

## Experimental renal osteodystrophy. The response to 25-hydroxycholecalciferol and dichloromethylene diphosphate therapy.

J E Russell, ... , J D Termine, L V Avioli

*J Clin Invest.* 1975;56(3):548-554. <https://doi.org/10.1172/JCI108123>.

### Research Article

Bone mineral and matrix maturation in chronically uremic, nonacidotic rats were investigated after 25-hydroxycholecalciferol (25OHD) and/or dichloromethylene diphosphonate (C12MDP) therapy utilizing bromoform-toluene density gradient fractionation and X-ray diffraction analyses. The bromoform-toluene density gradient analyses demonstrated that the progressive accumulation of less dense, more immature bone characteristic of progressive uremia was reversed by 25OHD and/or C12MDP therapy for a 2-wk period, and that after 4 wk of therapy the maturational profile of bones from chronically uremic animals treated with 25OHD and/or C12MDP was comparable to that from nonuremic littermates. X-ray diffraction analysis revealed that by the 4th wk of therapy with 25OHD and C12MDP both the degree of crystallinity and the crystal size/perfection parameters in the uremic bones were comparable to those of nonuremic, paired control littermates. Treatment for 4 wk with 25OHD resulted in enlarged and/or more perfect apatite crystallites, while C12MDP alone slightly inhibited crystal growth and/or perfection after 2 wk of treatment. Soft tissue calcification was diminished in uremic animals treated for 4 wk with C12MDP or a combined C2MDP/25OHD regimen, the latter being much more effective in this regard. The accumulated data in this study support the premise that the attendant accelerated bone resorption, soft tissue calcification, and abnormal mineralization and maturation of the skeletal tissue, well documented to characterize experimental renal insufficiency, may be alleviated with [...]

**Find the latest version:**

<https://jci.me/108123/pdf>



# Experimental Renal Osteodystrophy

## THE RESPONSE TO 25-HYDROXYCHOLECALCIFEROL AND DICHLOROMETHYLENE DIPHOSPHATE THERAPY

JEAN E. RUSSELL, JOHN D. TERMINE, and LOUIS V. AVIOLI

*From the Department of Medicine, The Jewish Hospital of St. Louis and Washington University School of Medicine, St. Louis, Missouri 63110, and the Molecular Structure Section, Laboratory of Biological Structure, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland 20014*

**ABSTRACT** Bone mineral and matrix maturation in chronically uremic, nonacidotic rats were investigated after 25-hydroxycholecalciferol (25OHD) and/or dichloromethylene diphosphonate (Cl<sub>2</sub>MDP) therapy utilizing bromoform-toluene density gradient fractionation and X-ray diffraction analyses.

The bromoform-toluene density gradient analyses demonstrated that the progressive accumulation of less dense, more immature bone characteristic of progressive uremia was reversed by 25OHD and/or Cl<sub>2</sub>MDP therapy for a 2-wk period, and that after 4 wk of therapy the maturational profile of bones from chronically uremic animals treated with 25OHD and/or Cl<sub>2</sub>MDP was comparable to that from nonuremic littermates.

X-ray diffraction analysis revealed that by the 4th wk of therapy with 25OHD and Cl<sub>2</sub>MDP both the degree of crystallinity and the crystal size/perfection parameters in the uremic bones were comparable to those of nonuremic, pair-fed control littermates. Treatment for 4 wk with 25OHD resulted in enlarged and/or more perfect apatite crystallites, while Cl<sub>2</sub>MDP alone slightly inhibited crystal growth and/or perfection after 2 wk of treatment. Soft tissue calcification was diminished in uremic animals treated for 4 wk with Cl<sub>2</sub>MDP or a combined Cl<sub>2</sub>MDP/25OHD regimen, the latter being much more effective in this regard.

The accumulated data in this study support the premise that the attendant accelerated bone resorption, soft tissue calcification, and abnormal mineralization and maturation of the skeletal tissue, well documented to

characterize experimental renal insufficiency, may be alleviated with therapeutic dosages of 25OHD and/or Cl<sub>2</sub>MDP.

### INTRODUCTION

Recent studies undertaken in this laboratory have sought to delineate more fully the defect in collagen metabolism in the experimental chronic uremic state, to relate this change to alterations in matrix mineralization, and to determine the compositional and structural alterations of the mineral components of skeletal tissue. We demonstrated alterations in bone collagen and mineral maturation in the experimental uremic state that progressed with advancing uremia, although insignificant changes were observed in plasma pH, calcium, and bone carbonate content (1). X-ray diffraction analyses substantiated density gradient measurements suggesting that the relatively "immature" bone tissue of uremic animals was characterized by both increased amounts of X-ray amorphous mineral and less perfect, smaller-sized, apatite crystallites (2).

It is thought that maturation and increased cross-linking of newly synthesized collagen precedes calcification in hard tissues (3, 4). Consistent with the deranged maturation of the skeletal tissue in progressive renal failure, the ratio of the NaB<sup>3</sup>H<sub>4</sub>-reducible cross-links, dihydroxylysinonorleucine to hydroxylysinonorleucine, in bones from uremic animals exhibited a progressive increase through the 9th wk of uremia (5). Because of the possible relationship between the uremic maturational defect in bone, reports of altered vitamin D metabolism in uremia (6), and defective collagen cross-linking in the vitamin D-deficient state (7), the

---

*Received for publication 30 December 1974 and in revised form 23 April 1975.*

present study was undertaken to determine whether the vitamin D metabolite 25-hydroxycholecalciferol (25-OHD)<sup>1</sup> alone or in concert with dichloromethylene diphosphonate (Cl<sub>2</sub>MDP), an agent potentially capable of stabilizing alterations in bone metabolism characterized by pathological resorption of the skeleton (8, 9), was effective in reversing the compositional and structural alterations in bone metabolism observed in experimental renal osteodystrophy.

## METHODS

Female Holtzman rats at 6 wk of age were unilaterally nephrectomized with contralateral segmental renal infarction, resulting in an impairment of seven-eighths normal kidney function as previously described (1). This ischemic infarction of functioning renal mass persisted during the subsequent 6 or 8-wk period of study. Control animals were pair-fed to maintain similar rates of growth between the two experimental groups. Pair-fed control and chronically (4 wk) uremic rats were maintained for an additional 2 and 4 wk on each of the following regimens: (a) cottonseed oil vehicle; (b) 25OHD (500 U/day, orally in cottonseed oil)<sup>2</sup>; (c) Cl<sub>2</sub>MDP (2 mg/kg per day, subcutaneously); and (d) 25OHD (500 U/day) and Cl<sub>2</sub>MDP (2 mg/kg per day). At the termination of the experimental period (18 h after the final dose of 25OHD and/or Cl<sub>2</sub>MDP): (a) serum calcium and phosphorus and urinary hydroxyproline and calcium analyses were made as previously described (1); (b) urine retentate hydroxyproline levels were measured (1), the latter comprised primarily of hydroxyproline-containing peptides with a mol wt > 13,000 (10, 11); and (c) soft tissue ash and mineral content were measured, the latter after subjecting the tissue to 600°C for 24 h. In vivo duodenal calcium absorption was measured by a modification of the techniques of Coates and Holdsworth (12). 0.5 μCi of <sup>45</sup>Ca containing 2.0 mg of <sup>45</sup>Ca in 0.1 ml vol was injected intraduodenally, and <sup>45</sup>Ca, <sup>45</sup>Ca, and phosphorus was determined in the serum 30 min after injection. Previous determinations in control and uremic rats have demonstrated a peak in <sup>45</sup>Ca absorption at 30 min; this time-point of <sup>45</sup>Ca absorption was unaltered by therapy with 25OHD and/or Cl<sub>2</sub>MDP.

Characterization of the changes in the bone mineral and matrix collagenous protein was accomplished utilizing bromoform-toluene density gradient fractionation (1) as well as X-ray diffraction analysis (2). Total skeletal calcium, phosphorus, and hydroxyproline levels were concomitantly determined, as previously described (1).

## RESULTS

As reported in earlier studies, the uremic state was attended by elevations in plasma, inorganic phosphate (controls, 6.68±0.13; uremics, 7.95±0.31 mg/100 ml),

<sup>1</sup>Abbreviations used in this paper: Cl<sub>2</sub>MDP, dichloromethylene diphosphonate; EHDP, ethane-1-hydroxy-1,1-diphosphonate; 25OHD, 25-hydroxycholecalciferol.

<sup>2</sup>Although no official definition of units has been formulated for 25OHD, 1 U of 25OHD in this study is defined to be 91 pmol. This potency is relative to a 1.4-fold stimulation of intestinal calcium transport compared to the parent vitamin (cholecalciferol).

TABLE I  
Urine Total and Retentate OH-Proline in Uremic Rats after 4 wk 25OHD and Cl<sub>2</sub>MDP Therapy\*

| Treatment group                            | Total          | Retentate      | Retentate as % of total |
|--|----------------|----------------|-------------------------|
|  | μg/mg Cr       |                |                         |
| Uremic + vehicle (17)                      | 51.69<br>±2.9  | 11.45<br>±1.2  | 22.94<br>±2.0           |
| Uremic + 25OHD (15)                        | 61.63<br>±7.6  | 17.58<br>±2.8  | 26.83<br>±2.4           |
| Uremic + Cl <sub>2</sub> MDP (19)          | 68.99‡<br>±7.8 | 17.09‡<br>±2.0 | 26.26<br>±2.5           |
| Uremic + 25OHD and Cl <sub>2</sub> MDP (4) | 54.40<br>±6.4  | 20.79‡<br>±4.2 | 37.45‡<br>±3.4          |

\* "Retentate OH-Proline" refers to hydroxyproline containing peptides with a mol wt > 13,000 (10, 11). Numbers represent mean±SEM for each respective group. Numbers of animals are represented in parentheses for each treatment group.

‡ *P* < 0.05, according to Student's *t* test, compared to the vehicle-treated group.

creatinine (controls, 0.60±0.05; uremics, 1.12±0.13 mg/100 ml), and urea nitrogen (controls, 20.2±0.19; uremics, 57.6±2.0 mg/100 ml). Plasma pH and calcium values in the uremic animals were 7.41±0.03 and 9.84±0.22 mg/100 ml, respectively, and comparable to those obtained in the control group, 7.40±0.02 and 9.44±0.16, respectively. After a 2-wk period of treatment with either 25OHD and/or Cl<sub>2</sub>MDP, reductions in serum calcium and phosphate concentrations were observed in both control and uremic animals. However, by the 4th wk of therapy, serum calcium and phosphorus concentrations were similar in both groups.

Therapy with 25OHD and/or Cl<sub>2</sub>MDP led to a rise in urinary hydroxyproline in the chronically uremic rats, primarily because of an increase in the retentate high molecular weight fraction (Table I). In contrast, no change was seen in either total or retentate hydroxyproline excretion in the nonuremic animals. Urinary calcium and phosphorus in both control and chronically uremic rats treated for 4 wk with 25OHD and/or Cl<sub>2</sub>MDP were unchanged.

The net intestinal absorption of calcium was unchanged by 2 wk of 25OHD and/or Cl<sub>2</sub>MDP therapy to both control and chronically uremic rats. At the termination of the 4-wk study the duodenal <sup>45</sup>Ca absorption in pair-fed control rats was still unchanged by the 25OHD/Cl<sub>2</sub>MDP treatment. While chronically uremic rats treated with 25OHD also evidenced no change in duodenal <sup>45</sup>Ca absorption, the Cl<sub>2</sub>MDP-treated group responded with a 50% increment; those animals on

**TABLE II**  
*Kidney and Muscle Ash Calcium and Phosphate Levels in Chronically Uremic Rats after 4 wk 25OHD and Cl<sub>2</sub>MDP Therapy\**

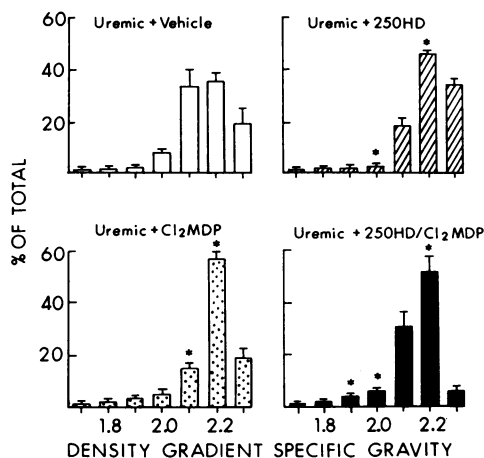
| Treatment group                            | Kidney                 |                 | Muscle         |                 |
|--|------------------------|-----------------|----------------|-----------------|
|  | Ca                     | PO <sub>4</sub> | Ca             | PO <sub>4</sub> |
|  | <i>mg/g dry tissue</i> |                 |                |                 |
| Uremic + vehicle (7)                       | 1.90<br>±0.08          | 14.82<br>±0.44  | 0.48<br>±0.02  | 10.39<br>±0.21  |
| Uremic + 25OHD (12)                        | 1.09‡<br>±0.20         | 16.04<br>±1.5   | 0.42<br>±0.04  | 9.45<br>±0.37   |
| Uremic + Cl <sub>2</sub> MDP (10)          | 0.94‡<br>±0.23         | 12.75<br>±1.2   | 0.23*<br>±0.01 | 7.77‡<br>±0.10  |
| Uremic + 25OHD and Cl <sub>2</sub> MDP (4) | 0.82‡<br>±0.24         | 6.43‡<br>±0.99  | 0.17*<br>±0.01 | 3.18‡<br>±0.45  |
| Nonuremic + vehicle (4)                    | 0.45<br>±0.02          | 8.59<br>±0.11   | 0.15<br>±0.01  | 5.50<br>±0.24   |

\* Numbers represent mean ± SEM for each treatment group. Numbers of animals are represented in parentheses.

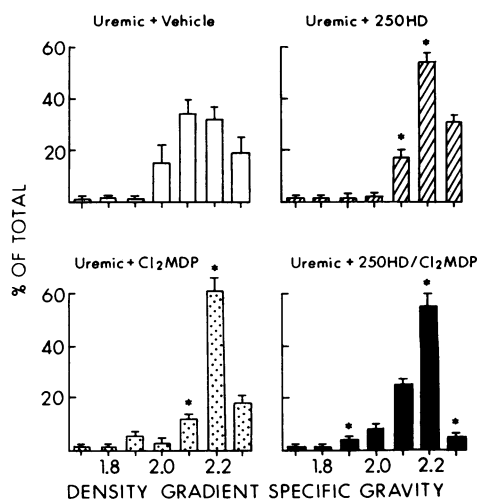
‡  $P < 0.001$ , according to Student's *t* test, compared to the vehicle-treated uremic group.

combined 25OHD/Cl<sub>2</sub>MDP therapy evidenced a 200% increase in calcium absorption, compared to the untreated chronically uremic rat.

Kidney or muscle ash calcium and phosphate concentrations, of the nonuremic animals, were unaffected by 25OHD and/or Cl<sub>2</sub>MDP treatment. In marked contrast, the significant degree of kidney and muscle calcification which was observed in the chronically uremic animals decreased during the 25OHD-Cl<sub>2</sub>MDP therapeutic interval (Table II). Cl<sub>2</sub>MDP alone reduced the



**FIGURE 1** Effect of 4 wk of treatment with 25OHD and Cl<sub>2</sub>MDP on bone calcium distribution in chronically uremic rats according to the bromoform-toluene fractionation technique (1). The (\*) in each instance signifies statistical significance between uremic and vehicle alone and the respective treated groups with  $P < 0.01$  according to the Student's *t* test.



**FIGURE 2** Effect of 25OHD and Cl<sub>2</sub>MDP therapy for 4 wk on bone collagen (hydroxyproline) distribution in chronically uremic rats according to the bromoform-toluene fractionation technique (1). The (\*) in each instance signifies statistical significance between uremic and vehicle alone and the respective treated group with  $P < 0.01$  according to the Student's *t* test.

muscle ash calcium and phosphorus by 30% while the combination of 25OHD and Cl<sub>2</sub>MDP resulted in a 50 and 70% diminution in the kidney and muscle concentration of these elements, respectively. In fact, the combined effect of 25OHD and Cl<sub>2</sub>MDP succeeded in lowering the soft tissue calcium and phosphorus levels to within the range of those measured for nonuremic animals (Table II).

Bromoform-toluene density gradient analysis revealed that the progressive accumulation of less dense, more immature bone mineral and collagen characteristic of progressive uremia was reversed by 25OHD and/or Cl<sub>2</sub>MDP therapy for a 2-wk period. This reversal was complete after 4 wk of therapy with either 25OHD, Cl<sub>2</sub>MDP, or a combination of the two (Fig. 1 and 2), and at which time the density gradient profiles of the 25OHD-Cl<sub>2</sub>MDP-treated uremic animals were comparable to those of their nonuremic pair-fed littermates. These agents had no effect on these parameters in normal, nonuremic animals. Quite striking was the observation that: (a) 25OHD administration elevated both the calcium and hydroxyproline content of the bone (per gram dry weight of bone); (b) Cl<sub>2</sub>MDP significantly elevated the skeletal phosphorus level; and (c) 25OHD in combination with Cl<sub>2</sub>MDP resulted in a marked elevation of skeletal calcium, phosphorus, and hydroxyproline (collagen) (Table III).

X-ray diffraction analyses revealed that after 2 wk of therapy the administration of 25OHD had not produced any change in the crystallographic properties of

TABLE III  
Tibial Calcium, Phosphate, and OH-Proline Levels in  
Chronically Uremic Rats after 4 wk 25OHD  
and Cl<sub>2</sub>MDP Therapy\*

| Treatment group                               | Calcium         | Phosphate       | OH-Proline     |
|---|-----------------|-----------------|----------------|
|   | mg/g dry tissue |                 |                |
| Uremic + vehicle (5)                          | 143.0<br>±5.8   | 83.6<br>±4.4    | 6.18<br>±0.06  |
| Uremic + 25OHD (8)                            | 180.4†<br>±9.1  | 92.3<br>±0.9    | 8.21†<br>±0.50 |
| Uremic + Cl <sub>2</sub> MDP (6)              | 181.99†<br>±7.8 | 109.6†<br>±5.0  | 7.60<br>±0.58  |
| Uremic + 25OHD and<br>Cl <sub>2</sub> MDP (4) | 180.88†<br>±8.1 | 111.66†<br>±6.1 | 9.32†<br>±0.60 |
| Nonuremic + vehicle (4)                       | 158.2<br>±2.3   | 95.3<br>±1.7    | 10.34<br>±0.40 |

\* Numbers represent mean ± SEM for each treatment group. Numbers of animals are represented in parentheses.

†  $P < 0.01$ , according to Student's *t* test, compared to the vehicle-treated uremic group.

the bone, relative to untreated uremics, while Cl<sub>2</sub>MDP alone or in combination with 25OHD decreased the crystal size/perfection measurements significantly (Table IV). By the 4th wk of therapy, however, the degree of crystallinity and the apatite crystal size/perfection of the bones in uremic animals treated with the 25OHD-Cl<sub>2</sub>MDP combination were comparable to those of non-

uremic littermates, while treatment for 4 wk with 25-OHD alone only increased apatite crystal size and/or perfection (Table IV). No alteration in X-ray diffraction properties of nonuremic rat bones was noted as a consequence of the therapeutic regimen employed.

## DISCUSSION

The accumulated data in this study support the premise that the attendant massive bone resorption, the soft tissue calcification, and the abnormal mineralization and maturation of skeletal tissue which characterize experimental renal insufficiency, may be alleviated with appropriate dosages of 25OHD and/or Cl<sub>2</sub>MDP. That little perturbation of circulating and/or excreted calcium and phosphorus levels occurred as a result of 25OHD and Cl<sub>2</sub>MDP treatment is in agreement with the studies of Younoszai and Schedl (13), Bonjour, Fleisch, and Copp (14), and Gasser, Morgan, Fleisch, and Richelle (9). The slight reduction in serum calcium and phosphorus concentrations which initially occurred at the 2-wk period has been noted previously in rats treated with Cl<sub>2</sub>MDP at varying doses and has been attributed to the decreased resorptive capability of the skeletal tissue as a result of the diphosphonate administration (9). Indeed, in the uremic rat there was a marked diminution in the rate of skeletal resorption as well as a stimulation of new bone collagen formation as a consequence of Cl<sub>2</sub>MDP and/or 25OHD. This was indicated by the 20% increment in the retentate urinary hydroxyproline fraction in the 25OHD and Cl-

TABLE IV  
XRD Data for Hydrazine Deproteinized Femurs Taken from Experimentally Induced  
Uremic Rats after 25OHD and Cl<sub>2</sub>MDP Therapy

| Treatment group                    | No. of animals | Peak broadening<br>$\beta_{\frac{1}{2}}(002) (^{\circ}2\theta)$ | Parameters*<br>$\beta_{\frac{1}{2}}(310) (^{\circ}2\theta)$ | Integrated diffraction<br>intensity† | X-ray amorphous<br>mineral<br>% |
|------------------------------------|----------------|---|---|--------------------------------------|---------------------------------|
| 2 wk therapeutic regimen           |                |   |   |                                      |                                 |
| Uremic + vehicle                   | 3              | 0.50 ± 0.01   | 1.48 ± 0.06   | 6.695 ± 0.289                        | 34.6 ± 2.8                      |
| Uremic + 25OHD                     | 3              | 0.52 ± 0.01   | 1.50 ± 0.05   | 6.705 ± 0.071                        | 34.5 ± 0.7                      |
| Uremic + Cl <sub>2</sub> MDP       | 3              | 0.52 ± 0.01   | 1.51 ± 0.02§  | 6.408 ± 0.112                        | 37.4 ± 1.0                      |
| Uremic + 25OHD/Cl <sub>2</sub> MDP | 3              | 0.52 ± 0.01   | 1.55 ± 0.02†  | 6.960 ± 0.265                        | 30.5 ± 2.5                      |
| Nonuremic + vehicle                | 4              | 0.50 ± 0.02   | 1.49 ± 0.06   | 6.812 ± 0.204                        | 33.9 ± 2.0                      |
| 4 wk therapeutic regimen           |                |   |   |                                      |                                 |
| Uremic + vehicle                   | 6              | 0.51 ± 0.01   | 1.58 ± 0.03   | 6.293 ± 0.141                        | 38.4 ± 1.4                      |
| Uremic + 25OHD                     | 7              | 0.52 ± 0.02   | 1.53 ± 0.05§  | 6.503 ± 0.168                        | 36.7 ± 1.6                      |
| Uremic + Cl <sub>2</sub> MDP       | 4              | 0.51 ± 0.01   | 1.55 ± 0.06   | 6.454 ± 0.228                        | 37.2 ± 2.2                      |
| Uremic + 25OHD/Cl <sub>2</sub> MDP | 4              | 0.52 ± 0.02   | 1.53 ± 0.02   | 6.615 ± 0.112                        | 35.0 ± 1.1                      |
| Nonuremic + vehicle                | 4              | 0.50 ± 0.01   | 1.50 ± 0.02   | 6.748 ± 0.114                        | 34.3 ± 1.0                      |

All values given are the mean ± SD of the mean.

\* These parameters are inversely proportional to crystal size and/or perfection.

†  $I \times \ln$  values (as defined in reference 2).

§  $P < 0.05$ , according to Student's *t* test, compared to respective uremic and vehicle control group.

||  $P < 0.02$ .

MDP-treated groups and by the 50% increment in that group subjected to 25OHD/C<sub>12</sub>MDP combination therapy (Table I).

The failure of the 25OHD therapy alone to alter <sup>45</sup>Ca absorption is not surprising in that animals in a vitamin D-repleted state have been shown to be relatively insensitive to exogenous administration of vitamin D and/or its metabolites (13). Although the chronically uremic rat is notoriously "vitamin D resistant" and has been shown to have decreased circulating levels of 25OHD, the body stores of vitamin D are obviously sufficient to blunt intestinal absorptive response to exogenous 25OHD. That the diphosphonate, C<sub>12</sub>MDP, did significantly increase the net duodenal <sup>45</sup>Ca absorption in the chronically uremic rat is in contrast to the findings of Bonjour et al. (15), Krawitt (16), and others who observed a reduction in intestinal calcium absorption after the administration of massive doses of diphosphonates. In agreement with observations on diphosphonate-treated patients suffering from Paget's disease and/or osteoporosis (17, 18), a lower dose of the compound does not markedly diminish duodenal calcium transport and may, in some osteoporotics (19), increase the rate of calcium absorption. Additionally, the observations by Gasser and coworkers (9), in rats given graded doses of C<sub>12</sub>MDP for 7 days, substantiate our observations in the uremic rat in that a small increase in the net calcium absorption does occur after a chronic administration of C<sub>12</sub>MDP (9).

In view of the enhanced duodenal <sup>45</sup>Ca absorption and unchanged calcium excretion of the 25OHD-C<sub>12</sub>MDP-treated uremic animal, compared to the untreated uremic rat, it is quite significant that the degree of kidney and muscle calcification which occurred in the untreated state was ameliorated by 25OHD and/or C<sub>12</sub>MDP therapy (Table II). Although the subcellular localization of the mineral in the kidney and muscle tissue is unknown, it may be that the 25OHD therapy results in a mobilization of mitochondrial calcium pools, thereby effecting a decrease in the tissue calcium concentration. Such a calcium-mobilizing action of vitamin D has been documented *in vitro* (20) and *in vivo* (21) in kidney tissue and may be operative in the 25OHD-treated uremic rats in the present study. A diphosphonate-related diminution in soft tissue calcification has been previously documented in vitamin D toxic animals (22) and in clinical disorders associated with ectopic calcification (23, 24), but no evidence has been presented to date correlating the degree of both soft tissue calcification and skeletal maturation in any one specific experimental model system using therapeutic dosages of the diphosphonates.

The accumulated bromoform-toluene density gradient profile and XRD data indicate that the bone being formed in the chronically uremic rat during the course

of therapy with 25OHD and/or C<sub>12</sub>MDP is more mature with respect to both its collagenous matrix and apatite crystals. Although the degree of skeletal resorption has been minimized (Table I), it is noteworthy that a corresponding decrease in accretion has not occurred. In fact, the net effect of the 25OHD/C<sub>12</sub>MDP treatment is probably one of uncoupling the processes of bone formation and resorption, with a resultant increment in bone mineral and/or collagenous matrix (Table III). These observations are in contrast with those of Kaye (25) who administered ethane-1-hydroxy-1,1-diphosphonate (EHDP) to chronically uremic rats from the start of renal infarction through the 4th wk of uremia and found no alteration in either the percent bone ash or degree of secondary hyperparathyroidism despite an increased retention of the EHDP compound compared to pair-fed, nonuremic littermates. Not only is it inappropriate to expect similar biological responses to diphosphonate compounds with different chemical composition (i.e. EHDP vs. C<sub>12</sub>MDP), but measurements of total bone mineral also provide little information as to the structural integrity or state of maturation of skeletal tissue that has been shown to be deranged as early as 2 wk after the induction of renal insufficiency (1). Moreover, the uremic rat of Kaye (25) exhibited hypocalcemia, a factor which is capable by itself of increasing parathyroid hormone secretion as well as decreasing bone collagen synthesis and mineralization.

Although it has been histologically demonstrated that C<sub>12</sub>MDP administration to rats diminishes bone resorption without impairing matrix mineralization (8), the physical effect of the diphosphonate upon bone mineral texture by XRD has not been previously investigated *in vivo*. Obviously, the short-term effects of C<sub>12</sub>MDP in decreasing apatite crystal size and/or perfection are quite distinct from the longer-term effects of 25OHD/C<sub>12</sub>MDP in reverting bone crystallinity and apatite crystal size/perfection to normal (Table IV). It is reasonable to assume that C<sub>12</sub>MDP acts through physicochemical mechanisms primarily and initially inhibits normal crystal growth in the uremic bones. Such a phenomenon has been substantiated *in vitro* (26) and indicated *in vivo* (27). That prolonged administration of the compound does not result in a significant compilation of smaller crystallites, and/or amorphous mineral may be related to the observations that bone and cartilage mineralization are not inhibited as markedly by C<sub>12</sub>MDP as is crystal dissolution (26, 28).

Alternatively, it may be argued that the transient hypocalcemia in the uremic rat, after 2 wk of 25OHD/C<sub>12</sub>MDP therapy, was sufficient stimulus to increase parathyroid hormone secretion. Although the interaction of parathyroid hormone with vitamin D metabolism is still controversial (29-31), some studies would suggest

(29, 32) that an increase in parathyroid hormone leads to an increase in conversion of 25OHD to 1,25(OH)<sub>2</sub>D. The resultant increased circulating levels of 1,25(OH)<sub>2</sub>D could thereby be the factor responsible for the observed increment in calcium absorption and bone collagen/mineral reorganization. However, since circulating levels of PTH and 1,25(OH)<sub>2</sub>D were not obtained in this study, theories as to their interactions on intestine and bone during either 25OHD or 25OHD/Cl<sub>2</sub>MDP therapy must remain conjectural.

In this regard it is most interesting that 25OHD therapy alone to the chronically uremic rat resulted in a reversion of the skeletal apatite crystal size/perfection to normal (Table IV). Previous studies have demonstrated that vitamin D and/or its metabolites are capable of promoting bone collagen synthesis and that the newly-formed matrix is capable of undergoing mineralization (33-35). Those uremic animals which received 25OHD did in fact exhibit an increased formation of bone collagen (hydroxyproline) (Table III). The rise in urinary nondialyzable (retentate) polypeptides, containing hydroxyproline, observed during treatment is consistent with this interpretation (Table I) since these fragments largely reflect collagen synthesis (10, 11). In a previous communication, alterations in collagen maturation were documented as early as the 2-wk period of experimentally induced uremia (1), well in advance of the inorganic structural alterations of the mineral phase alone (2). Since it is thought that maturation and increased cross-linking of newly synthesized collagen precedes calcification in hard tissues (3, 4), the partial restoration of the crystal maturational sequence may be secondary to the restoration of the bone collagen maturation. Indeed, the combined effects of 25OHD and Cl<sub>2</sub>MDP were to decrease the X-ray amorphous component of the bone mineral as well as increase the average crystal size-perfection parameters (Table IV). Furthermore, morphometric analyses have demonstrated that 25OHD administration to anephric patients does serve to ameliorate the attendant renal osteodystrophy (36). This latter observation in concert with in vitro (37) and in vivo (38) studies provides evidence that the 25OH-vitamin D metabolite may act directly on bone without first being hydroxylated in the 1-alpha position to 1,25(OH)<sub>2</sub>D. However, since the animals used in this study were not anephric, the potential contribution of 1,25(OH)<sub>2</sub>D to the observed effects in bone cannot be ignored.

The relationship of these data obtained from the experimental animals to reports of altered vitamin D metabolism in uremic man (6), response to 25OHD therapy (39), and to the pathogenesis of the defective collagen cross-linking in uremic (5) and vitamin D-deficient animals (7) are yet to be defined. It is interesting to note that experimental rachitic states are also

characterized by an increase in the X-ray amorphous bone mineral fraction (40), and that bone collagen synthesis in vitro, is influenced by fluctuations in media calcium and inorganic phosphate (41-43) as well as by the vitamin D status of the animals (33-35).

#### ACKNOWLEDGMENTS

The writers gratefully acknowledge The Proctor and Gamble Company for the gift of the diphosphonate compounds used in this study and the skilled assistance of Mrs. Shirley Peirick in preparation of this manuscript.

This work was supported by National Institutes of Health Contract 70-2219 and National Institutes of Health Grant 11674.

#### REFERENCES

1. Russell, J. E., and L. V. Avioli. 1972. Effect of experimental chronic renal insufficiency on bone mineral and collagen maturation. *J. Clin. Invest.* **51**: 3072-3079.
2. Russell, J. E., J. D. Termine, and L. V. Avioli. 1973. Abnormal bone mineral maturation in the chronic uremic state. *J. Clin. Invest.* **52**: 2848-2852.
3. Mills, B. G., and L. A. Bavetta. 1968. Bone collagen dynamics. *Clin. Orthop. Relat. Res.* **57**: 267-275.
4. Uitto, J., and O. Laitinen. 1968. Relation of collagen metabolism to calcium metabolism in the bone. *Acta Chem. Scand.* **22**: 1039-1040.
5. Russell, J. E., L. V. Avioli, and G. Mechanic. 1975. The nature of the collagen cross-links in bone in the chronic uraemic state. *Biochem. J.* **145**: 119-120.
6. Avioli, L. V., S. W. Lee, and H. F. DeLuca. 1971. The metabolism of 25-hydroxycholecalciferol-<sup>3</sup>H (25-HD-<sup>3</sup>H) in uremia. *J. Clin. Invest.* **50**: 4a. (Abstr.)
7. Mechanic, G. L., S. V. Toverud, and W. K. Ramp. 1972. Quantitative changes in bone collagen crosslinks and precursors in vitamin D deficiency. *Biochem. Biophys. Res. Commun.* **47**: 760-765.
8. King, W. R., M. D. Francis, and W. R. Michael. 1971. Effect of disodium ethane-1-hydroxyl-1, 1-diphosphonate on bone formation. *Clin. Orthop. Relat. Res.* **78**: 251-270.
9. Gasser, A. B., D. B. Morgan, H. Fleisch, and L. J. Richelle. 1972. The influence of two diphosphonates on calcium metabolism in the rat. *Clin. Sci. (Oxf.)*. **43**: 31-45.
10. Krane, S. M., A. J. Munoz, and E. D. Harris, Jr. 1970. Urinary polypeptides related to collagen synthesis. *J. Clin. Invest.* **49**: 716-729.
11. Haddad, J. G., Jr., S. Couranz, and L. V. Avioli. 1970. Nondialyzable urinary hydroxyproline as an index for bone collagen formation. *J. Clin. Endocrinol. Metab.* **30**: 282-287.
12. Coates, M. E., and E. S. Holdsworth. 1961. Vitamin D<sub>3</sub> and absorption of calcium in the chick. *Br. J. Nutr.* **15**: 131-147.
13. Younoszai, M. K., and H. P. Schedl. 1972. Influence of vitamin D on in vivo intestinal calcium transport in normal rats. *Proc. Soc. Exp. Biol. Med.* **140**: 496-501.
14. Bonjour, J-P., H. Fleisch, and D. H. Copp. 1973. Influence of a diphosphonate on serum calcium homeostasis. *Proc. Soc. Exp. Biol. Med.* **145**: 404-407.
15. Bonjour, J-P., H. F. DeLuca, H. Fleisch, U. Trechsel, L. A. Matejowic, and J. L. Omdahl. 1973. Reversal of EHDP inhibition of calcium absorption by 1,25-dihydroxycholecalciferol. *Eur. J. Clin. Invest.* **3**: 44-48.

16. Krawitt, E. L., H. W. Sampson, A. S. Kunin, and J. L. Matthews. 1974. Effect of ethane-1-hydroxy-1,1-diphosphonate (EHDP) administration on duodenal calcium transport. *Calcif. Tissue Res.* 15: 21-29.
17. Russell, R. G. G., R. Smith, C. Preston, R. J. Walton, and C. G. Woods. 1974. Diphosphonates on Paget's disease. *Lancet.* 5: 894-898.
18. Franck, W. A., N. A. Bress, F. R. Singer, and S. M. Krane. 1974. Rheumatic manifestations of Paget's disease of bone. *Am. J. Med.* 56: 592-603.
19. Szymedera, J., R. Heaney, and P. Saville. 1972. Intestinal calcium absorption: concurrent use of oral and intravenous tracers and calculation by the inverse convolution method. *J. Lab. Clin. Med.* 79: 570-586.
20. Engstrom, G. W., and H. F. DeLuca. 1962. The action of vitamin D in vivo and in vitro on the release of calcium from kidney mitochondria. *J. Biol. Chem.* 237: 974-975.
21. Engstrom, G. W., and H. F. DeLuca. 1964. Vitamin D-stimulated release of calcium from mitochondria. *Biochemistry.* 3: 203-209.
22. Fleisch, H. A., R. G. G. Russell, S. Bisaz, R. C. Muhlbauer, and D. A. Williams. 1970. The inhibitory effect of phosphonates on the formation of calcium phosphate crystals in vitro and on aortic and kidney calcification in vivo. *Eur. J. Clin. Invest.* 1: 12-18.
23. Schibler, D., and H. Fleisch. 1966. Inhibition of skin calcification (calciophylaxis) by polyphosphates. *Experientia (Basel).* 22: 367-369.
24. Francis, M. D., L. Flora, and W. R. King. 1972. The effects of disodium ethane-1-hydroxyl-1,1-diphosphonate on adjuvant induced arthritis in rats. *Calcif. Tissue Res.* 9: 109-121.
25. Kaye, M. 1973. The effect of a diphosphonate on uremic hyperparathyroidism. *Nephron.* 10: 188-194.
26. Fleisch, H., R. G. G. Russell, and F. Straumann. 1966. Effect of pyrophosphate on hydroxyapatite and its implications in calcium homeostasis. *Nature (Lond.).* 212: 901-903.
27. Russell, R. G. G., and H. Fleisch. 1970. Inorganic pyrophosphate and pyrophosphates in calcification and calcium homeostasis. *Clin. Orthop. Relat. Res.* 69: 101-117.
28. Schenk, R., W. A. Merz, R. Muhlbauer, R. G. G. Russell, and H. Fleisch. 1973. Effect of ethane-1-hydroxyl-1, 1-diphosphonate (EHDP) and dichloromethylene diphosphonate (Cl<sub>2</sub>MDP) on the calcification resorption of cartilage and bone in the tibial epiphysis and metaphysis of rats. *Calcif. Tissue Res.* 11: 196-214.
29. Garabedian, M., M. F. Holick, H. F. DeLuca, and I. T. Boyle. 1972. Control of 25-hydroxycholecalciferol metabolism by parathyroid glands. *Proc. Natl. Acad. Sci. U. S. A.* 69: 1673-1676.
30. Raisz, L. G., C. L. Trummel, and H. Simmons. 1972. Induction of bone resorption in tissue culture: prolonged response after brief exposure to parathyroid hormone and 25-hydroxycholecalciferol. *Endocrinology.* 90: 744-751.
31. Garabedian, M., Y. Tanaka, M. F. Holick, and H. F. DeLuca. 1974. Response of intestinal calcium transport and bone calcium mobilization to 1,25-dihydroxyvitamin D<sub>3</sub> in thyroparathyroidectomized rats. *Endocrinology.* 94: 1022-1027.
32. Rasmussen, H., M. Wong, D. Bikle, and D. B. P. Goodman. 1972. Hormonal control of the renal conversion of 25-hydroxycholecalciferol to 1,25-dihydroxycholecalciferol. *J. Clin. Invest.* 51: 2502-2504.
33. Morava, E., Z. Hegyi, M. Winter, and R. Trajan. 1971. Effect of Vitamin D on hydroxyproline production of rat bones. *Acta Physiol. Acad. Sci. Hung.* 39: 279-282.
34. Morava, E., R. Tarjan, and M. Winter. 1973. Lag period of action of 25-hydroxycholecalciferol on bone collagen metabolism in vitamin D deficient rats. *Experientia (Basel).* 29: 1225-1226.
35. Crenshaw, M. A., W. K. Ramp, W. A. Gonnerman, and S. V. Toveruda. 1974. Effects of dietary vitamin D levels on the in vitro mineralization of chick metaphyses. *Proc. Soc. Exp. Biol. Med.* 146: 488-493.
36. Witmen, G., and S. Balsan. 1971. 25-Hydroxycholecalciferol. Effects in renal osteodystrophy. European Society for Pediatric Research Working Party on Mineral Metabolism.
37. Trummel, C. L., L. G. Raisz, J. W. Blunt, and H. F. DeLuca. 1969. 25-hydroxycholecalciferol stimulation of bone resorption in tissue culture. *Science (Wash. D. C.).* 163: 1450-1451.
38. Pavlovitch, H., M. Garabedian, and S. Balsan. 1973. Calcium-mobilizing effect of large doses of 25-hydroxycholecalciferol in anephric rats. *J. Clin. Invest.* 52: 2656-2659.
39. Bordier, P. J., P. J. Marre, and C. D. Arnaud. 1975. Evolution of renal osteodystrophy: correlation of bone histomorphometry and serum mineral and immunoreactive PTH before and after treatment with calcium carbonate or 25-hydroxycholecalciferol. *Kidney Int.* 7: 5102-5112.
40. Termine, J. D., and A. S. Posner. 1967. Amorphous/crystalline interrelationships in bone mineral. *Calcif. Tissue Res.* 1: 8-23.
41. Cooper, C. W., and R. V. Talmage. 1965. A comparison of exogenous and endogenous parathyroid hormone effects on bone collagen synthesis. *Gen. Comp. Endocrinol.* 5: 534-541.
42. Russell, J. E., and R. V. Talmage. 1972. Differential effects of Ca and prior parathyroid state on proline-<sup>3</sup>H uptake by rat bone in vitro. *Am. J. Physiol.* 222: 1604-1609.
43. Asher, M. A., C. B. Sledge, and M. J. Glimcher. 1974. The effect of inorganic orthophosphate on the rates of collagen formation and degradation in bone and cartilage tissue in tissue culture. *J. Clin. Endocrinol. Metab.* 38: 376-389.