Studies on the Skeletal Tissues

3. THE RATE OF EXCHANGE OF THE INORGANIC PHOSPHATE IN DIFFERENT BONES AND PARTS OF BONES IN VARIOUS SPECIES OF MAMMAL

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Recent work has shown that there is considerable variation in the composition of the mammalian skeleton, according to the origin of the sample of bone and species of animal. In a number of species of animal, the degree of calcification of cleaned trabeculae from the metaphyses of the long bones is lower than that of cortical bone in the region of the middle of the shaft (Weidmann & Rogers, 1950; Rutishauser & Majno, 1951; Perrottet & Duckert, 1951). The degree of calcification varies in different parts of the cortex of the individual long bones from some species (Dallemagne, 1943; Strobino & Farr, 1949) and even greater variations have been shown to occur in cortical bone during growth of animals (Hammett, 1925; Logan, 1935; Burns & Henderson, 1936; Rogers, 1949; Rogers, Weidmann & Parkinson, 1952).

This variability in composition of the skeleton makes it seem likely that there may be similar differences in the rate of local metabolism of the inorganic salts in bones from different species of animal. Earlier studies of the skeleton using isotopes have already shown that the cancellous bones of various species exchange faster than the corresponding cortical bones (Hevesy, 1948). The recognition, however, that the ions of solid calcium phosphate can exchange with these ions in a contiguous liquid phase diverted attention from *in vivo* studies of exchange reactions of the skeleton. Some earlier studies (Hevesy, Levi & Rebbe, 1940; Volker, 1942; Volker & Ginn, 1942), suggested that there may be considerable differences in the rate of exchange of phosphate in different species of animal, but this work is difficult to interpret owing to the use of inadequate numbers of animals and to differences in technique or of expression of results. Very recently, during the course of preparation of this paper, the work of Amprino (1952), Zetterström (1952), Engfeldt, Engström & Zetterström (1952) and Lacroix, Devis & Schicks (1952) has again focused attention on the *in vivo* inorganic salt exchanges in the skeleton.

The present work is an attempt to study the relation between the rate of *in vivo* exchange of

phosphate and the type of bone, in different species of animal. It is thought that although ionic exchange between plasma phosphate and bone salts can take place, the operation of other more complicated mechanisms is not precluded.

METHODS

The isotope was administered to groups of healthy adult animals by intraperitoneal injection of approximately 30 $\mu\text{C.}/\text{kg.}$ body wt. of a carrier-free solution of $\text{Na}_2\text{H}^{32}\text{PO}_4$ in order to avoid altering the level of blood phosphate. Phenol (0.5%) was added to the stock isotope solution to prevent growth of micro-organisms which otherwise led to local concentrations of ^{32}P .

The measurements of radioactivity were made with a liquid counter using a standard scaling and counting apparatus. The results obtained were corrected for the decay of the isotope during the period between its standardization at the Atomic Energy Research Establishment and its experimental use. The activity of the solution was always checked before injection into animals. The results have been expressed throughout this paper as the following ratio (called specific activity):

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

The amount of ^{32}P in the inorganic fraction of the serum of the animals was measured as follows: to 5.0 ml. of serum or plasma from freshly drawn blood (containing 4 mg./ml. NaF) were added 5 ml. of water and 10 ml. of 20% (w/v) trichloroacetic acid (TCA). The precipitate obtained after 24 hr. at 0–4° was removed by centrifugation and the supernatant used for determinations of phosphate and radioactivity.

The bones were dissected, cleaned, powdered samples prepared and analysed by the methods previously described (Weidmann & Rogers, 1950). The same TCA extracts of the powders were used for determinations of radioactivity and phosphate content. This method of estimating ^{32}P in bone samples was compared with the methods used by Manly & Bale (1939) and by Hevesy *et al.* (1940). Both of these groups of workers first removed organic matter from the samples by heating with alkaline glycerol. Manly & Bale (1939) then dissolved the resulting ash in acid and estimated the radioactivity of the solutions. Hevesy *et al.* (1940) reprecipitated the phosphate first as molybdate and then as magnesium ammonium phosphate. The reliability of the method used by us clearly depends upon the completeness of removal of soft tissues, particularly marrow, which contain

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Table 1. *A comparison of the present method for the determination of the specific activity of bone samples with those of Manly & Bale (1939) and Hevesy et al. (1940)*

Species of animal	Type of bone	Exp. no.	Our method		Method of Manly & Bale		Method of Hevesy <i>et al.</i>	
			% P*	Specific activity†	% P*	Specific activity†	% P*	Specific activity†
Cat	Diaphyseal	1	12.0	25	10.8	19	—	—
Cat	Diaphyseal	2	11.8	7	8.1	10	10.9	6
Cat	Diaphyseal	3	12.0	52	11.4	48	10.9	47
Cat	Diaphyseal	4	12.1	37	12.0	33	11.5	39
Cat	Cancellous	3	11.0	400	10.8	360	11.0	410
Cat	Cancellous	4	11.4	300	11.1	300	11.0	310
Cat	Alveolar	1	11.3	54	9.8	54	10.4	47
Rabbit	Diaphyseal	10	12.9	22	9.6	24	—	—

* Measured in dry defatted bone powder.

† Expressed as $\frac{(\text{counts/min./mg. P}) \times 10^8}{\text{administered counts/min./kg. body wt.}}$.Table 2. *Specific activity of inorganic phosphorus of bones and serum, from various animals, measured 4 hr. after intraperitoneal injection of 30 $\mu\text{C.}$ /kg. body wt. of carrier-free ^{32}P as $\text{Na}_2\text{H}^{32}\text{PO}_4$*

Species of animal	Specific activity*						Relative activities for series 2 (cancellous bone = 100)	
	Series 1			Series 2			Cortical bone	Alveolar bone
	Cortical bone	Cancellous bone	Serum	Cortical bone	Alveolar bone	Cancellous bone		
Rabbit								
No. of animals	13	10	19	5	5	5	12	5
Mean value	42	330	19 600	60	290	450	15	63
S.D.	22	200	7 400	22	130	44	8	28
Cat								
No. of animals	7	7	7	6	6	6	7	6
Mean value	14	240	27 500	14	67	240	6.7	28
S.D.	4	91	13 200	4	21	100	3.5	5
Rat								
No. of animals	13	12	11	7	8	8	14	10
Mean value	46	320	10 900	45	160	310	19	49
S.D.	16	110	3 460	15	76	134	8	14
Ferret								
No. of animals	6	6	6	5	5	5	6	5
Mean value	35	230	15 400	38	99	240	16	42
S.D.	14	60	1 600	12	39	58	5	12

* Measured by the ratio $\frac{(\text{counts/min./mg. P}) \times 10^8}{\text{administered counts/min./kg. body wt.}}$.

highly radioactive compounds, during the preliminary cleaning of the bone. Table 1 shows results for the specific activity of samples examined by our own method in comparison with those obtained using the techniques of Manly & Bale (1939) and Hevesy *et al.* (1940). It will be seen that the measurements made with TCA solutions of the inorganic salts are in satisfactory agreement with those obtained by the other more complicated methods. Only in one instance is the result obtained using our technique higher than is obtained using the other methods—an adequate proof that the method for cleaning bones (Weidmann & Rogers, 1950) does not leave significant amounts of tissues containing radioactive organic phosphorus compounds attached to the bone.

RESULTS

The amount of ^{32}P appearing in various bones 4 hr. after injection

Rabbits, cats, rats and ferrets were injected with ^{32}P as previously described and killed 4 hr. later. The femoral cortical and trabecular metaphyseal bones and the mandibular molar alveoli were dissected, prepared and examined by the usual techniques. The results obtained are shown in Table 2. Preliminary inspection of this table shows that the relative activities of the three types of bone

Table 3. Statistical analysis of the differences between the activities of samples from diaphyseal cancellous and alveolar bones and serum from rabbits, cats, rats and ferrets 4 hr. after the intraperitoneal injection of ³²P

	Cortical bone			Alveolar bone			Cancellous bone			Serum		
	n	t	P	n	t	P	n	t	P	n	t	P
Rabbit/cat	18	3.28	<0.01	9	4.2	<0.01	15	1.18	0.24	24	1.94	0.07
Rabbit/ferret	17	0.7	0.5	8	2.98	0.017	14	12.8	0.22	23	1.26	0.21
Rabbit/rat	22	0.88	0.41	11	1.97	0.075	20	0.27	>0.5	26	3.02	<0.01
Cat/rat	16	5.85	<0.01	12	2.93	0.013	17	1.26	0.21	14	3.48	<0.01
Cat/ferret	11	3.82	<0.01	9	1.21	0.25	11	0.24	>0.5	11	2.07	0.06
Rat/ferret	15	1.85	0.09	11	1.57	0.15	16	1.86	0.08	13	1.49	0.17

n=degrees of freedom; t=Student's statistic; P=probability that the groups compared are drawn from identical populations.

are in the same order in all four species of animal. The cancellous trabecular bone is always the most active and the cortical bone is always the least. It is not possible, however, to decide upon the relation between the values for the same type of bone in the different species without the use of significance tests. This is due to the very large variation between individuals of the same groups, a deviation in marked contrast to the close agreement of the results for the chemical composition of bones from different individuals (Weidmann & Rogers, 1950). Statistical tests (Table 3) show that the value for the cortical bone of cats is different from that of rabbits, rats and ferrets ($P<0.01$), whereas the values for this bone in the latter three species show no differences which can be regarded as significant. The cancellous bones from the four species are all very similar in activity.

The interrelationship between the alveolar bones is more complex. A clear difference is apparent between this bone in the rabbit and the cat. Comparison of the rabbit with the ferret, and the cat with the rat, however, show differences with probabilities of 0.017 and 0.01 respectively, which make it likely that the results belong to different populations but are not sufficiently low to be convincing. Moreover, as has been previously pointed out (Weidmann & Rogers, 1950) it is difficult to be sure that the samples of alveolus are collected from one type of bone only, the lamina dura probably being different from the trabeculae within the alveolus.

The rate of appearance of the tracer in the bone

It had previously been shown (Manly, Hodge & Manly, 1940; Neuman & Riley, 1947) that the amount of radioactive phosphate increases rapidly in the cortical femoral bone of rats for the first few hours after parenteral administration of the isotope. At the same time the level of isotope in the inorganic fraction of the serum drops. After this initial period the amount of ³²P in the cortical bone remains constant within experimental error for 18 days and there is only a very slow fall in the serum value

during the latter period. Hevesy *et al.* (1940) also found a rapid increase of activity in the cortical bone of rabbits and frogs immediately following the start of a course of frequent injections of the isotope. In this work the authors were attempting to maintain the blood level of ³²P constant during the experimental period and using this technique the amount of tracer in the bones continued to increase at a slower rate after the initial rapid rise.

Fig. 1. Variation in the specific activity of the inorganic phosphorus in serum and cortical femoral bone from (a) rabbits, (b) rats, with time after intraperitoneal injection of carrier-free ³²P. Each point represents the mean value obtained for the numbers of animals indicated. The vertical lines represent the standard deviations. Specific activity expressed as

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

In the present work cats, rabbits and rats were given a single intraperitoneal injection of the isotope. At the time intervals indicated in Fig. 1a and b and in Table 4, suitable numbers of animals were killed and the isotope concentration in the cortical bones and serum was measured. It will be

seen that the rates of increase of activity in the femoral bones of both of these animals are similar. The only difference between the two species appears in the curves for the activity of the inorganic serum fraction which in the rabbit but not in the rat shows an increase for the first 30–60 min. following injection of the isotope. It is to be presumed that this difference is attributable either to the relative sizes of the two species of animals or to the speeds at which phosphate is absorbed from the peritoneal cavity.

The phosphate metabolism of the cat differs from that of the rabbit and rat as can be seen from Table 4. It has already been shown above that the amount of isotope appearing in the cortical, but not in the cancellous, bone of the cat 4 hr. after injection of the tracer is significantly smaller than for rats, rabbits and ferrets. Table 4 shows that this is

Table 4. *Influence of time elapsing between intra-peritoneal injection of 30 μ c./kg. body wt. 32 P as $\text{Na}_2\text{H}^{32}\text{PO}_4$ and the specific activity of the inorganic P in the femoral cortical bone of the cat and rabbit*

Time	Cat		Rabbit	
	Mean specific activity*	No. of animals	Mean specific activity*	No. of animals
10 min.	—	—	6.3	3
30 min.	10	1	22	3
1 hr.	—	—	25	2
4 hr.	14	7	42	13
2 days	12	2	35	2
9 days	61	4	45	5

* Expressed as

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

probably explained by a slower exchange of the phosphate in the former species. In the cat the amount of ^{32}P in the cortical bone is still increasing 9 days after injection of the tracer, this being the longest period examined. The activity of the serum shows an initial rise for the first 1–2 hr. after injection, followed by a rapid fall during the next 3 hr. During the subsequent experimental period there is a very slight fall in activity. Owing to the cost of cats, and the difficulty in obtaining them, it was impossible to examine sufficient animals to be able to draw a curve for the 9-day period, far from being able to extend the time of the experiment as was clearly desirable. It may be significant that the specific activity of the cortical bone phosphate 9 days after injection agrees with the values obtained for rats and rabbits after 4 hr. Thus it appears that the exchange of phosphate in the cat femoral bone is a very much slower process than in the rabbit or rat and that the smaller amount of ^{32}P found in

the cat bone 4 hr. after injection is a reflexion of this and not of the smaller amount of exchangeable ion present in the bone salt.

The amount of ^{32}P appearing in different parts of shaft bone

The results of Manly *et al.* (1940) and of Neuman & Riley (1947) for rats have been confirmed, showing that some fraction of the bone salts appears to exchange faster than the remainder and experiments with rabbits have yielded similar results. The former authors suggested that there is a labile fraction which amounts to about one-twelfth of the salts in cortical bone, and to about one-sixth in the epiphysis. The initial period of rapid uptake has also been interpreted (cf. Hevesy, 1948) as being due to exchange between the plasma and the ions on the surface of the bone salt crystals. Very recently Zetterström (1952) has used saturated ammonium sulphate and acetate buffers to extract fractions of high activity from bone obtained from animals treated with ^{32}P .

In the present study the activities of samples of bone powder from three arbitrarily selected regions of the shaft bone have been examined. These regions are (a) a thin layer immediately next to the marrow, (b) a thin layer immediately under the periosteum and (c) a layer from the 'middle' of the cortical bone. All the samples were taken from the region of the longitudinal mid-point of the femur and tibia of rabbits. If the period of rapid initial uptake is to be regarded as a saturation of the surface of all the crystals within the shaft we might expect the activities of the three samples examined to be similar. If, however, it corresponds to the rapid exchange of some small fraction of the bone salt due to either greater competence of blood circulation, or to the localization of some biological exchange mechanism in cells concentrated in this region, then the samples might show considerable differences in activity.

Table 5 shows the results obtained for the activities of these three samples taken from bones of animals killed at various periods after dosage with the tracer. It will be seen that the layer bordering on the marrow has a very much higher activity than the other two layers. In the animals examined 4 hr. after receiving the tracer the activity of the inner bone layer is 5–7 times that of the layer from immediately under the periosteum. As the period between injection of the tracer and examination of the bone extends, the very large difference tends to decrease. As might be expected with such arbitrarily selected regions there is even greater variation from individual to individual than when pooled samples of shaft bone are examined. Owing to these variations it is not possible to follow the course of activity changes with time after injection of tracer.

Table 5. *The specific activity of selected samples from cortical bone of adult rabbits. Sample (a) from the sub-periosteal layer; (b) from the 'middle' layer; (c) from the layer next to the marrow*

Time after intraperitoneal injection of ^{32}P (hr.)		Specific activity*					
		Femur			Tibia		
		a	b	c	a	b	c
4	Mean	75	58	520	72	68	350
	No. of animals	5	5	5	5	5	5
	Range	44-120	37-100	269-770	44-130	35-110	173-670
24	Mean	40	29	100	40	30	120
	No. of animals	6	6	6	5	6	6
	Range	9-69	11-58	25-280	15-86	10-49	31-200
48	Mean	45	32	150	74	49	170
	No. of animals	6	6	6	6	6	6
	Range	18-72	23-45	63-330	16-170	17-97	52-370
216	Mean	28	24	92	35	30	72
	No. of animals	3	4	4	4	4	4
	Range	17-38	8-48	48-330	20-47	12-50	49-97

* Expressed as $\frac{(\text{counts/min./mg. P}) \times 10^8}{\text{administered counts/min./kg. body wt.}}$

It is thought, moreover, that to attempt to do this by simply increasing the number of animals used would be unnecessarily cumbersome and that it is better to seek for some more exact method of sampling, or for some other way of examining the distribution of tracer within the bone. The activity of the layer next to the marrow remains higher than that for the pooled sample from the whole shaft, throughout the period of the experiment. The other two layers examined do not differ from the value for the pooled samples. These results are in good agreement with the very recent work of Engfeldt *et al.* (1952) whose autoradiographic pictures suggest qualitatively that the greatest density of ^{32}P is in the Haversian systems close to the marrow. Thus results based on an examination of samples of the whole shaft give an erroneous picture and the apparently steady state in the activity of the diaphyseal bone achieved 4 hr. after the injection of tracer may represent only the smoothing of a complicated pattern of internal fluctuations.

It may be noted that if the concentration of ^{32}P in the trabeculae (cf. Table 2) is compared with that in the inner layer of the shaft 4 hr. after injection of the isotope, the great difference which has hitherto always been found between cortical and cancellous bone disappears.

DISCUSSION

The work presented here together with that of Engfeldt *et al.* (1952), Amprino (1952) and Lacroix *et al.* (1952) emphasizes the degree to which the rates of exchange of inorganic phosphate between plasma and bone salt vary from one region of a bone to another and from one species of animal to another. The inner layer of the shaft preferentially

accumulates ^{32}P and Engfeldt *et al.* (1952) have shown that tracer localizes in the poorly calcified young osteons. It is known (Rogers *et al.* 1952) that the inner layer of shaft bone in some species of animal is relatively poorly calcified, and is therefore presumably rich in young osteons. These results might all be explained by assuming either differences in local blood supply, greater diffusion through freshly deposited bone, or the initial deposition of bone salt in some form which subsequently changes. The latter assumption would seem to be necessary to explain Amprino's (1952) observation that areas taking up large amounts of tracer *in vivo* also do so *in vitro* even after incineration. This assumption alone, however, seems unlikely to be sufficient to explain the finding (Neuman & Mulryan, 1950) that the amount of phosphate exchanged by young fresh periosteal bone *in vitro* is much greater than that by ash produced from the powder by treatment with alkaline glycerol; the extent of exchange by fresh bone powder is far greater than could be accounted for by superficial ions, is partially irreversible, and much affected by temperature. The authors explain their results as being due to 'recrystallization' of the bone salt. If, as seems probable, the most rapid exchange of phosphate takes place in forming osteons, even in adult animals, it seems reasonable to consider the possibility that, in addition to the direct exchange of plasma and bone salt ions, enzymic mechanisms of the type already involved in explaining the phenomena of calcification in young animals, may also be involved in controlling the rate of exchange of phosphate in adults.

The present results call attention to the danger of drawing conclusions from results for the exchange of

inorganic ions obtained using samples representing any considerable volume of bone. Many of the earlier results for the rate of exchange of the inorganic constituents of the skeleton represent only mean values for a series of areas in different states of equilibrium with the plasma.

SUMMARY

1. The rate of exchange of phosphate in the cortical bone of cats is very much slower than in this type of bone in rabbits and rats. This difference does not extend to the trabeculae of the cancellous metaphyseal bones.

2. The distribution of the tracer within the

femoral and tibial cortical bone of rabbits shows a high degree of heterogeneity. The layer next to the marrow is always very active compared with the rest of the cortex. The outer layers (sub-periosteal) have a relatively low activity but are slightly higher than those from the depth of the bone.

3. The significance of this heterogeneity of distribution is discussed.

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Bromide Determination in Body Fluids

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In a research involving the distribution of administered bromide between blood plasma and cerebrospinal fluid (c.s.f.), a method applicable to samples of about 1 ml. fluid containing from about 5 to 50 mg. bromine/100 ml. was required. The long-known colour reaction between bromide and auric chloride, used by Walter (1929), Wuth (1927) and others, was regarded as insufficiently accurate for our purposes. A number of methods depending on other principles seemed to be too elaborate or insufficiently proved.

A method based on the Van der Meulen (1931) reaction: $\text{Br}^- + 3\text{OCl}^- \rightarrow \text{BrO}_3^- + 3\text{Cl}^-$ has proved to

be suitable in most cases, and a preliminary report of this has already been given (Hunter, 1952). This principle was used by Dixon (1934) to determine bromide in normal blood, using 10 ml. The reaction was notably improved by D'Ans & Höfer (1934) who used phosphate instead of borate as buffer, and formate, instead of hydrogen peroxide, to destroy excess of hypochlorite. The reaction was further studied by Kolthoff & Yutzy (1937), and Friedman (1942) has used it to determine bromide in trichloroacetic acid filtrates from blood.

The present communication is based on the above principles, but the procedure has been modified and