# Skeletal Calcium Turnover in Non-Growing Rats.* (19717)

LEON SINGER, W. D. ARMSTRONG, AND MARY LOUISE PREMER.
*From the Department of Physiological Chemistry, University of Minnesota Medical School, Minneapolis.*

Singer and Armstrong(1) reported the existence in mature male rats of 2 kinds of skeletal calcium with markedly different rates of turnover. These forms of bone calcium were denoted as "mobile" and "fixed" fractions. Copp *et al.*(2) have also found evidence for the presence of a labile calcium fraction, amounting to approximately 15% of the total skeletal calcium, in the bones of young animals. Neuman and Mulryan(3) indicated that the mobile and fixed bone calcium fractions are the result of 2 distinct processes: (a) rapid ionic exchange of calcium for that on the surface of apatite crystals (the labile fraction), and (b) a slow recrystallization of the apatite crystals. The result of the latter process is to cause $Ca^{45}$ to become situated in relatively inaccessible locations in the interior of the crystals, thus producing the stable bone calcium.

This study, with mature female rats, was carried out in order to eliminate the influence of skeletal growth on the results. Skeletal accretion has the effect of reducing calcium specific activity of bones(1) and might also render $Ca^{45}$ in bones unavailable for removal by exchange through incarceration under layers of newly deposited bone or through deposition in older and more heavily mineralized Haversian systems(4,5). Unlike male rats, some female rats which can be selected by observation, attain a constant body weight. It was, therefore, assumed that such animals establish a nearly constant skeletal mass.

*Experimental.* Thirty female rats of the Sprague-Dawley strain whose weights had reached a plateau within the range 240-270 g were each given 3 intraperitoneal injections, at 2-hour intervals, of a high specific activity $Ca^{45}$ solution. A total of approximately one million counts of $Ca^{45}$ was administered. The animals were housed for 2 days following the injections in metabolism cages in order to permit the collection of the excreta and thus the estimation of the fraction of the injected $Ca^{45}$ which was promptly excreted. The fraction of the injected dose remaining in the animals (Table I) after 2 days was assumed to have been fixed in large part in the calcified tissues. The animals were sacrificed in groups of 2-4 animals at one to 210 days after the administration of the radioisotope. Two days before the animals were to be sacrificed they were again placed in metabolism cages and urine collections were made over the 24 hours prior to sacrifice. During this 2-day period the animals were fed a diet devoid of calcium, but otherwise adequate, in order to eliminate contamination of the urine with spilled food calcium.

The femora, humeri, teeth, pelt,† and 3 lower lumbar vertebrae were obtained for analysis. The 4 epiphyseal ends of the femora of a single animal were pooled as one sample and the 2 diaphyses of the same bones were combined as another sample. The carcass residue, which consisted of the remainder of the animal, was ground in a meat grinder and then converted into an homogenate with a small amount of water in a Waring blendor. Three large aliquots of this suspension were taken for analysis and the results were averaged. All samples were dried, ashed at 700°C, dissolved in hydrochloric acid solution, and diluted to known volumes. The ash of the feces and of the homogenate samples required fusion with sodium carbonate at 900°C be-

* This study was supported by grants from the Research Grants Division of the U. S. Public Health Service and the Graduate School of the University of Minnesota. The radiocalcium employed in the experiments was obtained from the Oak Ridge National Laboratory on allocation from the U. S. Atomic Energy Commission.

† At 28 days the pelt was found to contain negligible quantities of $Ca^{45}$. Accordingly this tissue from animals sacrificed at subsequent days was discarded without analysis. It appears probable that most of the $Ca^{45}$ associated with the pelt is derived from saliva or urine.

TABLE I. Turnover of Calcium Labeled with Radiocalcium in Non-Growing Female Rats.

| Day of sacrifice | No. of animals | Total calcium (mg) | % injected dose remaining: In animal after 2 days | In animal at sacrifice | In skeleton at sacrifice | Specific activities (% injected dose/mg Ca): Skeleton | Plasma | Urine |
|---|---|---|---|---|---|---|---|---|
| 1 | 2 | 3000 (242) | | 62.9 (1.4) | 53.8 (1.5) | .0199 (.0012) | .7631 (.0131) | 2.2380 (.0810) |
| 5 | 2 | 3203 (120) | 62.7 (3.9) | 51.7 (4 ) | 44.6 (5.3) | .0148 (.0023) | .0960 (.0327) | .1585 (.0946) |
| 10 | 2 | 2889 (166) | 67.1 (2.3) | 43 (3.7) | 29.2 ( .1) | .0109 (.0003) | .0778 (.0019) | .0760 (.0001) |
| 20 | 2 | 2821 (157) | 64.4 (2.8) | 42.4 (2.7) | 33.2 (2 ) | .0124 (.0004) | .0295 (.0012) | .0328 (.0070) |
| 28 | 4 | 2936 (168) | 64.1 (1 ) | 38.2 (5.1) | 29.7 (5.2) | .0122 (.0016) | .0250 (.0039) | .0291 (.0087) |
| 40 | 2 | 3255 (143) | 72.6 (1.4) | 38.8 ( .1) | 32.9 (0 ) | .0106 (.0005) | | .0154 (.0007) |
| 50 | 2 | 3210 (288) | 72.2 (6.2) | 33.8 ( .8) | 30.8 ( .9) | .0101 (.0012) | | .0536 (.0055) |
| 70 | 4 | 3254 (128) | 74.2 (2.2) | 35.7 (1.3) | 34.8 (2 ) | .0112 (.0006) | .0106 (.0009) | .0116 (.0025) |
| 90 | 3 | 2987 (40) | 74 (4.3) | 31.5 (7.7) | 29.2 (6.5) | .0102 (.0022) | .0083 (.0017) | .0095 (.0015) |
| 120 | 3 | 2943 (109) | 73.2 (1 ) | 23.3 (7.8) | 22.8 (8.7) | .0081 (.0025) | .0090 (.0021) | .0069 (.0014) |
| 154 | 2 | 3422 (107) | 75 (1.9) | 27.7 (2.4) | 27.3 (2.3) | .0083 (.0004) | .0069 (.0014) | .0111 (.0021) |
| 210 | 2 | 3223 (73) | 72.8 (1.3) | 23.6 (1.3) | 23.3 (1.3) | .0075 (.0002) | .0062 (.0001) | .0063 (.0006) |

Numbers within parentheses give the range of the results from the mean (2 animals) or average deviations from mean results (3 or 4 animals).

TABLE II. Calcium Specific Activities of Bones.

| Day of sacrifice* | No. of animals | Specific activities (% inj. dose/mg Ca): Vertebrae | Femur epiphysis | Femur diaphysis | Humeri |
|---|---|---|---|---|---|
| 1 | 2 | .0204 (.0014) | .0188 (.0004) | .0074 (.0003) | .0136 (.0002) |
| 5 | 2 | .0180 (.0026) | .0173 (.0008) | .0088 (.0021) | .0150 (.0027) |
| 20 | 2 | .0154 (.0010) | .0154 (.0017) | .0078 (.0010) | .0146 (.0010) |
| 210 | 2 | .0105 (.0015) | .0080 (.0002) | .0060 (.0004) | .0065 (.0002) |

Numbers within parentheses give range of results from the mean.
* To economize space, results for the selected days only are given.

fore solution in acid. The analytical methods and those used for the estimation of $Ca^{45}$ have been described(1).

*Results and discussion.* The means of the results obtained from the animals sacrificed at each time interval are given in the tables. The total calcium content of the animals and the fraction of injected $Ca^{45}$ present at sacrifice are the sums of total calcium and of $Ca^{45}$ found in the individual components of the animal bodies. The results in the column headed "In skeleton at sacrifice" (Table I) exclude $Ca^{45}$ in the teeth and pelt. The fractions of injected $Ca^{45}$ given in this column are attributed to the skeleton because most of the body calcium of the edentulous and skinned carcass is in the skeleton. The specific activities of the skeletons were calculated by excluding the total calcium and $Ca^{45}$ contents of the teeth and pelt.

The total calcium content of the animals exhibited some variation despite careful selection of animals of comparable age and weight. However, in contrast to the results obtained with male rats(1), there appears to have been no consistent increase of body calcium with increasing age of the animals, thus attesting

to the probable low order of bone accretion in individual animals during the experimental period. The percentage of the radioisotope retained after 2 days (difference between that injected and that excreted) was not closely correlated with the total calcium in the animals and showed an extreme variation between 62.7 ($\pm$3.9) and 75.0 ($\pm$1.9)%.

The percentage of injected $Ca^{45}$ present in the animals and in the skeletons at sacrifice decreased rapidly during the first 10 days after the injections. From Day 10 through Day 90 the quantity of radioisotope retained in the skeletons remained nearly constant. It appears that a further decline in the fraction of $Ca^{45}$ retained may have occurred after Day 90. In the light of our previous study(1) these findings are interpreted to indicate a rapid turnover and excretion of $Ca^{45}$ from a labile bone fraction during which time a large part (*circa* one-third) of the $Ca^{45}$ became incorporated into more stable locations from which it was only slowly released. These results are consistent with the concept(3) that calcium of the surface of bone crystals is rapidly exchangeable while that of the interior of crystals is made available for turnover more slowly by recrystallization.

The specific activities of the skeletons, plasma, and urines also furnish evidence of the existence of forms of skeletal calcium with different rates of turnover. The animals sacrificed on Day 1 excreted urine with a calcium specific activity many fold that of the plasma, due, undoubtedly, to early excretion of high specific activity calcium immediately after the injections and to a rapid decline during the first day in plasma calcium specific activity. By the 10th day the specific activities of the plasma and urine became equal, within the limit of experimental variation, and remained so throughout the rest of the experiment. At Day 10 the specific activity of the plasma was 7 times greater than that of the skeleton. Only after Day 50 did the plasma specific activity fall to values equal, within the experimental variation, to those of the total skeletal calcium. It is, therefore, suggested that through Day 50 a relatively rapidly exchangeable form of skeletal calcium was removed by excretion or incorporation into a stable form and attained equilibrium with the calcium of the plasma. Beginning with Day 70, $Ca^{45}$ in the plasma was maintained by the slow turnover of the more stable form of skeletal calcium.

In the previous study(1) with male rats whose skeletal calcium nearly doubled during the experimental period, 42-45% of the injected $Ca^{45}$ became incorporated in the stable bone fraction. In the present study with non-growing female animals, not more than about one-third of the administered $Ca^{45}$ was present in the stable bone fraction through Day 90 and this quantity declined to about one-fourth of the injected radioisotope from Day 120 through Day 210. It is suggested that the higher fraction of administered $Ca^{45}$ initially incorporated by the skeletons of male rats and the greater quantity of the radioisotope present in the "fixed" bone compartment of the male animals were due to their greater bone accretion. In the growing animal $Ca^{45}$ is accumulated in bones not only by exchange but in addition by deposition of bone salt from a medium containing the radioisotope.

The results presented in Table II show that various skeletal components differ in their abilities to accumulate $Ca^{45}$ and in turnover of the isotope. The vertebrae, femur epiphyses and humeri accumulated per unit weight of calcium relatively more $Ca^{45}$ than the femur diaphyses. Also, the femur diaphyses retained through Day 210 about 80% of their original $Ca^{45}$ content while the other bones allowed the removal of about 50% of the radioisotope present at Day 1. These findings indicate that the quantitative features of calcium turnover by exchange and recrystallization vary in different bones. They are also in agreement with the work of Comar, Latz, and Boyd(6) who also found that anatomical location in a given bone influences its turnover of calcium.

*Summary.* Total calcium and radioactive calcium contained in selected skeletal components and in the entire bodies was determined in non-growing female rats at intervals up to 210 days after administration of the radioisotope. Evidence for fractions of skeletal calcium with markedly different rates of

turnover was obtained from the changing rates with time in removal of radiocalcium from the skeleton and from comparisons of calcium specific activities of the skeleton and plasma of the animals sacrificed at increasing times after administration of the radiocalcium. In this study the effect of skeletal accretion was minimized. The results confirm those previously obtained when skeletal growth was a possible factor influencing the results. These findings and interpretations are consistent with the concept that the bone salt exchanges the calcium of crystal surfaces rapidly and that of the interior of crystals more slowly by recrystallization. The specific activities of different bones and the change in specific activity of the bones with time indicate that anatomical location of bone influences its calcium turnover.

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

2. Copp, D. H., Hamilton, J. G., Jones, D. C., Thompson, D. M., and Cramer, C., Third Conference on Metabolic Interrelations, Josiah Macy, Jr. Foundation, New York, 1951, p226.

3. Neuman, W. F., and Mulryan, B. J., *J. Biol. Chem.*, 1952, v195, 483.

4. Engfeldt, B., Enstrom, A., and Zetterstrom, R., *Biochimica et Biophysica Acta*, 1952, v8, 375.

5. Amprino, R., *Experientia*, 1920, v8, 20.

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

Received July 14, 1952. P.S.E.B.M., 1952, v80.

## A Rapid Method for Distinguishing D-Glucosamine from Galactosamine in Biological Preparations. (19718)

MILTON W. SLEIN. (Introduced by Riley D. Housewright)
*From the Chemical Corps Biological Laboratories, Camp Detrick, Frederick, Md.*

In the course of a study of the constituents of a polysaccharide isolated from *Shigella flexneri*(1), it became necessary to be able to distinguish between the 2 naturally-occurring aminosugars, glucosamine and galactosamine. These substances have very similar $R_f$ values and do not separate well in a number of solvent systems(2). The N-2,4 dinitrophenyl derivatives separate only slightly better(3) and methods for isolating derivatives involve more laborious procedures with relatively large amounts of aminosugars(4,5).

Brown reported the phosphorylation of D-glucosamine by hexokinase, and adenosinetriphosphate (ATP), the product being identified as D-glucosamine-6-phosphate(6). As is shown below, galactosamine does not function as a substrate for hexokinase. It is therefore, possible to differentiate between these aminosugars; and, in cases where these are the only 2 aminosugars present, the relative amounts of each may be determined.

*Materials.* The aminosugars used in the experiment described by Fig. 1 were partially purified from crude hydrolysates of the polysaccharide of *Sh. flexneri*(1) and bovine tracheae(7,8) by passage over a cation exchange resin and elution with HCl. The yeast hexokinase was also only partially purified(9).

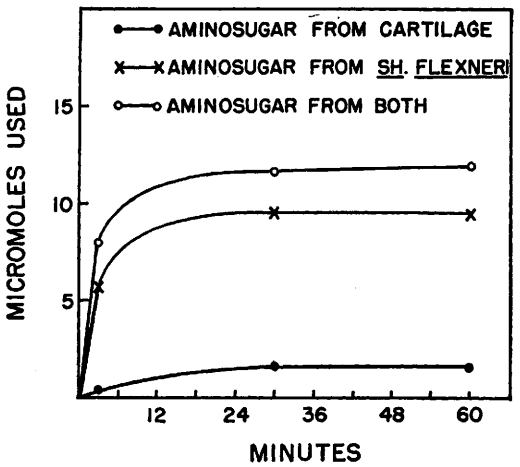


FIG. 1. Presence of D-glucosamine in hydrolysates of the polysaccharide of *Sh. flexneri*. The reaction mixture contained 33 micromoles ATP, 13 micromoles galactosamine from chondroitin sulfuric acid or 11 micromoles aminosugar from the polysaccharide of *Sh. flexneri*, or both, .006 M $MgCl_2$ and yeast hexokinase in .06 M veronal buffer, pH 8. Incubated at 30°C. The utilization of aminosugar corresponds to the D-glucosamine content of the reaction mixture.