Mobility of Skeletal Phosphorus in a Mature Dairy Cow as Determined with Radioactive Phosphorus.* (20081)

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The use of radioactive tracers to study skeletal mineral metabolism has resulted in a number of significant findings. It has been shown that the skeleton is not a stable structural tissue but continuously exchanges minerals with the tissue fluids of the body and is a very important reservoir of Ca and P. Several early workers, among them Manly and Bale(1), recognized that the skeleton was not a homogeneous system with respect to the behavior of its minerals but actually consisted of 2 fractions. One fraction of the skeleton they found in rapid exchange with the minerals of the plasma and they called this fraction "labile." The remaining minerals were held firmly by the skeleton and were slowly interchanged with plasma minerals only when apatite crystals were formed and resorbed. These minerals represented the "stable" fraction of the bone.

Several methods, *in vivo* and *in vitro*, have been used to estimate the size of this "labile" pool in bones and to determine its role in mineral exchange between the plasma and the skeleton. The estimates for the size of the labile calcium pool range from 15%(2) to 20%(4) of the total bone calcium. The labile phosphorus pool has been estimated to be 20%(3), 13%(5), and 17%(1) of the total bone phosphorus using different experimental methods in each case with small animals. Unless the magnitude of the "labile" exchange process is known it is difficult to interpret results obtained with radioactive tracers in studies of mineral metabolism. This report deals with the determination of the "labile" pool in the mature dairy cow and with an investigation of the problem of sampling skeletons of large size in isotope studies.

Experimental. An aged (10 years) lactating dairy cow weighing 354 kg was injected intravenously with 13.9 millicuries of phosphorus-32 and sacrificed 72 hours later by electrocution.‡ Bones were removed as rapidly as possible (all within 1 hr), scraped free of most adhering tissue, and placed in a boiling water bath for 5-10 minutes. This treatment simplified the removal of all remaining soft tissue from the bones. The bones were air-dried for 48 hours, weighed, and then samples of them were taken for analysis. Long bones were cut in half at the center of the shaft and then split lengthwise so that the marrow could be removed. Samples of cortical bone were obtained by filing across the cut surface of the shaft and samples of trabecular bone by scooping a small quantity out of the pocket at the ends of the long bones. Composite samples were collected as sawdust after sawing through the longest axis of the bone. For the flat bones of the skull, pelvic girdle, rib and scapula, holes were drilled at random through the bones and the borings collected for the composite sample of each bone. Each sample was dried at 105°C for 24 hours to determine the percentage dry matter and then ashed at 550°C until a white powder was obtained. The ash was dissolved in a few ml of 6N HCl and diluted to a known volume. Aliquots of this solution were assayed for phosphorus-32 by the method described by Kleiber(8). The total phosphorus of these same solutions was determined by the method of Fiske and SubbaRow(9). *Autoradiograms.* Bone sections from the desired location were cut as thin as possible with a fine bladed coping saw. These sections were further reduced in thickness by rubbing

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‡ After sacrifice the cow was found to have a fetus about 4 months old. The effect of this fetus on the phosphorus uptake of the maternal tissues is not known at present.

TABLE I. Specific Activity of Bone Samples from Different Parts of the Skeleton of a Mature Dairy Cow.

Sample	Stand. spec. activity, $\mu c/g$ P / mcP^{32} inj./kg B.W.	Spec. activity ratio (P^{32}/P^{31}) in skeleton / (P^{32}/P^{31}) in inorganic fraction
Composite bone sample		
3rd tail bone (1.6 g)	9.2	.66
9th " " (7.3 g)	19.6	1.4
Carpal	6.0	.43
Tarsal	12.0	.86
Metacarpal	8.9	.64
Metatarsal	9.3	.67
Radio-ulna	18.9	1.36
Humerus	11.0	.79
Tibia	16.6	1.2
Femur	16.3	1.17
Pelvic girdle (ilium)	11.0	.79
" " (pubis)	14.1	1.0
Skull	12.4	.89
Scapula (blade)	10.7	.77
" (distal end)	18.8	1.35
Rib (7th)	15.6	1.12
Vertebra (6th lumbar)	21.2†	1.53
" (9th thoracic)	25.6	1.84
" (3rd cervical)	50	3.6
Cortical bone		
Tibia shaft	7.2	.52
Femur shaft	8.7	.63
Radio-ulna shaft	7.8	.56
Humerus shaft	9.1	.65
Humerus (exterior surface of epiphysis)*	9.2	.66
		Avg = .6
Trabecular bone‡		
Tibia (distal end)	22.5	1.62
" (proximal end)	24	1.72
Femur	23.2	1.67
Radio-ulna	25.6	1.84
Humerus	20.8	1.5
Vertebra (6th lumbar)	16.1†	1.16
		Avg = 1.7
Marrow		
Tibia	256	18.4

* This sample of bone was filed from the outer surface of the proximal end of the humerus and sufficiently shallow so that no trabecular bone is included.

† We believe these samples may have been interchanged during processing which would account for the higher specific activity found in the composite sample as compared to the trabecular bone sample.

‡ Spongy cancellous bone found in the epiphysis of long bones and throughout the interior of flat bones and vertebra.

against sand paper of decreasing coarseness and finally on jeweler's rouge paper to obtain a smooth glossy surface. This surface was placed in direct contact with a piece of Ansco Triple S Pan film in a light-tight box and exposed for a predetermined period of time. The exposure period was determined by the time required for 8 to 10 million beta particles per sq cm to emerge from the bone surface in contact with the film. This exposure gave satisfactory contrast between regions of different phosphorus-32 concentration.

Results. The specific activity of each bone sample assayed is listed in Table I. In the first column the specific activity (microcuries P^{32}/g phosphorus) is expressed per unit of relative injected dose (millicurie phosphorus-32 injected per kg body weight). The advantage of expressing results in this manner (standard specific activity) arises when one compares data from this experiment with data obtained under similar conditions from animals of different body size and those which have received a different quantity of phosphorus-32. The second column in Table I lists the specific activity of each bone sample relative to the specific activity present in the inorganic fraction of the plasma. This expression reveals the extent to which the phosphorus of the bone has come into isotope equilibrium with the inorganic phosphorus of the plasma.

There is a large variation of the standard specific activities of the composite bone samples depending on the anatomical origin of the bone. In general, those bones in or near the vertebral column have the greatest P^{32} uptake and the small peripheral bones of the leg and foot the lowest P^{32} uptake. The large leg bones and the flat bones of the pelvic girdle, rib, skull, and scapula have an intermediate phosphorus turnover as indicated by their specific activities. The same variation of isotope uptake for different components of the skeleton has also been observed in rat (6,7,10) and human skeletons(11). This variation in phosphorus exchange for bones from different anatomical locations complicates skeletal metabolism studies in large animals because it is impractical to get an aliquot sample of the entire skeleton and individual bones must be used.

The sampling problem is further complicated by the variation in P^{32} uptake found in different anatomical regions of a single bone.

The specific activity of the cortex from the tibia shaft had 1/3 the specific activity of trabecular tissue from the epiphysis of the same bone. This variation is also found in samples taken from similar regions in other bones and confirms results obtained by several workers with other animals. Harrison(12) followed calcium-45 uptake by rats and found the specific activity 2 to 3 times greater in the epiphysis than the diaphysis 48 to 72 hours following the administration of isotope. Neuman(5) made an *in vitro* study of exchangeable P and found it to be 3 times as great in the epiphysis as in the diaphysis of fresh bone. Similar variations in phosphorus-32 uptake by different regions of the bone have been reported in skeletal studies of dogs(13), rabbits (14) and rats(15).

In small animals it is possible to radioassay the complete skeleton for mineral turnover studies but in the cow the size of the skeleton makes this an impractical approach and it is even difficult to obtain an aliquot sample of any single bone except the smallest foot and tail bones. The preliminary problem, then, was to establish a suitable method for obtaining a sample of the bones of the cow that would represent the average skeletal mineral turnover but would not require grinding up of the whole skeleton.

The specific activities of cortical bone from several different anatomical regions (Table I) are nearly constant. The trabecular bone samples also have rather constant specific activities but some 300% higher than that found for cortical bone. Each of the composite samples has a specific activity intermediate between the cortical and trabecular bone samples.§ This result suggests that the variation of specific activity observed for different skeletal components results from differences in the mass ratio of trabecular/cortical bone in different anatomical regions. The high specific activity always observed for bones in the vertebral column which have a large mass ratio of trabecular/cortical bone and the lower specific activity of peripheral bones that have a lower mass ratio of trabecular/cortical bone substantiates this relationship. The epiphysial region of long bones where trabecular bone is concentrated generally has a specific activity 2 to 3 times greater than the diaphysis(5,12-15) which contains mainly cortical bone. The specific P^{32} activity of cortical bone scraped from the exterior surface of the humeral epiphysis was the same as cortical bone from the shaft of the humerus (Table I) suggesting again that the controlling factor in P^{32} uptake is primarily the structural type of bone (trabecular or cortical) involved rather than anatomical location.

As a result of these observations it was decided that the most satisfactory method for sampling skeletal tissue in the cow would result from radioassaying a few samples of cortical bone from the shafts of long bones and trabecular bone from the epiphysis of long bones and using the average value for each. These average values establish the limits within which the specific activity for the skeleton as a whole should fall and by estimating the mass ratio of trabecular/cortical bone in the skeleton one may obtain an approximate value for phosphorus turnover.

The total weight of the skeleton for this cow was 29 kg and the average phosphorus content of the bones sampled was about 10%, thus giving a total skeleton content of 2.9 kg phosphorus. Assuming that the skeleton is 60% cortical bone and 40% trabecular bone, one would conclude that the cow has 1.7 kg phosphorus in cortical bone (specific activity = 0.325 μc P^{32}/gP) and 1.2 kg phosphorus in trabecular bone (specific activity = 0.91 μc P^{32} gP) which gives a total of 1.6 millicuries of P^{32} or 11.6% of the injected isotope in the skeletal tissue.

The relative specific activities listed in the second column of Table I show the extent to which bone phosphorus has equilibrated with plasma inorganic phosphorus and provide the data needed to calculate the size of the

§ The 3rd cervical vertebra is an exception and this may have been due to traces of marrow tissue which are difficult to remove completely from vertebral bones.

Some of the small foot bones were not included. Weight of ribs based on weight of 7th rib and total weight of each spinal section was based on the vertebra recovered.

"labile" phosphorus pool. It cannot be stated unequivocally that only the "labile" pool of the bone contains phosphorus-32 though it is necessary to make this assumption to calculate the size of the "labile" pool. However, studies that have been made on the 2 skeletal pools all indicate that the process of recrystallization is very slow compared with the surface exchange process(1,5,6,16). In addition, Neuman(16) produced evidence suggesting a decrease in the rate of recrystallization with increasing age of the animal. A 10-year-old cow is physiologically very old and thus recrystallization would be expected to constitute a negligible factor in P^{32} uptake by the skeleton during the 72-hour interval of the experiment. The "labile" fraction of the skeleton, on the other hand, is very rapidly exchanging with plasma minerals as indicated by the rapid skeletal deposition of isotopes in pigs as early as 2½ minutes after injection(4). The specific activity of the plasma for our cow decreased very slowly after 3 hours indicating that a state of near-equilibrium exists between the plasma and those pools rapidly exchanging phosphorus with the plasma. Data obtained from rat bones(5) indicate that an equilibrium state between the plasma and the bone labile phosphorus pool has been established within 2 days. The assumption is justified, therefore, that an equilibrium state has been established at 72 hours between the "labile" phosphorus pool of the skeleton and the plasma which means that the specific activity in each pool is the same. The measured specific activity of the bone will differ from that of the plasma due to the diluting effect of the stable bone which is not exchanging phosphorus and therefore the specific activity of the bone relative to that of the plasma (specific activity ratio—Column II, Table I) directly expresses the percent of the bone phosphorus in the labile fraction.

The specific activity for cortical bone is 0.6% and that for trabecular bone 1.7% of the plasma specific activity which means that the "labile" phosphorus pool is about 3 times more extensive in trabecular bone than cortical bone. The size of the "labile" phosphorus pool for the whole skeleton would be between 0.6% and 1.7% and for a mass ratio of 40% trabecular/60% cortical the value would be 1%. This "labile" pool would therefore amount to 29 g (1% of 2.9 kg phosphorus in total skeleton) of phosphorus.

The plasma contained 8.4 mg % inorganic phosphorus. In a total volume of 22.6 liters of plasma (determined with Evans Blue) this gives a circulating pool of 1.9 g phosphorus. The labile phosphorus pool in the skeleton therefore contains a phosphorus store some 15 times greater than that present in the circulating plasma pool.

It is not readily apparent why the labile pool constitutes only a small fraction (1%) of the skeletal phosphorus in the cow when values as high as 17%(1) to 20%(3) have been obtained for rat skeletal tissue. This difference between labile pool sizes does not arise from our assumption that recrystallization played a negligible part in P^{32} uptake. If this were a factor then the calculated value of 1% labile P in the cow would be too large and the discrepancy between cow and rat skeletal tissue would be even greater. The explanation may be that the tissue mass of the cow requires a relatively greater quantity of cortical bone for support and that this results in less vascularity for the skeletal tissue of the cow and therefore less surface for exchanging phosphorus with tissue fluids.

The autoradiograms in Fig. 1 and 2 give a picture of the anatomical distribution of the labile phosphorus pool. B, Fig. 1, is an autoradiogram of the smallest bone at the distal end of the tail. The marked deposit of P^{32}

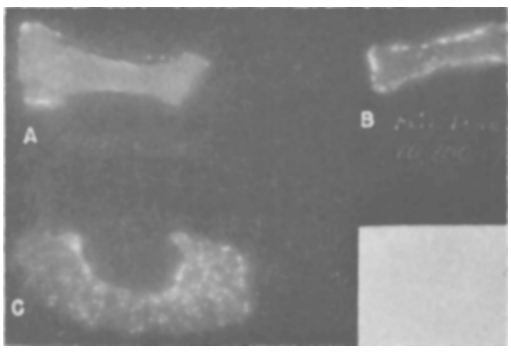

FIG. 1. Autoradiograms showing distribution of P^{32} in cow bones. A and B are longitudinal sections of tail bones and C is a cross section of half of the tibia shaft. Note the discrete deposition of P^{32} which occurs in cortical bone as represented in C.

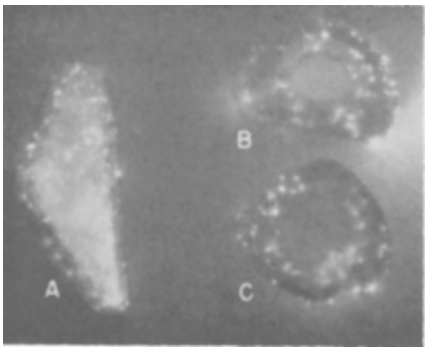

FIG. 2. Autoradiograms of foot bones from the cow. (A) a tarsal bone, (B) metacarpal; (C) metatarsal. Note the more uniform deposition of P^{32} in trabecular bone (central area in A) as compared to the discrete deposition in cortical bone (B, C, and outer area in A).

in this anatomically remote bone indicates that the labile phosphorus pool must extend through every part of the skeleton. This bone has no trabecular region and the P^{32} is all present in the outer cortical bone. The larger tail bone (A, Fig. 1) contains trabecular areas and shows a correspondingly greater P^{32} content inside the bone. The labile pool in cortical bone as illustrated by the cross section through the tibia shaft (C, Fig. 1) exists in discrete deposits and is apparently associated with the Haversian system that permeates bony tissue. This relationship would be expected since the small blood vessels of the bone pass along the Haversian canals and the "labile" pool must maintain intimate contact with these blood vessels to permit rapid mineral exchange.

Fig. 2 shows the distribution of this labile phosphorus pool in 3 bones of the foot, one of which, a tarsal bone (A), contains extensive trabecular bone. The general distribution of the labile pool throughout the trabecular region as opposed to the discrete distribution of this pool in cortical bone is apparent in these autoradiograms. This difference in distribution of the labile pool probably results from differences in the nature of the blood supply to these 2 types of bone. In cortical bone only the phosphorus along the Haversian canals has sufficient contact with the blood supply for rapid exchange while the less dense trabecular bone has a much larger surface in contact with the tissue fluids and thus a greater quantity of phosphorus may exchange per unit of time.

Summary. 1. Radioactive P^{32} was injected intravenously to measure the "labile" phosphorus pool in the skeleton of a mature, lactating dairy cow. This pool contains about 1% of the total skeletal phosphorus which was 15 times as large as the total circulating phosphorus pool in the plasma. 2. Autoradiograms and radioassays indicated that this labile pool extended throughout the skeleton but its distribution was determined by the type of bone involved. Cortical bone contains only one-third as much phosphorus in the labile state as does trabecular bone. 3. About 12% of the injected phosphorus-32 was present in the skeleton at the time of slaughter of the animal, 3 days after the injection.

1. Manly, R. S., Hodge, H. C., and Manly, M. L., *J. Biol. Chem.*, 1940, v134, 293.

2. Copp, D. H., Hamilton, J. G., Jones, D. C., Thompson, D. M., and Cramer, C., Metabolic Interrelations 3rd Conf. Jan. 8-9, 1951, p. 226-258.

3. Falkenheim, M., Neuman, W. F., and Hodge, H. C., *J. Biol. Chem.*, 1947, v169, 713.

4. Comar, C. L., Lotz, W. E., and Boyd, G. A., *Am. J. Anat.*, 1952, v90, 113.

5. Neuman, W. F., and Mulryan, B. J., *J. Biol. Chem.*, 1950, v185, 705.

6. Singer, L., and Armstrong, W. D., PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 229.

7. Singer, L., Armstrong, W. D., and Premer, M. L., PROC. SOC. EXP. BIOL. AND MED., 1952, v80, 643.

8. Kleiber, M., Smith, A. H., Ralston, N. P., and Black, A. L., *J. Nutr.*, 1951, v45, 253.

9. Fiske, C. H., and SubbaRow, Y., *J. Biol. Chem.*, 1925, v66, 375.

10. Bonner, J. F., Thesis University of Rochester (1948) as reported by Hodge, H. C., Metabolic Interrelations, First Conference, Feb. 7-8, 1949, p. 52.

11. Erf, L. A., PROC. SOC. EXP. BIOL. AND MED., 1941, v47, 287.

12. Harrison, H. E., and Harrison, H. C., *J. Biol. Chem.*, 1950, v185, 857.

13. Manly, M. L., Hodge, H. C., and Van Voorlis, S. N., PROC. SOC. EXP. BIOL. AND MED., 1940, v45, 70.

14. Hevesy, G. Ch., Levi, H. B., and Rebbe, O. H., *Biochem. J.*, 1940, v34, 532.

15. Manley, M., and Bale, W. F., *J. Biol. Chem.*, 1939, v129, 125.

16. Neuman, W. F., and Mulryan, B. J., *J. Biol. Chem.*, 1952, v195, 843.

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