dence is available to substantiate one or the other of these viewpoints.

Summary. The uptake of probenecid by kidney cortex slices was examined over a 100-fold concentration range. This study offers direct evidence implicating tissue binding in the slice uptake process. For example, the steady-state accumulation conformed to a modified "Scatchard plot". The data were interpreted to mean that two populations of binding sites exist. In addition it was shown that probenecid was bound by kidney cortex homogenates, but not by liver or renal medulla homogenates, an observation in keeping with slice uptake data reported previously.

The technical assistance of Mrs. Dace Grote is gratefully acknowledged.

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Ionic Interaction with Bone Mineral. III. Reversible Calcium Exchange with Bone Powder. (32383)

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In *in vivo* kinetic studies of intravenously administered radiocalcium in man(1-3), a change of slope in the specific activity (SA) curve of blood is sometimes encountered at about the 7th day after isotope is given, with a subsequent slower rate of decline. Some workers have attributed this phenomenon to the transfer of isotope from bone back into blood as a result of bone resorption(1,4).

An alternative explanation has been offered by Nordin and associates on the basis of *in vitro* studies with bone slices(5). They believed that the "bend" in the SA curve can be accounted for by "back diffusion" of radiocalcium from bone into solution rather than by physiologic bone resorption. The curve before the "bend" was considered to reflect only "diffusion" into bone.

The present experiments were designed to test the validity of the hypothesis of Nordin and associates(5). From the wash-out studies (6,7) of defatted bone powder and of synthetic hydroxyapatite labelled with ^{45}Ca, the release of radiocalcium from the sample was measured at various times after labelling. Contrary to the findings of Nordin and associates, our results suggest that "back-diffusion" is not limited to the period after the inflection in the SA curve, but occurs before the inflection as well.

Materials and methods. Fresh rat femur and tibia were cleaned of adhering tissue and freeze-dried. The specimen was then ground to powder in a mortar, defatted with ethanol-ether and dried. Bone powder obtained between 105 and 250 μ sieves (Central Scientific Co.) was employed in the study. The calcium-to-phosphate molar ratio of the sample was 1.70. Synthetic hydroxyapatite (B-R Apatite) was the same as that used previously(6,7).

The method for measuring ^{45}Ca exchange was reported previously(6,7) and will be described here only briefly. 500 mg of bone powder (or B-R Apatite) were preincubated

in 250 ml of solution which had been buffered to pH7.2 with 1.25 mmoles barbiturate and brought to ionic strength of 0.15 with NaCl. To facilitate the attainment of ionic steady state, the pre-incubation solution contained approximately the concentrations of calcium and phosphate which were expected at steady state. These were 0.63 mM calcium and 0.29 mM phosphate for bone powder(8) and 0.2 mM calcium and 0.2 mM phosphate for B-R Apatite(6,7). Ionic steady state was reached in about 36 to 72 hours, as shown by the lack of significant change in pH (7.2 ± .1), and in calcium and phosphate concentrations of the solution during the subsequent 3 to 14 days. (See legend, Fig. 1.)

After the attainment of ionic steady state, ^{45}Ca was added to the suspension. Isotope uptake by bone powder (or hydroxyapatite) was estimated by periodically removing one-ml aliquots of the suspension, and counting the filtrate in a scintillation counter. This procedure differs from that of Nordin and associates(5) who enclosed their bone sample in dialysis tubing; our procedure avoided possible Donnan membrane effects.

Wash-out experiments(6,7) were performed on the labelled crystals or bone powder to test the reversibility of uptake. After varying periods of exposure to ^{45}Ca, the samples were collected by filtration and resuspended in isotope-free buffer solution. The isotope release was followed by counting the filtrate. Except in those experiments which were designed to test the effect of varying the calcium concentration of solution, the pH, ionic strength and calcium and phosphate concentrations of the solution during the wash-out experiments were essentially identical to those of the steady state solution. The calcium concentration of the solution never changed by more than 4% during the course of wash-out.

Studies with bone powder were conducted at 37°C, those with B-R Apatite at 22°C.

Results. The disappearance of ^{45}Ca radioactivity from the solution as a result of uptake by bone powder is shown in Fig. 1. The SA of the solution as a function of time was comparable to that reported by Nordin and associates for bone slices(5). However, there was a gradual decrease in the rate of decline

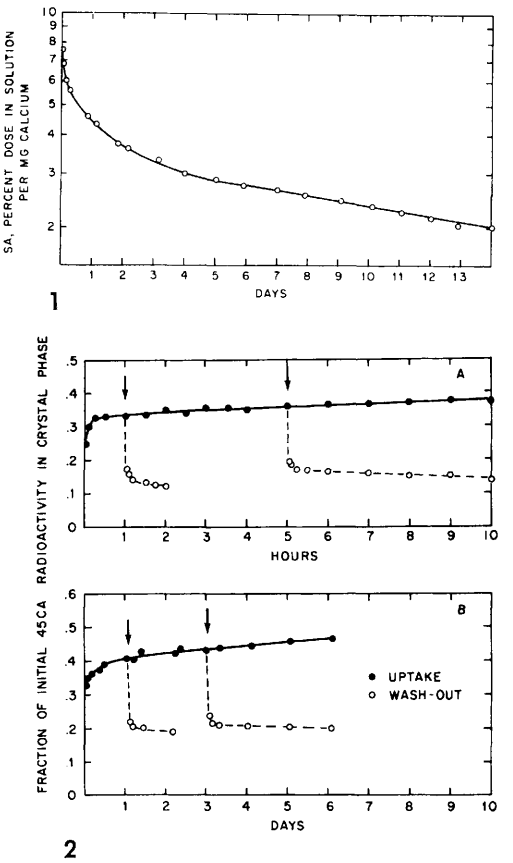


FIG. 1. ^{45}Ca uptake by bone powder as shown by decrease of SA of solution. Pre-incubation medium contained 0.63 mM calcium and 0.29 mM phosphorus (as orthophosphate). The steady-state solution contained 0.72 ± 0.02 (S. E. M.) mM calcium and 0.16 ± 0.02 mM phosphorus.

FIG. 2. ^{45}Ca uptake and release from the labelled B-R Apatite. B-R Apatite was labelled with ^{45}Ca from solution containing 1.6 mM calcium, and was washed in non-isotopic medium of identical chemical composition at one hour and 5 hours, and at one and 3 days (arrows).

of SA, followed by a monoexponential fall after 4 days. Thus, in contrast to the findings of Nordin and associates(5), the "bend" in the curve was not sharp, and SA did not reach a constant value during the 14 days of study.

By analogy with the exchange studies of synthetic hydroxyapatites(6,7,9), the monoexponential fall in SA may be considered to represent an intracrystalline exchange process. This view is supported by the observation that the rate of fall in SA after 4 days was not significantly altered by changing the ionic strength of the solution from 0.15 to 0.05 or

to 0.005. The portion of the curve between 0 and 5 days reflects primarily exchange between the solution on the one hand, and the crystal surface and "hydration shell"(7,9) on the other. The time course of the fall in SA of the solution for B-R Apatite differs from that for bone in that the curve for synthetic hydroxyapatite becomes monoexponential at approximately 12 hours(6,7), as opposed to 4 days in the case of bone.* Thus, the wash-out experiments were performed after 12 hours for B-R Apatite and after 4 days for bone powder to determine the reversibility of isotope uptake after the inflection in the curve. Similarly, ^{45}Ca release from B-R Apatite labelled for less than 12 hours, and from bone labelled for less than 4 days was evaluated to test the reversibility of isotope uptake before the inflection.

^{45}Ca release from labelled B-R Apatite. ^{45}Ca uptake and release by B-R Apatite from 1.6 mM calcium solution are shown in Fig. 2. The uptake curve shows a "bend" at about 0.5 day, with a slower increase in radioactivity of the crystal phase after the "bend" (Fig. 2B). Wash-out experiments were performed before (Fig. 2A) and after (Fig. 2B) the "bend." The wash-out curves were qualitatively the inverse of the uptake curve: the initial rapid fall in crystal phase radioactivity is followed by a continued decline at a slower rate.

The efficiency of wash-out procedure was calculated with the following formula:

Per cent reversibility of uptake $= (1 - R_2/R_1)\cdot[100/(1 - R_1)]$ where R_1 is the fraction of initial radioactivity in solid (crystal phase or bone) at time zero of wash-out.

R_2 is the fraction of initial radioactivity remaining in solid after the wash-out procedure.

$(1 - R_2/R_1)$ is the fraction of radioactivity in the solid which appears in solution during wash-out, and

$(1 - R_1)$ is the fraction of initial radioactivity in solution at time zero of wash-out.

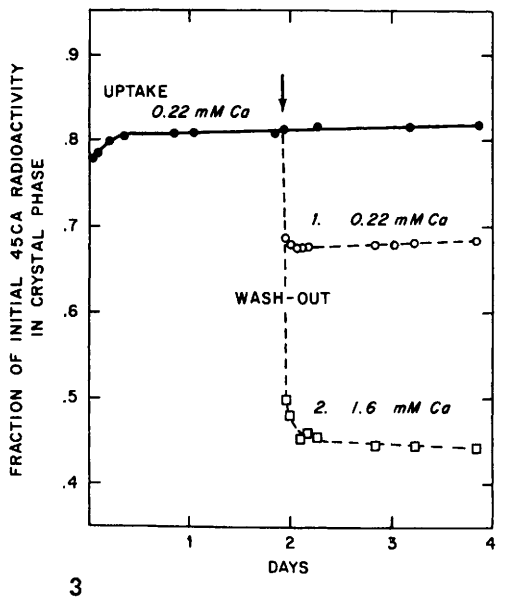
Table I shows greater than 93% reversibility of uptake at both before and after the "bend."

In Fig. 3, uptake and wash-out of B-R Apatite in 0.22 mM calcium solution are shown. The fraction of ^{45}Ca radioactivity of the crystal phase at this calcium concentration is greater than that in 1.6 mM calcium solution (compare Fig. 2 and 3). Further, the wash-out curve in 0.22 mM calcium solution is distinctly different: the initial rapid fall in crystal phase radioactivity was followed by a gradual rise (curve 1, Fig. 3). Further, there was only 83% reversibility (Table I). However, when the same preparation labelled in 0.22 mM calcium solution was washed in 1.6 mM calcium, much more isotope was lost from the crystal phase. Moreover, an initial rapid fall in crystal phase radioactivity was followed by a continued decline at a slower rate (curve 2, Fig. 3).

TABLE I. Per Cent Reversibility of ^{45}Ca Uptake. Column 1 refers to corresponding figures in text. Column 3 indicates initial calcium concentration of the wash-out solution. Per cent reversibility (column 6) was determined at the period of wash-out indicated on column 5.

1	2	3	4	5	6
Figure	Preparation	Ca of wash-out solution (mM)	Period of uptake	Period of wash-out	% Reversibility
2	B-R Apatite	1.6	1 hr	1 hr	95
		1.6	5 "	5 "	96
		1.6	1 day	1 day	93
		1.6	3 days	3 days	96
3	B-R Apatite	.22	2 "	2 "	83
4	Bone powder	.72	2 "	1/6 day	92
		.72	8 "	1 "	87
		.72	14 "	1 "	85

* This is probably a reflection of the surface area per gram of B-R Apatite which is very large compared to that of bone powder(10).



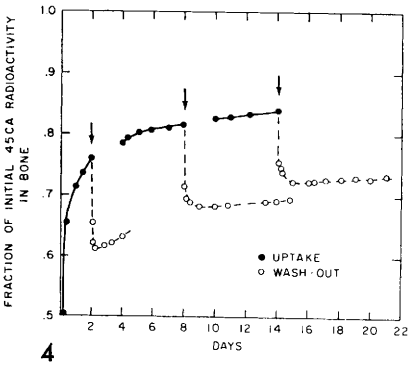


FIG. 3. ^{45}Ca uptake and release from B-R Apatite labelled from 0.22 mM calcium solution. Release into solutions containing 0.22 mM calcium (curve 1) and 1.6 mM calcium (curve 2) is shown.

FIG. 4. ^{45}Ca uptake and its release from labelled bone powder. Calcium concentration in the solution during uptake and during release was 0.72 ± 0.03 mM. The 3 pairs of uptake and wash-out curves represent results from 3 different samples of bone powder.

These results suggest that the wash-out curve is the inverse of uptake when there is a large fall in crystal phase radioactivity (approximately 45% or more) (Fig. 2; and curve 2, Fig. 3). However, when less than 25% of the crystal phase radioactivity appears in solution during wash-out (curve 1, Fig. 3), the initial fall in crystal phase radioactivity is followed by a slow net uptake of radiocalcium into the crystal phase. The wash-out curve therefore appears to reflect the combined influence of isotope release and isotope uptake. Where less than 25% of crystal phase radioactivity initially appears in solution, the crystal phase still retains greater than 75% of ^{45}Ca radioactivity. Thus, the quantity of isotope continuing to enter the "deeper calcium compartments" of the crystal phase would be considerable and a significant degree of uptake would persist during the wash-out. On the other hand, when the crystal phase radioactivity is reduced by more than 45%, much less radiocalcium is available for this uptake process. Thus, the quantity of ^{45}Ca leaving the crystal phase may be equal to or exceed that entering the deeper "compartments" of the crystal phase.

^{45}Ca release from labelled bone powder. In Fig. 4, the wash-out experiments on the labelled bone powder before and after the "bend" are shown. As in the wash-out from B-R Apatite in 0.22 mM calcium solutions (Fig. 3), the rapid fall in radioactivity of bone powder during the first 8 to 24 hours is followed by a gradual rise during the subsequent 2 to 7 days. The reversibility of uptake, calculated at the maximum of each wash-out curve, was 92% before the "bend" and 87 and 85% after the "bend" (Table I).

Discussion. Nordin and associates attempted to produce a model for studies of calcium dynamics in man(1-4) by performing "discard" experiments on the *in vitro* uptake of radiocalcium by bone slices(5). After adding radiocalcium to bone slices, they removed a fixed volume of fluid daily and replaced it with the same volume of isotope-free solution. A sharp "bend" was reported in the curve describing the disappearance of isotope from solution. These workers then compared the rate of "discard" of isotope expressed as per cent of "exchangeable" pool per day, with the rates of disappearance of isotope from solution. Since the rate of "discard" before the "bend" was lower than that of uptake, it was assumed that there was net "diffusion" of isotope into bone. Conversely, since the "discard" rate was greater than that of uptake after the "bend," it was assumed that there was net "back-diffusion" of radiocalcium from bone into solution.

We believe that this interpretation is mis-

leading. Our kinetic studies of the wash-out procedure on the labelled bone powder and on synthetic hydroxyapatite demonstrate an almost complete reversibility of ^{45}Ca uptake, regardless of the period of labelling (Table I). Thus, in contrast to the conclusions of Nordin and associates(5), "back-diffusion" (or release) is not confined to the period after the "bend" but occurs before it as well. The "discard" experiments of these workers represent a form of the wash-out procedure, but one less definitive than that employed in our study, since they removed only a portion of the labelling fluid and replaced it with non-isotopic medium. It is unfortunate that these workers did not follow the time course of isotope release after each "discard."

We believe that the SA curve of solution (Fig. 1) can best be explained as a reflection of exchange of calcium between solution and several "calcium compartments" of the crystal phase(6,7). The "bend" in the curve would then represent completion (or near completion) of rapidly-exchanging processes (exchange with hydration shell and crystal surface), followed by the predominance of slowly-exchanging (monoexponential) processes (intracrystalline exchange)(6,7). Under this interpretation, the timing of the "discard" procedure may alter the absolute values of the rates of decline in SA of solution, but it would not be expected to modify the number or the relative values of the rate constants. Specific assignment of "back-diffusion" or "diffusion" to certain parts of the SA curve therefore appears meaningless.

One may question also the validity of comparing SA curves in "discard" studies with the "corrected discard rate." Nordin and associates(5) calculated the "corrected discard rate" by expressing the actual discard rate as a percentage of "exchangeable calcium pool." Such comparison would be valid only if (1) the "exchangeable calcium pool" represents that quantity of calcium in the crystal phase which is influenced by the wash-out (or "discard") procedure, and (2) the size of the "pool" remains constant during the entire period. Neither of these assumptions was proven by these workers(5).

Although our experiments were performed with bone powder and with synthetic hydroxyapatite, there is no reason to believe that similar results would not have been obtained with bone slices. Studies with powder are technically considerably simpler, and avoid the use of dialysis tubing required in the studies with bone slices. Finally, considerable caution should be exercised in relating *in vitro* results with bone preparations directly to *in vivo* results. In the living organism, the physiological processes of bone formation and destruction *may* overshadow the physicochemical processes of the exchange reaction.

Summary. The curve describing the uptake of ^{45}Ca by bone powder or by synthetic hydroxyapatite shows an initial rapid fall in radioactivity of solution followed by a monoexponential decline at a slower rate. Wash-out experiments were performed on the labelled crystals before and during the period of monoexponential fall by resuspending the crystals, collected by filtration, in medium free of isotope. More than 80% of radiocalcium could be removed from the crystal phase by this procedure regardless of the period of labelling. Results from published reports are interpreted to show that "back-diffusion" of isotope from bone into solution is operative only at a certain region of the uptake curve; our studies contradict this interpretation.

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