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THE EFFECTS OF DIPHOSPHONATES AND DIETARY CALCIUM ON CALCIUM HOMEOSTATIC MECHANISMS OF THE COW.

The Ohio State University, Ph.D., 1976
Veterinary Science

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THE EFFECTS OF DIPHOSPHONATES AND DIETARY CALCIUM 
ON CALCIUM HOMEOSTATIC MECHANISMS OF THE COW

Dissertation

Presented in Partial Fulfillment of the Requirements for 
the Degree Doctor of Philosophy in the Graduate 
School of The Ohio State University

By 

* * * * *

The Ohio State University

1975

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ACKNOWLEDGMENTS

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PUBLICATIONS


FIELDS OF STUDY

Major Field: Veterinary Pathology

Studies in Endocrine Pathology. Professor Charles C. Capen


Studies in Intestinal Pathology. Professor Larry A. Nagode
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CHAPTER I

EFFECT OF ETHANE-1-HYDROXY-1,1-DIPHOSPHONATE (EHDP) ON THE ULTRASTRUCTURE OF PARATHYROID GLANDS AND PLASMA IMMUNOREACTIVE PARATHYROID HORMONE IN PREGNANT COWS FED A LOW CALCIUM DIET

INTRODUCTION

Ethane-1-hydroxy-1,1-diphosphonate (EHDP) is a synthetic analogue of pyrophosphate and has a P-C-P bond instead of the P-O-P bond of the naturally occurring compound (12, 14, 15). EHDP is a potent inhibitor of hydroxyapatite crystal precipitation (12, 14, 15) and dissolution (12, 13, 34). These effects of EHDP are due to its ability to chemisorb to the surface of apatite crystals (14).

EHDP has a potent effect on resorption of bone. EHDP can inhibit parathyroid extract-induced resorption of bone in mouse calvaria grown in tissue culture (13, 29) and in thyroparathyroidectomized rats in vivo (13, 34). High doses of EHDP (10 mg./kg./day) given subcutaneously to rats caused a reduced rate of bone resorption, diminished efflux of $^{45}\text{calcium}$ from bone, and decreased urinary excretion of hydroxyproline (16). Depending on dosage, EHDP may also have an effect on formation of bone. EHDP given to dogs (25), cats (23) and rats (16, 33) caused an increased accumulation of osteoid in metaphyseal and diaphyseal bone and an impaired mineralization and increased thickness of the epiphyseal plate in rats (35). The ability of EHDP at low levels to reduce turnover of bone (16) has been utilized to treat certain human
metabolic bone diseases, particularly Paget's disease (1, 24, 38).

High doses of EHDP (10 mg./kg./day) given subcutaneously to rats decreased the rate of net intestinal absorption of calcium (16). Less retention of calcium and magnesium in bone associated with increased fecal excretion of both calcium and magnesium was observed in rats given a moderate subcutaneous dose of EHDP (3 mg./kg./day) (17). This effect of EHDP has been suggested to be caused by impaired synthesis of the active metabolites of vitamin D (3, 21). Low doses of EHDP have been shown to stimulate intestinal calcium absorption by an apparent increase in intestinal 1,25-dihydroxycholecalciferol in rats (18).

Several studies have provided additional evidence for alterations in calcium homeostasis in animals and humans treated with EHDP. High doses of EHDP (50 or 500 mg./kg./day) given orally to cats and moderate doses of EHDP (10 or 20 mg./kg./day) administered orally to human patients have been associated with increases in serum total calcium but with normal ionized calcium levels (22, 23). An increase in blood calcium has also been reported in rats fed a high calcium diet with high vitamin D and administered subcutaneously high levels of EHDP (10 mg./kg./day) (3). EHDP (40 mg./kg./day) when given subcutaneously to rats prevents the minimal decrease (5 per cent) in plasma calcium caused by the injection of salmon calcitonin compared to a more significant decrease (25 per cent) in rats administered calcitonin but not receiving EHDP (41).

In contrast, the effect of EHDP administration on serum calcium in the rabbit was reported to be dose- and time-related (32). High doses of EHDP (10 mg./kg./day) given subcutaneously resulted in a decrease in
total serum calcium associated with a rapid but transient decrease in serum ionized calcium. The increase in blood calcium induced by parathyroid hormone in fasting thyroparathyroidectomized rats was prevented by EHDP (34, 40). An additional study evaluated the effects of EHDP in human patients with normal serum calcium and elevated serum phosphorus. EHDP (30 mg./kg./day orally) had no effect on the phosphaturia and urinary excretion of cAMP caused by the intravenous administration of 40 and 200 units of bovine parathyroid hormone (31).

The objectives of the present investigation were to determine the effect of EHDP on (1) the fine structure of the parathyroid glands, and (2) the synthesis and secretion of parathyroid hormone following a hypocalcemic challenge induced either by EDTA or associated with parturition and the initiation of lactation in pregnant cows fed a low calcium diet. Pregnant cows were used in this investigation because plasma levels of PTH can readily be monitored using sensitive radioimmunoassay techniques (37). The cow, because of lactation and the marked calcium drain into the milk, has very active and responsive calcium homeostatic mechanisms. A failure in these control mechanisms at parturition with the initiation of lactation often results in the development of a clinical syndrome characterized by an acute severe hypocalcemia and hypophosphatemia with associated muscular weakness, paresis, paralysis and, if not treated, death (10, 27). The feeding of low calcium diets prepartum has proven to be efficacious in decreasing the incidence of this hypocalcemic syndrome in cows (9).
MATERIALS AND METHODS

Experimental Design

Six Jersey cows (5 to 8 years of age) were obtained 84 days pre-partum from one farm. The cows were divided equally into two groups and were acclimated to laboratory conditions for a period of 14 days. During the acclimation period all the cows were fed a previously described experimental diet (2) which provided the National Research Council's recommended levels for pregnant cows of 25 gm. of calcium and 25 gm. of phosphorus per day and adequate amounts of vitamin D (30). Cows in group 1 (low calcium diet) were fed 12 pounds of the experimental diet that provided 9.5 gm. of calcium and 19.0 gm. of phosphorus per day. Monosodium phosphate was added to the experimental diet in amounts to increase the phosphorus intake to 25 gm. per day. The diet was supplemented by the addition of 4 pounds of oat straw per cow. Cows in group 2 were fed a similar low calcium diet and received 4 mg. of EHDP per kg. suspended in sterile physiological saline (0.9 per cent) as a daily subcutaneous dose.

Each experiment began 80 days prior to the calculated date of parturition. At ten day intervals each cow was placed in a metabolism stall for the collection of serum, plasma, and urine samples. Plasma was collected in precooled centrifuge tubes and frozen (-20° C.) within 20 minutes. Duplicate 24 hour urine samples were collected using indwelling catheters (Bardex-Foley with a 75 ml. inflatable, fluted oval balloon, sizes 26, 28 or 30 French). A four foot rubber tube (0.25 inch diameter) attached to the indwelling catheter led to a Y-tube which funneled urine into two stainless steel collection vessels.
Toluene was added to one container to form a layer over the urine and preserve it for hydroxyproline determinations. Glacial acetic acid (5 ml.) was added to the second container to preserve urine for cyclic adenosine monophosphate (cAMP) assays. Aliquots of urine were stored at -20°C.

Immediately Available Calcium Reserves

The immediately available calcium reserves were evaluated by two ethylenediaminetetraacetic acid (EDTA) infusion studies. The first EDTA infusion (baseline) was performed after 7 days of the acclimation period. A second EDTA infusion (experimental) was performed after 60 days of the experiment. Forty-nine grams of EDTA in 5 per cent dextrose solution was administered intravenously to each cow over a 4 hour period or until the serum calcium level dropped below 7 mg. per 100 ml. Plasma and serum samples were collected prior to infusion and at 0.5 hour intervals for the next 16.0 hours. Urine samples were collected at hourly intervals. Blood and urine samples also were collected 24 and 48 hours from the start of the infusion.

Serum and Urinary Electrolyte Determinations

Serum and urinary calcium and serum magnesium were determined by atomic absorption spectrophotometry (Perkin-Elmer 303). Serum calcium concentrations were determined by the citric acid titration method (Harleco, Philadelphia, Pennsylvania) before, during and immediately after EDTA infusion. Serum and urinary phosphorus were determined by the method of Fiske and Subbarow (11). Urinary calcium and phosphorus were expressed as a ratio to creatinine.
Plasma Parathyroid Hormone Determination

Plasma samples from each collection period were frozen within 20 minutes for determination of parathyroid hormone concentration by radio-immunoassay (19, 36). Plasma samples were assayed employing an antiserum (GP-1) that detects both the amino terminal end (1-34 chain) of parathyroid hormone (the biologically active portion) and the carboxyl portion (inactive segment).

Urinary Hydroxyproline and Cyclic Adenosine Monophosphate

Urinary total hydroxyproline was determined by the method of Kivirikko, Laitinen and Prockop (26) and creatinine by the method of Clarke (7). Urinary cyclic adenosine monophosphate was determined as an index of the renal response to parathyroid hormone (28) by the method of Steiner et al. (39). Urinary hydroxyproline and cyclic adenosine monophosphate were expressed as a ratio to creatinine.

Bioassay of Thyroid Glands for Calcitonin Activity

Thyroid glands collected at the termination of the experiment from both groups of cows were frozen in liquid nitrogen (-190° C.) and stored at -20° C. for bioassay of calcitonin activity. Extracts of thyroid glands were prepared individually from each cow, lyophilized, and stored at -20° C. prior to assay. The extracts were reconstituted with a diluent composed of 0.01 M sodium acetate trihydrate and 20 mg. of bovine serum albumin. The reconstituted extracts were adjusted to pH 4 and assayed in 35-day-old male Holtzman rats according to the method of Cooper et al. (8) against a bovine thyrocalcitonin standard. The rats were exsanguininated from the abdominal aorta at 65 minutes after subcu-
taneous injection. In all of the four-point assays the high dose was 4 times the low dose. Serum was collected and analyzed for calcium by atomic absorption spectrophotometry. The logarithmic dose response was plotted for each assay and the relative potency of the extracts was determined.

**Light and Electron Microscopy**

Forty-eight hours postpartum or when the serum calcium dropped below 6 mg. per 100 ml. postpartum, each cow was euthanatized by electrocution. Sections of parathyroid and thyroid glands for electron microscopic evaluation were immediately immersed in fixative and cut into 0.5 cm. cubes, fixed in 3 per cent glutaraldehyde in 0.1 M sodium cacodylate, washed twice in cacodylate buffer, and postfixed with 1.33 per cent osmium tetroxide in s-collidine at pH 7.4. Tissues were dehydrated through ascending concentrations of ethyl alcohol, transferred to propylene oxide, and embedded in Epon 812 (Shell Oil Company, New York, New York). Sections were cut at 600 to 800 Å on a Reichert OmU-2 ultramicrotome and mounted on 300-mesh copper grids. The sections were stained with uranyl acetate and lead citrate, and examined with a Philips 200 electron microscope. Representative tissues from all major organ systems were fixed in 10 per cent phosphate-buffered formalin for histopathologic evaluation.
RESULTS

Serum Electrolytes

There were no significant differences in serum calcium between the two groups of cows during the prepartum period of 70 days. Average serum calcium for both groups of cows remained in the normal range (Fig. 1). At parturition serum calcium in cows fed the low calcium diet moderately declined to 8.05 mg. per 100 ml. Serum calcium dropped to 5.44 mg. per 100 ml. (P < 0.05) for cows fed the low calcium prepartal diet and administered EHDP. When serum calcium values fell below 6.00 mg./100 ml. the cows showed clinical signs varying from muscular weakness and incoordination to paresis. By one day postpartum serum calcium for cows fed the low calcium diet and administered EHDP further declined (P < 0.05) to 4.80 mg. per 100 ml. whereas for cows fed only the low calcium diet it remained unchanged (Fig. 1).

Serum phosphorus levels for both groups of cows remained within the normal range prepartum but declined with the approach of parturition. At parturition cows fed the low calcium diet had a serum phosphorus of 4.03 mg. per 100 ml. whereas in EHDP-treated cows it declined to 1.49 mg. per 100 ml. (P < 0.05). By one day postpartum the serum phosphorus in the cows fed the low calcium diet increased to 5.86 mg. per 100 ml. but it remained unchanged in cows administered EHDP (P < 0.01) (Fig. 2).

The levels of serum magnesium did not change prepartum, at parturition or postpartum in the experimental cows. Mean values for serum magnesium in the EHDP-treated cows tended to be lower but not significantly different from levels in cows fed the low calcium diet.
Fig. 1. Serum concentration of calcium in cows fed a low calcium diet (—) and cows fed a low calcium diet and administered EHDP (---) prepartum. Values for each interval represent the mean ± standard error.
FIG 1

- LOW Ca DIET
- LOW Ca DIET + EHDP (4mg/kg)
Fig. 2. Serum concentration of phosphorus in cows fed a low calcium diet (---) and cows fed a low calcium diet and administered EHDP (---) prepartum. Values for each interval represent the mean ± standard error.
EHDP EFFECT ON SERUM PHOSPHORUS

FIG 2

- LOW Ca DIET
- - LOW Ca DIET + EHDP (4 mg/kg)
Plasma Parathyroid Hormone

Plasma immunoreactive parathyroid hormone (iPTH) levels were consistently higher prepartum in EHDP-treated cows compared to cows fed the low calcium diet (Fig. 3). The iPTH levels increased from 1.4 ng. per ml. at parturition to 2.4 ng. per ml. one day postpartum in cows receiving EHDP. Plasma iPTH levels in cows fed the low calcium diet increased from 1.0 ng./ml. at parturition to 1.1 ng./ml. one day postpartum.

Urinary Phosphorus

Values of urinary phosphorus for the EHDP-treated cows was consistently higher than values for cows fed the low calcium diet (Fig. 4). Changes in urinary phosphorus accompanied similar fluctuations in iPTH levels in the cows administered EHDP (Fig. 3). A small decline in urinary phosphorus occurred at one day postpartum in this group of cows. Urinary phosphorus excretion by cows fed the low calcium diet did not change significantly prepartum. Excretion of urinary calcium prepartum was consistently higher for the EHDP-treated cows. During the experimental EDTA infusions urinary calcium was usually lower for the EHDP-treated cows compared to cows fed the low calcium diet.

Urinary Hydroxyproline

The urinary excretion of hydroxyproline increased initially at 60 and 50 days prepartum in the EHDP-treated cows. Urinary hydroxyproline excretion did not markedly change during the same interval for cows fed the low calcium diet. The difference in urinary hydroxyproline excretion between the two groups of cows was significant at both 60 days
Fig. 3. Plasma concentration (ng. per ml.) of immunoreactive parathyroid hormone in cows fed a low calcium diet (——) and cows fed a low calcium diet and administered EHDP (---) for 70 days prepartum and one day postpartum.
EHDP EFFECT ON PLASMA PTH

- LOW Ca DIET
- LOW Ca DIET + EHDP (4mg/kg)

FIG. 3
PLASMA IMMUNOREACTIVE PARATHYROID HORMONE (ng/ml)

DAYS PREPARTUM

PARTUITION
Fig. 4. Urinary concentration of phosphorus in cows fed a low calcium diet (—) and cows fed a low calcium diet and administered EHDP (---) for 70 days prepartum and one day postpartum. Urinary phosphorus concentrations are expressed as a ratio to creatinine in mg. per 100 ml. of urine.
LOW CALCIUM DIET

LOW CALCIUM DIET + EHDP
(4mg/kg)

PHOSPHORUS:CREATININE (mg/100ml)

DAYS PREPARTUM

PARTURITION

FIG 4
(P<0.05) and 50 days (P<0.01) prepartum. Urinary hydroxyproline excretion for both groups of cows during the final 50 days prepartum were not significantly different. At the time of parturition urinary hydroxyproline, expressed as a ratio to creatinine, was 0.067 for EHDP-treated cows and 0.040 for cows fed the low calcium diet. At one day postpartum urinary hydroxyproline excretion for the EHDP-treated cows remained unchanged. However, urinary hydroxyproline excretion by the cows fed the low calcium diet increased 207 per cent compared to the level at parturition (Fig. 5).

**Urinary Cyclic Adenosine Monophosphate**

Differences in the urinary excretion of cyclic adenosine monophosphate (cAMP) prepartum by both groups of cows were not significant although values in cows receiving EHDP were consistently higher. Urinary cAMP excretion increased between parturition and one day postpartum in the EHDP-treated cows (Fig. 6). Fluctuations in urinary excretion of cAMP correlated with similar changes in plasma iPTH, especially in the cows receiving EHDP (Figs. 3 and 6).

**Immediately Available Calcium Reserves by EDTA Infusion**

During the EDTA infusion after 60 experimental days 140 per cent more EDTA was required to lower serum calcium levels to 7 mg. per 100 ml. or less in cows fed the low calcium diet compared to the EHDP-treated cows. The decrease in serum calcium was greatest between 3.5 and 4.0 hours in both groups. Serum calcium returned to normal levels at approximately the same rate during the experimental and baseline EDTA infusions in cows fed the low calcium diet (Fig. 7). However, serum calcium levels
Fig. 5. Urinary hydroxyproline excretion in cows fed a low calcium diet (---) and cows fed a low calcium diet and administered EHDP (---) for 70 days prepartum. The mean levels of hydroxyproline (mg. per 100 ml.) are expressed as a ratio to creatinine (mg. per 100 ml.) ± standard error in the urine.
LOW Ca DIET

LOW Ca DIET + EHDP (4 mg/kg)

HYDROXYPROLINE : CREATININE (mg/100ml)

DAYS PREPARTUM

PARTURITION
Fig. 6. Urinary cyclic adenosine monophosphate (cAMP) excretion in cows fed a low calcium diet (——) and cows fed a low calcium diet and administered EHDP (---) for 70 days prepartum. The mean levels of cAMP are expressed as a ratio to creatinine ± standard error.
FIG 6

LOW Ca DIET

LOW Ca DIET + EHDP (4 mg/kg)
Fig. 7. Immediately available calcium reserves determined by the response of serum calcium to a 4-hour EDTA infusion administered prior to experimentation (baseline) (-----) compared to values after 60 days of feeding the low calcium diet prepartum (---). Values for each interval represent the mean ± standard error.
FIG 7
were consistently higher at most intervals following EDTA infusion after 60 days of feeding the low calcium diet. In the EHDP-treated cows, serum calcium declined with the infusion of EDTA and remained essentially unchanged during the subsequent 20 hours. Differences in serum calcium values were significant ($P < 0.05$) after 10.5 hours when values of the experimental EDTA infusion were compared to corresponding levels obtained during the baseline infusion (Fig. 8).

A marked elevation of plasma iPTH was demonstrated from the 12th to the 16th hour of the baseline EDTA infusion in control cows (Fig. 9). This peak increase of iPTH was absent after the cows were fed the low calcium diet for 60 days. A similar response occurred in plasma iPTH levels during the baseline and experimental EDTA infusions in EHDP-treated cows (Fig. 10). A moderate increase in iPTH occurred between 12 and 16 hours in both baseline and experimental EDTA infusions.

The rise in serum calcium following the baseline EDTA infusion in both groups of cows was accompanied by an increase in urinary hydroxyproline excretion. During the experimental EDTA infusion, urinary hydroxyproline excretion was consistently higher in the cows fed the low calcium diet compared to corresponding values during the baseline infusion ($P < 0.05$) (Fig. 11). The urinary hydroxyproline excretion by the EHDP-treated cows was consistently higher during the baseline infusion than after the EDTA infusion 60 days later (Fig. 12). Excretion of urinary cyclic adenosine monophosphate was similar comparing experimental EDTA infusions in both groups of cows (Fig. 13). However, during 10 to 24 hours of the experimental EDTA infusion consistently higher values of urinary cAMP correlated with higher levels of plasma para-
Fig. 8. Immediately available calcium reserves determined by the response of serum calcium to a 4-hour EDTA infusion administered prior to experimentation (baseline) (——) compared to values after 60 days of feeding the low calcium diet and administering EHDP (---). Values for each interval represent the mean ± standard error.
Fig. 9. Response of plasma concentration of immunoreactive parathyroid hormone to a 4-hour EDTA infusion administered prior to experimentation (---) compared to values after 60 days of feeding a low calcium diet (---).
FIG 9

PLASMA IMMUNOREACTIVE PARATHYROID HORMONE

HOURS

0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

LOW Ca Diet (60 DAYS)

BASELINE
Fig. 10. Response of plasma concentration of immunoreactive parathyroid hormone to a 4-hour EDTA infusion given prior to experimentation (-----) compared to values after 60 days of feeding a low calcium diet and administering EHDP (---). Values for each interval represent the mean ± standard error.
FIG 10

PLASMA IMMUNOREACTIVE PARATHYROID HORMONE (ng/ml)

LOW Ca DIET + EHDP (60 DAYS) (4mg/kg)

HOURS

0 2 4 6 8 10 12 14 16 18 20 22 24 48
Fig. 11. Urinary hydroxyproline excretion after a 4-hour EDTA infusion given prior to experimentation (——) compared to values after 60 days of feeding a low calcium diet prepartum (---). Values for each interval represent the mean ± standard error.
Fig. 12. Urinary hydroxyproline excretion after a 4-hour EDTA infusion given prior to experimentation (---) compared to values after 60 days of feeding a low calcium diet and administering EHDP prepartum (---). Values for each interval represent the mean ± standard error.
BASELINE

LOW Ca DIET + EHDP
(60 DAYS, 4mg/kg)
Fig. 13. Urinary cyclic adenosine monophosphate excretion after a 4-hour EDTA infusion given after 60 days of feeding a low calcium diet (----) compared to values in cows fed a low calcium diet prepartum and administered EHDP (---). Values for each interval represent the mean ± standard error.
LOW Ca DIET (60 DAYS)
LOW Ca DIET + EHDP (60 DAYS) (4mg/kg)

FIG 13
thyroid hormone for the EHDP-treated cows.

**Light Microscopy and Ultrastructure of the Parathyroid Glands**

Parathyroid glands from both groups of cows were evaluated histologically and ultrastructurally at the termination of the experiment at 48 hours postpartum or when the serum calcium dropped to under 7 mg. per 100 ml. Histological evaluation revealed no apparent abnormalities in the parathyroid glands of cows of either group. Ultrastructurally, the parathyroid glands from cows receiving EHDP and fed the same low calcium diet had a population of active chief cells with interspersed oxyphils, inactive chief cells, and transitional forms. The chronically stimulated chief cells were degranulated and contained fewer secretory granules in their cytoplasm than chief cells in control cows fed only the low calcium diet. The plasma membranes of adjacent active chief cells often had intricate interdigitations (Fig. 14). When secretory granules were present in the EHDP-treated cows they usually were situated more peripherally in chief cells near the plasma membrane (Fig. 15). The large cytoplasmic area of these chronically stimulated chief cells had numerous clusters of polyribosomes, large mitochondria, and prominent Golgi apparatuses associated with many prosecretory granules. In addition, there were more prominent clusters of fine microfilaments throughout the cytoplasm of chief cells from cows in this group (Fig. 15). Large bundles of microfilaments often extended from the perinuclear region into the cytoplasm between organelles (Fig. 16). The occurrence of mitochondrial vacuolation with disruption of cristae and large lysosome-like bodies was more frequent in chief cells of pregnant cows receiving EHDP (Fig. 15).
Fig. 14. Chronically stimulated chief cell in the parathyroids of a cow fed a low calcium diet and administered EHDP prepartum. Within the abundant cytoplasmic area are a prominent Golgi apparatus (G) and numerous microfilaments located in the perinuclear area. The cells are degranulated and plasma membranes are interdigitated (arrowheads). X 8,600.
Fig. 15. A partially degranulated chief cell in the parathyroids of a cow fed a low calcium diet and administered EHDP prepartum. Secretory granules (S) are peripherally situated and clusters of microfilaments (arrowheads) are present in the cytoplasm. Lipofuscin granules (L) also are present. X 32,400.
Fig. 16. Active chief cell in the parathyroid glands from a cow fed a low calcium diet and administered EHDP prepartum. The abundant cytoplasmic area was depleted of mature secretory granules. Prominent accumulations of microfilaments (F) extend from the perinuclear region into the cytoplasm. The occurrence of mitochondrial vacuolation with disruption of cristae (M) was more frequent in chief cells of pregnant cows receiving EHDP. X 10,600.
The predominant chief cells in parathyroids of cows fed a low calcium diet prepartum were primarily in the active stage of their secretory cycle. The large cytoplasmic area of active chief cells had well developed secretory organelles. The endoplasmic reticulum often was aggregated into large lamellar arrays with numerous attached ribosomes and clusters of free polyribosomes were present in the cytoplasm (Fig. 17). Large Golgi complexes were situated in a perinuclear region and were associated with prosecretory granules. Many large mitochondria with transverse cristae were present throughout the cytoplasm. The plasma membranes of adjacent active chief cells often were intricately inter-digitated. Small secretory granules were either depleted or when present were particularly numerous near the Golgi apparatus and near the plasma membrane. The limiting membrane of an occasional secretory granule appeared to be fused with the plasma membrane (Fig. 18). Lipid bodies and lipofuscin granules were observed occasionally in the cytoplasm. Inactive chief cells, oxyphil cells, and transitional forms were interspersed between the more numerous active chief cells in the parathyroid glands.

**Ultrastructure of Thyroid C- (Parafollicular) Cells**

No significant difference was observed in the fine structure of parafollicular cells in thyroids of either group of cows. Thyroid C-cells appeared to be in a storage phase of their secretory cycle. Most of the cytoplasmic area was occupied by numerous secretory granules and scattered mitochondria (Fig. 19). Other cytoplasmic organelles were poorly developed.

Consistent with these ultrastructural findings was the finding that the thyroid calcitonin content of EHDP-treated cows was 610 MRC mU/gm.
Fig. 17. Chief cell in active stage of secretory cycle from a pregnant cow fed a low calcium diet prepartum. The abundant cytoplasmic area contains prominent lamellar arrays of endoplasmic reticulum (E) and large mitochondria (M). Secretory granules (S) were predominately situated peripherally near the plasma membrane. X 50,000.
Fig. 18. Active chief cell in the parathyroids from a cow fed a low calcium diet prepartum. Golgi complexes (G) in a perinuclear region are associated with prosecretory granules (arrowhead). Many large mitochondria (M) are present throughout the cytoplasm. The plasma membranes of adjacent active chief cells are interdigitated (arrow). Small membrane-limited secretory granules (S) were present throughout the cytoplasm of some active chief cells. X 17,800.
Fig. 19. Thyroid C-cell in storage phase from a cow fed a low calcium diet prepartum. The cytoplasm is packed with many large secretory granules (S) but organelles other than mitochondria (M) are not well developed. X 15,000.
and for control cows fed only a low calcium diet was 655 MRC mU/gm.
These differences of calcitonin content in the thyroid gland determined
by bioassay were not significant.

Amyloid fibrils near C-cells were observed as an incidental finding in the thyroid glands of a cow fed the low calcium diet. Prominent aggregations of fine amyloid fibrils were present around interfollicular capillaries often adjacent to thyroid C-cells situated within the follicular basement membrane (Fig. 20).
Fig. 20. Amyloid fibrils (F) in perivascular space of thyroid from a cow fed a low calcium diet prepartum. Thyroid C-cell with many storage granules is situated within follicular basement membrane (B). X 10,000.
DISCUSSION

The results of this investigation demonstrated that the parathyroid glands of pregnant cows fed a low calcium diet prepartum and administered EHDP for 70 days were able to secrete as high or higher levels of immunoreactive parathyroid hormone as pregnant control cows fed a low calcium diet. Plasma parathyroid hormone levels were consistently higher at parturition and at one day postpartum in response to hypocalcemia. The postpartum hypocalcemia in the EHDP-treated cows was severe in comparison to previously reported reductions in blood calcium induced by the administration of EHDP (32, 33). Ultrastructurally, chief cells in the active stage of the secretory cycle predominated in the parathyroids of both groups. Active chief cells were characterized by well developed Golgi apparatuses, large mitochondria, and prominent lamellar arrays of rough endoplasmic reticulum. The chief cells in cows administered EHDP either were degranulated or had only a few secretory granules aligned along the plasma membrane. Chief cells in EHDP-treated cows had the additional finding of prominent perinuclear arrays of microfilaments, vacuolations of some mitochondria, and occasional lysosomal bodies. Despite these fine structural alterations, the secretion of parathyroid hormone by chief cells was not impaired.

Ultrastructural findings in thyroid C- (parafollicular) cells were similar in both groups of cows. Thyroid C-cells appeared to be predominantly in a storage phase since the cytoplasm was densely packed with mature secretory granules. Biologic assay of thyroids revealed a similar calcitonin content in both groups. It seems unlikely that an increased secretion of calcitonin was a factor in the pathogenesis of the
hypocalcemia and hypophosphatemia in the EHDP-treated cows following
parturition and the initiation of lactation.

Urinary excretion of cAMP was not significantly different between
the two groups of cows although values increased more markedly post-
partum in cows administered EHDP. These findings were consistent with
the ultrastructural evidence of parathyroid stimulation and elevation
of IPTH in both groups. Parathyroid hormone stimulation of target cells
in the kidney has been shown to increase the concentration of cAMP in
the renal parenchyma as well as increase urinary excretion of the nuc-
leotide (4-6). The increase in cAMP excretion postpartum in the cows
administered EHDP suggested a greater stimulation of the kidney by the
increased circulating levels of IPTH in response to the severe hypocal-
cemia. A similar increase in urinary cAMP from 10 to 24 hours of the
experimental EDTA infusions coincided with a reduction of urinary cal-
cium in cows receiving EHDP. Therefore, EHDP did not appear to inter-
fere with the mechanism of action of parathyroid hormone and the forma-
tion of cAMP by target cells. A similar increase of urinary cAMP excre-
tion has been reported in human patients treated with EHDP and adminis-
tered exogenous PTH (31).

Despite the increased parathyroid hormone levels and urinary cAMP
in response to severe hypocalcemia, bone resorption appeared to be im-
paired postpartum in the EHDP-treated cows. The urinary hydroxyproline
excretion remained unchanged one day postpartum compared to a 207 per-
cent increase for the control cows. The results of the experimental
EDTA infusions after 60 days of EHDP and feeding the low calcium diet
indicated that the immediately available calcium reserves were reduced
significantly in the cows. Surfaces on trabecular and Haversian bone undergoing resorption, as determined by microradiography, were significantly less in the EHDP-treated cows (43). These findings suggest a defect in bone matrix catabolism that is not due to a deficient formation of second messenger (cAMP) by target cells in response to parathyroid hormone. Previous in vitro and in vivo studies have reported that osteocytic cells in the presence of EHDP are less responsive to stimulation by PTH (13, 29, 40). Osteolytic cells in cows administered EHDP appeared to be unable to rapidly mobilize calcium from bone and effectively restore homeostasis after a hypocalcemic challenge.

Additional studies on this group of cows administered EHDP have demonstrated the presence of widened osteoid seams on bone surfaces (43). This thick osteoid layer may be related in part to an alteration in the formation of active vitamin D metabolites by EHDP (42). Previous reports have emphasized that excessive osteoid accumulation can interfere with the effective mobilization of bone mineral by osteoclasts (20). However, bone of cows treated with dichloromethane diphosphonate (CL₂MDP) also is refractive to parathyroid hormone-stimulated resorption despite the absence of large accumulations of osteoid on bone surfaces (43). It appeared that the refractiveness of bone following the administration of EHDP is not dependent on the accumulation of osteoid but rather an impaired dissolution of hydroxyapatite crystals coated by the diphosphonate (12, 13).

The uptake of ⁴⁵Ca into duodenal mucosa incubated in vitro was essentially similar in both groups of cows fed the low calcium diet but was considerably lower than in cows fed the required amounts of cal-
cium and phosphorus (44). These findings suggest that calcium absorption from the intestine does not increase in response to EHDP administration to fulfill the requirements for the maintenance of maternal calcium homeostasis and mineralization of the fetal skeleton. Therefore, an inability to rapidly mobilize bone mineral following a challenge associated either with EDTA infusion or parturition and the initiation of lactation in cows receiving EHDP appeared to be primarily responsible for the development of severe hypocalcemia and hypophosphatemia. The ability of the parathyroid glands to synthesize and secrete parathyroid hormone in response to hypocalcemia was not impaired by the administration of EHDP.
ABSTRACT

The long term (70 days) effect of administering ethane-1-hydroxy-1,1 diphosphonate (EHDP) (4 mg/kg/day) on parathyroid function was investigated in pregnant cows fed a low calcium diet and compared to findings in control cows receiving only a low calcium diet. Serum calcium and phosphorus were significantly lower near parturition in EHDP-treated cows. In response to the hypocalcemia plasma immunoreactive parathyroid hormone was consistently higher prepartum, at parturition, and postpartum in cows administered EHDP. Elevations in urinary cyclic AMP and phosphorus correlated with the increased plasma parathyroid hormone levels, especially in EHDP-treated cows. Urinary hydroxyproline did not change near parturition in cows administered EHDP but an increase of 207 per cent occurred one day postpartum in control cows. Immediately available calcium reserves were greater prepartum in cows fed the low calcium diet than in cows receiving EHDP as indicated by a more rapid rate of return of serum calcium to normal levels following ethylenediaminetetraacetic acid (EDTA)-induced hypocalcemia. EHDP-treated cows responded to the hypocalcemic challenge with a similar rapid increase in plasma parathyroid hormone but an increase in urinary hydroxyproline did not occur as in cows receiving only the low calcium diet.

Ultrastructurally, chief cells in parathyroid glands of both groups of cows were in an active stage of the secretory cycle. Organelles concerned with hormonal synthesis were well developed and the
remaining secretory granules were aligned peripherally near the plasma membrane. Chief cells in EHDP-treated cows often had prominent perinuclear accumulations of microfilaments, scattered vacuolated mitochondria, and lysosomal bodies in the cytoplasm. Thyroid C-cells were densely granulated and thyroid calcitonin content was similar in both groups of cows.

The principal defect in calcium homeostasis of EHDP-treated cows appeared to be a refractiveness of bone to the induction of matrix catabolism stimulated by parathyroid hormone. The ability of the parathyroid glands to synthesize and secrete parathyroid hormone in response to hypocalcemia induced either by EDTA or associated with parturition was not impaired by the administration of EHDP.
CHAPTER II

EFFECTS OF TWO DIPHOSPHONATES ON INTESTINAL CALCIUM UPTAKE
IN PREGNANT AND NONPREGNANT COWS

INTRODUCTION

The diphosphonates are a group of synthetic analogues of pyrophosphate having a P-C-P rather than a P-O-P bond. The diphosphonates are resistant to both chemical and enzymatic hydrolysis (1, 2). Ethane-1-hydroxy-1,1-diphosphonate (EHDP) has been shown to be effective in reducing bone turnover in animals (3, 4) and, depending on dosage, may produce alterations in both bone formation and resorption (3). A second diphosphonate (CL₂MDP) has been shown to have a potent but more selective effect on bone remodeling, by inhibiting bone resorption with little effect on bone formation (5, 6).

There is a paucity of information regarding the mechanisms of these drugs on calcium homeostatic mechanisms, particularly their effects on intestinal calcium transport. At doses up to 1 mg P/kg/day, EHDP given to intact rats fed a normal calcium-phosphorus diet caused increased absorption of dietary calcium and a decreased endogenous fecal calcium (3, 7). EHDP at doses ranging from 3 to 10 mg P/kg/day has been shown to decrease net retention of calcium associated with a diminished intestinal calcium absorption and an increased urinary excretion of calcium (3, 7, 8).

Calcium transport in the small intestine of rats fed either a normal calcium diet or a high calcium-vitamin D diet and given 10 mg P/kg/day of
EHDP for 7 days has been studied by means of in vivo calcium perfusions of the intestine. These studies revealed a reduction in net calcium absorption which was not associated with an increased endogenous intestinal excretion of calcium (7, 9, 10). Intestinal calcium-binding protein, Ca-ATPase and alkaline phosphatase were also decreased in the EHDP-treated rats.

Intestinal calcium-binding protein (11, 12), Ca-ATPase (13) and alkaline phosphatase (14) have all been proposed as components of vitamin D-mediated active transport of calcium. Large doses of vitamin D (2000 IU/day for 7 days) or 1,25-dihydroxycholecalciferol (325 pmol) reverse the EHDP-induced inhibition of duodenal calcium absorption (9, 10). High dose levels of EHDP (10 mg P/kg/day) may directly reduce the renal formation of 1,25-dihydroxycholecalciferol in rats (15). Paradoxically, recent reports indicate that low doses of EHDP (1 mg P/kg/day) may increase the rate of intestinal absorption of calcium and enhance the accumulation within the intestinal mucosa of a vitamin D metabolite with chromatographic characteristics of 1,25-dihydroxycholecalciferol (16).

EHDP may interfere in the transport of calcium from intestinal microvilli to other intracellular sites (17). A recent electron microscopic study revealed that rats given high doses of EHDP (10 mg P/kg/day) appeared to accumulate electron-dense granules in duodenal microvilli but few mineral granules in mitochondria of intestinal absorptive cells (17).

In contrast little is known of the effect of CL2MDP on intestinal calcium transport. One recent study suggested that CL2MDP (10 mg P/kg/
day) may cause a small increase in retention of calcium as evidenced by a decrease in the endogenous excretion of fecal calcium (3). At this dose level \( \text{CL}_2\text{MDP} \) caused an increase in calcium-ATPase activity while at a similar dose level \( \text{EHDP} \) caused a decrease in activity (7).

Because of this paucity of information on the effects of diphosphonates on intestinal calcium transport, this study was undertaken with the objective of determining and comparing the effects of \( \text{EHDP} \) and \( \text{CL}_2\text{MDP} \) on intestinal calcium uptake in nonpregnant and pregnant cows.

**METHODS**

**Experimental Animals**

A total of 15 nonlactating pregnant cows, ranging from 5 to 8 years of age, with no known previous history of parturient paresis (18) were used in this study. An additional 7 nonlactating and nonpregnant cows, ranging from 5 to 8 years of age, which had no previous history of parturient paresis, were utilized for studies to determine the influence of pregnancy. All cows were purchased from the same farm and were raised under similar environmental conditions.

**Experimental Design**

Pregnant cows were acclimated to laboratory conditions for 14 days before initiation of the experiments. During this time each cow was fed the previously described basal diet (19) supplemented with the daily recommended requirements for calcium (25 gm) and phosphorus (25 gm) (control diet). At the onset of the experiment 15 pregnant cows were divided into 5 groups of 3 cows each. Three groups were fed the basal calcium-deficient diet that provided 12.5 gm of calcium per day supple-
mented with phosphorus (25 gm/day). Two groups of cows were fed the control diet containing the required daily amounts of calcium and phosphorus.

The three groups of pregnant cows fed the low calcium prepertal diet were treated as follows: (1) no diphosphonate treatment, (2) EHDP administered subcutaneously twice daily at 4 mg/kg/day and (3) CL₂MDP administered subcutaneously twice daily at 4 mg/kg/day. The two groups of pregnant cows fed the control diet were treated as follows: (1) no diphosphonate treatment and (2) EHDP administered subcutaneously twice daily at 4 mg/kg/day. The EHDP and CL₂MDP solutions were made by dissolving purified diphosphonate powder in sterile physiological saline. One subcutaneous injection of 10 ml of solution provided a diphosphonate dose of 2 mg/kg/day. The dose level of 4 mg/kg/day was chosen because a pilot study had demonstrated that this level was potent yet nontoxic to the calcium homeostatic mechanism of the cow (20). The subcutaneous route was chosen to eliminate possible problems associated with variations in blood levels of the drug that could result from poor or variable gastro-intestinal absorption if the diphosphonate was administered orally.

The pregnant cows were on the experiment for an average 80 days prepartum, through parturition, and up to 2 days postpartum unless serum calcium levels fell below 6.5 mg/100 ml postpartum. At the termination of the experiment each cow was euthanatized by electrocution. Further details of the experimental design are described elsewhere (21).

An additional 7 nonpregnant cows were used to determine the effects of varying dose levels of EHDP and CL₂MDP on intestinal calcium uptake.
The cows were acclimated to laboratory conditions for 14 days and were fed the control diet during both the acclimation and experimental periods. The cows were treated as follows: (1) 1 mg/kg/day EHDP (1 cow), (2) 4 mg/kg/day EHDP (1 cow), (3) 10 mg/kg/day EHDP (2 cows), (4) 1 mg/kg/day CL₂MDP (1 cow), (5) 4 mg/kg/day CL₂MDP (1 cow), and (6) 10 mg/kg/day CL₂MDP (1 cow). Each cow was administered the dose of diphosphonate for 60 days and then euthanatized by electrocution.

**Calcium Uptake by Duodenal Mucosa**

Immediately following euthanasia a 10 cm section was removed from that segment of the duodenum that crosses the right paralumbar fossa. The duodenum was opened and placed in an aerated prewarmed isotonic wash solution (22). Estimation of intestinal uptake utilized $^{45}$calcium with *in vitro* incubations of intestinal segments mounted on microslides essentially as described by Martin and DeLuca (22). Modifications of this method included the use of three intestinal segments for each of three times of incubation (1, 4 and 7 minutes) and dissection of exposed areas of intestinal mucosa from underlying muscle layers prior to solubilization in N-chlorosuccinimide (NCS Tissue Solubilizer, Amersham/Searle Corporation, Arlington Heights, Illinois). Counting was in a liquid scintillation counter (Packard Tri-Carb, Model 3375). Calcium uptake was expressed as nmoles of calcium per square centimeter of surface area per specific time. Normal ranges for calcium uptake in cows in our laboratory are 1 minute (1.6 - 2.7), 4 minutes (3.3 - 5.1) and 7 minutes (5.7 - 8.3).
Calcium-binding Protein (CaBP) Activity in Duodenal Mucosa and Renal Cortex

A small section of each renal cortex (3 grams) was collected and a section of excised duodenum (approximately 5 cm) was split and rinsed thoroughly with cold physiologic saline solution. The duodenal mucosa was removed from the underlying muscle layer by scraping with a glass microslide. Both the renal cortical and duodenal tissue were homogenized at 4°C in Tris buffer (20% w/v, pH 7.4). The homogenate was centrifuged for 20 minutes at 39,000 x g in a refrigerated centrifuge. Supernatant fluids were collected, heated to 60°C for 10 minutes, and re-centrifuged at 39,000 x g for 20 minutes. The protein content of the supernatant fluid was determined by the method of Lowry et al. (23).

The calcium-binding protein was assayed by the ion exchange procedure of Wasserman, Corradino and Taylor (11) using Chelex-100 resin. The calcium-binding activity in the duodenal mucosa and renal cortex was expressed as a ratio of protein-bound calcium to resin-bound calcium per milligram of protein in the supernatant fluid. Normal ranges for calcium-binding protein in the duodenal mucosa and renal cortex of cows determined in our laboratory are duodenum (0.079 - 0.471) and renal cortex (0.106 - 0.176).

Intestinal Alkaline Phosphatase Assay

Intestinal alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1.) was measured in 20% butanol extracts of duodenal mucosa by the method of Hausamen et al. (24). The activity of alkaline phosphatase was expressed as milli-international units of enzyme activity per milligram of protein.
Statistical Analysis

The results were analyzed by the Student t test for samples with unequal variances (25).

RESULTS

The in vitro calcium uptake by the duodenal mucosa of parturient cows administered 4 mg/kg/day of CL₂MDP and fed the low calcium diet prepartum was greater at 1 minute (P < 0.05), 4 minutes (P < 0.001), and 7 minutes (P < 0.01) than in cows fed only the low calcium diet prepartum (Fig. 1). Calcium uptake by cows administered 4 mg/kg/day of CL₂MDP and fed the low calcium diet prepartum was greater at 4 minutes (P < 0.01) and 7 minutes (P < 0.01) of incubation than uptake by the duodenal mucosa from cows fed the control diet prepartum.

No significant differences in in vitro calcium uptake by the duodenal mucosa was demonstrated between cows administered 4 mg/kg/day of EHDP and fed the low calcium diet prepartum compared to cows fed only the low calcium diet (Fig. 2). Similarly, no differences were demonstrated between cows administered EHDP (4 mg/kg/day) and fed the control diet compared to cows fed only the control diet prepartum (Fig. 2). The in vitro calcium uptake by the duodenal mucosa of cows fed the control diet was greater after 7 minutes (P < 0.05) of incubation than that of cows fed the low calcium diet prepartum (Fig. 2).

The effect on in vitro intestinal calcium uptake was quite different when three different dose levels of EHDP or CL₂MDP was administered for 60 days to nonpregnant cows. Linearity of calcium uptake over a 7 minute period of incubation was evident for both the EHDP-treated (Fig. 3) and CL₂MDP-treated (Fig. 4) cows with the exception of the high dose of
Fig. 1. Long term effect of CL₂MDP (4 mg/kg/day) on in vitro intestinal calcium uptake in postparturient cows fed a low calcium diet prepartum.
FIG 1

- LOW CALCIUM DIET
- LOW CALCIUM DIET + CL₂ MDP

\( (4 \text{mg/kg/day}) \)

\[\text{CALCIUM UPTAKE (m Moles/cm²)}\]

\[\text{MIN. OF INCUBATION}\]

\[\Delta P < .001\]
\[\ast P < .01\]
Fig. 2. Long term effect of EHDP on \textit{in vitro} intestinal calcium uptake in postparturient cows fed either a low or control calcium diet prepartum.
GRAPHICAL ABSTRACT

- CONTROL DIET
- CONTROL DIET + EHDP (4mg/kg/day)
- LOW CALCIUM DIET
- LOW CALCIUM DIET + EHDP (4mg/kg/day)

Calcium Uptake (mMoles/cm²) vs. Min. of Incubation

FIG 2
Fig. 3. Effect of varying doses of EHDP on \textit{in vitro} intestinal calcium uptake in nonpregnant cows fed a control diet providing the required daily amounts of calcium and phosphorus.
Fig. 4. Effect of varying doses of CL₂MDP on *in vitro* intestinal calcium uptake in nonpregnant cows fed a control diet providing the required daily amounts of calcium and phosphorus.
Calcium uptake appeared to plateau after 4 minutes of incubation in the cow administered 10 mg/kg/day of CL$_2$MDP (Fig. 4).

The *in vitro* uptake of calcium by the duodenal mucosa of the cow administered 1 mg/kg/day of CL$_2$MDP was 3, 3 and 4 times greater at 1, 4 and 7 minutes of incubation than the uptake of the cow administered 1 mg/kg/day of EHDP. Furthermore, calcium uptake by the nonpregnant cow administered 1 mg/kg/day of CL$_2$MDP was the highest of all cows in the entire study. The *in vitro* calcium uptake by the nonpregnant cow administered 4 mg/kg/day of CL$_2$MDP was consistently 2 times higher at each interval of incubation than values from the cow administered EHDP. At the 10 mg/kg/day dose level, the duodenal calcium uptake at all intervals of incubation was 3.5 times greater for the CL$_2$MDP-treated cow compared to the two nonpregnant cows administered EHDP.

Duodenal and renal CaBP activity was significantly higher (P<0.05) in pregnant cows fed the low calcium diet compared to cows fed the control diet prepartum (Table 1). In the EHDP-treated cows average values for CaBP activity were consistently higher than values from cows fed similar diets without diphosphonates. No significant differences in intestinal alkaline phosphatase activity were demonstrated between any of the groups of cows regardless of the dietary calcium content or diphosphonate administration.

**DISCUSSION**

The results of this study demonstrate that the administration of CL$_2$MDP to cows causes increased *in vitro* intestinal uptake of calcium. Consistently greater uptakes of calcium (2- to 4-fold) were observed in the CL$_2$HDP-treated cows. The greatest uptake of calcium (13 nmoles/cm$^2$)
TABLE I.

Effect of EHDP and CL₂MDP on Serum Calcium, Intestinal Calcium Uptake, Duodenal and Renal Cortical CaBP, and Intestinal Alkaline Phosphatase in Parturient Cows Fed Either a Low or Control Calcium Prepartal Diet.

<table>
<thead>
<tr>
<th>Diet + Treatment</th>
<th>Serum Calcium (mg/100 ml.)</th>
<th>Calcium Uptake (nmoles/cm²) at 7 min.</th>
<th>CaBP (Sp. activity/mg protein)</th>
<th>Intestinal Alkaline Phosphatase (mIU/mg protein butanol extract)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Calcium</td>
<td>$8.63 \pm 0.31^a$</td>
<td>$6.933 \pm 0.734^b$</td>
<td>$0.282 \pm 0.114^b$</td>
<td>$0.148 \pm 0.021^b$</td>
</tr>
<tr>
<td>Control Calcium + EHDP (4 mg/kg)</td>
<td>$6.35 \pm 1.61^e$</td>
<td>$7.431 \pm 1.301$</td>
<td>$0.544 \pm 0.169$</td>
<td>$0.367 \pm 0.211$</td>
</tr>
<tr>
<td>Low Calcium</td>
<td>$8.24 \pm 0.51$</td>
<td>$4.265 \pm 0.935$</td>
<td>$0.609 \pm 0.176$</td>
<td>$0.405 \pm 0.059$</td>
</tr>
<tr>
<td>Low Calcium + EHDP (4 mg/kg)</td>
<td>$4.80 \pm 0.78^b$</td>
<td>$4.227 \pm 1.805$</td>
<td>$0.753 \pm 0.155$</td>
<td>$0.523 \pm 0.153$</td>
</tr>
<tr>
<td>Low Calcium + CL₂MDP (4 mg/kg)</td>
<td>$5.07 \pm 1.15^b$</td>
<td>$12.971 \pm 1.199^c,d$</td>
<td>$0.437 \pm 0.146$</td>
<td>$0.147 \pm 0.025$</td>
</tr>
</tbody>
</table>

$^a$ ± standard error of mean.
$^b$ $P<0.05$ when compared to low calcium group.
$^c$ $P<0.01$ when compared to low calcium group.
$^d$ $P<0.01$ when compared to control calcium group.
$^e$ $P<0.05$ when compared to control calcium group.
was observed in cows treated with CL₂MDP. Despite this positive \textit{in vitro} effect, the increased intestinal uptake of calcium was unable to prevent the development of severe hypocalcemia in parturient cows treated with CL₂MDP.

Intestinal CaBP and alkaline phosphatase were higher in the EHDP-treated (4 mg/kg/day), parturient cows compared to control cows fed the same diet. These same parameters were lower in the cows treated with an equivalent dose of CL₂MDP. These results are in contrast to an earlier study which indicated that at equivalent dose levels intestinal CaBP was higher in CL₂MDP-treated compared to EHDP-treated rats (12). However, in that study a high dose of diphosphonate (10 mg/kg/day) was used in comparison to the lower dose (4 mg/kg/day) administered to the pregnant cows.

Since both intestinal CaBP and alkaline phosphatase are thought to be related to vitamin D-mediated active transport of intestinal calcium, the increased calcium uptake by duodenal mucosa in this study associated with CL₂MDP at dosages of 4 mg/kg/day or less was believed not to be related to the actions of vitamin D metabolites. The action of CL₂MDP may be due, in part, to a physical-chemical chelation of calcium on microvillar surfaces as suggested by a recent electron microscopic study (17).

The present observations on intestinal uptake of calcium in CL₂MDP-treated cows suggest that calcium homeostasis of the parturient, diphosphonate-treated cow is dependent on target organs other than the intestine for the mobilization of calcium. Additional studies indicate that the defect in mobilization of extracellular calcium in these diphosphonate-treated parturient cows is not due to an insufficiency of parathy-
roid hormone secretion or mediation of the action of the hormone by cyclic AMP (21). Since the present study suggests a positive effect on intestinal uptake of calcium by $\text{CL}_2\text{MDP}$, bone appears to be the principal target organ for the effects of the diphosphonates. Subsequent studies have demonstrated a lower urinary excretion of hydroxyproline and a decrease in bone resorptive surfaces in these diphosphonate-treated parturient cows (21, 26).

Rates of intestinal uptake of calcium also decreased progressively as the dose level (1, 4 and 10 mg/kg/day) of $\text{CL}_2\text{MDP}$ and EHDP increased. High doses of EHDP (10 mg/kg/day) given to two nonpregnant cows caused lower intestinal CaBP and alkaline phosphatase activity when compared to values from a cow given an intermediate dose (4 mg/kg/day). Serum calcium levels in these cows were normal. Lower intestinal CaBP and alkaline phosphatase values have been reported in rats treated with a high dose (10 mg/kg/day) of EHDP (9). The decrease in EHDP-treated rats appeared to be due to an impaired formation of active vitamin D metabolites (15). A decreased formation of 1,25-dihydroxycholecalciferol may also be the cause of the lowered intestinal uptake of calcium in the cows given a high dose of diphosphonate (10 mg/kg/day).

These studies suggesting that there is greater intestinal uptake of calcium with low doses of $\text{CL}_2\text{MDP}$ may have possible clinical and therapeutic implications. This uptake was associated with normal serum calcium in nonpregnant cows given $\text{CL}_2\text{MDP}$ at dosages of 4 mg/kg/day or less. A recent study also indicated that $\text{CL}_2\text{MDP}$ treatment (10 mg/kg/day) in rats caused an increased net intestinal retention of calcium compared to a decreased retention in rats treated with an equivalent dose of EHDP (3).
Therefore, it appears that low dosages of CL₂-MDP may be efficacious in treating certain metabolic diseases associated with a negative calcium balance. Although a similar increase in intestinal calcium uptake was observed in pregnant cows, the administration of diphosphonates near parturition may be contraindicated due to an interference in bone resorption. Parturient cows were unable to maintain blood levels of calcium at the time of maximal challenge to their calcium homeostatic mechanisms imposed by the initiation of lactation.
ABSTRACT

In vitro calcium uptake by the duodenal mucosa after 1, 4 and 7 minutes of incubation was consistently 2 to 4 times greater in CL₂MDP-treated nonpregnant cows compared to calcium uptake in EHDP-treated cows given equivalent doses (1, 4 or 10 mg/kg/day). The calcium uptake for each cow approached linearity over 7 minutes of incubation.

Serum calcium was significantly lower in parturient cows treated with either diphosphonate (4 mg/kg/day) than in parturient control cows (P < 0.05). Feeding control or low calcium diets appeared to have no significant influence on the hypocalcemia induced in the diphosphonate-treated cows. The greatest intestinal uptake of calcium in parturient cows was observed in cows administered CL₂MDP (P < 0.001). No significant difference in intestinal calcium uptake was observed in the EHDP-treated cows compared to nontreated cows fed the same diet. Intestinal calcium uptake was significantly lower after 7 minutes incubation in cows fed the low calcium diet (P < 0.05) compared to cows fed the control diet. No correlation between intestinal calcium uptake and intestinal CaBP and alkaline phosphatase could be made in the diphosphonate-treated parturient cows. These parameters were higher but not significantly so in EHDP-treated cows compared to nontreated cows fed the same diet.

These results suggest that the greater in vitro intestinal calcium uptake observed after administration of 4 mg/kg/day or less of CL₂MDP may be the result of some mechanism other than vitamin D-mediated, active calcium transport. Despite the significant increase (P < 0.01) in vitro of calcium uptake by the intestine, this effect in vivo did not
prevent the development of severe hypocalcemia in the \( \text{CL}_2\text{MDP} \)-treated cows. The relative decrease in calcium uptake in the nonpregnant cows administered high doses (10 mg/kg/day) of either diphosphonate was much greater for \( \text{EHDP} \) and may be explained by an impaired formation of active vitamin D metabolites.
CHAPTER III

EXPERIMENTAL PARTURIENT HYPOCALCEMIA IN COWS FOLLOWING PREPARTAL ADMINISTRATION OF ETHANE-1-HYDROXY-1,1-DIPHOSPHONATE (EHDP)

Introduction

The pregnant dairy cow is a unique animal model in which to study calcium homeostasis. Plasma levels of parathyroid hormone (PTH) in the cow can be monitored readily using sensitive radioimmunoassay techniques. Because of lactation and the marked drain of calcium into the milk, the cow has active and responsive calcium homeostatic mechanisms. A failure in these control mechanisms at parturition and the initiation of lactation results in the development of a clinical syndrome characterized by severe hypocalcemia and hypophosphatemia associated with muscular weakness, paresis, and death if appropriate treatment is not initiated rapidly. Investigations into the cause and prevention of the syndrome have shown that PTH synthesis and secretion is normal but trabecular and Haversian bone surfaces are predominately inactive. The administration of high levels of vitamin D or feeding low calcium diets prepartum reduce the incidence while high calcium diets increase the incidence of the disease.

Disodium ethane-1-hydroxy-1,1-diphosphonate (EHDP) is a compound which has a P-C-P bond and shares many properties with natural pyrophosphate. In vitro studies using EHDP have demonstrated that it inhibits
the growth of hydroxyapatite crystals and retards the dissolution of previously formed hydroxyapatite crystals. Experiments using living bone in tissue culture have demonstrated that EHDP is an effective inhibitor of the resorption of living bone and produces a significant reduction of bone resorption when parathyroid hormone is added to the medium.11,12

Although the administration of EHDP to pregnant cows fed a control diet has been shown to cause reduced bone turnover by decreasing the rates of bone formation and resorption,13 the effects on parathyroid function have not been investigated. The objectives of the present investigation were (1) to determine the effects of daily long-term administration of EHDP on the fine structure of parathyroid chief cells and thyroid C-cells, (2) to investigate the secretion of parathyroid hormone and the response of target cells in bone and kidney to a hypocalcemic challenge induced prepartum by EDTA or associated with parturition and the initiation of lactation, and (3) to determine if blocking bone resorption by the prepartal administration of diphosphonates will result in the development of a profound hypocalcemia at parturition in cows fed the required amounts of dietary calcium.

MATERIALS AND METHODS

Experimental Design

Jersey cows ranging in age from 5 to 8 years of age were brought to the laboratory 104 days prior to the calculated date of parturition. The cows were obtained from the same farm and were acclimated to labo-
ratory conditions for 14 days. During acclimation and experimentation periods all cows received a diet which provided the National Research Council's recommended levels of 25 gm of calcium and 25 gm of phosphorus per day for pregnant cows. Feeding 12 pounds of the experimental diet provided 9.2 gm of calcium and 19.0 gm of phosphorus. Calcium carbonate and monosodium phosphate were added in amounts to give the desired calcium and phosphorus levels.

Six cows were divided equally into two groups starting at 90 days from the calculated date of parturition. One group of cows was administered subcutaneously EHDP at a dose of 4 mg/kg/day. This dose level was chosen because a previous pilot study demonstrated that this level in cows was biologically active but non-toxic. A solution was prepared by suspending purified EHDP in sterile physiological saline (0.9 per cent) so that 10 ml provided a dose of 2 mg/kg. The subcutaneous rather than the oral route was chosen in order to avoid possible variations in blood levels of the EHDP that might result from variable gastrointestinal adsorption.

From the beginning of the experiment and at subsequent 10 day intervals each cow was placed in a metabolism stall where serum, plasma and urine samples were collected. Blood samples for serum ionized calcium determinations also were drawn and immediately placed in a 7 ml vacutainer without anticoagulant (Becton-Dickinson, Rutherford, New Jersey). After clotting the serum was collected, immediately placed in a 2 ml vacutainer, and frozen at -20°C until assayed. Plasma for immunoreactive parathyroid hormone determinations was collected in precooled centrifuge tubes and frozen (-20°C) within 20 minutes.
Duplicate 24-hour urine samples were collected using indwelling catheters (Bardex-Foley with a 75 ml inflatable, fluted, ovoid balloon (size 26, 28 or 30 French). A 4 foot rubber tube (0.25 inch diameter) attached to the indwelling catheter led to a Y-tube which funneled urine into two stainless steel collection vessels. Toluene was added to one container as a preservative for hydroxyproline determinations. The second collection vessel contained glacial acetic acid (5 ml) to preserve urine for cyclic adenosine monophosphate (cAMP) assays. Aliquots of urine preserved by both methods were stored at -20°C until the assays were performed.

**Serum and Urine Electrolytes**

Serum total calcium and magnesium were determined by atomic absorption spectrophotometry (Perkin-Elmer 303). Serum calcium concentrations before, during, and after EDTA infusion were determined by the citric acid titration method (Harleco, Philadelphia, Pennsylvania). Serum ionized calcium was analyzed by a calcium ion-selective electrode (model 801, Orion Research, Inc., Cambridge, Mass.). Serum and urine phosphorus were determined by the method of Fiske and Subbarow. Values of urinary phosphorus were expressed as a ratio to creatinine.

**Plasma Parathyroid Hormone**

Plasma samples were analyzed for parathyroid hormone concentration by means of radioimmunoassay. The assay employed an antisera (GP-1) that detects both the amino terminal end (biologically active portion) and the carboxyl portion (inactive portion) of parathyroid hormone.
Urinary Hydroxyproline and Cyclic Adenosine Monophosphate

Urinary total hydroxyproline was determined by the method of Kivirikko, Laitinen and Prockop\textsuperscript{19} and creatinine by the method of Clarke.\textsuperscript{20} Urinary cyclic adenosine monophosphate was determined as an index of the renal response to parathyroid hormone\textsuperscript{21} by the method of Steiner et al.\textsuperscript{22} Values of urinary hydroxyproline and cyclic adenosine monophosphate were expressed as a ratio to creatinine.

Immediately Available Calcium Reserves

The immediately available calcium reserves were evaluated by two ethylenediaminetetracetic acid (EDTA) infusion studies. The first EDTA infusion (baseline) was given after 7 days of acclimation to the laboratory. A second infusion (experimental) was administered to each cow after 60 experimental days. Forty-nine grams of EDTA in 5 per cent dextrose solution was administered intravenously to each cow over a period of 4 hours or until the serum calcium was below 7 mg per 100 ml. Samples of plasma and serum were collected prior to infusion and at 0.5 hour intervals for the next 160 hours. Urine samples were collected at hourly intervals. Blood and urine samples were also collected 20 and 44 hours postinfusion. During the 16th hour of the experimental EDTA infusion, 2 cows treated with EHDP were administered intravenously calcium gluconate when the serum calcium fell below 5.00 mg/100 ml. Therefore, the EDTA studies reported for these 2 cows terminated at 16 hours.
Bioassay of Thyroid Glands for Calcitonin Activity

Thyroid glands collected at the termination of the experiment from both groups of cows were frozen in liquid nitrogen and stored at -20°C. Extracts of thyroid glands were prepared individually from each cow, lyophilized and stored at -20°C prior to assay. The extracts were reconstituted with a diluent composed of 0.01 M sodium acetate trihydrate and 20 mg of bovine serum albumin. The reconstituted extracts were adjusted to pH 4 and assayed in 25-day-old male Holtzman rats according to the method of Cooper et al. against a bovine thyrocalcitonin standard. The rats were exsanguinated from the abdominal aorta at 65 minutes after subcutaneous injection. In all of the four-point assays the high dose was 4 times the low dose. Serum was collected and analyzed for calcium by atomic absorption spectrophotometry. The logarithmic dose response was plotted for each assay and the relative potency of the extracts was determined.

Light and Electron Microscopy of Parathyroid Glands and Thyroid C-Cells

Forty-eight hours postpartum or when the serum calcium fell below 6 mg/100 ml postpartum each cow was euthanatized by electrocution and a complete necropsy was performed. Sections of parathyroid and thyroid glands were collected for electron microscopic evaluation. The tissues were cut into 1.0 mm cubes, fixed in 3 per cent glutaraldehyde in 0.1 M sodium cacodylate, washed twice in cacodylate buffer, and postfixed with 1.33 per cent osmium tetroxide in s-collidine at pH 7.4. Tissues were dehydrated through ascending concentrations of ethyl alcohol, transferred to propylene oxide, and embedded in Epon 812 (Shell Oil
Company, New York, New York). Sections were cut at 600 to 800 Å on a Reichert OmU-2 ultramicrotome and mounted on 300-mesh copper grids. The sections were stained with uranyl acetate and lead citrate and examined with a Philips 200 electron microscope. Representative tissues were fixed in 10 per cent phosphate-buffered formalin for histological evaluation.

RESULTS

Serum Electrolytes

Serum total calcium for both groups of cows was similar during 80 days prepartum (Figure 1). However, mean ionized calcium values for cows administered EHDP were consistently lower for 70 days prepartum (Figure 1). Differences in ionized calcium at 30 and 20 days prepartum were significant (P<0.05) between the two groups of cows. At parturition total serum calcium values (mg/100 ml) for the control cows were 8.70 while serum calcium fell to 6.35 (P<0.05) in EHDP-treated cows. At 1 day postpartum serum total calcium remained unchanged in the control cows (8.63) while cows administered EHDP continued to decline (5.66) (P<0.05). When serum total calcium fell under 6.00 mg/100 ml the EHDP-treated cows developed clinical signs of muscular weakness, incoordination, and paresis. Serum ionized calcium (mg/100 ml) remained relatively unchanged at parturition (3.73) and one day postpartum (3.60) in cows fed the control diet. The mean ionized calcium value for cows receiving EHDP was 1.85 mg/100 ml at parturition and declined significantly (P<0.05) at one day postpartum to 0.49 mg/100 ml. In cows ad-
Figure 1 - Serum total and ionized calcium (mg/100 ml) prepartum, at parturition, and postpartum for cows fed a control diet and cows fed a control diet and administered EHDP.
FIG 1

SERUM CALCIUM (mg/100ml)

TOTAL CALCIUM, CONTROL DIET
IONIZED CALCIUM, CONTROL DIET
TOTAL CALCIUM, CONTROL DIET + EHDP
IONIZED CALCIUM, CONTROL DIET + EHDP

TIME - DAYS

80 70 60 50 40 30 20 10 PARTURITION 1 DAY POSTPARTUM

PARTURITION 1 DAY POSTPARTUM
ministered EHDP the serum total calcium declined 16% and ionized calcium fell 74% at 1 day postpartum.

Serum phosphorus levels were not significantly different from 90 to 50 days prepartum between the two groups of cows (Figure 2). During the final 50 days prepartum serum phosphorus was consistently lower in cows administered EHDP. At parturition serum phosphorus (mg/100 ml) was higher for the control cows (5.13) compared to cows receiving EHDP (4.25). One day postpartum serum phosphorus declined to 4.41 in control cows but decreased significantly (P < 0.05) to 2.27 mg/100 ml in cows administered EHDP.

Serum magnesium levels were similar for both groups of cows for 90 days prepartum, at parturition, and one day postpartum.

**Plasma Immunoreactive Parathyroid Hormone (iPTH)**

Plasma iPTH levels during the period of 90 to 20 days prepartum were similar for both groups of cows (Figure 3). The greater increase in iPTH from 10 days prepartum to 1 day postpartum correlated with the lower serum ionized calcium levels in cows administered EHDP compared to control cows (Figure 1).

**Urinary Phosphorus, cAMP and Hydroxyproline**

Peaks in urinary phosphorus excretion (mg/mg creatinine) occurred at 70 and 30 days during the prepartum period in control cows and at 80, 60 and 20 days prepartum in cows receiving EHDP (Figure 4). An increase in the urinary excretion of cAMP appeared to coincide with the peaks of urinary phosphorus in both groups of cows (Figure 5). At parturition urinary phosphorus values were similar for both groups of cows while...
Figure 2 — Serum phosphorus prepartum, at parturition and postpartum for control cows (---) and cows given EHDP (---). Values are expressed as mg per 100 ml ± standard error.
FIG 2

SERUM PHOSPHORUS (mg/100ml)

CONTROL DIET
CONTROL DIET + EHDP
(4 mg/kg)

DAYS PREPARTUM
PARTURITION
Figure 3 - Plasma immunoreactive parathyroid hormone prepartum, at parturition, and postpartum for control cows (---) and cows given EHDP (---). Values are expressed as ng per ml ± standard error.
EFFECT OF EHDP ON PLASMA IMMUNOREACTIVE PARATHYROID HORMONE

---

CONTROL DIET

CONTROL DIET + EHDP (4mg/kg)

FIG 3

PLASMA IMMUNOREACTIVE PARATHYROID HORMONE (ng/ml)

DAYS PREPARTUM

PARTURITION
Figure 4 - Urinary excretion of phosphorus prepartum, at parturition and postpartum for cows fed a control diet (—) and cows fed a control diet and administered EHDP (---). Values are expressed as a ratio of phosphorus (mg) to creatinine (mg).
FIG 4
Figure 5 - Urinary excretion of cAMP prepartum, at parturition and postpartum for control cows (——) and EHDP-treated cows (---).

Values are expressed as a ratio of cAMP (pmoles) to creatinine (mg).
FIG 5

- CONTROL DIET
- CONTROL DIET + EHDP (4mg/kg)
Urinary excretion of cAMP (806 pmoles/mg creatinine) was higher in control cows. Urinary phosphorus increased from parturition to one day postpartum in cows administered EHDP but it declined in control cows (Figure 4). During this same interval urinary cAMP declined in both groups of cows (Figure 5); however, values were consistently lower in the cows receiving EHDP.

Urinary hydroxyproline was consistently lower in the EHDP-treated cows during the 90 days prepartum (Figure 6). At parturition the mean urinary excretion of hydroxyproline (mg/mg creatinine) was .058 for control cows compared to 0.026 in experimental cows. By one day postpartum urinary hydroxyproline had increased in both groups but levels remained lower in cows administered EHDP.

Immediately Available Calcium Reserves

During the experimental EDTA infusion 295 per cent more EDTA was required to lower serum calcium below 7.00 mg/100 ml in control cows than in cows given EHDP. Serum calcium after 3 hours of EDTA was under 7.00 mg per 100 ml in both groups of cows (Figure 7). The serum calcium returned to near normal levels at a similar rapid rate during the next 4 to 16 hours in cows during the baseline infusion and after feeding the control diet for 60 days (Figure 7). Following the initial decline serum calcium remained unchanged and significantly lower (P<0.05) after the experimental EDTA infusion in cows administered EHDP (Figure 8). Serum calcium (mg/100 ml) was 6.30 by 12 hours post-EDTA infusion for cows receiving EHDP whereas serum calcium had returned to near normal levels (8.94) by 6 hours postinfusion in control cows.
Figure 6 - Urinary excretion of hydroxyproline prepartum, at parturition, and postpartum for control cows (—) and EHDP-treated cows (---). Values are expressed as a ratio of hydroxyproline (mg) to creatinine (mg) ± standard error.
FIG 6

HYDROXYPROLINE: CREATININE

- CONTROL DIET
- CONTROL DIET + EHDP
(4mg/kg)

DAYS PREPARTUM
PARTURITION
Figure 7 - Immediately available calcium reserves determined by EDTA infusion prior to the experiment (baseline —) and after 60 days of feeding cows a control diet (---). Values of serum calcium are expressed as mg per 100 ml ± standard error.
Figure 8 - Immediately available calcium reserves determined by EDTA infusion prior to the experiment (baseline —) and after 60 days of feeding a control diet plus administering EHDP. Values of serum calcium are expressed as mg per 100 ml ± standard error.
During the experimental EDTA infusion plasma iPTH levels were consistently higher from the 6th to 24th hour for cows administered EHDP with a prominent peak at twelve hours (Figure 9). The greater elevation of plasma iPTH during this interval correlated with the persistently lower serum calcium following EDTA infusion in cows receiving EHDP (Figure 8).

Urinary cAMP was consistently higher from 3 to 24 hours of the experimental EDTA infusion in control cows compared to cows receiving EHDP for 60 days (Figure 10). These changes in urinary cAMP excretion did not appear to coincide with fluctuations in serum calcium or iPTH during the EDTA infusions in either group of cows.

Urinary hydroxyproline excretion was consistently lower after the experimental EDTA infusion in cows administered EHDP for 60 days (Figure 11). Hydroxyproline levels increased to a maximum (0.036 mg/mg creatinine) in control cows at 8 and 11 hours of the EDTA infusion. In cows receiving EHDP urinary hydroxyproline increased to a maximum of 0.016 after 48 hours. The urinary hydroxyproline values from 6 to 16 hours after EDTA infusion for the control cows were significantly (P > 0.001) higher than in cows receiving EHDP.

Urinary phosphorus excretion during the experimental EDTA infusion by the EHDP-treated cows was consistently higher than in control cows. The increased urinary excretion of phosphorus coincided with the elevation of iPTH levels following EDTA infusion in cows administered EHDP for 60 days.
Figure 9 - Plasma immunoreactive parathyroid hormone (ng/ml) determined during an EDTA infusion given after 60 days of feeding a control diet to pregnant cows (-----) and feeding the control diet plus administering EHDP (---).
EFFECT OF EHDP ON PLASMA IMMUNOREACTIVE PARATHYROID HORMONE DURING EDTA INFUSION

- CONTROL DIET (60 DAYS)
- CONTROL DIET+EHDP (60 DAYS) (4mg/kg)

**Fig 9**
Figure 10 - Urinary cAMP determined during EDTA infusions given after 60 days of feeding a control diet to pregnant cows (---) and feeding the control diet plus administering EHDP to pregnant cows (----). Values of urinary cAMP (pmoles) were expressed as a ratio to creatinine (mg).
FIG 10

- CONTROL DIET (60 DAYS)
- CONTROL DIET (60 DAYS) + EHDP (4 mg/kg)
Figure 11 - Urinary excretion of hydroxyproline determined during an EDTA infusion given after 60 days of feeding a control diet to pregnant cows (—) and feeding the control diet plus administering EHDP (---). Values of urinary hydroxyproline (mg) were expressed as a ratio to creatinine (mg) ± standard error.
EHDP EFFECT ON URINARY HYDROXYPROLINE IN PREGNANT COWS FED A CONTROL DIET

- CONTROL DIET (60 DAYS)
- CONTROL DIET + EHDP (60 DAYS) (4mg/kg)
Ultrastructural Evaluation of Parathyroid Glands and Thyroid C-Cells

The parathyroid glands from parturient cows fed the control diet with the required amounts of calcium and phosphorus had a varied population of chief cells and transitional forms. Chief cells were predominantly in the active phase of the secretory cycle. They were characterized by a prominent Golgi apparatus, large mitochondria, a few peripherally situated secretory granules, and intricately inter-digitation plasma membranes (Figure 12). An occasional chief cell in control cows had more numerous secretory granules, profiles of rough endoplasmic reticulum, prominent microfilaments and microtubules in close proximity to the Golgi apparatus (Figure 13).

Chief cells from the parathyroid glands of cows administered EHDP also appeared to be predominantly in the active phase of the secretory cycle. However, chief cells were more consistently degranulated in response to the severe hypocalcemia that developed near parturition. Mitochondria were large and the rough endoplasmic reticulum often was aggregated into lamellar arrays (Figure 14). Intercellular spaces between chief cells were more prominent in parathyroids of cows receiving EHDP. Other chief cells appeared to be hyperactive with a large cytoplasmic area that contained multiple Golgi apparatuses, prominent lamellar arrays of rough endoplasmic reticulum, large mitochondria, and scattered secretory granules in close apposition to the plasma membrane (Figure 15). An occasional degranulated chief cell in cows administered EHDP had prominent cytoplasmic microfilaments, lipid droplets, and vacuolation of some mitochondria (Figure 16).
Figure 12 - Chief cell in the active phase of the secretory cycle from the parathyroids of a parturient cow fed a control diet with the required amounts of calcium and phosphorus. The cytoplasm contains prominent Golgi apparatuses (G) and mitochondria (M) and a few peripherally situated secretory granules (S). Plasma membranes of adjacent chief cells are intricately interdigitated (arrow). X 14,800
Figure 13 - Chief cell from the parathyroids of a control parturient cow with numerous secretory granules (S), prominent profiles of rough endoplasmic reticulum (E), microfilaments (arrow) and microtubules (arrowhead) in close proximity to the Golgi apparatus (G). X 22,500
Figure 14 - Degranulated chief cell from the parathyroids of a parturient cow administered EHDP. In response to the severe hypocalcemia there is a paucity of secretory granules (S) but there are prominent mitochondria (M) and lamellar arrays of rough endoplasmic reticulum (E). Intercellular spaces (arrow) are enlarged. X 14,800
Figure 15 - Hyperactive chief cell from a cow administered EHDP and fed a prepartal diet with the required amount of calcium and phosphorus. The large cytoplasmic area contains large Golgi apparatuses (G), lamellar arrays of rough endoplasmic reticulum (E), and prominent mitochondria (M). Secretory granules (S) are present near the Golgi apparatus and aligned along the plasma membrane. X 19,800
Figure 16 - Degranulated chief cell from an EHDP-treated cow with prominent microfilaments (arrow), large lipid droplets (L), and occasional vacuolated mitochondria (M). X 11,800
Ultrastructural characteristics of thyroid C-cells were similar for both groups of cows fed the control diet with the required amounts of calcium and phosphorus. Although C-cells were observed in different stages of the secretory cycle, the predominant C-cell had a prominent Golgi apparatus and endoplasmic reticulum with moderate numbers of secretory granules in the basal part of the cell facing the interfollicular capillaries (Figure 17). Thyroid calcitonin activity (MRC milli-units per gm of thyroid) determined by bioassay was 241 for control cows and 253 for cows receiving EHDP prepartum. These differences were not significant.

DISCUSSION

The present investigation demonstrated that cows administered EHDP and fed a balanced diet with adequate calcium and phosphorus prepartum consistently developed severe hypocalcemia and hypophosphatemia near parturition. Control cows fed the same diet did not develop acute hypocalcemia at parturition. The profound reduction in both serum total and ionized calcium was associated with the development of progressive muscle weakness, paresis, and other functional disturbances identical to those associated with naturally occurring parturient paresis. A similar magnitude of reduction in serum total and ionized calcium occurs in cows with the naturally occurring disease.

Chief cells in parathyroid glands were predominately in the active stage of their secretory cycle in both groups of parturient cows, because of the great demand on calcium reserves at the onset of lactation.
Figure 17 - Typical thyroid C-cell in the follicular wall of a parturient cow fed a control diet with or without EHDP administration. The cytoplasm contains a prominent Golgi apparatus (G), endoplasmic reticulum (E), and moderate numbers of secretory granules (S) in the basal part of the cell facing interfollicular capillaries. X 15,000
Active chief cells from the EHDP-treated cows were more degranulated of mature secretory granules in response to the severe hypocalcemia than in control cows and had prominent microfilaments and microtubules in the cytoplasm. Occasional chief cells in cows administered EHDP had vacuolated mitochondria; however, this did not appear to interfere with the secretion of parathyroid hormone either in cows fed a control or low calcium diet. The increase of plasma immunoreactive parathyroid hormone levels in response to hypocalcemia induced either by EDTA or lactation was as great or greater in cows receiving EHDP as in control cows. Therefore, it appeared that an interference in the secretion of parathyroid hormone was not an important factor in the pathogenesis of severe hypocalcemia at parturition induced by EHDP. A normal or exaggerated parathyroid hormone secretion also has been demonstrated near parturition in cows with naturally occurring parturient hypocalcemia.

Urinary excretion of cAMP in EHDP-treated cows was not significantly different than in control cows. This finding suggests that the failure to mobilize calcium reserves and development of severe hypocalcemia near parturition in the cows administered EHDP was not due to an impaired production of cAMP by target cells in the kidney and elsewhere. A similar reduction in urinary cAMP excretion has been reported in cows that develop parturient hypocalcemia compared to control parturient cows. The fluctuations in urinary phosphorus excretion appeared to coincide with changes in cAMP in both groups of cows.

The decreased urinary excretion of hydroxyproline prepartum and during the experimental EDTA infusion suggested an impairment of bone matrix catabolism in cows administered EHDP. A diminished excretion of
hydroxyproline also has been reported previously in cows with naturally occurring parturient paresis and parturient hypocalcemia induced by the prepartal feeding of high calcium diets.\textsuperscript{27,28} These findings suggest that an interruption in bone resorption and mobilization of calcium from skeletal reserves occurs during the development of spontaneous, diet- and diphosphonate-induced hypocalcemia near parturition and the initiation of lactation in dairy cows. Additional studies on the cows receiving EHDP have demonstrated that the per cent of bone surfaces undergoing resorption on microradiographs was significantly reduced compared to control cows.\textsuperscript{13} These findings are similar to previous observations that cows with naturally occurring parturient hypocalcemia have reduced bone resorptive surfaces with few osteoclasts.\textsuperscript{6} Morphometric evaluation of bone demonstrated that cows administered EHDP had decreased appositional and radial closure rates but an increased osteon formation time and numbers of osteoid seams compared to control cows.\textsuperscript{13} The intestinal uptake of calcium \textit{in vitro},\textsuperscript{29} thyroid calcitonin activity, and granulation of C-cells was similar for both groups of cows. Therefore, mobilization of extracellular calcium in cows receiving EHDP appeared to be primarily the result of a direct effect of the diphosphonate on bone by preventing dissolution of hydroxyapatite crystals.\textsuperscript{11,12} In addition, an impairment in the ability to form 1,25 dihydroxycholecalciferol may have contributed to the development of severe hypocalcemia near parturition in experimental cows receiving EHDP. A recent study indicated that high levels (10 mg/kg) of EHDP decrease the formation of active vitamin D metabolites.\textsuperscript{30}
The results of this investigation suggest that the pathogenesis of parturient hypocalcemia is similar in experimental cows receiving EHDP and those with the naturally occurring diseases. Neither an interference in parathyroid hormone secretion, impaired intestinal uptake of calcium nor a lack of formation of second messenger (cAMP) appear to be significant factors in the development of the hypocalcemia near parturition. The principal defect in both experimentally induced and naturally occurring parturient hypocalcemia appears to be a refractiveness of bone to PTH-stimulated resorption as well as a reduction in the immediately available calcium reserves. There appears to be a failure of rapid mobilization of calcium reserves due to a small pool of active bone resorbing cells capable of responding to the increased parathyroid hormone secretion during hypocalcemic challenges imposed either by parturition or EDTA infusion. The rapid mobilization of skeletal reserves appears to contribute significantly to calcium homeostasis near parturition in cows and factors that interrupt bone resorption result in the development of profound hypocalcemia.
ABSTRACT

Cows administered ethane-1-hydroxy-1,1-diphosphonate (EHDP) (4 mg/kg) daily for 90 days prepartum and fed a balanced diet with the required amounts of calcium and phosphorus developed severe hypocalcemia and hypophosphatemia either at parturition or immediately postpartum. When serum total and ionized calcium declined below 6.5 and 1.0 mg/100 ml, respectively, the cows developed similar clinical signs as cows with naturally occurring parturient paresis. In response to the hypocalcemia plasma immunoreactive parathyroid hormone levels were as high or higher prepartum, at parturition, and one day postpartum in cows administered EHDP as in control cows. Parathyroid chief cells were predominately in the actively synthesizing phase of the secretory cycle with a prominent Golgi apparatus and lamellar arrays of rough endoplasmic reticulum in cows receiving EHDP. Many chief cells were degranulated of mature secretory granules in response to the severe hypocalcemia. Calcitonin activity in thyroid extracts determined by bioassay and the numbers of secretory granules in thyroid C-cells were similar in both groups of cows. EDTA infusion after 60 days of the experiment demonstrated that the immediately available calcium reserves were reduced in EHDP-treated cows. The serum calcium remained significantly lower and did not return to preinfusion levels by 24 hours in cows administered EHDP even though parathyroid hormone levels were elevated compared to control cows. Serum calcium in control cows returned to within the normal range by 6 hours after EDTA infusion. The urinary excretion of hydroxyproline was consistently reduced prepartum and fol-
lowing EDTA infusion in cows receiving EHDP. These findings suggest that bone catabolism was impaired in cows administered EHDP in spite of an increased secretion of parathyroid hormone. The experimental induction of parturient hypocalemia by the prepartal administration of EHDP may provide a valuable model for studies to investigate the pathogenic mechanisms in bone responsible for the development of severe hypocalemia at parturition.
CHAPTER IV

MORPHOMETRIC AND MICRORADIOGRAPHIC EVALUATION OF THE EFFECTS
OF DIPHOSPHONATES AND DIETARY CALCIUM ON BONE OF PREGNANT COWS

Introduction

Disodium ethane-1-hydroxy-1,1-diphosphonate (EHDP) and dichloro-
methane diphosphonate (CL₂MDP) have many similar properties as pyro-
phosphate (Fleisch et al., 1968) but differ by having a P-C-P instead
of a P-O-P bond and thereby are more resistant to both chemical and
enzymatic hydrolysis (Francis, 1969). EHDP inhibits the precipitation
of calcium phosphate from solution in vitro (Fleisch et al., 1968 and
Fleisch et al., 1970), blocks the transformation of amorphous calcium
phosphate into hydroxyapatite (Francis, 1969), binds strongly to crys-
tals of hydroxyapatite and displaces orthophosphate (Juny et al., 1973),
and blocks crystal growth and dissolution (Fleisch et al., 1969).
Juny et al. (1973) have shown that the binding of EHDP to the surface
of hydroxyapatite is greater than with CL₂MDP.

Jowsey et al. (1970) demonstrated by autoradiography that EHDP was
present on bone surfaces in vivo. EHDP given either orally or subcu-
taneously (10 mg P/kg/day) to several species including the dog
(King et al., 1971), cat (Jowsey et al., 1970), and rat (Russell et al.,
1973) caused an increase in the amount of osteoid in bone and a thick-
ened epiphyseal plate that consisted of poorly mineralized physeal car-
tillage (Schenk et al., 1973). At an equivalent dose, CL$_2$MDP did not impair mineralization of bone matrix in rats (Russell et al., 1973) but caused marked impairment of normal metaphyseal remodeling as well as periosteal resorption (Schenk et al., 1973).

Reduced rates of bone remodeling have been reported in animals treated with both EHDP and CL$_2$MDP as determined by $^{45}$calcium kinetics and urinary hydroxyproline (Gasser et al., 1972; Cabenela and Jowsey, 1974). The cause of altered bone remodeling appeared to be the effect of the two diphosphonates not only on mineralization of bone but also on bone resorption. CL$_2$MDP has been shown to be a more potent inhibitor of bone resorption in vitro (Minkin et al., 1974) as well as parathyroid hormone-induced bone resorption in vitro or in vivo (Russell et al., 1970 and Morgan et al., 1973). CL$_2$MDP has been shown to prevent parathyroid hormone induced $^{45}$calcium release and lactate production from mouse calvaria similar to calcitonin but is unable to prevent the increase in acid phosphatase and pyrophosphatase as has been reported for calcitonin (Morgan et al., 1973).

The selective effects of EHDP on bone dynamics are being utilized in the treatment of Paget's disease (Smith et al., 1971). CL$_2$MDP has not been used in clinical trials because, in part, of a paucity of information regarding its long-term effects on bone remodeling and on calcium homeostasis. Recently, the diphosphonates have been shown to significantly affect calcium homeostatic mechanisms by blocking bone resorption resulting in the development of severe hypocalcemia in response to the challenge associated with parturition and the initiation of lactation in cows (Yarrington et al., 1976a). The objective of the
present investigation was to determine the long-term effects of EHDP, 
CL$_2$HDP, and dietary calcium on bone dynamics and surface characteris-
tics. Previous studies have indicated that the prepartal feeding of 
low calcium diets prevents and high calcium diets promote the develop-
ment of parturient hypocalcemia in the cow (Black et al., 1973; Ender 
and Dishington, 1970). However, the influence of prepartal dietary 
calcium content on Haversian bone remodeling dynamics have not been 
determined in order to explain these preventative or promotional ef-
fects.

Materials and Methods

Experimental Design

Pregnant Cows. A total of 18 nonlactating, pregnant Jersey cows, 
ranging in age from 5 to 8 years of age, were brought to the labora-
atory on an average of 94 days prepartum. They were acclimated to lab-
oratory conditions for a period of 14 days before the initiation of 
the experiments. During the acclimation period each cow was fed a 
balanced control diet (Black et al., 1973a) supplying the daily re-
commended requirements for calcium (25 gm) and phosphorus (25 gm).

At the onset of the experiment, the 18 cows were divided into 6 
groups of 3 cows each. Three groups of cows were fed a low calcium 
diet of similar composition as the control diet in amounts to provide 
12.5 gm of calcium per day. The three groups of cows fed the low cal-
cium prepartal diet either were administered EHDP (4 mg/kg/day) subcu-
taneously twice daily, received CL$_2$HDP (4 mg/kg/day) subcutaneously 
twice daily, or were fed only the low calcium diet. The dose level of
4 mg/kg/day for the diphosphonates was chosen because a pilot study (Yarrington et al., 1974) had demonstrated this level had potent yet nontoxic effects on calcium homeostasis in cows. Both EHDP and CL₂MDP were suspended in sterile physiological saline so that one subcutaneous injection of 10 cc of solution provided a dose of 2 mg/kg body weight. The subcutaneous rather than the oral route of administration was chosen to eliminate possibilities of enzymatic degradation and variable absorption in the gastrointestinal tract.

Two groups of cows fed the balanced control diet prepartum either were administered EHDP (4 mg/kg/day) subcutaneously twice daily or were fed only the control diet. A sixth group of pregnant cows were fed a high calcium diet prepartum that supplied 150 gm of calcium and 25 gm of phosphorus per day. The cows were carefully monitored during the experiment until parturition and up to two days postpartum or when serum calcium levels fell under 6.5 mg per 100 ml. Further details of the experimental design are described elsewhere (Yarrington et al., 1976).

Double Tetracycline Labeling of Bone

For the purpose of determining the dynamics of appositional bone growth, each cow was administered intravenously oxytetracycline after ten days of the experiment (Liquamycin, Charles Pfizer Co., Inc., New York, N.Y.) at therapeutic levels (5 mg/lb/day) for three consecutive days. The same dose level of oxytetracycline was again given to each cow after 40 days from the end of the first label.
A section of the right tenth rib was removed at the termination of the experiment when each cow was euthanatized by electrocution. The bone was fixed in ten per cent buffered formalin. Unstained, ground bone sections (80-100 microns) were viewed under a Zeiss fluorescent microscope for determination of bone morphometric analysis.

**Microradiographic Evaluation of Bone**

A section of the right tenth rib from each cow was fixed in 70 per cent ethanol (Jowsey et al., 1965) for microradiography. Bone sections were embedded in methyl methacrylate and cut at approximately 180 microns with a Gillings-Bronwill thin sectioning machine (Hamco Machines, Inc., Bronwill Scientific Division, Rochester, New York) at a reduced speed (500 r.p.m.). A 4 inch diamond cutting wheel was used with a thickness of 340 microns. The sections were ground under glass to obtain a uniform thickness of 100 microns. Section thickness was critically measured by a Mikrokator (Mikrokator, no. 7VZ509-4, C. E. J. Gage Company, Dearborn, Michigan, subsidiary of C. E. Johnson, Sweden) with adjustable measuring pressure.

The sections were exposed to Kodak 649 emulsion on 1- by 3-inch microslides in a continuously evacuated removable camera using a Philips X-ray diffraction unit with full wave rectification. The diffraction unit was operated at 20 kv and 20 ma with a copper target (focal spot 1 mm) and a target to specimen distance of 20 cm.

Bone remodeling was assessed directly by viewing the microradiographs using a light microscope. Bone surfaces concerned with formation and resorption were interpreted according to characteristics es-
established by Jowsey et al. (1965). Formation and resorption surfaces were marked on photomicrographs that were enlarged 30 times. These surfaces were measured with a K and E map measurer (model 620350, Minneapolis Blue Printing Company, South Minneapolis, Minnesota) and expressed as a percentage of the total surface area on the section.

**Light Microscopy and Osteoid Staining**

Bone sections from the right tenth rib, third lumbar vertebrae, right humerus and right femur from each cow were fixed in 10 per cent phosphate-buffered formalin. The undecalcified sections were cut with a jeweler's saw, ground to 80-125 microns by hand grinding under running water on waterproof carborundum sandpaper, and stained with Villanueva's Osteochrome stain (Villanueva, 1967).

**Morphometric Analysis of Bone**

Osteoid seam analysis was performed on osteochrome-stained, undecalcified right tenth rib. The evaluation was performed on cortical bone surfaces because of the variability of trabecular surfaces between each cow. By means of a conventional light microscope utilizing the 10x and 25x objectives, the number of osteoid seams, resorptive spaces, and the mean wall thickness of completed osteons (MWT) were determined (Schock et al., 1972). The number of osteoid seams (Af) and resorptive spaces (Ar) were expressed as a ratio to the mean cortical cross sectional area (Ac) of rib which was determined in accordance with the method of Schock et al. (1972). The mean circumference of an osteoid seam (Sf) and the thickness of osteoid seams (OST) (μ) were determined utilizing a 25x objective (Schock et al., 1972). Approximately 150
osteoid seams were selected at random and measured for each group of cows. The relationship of a osteoid seam to an osteon (OST/MST) was expressed as a ratio of the width (μ) of the seam to the mean wall thickness (μ).

Undecalcified and unstained sections of right tenth rib were examined under a Zeiss fluorescent microscope utilizing a 25x objective. Active centers of bone formation were identified clearly by the presence of a tetracycline label at the periphery of the osteoid seam. The percent of labeled osteoid seams was determined by comparing the number of osteoid seams with at least one tetracycline label to the total number of osteoid seams. In each cow given a double label of tetracycline, only those osteons in cortical bone containing two fluorescent bands separated by a nonfluorescent space were used for further morphometric analysis. The mean appositional rate (M), radial closure rate (Mf), osteon formation time (σf) and bone formation rate were determined (Schock et al., 1972). The quantitative histologic measurements of Haversian bone remodeling used in this study are summarized in Table I.

**Alkaline Pyrophosphatase Analysis**

Estimations of inorganic pyrophosphatase were made using trabecular bone from the right tenth rib of each cow. The assay technique has been previously described (Holdsworth et al., 1973). Activity was expressed as nmoles of pyrophosphatase split products in 30 minutes per milligram of protein.
Table 1. Summary of histologic measurements of Haversian bone remodeling.
### QUANTITATIVE HISTOLOGIC MEASUREMENT OF HAVERSIAN BONE REMODELING

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unit</th>
<th>Formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. Osteoid Seams</td>
<td>/mm²</td>
<td>( A_F )</td>
</tr>
<tr>
<td>Osteoid Seam Thickness</td>
<td>( \mu )</td>
<td>( \text{OST} )</td>
</tr>
<tr>
<td>Osteoid Seam Thickness Ratio</td>
<td></td>
<td>( \text{OST}/\text{MST}_{OS} )</td>
</tr>
<tr>
<td>Circumference of Osteoid Seam</td>
<td>mm</td>
<td>( S_F )</td>
</tr>
<tr>
<td>Mean Cortical Area</td>
<td>mm²</td>
<td>( A_C )</td>
</tr>
<tr>
<td>Cortical to Total Area Ratio</td>
<td></td>
<td>( C/T )</td>
</tr>
<tr>
<td>Mean Osteon Wall Thickness</td>
<td>mm</td>
<td>( MWT )</td>
</tr>
<tr>
<td>Appositional Rate</td>
<td>( \mu/\text{day} )</td>
<td>( M )</td>
</tr>
<tr>
<td>Labeled Osteoid Seams</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>Radial Closure Rate</td>
<td>mm/yr</td>
<td>( M_F )</td>
</tr>
<tr>
<td>Osteon Formation Time</td>
<td>years</td>
<td>( \sigma_F )</td>
</tr>
<tr>
<td>No. Resorption Spaces</td>
<td>/mm²</td>
<td>( A_r )</td>
</tr>
<tr>
<td>Resorption to Formation Ratio</td>
<td></td>
<td>( A_r/A_F )</td>
</tr>
<tr>
<td>Bone Formation Rate</td>
<td>mm²/mm²/yr</td>
<td>( V_F )</td>
</tr>
<tr>
<td>Activation Frequency</td>
<td>foci/mm²/yr</td>
<td>( \mu_F )</td>
</tr>
<tr>
<td>Mean Total Area</td>
<td>mm²</td>
<td>( A_t )</td>
</tr>
</tbody>
</table>
Serum Calcium and Urinary Hydroxyproline Determinations

Terminal values for serum calcium were determined by atomic absorption spectrophotometry (Perkin-Elmer 303). Urinary total hydroxyproline was determined by the method of Kivirikko, Laitinen, and Prockop (1967) and creatinine by the method of Clarke (1961). Hydroxyproline was expressed as a ratio to creatinine.

Statistical Analysis

The data was analyzed for significance using the Student's t test for samples of equal size with unequal variances (Snedcor and Cochran, 1971). Actual probability of significance is expressed within the text.

Results

Effects of EHDP and CL₂HDP on Bone Formation

The administration of EHDP to pregnant cows fed a low calcium prepartal diet caused a significant ($P < 0.05$) increase in the number of osteoid seams/mm² of cortical area ($A_f$) (Fig. 1). In contrast a paucity of osteoid seams were observed in cortical areas of rib from cows only fed the low calcium prepartal diet (Fig. 2). Similar differences were observed in cows fed the control diet and administered EHDP (8.95/mm²) when compared to cows fed the control diet (1.43/mm²) (Table 2). The administration of EHDP caused a significant ($P < 0.01$) increase in the thickness ($OST$) and an enlarged circumference ($S_f$) of osteoid seams in cows fed either a low or control calcium diet (Table 2). The ratio of mean osteoid seam thickness to the mean wall thickness ($OST/MWT$) were also significantly increased ($P < 0.05$) in
Fig. 1. Ground section of cortical bone of right tenth rib from a cow given EHDP and fed a low calcium diet. Note the presence of numerous prominent osteoid seams (arrow). Osteochrome stain. X 50
Fig. 2. Ground section of cortical bone of right tenth rib from a cow fed a low calcium diet. Note the absence of prominent osteoid seams. Osteochrome stain. X 50
Table 2. Quantitative histologic measurements of Haversian bone remodeling.
## Quantitative Histologic Measurement of Haversian Bone Remodeling in Cows

<table>
<thead>
<tr>
<th>HISTOLOGIC INDEX</th>
<th>CONTROL DIET</th>
<th>HIGH CA DIET</th>
<th>LOW CA DIET</th>
<th>LOW CA DIET +EHDP</th>
<th>CONTROL DIET +EHDP</th>
<th>LOW CA DIET +CL₂MDP</th>
</tr>
</thead>
<tbody>
<tr>
<td>A₀ (mm²)</td>
<td>1.43</td>
<td>2.81</td>
<td>0.81</td>
<td>11.30⁺</td>
<td>8.95*</td>
<td>3.50</td>
</tr>
<tr>
<td>Osteoid Seam Thickness (OST)</td>
<td>5.30</td>
<td>8.10</td>
<td>7.57</td>
<td>14.70*⁺</td>
<td>16.10*</td>
<td>9.77</td>
</tr>
<tr>
<td>Thickness Ratio (OST/MWT)</td>
<td>0.11</td>
<td>0.16</td>
<td>0.10</td>
<td>0.26**⁺⁺</td>
<td>0.30*</td>
<td>0.16</td>
</tr>
<tr>
<td>S₀ (mm)</td>
<td>0.09</td>
<td>0.12</td>
<td>0.10</td>
<td>0.16**⁺⁺</td>
<td>0.18**</td>
<td>0.12</td>
</tr>
<tr>
<td>A₀ (mm²)</td>
<td>11.8</td>
<td>10.5</td>
<td>10.6</td>
<td>10.9</td>
<td>11.7</td>
<td>16.2</td>
</tr>
<tr>
<td>C/T</td>
<td>0.84</td>
<td>0.81</td>
<td>0.77</td>
<td>0.88</td>
<td>0.84</td>
<td>0.85</td>
</tr>
<tr>
<td>A₀ (mm²)</td>
<td>1.06</td>
<td>0.85</td>
<td>2.87**⁺⁺</td>
<td>0.56**⁺⁺</td>
<td>0.41**⁺⁺</td>
<td>0.44**⁺⁺</td>
</tr>
</tbody>
</table>

N = 3 cows in each group
* = P < 0.01 compared to control group
** = P < 0.05 compared to control group
⁺ = P < 0.01 compared to low calcium group
⁺⁺ = P < 0.05 compared to low calcium group
both groups of EHDP-treated cows compared to cows fed the same diet but not administered the diphosphonate (Table 2).

The administration of an equivalent dose of CL$_2$MDP (4 mg/kg/day) to pregnant cows fed a low calcium prepartal diet caused a slight increase in $A_f$ (3.50/mm$^2$), OST (9.77 µ) and OST/MWT (0.16) values when compared for cows only fed a low calcium diet. However, these differences were not significant.

The dynamics of bone formation were determined by means of double tetracycline labeling. Prolonged administration of EHDP caused a significant reduction in the number of osteoid seams labeled with at least one tetracycline band in cows fed either a low calcium (41.9%) or control calcium diet (29.7%) (Table 3). The number of labeled osteoid seams from nontreated cows was two to three times as great compared to values in cows administered EHDP and fed the same diet. Furthermore, the number of osteoid seams that had distinct double tetracycline labels was greatly diminished in cows receiving EHDP. A similar dose of CL$_2$MDP given to pregnant cows fed the low calcium diet caused no effect on the per cent of labeled osteoid seams compared to cows fed only the low calcium diet (Table 3).

The appositional rate ($M$) and radial closure rate were significantly reduced whereas osteon formation rates ($\alpha_f$) were significantly increased in cows administered EHDP and fed either a low or control calcium diet (Table 3). Compared to corresponding values for cows fed the control diet the values for $M$, $M_f$ and $\alpha_f$ were about one half or less of cows given EHDP. Similar parameters in CL$_2$MDP-treated cows fed the low calcium diet were about twice as great as cows fed the low
Table 3. Quantitative histologic measurements of Haversian bone remodeling in experimental cows given double tetracycline labels.
### QUANTITATIVE HISTOLOGIC MEASUREMENT OF HAVERSIAN BONE REMODELING IN COWS

<table>
<thead>
<tr>
<th>HISTOLOGIC INDEX</th>
<th>CONTROL DIET</th>
<th>HIGH CA DIET</th>
<th>LOW CA DIET</th>
<th>LOW CA DIET +EHDP</th>
<th>CONTROL DIET +EHDP</th>
<th>LOW CA DIET +CL₂MHP</th>
</tr>
</thead>
<tbody>
<tr>
<td>MWT (mm)</td>
<td>0.087</td>
<td>0.089</td>
<td>0.075</td>
<td>0.079</td>
<td>0.085</td>
<td>0.082</td>
</tr>
<tr>
<td>M (µ/day)</td>
<td>0.573</td>
<td>0.274*</td>
<td>0.215**</td>
<td>0.350**</td>
<td>0.285**</td>
<td>0.429</td>
</tr>
<tr>
<td>Labeled Osteoid Seams (%)</td>
<td>99.0</td>
<td>99.0</td>
<td>94.5</td>
<td>41.9**,+</td>
<td>29.7*</td>
<td>99.0</td>
</tr>
<tr>
<td>M_f (mm/yr.)</td>
<td>0.209</td>
<td>0.090*</td>
<td>0.079*</td>
<td>0.048*</td>
<td>0.030*</td>
<td>0.153</td>
</tr>
<tr>
<td>σ_f (years)</td>
<td>0.484</td>
<td>1.016**</td>
<td>1.008**</td>
<td>1.882*</td>
<td>3.454*</td>
<td>0.550</td>
</tr>
</tbody>
</table>

N = 3 cows in each group  
* = P < 0.01 compared to control group  
** = P < 0.05 compared to control group  
+ = P < 0.01 compared to low calcium group
calcium diet alone and were similar to values in cows fed the control calcium diet (Table 3).

Effects of EHDP and CL₂MDP on Bone Resorption

Morphometric analysis revealed that resorptive spaces ($A_r$) in cortical bone of rib were significantly reduced in cows given either EHDP (0.56/mm$^2$) or CL₂MDP (0.44/mm$^2$) and fed a low calcium diet (Table 2). The $A_r$ value was significantly ($P < 0.05$) increased in cows fed the low calcium diet (2.87/mm$^2$) compared to cows fed the control calcium diet (Table 2). While the $A_r$ value was slightly higher for EHDP-treated cows fed the low calcium diet, it was not significantly different from the corresponding value in cows fed the control diet and administered EHDP. However, the $A_r$ for each of the diphosphonate-treated groups of cows was significantly lower than the mean value for the corresponding cows fed the same diet but not receiving EHDP (Table 2). The changes in $A_r$ in experimental cows were consistent with the results of the per cent resorptive surfaces as determined by micro-radiography (Table 4). There was a significant decrease in resorptive surface on microradiographs of both cortical and trabecular bone in EHDP or CL₂MDP-treated cows fed the low calcium diet (Fig. 3) when compared to cows fed only the low calcium diet (Fig. 4). Resorptive surfaces were also significantly ($P < 0.01$) decreased in cows fed the control diet and administered EHDP compared to cows fed the same diet but not receiving diphosphonate.

Terminal urinary hydroxyproline values correlated well with results of microradiographic analysis of resorptive bone surfaces
Table 4. The effect of dietary calcium and diphosphonates on serum calcium and phosphorus, urinary hydroxyproline, microradiographic evaluation of resorptive surfaces, and bone alkaline pyrophosphatase in experimental cows.
THE EFFECT OF DIETARY CA AND DIPHOSPHONATES ON SERUM CA AND P, URINARY HYDROXYPROLINE, MICRORADIOGRAPHIC RESORPTIVE SURFACES AND BONE ALKALINE PYROPHOSPHATASE IN COWS

<table>
<thead>
<tr>
<th>PARAMETER</th>
<th>CONTROL DIET</th>
<th>HIGH CA DIET</th>
<th>LOW CA DIET</th>
<th>LOW CA DIET +EHDP</th>
<th>CONTROL DIET +EHDP</th>
<th>LOW CA DIET +CL₂MDP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum CA (mg/100 ml)</td>
<td>8.63</td>
<td>9.33</td>
<td>8.24</td>
<td>4.80**</td>
<td>6.35**</td>
<td>5.07**</td>
</tr>
<tr>
<td>Serum P (mg/100 ml)</td>
<td>5.29</td>
<td>4.90</td>
<td>5.86</td>
<td>1.47**</td>
<td>2.27**</td>
<td>2.88**</td>
</tr>
<tr>
<td>Urinary HOP:CR</td>
<td>0.076</td>
<td>0.026</td>
<td>0.083</td>
<td>0.066</td>
<td>0.058</td>
<td>0.040**</td>
</tr>
<tr>
<td>Microradiographic Resorptive Surface (%)</td>
<td>4.8</td>
<td>3.5</td>
<td>9.4*</td>
<td>2.9+</td>
<td>2.6*</td>
<td>1.8+</td>
</tr>
<tr>
<td>Pyrophosphatase</td>
<td>39.9</td>
<td>-</td>
<td>42.9</td>
<td>65.0</td>
<td>48.4</td>
<td>47.7</td>
</tr>
</tbody>
</table>

nMoles PP split/30 min/mg. protein

N = 3 cows in each group
* = P<0.01 compared to control group
** = P<0.05 compared to control group
+ = P<0.01 compared to low calcium group
++ = P<0.05 compared to low calcium group
Fig. 3. Microradiograph of trabecular bone of the right tenth rib from a cow given EHDP and fed a low calcium diet. Note the smooth, radiodense trabecular bone surfaces (arrowhead). X 120
Fig. 4. Microradiograph of trabecular bone of the right tenth rib from a cow fed a low calcium diet. Note the prominent irregular surfaces, suggestive of areas of bone resorption (arrows).
There was a decreased excretion of urinary hydroxyproline in cows administered diphosphonates that was consistent with lower values for resorptive surfaces. Furthermore, the impaired bone matrix catabolism in diphosphonate-treated cows was associated with the development of profound hypocalcemia and hypophosphatemia near parturition and the initiation of lactation (Table 4). The serum calcium and phosphorus levels postpartum for the cows receiving either EHDP or CL₂MDP were significantly (P < 0.05) lower compared to values for cows fed either the low or control calcium diet.

**Effect of High or Low Calcium Prepartal Diets on Bone Formation and Resorption in Parturient Cows**

Analysis of Haversian bone remodeling in adult Jersey cows (5 to 8 years) fed a control calcium diet demonstrated that the mean osteoid seams/mm² (A_f) was 1.43, circumference of osteoid seams (S_f) was 0.09 mm, appositional rate (M) was 0.57 (μ/day), and the radial closure rate (M_f) was 0.21 (mm/year) (Table 2). Additional parameters of Haversian bone remodeling in cows are given in Table 5. The values of aged human beings at 59 years of age (Frost, 1964) the M_f, C/T, percent labeled osteoid seams, and V_f were similar to those of adult cows (Table 5). One striking difference was that in human beings there were fewer osteoid seams (A_f) that were larger in size (S_f).

The number of osteoid seams (A_f) were increased in cows fed the high calcium diet and decreased in cows fed the low calcium diet compared to control cows (Table 2). While the average osteoid seam thickness was 5.30 μ for control cows, the OST increased to 8.10 μ in cows fed the high calcium diet. Mean appositional (M) and radial closure rates
Table 5. Comparison of normal parameters of Haversian bone remodeling in human beings and experimental cows.
NORMAL PARAMETERS OF HAVERSIAN BONE REMODELING IN HUMAN BEINGS AND COW

<table>
<thead>
<tr>
<th>MEAN HISTOLOGIC INDEX</th>
<th>NORMAL HUMAN BEING (MEAN AGE 59 YR.)</th>
<th>COW (AGE 5-8 YR.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$A_f$</td>
<td>$0.39 \pm 0.04$</td>
<td>$1.43 \pm 0.90$</td>
</tr>
<tr>
<td>$S_f$</td>
<td>$0.32 \pm 0.01$</td>
<td>$0.09 \pm 0.01$</td>
</tr>
<tr>
<td>$M$</td>
<td>$0.80 \pm 0.06$</td>
<td>$0.57 \pm 0.14$</td>
</tr>
<tr>
<td>$M_f$</td>
<td>$0.28 \pm 0.02$</td>
<td>$0.21 \pm 0.05$</td>
</tr>
<tr>
<td>$\sigma_f$</td>
<td>$0.24 \pm 0.02$</td>
<td>$0.48 \pm 0.14$</td>
</tr>
<tr>
<td>$\mu_f$</td>
<td>$1.7 \pm 0.02$</td>
<td>$4.3 \pm 3.3$</td>
</tr>
<tr>
<td>$C/T$</td>
<td>$0.80 \pm 0.08^*$</td>
<td>$0.84 \pm 0.04$</td>
</tr>
<tr>
<td>Labeled Osteoid Seams (%)</td>
<td>95</td>
<td>99</td>
</tr>
<tr>
<td>$V_f$</td>
<td>$0.035 \pm 0.003$</td>
<td>$0.035 \pm 0.028$</td>
</tr>
<tr>
<td>$MWT$</td>
<td>$0.06 \pm 0.00^*$</td>
<td>$0.09 \pm 0.02$</td>
</tr>
</tbody>
</table>

* Mean indices for human beings age 10-19 yr.
(Mf) were consistently lower and osteon formation rates \((\sigma_f)\) were consistently higher in parturient cows fed either a high or low calcium prepartal diet compared to control cows (Table 3).

The number of active resorptive spaces \((A_r)\) in cortical bone \((2.87/\text{mm}^2)\) and the total resorptive surfaces of cortical and trabecular bone \((0.4\%)\) increased in parturient cows fed a low calcium prepartal diet and decreased \((0.85/\text{mm}^2\) and \(3.5\%)\) in cows fed a high calcium diet (Tables 2 and 4). The \(A_r\) value and total resorptive surfaces on micro-radiographs were \(1.06/\text{mm}^2\) and \(4.8\%\) for cows fed the control calcium diet. Urinary excretion of hydroxyproline at one day postpartum was correspondingly higher in cows fed a low calcium diet \((0.083)\) and lower in cows fed a high calcium diet prepartum \((0.026)\) (Table 4). The changes of urinary hydroxyproline in response to feeding prepartal diets with high or low calcium correlated well with values of \(A_r\) and percent of bone resorptive surfaces in experimental cows.

Discussion

The long-term prepartal administration of EHDP caused impaired bone formation and an impairment in mineralization at active bone forming sites as indicated by the large numbers of thickened osteoid seams. Large numbers of osteoid seams were not observed in control parturient cows fed either high, normal or low calcium prepartal diets. Therefore, the increased numbers of osteoid seams in the EHDP-treated cows appeared to be due to an effect of the diphosphonate and not the result of dietary calcium levels. The presence of large numbers of osteoid seams in bones of cows receiving EHDP is consistent with previous re-
ports (King et al., 1971; Jowsey et al., 1970).

Several possible causes have been proposed for the impaired mineralization by diphosphonates. In vitro studies have suggested that EHDP prevents the precipitation of hydroxyapatite crystals by coating active sites of bone mineralization (Fleisch et al., 1968). EHDP has also been shown to alter the synthesis of active vitamin D metabolites (Von Herrath et al., 1972). Either an absolute deficiency or impaired action of 1,25-dihydroxycholecalciferol in bone may have contributed to the formation of wide osteoid seams in experimental cows. A recent ultrastructural study indicated that the intraperitoneal administration of EHDP (7.5-10 mg P/kg/day) to rats caused disturbances in the formation of collagen fibrils during early dentin formation (Larsson, 1974). These findings were interpreted to suggest that an interference in precollagen synthesis is important in the pathogenesis of the interference in mineralization induced by EHDP. Other investigations have reported that EHDP (> 5.0 mg/kg/day) caused no alterations in the fine structure of osteoblasts and osteocytes of rats despite the presence of widened osteoid seams (Doty et al., 1972). EHDP caused a significant loss of lysosomal activity with an associated increase in amorphous, osmiophilic material on bone cell surfaces. Therefore, it appears that the effect of EHDP on bone formation in experimental cows was at the subcellular level and resulted in an impairment of mineralization of osteoid by one of several possible mechanisms.

An equivalent dose of Cl₂HDP given to cows fed a low calcium diet enhanced the osteon formation rate when compared to either cows receiving EHDP and fed the same diet or cows only fed the low calcium
diet. This finding is in contrast to a previous study that reported \( \text{CL}_2\text{MDP} \) decreased bone formation in rats (Gasser et al., 1972). The administration of \( \text{CL}_2\text{MDP} \) to pregnant cows caused less significant increase in number of osteoid seams compared to cows receiving EHDP and fed the same diet. This observation suggests \( \text{CL}_2\text{MDP} \) has less profound effects on mineralization of osteoid than an equivalent dose of EHDP in the cow. These findings are consistent with a previous report which demonstrated that \( \text{CL}_2\text{MDP} \) caused a diminution of osteoid formation compared to the effects of EHDP (Minkin et al., 1974). No significant increases in alkaline pyrophosphatase activity were observed in pregnant cows given either EHDP or \( \text{CL}_2\text{MDP} \). These findings are in agreement with previous in vitro observations that demonstrated diphosphonates prevent an increase in pyrophosphatase activity (Woltgens et al., 1973; Morgan et al., 1973). The P-C-P bond of the diphosphonates is known to be resistant to pyrophosphatase activity (Fleisch et al., 1968). If the diphosphonates coated active sites of mineralization, the organic matrix would be refractive to the pyrophosphatase activity of bone forming cells. Therefore, the precipitation of hydroxyapatite crystals on bone forming sites may have been prevented in the cows administered diphosphonates, as has been reported with in vitro studies (Fleisch et al., 1968).

Both diphosphonates proved to be potent inhibitors of bone resorption. At equivalent dose levels cows administered \( \text{CL}_2\text{MDP} \) had fewer resorptive surfaces than those receiving EHDP. These findings are consistent with previous reports that \( \text{CL}_2\text{MDP} \) is a more potent inhibitor of bone resorption (Gasser et al., 1972; Minkin et al., 1974).
lower values of urinary hydroxyproline excretion correlated well with
the decreased resorptive surfaces in cows following the administration
of diphosphonates. The stimulation of bone resorption by an increased
parathyroid hormone secretion in response to hypocalcemia near parturi-
tion and the initiation of lactation was prevented in parturient cows
receiving diphosphonates. Additional studies have demonstrated that
the experimental cows administered either EHDP or CL₂MDP do not have
an interference of parathyroid hormone secretion, deficient production
of cAMP or impaired intestinal calcium uptake in vitro (Yarrington
et al., 1976a,b). It appeared that the refractiveness of bone to para-
thyroid hormone-stimulated resorption is a major factor in the patho-
genesis of the severe hypocalcemia and hypophosphatemia that developed
near parturition in diphosphonate-treated cows. Although the mecha-
nisms by which bone resorption is impaired by diphosphonates are still
not completely defined, previous studies have suggested that hydroxy-
apatite crystals are resistant to dissolution in vitro after exposure
to diphosphonates (Fleisch et al., 1969). At present there is a pauci-
ity of information regarding the effects of diphosphonates on subcellular
mechanisms and fine structure of resorptive bone cells.

The results of this investigation clearly demonstrated that the
numbers of resorptive surfaces and urinary hydroxyproline excretion in-
creased markedly in cows fed a low calcium prepartal diet while these
parameters were decreased in cows fed a high calcium diet. These find-
ings help explain why cows fed low calcium prepartal diets have more
responsive calcium homeostatic mechanisms and are less likely to devel-

op severe hypocalcemia and hypophosphatemia near parturition and the
initiation of lactation than cows fed high calcium diets (Black et al., 1973).
Abstract

The prepartal administration of EHDP (4 mg/kg/day) resulted in an impairment of bone formation as reflected by an increased number of osteoid seams, greater circumference of osteoid seams, and an increased osteon formation rate in the pregnant dairy cow. The number of tetracycline-labeled osteoid seams, appositional rate, and radial closure rate were significantly decreased compared to pregnant cows fed the same diet but not receiving EHDP. By comparison, the administration of an equivalent dose of CL₂MDP to cows fed a low calcium diet enhanced bone formation. EHDP and CL₂MDP both resulted in impaired bone resorption as determined by microradiographic and quantitative measurements of Haversian bone in pregnant cows fed either a low or control calcium diet. The decreased numbers of resorption spaces in cows receiving diphosphonates was associated with decreased urinary hydroxyproline and lower values of serum calcium and phosphorus at one day postpartum. The lowest per cent of resorptive surfaces in bone and urinary hydroxyproline excretion occurred in cows given CL₂MDP and fed a low calcium diet. A major factor in the pathogenesis of the severe parturient hypocalcemia and hypophosphatemia that developed in cows administered diphosphonates was an inhibition of bone matrix catabolism and resorption on bone surfaces despite an increased secretion of parathyroid hormone in response to the challenge for calcium mobilization imposed by parturition and the initiation of lactation.

Cows fed a low calcium prepartal diet had increased numbers of resorptive spaces and urinary hydroxyproline whereas these parameters
were decreased by feeding a high calcium diet. These alterations in bone resorptive surfaces and matrix catabolism appear to have an important role in determining the protective effects exerted by low calcium prepartal diets and the provocative effects of feeding high calcium diets on the development of parturient hypocalcemia in dairy cows.

Key words: Diphosphonates - Bone - Cows - Diet
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