ENZOOTIC CALCINOSIS IN SHEEP CAUSED BY THE INGESTION OF *Nierembergia veitchii* (Solanaceae)[1]

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Descreve-se em ovinos, no Rio Grande do Sul, uma doença caracterizada por emagrecimento progressivo, hipercalcemia, atrofia das células das paratireóides, hiperplasia das células C da tireóide, osteopetrose, osteonecrose e calcificação dos tecidos moles. Ovinos de ambos sexos e todas as idades exceto lactentes são afetados. A hipercalcemia ocorre desde outubro a fevereiro, época em que os ovinos consomem a planta calcinogênica *Nierembergia veitchii*. A incidência da doença é variável entre anos e fazendas, com aparente correlação com a disponibilidade da planta nas pastagens. Experimentos em pintos raquíticos demonstraram que *N. veitchii* possui ação biológica similar à vitamina D, já que a adição da planta na dieta estimulou a absorção de cálcio, a deposição de cálcio na tíbia, e também aumentou a síntese de calbindin-D (proteína complexante do cálcio induzida pela vitamina D sintetizada no intestino) e os níveis plasmáticos de cálcio e fósforo.

Pintos foram alimentados com dietas contendo altas concentrações de $SrCl_2$ que inibe o sistema de hidrolase renal. Uma partida de *N. veitchii* secada e moída, agregada à dieta com $SrCl_2$, estimulou a síntese de pequenas quantidades de calbindin-D, mas não causou aumento na absorção de cálcio. Outra partida de planta foi totalmente inativa. Esses dados sugerem que *N. veitchii* contém um composto similar a 1,25 $(OH)_2D_3$. Os resultados variáveis entre as duas partidas poderiam ser devidas a instabilidade da substância na planta, diferenças na sua preparação ou outro fator desconhecido. As dificuldades em demonstrar em forma clara e constante a presença de uma substância de ação biológica similar a 1,25 $(OH)_2D_3$ em *N. veitchii*, poderia ser devido à administração de doses insuficientes da planta. As possibilidades de que a planta no campo contenha suficientes quantidades de um composto similar a 1,25 $(OH)_2D_3$ para causar calcinose, ou de que a calcinose seja causada por outro fator da planta requerem posterior atenção.

TERMOS DE INDEXAÇÃO: Ovinos, intoxicação por planta, *Nierembergia veitchii*, hipercalcemia, hipoparatireoidismo, hipercalcitoninismo, osteopetrose, calcificação dos tecidos moles.

ABSTRACT.- A chronic debilitating disease with hypercalcemia, parathyroid cell atrophy, C cell hyperplasia, osteopetrosis, osteonecrosis, and soft tissue calcinosis is described in sheep in southeastern Rio Grande do Sul, Brazil. Sheep of both sexes and all ages, except suckling lambs, are affected. Hypercalcemia occurs from October through February during which time sheep on pasture have access to the calcinogenic plant *Nierembergia veitchii*. The incidence of the disease varies between years and farms with apparent correlation to the availability of the plant. Experiments in rachitic chicks demonstrated a vitamin D-like activity in *N. veitchii* since the addition of the plant to the diet stimulated calcium absorption, uptake of calcium by the tibia and also increased the amount of calbindin-D (the vitamin D induced protein sinthesized by the intestinal mucosa) and the calcium and phosphorus plasma levels. Chicks were fed a diet containing a high concentration of $SrCl_2$ which inhibits the formation of $1,25(OH)_2D_3$ by the renal hydroxylase system. Adding a dried, ground preparation of one batch of *N. veitchii* to the $SrCl_2$ diet stimulated the synthesis of small quantities of intestinal calbindin-D (CaBP), but did not cause an increase in calcium absorption. Another batch of the plant was totally inactive. These data suggest that *N. veitchii* does contain a $1,25(OH)_2D_3$–like compound. The variable results between the two batches might be explained on the basis of lability of the substance in these plants, differences in preparation or other unknown reasons. The failure to demonstrate the presence of $1,25(OH)_2D_3$–like activity in *N. veitchii* in a clear and constant way may be that an insufficient amount of the plant was fed. Whether, in the field, *N. veitchii*

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contains sufficient amounts of the $1,25(OH)_2D_3$ – like substance to cause calcinosis or that calcinosis is due to other factors in the plant, requires further attention.

INDEX TERMS: Sheep, plant poisoning, *Nierembergia veitchii*, hypercalcemia, hypoparathyroidism, hypercalcitoninism, osteopetrosis, soft tissue calcinosis.

INTRODUCTION

Enzootic calcinosis in domestic animals has been reported from many parts of the world. The disease in cattle has been shown to be caused by ingestion of *Solanum malacoxylon* in Argentina (Carrillo & Worker 1967), Brazil (Döbereiner et al. 1971) and Uruguay (Riet-Correa et al. 1975). *Trisetum flavescens* causes an enzootic calcinosis in Germany (Dirksen et al. 1972) and in Austria (Libiseller et al. 1976) and *Cestrum diurnum* ingestion affects cattle (Krook et al. 1975b) and horses (Krook et al. 1975a) in Florida, USA. *Solanum torvum* has been associated with calcinosis in cattle in Papua, New Guinea (Copland 1975, Morris et al. 1979).

Similar conditions of uncertain etiology have been described in cattle in Hawaii (Lynd et al. 1965) and Jamaica (Arnold & Bras 1956), in sheep in South Africa (Tustin et al. 1973) and India (Gill et al. 1976) and in goats and sheep in Israel (Newman et al. 1973, 1977).

Cestrum diurnum has been suggested as the cause of the disease in Hawaii and Jamaica (Gimeno 1977) and *Solanum sodomaeum* has been mentioned in connection with the disease in Hawaii (Ross et al. 1971).

Excessive feeding with poultry waste has been related to an outbreak of enzootic calcinosis in sheep in Israel (Newman et al. 1977).

Enzootic calcinosis in Brazilian sheep has been recognized since 1968 as described in farms in the municipality of Julio de Castilhos, Rio Grande do Sul (Barros et al. 1970). The disease has been reproduced experimentally in rabbits (Riet-Correa et al. 1981) and sheep (Riet-Correa et al. 1980) by administration of *Nierembergia veitchii*[5].

The objectives of the present work were to determine the epidemiology and pathology of enzootic calcinosis caused by *N. veitchii* in sheep in Rio Grande do Sul, Brazil, and to study the calcinogenic properties of the plant.

MATERIAL AND METHODS

The study concerned 12 farms in the municipalities of Piratini, Pinheiro Machado and Lavras do Sul, Rio Grande do Sul. This area is a hilly country with variable amounts of bush; the soil is described as *Haplumbrept* by the US Department of Agriculture and as *Rankers* by the FAO classification (Lemos et al. 1973).

Epidemiologic data were obtained annualy by visits to each farm in which the disease was diagnosed, from 1978 to 1985.

Jugular vein blood samples from 16 grazing sheep in a paddock with a history of calcinosis were collected monthly from September 1978 to October 1979. Serum calcium was determined by the method of Ferro and Ham as modified in commercial kits (Lab Test(R)). Serum magnesium was determined colorimetrically with commercial kits (Merckotest(R)). Whole blood inorganic phosphorus was determined by the method of Fiske and Subbarow as modified by Gomori (1942).

Six sheep with clinically diagnosed disease were used for pathology examinations. They included 4 wethers, 3 of which were approximately 2 years old, one about 1 year old, and 2 ewes about 4 to 5 years old. They were terminated by exsanguination.

Soft tissues were fixed in 10% formalin, embedded in parafin, sectioned at 6 μm and stained with hematoxylin and eosin (HE), von Kossa, PAS-Alcian Blue and van Gieson stains (Luna 1968). Two millimeter sections were excised mid-sagitally from the proximal humerus and femur epiphysis-metaphysis, and transversely from the mid-diaphysis of these bones. The slices were fixed in 10% formalin and demineralized under partial vacuum in a solution of formic acid and sodium citrate (Luna 1968). Paraffin embedded sections were cut at 6 μm and stained with HE.

Thick slices of formalin fixed bones were excised as above, re-fixed in absolute alcohol for 1 week with 3 changes, embedded in methylmethacrylate and ground to 60 μm.

The ground sections were taped to Kodak High Resolution Plates (Eastman Kodak Rochester, NY, USA) and exposed in a custombuilt microradiographic unit (Westenskow Co., Salt Lake City, Utah, USA) at 10 kVp and 10 mA for 10 minutes.

Quantitative morphometry of the parathyroid glands was done by projecting medium-high magnification color slides of the glands on a paper with 50 rows of 40 dots equally spaced. Cystoplasmic and nuclear "hits" were scored for each row and the cytoplasm to nuclear ratio was recorded. The x ± s.e.m. of the 50 rows was then calculated and the data from different sheep were compared by the test.

Selected specimens of formalin fixed thyroid glands were cut into small blocks, washed in buffer, postfixed in 1.5% osmium tetroxide, dehydrated through alcohols and propylene oxide and embedded in epon. One-micron sections were stained with toluidine blue and from these, areas were selected for ultra thin sectioning. Ultra thin sections were stained with uranyl acetate and lead citrate for electron microscopy.

Four experiments were developed in order to elucidate the calcinogenic properties of *Nierembergia veitchii* (NV). The plant administrated in experiments I and II was collected during November 1978, dried in the shade and ground; NV used in experiments III and IV was collected during November 1979, deep frozen at – 30°C, ground and lyophilized.

Experiment I. One-day-old chicks were raised on Cornell rachitogenic diet for 20 days. At this time they were divided in 3 groups of 5 chiks each. Group 1 was maintained on the rachitogenic diet, in group 2 the diet was supplemented with 1200 international units (IU) of vitamin D_3, and in group 3 with 2% of NV. At 8 days, absorption of ^{47}Ca from a ligated duodenal loop, tibia uptake of ^{47}Ca and mucosal calbindin-D_{28} (CaBP) were determined by previously described methods (Wasserman et al. 1976).

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5 Description of the plant: *Nierembergia veitchii* Hook, family Solanaceae. Reference: Sacco et al. 1982. Ervas daninhas do Brasil. Embrapa, DID, Brasília, p. 30.

Material examined: Rio Grande do Sul, Pinheiro Machado, F. Riet s.n(R); Piratini: J. Sacco 2.573 et al. (R).

The plants are prostated, beardles or pilous. Their leaves are pilous, alternated or opposited, isomorphous, ovates, obovates, elliptical or oblonged, atenuated in the base, measuring 1 to 3.5 cm long and 0.4 to 1.7 cm wide. The flowers are solitaries, axilaries, white bluish or lilac, measuring until 6 cm long; the chalice is pilous, with 5 foliaceous lanceolated or obovated lacinia; the corolla is pilous-glandulous, with a largely campanulate leafe-blade and 5 rounded lobes with simple hairs wich are thickened in the apex of the median nervures; the filaments are connivant and the stigma has a semilunar shape and is not embracing the stamen.

Experiment II. Chicks were raised for 3 weeks on a commercial chick starter diet (Agway) and then divided into 3 groups, 5 chicks per group. The control group received a complete semi-purified diet, containing 1.3% Ca, 0.8% phosphorus and 1200 IU vitamin D_3 per kg, group 2 received a diet containing 0.1% Ca, 0.85% phosphorus, 2.7% strontium as $SrCl_2$ and 1200 IU vitamin D_3/kg and group 3 received a diet containing 0.1% Ca, 0.85% P, 2.7% strontium and 2% of NV. After a 5-day feeding period the same parameters were determined as in experiment I.

Experiment III. Four groups of 6 chicks each, were fed with Cornell rachitogenic diet from age 1 day to age 24 days. Then the diet was supplemented in the following way: group 1, control; group 2, 1200 IU vitamin D_3/kg; group 3, 2% dried ground *Cestrum diurnum*; and group 4, dried ground NV. After 7 days feeding, duodenal ^{47}Ca absorption and duodenal CaBP were determined as previously mentioned. Blood was drawn from the bifurcation of the aorta; plasma Ca was determined by atomic absorption, and plasma phosphorus by a modification of the procedure of Fiske and Subbarow with the use of an autoanalyzer (Technicon).

Experiment IV. One-day-old chicks were fed commercial starter ration for 24 days and then divided into 4 group of six or seven chicks per group. Group 1 received a complete semipurified diet, containing 1.2% Ca, 0.8% P and 1200 IU vitamin D_3/kg. Group 2 received a diet containing 1.2% Ca, 0.8% P, 1,4% strontium and 1.200 IU vitamin D_3/kg. Groups 3 and 4 received the same diet as group 2 supplemented with 2% *Cestrum diurnum* and 10% NV, respectively, but without vitamin D_3. After a 5 day feeding period, the same parameters were determined as in experiment III.

RESULTS

1. *Epidemiology and clinical signs*

The disease was seasonal with the first clinical cases occurring in October and the last ones in January or February.

Sheep of both sexes were affected and the disease occurred in sheep of all ages except in suckling lambs.

The frequency of calcinosis varied between farms and between years. There was also variation within farms, *i.e.*, between different paddocks within farms. Deaths never occurred in some paddocks while mortality as high as 61.5% was recorded in one camp, in farm 1, during 1977-78. Occurrence, morbidity and/or mortality rates are presented in Table 1.

Nierembergia veitchii (Hook), a plant of the *Solanaceae* family (Fig. 1 and 2), was constantly present in amounts proportional to the severity of the disease in each camp.

Affected sheep suffered a pronounced weight loss. There was extreme retraction of the ventral abdominal wall which resembled a Greyhound belly. The gait was stiff and more severe in the pelvic than in the thoracic limbs. The stiffnes was most severe when the animals tried to get up. The sheep exhibited a mild kyphosis.

Death could occur after a progressive course of 2 to 3 months or suddenly after the animals were handled during dippings, shearing or deworming.

The animals seemed to improve when changed to a paddock where the disease did not occur, or after February. Clinical signs could, however, be permanent.

Table 1. *Occurrence, morbidity and/or mortality of calcinosis in sheep from 1977 to 1986 in 12 farms* [a]

Season	Farm 1		2		3		4		5		6		7		8		9		10		11		12	
	MB[b]	MT[c]	MB	MT	MB	MT	MB	MT	MB	MT	MB	MT	MB	MT	MB	MT	MB	MT	MB	MT	MB	MT	MB	MT
77-78[d]	80	22	80	ND[e]	70	25	ND	3		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
78-79	78	16	NO[f]		1	ND	6	2	6	2	2	ND	3	1	–	–	–	–	–	–	–	–	–	–
79-80	2.5	0.5	NO		2	ND	NO		80	20	4	ND	3	1	20	6	ND	7.5	50	7.5	–	–	–	–
80-81	NO		NO		NO		NO		5	ND	NO		NO		NO		NO		NO		–	–	–	–
81-82	NO		NO		NO		NO		NO		ND	15	NO		NO		NO		NO		–	–	–	–
82-83	10	5	NO		3	1	NO		85	25	NO		2	1	25	0.5	NO		NO		–	–	–	–
83-84	NO		NO		3	0.5	NO		NO		NO		4	2	ND	2.5	NO		NO		–	–	–	–
84-85	NO		NO		NO		NO		NO		NO		NO		ND	1	NO		NO		10	2	ND	6
85-86	NO		NO		1	ND	NO		18	13	NO		1	ND	ND	10	2		NO		NO		ND	5
Nº of sheep	900		2000		250		260		270		100		1100			570	400		1000		300		400	
Paddocks affected[g]	6/6		1/12		2/5		1/5		1/1		1/3		2/6			4/4	2/6		2/3		1/2		6/6	
County	PM[h]		PM		PM		PM		PIR[i]		PIR		PIR			PIR	PIR		PM		PM		LAV[j]	

[a] In farms 1,2,3 and 4 the disease was diagnosed for the first time in 1977-78; in farms 5,6 and 7 in 1978-79; in farms 8,9 and 10 in 1979-80 and in farms 11 and 12 in 1984-85.
[b] Morbidity %.
[c] Mortality %.
[d] Spring 1977 and Summer 1978.
[e] Not determined.
[f] The disease did not occur.
[g] *Total of paddocks* Paddocks affected.
[h] Pinheiro Machado.
[i] Piratini.
[j] Lavras do Sul.

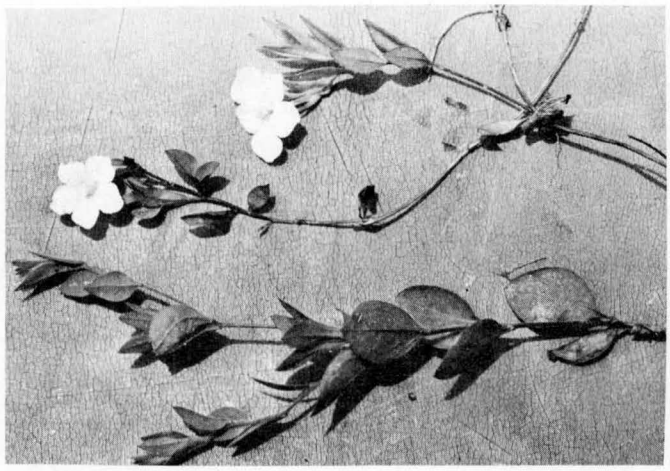


Fig. 1. Nierembergia veitchii. *Municipality of Pinheiro Machado, Rio Grande do Sul.*

Fig. 2. *Flowers of* Nierembergia veitchii *growing in the pasture. Pinheiro Machado, Rio Grande do Sul.*

2. *Clinical pathology*

Monthly means of serum calcium are presented in Fig. 3.

Serum calcium rose sharply and significantly from September and October and remained high through February. There was then a more gradual return to the September lower values.

Serum magnesium and whole blood inorganic phosphorus concentrations underwent no significant changes during the year.

3. *Anatomic pathology*

a. *Macroscopic examination.* The fat depots were depleted but there was no serous atrophy. The major arteries, with the exception of the pulmonary arteries, were hard, thick and inelastic along their entire length. The intimal surface was rough and covered by mineralized plaques of irregular shape and size (Fig. 4). The degree of mineralization was less severe in the thoracic than in the abdominal aorta in two sheep.

Three sheep had mineralization of the jugular veins at sites of previous venipuncture. All sheep showed mineralization of the bicuspid valves which were hard and thick. The aortic valves were also thick with loss of elasticity and appeared as rigid plaques. In one case endocardial mineralization was observed in the left ventricular wall.

In the lungs of five sheep, and mainly around the acute margin of the diaphragmatic lobes, there were areas where the tissue was inelastic, with a pumice stone-like appearance. The surface and cut surface of affected areas were grey and dry. The remaining lung showed a moderate emphysema.

The kidneys of all sheep showed focal, whitish areas of mineralization in the cortex and homogenous, whitish streaks in the medulla.

Many tendons and ligaments were calcified and the tendinous part of the diaphragm showed opaque and rough plaques of mineralization.

The thyroid glands of two sheep showed white spots of varying size on the cut surface.

The wall of the uterus of two ewes was markedly thickened, hard and inelastic because of severe mineralization.

The articular cartilage of many joints was eroded and showed white, granular areas. The coxo-femoral and carpometacarpal joints were most commonly affected.

The liver of one sheep showed pronounced chronic passive hyperemia and in 2 sheep there were moderate hydrothorax and ascites.

b. *Microscopic examination of soft tissues.* In the intima and media of the aorta, the elastic fibers were swollen, eosinophilic and fragmented. These degenerated fibers showed an increase in acid and neutral mucopolysaccharides. Such fibers were the site of mineralization, first as fine granular deposits which increased in amounts; subsequently the fibers could not be visualized due to large plaques that were structureless and completely mineralized. The collagen fibers and the ground substance were also involved in the same processes.

The degenerate fibers underwent chondroid metaplasia in rare instances.

The mineralized areas were usually surrounded by granulation tissue with fibroplasia and infiltration of lymphocytes, plasma cells and multinucleated giant cells, the latter sometimes in large numbers. In this fibrous tissue, chondroid and osseous metaplasia occurred and the osseous plaques were ocasionally of impressive size (Fig. 5).

Other elastic and muscular arteries exhibited lesions similar to those of the aorta. The internal elastic membrane was

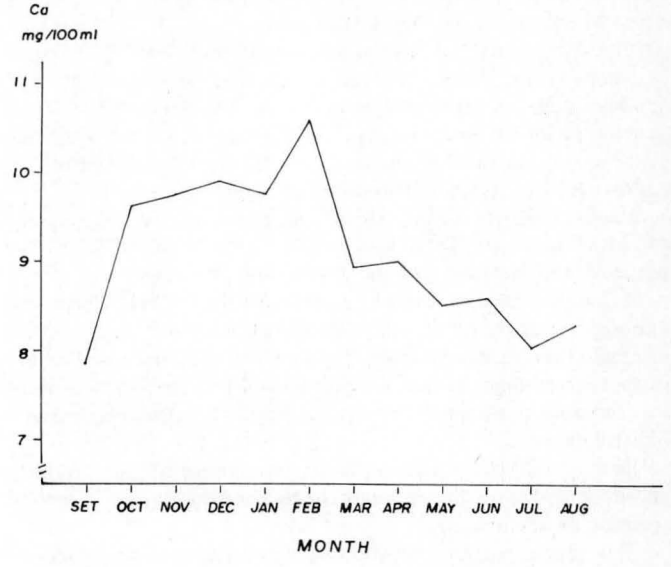
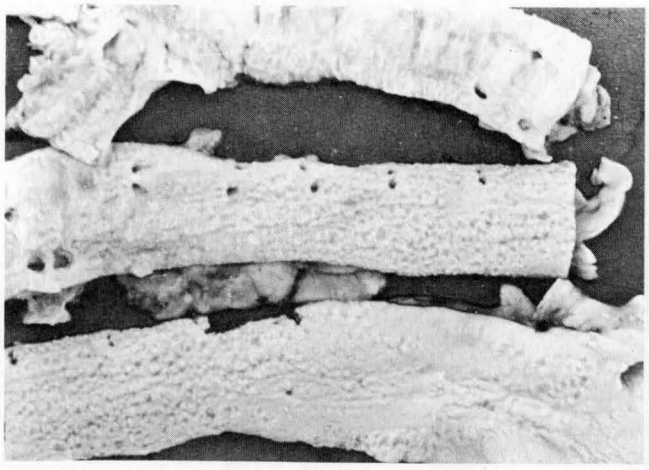


Fig. 3. *Monthly means of serum calcium from September 1978 to October 1979 of 16 sheep from an affected farm.*



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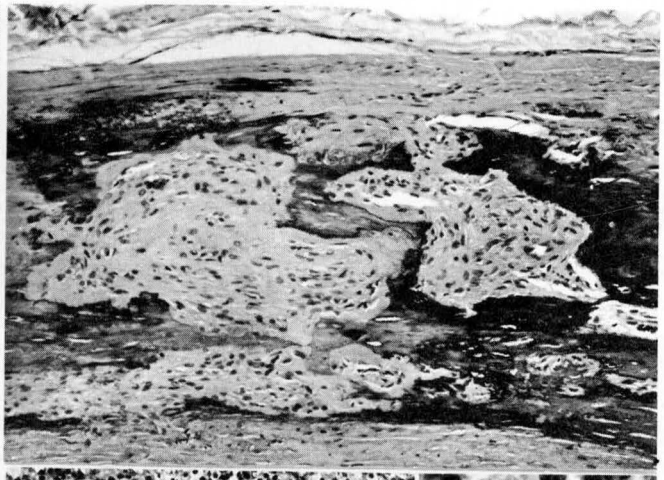

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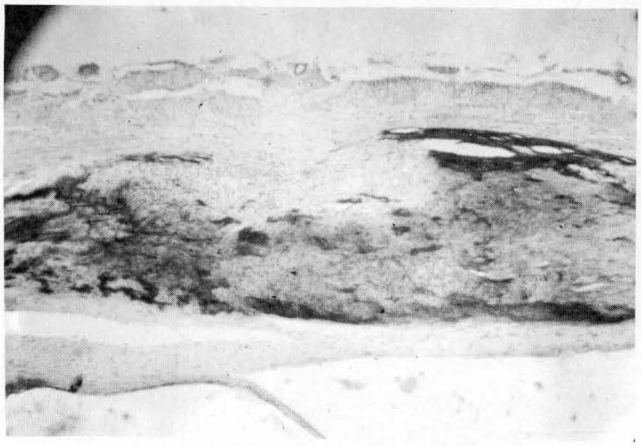

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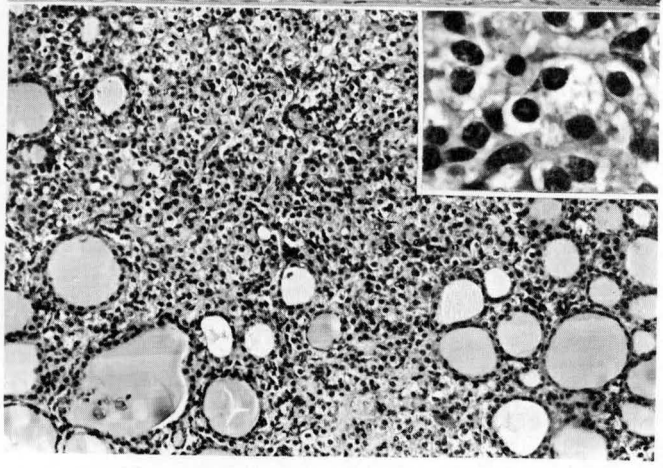

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Fig. 4. *Aorta from an affected sheep with severe mineralization of the intimal surface.*

Fig. 5. *Aorta of an affected sheep with osseous metaplasia into a large calcified plaque. HE, obj. 10.*

Fig. 6. *Tendon from an affected sheep showing severe calcification. HE, obj. 4.*

Fig. 7. *Thyroid from an affected sheep with C cell hyperplasia. HE, obj. 10. Insert higher power of C cells.*

degenerated and calcified. Some muscle fibers showed the same changes. The arterial lesions were observed in severe degree in the uterus and to a variable degree in skeletal muscle, forestomach, abomasum, intestine, thyroid gland, lymph nodes and in the spleen.

The heart of one sheep showed degeneration, necrosis and calcification of muscle fibers and replacement by fibrous tissue.

The changes in tendons and ligaments were comparable to those in the arteries. Both elastic and collagen fibers were involved (Fig. 6).

Interlobular septae of the lungs were severely swollen, degenerated and calcified. In some areas, the alveolar epithelium was of the cuboidal type. Bronchial and tracheal cartilage showed restricted areas of dystrophic calcification.

The kidneys showed severe calcinosis of the arteries. There was fibrous thickening of Bowman's capsule. The interstitium was increased and sometimes the site of dystrophic calcinosis. The basal membrane with the tubular epithelium, mainly of collecting tubules, was degenerated. Calcification also involved the epithelium which was sometimes desquamated; calcified casts sometimes obliterated the tubular lumen.

The parathyroid glands examined were from three sheep; one terminated in early January, one at the end of February and one in May. The first parathyroid glands were collected at a time of abundant supply of *N. veitchii*, the second when the amount of the plant in the pastures had decreased, and the third set was collected after 2-3 months without access to the plant.

In the May parathyroid glands, the cells were large with extensive cytoplasm. The cytoplasm to nucleus ratio was 2.87 ± 0.18 ($\bar{x}$ ± s.e.m.). In the January parathyroids, there was a severe reduction in the amount of cytoplasm and the nuclei were, accordingly, spaced much more closely together. The cytoplasm to nucleus ratio was 1.71 ± 0.10. The difference between the May and January ratios was significant at $P<0,001$.

The parathyroid glands collected at the end of February had a cytoplasm to nucleus ratio of 2.39 ± 0.15. The mean was approaching that of the May parathyroid gland but was still significantly lower ($P<0.05$).

The seasonal changes in the cytoplasm to nucleus ratio of the parathyroid chief cells were caused by changes in the amount of cytoplasm. The mean diameter of the January and February chief cells was not significantly different from the May value.

Five sheep showed pronounced C cell hyperplasia (Fig. 7). In 3 animals hyperplastic C cells were observed mainly in multiple layers around the follicles or distributed between them. In the 2 sheep with macroscopic white spots in the thyroids, such lesions were constituted by hyperpalstic C cells, sometimes arranged in

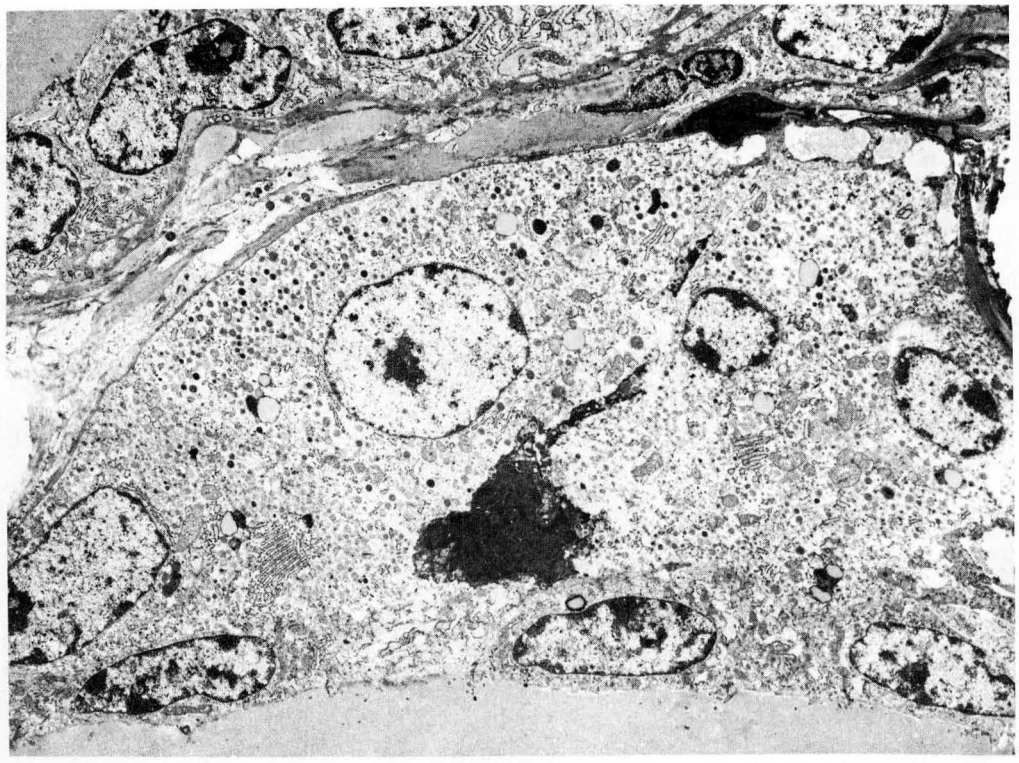


Fig. 8. *Electron micrograph of the thyroid from an affected sheep. Hypertrophic C cells are present in the wall of the follicle but do not contact the follicular lumen. Some C cells have numerous secretory granules, others few. x 6250.*

Table 2. *The effect o* Nierembergia veitchii *on duodenal ^{47}Ca absorption, tibia uptake of ^{47}Ca and mucosal CaBP in chicks* (Experiment I)[a]

Group	Addition to rachitogenic diet	Absorption of ^{47}Ca from duodenal loop (% dose)[b]	Tibia uptake of ^{47}Ca (% dose)	Mucosal CaBP (μ g/mg protein)
1	–	28.9±5.6	1.6±0.24	1.3±0.3
2	Vitamin D_3	90.4±1.6	3.39±0.07	54.8±2.1
3	NV (2%)	57.9±7.8	2.24±0.30	6.7±0.2

[a] Values represented mean±SEM.
[b] Absorption period = 20 min.

nodules, measuring 0,1 to 0,3 mm wide and separated by fibrous tissue.

c. *Ultrastructural alterations in the thyroid.* C cells were observed distending the parafollicular space and also within the basement membrane of the follicle but not in contact with the follicular lumen (Fig. 8). Some C cells had numerous granules whereas others contained very few. Some C cells were hypertrophic and others had degenerated. Stacks of rough endoplastic reticulum were present in many C cells. The follicular epithelium was flattened and atrophic and contained no secretory granules.

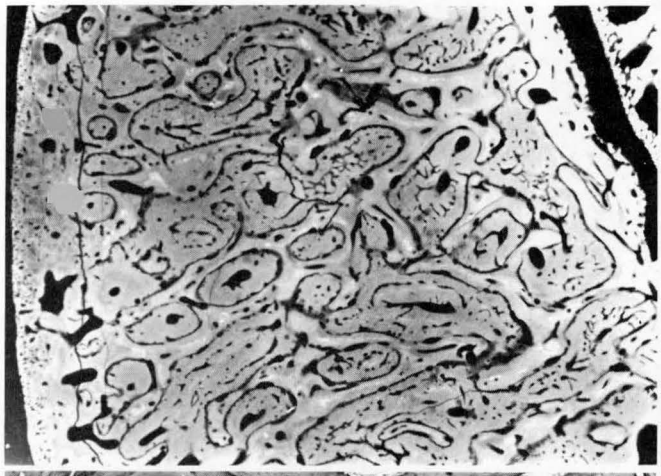
d. *Microscopic and microradiographic examination of bone.* All animals had severe osteopetrosis (Fig. 9, 10 and 11), characterized by enlargment and confluence of trabeculae in the epiphysis and metaphysis. The epiphysis showed a mixture of osteonic and trabecular bone. Transverse bands of bone, parallel to the epiphyseal plate were observed in the metaphysis (Fig. 12). In diaphyseal bone the cortex was very thick, with small Haversian canals and poor differentiation between primary and secondary osteons.

Another striking alteration was the presence of wide basophilic lines within, or at the surfaces of bones (Fig. 10 and 11). Such lines were more common in trabecular bone, but were also observed parallel to the periosteum or concentrically located around Haversian canals. These were characterized by a fibrillar material with lack of birefringency and in some cases with trapped osteocytes and osteocytic lacunae. In microradiographs, these lines appeared as black lines of low mineral density, causing a mosaic pattern (Fig. 9). Such structures were different from cementing lines which were also observed (Fig. 12).

Osteocytes in the center of the wide trabecular and in interstitial lamellae were frequently dead with empty lacunae; this osteonecrosis sometimes resulted in desintegration of bone tissue (Fig. 12).

Articular cartilage showed poor differentation into vesicular cartilage, with a dense layer of compact bone attached directly to the cartilage cells. Near half of the epiphyseal plate was resting cartilage, and differentiation into columnar and vesicular cartilage was very poor. There was very little primary spongiosa and, in some occassions, a layer of bone, forming the distal terminal plate, was attached directly to the cartilage. Dense transverse trabeculae were frequently observed in secondary spongiosa (Fig. 12).

Retention of chondroid core was also observed in epiphyseal and metaphyseal trabeculae (Fig. 12). Occasionally large areas of cartilage were trapped in bone (Fig. 10). In metaphysis and on some ocassions in epiphysis, the presence of such cartilage was due to downgrowth of articular and epiphyseal cartilage, but in



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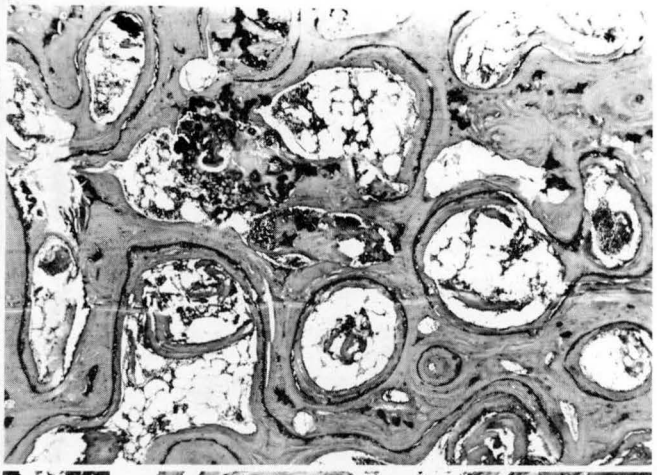

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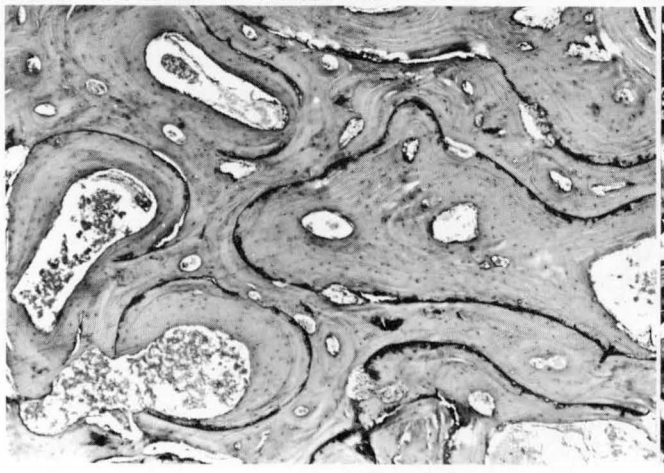

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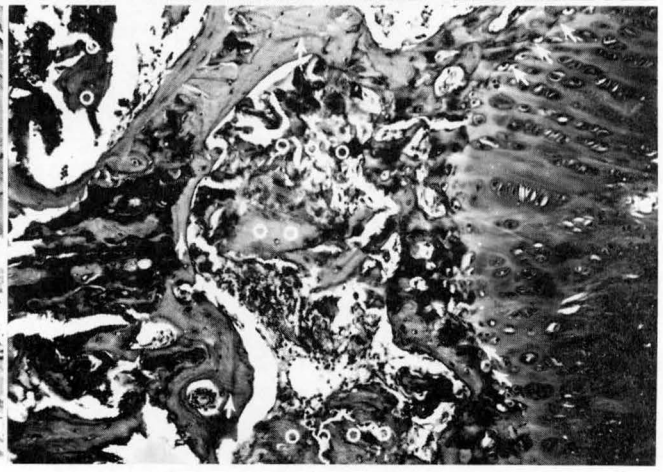

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Fig. 9. *Microradiograph of the proximal humerus epiphysis of an affected sheep. Very severe osteopetrosis. Numerous black lines of low mineral density in the periphery of osteons and in trabeculae cause a mosaic pattern.*

Fig. 10. *Proximal humerus epiphysis of an affected sheep showing osteopetrosis, basophilic lines in the osteons and trabeculae, a piece of cartilage trapped in bone (C) and osteonecrosis in some areas. HE, obj. 4.*

Fig. 11. *Basophilic lines in the proximal humerus epiphysis of an affected sheep. HE, obj. 10.*

Fig. 12. *Proximal epiphyseal cartilage and metaphysis of an affected sheep. Epiphyseal cartilage at top showing very poor differentiation of cartilage cells with no vesicular cartilage cells. Distal terminal plate is formed (in front of oblique arrows). Necrotic bone indicated by open circles. In the transverse trabeculae in the lower part are large amounts of chondroid core and cementing lines in front of horizontal arrows. HE, obj. 10.*

the epiphysis there was also cartilage trapped in bone near or in connection with the epiphyseal plate, indicating that it originated from resting cartilage of the epiphyseal plate.

4. *Calcinosis in cattle*

On farm 10 the disease was observed in cattle during February 1980. In a herd of 300 cows, 10 showed a considerable loss of weight. Affected animals were removed to another farm without *N. veitchii*. After transportation, one cow which had its limb accidentally broken was slaughtered; severe mineralization of the aorta was found. The mean serum calcium of the 10 affected animals in February was 9,7 mg/100 ml; in May, 3 months after removal, the mean serum calcium of the same animals was of 8,3 mg/100 ml, significantly lower (P 0.05) than the previous determination. Mean serum calcium of 10 clinically unaffected cows which stayed on the farm were 9,55 mg/100 ml in February and 9,0 mg/100 ml in April.

5. *Effect of* N. veitchii *on calcium absorption and CaBP synthesis in chicks*

As shown in Table 2, the addition of vitamin D_3 to the diet of rachitic chicks resulted in an increased absorption of the test dose of ^{47}Ca, which was also reflected in the greater uptake of ^{47}Ca by the tibia in the vitamin D_3–supplemented chicks. The addition of *N. veitchii* to the diet similary increased ^{47}Ca absorption, tibia uptake of the absorbed ^{47}Ca and stimulated the synthesis of CaBP, as did vitamin D_3 supplementation. These results clearly indicated that *N. veitchii* contains a vitamin D-like substance since for the induction of CaBP synthesis there is an absolute requirement for vitamin D through its metabolites.

It was previously shown that the addition of high concentrations of $SrCl_2$ to low calcium diets produced a physiological vitamin D deficiency even in the presence of dietary vitamin D_3. This treatment inhibits the synthesis of the vitamin D hormone, 1,25-dihydroxycholecalciferol, by the renal

Table 3. *The effect of* Nierembergia veitchii *on duodenal* ^{47}Ca *tibia uptake of* ^{47}Ca *and mucosal CaBP in Strontium chicks* (Experiment II)[a]

Group	Sr diet	Addition to diet	Absorption of ^{47}Ca from duodenal loop (% dose) [b]	Tibia uptake of ^{47}Ca (%dose)	Mucosal CaBP (μ g/mg protein)
1	–	Vit. D_3	65.4±3.9	2.08±0.15	10.2±0.7
2	+	Vit. D_3	40.0±2.0	1.22±0.06	(0.18)[c]
3	+	NV (2%)	40.4±2.2	1.20±0.08	1.83±0.2

[a] Values represented mean±SEM.
[b] Absorption period = 20 min.
[c] All CaBP in this group were "zero" except one value of 1.08 μ g/mg protein.

enzyme system and thereby depresses calcium absorption and inhibits CaBP synthesis. As shown in Table 3, the addition of $SrCl_2$ to the diet significantly decreased ^{47}Ca absorption, the uptake of absorbed ^{47}Ca by the tibia and inhibited CaBP synthesis by the enterocytes. The supplementation of the diet with 2% *N. veitchii* did not enhance ^{47}Ca absorption and therefore there was no increase in tibia ^{47}Ca uptake. However, there was a small but significant stimulation of CaBP synthesis by the plant. This observation strongly suggests that *N. veitchii* does contain a 1,25-dihydroxycholecalciferol-like substance. However, the amount of the putative vitamin D-like hormone in the plant was apparently insufficient to elicit a change in ^{47}Ca absorption.

In experiments with another batch of *N. veitchii*, the stimulation of the ^{47}Ca absorption process and CaBP synthesis in vitamin D-deficient chicks was demonstrated (Table 4). In the same experiment, it was also shown that another calcinogenic plant, *Cestrum diurnum* was also effective. When this batch of *N. veitchii* was fed to strontium-fed chicks, no effect on ^{47}Ca absorption or CaBP synthesis was observed whereas *C. diurnum* increased ^{47}Ca absorption and CaBP synthesis, demonstrating the presence of the $1,25(OH)_2D_3$–like substance contained there in (Table 5).

The differences in the response of the strontium-fed chicks to the two batches in terms of inducing the synthesis of CaBP cannot be readily explained but could be due to differences in the methods of preparation of the plant material or year-to-year differences (cf. Material and Methods section) in the content of the $1,25(OH)_2D_3$-like substance in theses plants. In any event, the data on CaBP synthesis by strontium-fed chicks (Table 3) does suggest that *N. veitchii*, under certain growth and/or preparatory conditions, does contain a small quantity of a $1,25(OH)_2D_3$-like substance.

DISCUSSION

The clinical and pathological characteristics of the disease was similar to those described for other calcinosis in sheep, goats, cattle and horses as mentioned in the Introduction. The experimental reproduction of the intoxication in sheep (Riet-Correa et al. 1980) and rabbits (Riet-Correa et al. 1981), as well as the demonstration of vitamin D like activity in *Nierembergia veitchii* (NV), confirmed this plant as the causative agent of the disease.

The intoxication was characterized by seasonal hypercalcemia. In the experiment during 1979-80, serum calcium increased in October, remained high until February and decreased from March. The first clinical cases coincided with the increase in serum calcium and new cases were observed through February . The seasonal occurrence of the disease coincided with the biologic cycle o NV. At the end of August, small amounts of the plant were observed on pastures, the amounts were considerable by October, persisted at high levels during spring and disappeared in March.

In the following years morbidity and mortality were very variable depending always on the amount of plant present in pastures, which is probably related to climatic variations.

In many farms, there was a decrease in the incidence of the disease after its first diagnosis. This was probably due to the management measures which consisted in the withdrawal of sheep from the camps with large quantities of NV from November to February.

The intoxication occurred almost exclusively in sheep, but cattle could be also be affected, as it was observed in farm 10 in February 1980. The difference in morbidity between sheep and cattle is apparently based in the different pasture habits. NV is a

Table 4. *Effect of* Nierembergia veitchii *on duodenal* ^{47}Ca *absorption, CaBP synthesis, and plasma Ca and P levels in rachitic chicks* (Experiment III)[a]

Group	Addition to rachitogenic diet	Duodenal ^{47}Ca absortion (%)[b]	Duodenal CaBP (μ g/mg protein)	Plasma Ca(mg/100 ml)	Plasma P(mg/100 ml)
1	–	28.8±4.9	0	6.6±0.2	3.6±0.2
2	Vitamin D_3	92.4±1.6	48.3±4.5	11.0±0.2	4.7±0.2
3	*Cd* (2%)	86.5±1.5	52.4±2.3	10.2±0.2	4.8±0.2
4	*Nv* (10%)	92.2±1.0	52.7±2.4	10.8±0.3	5.4±0.2

[a] Data given as mean±SEM.
[b] Absorption period = 20 min.

Table 5. *Effect of* Nierembergia veitchii *on duodenal ^{47}Ca absorption, CaBP synthesis, and plasma Ca and P levels in Strontium-fed chicks* (Experiment IV)[a]

Group	Sr	Addition to diet	Duodenal ^{47}Ca absorption[b](%)	Duodenal CaBP (μ g/mg protein)	Plasma Ca (mg/100 ml)	Plasma P (mg/100 ml)
1	–	Vit. D_3	74.9±2.9	11.3±1.0	11.5±0.2	5.4±0.2
2	+	Vit. D_3	38.6±4.2	0.4±0.3	9.8±0.1	5.0 0.2
3	+	Cd (2%)	53.0±4.4	8.5±1.0	9.8±0.1	5.3±0.3
4	+	Nv (10%)	42.1±2.5	0.6±0.3	9.8±0.2	4.8±0.1

[a] Data given as mean±SEM.
[b] Absorption period = 20 min.

plant that creeps between the pasture grasses, and is thus more likely to be consumed by sheep than cattle. In addition, large doses of NV are necessary to cause intoxication in experimental animals (Riet-Correa et al. 1980), which means that cattle probably do not eat enough of the plant to become intoxicated.

The economical impact of the intoxication to the sheep industry of Rio Grande do Sul is indicated by the geographic distribution of the disease, observed in 3 of the 13 counties which is the area of influence of the Pelotas Regional Diagnostic Laboratory. Besides, the intoxication occurs also in at least another county in the central area of the State (Barros et al. 1970).

The soft tissue changes observed in the present material were closely similar to those described in hypervitaminosis D, both from ingestion of excessive amounts of cholecalciferol (Hass et al. 1958, Capen et al. 1966, Chineme et al. 1976, Simesen et al. 1978) and from intoxication by calcinogenic plants (Carrillo & Worker 1967, Dirksen et al. 1971, Krook et al. 1975a,b, Morris et al. 1979). Two factors seem to be responsible for the soft tissue calcification: 1) the direct toxic effect of the active principle on elastic tissues as it was mentioned in the intoxication by *Solanum malacoxylon* (Barros et al. 1981), *Cestrum diurnum* (Krook et al. 1975a, Simpson & Bruss 1979) and vitamin D (Haschek et al. 1978); and 2) the hypercalcemia as it was mentioned by Wasserman (1978).

In bone tissue, osteopetrosis was constantly observed. A similar lesion was described in intoxication by vitamin D (Storey 1960, Capen et al. 1966, Chineme et al. 1976) and calcinogenic plants (Carrillo 1973, Dirksen et al. 1972, Krook et al. 1975 a,b; Dämmrich et al. 1975). Hypercalcitoninism, induced by the hypercalcemia, and evidenciated by the presence of hyperplasic C cells, is obviously causing osteopetrosis through the inhibition of osteoclastic and osteocytic resorption. Calcitonin is a potent inhibitor of osteoclastic resorption and increases mineral deposition in perilacunar bone (Jubb et al. 1985).

Hypoparathyroidism indicated by the histological picture of the parathyroid glands should contribute to the inhibited bone resorption. This hypoparathyroidism is commonly interpreted as a consequence of hypercalcemia. On the other hand, it has been shown in short term experiments with very high dietary cholecalciferol in young pigs that parathyroid atrophy precedes hypercalcemia (Haschek et al. 1978).

The direct effect of the active principle of the plant on bone cells could be also responsible for the osteopetrosis. A clear evidence of this effect on osteocytes is the presence of osteonecrosis, an alteration decribed also in the intoxication by calcinogenic plants (Santos et al. 1976, Krook et al. 1975a,b) and vitamin D (Chineme et al. 1976).

The presence of wide basophilic lines in the skeleton, similar to the observed in the present material, has been described in the intoxication by *Solanum malacoxylon* (Carrillo 1973, Dämmrich et al. 1975) and vitamin D (Hass et al. 1958, Storey 1960, Stevenson et al. 1976, Simesen et al. 1978), and are probably related to the production o abnormal osteoid tissue by osteoblasts (Hass et al. 1958, Storey 1960). The formation of more than one layer of such basophilic lines probably represents the periodical remissions of the disease.

The lack of differentiation of articular and epiphyseal cartilages, and the presence of transversal trabeculation occurred as a consequence of the effect of NV toxic principle in the cartilage. Similar lesions were described in the intoxication by calcinogenic plants (Santos et al. 1976, Kasali et al. 1976) and vitamin D (Storey 1960, Haschek et al. 1978).

Another evidence of the interference of the intoxication in endochondral ossification was the presence of cartilage trapped in epiphyseal and metaphyseal bone.

The calcinogenic mechanism of some calcinogenic plants has been determined. The toxic principle of *Solanum malacoxylon* and *Cestrum diurnum* is a glycosidic steroid that, after hydrolysis, acts biologically and chromatographically like authentic 1,25 $(OH)_2D_3$. Mass spectrometry by direct probe analysis and gas chromatography mass spectometry showed that the spectral pattern of these steroids is very similar to the authentic 1,25 $(OH)_2D_3$ (Wasserman 1978). In *Trisetum flavescens*, a 1,25 $(OH)_2D_3$ like activity has been also detected (Rambeck & Zucker 1982).

Cholecalciferol is first hydroxylated in the liver to form 25 $(OH)D_3$ and further hydroxylation occurs in the kidney which is controlled by a feed back system to form $1,25(OH)_2D_3$. Hypercalcemia and low plasma parathormone decrease the formation of $1,25(OH)_2D_3$. When the $1,25(OH)_2D_3$ is ingested with calcinogenic plants, the feed back system is by passed, as a consequence there is an excessive synthesis of calbidin-D (CaBP) which increases calcium absorption causing an uncontrolled hypercalcemia (Wasserman 1978).

In the present work, the results obtained in experiments I and III demonstrated that NV does act as vitamin D since the addition of the plant to the rachitogenic diet stimulated calcium (^{47}Ca) absorption, uptake of ^{47}Ca by the tibia, and also increased the amount of CaBP synthesized by the duodenal mucosa, and the calcium an phosphorus plasma levels.

In the strontium experiments, contradictory results were obtained. In experiment II the results showed that the presence of the plant in the diet did not elevate calcium absorption or the uptake of ^{47}Ca by the tibia. However, there was an increase of CaBP synthesized by the duodenal mucosa of the chicks fed the diet containing NV. These results can be interpreted as indicating

that NV does contain 1,25 $(OH)_2D_3$-like activity, although not in sufficient amount to stimulate the intestinal absorption of calcium. In contrast, in experiment IV the response of the chicks to a diet containing 10% NV, compared with a diet with 2% *Cestrum diurnum*, showed that NV was unable to overcome the inhibitory effect of the strontium diet, whereas the *Cestrum* plant was able to do so.

At this point, it can be concluded that NV does contain vitamin D-like activity; in one batch of NV the presence of a small quantity of 1,25 $(OH)_2D_3$ – like substance was demonstrated. The failure to demonstrate its presence in a clear and constant way in the experiments in chicks may be that an insufficient amount of NV was fed.

It has been shown that large doses of NV (116 to 338 g per kg of body weight) are necessary to induce mild to severe calcinosis in sheep (Riet-Correa et al. 1980). *Trisetum flavescens* is another calcinogenic plant which needs to be ingested in large quantities in order to induce calcinosis (Simpson & Bruss 1979), and it was difficult to demonstrate 1,25 $(OH)_2D_3$ activity in chicks when it was added at 10% in the diet (Wasserman et al. 1977). However, Rambeck an Zucker (1982) were able to demonstrate 1,25 $(OH)_2D_3$-like substance in an ether extract of the plant. Köhler et al. (1978) and Peterlik et al. (1977) also showed the presence of a 1,25 $(OH)_2D_3$-like substance in *T. flavescens*. Other possibilities are that the active principle in NV is considerably more labile than the 1,25 $(OH)_2D_3$-like substance in the *Cestrum* plant or that there is another mechanism involved in the production of calcinosis by NV.

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