The Fate of Trenbolone Acetate and Melengestrol Acetate after Application as Growth Promoters in Cattle: Environmental Studies

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The steroids trenbolone acetate (TbA) and melengestrol acetate (MGA) are licensed as growth promoters for farm animals in several meat-exporting countries. Although many studies have explored their safety for both animals and consumers, little is known about their fate after excretion by the animal. Our study aimed to determine the residues and degradation of trenbolone and MGA in solid dung, liquid manure, and soil. In animal experiments lasting 8 weeks, cattle were treated with TbA and MGA. Solid dung and, in case of trenbolone, liquid manure were collected and spread on maize fields after 4.5 and 5.5 months of storage, respectively. Determination of the hormone residues in all samples included extraction, clean-up (solid-phase extraction), separation of metabolites and interfering substances by HPLC (RP-18), and quantification by sensitive enzyme immunosay. Procedures were validated by mass spectrometry (MS) methods. During storage of liquid manure the level of trenbolone decreased from 1,700 to 1,100 pg/g (17α-isomer), corresponding to a half-life of 267 days. Before storage, the concentrations in the dung hill ranged from 5 to 75 ng/g TbOH and from 0.3 to 8 ng/g MGA. After storage, levels up to 10 ng/g trenbolone, and 6 ng/g MGA were detected. In the soil samples trenbolone was traceable up to 8 weeks after fertilization, and MGA was detected even until the end of the cultivation period. The results show that these substances should be investigated further concerning their potential endocrine-disrupting activity in agricultural ecosystems. Key words: degradation, dung, growth promoter, manure, melengestrol acetate, soil, trenbolone. Environ Health Perspect 109:1145–1151 (2001). [Online 2 November 2001] http://ehpnet1.nih.gov/docs/2001/109p1145-1151schiffer/abstract.html

For several years we have known that sex hormones excreted via human and/or animal feces can exhibit endocrine-disrupting activity; for example, estrogens present in chicken manure caused hyperestrogenism when fed to cattle (1). Natural and synthetic estrogens such as estradiol-17β and ethinylestradiol-17α were frequently detected in lower nanogram per liter ranges in discharges of sewage treatment plants, caused by their incomplete removal during passage through the sewage treatment plants (2). Exposure of fish to sewage treatment plant effluents increased plasma levels of vitellogenin, a protein synthesized by the liver of oviparous fish in response to estradiol stimulation (3). Public concern focuses especially on the synthetic estrogen and progestin components of oral contraceptives, which have high physiologic activity at low doses. Compared with the natural hormones, they show a relatively greater stability in aqueous media (4) and a greater resistance to microbial degradation (5). These properties pose the potential for accumulation and persistence in the environment. It can be presumed that other structurally related xenobiotic hormones that are used for veterinary treatment show a similar behavior.

The synthetic steroids trenbolone acetate (TbA (17β-acetoxyestra-4,9,11-triene-3-one); Figure 1) and melengestrol acetate [MGA (17α-acetoxy-6-methyl-16-methylene-pregna-4,6,14-diene-3,20-dione); Figure 2] are licensed as growth promoters for farm animals in the United States and Canada. TbA is administered as a subcutaneous implant either alone or in combination with an estrogenic compound. The anabolic effect of TbA, which is 8–10 times stronger than that of testosterone propionate (6), is based on androgenic and antiglucocorticoid activity (7,8). After its release from the depot into the blood circulation, TbA is hydrolyzed to the active trenbolone-17β (TbOH-17β). In the heifer, only one major metabolic route occurs, oxidation of TbOH-17β to tren-dione (TbO), followed by reduction to TbOH-17α (Figure 1). The epimerization strongly decreases the compound’s biologic efficacy; the anabolic potency of TbOH-17α is only about 5% of that of TbOH-17β (9), and the affinity to the recombinant human androgen receptor (rhAR) is reduced to about 4% (10).

Melengestrol acetate (MGA), an orally active gestagen, can be used for estrus synchronization and/or induction in cattle (11). It is also marketed as a feed additive for feedlot heifers to improve feed efficiency and rate of weight gain. The administered daily dose of 0.5 mg per cow allows ovarian follicular development while inhibiting estrus and ovulation (12). MGA exerts both progestational and glucocorticoid activity (13). Its progestational activity was about 125 times greater than that of progesterone as measured by estrus cycle inhibition in cattle (13); anti-inflammatory assays in rats showed that its glucocorticoid activity was comparable with that of hydrocortisone (14). The anabolic mode of action of MGA is assumed to be due to stimulation of the ovarian synthesis of endogenous estradiol (15). Androgenic side effects are probably not of concern because a recent study has shown that the binding affinity of MGA to the rhAR is only about 1% of testosterone and 0.3% of dihydrotestosterone (10).

Although many studies have been performed on the safety of TbA and MGA for both animals and consumers (11,13,16), little is known about their fate after excretion by animals. It is possible that these substances and/or their metabolites accumulate in soil or find their way into surface or even ground water via dung or manure. The intention of our studies was to determine the residues and degradation of TbOH and MGA, respectively, in solid dung, liquid manure, and soil.

Material and Methods

Animal Experiments, Manure Collection, and Field Experiments

All animals used in our research have been treated according to the Code of Ethics of the World Medical Association (Declaration of Helsinki) and the guiding principles in the Guide for the Care and Use of Laboratory Animals, National Institutes of Health (17). Study I: Degradation of TbOH in liquid manure. We implanted 41 cattle (Holstein Friesian) with commercially available anabolic preparations containing TbA. The total amount of TbA applied to the animals was 3,340 mg. The liquid manure was collected in a manure collection canal and pumped into the cylindrical manure storage pit, open at the top, every 2 weeks. In the collection canal the material was not homogeneous, whereas in the manure storage a stirring propeller achieved good homogeneity before sampling. The manure was stored

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under anaerobic conditions. After the end of the animal experiments the total volume of the manure in the storage was about 170 m³ and contained all animal excrement, the stable-cleaning water, and atmospheric precipitations (rain, snow) that also reached the storage. The estimated mass of excrement was 100 tons.

Samples of liquid manure were taken every second week from the collection canal (to survey the conditions immediately after the manure formation), every 2 or 4 weeks from the manure storage tank, and before spreading on the fields. A small fraction of the total amount was spread on an experimental field in November after the end of the animal experiments. The majority was used for fertilization in spring after 5.5 months of storage. We took samples of the stored manure every month. All samples were stored at –25°C.

Study II: Stability of TbOH in solid dung. We implanted 12 Holstein Friesian heifers with commercially available TbA preparations. The total amount of TbA applied in the experiment was 5,600 mg. For cleaning of the stables, the excrement of the animals was removed in a traditional procedure with the help of straw, and a dung hill was erected. After the end of the animal experiments, the dung hill contained the excrement of the 12 animals from day 31 before treatment to day 56 after treatment. The estimated total volume of the dung hill was 40 m³; the estimated mass of excrement was 20 tons.

After finishing the animal experiments, we took samples of solid dung from four different locations of the dung hill, representing different regions (top, middle, bottom, and liquid effluent). In November the solid dung was transferred to a sealed storage ground. Mixing of the dung hill during transportation was inevitable. After 4.5 months of storage, samples were again taken from different regions (top, middle, and bottom). All samples were kept at –25°C.

Study III: Residues of melengestrol acetate in feces and solid dung. We treated 13 Holstein Friesian heifers with MGA, served as feed premix that was prepared from reference material (ICN Biomedicals, Eschwege, Germany) at the Institute of Animal Nutrition at the Technical University of Munich-Weihenstephan, Germany. The total amount of MGA applied in the experiment was 840 mg. The excrement was removed with the help of straw similar to study II, but the dung hill was erected automatically by pressing the fresh dung from the bottom of the dung plate into the dung hill. After the end of the animal experiments, the dung hill contained about 20 tons of excrement in an estimated total volume of 60 m³.

Samples of feces were taken twice per week from each animal. Sampling and storage of solid dung were performed analogously to study II.

Studies IV and V: Steroid residues and stability in soil. At our experimental farm the liquid manure and solid dung from the hormone treatment experiments were used to fertilize fields on which maize was cultivated according to good agricultural practices.

Liquid manure containing TbOH was spread on one section of the fields in November (fresh manure) and on another section in March (stored manure). Solid dung from studies II and III was brought out also in March. Soil samples were collected from three representative locations of each experimental section of the fields, some immediately after fertilization, and regularly every month (first 3 months) or every second month, respectively, until the end of the cultivation period (i.e., ploughing of the fields in October). All samples were stored at –25°C.

Chemicals

All solvents and chemicals used during extraction and quantification were at least of analytic-reagent grade. TbOH-17α and TbOH-17β were provided by Roussel-Uclaf (Paris, France), TbOH-17β was purchased from Sigma (Deisenhofen, Germany), and MGA from ICN. Testosterone-d₃ and MGA-d₃ were provided by RIVM (Bilthoven, Netherlands).

Equipment

The HPLC system used for studies I, II, IV, and V included a pump module (model 420; Kontron Instruments, Neufrunh, Germany), an injector (model 210-A Valve; Beckman, München, Germany), a column oven (Jetstream Plus; Beckman), a fraction collector (model Frac-100; Pharmacia, Uppsala, Sweden), and an RP-18 column (studies I, II and IV; LUNA, 250 mm × 4.6 mm, 5 µm, Phenomenex, Aschaffenburg, Germany; study V; NUCLEOSIL EC 150/4.6, 100-5 C18, Macherey-Nagel, Düren, Germany).

We performed liquid chromatography (study I) using a GC-8000 apparatus (Fisons/Carlo-Erba, Altrincham, UK) and a DB-5 column, size 15 m × 0.25 mm, 0.25 µm film thickness, (J&W Scientific, Folsom, CA, USA) were used with helium (5.0; Linde, Wiesbaden, Germany) as carrier gas.

We performed mass spectrometry (study I and III) using a photometer (model Spectra Image) from Tecan (Crailsheim, Germany).

Quantification of TbOH in Liquid Manure and Solid Dung

We performed steroid extraction and purification using a method previously described for feces (18). The eluate of the solid-phase extraction was completely dried in vacuum, and the residue was resolved in 600 µL 20% methanol.

We separated TbOH-17α from its metabolites TbOH-17β and TbO by HPLC on a C18 reverse-phase column. The injection volume was 500 µL (of the purified extract) and the column was eluted at 25°C with a mixture of methanol/water (65/35, v/v) at a flow rate of 1 mL/min. The fraction size was 330 µL.

We quantified the hormone concentration in the HPLC fractions by enzyme immunoassay following the procedure...
described in literature (18, 19). In liquid
manure and solid dung samples before
storage, we calibrated the assay for the main
metabolite TbOH-17α. We calculated the
concentrations of TbOH-17β and TbO by
their cross-reaction in relation to TbOH-17α
e.g., a measured concentration of 45 pg/g
TbOH-17β corresponded to an actual con-
tent of 31 pg/g; the cross-reaction of TbOH-
17β compared to TbOH-17α was 144%). In
solid dung samples after storage, we quanti-
fied TbOH-17α, TbOH-17β, and TbO by
using the corresponding specific calibration
curves.

Quantification of TbOH in Soil
Because of the dilution effect when manure
or dung is spread on the fields, only low con-
centrations of TbOH and its metabolites
could be expected in soil, and analyte enrich-
ment had to be performed. Therefore, 50 g
of soil were suspended in 25 mL water and
evacuated twice with 15 mL tert-butyl methyl
ether (TBME) (overnight at 20°C, using an
overhead shaker). The TBME phases of both
extractions were combined and completely
evaporated (60°C, shaking water bath), and
the residue was resolved in 0.5 mL 80% methanol. Purification and quantification
proceeded as described above. We measured
the concentrations of TbOH-17α and
TbOH-17β using the corresponding specific
calibration curves, and we determined TbO by its cross-reaction in relation to
TbOH-17β.

Validation of TbOH Determination
Validation was performed for the major
metabolite TbOH-17α. We determined the
limit of detection, which corresponds to the
smallest measurable content of analyte, by
analyzing negative samples and calculating
the mean plus the 3-fold standard deviation
of the resulting values. Accuracy and preci-
sion were determined as the recovery in for-
tified blank samples (carried out in triplicate)
and the variation coefficient of these recovery
experiments, respectively (Table 1).

The poor and varying recovery rates
demanded internal standardization, but it
was not possible to find standard substances
that behaved proportionally to the analyte
during extraction and purification. Therefore
we had to perform external standardization
by the mean recovery rates of 42, 32, or
30%, depending on the matrix.

Confirmation Analysis
To confirm the identity of TbOH residues,
we analyzed two representative liquid manure
samples with gas chromatography-mass spec-
trometry (GC-MS): one sample from the
manure canal and one from the manure stor-
age. Extraction and clean-up occurred as
described above; we determined the heptaflu-
oroxybutyl derivatives similarly to a method
described elsewhere (20). From the manure
sample from the canal we measured the fol-
lowing concentrations: 6.7 ng/g TbOH-17α and
0.20 ng/g TbOH-17β with GC-MS compared to 4.1 ng/g TbOH-17α and 0.18
ng/g TbOH-17β with HPLC/enzyme
immunoassay. In the manure sample from
the storage tank, the agreement of the results
was just as satisfactory: 3.1 ng/g TbOH-17α
and 0.065 ng/g TbOH-17β detected with
GC-MS, compared to 1.6 ng/g TbOH-17α
and 0.055 ng/g TbOH-17β detected with
HPLC/enzyme immunoassay. Interferences
made us unable to determine the TbO con-
tents with GC-MS.

Quantification of Melengestrol
Acetate in Feces
We analyzed fecal samples from study III by
liquid chromatography (LC)-MS to identify
the parent compound MGA excreted in feces.
After addition of 5 ng internal standard
(MGA-d3) per gram of sample, an aliquot of
4 g feces was suspended in 6 mL water and
extracted twice with 10 mL petroleum ether
(PE) under gentle rotation (at 40°C
overnight and for 1 hr, respectively). The
residue of the combined PE phases was
resolved in 1 mL acetonitrile/water (95/5,
v/v) and defatted twice with 2 mL PE. After
the acetonitrile phase was evaporated (in vac-
um) and the residue was resolved in 0.5 mL
80% methanol, purification proceeded as
described above. The eluate of the solid
phase extraction was evaporated to dryness
(in vacuum) and the residue resolved in 30
µL acetonitrile. For LC-MS analysis we
injected 20 µL of the purified steroid
eextract under the following conditions:
Acetonitrile/water/formic acid (50/50/1,
v/v/v) served as mobile phase at a flow rate of
0.6 mL/min. Retention times were 6.72
min for MGA and 6.70 min for MGA-d3.
The monitored ions after electrospray ion-
ization were 397, 438, and 337 for MGA
and 400, 441, and 340 for MGA-d3. We
identified the substances by the corre-
ponding retention time and the relative peak area
of selected ions. For quantification we cal-
culated the area of the base peak of MGA (m/z
397) and MGA-d3 (m/z 400) and compared
their ratio to a linear calibration curve,
which we obtained by measuring a range of
at least five standards.

Quantification of Melengestrol
Acetate in Solid Dung
We determined MGA in solid dung analo-
gously to its determination in feces, but with
some modifications: We extracted 3 g of
solid dung; after evaporation of the com-
bined PE extracts we redissolved the residue
in 0.5 mL 80% methanol and then defatted
it twice; we eluted the solid-phase extraction
columns with 1.5 mL 80% methanol; the eluate
was evaporated to dryness and the residue
resolved in 15 µL acetonitrile; injection
volume for LC-MS analysis was 10 µL,
and flow rate was 0.3 mL/min.

Validation of LC-MS Analysis
Accuracy, precision, and limit of detection
followed the principles described for TbOH
determination. The detection limit was 0.2
ng/g (signal to noise ratio 5:1). For valida-
tion parameters, see Table 1.

Quantification of MGA in Soil
Analysis of soil samples focused on MGA.
Only small concentrations of MGA could be
expected in soil, and analyte enrichment had
to be performed. Because the sensitivity of
LC-MS for the determination of MGA in soil
was not sufficient, we had to apply
enzyme immunoassay for quantification.

We extracted 50 g of soil with 30 mL
methanol overnight and afterward cen-
trifuged the sample. The supernatant was
transferred to new extraction vials, diluted
with water to a final concentration of 40% methanol, and extracted overnight with 15
mL PE. After the emulsion was centrifuged
and frozen, the PE phase was decanted and
evaporated to dryness in a shaking water
bath (at 70°C). The residue was resolved in
1 mL 80% methanol. After adding 2 mL

Table 1. Determination of TbOH-17α and MGA: validation parameters.

| Parameter                  | Liquid manure TbOH-17α | Solid dung TbOH-17α | Soil MGA | Feces MGA |
|----------------------------|------------------------|---------------------|---------|----------|
| Detection limit (pg/g)     | 4                      | 5                   | 0.4     | 0.2      |
| Spikes (pg/g)              | 100/450/1,800          | 5,000/1,750/18,500  | 3/45/2400| 4/20/40  |
| Mean recovery (%)          | 42                     | 102.6               | 20/25/25| 100/80   |
| Mean precision (%)         | 30                     | 2.8                 | 12      | 5        |

*Performed with enzyme immunoassay. aLC-MS analysis. bPerformed in triplicate. cPerformed in quintuplicate. dPerformed in quadruplicate. eNot corrected by standardization. fCorrected by internal standardization.
water, we performed solid-phase extraction as described for solid dung samples. The eluate was evaporated to dryness (in vacuum) and resolved in 600 µL 20% methanol. We separated MGA from interfering substances by HPLC on a C18 reverse-phase column. HPLC conditions (injection volume, mobile phase, flow rate, and column temperature) were the same as applied for analysis of TbOH samples; however, the fraction size was 250 µL. The MGA content in the HPLC fractions was quantified by enzyme immunoassay (21) with a commercially available EIA-Kit (R-Biopharm, Darmstadt, Germany).

Validation of MGA Determination in Soil

The validation followed the principles described for TbOH determination. Table 1 shows the validation parameters. Similar to TbOH, internal standardization with structurally related steroids was not possible because all tested substances showed a different extraction effectiveness compared to MGA. For external standardization, all results were corrected by the mean recovery rate of 25%.

Results

TbOH

Residues in liquid manure. Figure 3 shows the residues of TbOH in liquid manure during collection in the manure canal (all values were corrected by the recovery rate). In the canal the manure was heterogeneous, and two samples collected at the same date were corrected for analysis of TbOH samples; however, the fraction size was 250 µL. The MGA content in the HPLC fractions was quantified by enzyme immunoassay (21) with a commercially available EIA-Kit (R-Biopharm, Darmstadt, Germany).

Figure 3. Residues of TbOH in liquid manure during collection in the manure canal.

Residues in solid dung. Figure 4 shows the degradation of TbOH during 5.5 months of manure storage is illustrated in Figure 4 (all results were corrected by the recovery rate). The level of TbOH decreased from 1,700 pg/g in the beginning to 1,100 pg/g from 17α-isomer and from 160 pg/g to 100 pg/g from 17β-isomer. These values corresponded to a half-life of 267 and 257 days, respectively, whereas for TbO we observed no decline, possibly because of oxidation of TbOH-17α and -17β.

The half-lives of TbOH-17α and TbOH-17β were calculated according to the following formula, usually applied for radioactive decay kinetics:

\[ c(t) = c(0) \times e^{-\lambda t}, \]

where \( t \) is time; \( c(0) \) is concentration at time 0; \( c(t) \) is concentration at time \( t \); and \( \lambda \) is the constant of decay. Thus, the half-life is given by

\[ t_{1/2} = \frac{-\ln(1/2)}{\lambda}. \]

Contents in Solid Dung. As in liquid manure, TbOH-17α was the main metabolite of TbOH in solid dung. However, in 4 of 10 analyzed samples the amount of TbO exceeded that of TbOH-17β (Table 2). Compared to liquid manure the TbOH contents in solid dung before storage were 5–70 times higher, depending on the position in the dung hill. TbOH was eluted with rainwater passing the dung hill and gathering at the effluent. Although TbOH was partly degraded during 4.5 months of storage, it could be detected in four of six samples (levels up to 10 ng/g TbOH-17α, 0.3 ng/g TbOH-17β, and 0.8 ng/g TbO).

The huge variation of the measured concentrations reflected the heterogeneity of the dung hill caused by erection and transportation procedures.

Residues in Soil. TbOH-17α, TbOH-17β, and TbO could be detected in soil fertilized with liquid manure and solid dung. The dilution effect when manure and dung were spread on the fields made the maximum concentrations in soil markedly lower (Table 3). The first soil samples were taken 31 days after fertilization with fresh liquid manure in autumn. Assay of these samples indicated that TbOH residues originating from liquid manure were stable for less than a month. We confirmed this result by analyzing the soil samples fertilized in spring with stored manure. TbOH was traceable 8 days after spreading on the fields, but could not be quantified after 40 days.

TbOH concentrations in soil fertilized with stored solid dung were lower than in soil fertilized with liquid manure. However, residues were detectable 58 days after fertilization. This potential greater stability might be caused by adsorption of TbOH to straw material, which possibly protected the substances from degradation or leaching.
MGA

Residues in feces. The data in Table 4 demonstrate that MGA residues in feces were clearly dose dependent. Average levels during 1-, 3-, and 10-fold treatment were 2.1, 5.9, and 16.2 ng/g, respectively. The concentrations 24 hr after feeding were approximately 1.4 times higher than after 12 hr, reflecting the passage through the digestive tract.

Contents in solid dung. The MGA amounts in solid dung before storage ranged between 260 and 7,760 pg/g. After 4.5 months of storage the concentrations still ranged between 420 and 6,030 pg/g (Table 5). In comparison with TbOH the decrease was not so clear, owing to a greater stability of MGA. But like TbOH, the varying MGA concentrations reflected the heterogeneity of the dung hill caused by erection and transportation conditions.

Residues in Soil. In soil samples MGA originating from solid dung was traceable from spring until the end of the cultivation period in October (Table 3). The experimental fields were thoroughly ploughed 195 days after fertilization; thus continuation of sampling seemed inappropriate. As described for TbOH, the maximum MGA concentrations in soil were definitely lower than for solid dung because of the dilution effect when dung was spread on the fields.

Discussion

After the use of TbA and MGA as growth promoters, we analyzed the degradation of their residues in solid dung and liquid manure. In soil fertilized with solid dung, TbOH and MGA were traceable for 58 and 195 days, respectively.

Studies I and II

After excretion via feces, TbOH could be detected in liquid manure and solid dung in significant concentrations. With the help of a simplified model calculation illustrated in Table 6, we tried to estimate the recovered fraction of the total applied dose. The determined values between 3 and 42% are significant, considering the fact that in the United States, for example, presumably several tons of TbA are applied every year. In some circumstances, discussed below, the total concentration of TbOH metabolites was probably even higher.

In cows the biliary excretion of TbOH predominates. Ten metabolites with 3-oxotriene-structure and three additional compounds that had lost their 3-oxotriene-structure could be identified in cow bile (9). However, our quantification system was validated and suitable only for the metabolites TbOH-17α, TbOH-17β, and TbO.

TbOH is known for its ability to bind to biologic macromolecules, especially proteins. Studies with radiolaabeled TbA implants in heifers proved that about 90% of the total radioactivity could not be extracted with commonly used organic solvents and either was water-soluble or an insoluble tissue-bound residue (24). If TbOH residues are also bound to fecal compounds, the extraction and measuring methods we applied in our studies underestimated the actual concentrations in liquid manure and solid dung.

Studies on the stability of TbOH in bovine urine showed that storage of urine samples in direct sunlight led to decreased TbOH concentrations (25). Storage of feces samples at room temperature in some cases caused partial or complete loss of the TbOH-17α content (26). Throughout our experiments, dung hills, the manure collection canal, and storage pit were neither cooled nor protected from sunlight.

Because other steroid hormones (e.g., estrone) can be catabolized by microorganisms (27), microbial degradation of TbOH is conceivable as well. However, knowledge of microbial metabolism of steroids is still scarce. In an in vitro study performed with *Escherichia coli* and *Clostridium perfringens* as representative intestinal microorganisms, no specific effect on the concentration of the hormone 4-pregnene-20β-ol-3-one was observed (28).

Finally, the actual amount of metabolites may have been higher because small amounts of TbOH were eluted with rainwater passing over the dung hill, and the adsorption of TbOH to straw material cannot be excluded.

Previous studies performed by Haase et al. (29) and Rumsey et al. (30) demonstrated that synthetic hormones (namely, diethylstilbestrol) were stable in liquid manure stored under anaerobic conditions. Similarly, degradation of TbOH occurred rather slowly. Its half-life in liquid manure without ventilation was approximately 260 days.

In a dung hill erected with excrement from pregnant heifers and stored for several

### Table 2. Residues of trebolone in solid dung before and after storage

| Sample (position within dung hill) | TbOH-17α (pg/g) | TbOH-17β (pg/g) | TbO (pg/g) | MGA (pg/g) |
|-----------------------------------|-----------------|-----------------|------------|------------|
| Fresh (~1 m below top)            | 13,820          | 1,000           | 1,225      | ND         |
| Medium (height 2.5 m)             | 75,400          | 4,265           | 4,700      | ND         |
| Old (height 0.5 m)                | 4,726           | 484             | 405        | ND         |
| Effluent                          | 227             | 19              | 10         | ND         |

**Notes:**
- ND, not detectable (below limit of detection).
- *a* Values were corrected by the recovery rate.

### Table 3. Residues of trebolone and MGA in soil

| Sample (days after fertilization) | TbOH-17α (pg/g) | TbOH-17β (pg/g) | TbO (pg/g) | MGA (pg/g) |
|-----------------------------------|-----------------|-----------------|------------|------------|
| Soil fertilized in autumn with fresh liquid manure | ND | ND | ND | ND |
| Soil fertilized in spring with stored liquid manure | A–B(1)* | 24/16 | 8.1/5.1 | 21/18 |
| Soil fertilized in spring with stored solid dung | A–C(26)* | 5.8/3.3/11 | 0.7/0.4/1.0 | 2.6/1.3/4.1 | 34/11/17 |

**Notes:**
- ND, not detectable (below limit of detection).
- *a* Values were corrected by the recovery rate. *Capital letters represent samples taken from different locations of the same field. *n* = 2, *m* = 3.
months, estrogen concentrations up to 780 ng/g were measured (31). In this study, however, more degradation was observed in the anaerobic lower layers of the dung hill.

Study III
As for TbOH, we attempted to evaluate the recovered fraction of the total applied dose of MGA using a simplified model (Table 7). The calculated excretion rate via feces (12%) confirmed preceding observations that about 15% of the daily administered dose passed through the gastrointestinal tract unabsorbed. Bile cannulation studies showed that the primary route of excretion from the body was via the bile. However, the metabolic fate of MGA in heifers has not been investigated in detail until now. Although MGA was primarily excreted unmodified, several metabolites were found in the non-MGA fraction in liver (71). At least three of them are hormonally active substances; they exhibited binding affinities to the bovine uterine progesterin receptor (bPR) between 28 and 85% in comparison to progesterone (10). Because our measurement method was specific only for the parent compound, our results cannot give a complete picture concerning the actual total residues. Microbial degradation and adsorption to straw might also have contributed to a reduced recovery of the parent substance in relation to the total applied dose.

Studies IV and V
TbOH and MGA originating from contaminated excrement were detectable in soil up to several months after fertilization. From our field experiments we cannot deduce the mechanisms of how these hormones disappear from soil, but it is known that steroids can interact with humic substances and form stable products (32). By comparing to the behavior of other well-known agricultural or industrial soil pollutants, we can speculate on the fate of TbOH and MGA. They can be degraded by soil bacteria and/or photochemical reactions (UV light). Rain might wash the substances into lower soil horizons or directly into surface water without passing the soil column. Both processes might be promoted by dissolved organic matter that can bind the steroids and enhance their solubility and mobility in the aqueous phase. Strong adsorption of hydrophobic compounds to soil particles is well documented for many agricultural and industrial chemicals. Thus, persistent organic chemicals accumulate, whereas weaker adsorption might result in transposition to ground and surface water. Big hydrophobic molecules are generally more strongly adsorbed than small hydrophilic molecules (33,34).

In conclusion, research on the stability and degradation of sex hormones should be a crucial element of an environmental risk evaluation.

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### Table 5. Residues of MGA in solid dung samples before and after storage

| Sample (position within dung hill) | MGA (pg/g) |
|-----------------------------------|------------|
| Top (n = 4)                        | 380 ± 658/3,419/731 |
| Higher middle (n = 4)              | 260 ± 4,200/5,104/3,037 |
| Lower middle (n = 4)               | 7,760 ± 417/1,351/741 |
| Bottom (n = 3)                     | 6,524 ± 2,619/4,076 |

### Table 6. Model calculation for the recovered fraction of trenbolone in liquid manure and solid dung.

| Sample            | Liquid manure | Solid dung |
|-------------------|---------------|------------|
| Total amount of applied TbA | 3,340 mg | 5,600 mg |
| TbA remaining in implantation sites | 1,000 mg | 1,400 mg |
| TbA equivalents excreted | 2,340 mg | 4,200 mg |
| Trenbolone excreted | 2,025 mg | 3,635 mg |
| TbOH concentration | 1,700 pg/g | 4,730 pg/g |
| Total amount of excrement | 420 tons | 1,700 pg/g |
| Total trenbolone | 170 mg | 95 ± 1,510 mg |
| Estimated recovery | 8% | 3/42% |

### Table 7. Model calculation for the recovered fraction of MGA in feces and solid dung.

| Feces                  | MGA concentration (mg/cow) |
|------------------------|-----------------------------|
| Daily administered dose of MGA | 0.5 mg/cow (1-fold treatment) |
| MGA concentration in feces | 2 mg/g |
| Estimated production of feces per cow | 20 kg/day |
| MGA excreted per cow | 60 kg/day |
| Excretion rate via feces | 12% |

| Solid dung | MGA concentration | Total amount of excrement | Total MGA | Estimated recovery |
|------------|-------------------|---------------------------|-----------|-------------------|
| Total amount of applied MGA | 840 mg | 20 tons | 5/155 mg | 0.6/18% |

*Values were corrected by internal standardization.

Data from a study based on the same animal experiments (23). Values refer to TbOH-17αc. Concentration at the beginning of storage. Minimum concentration before storage. Maximum concentration before storage.

*Average level during 1-fold treatment. Minimum concentration before storage. Maximum concentration before storage.
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