JCI The Journal of Clinical Investigation

Extraction of Vitamin D Metabolites by Bones of Normal Adult Dogs

K. Olgaard, … , S. Klahr, E. Slatopolsky

J Clin Invest. 1982;69(3):684-690. https://doi.org/10.1172/JCI110496.

Research Article

Using the isolated perfused canine tibia we examined the extraction of [^{3}H]25(OH)D_3, [^{3}H]1,25$(OH)_2D_3$ and [^{3}H]24,25$(OH)_2D_3$ by bone of normal adult dogs. The studies were performed with and without vitamin D binding protein (DBP) in the perfusate to examine the effect of protein binding on the extraction of the vitamin D metabolites. An average of 48±2% of [^{3}H]25(OH)D_3 was extracted by bone, when no DBP was present. However, addition of only a small amount of DBP (~720 ng/ml of perfusate) nearly completely abolished the extraction of [^{3}H]25(OH)D_3 by bone. No degradation and/or transformation of the labeled 25(OH)D_3 could be demonstrated during passage through the isolated perfused bone. The extraction of [^{3}H]24,25$(OH)_2D_3$ in a DBP-free medium averaged 33±5%. Addition of 720 ng of DBP/ml of perfusate completely inhibited the extraction of this metabolite. The extraction of [^{3}H]1,25$(OH)_2D_3$ averaged 30±3% in a DBP free medium and no inhibition of the extraction was demonstrated after addition of DBP (720 ng/ml of perfusate). However, addition of DBP in a concentration of 14.4 μg/ml of perfusate reduced the extraction of 1,25$(OH)_2D_3$ to 8±2%, a value still significantly higher than that seen after addition of 20 times less DBP to perfusions with 25(OH)D_3 and 24,25$(OH)_2D_3$. It is concluded that the isolated perfused bone of normal dogs can extract significant amounts of 25(OH)D_3, 1,25$(OH)_2D_3$, and 24,25$(OH)_2D_3$. Small concentrations […]

Extraction of Vitamin D Metabolites by Bones of Normal Adult Dogs

K. OLGAARD, J. SCHWARTZ, D. FINCO, M. ARBELAEZ, J. HADDAD, L. AVIOLI, S. KLAHR, and E. SLATOPOLSKY, *Renal Division and the Division of Bone and Mineral Metabolism, Department of Medicine, Washington University School of Medicine, St. Louis, Missouri 63110*

ABSTRACT Using the isolated perfused canine tibia we examined the extraction of [^{3}H]25(OH)D_3, [^{3}H]1,25$(OH)_2D_3$ and [^{3}H]24,25$(OH)_2D_3$ by bone of normal adult dogs. The studies were performed with and without vitamin D binding protein (DBP) in the perfusate to examine the effect of protein binding on the extraction of the vitamin D metabolites. An average of 48±2% of [^{3}H]25(OH)D_3 was extracted by bone, when no DBP was present. However, addition of only a small amount of DBP (~720 ng/ml of perfusate) nearly completely abolished the extraction of [^{3}H]25(OH)D_3 by bone. No degradation and/or transformation of the labeled 25(OH)D_3 could be demonstrated during passage through the isolated perfused bone. The extraction of [^{3}H]24,25$(OH)_2D_3$ in a DBP-free medium averaged 33±5%. Addition of 720 ng of DBP/ml of perfusate completely inhibited the extraction of this metabolite. The extraction of [^{3}H]1,25$(OH)_2D_3$ averaged 30±3% in a DBP free medium and no inhibition of the extraction was demonstrated after addition of DBP (720 ng/ml of perfusate). However, addition of DBP in a concentration of 14.4 μg/ml of perfusate reduced the extraction of 1,25$(OH)_2D_3$ to 8±2%, a value still significantly higher than that seen after addition of 20 times less DBP to perfusions with 25(OH)D_3 and 24,25$(OH)_2D_3$. It is concluded that the isolated perfused bone of normal dogs can extract significant amounts of 25(OH)D_3, 1,25$(OH)_2D_3$, and 24,25$(OH)_2D_3$. Small concentrations of DBP (720 ng/ml) in the perfusate significantly inhibited the extraction of 25(OH)D_3 and 24,25$(OH)_2D_3$. A carrier role for DBP is suggested and it is proposed that the levels of free vitamin D are important for extraction of the metabolites by bone. Therefore, due to the different affinities of DBP for the various metabolites of vitamin D, only 1,25$(OH)_2D_3$ is extracted in vitro in significant amounts by bone of normal adult dogs, in the presence of DBP.

INTRODUCTION

The metabolism and fate of the different vitamin D metabolites, 25-hydroxy vitamin D_3(25[OH]D_3), 1,25-dihydroxy vitamin D_3 (1,25$[OH]_2D_3$), and 24,25-dihydroxy vitamin D_3 (24,25$[OH]_2D_3$), in the skeleton is still a matter of debate. Specific cytosolic receptors have been demonstrated in bone for 1,25$(OH)_2D_3$ (1–6) and 25(OH)D_3 (7). Furthermore, conversion of 25(OH)D_3 to 24,25$(OH)_2D_3$ (8) and to 1,25$(OH)_2D_3$ (9) has been suggested to take place in bone cells. However, most of these studies have been performed in isolated bone cells, often from fetal rats (2, 3, 7, 8) or from vitamin D-deficient animals (1). The handling of the different vitamin D metabolites by the bone of normal, adult vitamin D-repleted animals has not been studied.

The present studies, therefore, were designed to examine the extraction of 25(OH)D_3, 1,25$(OH)_2D_3$, and 24,25$(OH)_2D_3$ by bone of normal adult dogs. The study was performed using the isolated canine tibia, which was perfused with Krebs-Henseleit bicarbonate buffer containing one of the different vitamin D metabolites labeled with tritium. Furthermore, in order to examine the effect of vitamin D binding protein (DBP)[1] on the extraction of the different vitamin D metabolites by bone, experiments were performed either with the vitamin D metabolites present in the buffer in a free, nonprotein bound form, or when serum (containing DBP) was added to the buffer together with the vitamin D metabolite.

Received for publication 23 April 1981 and in revised form 19 August 1981.

[1] *Abbreviation used in this paper*: DBP, vitamin D binding protein.

METHODS

Isolated perfused canine tibia. The canine tibia was prepared for perfusion in vitro as previously described (10). In brief, after pentobarbital anesthesia (20–30 mg/kg), and heparin 3,000 U, i.v., both tibiae were removed from the dog. The anterior tibial artery was identified and the nutrient artery dissected and cannulated with PE 50 tubing. The remaining adherent muscles were removed. After cannulation of the nutrient artery, the bone was washed with 10 ml of Krebs-Henseleit bicarbonate buffer. The bone was then placed in a specially designed apparatus (10) and perfused at 37°C. The perfusate, a Krebs-Henseleit bicarbonate buffer (Na^+ 135 mM, K^+ 5.0 mM, Cl^- 115 mM, HCO_3^- 25 mM, Mg^{++} 0.6 mM, Ca^{++} 1.2 mM, phosphorus 2 mM, glucose 5.6 mM, bovine serum albumin 10 g/l, pH 7.40), was continuously gassed with 95% oxygen and 5% carbon dioxide and pumped into the bone at a pressure of 90–100 mm Hg. The flow rate was 2±0.2 ml/min. The venous effluent was allowed to seep from the bone surface and was subsequently collected for analysis. Once the perfusion system was established, the bone was perfused with buffer alone for 30 min. After this equilibration period, four basal control periods of 5-min duration were obtained. Then the tritium-labeled vitamin D compound to be tested was added to the arterial side of the perfusion system. Following this, both arterial and venous samples were collected at 5-min periods for 80 min. The samples were kept at 4°C, centrifuged and aliquots transferred to counting vials. Based on the counts, the arteriovenous (A-V) difference of the vitamin D compound was calculated for the different time periods and the extraction expressed in percent: $A - V/A \times 100$. The vitamin D compounds to be tested were all labeled with tritium in the 23,24 position. The binding of tritium in this position has previously been shown to be very stable in both vitamin D compounds and other steroids, labeled at the same position.

Experimental design

68 bones obtained from 44 normal adult mongrel dogs were used in this study. Measurements of serum concentrations of ionized calcium, total calcium, phosphorus, creatinine and immunoreactive PTH (iPTH) were obtained in all dogs before study. The dogs were fed 400 g of normal dog chow daily and tap water ad lib. The following studies were performed.

Extraction of 25(OH)D_3. (*a*) 16 bones obtained from 11 dogs were perfused with [^{3}H]25(OH)D_3 (sp act 102 Ci/mmol) at a concentration of 80 pg of 25(OH)D_3/ml of perfusate. In all these experiments the buffer contained bovine serum albumin (fraction V, Pentex) from Miles Laboratories, Elkhart, Ind. No serum was added to the buffer in these experiments. (*b*) The effect of DBP on the extraction of 25(OH)D_3 by bone was studied by adding freshly obtained dog serum to the perfusate containing [^{3}H]25(OH)D_3 as above. In five experiments 500 µl of serum (~180 µg of DBP) were added to 250 ml of perfusate. In three experiments progressively increasing amounts of serum were added to the perfusate resulting in approximate concentrations of 0, 36, 54, 144, and 720 ng of DBP/ml buffer. (*c*) In eight experiments thin-layer silica gel G chromatography was performed on both the arterial (before entering the bone) and venous samples (after leaving the bone) to check for degradation of [^{3}H]25(OH)D_3 and/or production of new metabolites. Lipid extraction of the samples was performed in $CHCl_3$:CH_3OH (11). $CHCl_3$ layers were dried under a N_2 stream. The thin-layer chromatography (12) was then performed with *n*-hexane/acetone (8:2, vol/vol) solvent on silica gel G sheets (Gelman Instrument Co., Ann Arbor, Mich.). Unlabeled markers of cholecalciferol, 25(OH)D_3, 1,25(OH)$_2D_3$, and 24,25(OH)$_2D_3$ were cochromatographed and detected by their absorbance of ultraviolet light. (*d*) In two experiments the bone marrow was scraped out of the bone after perfusion with [^{3}H]25(OH)D_3. The bone marrow was checked for radioactivity after extraction with acetone and further examined for the presence of vitamin D metabolites by high-pressure liquid chromatography. (*e*) To examine for a possible noncellular distribution of [^{3}H]25(OH)D_3 in the bone and thus a noncellular extraction of [^{3}H]25(OH)D_3, four bones were perfused with buffer containing [^{3}H]25(OH)D_3 without DBP for 80 min. The perfusate was then exchanged to buffer without [^{3}H]25(OH)D_3 and without DBP and the perfusion continued for 30 min. The perfusion was then stopped and the perfusate exchanged with a buffer containing 10 ml of serum (providing a concentration of ~14.4 µg of DBP/ml of buffer) but no radioactive or "cold" 25(OH)D_3 in order to examine how much of the previously extracted [^{3}H]25(OH)D_3 could be mechanically "washed out" of the bone. It was expected that the high affinity of DBP for 25(OH)D_3 would result in efflux of non-intracellularly deposited labeled 25(OH)D_3 from the bone during perfusion with a buffer containing serum (and DBP).

Extraction of 1,25(OH)$_2D_3$. In another series of dogs, the extraction of [^{3}H]1,25(OH)$_2D_3$ by bone was examined. [^{3}H]1,25(OH)$_2D_3$ (sp act 102 Ci/mmol) was added to the perfusate at a concentration of 40 pg of 1,25(OH)$_2D_3$/ml of buffer. Bone perfusion was performed with (n = 8) and without (n = 3) addition of 500 µl of serum to the perfusate (~720 ng of DBP/ml of buffer) and in other experiments (n = 3) with the addition of 10 ml of serum (~14.4 µg of DBP/ml of buffer).

Extraction of 24,25(OH)$_2D_3$. In another series of dogs, the extraction of [^{3}H]24,25(OH)$_2D_3$ by bone was examined. [^{3}H]24,25(OH)$_2D_3$ (sp act 68 Ci/mmol) was added to the perfusate at a concentration of 59 pg of 24,25(OH)$_2D_3$/ml of buffer. Bone perfusion was performed without (n = 5) and with (n = 3) addition of 500 µl of serum to the perfusate (~720 ng of DBP/ml of buffer).

Extraction of other compounds. (*a*) The extraction of vitamin D binding protein by bone was examined by adding ^{125}I-human(h)DBP (sp act 50–100 µCi/µg) to the perfusate resulting in a concentration of hDBP of 7–14 ng/ml of perfusate. The bone perfusion was performed without any vitamin D metabolite added (n = 6). In two experiments ^{125}I-hDBP (~10 ng/ml) was added to the perfusate together with 15% normal human serum. The concentration of nonlabeled hDBP in the perfusate was measured before and after passage across the bone by an immunodiffusion technique. (*b*) Finally, the "specificity" of the extraction by bone was examined using a steroid such as [^{3}H]aldosterone, which does not have known effects on bone and by means of ^{14}C-inulin. These compounds were added to the perfusate in eight different experiments.

Compounds

The vitamin D compounds, 25-hydroxy[23,24(n)^{3}H]-cholecalciferol (sp act 102 Ci/mmol), 1,25-dihydroxy-[23,24(n)-^{3}H]cholecalciferol (sp act 102 Ci/mmol), and 24,25-dihydroxy[23,24(n)-^{3}H]cholecalciferol (sp act 68 Ci/mmol),

were all purchased from Amersham Corp., Arlington Heights, Ill. and further purified on a LH-20 Sephadex column before use (13). [1,2-^{3}H]Aldosterone (sp act 40 Ci/mmol) and [^{14}C]inulin (sp act 11.3 mCi/mmol) were both obtained from Amersham Corp. and were used without further purification. h-DBP was labeled by the lactoperoxidase method (sp act 50–100 μCi/μg) (14). The concentration of nonlabeled hDBP was measured in the perfusate by an immunodiffusion analysis (15). This compound was found to be free of contamination by DBP. Since purified canine DBP was not available, freshly obtained dog serum was used. As the immunodiffusion analysis used for measuring the hDBP concentration did not crossreact with dog serum, no exact concentration of DBP in dog serum can be given. However, saturation analysis of dog serum by 25(OH)D_3 resulted in an estimated concentration of DBP in dog serum of 360 μg/ml, a value similar to that seen in other mammals (16, 17).

RESULTS

Fig. 1 demonstrates the extraction of [^{3}H]25(OH)D_3 by bone during 80 min of perfusion. The extraction was highest (mean, 64±2%) during the first 20 min of perfusion (not shown), possibly due to the distribution of the D metabolite in a certain volume of bone. Thereafter, a relatively stable extraction was seen during the remaining 60 min (mean, 48±2%). In the experiments illustrated by the upper curve no DBP was added to the perfusate, while the lower curve demonstrates the effect of addition of DBP (720 ng/ml of perfusate) on the extraction of [^{3}H]25(OH)D_3. Fig. 2 demonstrates the effect of adding increasing amounts of DBP to the perfusate on the extraction of [^{3}H]25(OH)D_3 by bone. The concentration of [^{3}H]25(OH)D_3 was the same in all of these experiments. Addition of 36 ng of DBP/ml of buffer resulted in a mean uptake of [^{3}H]25(OH)D_3 of 32±2%; 54 ng of DBP/ml of perfusate reduced the uptake further to 27±2%; 144 ng of DBP/ml of perfusate decreased extraction to 10±2% and finally addition of 720 ng of DBP/ml of buffer decreased extraction of [^{3}H]25(OH)D_3 to 2±1%. Thus, it was clearly shown that the extraction of [^{3}H]25(OH)D_3 by bone

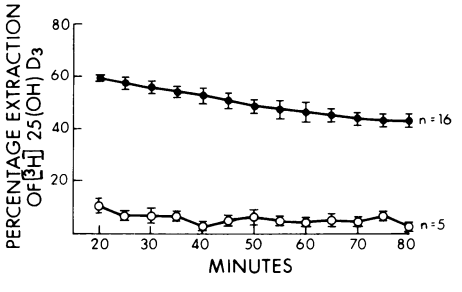

FIGURE 1 Percentage extraction of [^{3}H]25(OH)D_3 by bone (mean±SEM), without serum containing DBP in the perfusate (upper curve) and with serum containing an estimated DBP concentration of 720 ng/ml added to the perfusate (lower curve).

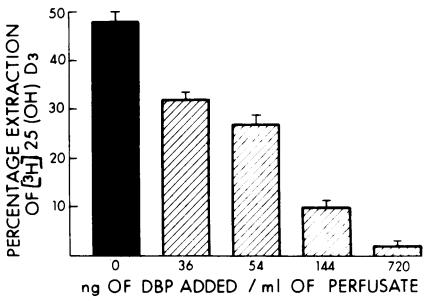

FIGURE 2 Mean percentage extraction by bone of [^{3}H]25(OH)D_3 during 1 h of bone perfusion (from 20 to 80 min). In experiments depicted by the first column to the left, no serum and thus no DBP was added to the perfusate (n = 16). The next four columns indicate the effect of increasing amounts of dog serum containing the indicated estimated amounts of DBP on the percentage extraction of [^{3}H]25(OH)D_3 by bone (n = 3).

diminished by adding increasing amounts of DBP to the perfusate and that a concentration of 720 ng of DBP/ml of perfusate resulted in a nearly total cessation of extraction of 25(OH)D_3 by bone. 5 μCi [^{3}H]25(OH)D_3 (sp act 102 Ci/mmol) were added to 250 ml of perfusate in each experiment, resulting in a concentration of 25(OH)D_3 of ~80 pg/ml of buffer. Thus, in the experiments using a DBP-free buffer, the bone extracted ~77 pg of 25(OH)D_3/min, when the flow rate was 2 ml/min. In the experiments with 720 ng of DBP added/ml of perfusate, the extraction of [^{3}H]25(OH)D_3 was 1.6 pg/min, a value that, due to the low percent extraction, was not significantly different from zero.

The possibility of degradation and/or transformation of the labeled 25(OH)D_3 during the passage through the isolated perfused bone was examined. Fig. 3 demonstrates by use of thin-layer silica gel chromatography that the products coming out of the bones were not distinguishable from the vitamin D metabolites entering the bone and that no new peaks appeared. However, the method of thin-layer chromatography might be too insensitive to demonstrate minor changes of a few percent in the vitamin D pattern of the venous effluent. Furthermore, the [^{3}H]25(OH)D_3 was diluted by the much greater pool in the bone of unlabeled 25(OH)D_3, which also may mask eventually small changes in the venous pattern of vitamin D metabolites. During the extraction no tritium was detectable in the water phase, indicating no degradation of [^{3}H]25(OH)D_3 or release of tritium. It was, therefore, concluded that by the use of thin-layer chromatography no new metabolites appeared in the effluent fluid.

Examination of the bone marrow after 80 min of perfusion with [^{3}H]25(OH)D_3 containing buffer in two

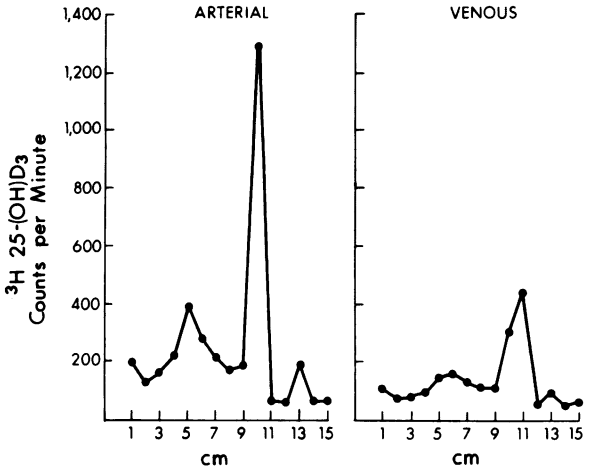

FIGURE 3 Thin-layer silica gel G chromatography of samples obtained from the arterial inflow and the venous outflow of a bone perfused with [^{3}H]25(OH)D$_3$ (n = 8). This figure, showing a representative experiment, demonstrates that the pattern of peaks in arterial and venous samples was the same and only the greater height of the peaks, on the arterial side, was different as would be expected due to extraction of [^{3}H]25(OH)D$_3$ during its passage through bone. Thus, no metabolism of [^{3}H]25(OH)D$_3$ by bone could be demonstrated.

experiments revealed no signs of radioactivity or vitamin D metabolites and thus excluded the possibility that the extraction of 25(OH)D$_3$ by the bone was due to extraction of this metabolite by the fat present in the bone marrow.

The "nonspecific" extraction of [^{3}H]25(OH)D$_3$ by bone was examined using the high affinity of DBP for 25(OH)D$_3$. After 80 min of bone perfusion with buffer containing [^{3}H]25(OH)D$_3$, the total amount of [^{3}H]25(OH)D$_3$ taken up by the bone was calculated and expressed as total counts per bone. A total of 58% of the [^{3}H]25(OH)D$_3$ entering the bone was extracted. Then the perfusate was exchanged to a buffer containing no vitamin D and no DBP. After an additional 30 min of perfusion the amount of radioactivity remaining in the bone was reduced by 11%. The buffer was then exchanged to a new buffer without vitamin D, but containing 14.4 μg of DBP/ml of perfusate. After 30 min of further bone perfusion the amount of [^{3}H]25(OH)D$_3$ remaining in the bone was further reduced by 13%. Thus, 24% of the labeled 25(OH)D$_3$ was "washed out" of the bone by means of a high DBP concentration in the perfusate. This amount, therefore, accounts for the "minimal" amount of [^{3}H]25(OH)D$_3$, which was extracted by the bone but not deposited intracellularly, while 34% of the extracted 25(OH)D$_3$ by bone could not be removed by a high DBP concentration in the perfusate.

Fig. 4 presents data on the extraction of [^{3}H]-1,25(OH)$_2$D$_3$ by bone. The mean extraction of this compound when no DBP was present in the perfusate averaged 30±3%. Addition of DBP at a concentration of 720 ng/ml of perfusate did not change significantly the extraction of [^{3}H]1,25(OH)$_2$D$_3$ (extraction 28±3%). This was in contrast to the nearly total inhibition of the uptake of [^{3}H]25(OH)D$_3$ seen when this concentration of DBP was used (Fig. 1). However, addition of 14.4 μg of DBP (20 times as much) resulted in a clear inhibition of the uptake of 1,25(OH)$_2$D$_3$ by bone (extraction 8±2%). 2.5 μCi of [^{3}H]1,25(OH)$_2$D$_3$ (sp act 102 Ci/mmol) were added to 250 ml of perfusate in each experiment, resulting in a concentration of ~40 pg of 1,25(OH)$_2$D$_3$/ml of buffer. Thus, in the experiments using a DBP-free buffer, the bone extracted ~24 pg of 1,25(OH)$_2$D$_3$/min at a flow rate of 2 ml/min. When 720 ng/ml of DBP was added to the perfusate, the extraction of 1,25(OH)$_2$D$_3$ averaged 22 pg/min, while a concentration of 14.4 μg of DBP/ml resulted in an average extraction of 1,25(OH)$_2$D$_3$ of 6 pg/min.

The mean extraction of [^{3}H]24,25(OH)D$_3$ by bone averaged 33±5% when no DBP was added to the medium (Fig. 4). When 720 ng of DBP was added/ml of perfusate, the extraction of [^{3}H]24,25(OH)$_2$D$_3$ was totally inhibited (Fig. 4), similar to the effect observed with [^{3}H]25(OH)D$_3$. 2.5 μCi of [^{3}H]24,25(OH)$_2$D$_3$ (sp act 68 Ci/mmol) were added to 250 ml of perfusate in each experiment, resulting in a concentration of ~59 pg of 24,25(OH)$_2$D$_3$/ml of buffer. Therefore, in experiments using DBP-free buffer, the bone extracted ~39 pg of 24,25(OH)$_2$D$_3$/min at a flow rate of 2 ml/min. Addition of 720 ng of DBP/ml of buffer reduced the amount of 24,25(OH)$_2$D$_3$ extracted to ~2 pg/min, not significantly different from zero, due to the low percent extraction.

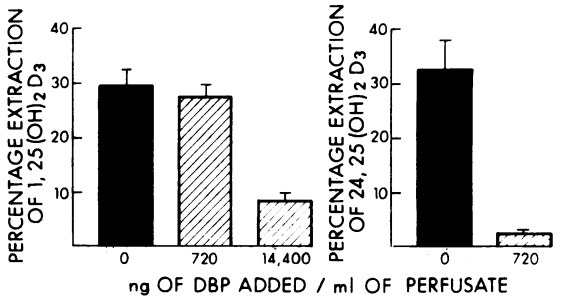

FIGURE 4 Mean percentage extraction by bone of [^{3}H]1,25(OH)$_2$D$_3$ and [^{3}H]24,25(OH)$_2$D$_3$. Extraction of [^{3}H]1,25(OH)$_2$D$_3$ was examined without (n = 3) dog serum (no DBP) and in the presence of dog serum in the perfusate with an estimated DBP concentration of 720 ng (n = 8) and 14.4 μg (n = 3)/ml. It is seen that the extraction of 1,25(OH)$_2$D$_3$ is less sensitive to the presence of dog serum containing DBP in the perfusate than the extraction of 24,25(OH)$_2$D$_3$.

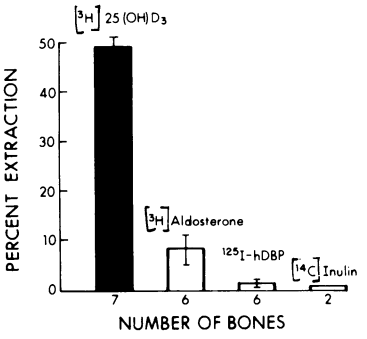

FIGURE 5 Mean percentage extraction of [^{3}H]aldosterone, ^{125}I-hDBP and [^{14}C]inulin by bone compared with the extraction of [^{3}H]25(OH)D_3.

Fig. 5 contrasts the extraction of [^{3}H]25(OH)D_3 with those of ^{125}I-hDBP, [^{3}H]aldosterone and ^{14}C-inulin. The extraction of aldosterone and DBP by bone was quite low and no extraction of inulin was detected.

In two experiments, where ^{125}I-hDBP was added to the perfusate together with 15% normal human serum, an extraction of labeled DBP of 1.7±0.4% was found across the bone. Furthermore, the concentration of hDBP in the perfusate was not different before and after passage across the bone (mean 78.5 and 82.0 μg/ml, respectively).

DISCUSSION

Although the relationship between vitamin D and bone is well established in treatment studies (18–21), the actual handling of the different vitamin D metabolites by bone has not been clarified. Most studies on this topic have been performed in either animals with vitamin D deficiency or in fetal cell preparations often from the rat calvaria (1–3, 7, 8). To date the handling of vitamin D metabolites by bone of normal adult animals without bone disease has not been examined. The present investigation, therefore, has used the previously published model (10) for perfusion of the isolated dog tibia to get further information on the handling of 25(OH)D_3, 1,25$(OH)_2D_3$, and 24,25$(OH)_2D_3$ by normal adult canine bone.

The present investigation clearly demonstrates that the extraction of 25(OH)D_3 by bone is high in the presence of a DBP-free medium. However, this situation probably never occurs in vivo, since no condition has yet been described wherein the concentration of DBP in plasma is zero (16, 22). The use of subphysiological concentrations of DBP in the perfusate was necessitated by the use of the isolated perfused canine tibia model. Since purified dog DBP was not available, it would have been necessary to use serum exclusively for the perfusion instead of the Krebs-Henseleit bicarbonate buffer to obtain a "normal" concentration of DBP. The bone model has, however, not been optimized for this purpose. Furthermore, the concentration of 25(OH)D_3 used in the perfusate was reduced ~1:375, when compared with normal plasma levels. This was similar to the reduction in DBP concentration, when 720 ng/ml was used in the perfusate. Therefore, the demonstration that even low concentrations of DBP in the perfusate (compared to the normal concentration in plasma, ~360 μg/ml) nearly completely inhibited the extraction of 25(OH)D_3 by bone is important. This suggests, together with the finding that DBP is not extracted by the bone, that it is the concentration of free, non-DBP bound, 25(OH)D_3 that is of significance for the uptake of this metabolite by bone. The concentration of the dihydroxylated vitamin D compounds used was similar to the concentration of 25(OH)D_3. The metabolite 24,25$(OH)_2D_3$ demonstrated a similar relationship between its affinity for DBP and its extraction by bone as seen for 25(OH)D_3. However, the extraction of 1,25$(OH)_2D_3$ by bone was much less affected by increasing concentrations of DBP in the perfusate than 25(OH)D_3 and 24,25$(OH)_2D_3$ in accordance with the much lower affinity of 1,25$(OH)_2D_3$ for DBP (17). Unfortunately, it has not yet been possible to measure the "free" concentration of the different vitamin D metabolites in plasma, although a calculation of the free vitamin D index, based on the molar ratio between vitamin D and DBP (23) has been used to approximate the "real" free concentration in plasma. However, the present study suggests that vitamin D binding protein has a transport and a carrier role for vitamin D and is not extracted by the bone together with the vitamin D metabolite in question. Thus, it is only the free unbound form of the different vitamin D compounds that is available for extraction. The present results emphasize the importance of knowing exactly what type of medium has been used in experiments on the effect of vitamin D metabolites. Several bovine serum albumin preparations (except fraction V-Pentex) are to some extent contaminated by DBP (unpublished observation). In many cell preparations fetal calf serum is used. Thus, different results in the literature on, e.g., the effect of vitamin D metabolites on the secretion of parathyroid hormone from the parathyroid glands (24, 25, 26) may result from the fact that the medium has contained variable concentrations of DBP and, therefore, influenced the concentration of the free vitamin D metabolites present in the medium.

The present study does not clarify the further handling of the vitamin D metabolites by the bone. It is not known by what cells the metabolites are taken up. However, the vitamin D compounds were not extracted by the bone marrow. Bone perfusion with other compounds with no supposed relationship to bone

demonstrated no extraction and thus demonstrated that the extraction of the vitamin D metabolites was not nonspecific. By the "wash out" experiments involving high concentration of DBP in the perfusate, ~34% of the extracted $25(OH)D_3$ was not available for removal by DBP, indicating the amount of $25(OH)D_3$ specifically bound to the bone.

The present studies were carried out in normal adult vitamin D-repleted dogs. This approach may have resulted in calculation of minimal values for the extraction of the different vitamin D metabolites by bone, since the bones in vivo already had been exposed for a "long term" to vitamin D and DBP. If similar experiments would have been performed in vitamin D-depleted dogs, it is possible that the extraction of the different D metabolites would increase. Furthermore, it cannot be excluded by the results of the present investigation that the extraction of DBP could be significantly greater in vitamin D-depleted dogs.

Although the isolated perfused bone does not provide information as to the handling of different vitamin D metabolites at the cellular level of bone, it clearly demonstrates that the bone has a higher affinity for vitamin D than for the other compounds tested (e.g. inulin, aldosterone, and h-DBP) and that the affinity is different for the different vitamin D metabolites. It is also clearly demonstrated in the present investigation that it probably is the free and not the DBP bound concentration which is of importance for the extraction of vitamin D metabolites by normal bone.

In conclusion, the isolated perfused bone of normal adult dogs extracted significant amounts of $25(OH)D_3$, $1,25(OH)_2D_3$, and $24,25(OH)_2D_3$. DBP was not apparently extracted by bone, but small quantities of DBP in the perfusate significantly inhibited the extraction of $25(OH)D_3$ and $24,25(OH)_2D_3$ by bone of normal adult dogs. A carrier role for DBP in the handling of vitamin D metabolites is therefore suggested. It seems unlikely that DBP takes part in the cellular uptake of vitamin D by bone. Since the plasma half-life of rabbit DBP is 40 h (27), it is possible that an active entry of DBP into tissues may not be clearly seen in the short term experiments described in the present work. Although sterol entry may be facilitated by the absence of DBP in the perfusing medium the precise role of DBP in vivo is not presently clear. Thus, the free (nonprotein bound) concentration of the vitamin D metabolites could be a limiting factor in the extraction of these compounds by bone of normal adult dogs. Therefore, due to different affinities of DBP for $25(OH)D_3$, $24,25(OH)_2D_3$, and $1,25(OH)_2D_3$, only $1,25(OH)_2D_3$ is extracted in vitro in significant amounts by the bone of adult normal dogs, in the presence of DBP.

ACKNOWLEDGMENTS

The secretarial assistance of Mrs. Pat Verplancke in the preparation of this manuscript is gratefully acknowledged. The authors wish to express their appreciation to Dr. Dean L. Griffith and Timothy Vander Meulen from the Upjohn Co., Kalamazoo, Mich., for supplying the $25(OH)D_3$.

This work was supported by U. S. Public Health Service National Institutes of Arthritis, Metabolism, and Digestive Diseases grants AM-09976 and AM-07126, and AM-11674. Dr. Klaus Olgaard received partial support by grants from The Danish Medical Research council, The Guldborg and Carlo B. Hansen Foundation, the Nato Science Foundation, The Dagmar Marshall Foundation, the Foundation of Leo Pharmaceutical, and the Shriners Hospital of St. Louis. Dr. Mario Arbelaez received partial support by grants from the University of Antioquia, Colombia.

REFERENCES

1. Weber, J. C., V. Pons, and E. Kodicek. 1971. The localization of 1,25-dihydroxycholecalciferol in bone cell nuclei of rachitic chicks. *Biochem. J.* **125:** 147–153.
2. Kream, B. E., H. Jose, S. Yamada, and H. F. DeLuca. 1977. A specific high-affinity binding macromolecule for 1,25-dihydroxyvitamin D_3 in fetal bone. *Science (Wash. D. C.).* **197:** 1086–1088.
3. Manolagas, S. C., C. M. Taylor, and D. C. Anderson. 1979. Highly specific binding of 1,25-dihydroxycholecalciferol in bone cytosol. *J. Endocrinol.* **80:** 35–39.
4. Mudher, S. A., S. C. Manolagas, and D. C. Anderson. 1980. Comparison of binding of 25 hydroxy and 1,25 dihydroxy vitamin D_3 to intact tissue and pre-prepared cytosol from bone and non-target organs. Abstract no. 215, VI International Congress on Endocrinology, Melbourne.
5. Colston, K., M. Hirst, and D. Feldman. 1980. Organ distribution of the cytoplasmic 1,25-dihydroxycholecalciferol receptor in various mouse tissues. *Endocrinology* **107:** 1916–1922.
6. Wecksler, W. R., and A. W. Norman. 1980. Biochemical properties of 1,25-dihydroxyvitamin D receptors. *J. Steroid Biochem.* **13:** 977–989.
7. Mellow, A. M., G. V. Stosich, and P. H. Stern. 1978. Dissociation of specific binding of 25-OH-D_3 and resorption in fetal rat bones. *Mol. Cell. Endocrinol.* **10:** 149–158.
8. Garabedian, M., M. Lieberherr, T. M. N'Guyen, M. T. Corvol, M. B. Du Bois, and S. Balsan. 1978. The in vitro production and activity of 24,25-dihydroxycholecalciferol in cartilage and calvarium. *Clin. Orthopaed. Rel. Res.* **135:** 241–248.
9. Keck, E., R. Durdel, H. U. Schweikert, W. Meier, V. Hennes, F. Kruck, and H. L. Kruskemper. 1980. Metabolism of 25-hydroxycholecalciferol in human spongiosa. Abstract 89, VI International Congress on Endocrinology, Melbourne.
10. Martin, K. J., J. J. Freitag, M. B. Conrades, K. A. Hruska, S. Klahr, and E. Slatopolsky. 1978. Selective uptake of the synthetic amine terminal fragments of bovine parathyroid hormone by isolated perfused bone. *J. Clin. Invest.* **62:** 256–261.
11. Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37:** 911–917.
12. Haddad, J. G., Jr., and J. Walgate. 1976. 25-hydroxy-

vitamin D transport in human plasma. *J. Biol. Chem.* **251:** 4803–4809.
13. Haddad, J. G., Jr., and S. Rojanasathit. 1976. Acute administration of 25-hydroxycholecalciferol in man. *J. Clin. Endocrinol. Metab.* **42:** 284–290.
14. Miyachi, Y., J. L., Vaitukaitis, E. Nieschlag, and M. B. Lipsett. 1972. Enzymatic radioiodination of gonadotropins. *J. Clin. Endocrinol. Metab.* **34:** 23–28.
15. Cooke, N. E., J. Walgate, and J. G. Haddad, Jr. 1979. Human serum binding protein for vitamin D and its metabolites. *J. Biol. Chem.* **254:** 5965–5971.
16. Bouillon, R., H. van Baelen, and P. de Moor. 1977. The measurement of the vitamin D-binding protein in human serum. *J. Clin. Endocrinol. Metab.* **45:** 225–231.
17. Bouillon, R., H. van Baelen, and P. de Moor. 1980. Comparative study of the affinity of the serum vitamin D-binding protein. *J. Steroid Biochem.* **13:** 1029–1034.
18. Witmer, G., A. Margolis, O. Fontaine, J. Fritsch, G. Lenoir, M. Broyer, and S. Balsan. 1976. Effects of 25-hydroxycholecalciferol on bone lesions of children with terminal renal failure. *Kidney Int.* **10:** 395–408.
19. Russell, J. E., M. L. Roberts, S. L. Teitelbaum, P. M. Stein, and L. V. Avioli. 1978. Therapeutic effects of 25-hydroxyvitamin D_3 on renal osteodystrophy. *Min. Electrolyte Metab.* **1:** 129–138.
20. Fournier, A., P. Bordier, J. Gueris, J. L. Sebert, P. M. C. Ferriere, J. Bedrossian, and H. F. DeLuca. 1979. Comparison of 1-hydroxycholecalciferol in the treatment of renal osteodystrophy: greater effect of 25-hydroxycholecalciferol on bone mineralization. *Kidney Int.* **15:** 196–204.
21. Bordier, P., H. Rasmussen, P. M. L. Miravet, J. Gueris, and A. Ryckwaert. 1978. Vitamin D metabolites and bone mineralization in man. *J. Clin. Endocrinol. Metab.* **46:** 284–294.
22. Bouillon, R., and H. van Baelen. 1979. The transport of vitamin D. *In* Vitamin D, Basic Research and Its Clinical Application. A. W. Norman, K. Schaefer, D. v. Herrath, H-G. Grigoleit, J. W. Coburn, H. F. DeLuca, E. B. Mawer, and T. Suda, editors. Walter de Gruyter and Co., New York. 137–143.
23. Bouillon, R., F. A. van Assche, H. van Baelen, W. Heyns, and P. de Moor. 1981. Influence of the vitamin D-binding protein on the serum concentration of 1,25-dihydroxyvitamin D_3. *J. Clin. Invest.* **67:** 589–596.
24. Henry, H. L., A. N. Taylor, and A. W. Norman. 1977. Response of chick parathyroid glands to the vitamin D metabolites, 1,25-dihydroxycholecalciferol and 24,25-dihydroxycholecalciferol. *J. Nutr.* **107:** 1918–1926.
25. Canterbury, J. M., S. Lerman, A. J. Claflin, H. Henry, A. Norman, and E. Reiss. 1978. Inhibition of parathyroid hormone secretion by 25-hydroxycholecalciferol and 24,25-dihydroxycholecalciferol in the dog. *J. Clin. Invest.* **61:** 1375–1383.
26. Dietel, M., G. Dorn, R. Montz, and E. Altenahr. 1979. Influence of vitamin D_3, 1,25-dihydroxyvitamin D_3, and 24,25-dihydroxyvitamin D_3 on parathyroid hormone secretion, adenosine 3′,5′-monophosphate release, and ultrastructure of parathyroid glands in organ culture. *Endocrinology.* **105:** 237–245.
27. Haddad, J. G., Fraser D. R., and D. E. M. Lawson. 1981. Vitamin D plasma binding protein. Turnover and fate in the rabbit. *J. Clin. Invest.* **67:** 1550–1560.