CONCURRENT USE OF RADIOISOTOPES OF CALCIUM AND PHOSPHORUS IN THE STUDY OF THE METABOLISM OF CALCIFIED TISSUES*

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Important results have been obtained with tracer isotopes, particularly radiophosphorus, in the study of calcified tissues. Tracer studies with calcium have, however, been limited by the scarcity of the only suitable radioisotope of calcium, namely Ca$^{45}$ with a half life of 180 days. The radiocalcium employed in previous studies (1-4) and in the present investigation was prepared with difficulty by bombardment of calcium metal with deuterons in a cyclotron (1, 2). At the present time it appears that insufficient amounts of radiocalcium will be available from neutron-induced nuclear transformations in the uranium pile for use in extensive studies with intact animals (5). Because of these circumstances it is unlikely that experiments such as that described in this paper can be extended in the near future. For this reason the work is now reported in its present state of progress.

Campbell and Greenberg (1) studied the distribution of Ca$^{45}$ in the tissues and excreta of a rat following the intragastric administration of the isotope as the lactate. Their findings of a nearly equal specific retention of Ca$^{45}$ by bones and teeth (pooled incisors and molars) on a dry weight basis does not give the correct impression as to the relative rates of turnover of calcium by bone and dental hard tissues. No distinction was made between the continuously growing incisor teeth and the non-growing molars nor between the enamel and dentin. Also, the Ca$^{45}$ retentions were not recorded in relation to the calcium contents of the tissues. Incidental to another study Greenberg (4) examined the molar and incisor teeth of rats but did not separate the enamel and dentin of either type of teeth and again reported the results on a dry weight basis. The uptake of Ca$^{45}$ by the femurs was greater than that of the molar teeth but was less than that of the incisors. On a priori grounds it would be expected that dental hard tissues would exchange calcium to a lesser degree than bone, a situation which has been demonstrated with respect to the exchange of phosphorus labeled with P$^{32}$ (6, 7).

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The experiments reported in this paper were carried out in order to obtain data which would allow a comparison of the turnover of calcium in the normal calcified tissues and to compare in the same subject the turnover of calcium with that of phosphorus. To this end both Ca\textsuperscript{45} and P\textsuperscript{32} were administered to the animal and a chemical separation of calcium and phosphorus effected before the radioactivity measurements were made.

**Experimental**

A solution containing Ca\textsuperscript{45} and P\textsuperscript{32} was prepared by adding sufficient hydrochloric acid to a suspension of calcium lactate and sodium phosphate to dissolve the solids. 8 cc. of the solution containing about \(0.6 \times 10^6\) counts per minute of Ca\textsuperscript{45} (122.3 mg. of Ca\textsuperscript{1}) and nearly \(9 \times 10^6\) counts per minute of P\textsuperscript{32} (6.7 mg. of P) were administered to a male rat weighing 238 gm. The syringe and stomach tube were washed and the Ca\textsuperscript{45} and P\textsuperscript{32} contents of the washings taken into account in the calculation of the actual administered doses of the radioisotopes. The animal was fed a normal diet and the urine and feces separately collected for 5 days, at the end of which period the animal was sacrificed and a blood sample obtained.

The contents of the intestine were added to the feces. The animal was skinned and the femurs and teeth removed. The remainder of the carcass (hereafter referred to as carcass residue) was ground in a food chopper and, with added water, converted into a suspension in a Waring blender. Both epiphyseal ends of the femurs were cut off and the marrow removed from the diaphyses. The marrow, contained in a platinum dish, was extracted repeatedly with a mixture of alcohol and ether in equal parts. The epiphyses and diaphyses of the femurs were broken into fragments and extracted overnight in a Soxhlet apparatus with an alcohol-ether mixture. The molar teeth were pooled as one sample and the incisor teeth as another sample. After the teeth had been broken into bits and fat extracted, the enamel and dentin were separated by the method of Manly and Hodge (8). The serum proteins, after precipitation with trichloroacetic acid, were combined with miscellaneous fractions such as the red blood cells, the residue from the evaporation of the solvents of the alcohol-ether extractions, the fractions of the teeth not pure enamel or dentin, etc. This mixture was treated as a separate sample labeled debris. All samples, other than the protein-free serum, were dried and ashed at 500°C to constant weight. The ash of each sample was dissolved in hydrochloric acid and diluted to a convenient volume. From this point all samples were treated in a uniform manner.

An excess of ammonium oxalate was added to an aliquot of the ash

\(^1\) The large dose of calcium given the animal was necessitated by the low specific activity of the preparation of Ca\textsuperscript{45}.\textsuperscript{1}
solution in a centrifuge tube and the resulting precipitate centrifuged. Ammonium hydroxide was added to the supernatant liquid until the pH was about 4 (brom-cresol green) and the solution of the newly formed precipitate centrifuged. The pH of the supernatant liquid was then carefully adjusted to 5 and the tube allowed to stand overnight and again centrifuged. The supernatant liquid and two washings of the precipitate were transferred to a volumetric flask. The contents of this flask, after being diluted to volume, were used for total phosphorus analyses and for \( \text{P}^{32} \) measurements.

The precipitate of calcium oxalate contained in the centrifuge tube was dissolved in hydrochloric acid and reprecipitated by the addition of ammonium oxalate at pH 5. The supernatant liquid was poured off, the precipitate again dissolved, and the calcium precipitated a third time as the oxalate. This precipitate was dissolved in hydrochloric acid and made to a convenient volume for subsequent determinations of total and radio-calcium.

Phosphorus was chemically determined by the method of Fiske and Subbarow (9) adapted to the Evelyn photoelectric colorimeter. Radioactive phosphorus was measured with a dipping Geiger-Müller counter tube (10) coupled to a variable scaling unit. Calcium was determined chemically by the method of Clark and Collip (11). Radioactive calcium was counted in precipitates of calcium oxalate with a modified Libby (12) screen-walled counter. This counter tube was arranged so that a standard and a sample prepared in the same manner could alternately be brought under the sensitive region of the counter. In this way the activities of the standard and unknown were measured under identical conditions of counter characteristics. The background was determined, without opening the counter, by moving both samples away from the sensitive region of the counter.

Aliquots of the calcium solutions, after the determination of total

2 The supernatant liquids from the second precipitation of calcium oxalate from all samples except serum were examined for \( \text{P}^{32} \). These liquids contained, on an average, 0.2 per cent of the \( \text{P}^{32} \) present in the supernatant fluid and washings of the first precipitation of calcium oxalate. It thus appears that essentially all of the phosphorus in the samples was present in the solutions employed for the analyses for total phosphorus and \( \text{P}^{32} \).

3 In an experiment in which a large amount of the \( \text{P}^{32} \) was added to a solution of non-radioactive calcium it was found that the calcium oxalate after three precipitations contained 0.0001 per cent of the original \( \text{P}^{32} \). It thus appears that the treatment described in the text effectively separated the phosphorus from the calcium and assured that the observed activity of the calcium oxalate precipitates was not due to the contamination with \( \text{P}^{32} \). This probability is further increased by the fact that the calcium was precipitated a fourth time (see the text) as the oxalate before the \( \text{Ca}^{46} \) was counted in the screen wall counter.
calcium, were taken so as to contain 8 mg. of calcium. Calcium oxalate was precipitated and collected by gentle suction on filter paper over a uniform area and washed with water and with acetone and ether. After the ether had evaporated, the precipitate and paper were transferred to the counting tube which was exhausted of air and left overnight in communication with a container of phosphorus pentoxide.

All radioactivity measurements were corrected for the resolving time losses and were calculated to a given date. An effort was made to keep the statistical error of the radioactivity measurements to less than 2 per cent of the observed counting rate but this was not feasible in the case of the measurements of Ca\textsuperscript{45} in serum, femur marrow, molar dentin, and molar enamel because of the low activities observed. The reproducibility of the technique of Ca\textsuperscript{45} determination is indicated by duplicate determinations of the activity of the calcium in feces, which agreed within 2.8 per cent. Duplicate Ca\textsuperscript{45} determinations in the urine agreed within 2.2 per cent.

RESULTS AND DISCUSSION

Of the administered radioisotopes 91.2 per cent of the Ca\textsuperscript{45} and 95.1 per cent of the P\textsuperscript{32} were recovered in the tissues and excreta of the animal. The lower recovery of Ca\textsuperscript{45} is possibly to be accounted for by the losses in the multiple precipitations of calcium oxalate. The feces contained 64.5 per cent of the administered Ca\textsuperscript{45} and 33.2 per cent of the P\textsuperscript{32}. The urine contained 2.05 per cent of the administered Ca\textsuperscript{45} and 7.42 per cent of the P\textsuperscript{32}. The large fecal output of both of the radioisotopes is probably a result of the very large amount of calcium which had to be administered. Campbell and Greenberg (1) who were able to give much less calcium as the lactate, with a higher specific activity than that employed in this study, found only 10.8 per cent of the isotope in the feces of a rat.

The results obtained with the tissues and fractions of the body are shown in Table I. Because of the unequal excretion of the isotopes and because of their unequal recoveries these data are not referred to the administered doses of the isotopes but are recorded as the actual recovered activities found in the several fractions of the animal and in the body as a whole. In the columns headed "Relative specific activity" the specific activities (i.e. counts per minute per mg. of P\textsuperscript{32} or Ca\textsuperscript{45}) of the various fractions are compared with the average specific activities of these elements in the body.

The results presented show that the uptake and retention of both P\textsuperscript{32} and

\footnote{In certain cases such as serum calcium, bone marrow calcium, and standards it was necessary to add normal calcium to the solution to make the total 8 mg. before precipitating the calcium oxalate for counting.}
Ca\textsuperscript{45} by calcified tissues stand in the following decreasing order:\textsuperscript{5} bone marrow, skeletal epiphysis, skeletal diaphysis, incisor dentin, incisor enamel, molar dentin, and molar enamel. The higher uptake of the radioisotopes by the fractions of incisor teeth than by those of molar teeth is due in large part to the fact that the incisor teeth of the rat in contrast to the molar teeth are organs of continuous growth and eruption. The tracer isotopes present in the molar teeth were acquired by exchange of normal isotopes of the elements for the labeled isotopes. The incisor enamel and

\textbf{Table I}

\begin{table}
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\begin{tabular}{lllllll}
\hline
\textbf{Fraction}   & \multicolumn{2}{c}{\textbf{Phosphorus}} & \multicolumn{2}{c}{\textbf{Calcium}} & \\
                    & \textbf{Total P in fraction} & \textbf{Specific activity of P\textsuperscript{32}} & \textbf{Relative specific activity} & \textbf{Total Ca in fraction} & \textbf{Specific activity of Ca\textsuperscript{45}} & \textbf{Relative specific activity} \\
\hline
Skin and hair       & 37.0 & 6245.3 & 168 & 5.9  & 100.1 & 163  \\
Carcass residue     & 1090.8 & 3763.1 & 101 & 2030.3 & 63.5  & 103  \\
Serum               & 0.05 & 3494.3 & 94  & 0.14 & 84.2  & 137  \\
Femur marrow        & 0.56 & 5325.0 & 143 & 0.60 & 55.7  & 91   \\
`` epiphyses       & 16.3 & 2927.7 & 80  & 38.9 & 56.7  & 92   \\
`` diaphyses       & 55.8 & 2785.2 & 75  & 150.9 & 50.6  & 82   \\
Incisor dentin      & 18.4 & 1940.9 & 52  & 37.9 & 33.8  & 55   \\
`` enamel           & 3.3  & 992.4  & 27  & 9.8  & 16.4  & 27   \\
Molar dentin        & 5.3  & 536.0  & 14  & 13.2 & 7.2   & 12   \\
`` enamel           & 4.3  & 100.2  & 2.7 & 11.0 & 0.75  & 1.2  \\
Débris             & 12.9 & 2097.1 & 78  & 17.8 & 74.6  & 116  \\
\hline
Total body          & 1245.7 & 3712.5 & 100 & 2316.4 & 61.4  & 100   \\
\hline
\end{tabular}
\end{table}

\* Counts per minute per mg. of P.
\textsuperscript{t} Ratios of relative specific activities of P\textsuperscript{32} and Ca\textsuperscript{45}.

dentin acquired the tracer elements by the same process of exchange and also by deposition of newly formed mineral phase from a medium containing the radioelements.

The figures given in the last column of Table I are not a measure of the absolute relationship of P\textsuperscript{32} and Ca\textsuperscript{45} uptake. Knowledge as to the number of phosphorus atoms turned over in relation to the number of calcium atoms turned over cannot be obtained from this experiment because there is no way of knowing the manner in which the specific activity of the plasma inorganic phosphate and ionized calcium varied with respect to one another. These calculated results do afford a comparison of the relative uptake of

\textsuperscript{5} Except for Ca\textsuperscript{45} uptake in marrow and epiphysis which was nearly identical.
and Ca\textsuperscript{46} by the tissues studied in \textit{relation} to the average distribution of the isotopes throughout the body. Since the apatite mineral phase is essentially the same in all calcified tissues (13, 14), the difference in rates of exchange of calcium and phosphorus is probably due to physiological or structural factors which produce differences in rates of migration of calcium and phosphate ions into the various calcified tissues. A suggestion that such factors operated is seen from the fact that the relative uptake of P\textsubscript{32} to Ca\textsuperscript{46} by the calcified tissues stands in an inverse relationship to the order of the uptake of the radioelements.

\textbf{SUMMARY}

Methods have been presented for the separation of radiocalcium and radiophosphorus in tissues which allow the concurrent use of these isotopes. The uptake and retention of both radiocalcium and radiophosphorus by calcified tissues stand in the following decreasing order: femur, marrow, femur epiphysis, femur diaphysis, incisor tooth dentin, incisor tooth enamel, molar tooth dentin, and molar tooth enamel. Evidence has been presented that under conditions \textit{in vivo} calcified tissues do not all exchange calcium and phosphorus in the same proportion.

We wish to express our thanks to Dr. D. M. Greenberg who generously shared his supply of radiocalcium with us and to Dr. E. O. Lawrence who donated the radiophosphorus used in this study.

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