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# Pathologic changes, tissue distribution, and extent of conversion to ethylenethiourea after subacute administration of zinc ethylene-bis-dithiocarbamate (zineb) to calves with immature rumen function

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## SUMMARY

The toxicity of zinc ethylene-bis-dithiocarbamate (zineb), a widely used fungicide, was studied in four 4-week-old Friesian calves with immature rumen function. Calves were first subjected to liver biopsy, and thereafter, 3 of them were orally administered 200 mg of zineb/kg of body weight daily for 80 days, whereas the fourth calf served as control and remained untreated. Clinical, hematologic, and pathologic (including ultrastructural) findings were recorded. The distribution in body fluids and tissues of the parent compound and one of its main metabolites, ethylenethiourea (ETU), also was examined. Treated calves had unthrifty appearance and reduction in weight gain. They also had remarkable impairment of thyroid function, as reflected by reduction in serum concentrations of triiodothyronine and thyroxine and increase in weight of the thyroid gland associated with epithelial vacuolization and foci of hyperplasia. Moderate increase in liver glycogen content and impairment in maturation of germ cells were recorded consistently. Whereas zineb was widely distributed in body tissues, ETU accumulated mainly in the liver and the thyroid gland, although noticeable concentrations also were attained in muscle. Data were consistent with involvement of ETU mainly in the pathogenesis of thyroid gland lesions, and indicate that unweaned calves given zineb develop a clinicopathologic syndrome that does not differ qualitatively from that already described in adult cattle exposed to zineb.

Since its discovery, zinc ethyylene-bis-dithiocarbamate (zineb) has gained considerable popularity as a fungicide in agricultural practice. Zineb and other ethylene-bis-dithiocarbamates (EBDC), however, cause a host of undesirable effects in mammals and birds; they can decrease thyroid function, fertility, and drug metabolism.[1-3] Moreover, ethylenethiourea (ETU), a chemical and metabolic breakdown product of several EBDC, induces teratogenic, mutagenic, and tumorigenic effects in laboratory animals.[4-6] These findings have resulted in severe restrictions on the use of EBDC fungicides in several countries.[7] Despite this, they are still extensively used in Italy; as much as 18,000 tons were used in 1983.[a]

Effects of the subchronic administration of zineb were previously investigated in Friesian cattle.[8] The objective of the study reported here was to assess whether the young age and the related lack of completely developed ruminal function could influence zineb distribution and toxicity in Friesian calves. Because the authors were unaware of any evidence on the metabolic conversion of zineb to ETU in cattle, concentrations of this metabolite were also determined.

## Materials and Methods

*Calves and facilities*—Four 4-week-old healthy Friesian calves (A, B, C, and D) were weighed, housed in individual manure-pack pens, and maintained under identical stable conditions. They were fed a commercially available milk replacer as the sole ration throughout the study. Calves were allowed ad libitum access to tap water.

*Liver biopsy*—One week before the study began, calves were subjected to liver biopsy as described[9] to provide pretreatment samples for morphologic examination.

*Dosing regimen*—Zineb of technical grade (92% purity) was supplied.[b] Throughout the study, the ETU content of this preparation was periodically determined and did not exceed 0.25%. The fungicide was suspended in milk and given to calves A, B, and C by the oral route at daily dosage of 200 mg/kg of body weight for 80 days. Calf D was fed nontreated milk and was regarded as a control. Calves were weighed every 8 days and the dose of the administered fungicide was modified accordingly.

*Blood sample collection and analysis*—Blood samples were obtained by jugular venipuncture the day before

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[a] Annuario di statistica agraria, Istituto Centrale di Statistica, Roma, Italy, 1985;544.
[b] Farmoplant, Milano, Italy.

treatment (day 0) and every 20 days thereafter during the study. The anticoagulant EDTA was used for hematologic examination, which was performed the day after collection of blood samples. Hematologic evaluation consisted of RBC count, total and differential WBC counts, and determination of PCV and hemoglobin concentration performed in routine manner. Serum triiodothyronine ($T_3$) and thyroxine ($T_4$) concentrations were measured by use of a radioimmunoassay, as described.[8]

*Necropsy and morphologic examination*—After 80 days from the start of the study, all calves were deprived of food overnight. On the subsequent morning, they were euthanatized. Complete necropsy was performed on each calf and the wet weight of the thyroid gland, testes, and liver was recorded. Samples of brain, lungs, thymus gland, heart, small intestine, cecum, colon, spleen, liver, testes, thyroid gland, adrenal glands, pancreas, mesenteric lymph nodes, and skeletal muscle (quadriceps femoris) were fixed in neutral buffered 10% formalin. Sections were prepared in routine manner for histologic examination, then were stained with H&E. Small portions of the thyroid gland, liver, and kidneys, as well as liver biopsy specimens were fixed in 2.5% glutaraldehyde and processed for ultrastructural examination as described.[8]

*Analysis of zineb and ETU*—Immediately after death, specimens of tissues (thyroid gland, pancreas, quadriceps femoris muscle, kidneys, liver, testes) and aliquots of blood, urine, bile, and feces were collected, weighed, and stored frozen at −20 C.

Zineb determination was carried out by the method of Cullen[10] as modified by Keppel.[11] These techniques are based on hot acid decomposition of tissues and subsequent titration of the evolved carbon disulfide. The sensitivity of the assay, using a standard solution, was 1 μg of $CS_2$/ml.

Determination of ETU was performed, using a modification of a high-performance liquid chromatography method as described.[12,13] Briefly, this procedure involved extraction, cleanup on an alumina column, and vacuum evaporation to dryness. Residues were dissolved in the mobile phase (methanol-water, 1:9) and injected into a liquid chromatograph[c] equipped with a C8-bonded silica column.[d] The UV detector was set at 240 nm. The detection limit of ETU, using a standard solution, was 0.05 μg/ml.

## Results

*Clinical and hematologic observations*—The zineb suspension appeared to be palatable, so force-feeding during the trial was not necessary. Zineb administration did not significantly affect food intake (data not shown). Treated calves did not appear to thrive and, at the end of the treatment period, total weight gain was greatly reduced, compared with that of the control calf (Fig 1). At the same time, treated calves also had modest decrease in hemoglobin concentration and hematocrit, with respect to pretreatment values; RBC and total and differential WBC counts were not appreciably changed (data not shown).

[c] SP 8100, Spectra Physics, San Jose, Calif.
[d] Chromsep 028430 packed with Lichrosorb RP8, Chrompack, Middelburg, The Netherlands.

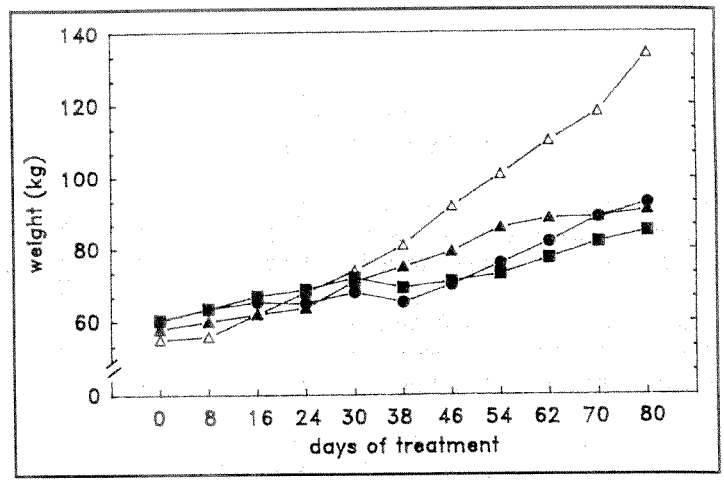


Figure 1—Changes in body weight of calves administered 200 mg of zinc ethylene-bis-dithiocarbamate (zineb)/kg of body weight daily during a 80-day feeding trial (▲, ●, ■ = treated calves; △ = control calf).

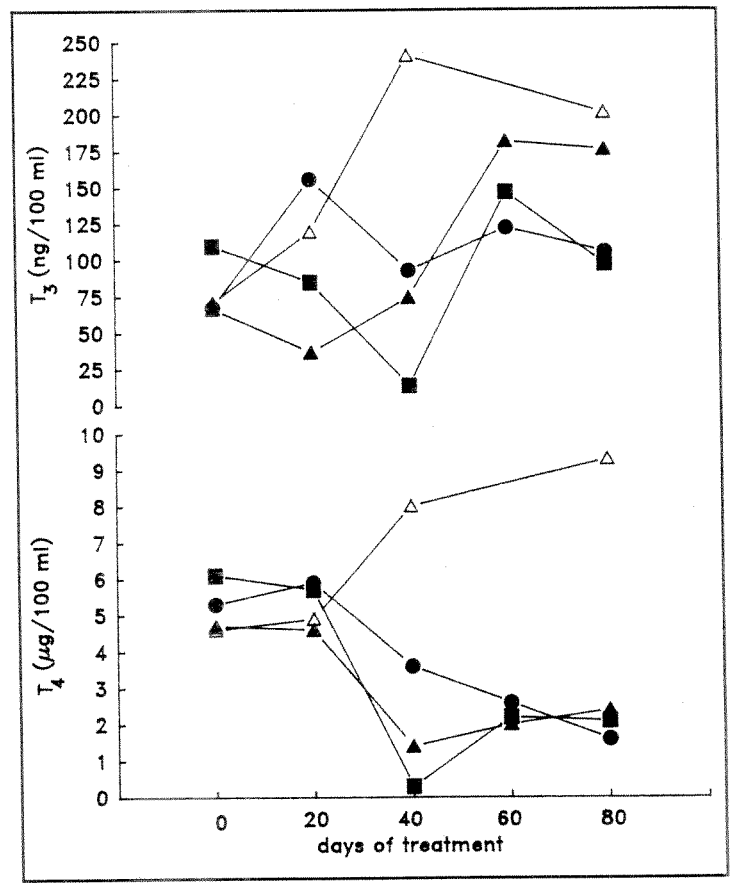


Figure 2—Effects of zineb on serum $T_3$ and $T_4$ concentrations in calves (▲, ●, ■ = treated calves; △ = control calf).

*Effects on circulating thyroid hormone concentrations*—Although serum $T_3$ and $T_4$ concentrations had wide time-related variability, each seemed to be affected to a different extent (Fig 2). At 60 and 80 days, serum $T_3$ concentration of the zineb-treated calves increased, resulting in values at the end of the study that exceeded those obtained before treatment. Serum $T_4$ values of treated calves decreased and appeared to stabilize at concentration well below initial values. In contrast, serum $T_4$ concentration in the control calf increased to approximately

twice the pretreatment value and was 3 to 6 times the values in zineb-treated calves.

*Gross pathologic findings*—The absolute and relative thyroid gland weights for the control calf were 13 g and 0.011%, respectively. Both variables markedly increased in the zineb-exposed calves, ranging from 2.2 to 6 times the former and from 3 to almost 9 times the latter. The liver and kidneys appeared paler than normal, and moderate quantity of transudate was detected in the pericardial sac. Such alterations were not found in the control calf.

*Histologic and ultrastructural changes*—Relevant changes were observed in the thyroid gland, liver, and testes. Sections of the thyroid gland were characterized by increased numbers of macrofollicles, mostly lined by flat epithelium, and scant microfollicles. Some foci of follicular hyperplasia were also detectable (Fig 3). Examination of semithin sections and transmission electron micrographs of the epithelial cells revealed remarkable intracytoplasmic vacuolization, localized mainly in the apical part of the cell between the nucleus and the cytoplasmic membrane. The vacuoles were membrane-bound and filled with flocculent homogeneous material (Fig 4). Less intense vacuolization was observed in flat follicular cells where chromatin margination was frequently detected.

Many hepatocytes had a foamy appearance, which was attributable to increased storage of glycogen as viewed by electron microscopy. Indeed, all sections from treated calves had moderate increase of glycogen deposition, in comparison with findings for pretreatment biopsy specimens (Fig 5). Glycogen is stored free in the cytoplasm in typical rosette form (α particles), which are well stained by lead citrate. Some swelling of mitochondria and chromatin margination was detected, but smooth endoplasmic reticulum hyperplasia was not observed.

Testicular alterations included slight-to-moderate interstitial edema associated with apparent reduction in the proliferation of supporting cells and spermatogonia (Fig 6A). The testes of the control calf, which by the end of the trial still had not reached sexual maturity, were characterized by normal spermatogenetic line (Fig 6B).

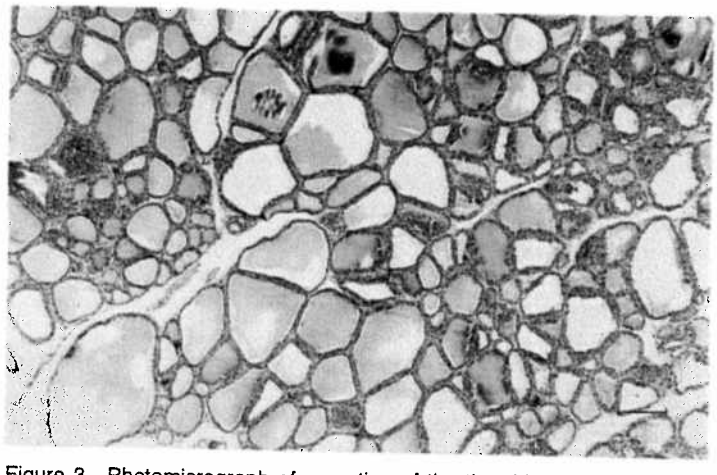

Figure 3—Photomicrograph of a section of the thyroid gland from zineb-exposed calf B. Notice micro- and macrofollicles with scattered foci of epithelial hyperplasia. H&E stain; bar = 200 μm.

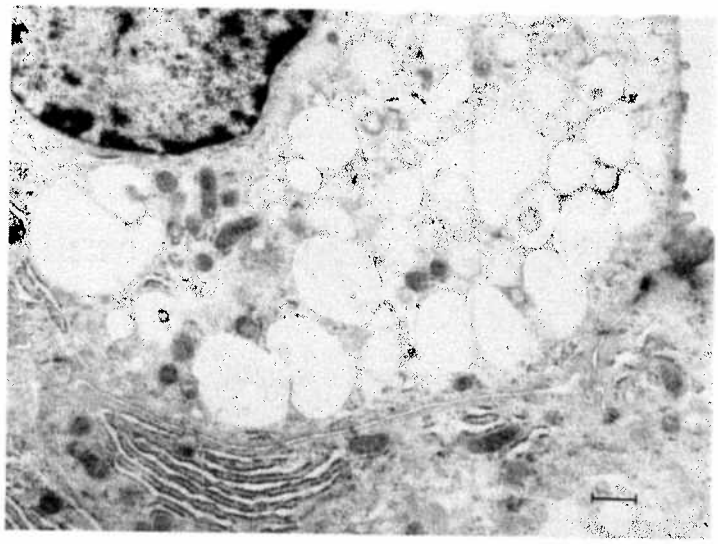

Figure 4—Electron micrograph of a section of the thyroid gland from zineb-exposed calf B. Notice extensive vacuolization in a follicular cell; bar = 0.4 μm.

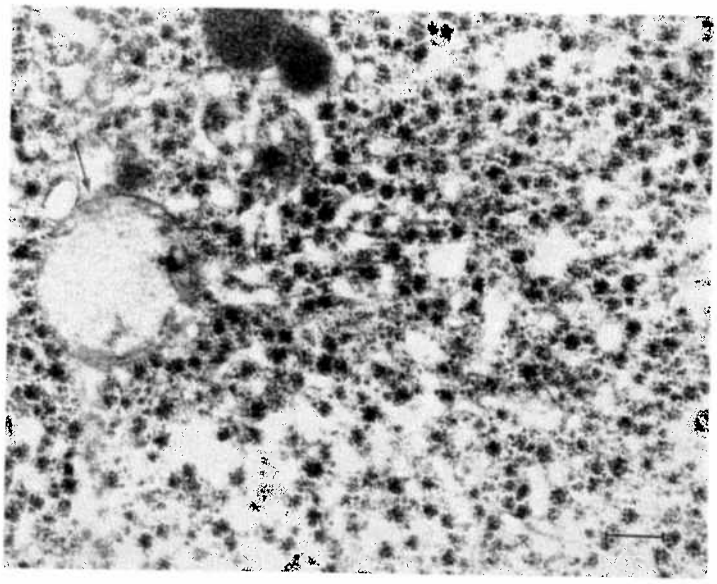

Figure 5—Electron micrograph of a section of the liver from calf C treated with zineb. Notice increased amount of stored glycogen and a swollen mitochondrion (arrow). Uranyl acetate and lead citrate; bar = 0.4 μm.

The thymus gland from treated calves was characterized by moderate interstitial edema, but the cellular density and the demarcation between cortex and medulla appeared to be within normal limits. Slight rarefaction of the lymphoid tissue was observed in the spleen, but relevant changes were not detected in the examined lymph nodes.

Finally, slight hyalinization of the arteriolar wall of the media was apparent in the myocardium of 2 of the 3 zineb-treated calves and scant Anitschkow cells were observed. Relevant histologic lesions were not detected in the remaining organs or in the control calf.

*Distribution of zineb and ETU*—Zineb concentration was highest in testes and liver, but considerable amounts were also detected in all examined organs (Fig 7). Zineb administration resulted in its extensive conversion to ETU. Com-

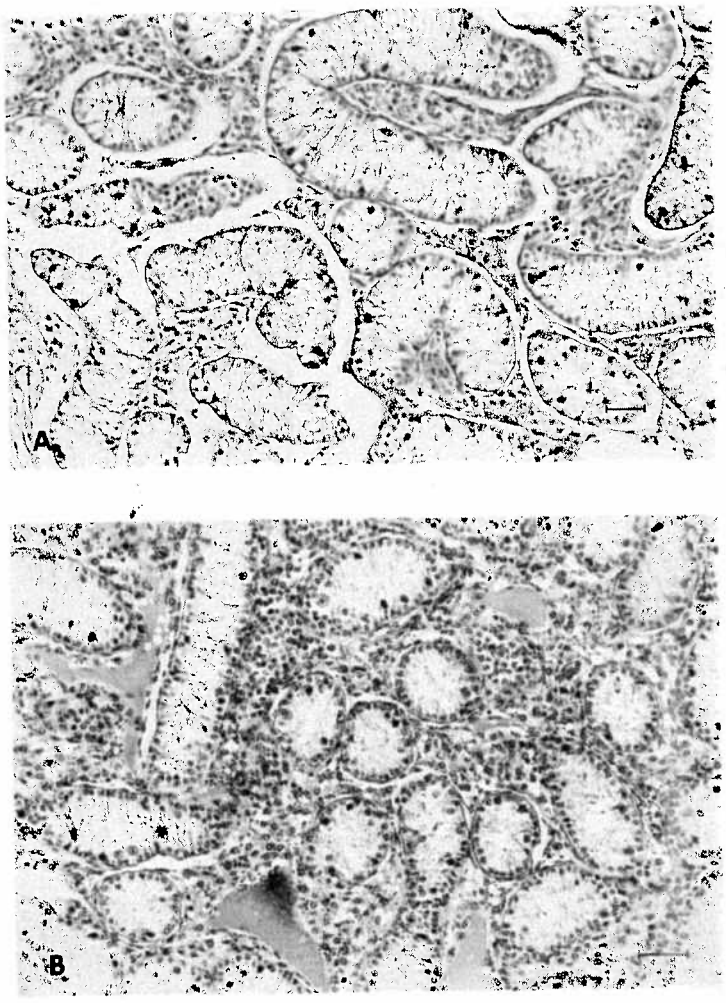

Figure 6—Photomicrograph of a section of a calf testis.
A—Section from treated calf B. Notice slight intertubular edema and marked depletion of the maturative line. Many spermatogonia have pyknotic nuclei. H&E stain; bar = 100 μm.
B—Section from control calf D. The spermatogenetic epithelium is normal. H&E stain; bar = 100 μm.

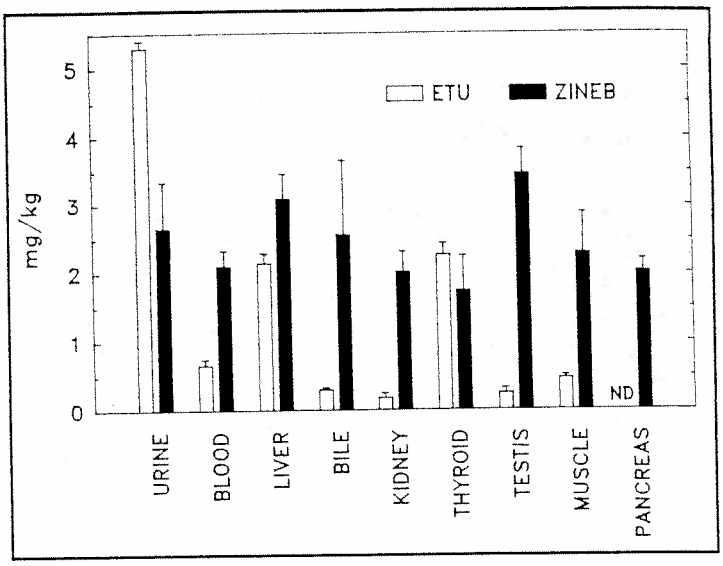


Figure 7—The zineb and ethylenethiourea (ETU) distribution in tissues and body fluids from calves subacutely exposed to zineb. Blood, urine, and feces were obtained on the day of euthanasia. Values are mean ± SD (n = 3); ND = not detectable, beyond the resolution limits of the analytic method.

pared with the parent compound, the metabolite had a lesser tendency to build up in tissues, other than in the liver and the thyroid gland. Interestingly, the latter was the only organ in which the amount of ETU exceeded that of zineb. Measurable concentrations of ETU were not detected in the pancreas.

Both compounds appeared to undergo urinary and fecal excretion. Mean ± SD fecal contents of zineb and ETU were 42.19 ± 1.45 mg/kg and 1.24 ± 0.19 mg/kg.

## Discussion

One goal of the study, was to assess whether age would render calves with immature rumen function more susceptible to zineb toxicosis, but such did not appear to be the case. Unweaned calves had more intense thyroid involvement, as reflected by the marked epithelial vacuolization and foci of hyperplasia seen in the gland of calves, but not of adult cattle. On the whole, poor growth, increased glycogen storage in the liver, and appreciable impairment of testicular and thyroid function may be regarded as the main features of zineb exposure in cattle, regardless of age.[8]

The high amount of fungicide that was found in feces, although partly attributable to biliary excretion, would reasonably support low gastrointestinal tract uptake of zineb. Poor gastrointestinal tract absorption is generally considered to be a feature of EBDC.[14]

In this study, zineb was uniformly distributed among the examined tissues. The slightly higher amount that was recovered in the testes and pancreas might be ascribed to the known ability of these organs to concentrate zinc.[15] An unexpectedly high concentration of zineb was found in the bile. High concentrations of zineb and ziram were also found in the gallbladder of experimentally dosed rainbow trout.[16] This phenomenon may be at least partly attributable to the well-known chelating properties of EBDC.[17] Zineb was shown to form complexes with copper, a metal that is largely excreted via the bile.[17,18]

Another aim of this study was to examine whether and at what rate orally administered fungicide could be converted to ETU. Our results clearly indicate that unweaned calves are able to extensively transform zineb to ETU. The EBDC are known to yield ETU spontaneously under conditions of high humidity and slight alkalinity.[19] Such conditions are normal in the gastrointestinal tract or in the urinary bladder. Considerable amounts of ETU were detected in urine. This, however, may be also explained by the high water solubility of the imidazolidine derivative.[4] The marked difference between urinary and fecal ETU contents, as well as the low biliary concentration of the imidazolidine derivative indicates that ETU generation in zineb-exposed calves may at least partly reflect a true metabolic conversion, although the role of spontaneous breakdown should not be overlooked.

Although ETU formation as a consequence of EBDC administration has been reported in several species including rats, common marmosets and mice, the metabolic pathways underlying this process are still poorly understood.[20,21] Zineb appears to be a substrate for the liver microsomal flavin-containing monooxygenase, the oxy-

gen atom being most likely added to a thione sulphur.[e] On the other hand, scant evidence is available to support substantial involvement of cytochrome P-450-mediated metabolism of the fungicide. Microsomal cytochrome P-450-dependent drug metabolism was severely depressed in the calves of this study.[22]

Compared with zineb, ETU was detected at low concentrations in tissues, except in liver and thyroid gland. In rats, the latter organ was also reported to accumulate ETU.[23] Although achieved by different mechanisms, zineb and ETU interfere with various steps of thyroid hormone biosynthesis.[1,6] A comparison between the thyroid concentration of ETU and that of zineb leads us to conclude that the antithyroid effects of the fungicide may likely result from the combined action of both compounds. A similar conclusion might also hold true for liver changes, although the observed increase in glycogen storage could also be the result of extrahepatic effects, such as depression of thyroid function. Thyroxine is believed to enhance indirectly the rate of glycogenolysis[24]; increased accumulation of liver glycogen was reported after administration of the antithyroid drug propylthiouracil to chicks.[25]

In contrast, the marked difference between zineb and ETU testicular concentrations might account for involvement of the former in the induction of the aforementioned histologic changes. Interestingly, zineb proved to be more effective than ETU in inhibiting lactate dehydrogenase activity and the testis-specific isoenzyme lactate dehydrogenase-X in bovine testicular subfractions.[26] The mechanisms underlying the toxic effects of EBDC on male gonads have not been fully elucidated. The depression of thyroid function may be involved in the pathogenesis of the testicular alterations. Hypothyroidism is known to reduce androsterone synthesis, and male goats experimentally dosed with thiourea, a potent goitrogen, had germ cell rarefaction, reduction in sperm viability, and impairment of the secretory activity of the accessory sex glands.[27,28]

Despite lack of consistent histologic lesions, appreciable amounts of zineb and ETU were recovered in skeletal muscle. This finding should be regarded with particular concern in association with human meat consumption. Evidence is growing that at least in Italy, EBDC should be considered as environmental pollutants and food contaminants. During a field survey conducted in an agricultural area of Northern Italy, EBDC residues ranging from 0.13 to 2.04 mg/kg were found in about 90% of the fruits examined.[29] Moreover, results of a recent study performed in the same area on forage crops grown close to poplar groves treated with mancozeb indicated that residues of the fungicide (up to 84 mg/kg) were detected in > 60% of the samples examined.[f] Data pertaining to EBDC contamination of meat products are, to our knowledge, not available, but it is worth noting that EBDC residues were found in the thyroid gland of aborted bovine fetuses.[30]

Provided that farm animals may be fed with contaminated foodstuffs, however, results of this study would indicate that calves did not appear to accumulate zineb extensively in any edible tissue.

Further research is in progress to ascertain whether environmental exposure of cattle to EBDC could result in accumulation of concentrations in excess of the estimated acceptable daily human intake. It should be remembered that the estimated acceptable daily human intake of zineb and ETU is as little as 0.05 and 0.002 mg/kg, respectively.[7]

In conclusion, calves with immature rumen function do not appear to be more susceptible than adult cattle to the toxic effects of zineb, possibly with the exception of its antithyroid effects, which likely are attributable to a mutual interaction between the fungicide and ETU, one of its main metabolic products.

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