium magnesium phosphate and its radioactivity measured with a Geiger counter. In prolonged experiments the analysis of the plasma inorganic P is conveniently replaced by that of the urine P. In Tables 3 and 4 the specific activities of the different bone P fractions are recorded.

Table 3. *Extent of rejuvenation of the tibia in the course of 9 days*

Wt. of rabbit: 2 kg.

Fraction	% P rejuvenated (specific activity)
Epiphysis P	11.2
Diaphysis P	3.2
Tibia phosphatide P	74.8
Marrow phosphatide P	80.1

In the course of 9 days, therefore, only 11 % of the epiphysis and 3 % of the diaphysis are rejuvenated, while most of the phosphatide molecules present in the marrow and in the bone are newly formed.

Table 4. *Extent of rejuvenation of the skeleton of a rabbit in the course of 50 days*

Fraction	% P rejuvenated
Femur epiphysis inorganic P	29.7
Femur epiphysis phosphatide P	100
Femur epiphysis glycerol extract* P	51.0
Femur diaphysis inorganic P	6.7
Femur diaphysis phosphatide P	ca. 100
Femur diaphysis glycerol extract* P	84.5
Tibia epiphysis inorganic P	28.6
Tibia diaphysis inorganic P	7.6
Costa	27.5
Scapula	43.8
Incisor dentine, apical	103
Incisor dentine, medial	98.5
Incisor dentine, incisal	41.2
Incisor enamel, apical + medial	82.0
Incisor enamel, incisal	6.6

* This fraction presumably contains some mineral P.

In the course of 50 days, only 29 % of the epiphysial and 7 % of the diaphysial mineral constituents were replaced (Table 4). The tibia and femur show about the same behaviour. About half of the scapula remained unchanged. The almost complete replacement of the apical and medial parts of the incisor dentine P can hardly be interpreted as due to an exchange between dentine P and plasma P, since the replacement rate of the dentine P was found to be lower than that of the tibia P [Hevesy *et al.* 1937; Lefevre & Bale, 1939] and since the tibia P, as seen above, was replaced only to a restricted extent. The high ^{32}P content of the incisor dentine must be due to an actual growth, to a formation by a calcification process. As a plasma containing ^{32}P was instrumental in calcifying the newly grown parts of the incisor, the P of the latter was bound to have the same specific activity as shown by the plasma P. As seen in Table 4, the P of the apical part of the incisor dentine investigated has, within the errors of experiment ($\pm 5\%$), the same specific activity as that of the plasma P. This part, having a length of about 0.9 mm., is entirely newly formed during the experiment. The bulk of the medial part of the dentine was freshly grown as well, while the incisal part, having a length of 1.2 mm., is only partially newly formed with participation of the labelled plasma. About half of the P atoms present in the incisal part of the dentine were not replaced; they must thus be those which were located in the apical or medial region of the incisor before the start of the experiment. The tissue containing these atoms was pushed forward *in toto*. Partly before this "slipping" process and partly during it, some of the P atoms of the dentine have the opportunity to exchange with labelled P atoms and, therefore, the P of the incisal part of the dentine shows an activity which amounts to about 1/3 of the specific activity of the plasma P. We see here an interesting case of tissue formation in which macroscopic aggregates are "slipped" from one place to another *in toto*, experiencing only a restricted atomic or molecular replacement. This effect is much more clearly shown in the growth of the enamel.

The apical and medial parts of the enamel are formed by a calcification process from labelled plasma and, therefore, these parts of the enamel became strongly active. From the fact that the incisal part of the enamel is only slightly active, we have to conclude that this fraction is not formed in the course of the experiment through a calcification process. Its crystals were formed at an earlier date

from non-labelled plasma and the whole fraction "slipped" *in toto* during the course of the experiment from the position in which it was calcified into the place it took up at the end of the experiment. The incisal end of the dentine is probably to a large extent also formed by "slip" *in toto* of the medial parts, though this conclusion is not supported as clearly by the activity figures arrived at in the case of the enamel. A part of the incisal dentine P had an opportunity to exchange to an appreciable extent before the "slip" took place and also during that process. Enamel P exchanges only to a minute extent [Armstrong, 1940]. It is also of interest to note that the activity figures exclude the possibility that the incisal part of the enamel is formed by extensive calcification of the outer region of the dentine. In that case, the enamel could not be much less active than the corresponding dentine part. The foundation of the incisal enamel is laid in the apical end and reaches its final position without interchange with the dentine. As both the P and Ca of the teeth have their origin in the plasma, the application of labelled Ca as an indicator can be expected to lead to similar results to those found above.

Epiphysial and diaphysial bone tissues

The epiphysis was found to exchange the P content of its mineral constituents at a higher rate than the diaphysis, as seen in Table 5.

Table 5. *Ratio of the specific activities of the epiphysial and diaphysial P of the tibia of rabbits*

The level of the activity of the plasma inorganic P was kept constant all through the experiment.

Time	Ratio
2 hr.	3.2
4 "	1.9
10 days	3.5
50 "	3.8

In experiments with frogs, in which the labelled phosphate was injected into the lymph sac at the start of experiments, taking 1-22 days, the ratio 1.3-1.6 was found. In these experiments, the tibia and femur were both investigated and the average was taken. In an investigation of the tibia P of rats, to which the labelled phosphate was administered at the start of the experiment, the ratios 3.1, 2.9, 2.5, 1.7 and 1.8 were found after $\frac{1}{2}$ hr., 4, 10, 50 and 110 days, respectively. It is of interest to remark that from the finding that the diaphysial P is only about half as active as the epiphysial P 110 days after the start of the experiment, we can conclude that an appreciable part of the skeleton has not been renewed within 110 days.

In experiments on chickens, Dols *et al.* [1939] found the above ratio to be 3, 22 hr. after administration of labelled phosphate; rachitic chickens gave the ratio 2.5.

The more rapid exchange of the epiphysial P is just what would be expected. The epiphysis is characterized by a poorer mineralization of the matrix than is the diaphysis and contains more organic matter and water than the diaphysis. The circulating lymph, containing the labelled P, will therefore reach the apatite surface more easily in the first-mentioned case. Should the size of the apatite crystals be smaller in the epiphysis than in the diaphysis and therefore the ratio surface: volume be larger in this type of bone tissue, one would also expect a more rapid exchange of the mineral constituents. Whether such a difference in the size of the apatite crystals actually occurs is not known. X-ray

investigations [Bale *et al.* 1934] lead to the result that the size of the ultimate crystals of dentine and bone is about 10^{-6} cm., while the very effectively mineralized enamel contains larger crystals (10^{-5} cm.). It is, therefore, quite probable that the difference in the extents of mineralization of the epiphysis and diaphysis manifests itself in a moderate difference in the sizes of the ultimate crystals in the two types of bone tissue. It is of interest to note that the difference in hardness of the different types of bones is, to a large extent, the result of a different degree of orientation of the crystallites of the bone. X-ray patterns indicate that orientation occurs during growth and first in those bones where the need for solidity (e.g. leg bones) is greatest [Caglioti & Gigante, 1936].

SUMMARY

Labelled (radioactive) phosphate was administered to rabbits and frogs repeatedly during the experiment in order to keep the radioactive plasma inorganic phosphorus at a constant activity level. The comparison of the activity of 1 mg. bone inorganic P with that of the plasma inorganic P permits us to conclude to what extent the mineral constituents of the bone were renewed during the experiment.

Within 50 days, 29% of the femur and tibia epiphysis were found to be renewed, while the corresponding figure for the diaphysis amounted to 7%. Only half of the mineral constituents of the scapula were found to be unchanged. The phosphatides of the bones and the marrow were entirely renewed.

The phosphorus of the apical part of the dentine of the rabbit's incisor was found to have the same activity as the plasma phosphorus. From this result it follows that this part of the dentine was grown with the participation of labelled plasma phosphorus during the experiment. The greater part of the incisal end of the dentine was not formed by a calcification process *in situ* but by a "slip" of the apical part of the dentine.

The same behaviour is shown even more pronouncedly by the enamel. No interaction of any significance takes place between the incisal part of the enamel and the dentine either during their formation or at a later date. The atoms present in the former are to a very large extent those which were previously located in the apical (medial) part of the enamel.

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