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OPINION OF THE

SCIENTIFIC COMMITTEE ON VETERINARY MEASURES RELATING TO PUBLIC HEALTH

ON

Review of previous SCVPH opinions of 30 April 1999 and 3 May 2000 on the potential risks to human health from hormone residues in bovine meat and meat products

(adopted on 10 April 2002)

Table of contents

GLOSSARY

A→G: adenine replaced by guanine
A→T: adenine replaced by thymine
ADI: acceptable daily intake
ATBO: intestinal amino acid transporter BO
ATP-ase: adenosine-triphosphatase
bp: base pairs of DNA
bPR: bovine progesterone receptor
b.w.: body weight
COMT: catechol-o-methyl-transferase
COS-7: cell line
CSTEE: Scientific Committee on Toxicity, Ecotoxicity and the Environment
CVMP: Committee on Veterinary Medical Products
DES: diethylstilboestrol
DGEL: degree of germinal epithelial loss
DHT: dehydrotestosterone
DNA: deoxyribonucleic acid
ED_{50}: dose inducing 50% of the maximum effect
EIA: enzyme immuno assay
FAO: Food and Agriculture Organization
G 2 phase: phase in the cell cycle
G→T: guanine replaced by thymine
GC-MS: gas chromatography mass spectrometry
G-specific: guanine-specific
GTSμ3: glutathione transferase μ3
GVP: Good Veterinary Practice
HPLC/MS: high pressure lipid chromatography/mass spectrometry
HPRT: hypoxanthine-phosphoribosyl-transferase
hprt (loci): gene locus of HPRT (*see above*)
H-ras DNA: DNA fragment encoding for H-ras (oncogene)
IARC: International Agency for Research in Cancer (Lyon)
ICI 182,780: fulvestrant (Faslodex)
JEFCA: Joint FAO/WHO Expert Committee on Food Additives
LC-MS: liquid chromatography mass spectrometry
MCF 7: human breast cancer cell line
MCF 7-AR 1: MCF 7-amphiregulin
MGA: melengestrol acetate
MRG1= MRG1/p35rsj = Cited2: gene family expressed during mouse development
MRL: maximum residue level
PG-2: germ cell marker

PS2: presenilin 2
RBA: relative binding affinity
RhAR: recombinant human androgen receptor
RPPs: relative proliferative potencies
SBG: steroid binding globulin
SBP: steroid binding protein
SCVPH: Scientific Committee on Veterinary Measures relating to Public Health
SENCAR: strain of mice
SHBG: sex hormone binding globulin
SHBP: sex hormone binding protein
Tb OH-17α: trenbolone-17a
Tb OH-17β: trenbolone-17b
TBA: trenbolone acetate
TGF-beta3: transforming growth factor beta-3
US-FDA: United States-Food and Drug Administration
VPC: Veterinary Products Committee
WHO: World Health Organization
WTO: World Trade Organization
ZER: zeranol

mg: milli-gram
µg: micro-gram
ppb: parts per billion
ppt: parts per trillion
nM: nanomole per liter

1. BACKGROUND

1.1. Previous opinions

The Scientific Committee on Veterinary Measures relating to Public Health (SCVPH), issued an opinion[1] in April 1999. The major conclusions were:

- "As concerns excess intake of hormone residues and their metabolites, and in view of the intrinsic properties of hormones and epidemiological findings, a risk to the consumer has been identified with different levels of conclusive evidence for the 6 hormones in question.

- In the case of 17-β oestradiol there is a substantial body of recent evidence suggesting that it has to be considered as a complete carcinogen, as it exerts both tumour initiating and tumour promoting effects. The data available does not allow a quantitative estimate of the risk.

- For the other 5 hormones, in spite of the individual toxicological and epidemiological data described in the report, the current state of knowledge does not allow a quantitative estimate of the risk.

- For all six hormones endocrine, developmental, immunological, neurobiological, immunotoxic, genotoxic and carcinogenic effects could be envisaged. Of the various susceptible risk groups, prepubertal children is the group of greatest concern. Again the available data do not enable a quantitative estimate of the risk.

- In view of the intrinsic properties of the hormones and in consideration of epidemiological findings, no threshold levels can be defined for any of the 6 substances."

The Committee was asked to review its opinion in the light of the scientific information published after 30 April 1999 and in particular:

- the report of December 1999, from the Committee on Veterinary Medical Products (CVMP) "opinions and summary reports on 17β-oestradiol and progesterone for zootechnical and therapeutics purposes"

- the October 1999 report of the UK's Veterinary Products Committee (VPC) sub-group on the SCVPH opinion of 30/04/99

- the 1999 re-evaluation of some hormones by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) for Codex Alimentarius.

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[1] Opinion of the Scientific Committee on Veterinary Measures relating to Public Health on "Assessment of potential risks to human health from hormone residues in bovine meat and meat products, adopted on 30 April 1999. http://europa.eu.int/comm/food/fs/sc/scv/outcome_en.html

In its opinion of 3 May 2000[2], the Committee concluded that none of the new information would lead it to amend its previous opinion as the information submitted "did not provide convincing data and arguments demanding revision of the conclusions drawn in the opinion of the SCVPH of April 30th 1999".

1.2. The 17 studies

Early in 1998, the Commission launched 17 specific studies to address certain gaps in research identified by the Commission following the ruling of the WTO Appellate Body concerning the scientific basis of the EC import ban on meat and meat products and animals treated with hormones for growth promotion purposes. The studies addressed toxicological and carcinogenicity aspects, residue analysis, potential abuse and control problems, environmental aspects, etc. on six hormones (oestradiol 17-β, progesterone, testosterone, zeranol, trenbolone acetate and melengestrol acetate and their metabolites for growth promotion purposes).

The Committee was kept informed of progress in the studies, as results became available.

All the studies have now been completed, final reports have been submitted to the Commission and a substantial number of them have been the subject of publications in peer reviewed scientific journals.

2. MANDATE

The Scientific Committee on Veterinary Measures relating to Public Health is asked to review and, if appropriate, amend its previous opinions on the potential risks to human health from hormone residues in bovine meat and meat products in the light of the studies and taking into account recent scientific data from any source.

The Committee is asked to give its advice as soon as is reasonably possible, preferably by the Plenary meeting of April 2002.

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[2] Opinion of the Scientific Committee on Veterinary Measures relating to Public Health on "Review of specific documents relating to the SCVPH opinion of 30 April 99 on the potential risks to human health from hormone residues in bovine meat and meat products, adopted on 3 May 2000. http://europa.eu.int/comm/food/fs/sc/scv/outcome_en.html

3. INTRODUCTION TO THE PRESENT OPINION

The risk assessment of endogenous compounds, like steroid hormones, is a matter of controversial debate. Hormones fulfil an essential and indispensable role in the maintenance of vital body functions, including sexual development, maturation and reproduction. Detrimental effects are the consequence of imbalances that might not only be attributed to organ specific diseases but also to physiological alterations during the life cycle of vertebrates. The delicate balance in the endocrine regulation of virtually all body functions makes it even difficult to classify certain effects; for example, the obvious protective effect of oestrogens against cardiovascular diseases that contrasts with the increased risk of hormone dependent cancers.

The previous opinion of the SCVPH of April 30th, 1999, aimed to provide an integrated approach towards the different aspects of risk assessment. It followed not only the established pathways of standardised risk assessment procedures for chemicals which might contaminate food commodities, but also provided an overview of the emerging concerns related to hormonally active substances. This approach is clearly different from the institutionally applied risk assessment procedure and was inspired by the definition presented by the WTO Appellate Body in stating that a risk to be evaluated is "not only risk ascertainable in a laboratory operating under strictly controlled conditions, but also risk in human societies as they actually exist, in other words the actual potential of adverse effects in human health in the real world where people live and work and die".

In considering these various aspects, the SCVPH stated (see page 2/3 of 1999 opinion) that for the steroid hormones (and the synthetic hormonally active substances) the potential risk should be discussed in the view of:

- "general concerns related to hormonally active substances evaluating the potential effects of endogenous and exogenous hormone exposure at all stages of life;

- factors affecting the outcome of exposure to hormones during life span;

- hormonal and non-hormonal toxicological effects of endogenous and exogenous hormones and metabolites thereof, taking into account the present *state of art* in the understanding of biotransformation mediated genotoxicity".

As mentioned above in the mandate, the present opinion should review the previous opinions on the potential risks to human health from hormone residues in bovine meat and meat products in the light of the 17 studies initiated by the Commission and any other scientific data that have become available recently.

The 17 studies were launched early in 1998, prior to the initial SCVPH Opinion of April 1999 and provide an independent scientific view rather than addressing specific items identified in the SCVPH Opinion. Following the mandate given to SCVPH, the results of these 17 studies (an overview of these studies is provided in an Annex to this report) will be discussed in the light of the major conclusions presented in the Opinion of April 30th 1999.

4. RECENT SCIENTIFIC DATA RELATING TO THE POTENTIAL RISKS TO HUMAN HEALTH FROM HORMONE RESIDUES IN BOVINE MEAT AND MEAT PRODUCTS

4.1. Recent data on exposure

Consumers are exposed to various levels of endogenous hormones in bovine meat and meat products and other animal derived products, including milk and eggs. The level of exposure is determined by individual consumption habits as well as by the variability in the endogenous hormone levels in slaughter animals and animal derived products (for review see WHO Food Series 43; FAO Food and Nutrition 41/12, 2000).

Data obtained in the frame of the 17 studies add to this information as follows:

4.1.1. Analytical techniques

A new method to detect trace amounts of hormones in meats was developed comprising a multi step extraction/enrichment followed by derivatisation and analysis by gas chromatography linked to mass spectrometry (**study 6/7**). This method has a detection limit of 2-10 ppt. The details of this method have been published (Marchand *et al.*, 2000; Le Bizec, 2000). A different method based on HPLC analysis with electrochemical detection and with a limit of detection of 50 ppt has been developed in a comparable study and been applied in a small number of at random samples of bovine liver and meat from the United States (U.S.) market (**study 8**). The analyses included conjugated metabolites. Despite a number of positive analytical results in this study, the low number of samples does not allow a qualified validation of typical characteristics such as sensitivity, specificity, accuracy and reproducibility (**study 1, study 8**).

4.1.2. Bioassays for screening and determination of oestrogenic potency of substances used as growth promoters

Three complementary bioassays involving different recombinant-DNA technology were tested for their suitability to screen known oestrogenic and androgenic compounds (**study 9**). In the first, rainbow trout oestrogen receptors and human oestrogen receptors were expressed in yeast. The second involved the expression of vitellogenin in rainbow trout hepatocyte culture. The third has to be considered as a reference method and involved the use of the Ishikawa endometrial cells expressing a natural oestrogen-inducible alkaline phosphatase gene. The sensitivity of the different assays varied significantly from very sensitive (Ishikawa cell line) to rather insensitive (yeast and rainbow hepatocytes). (**study 9**)

The obtained results suggest that the use of recombinant yeast and rainbow trout hepatocytes to detect oestrogenic compounds is not justified in view of their lack of sensitivity.

4.1.3. Bovine metabolism of 17β-oestradiol and quantitative analysis of oestrogen residues in edible tissues from treated steers

In an exposure assessment, not only residues of the parent compound need to be evaluated but also the exposure to biologically active metabolites. Previous studies on the biotransformation of oestradiol focussed on major, readily extractable metabolites. Hence, additional experiments were conducted with ^{3}H-labelled 17β-oestradiol (17β-E_2) to re-assess the bovine biotransformation by applying sophisticated analytical procedures (**study 3**).

In these studies and in accordance with earlier studies, it was shown that in muscle tissue and fat of cattle treated with ^{3}H-labelled 17β-E_2 the major residues were identified as 17β-E_2 and oestrone, whilst 17α-E_2 was found to be a minor metabolite. In contrast, in kidney and liver tissues 17α-E_2 was the major metabolite and 17β-E_2 and oestrone were minor components. These analyses included both free compounds and glucuronic- and glycoside conjugates. No catechol metabolites were detected. A considerable part of the residues seemed to comprise non-extractable, protein-bound metabolites of unknown structure.

These data were supported by *in vitro* studies using bovine liver and kidney slices. In these studies the formation of lipoidal esters (25-40% saturated fatty acid esters, see also Larner *et al.*, 1985) was demonstrated. Subsequently, the lipoidal esters were also isolated from adipose tissue and found to account for 5-75% of total oestradiol residues in fat and 1-18% in liver and kidney, respectively.

In a preliminary validation experiment, four groups of five steers were treated with commercial formulations of growth promoters to study residue disposition *in vivo*. The first group served as untreated control, the second group received one implant of Revalor®-S (24 mg 17β-E_2 +120 mg Trenbolone) the third group received two implants of Revalor®-S (at the beginning and again on day 45), and the fourth group received four implants of Revalor®-S at the beginning of the experiment. Animals were slaughtered on day 90 and muscle meat, fat, liver and kidneys were analysed for oestradiol and its metabolites (Maume *et al.* 2001).

The results indicated considerable increases of oestrogen residue levels in all tissues of treated animals, with a clear dose-response. Levels of 17β-E_2 increased up to 7-fold, lipoidal esters increased up to 20-fold in fat, but not in the liver.

Levels of 17α-E_2 were not significantly increased in muscle tissue and fat, but were found to be up to several hundred times increased in liver and kidneys of treated animals.

In conclusion, these data indicate that lipoidal esters form a sizeable part of the total residue level and thus may contribute considerably to an additional oestrogen exposure via meats. Lipoidal esters have neither been included in previous analysis of oestrogen residues, nor in any exposure assessment study.

4.1.4. Multiple implanting, multiple dosing

Multiple implanting occurs in daily routine of cattle husbandry, despite various guidelines defining the principle of Good Veterinary Practice (GVP). As all commercial growth promoters are over-the-counter products, correct implantation can neither be guaranteed nor expected. This also implies that implants might not be positioned correctly (commonly the subcutaneous application in the ear-region is recommended) and thus might be missed during post-mortem meat inspection. This disregard for the requirements as defined by GVP was simulated in a study in which 71 heads of cattle were treated in typical, but non-approved dose regimes (**study 5**). These studies revealed not only noticeable irritations at the injection sites following misplacing of implants, but also very high residual amounts of active substances such as oestradiol, trenbolone and zeranol at these injection sites. The authors conclude that melengestrol acetate applied in concentrations exceeding the licensed doses by a factor of 3 would result in a violation of the tolerance levels as proposed by US-FDA.

Model calculations indicated that, depending on the actual implanted total dose, processing of such injection sites can contaminate tons of (minced) meat or meat products with hormone concentrations violating the ADI/MRL levels as proposed by JECFA and other regulatory bodies. Assuming that a non-removed injection site reaches one individual meat portion of 250g (the probability is according to the authors in the order of 1:500 – 1:1000) the hormone concentration in this portion may even induce acute hormonal effects in a consumer.

If injection sites are removed as being unfit during inspection and/or cutting of meat, the discharged tissues may be used for pet food production with similar undesirable consequences.

Overdosing might also be considered with respect to melengestrol acetate (MGA). MGA is added to the feed of heifers at a dose of 0.5 mg/day to promote growth. A recent study investigated the levels of MGA in the plasma and muscle, kidney, liver and fat after feeding MGA for 8 weeks at doses of 0, 0.5, 1.5 or 5.0 mg/day; each group contained 2 heifers (Daxenberger *et al.*, 1999). Another group of heifers fed MGA at 0.5 mg/day was slaughtered 48 hrs after the last dosing to determine the effects of withdrawal (Hageleit *et al.*, 2000). MGA levels in plasma were determined using an enzyme immunoassay (Hageleit *et al.*, 2001). Concentrations in kidney, liver and muscle were determined by LC-MS and concentrations in fat by GC-MS (Daxenberger *et al*., 1999). MGA levels in plasma followed a dose-response increase and were 40, 128, and 280 ng/L after dosing at 0.5, 1.5 and 5.0 mg/day, respectively. Among the liver, kidney and muscle tissues, increased levels were detected at the two highest doses. However, the greatest increases were observed in fat, which at a dosing level of 1.5 mg/day reached the level of 29 μg/kg. The authors indicated that this level exceeds the USA regulatory limit of 25 ppb.

In conclusion, these experiments clearly identify a risk for excessive exposure of consumers to residues from misplaced or off-label used implants and incorrect dose regimes. In these cases, levels of oestradiol and its

metabolites in muscle, fat, liver and kidney from hormone treated cattle may be 2-fold up to several hundred folds higher as compared to untreated meat. The level of increase depends on the treatment regime and the actual hormone levels in the implants used.

Multiple applications need to be considered as unavoidable under practical conditions. This practice is also advertised (NebGuide Beef Cattle Update 1997, see also http://www.ext.nodak.edu/extpubs/ansci/beef/as1178w.htm) and even recommended in scientific publications (for example: Duckett *et al.*, 1999; Paisley *et al.*, 1999, Rumsey *et al.*, 1999, Smith *et al.*, 1999) to achieve optimal economic results. Therefore, these data have to be considered in any quantitative exposure assessment exercise.

4.1.5. Oestrogenic potencies of residues

In consideration of the studies of the metabolism of oestradiol, 17β-E_2 and its metabolites were evaluated with regard to their oestrogenic activity in a traditional *in vivo* assay (uterotrophic effect in rats following oral administration)**(study 3)**.

The obtained results indicate that the potency of 17α-E_2 is approximately 10% of 17β-E_2. However, the potency of the lipoidal esters exceeded the effect of 17β-E_2 in the *in vivo* assay by approximately a factor of 10 (Paris *et al*., 2001). Furthermore, lipoidal esters appear to have an even higher effect on the mammary gland in experimental animals (Mills *et al*., 2001). The high potency of lipoidal esters after oral applications might be explained by the fact that they reach systemic circulation via the lymphatic system, as suggested by preliminary data. These findings warrant further investigation, as a high bioavailability of biologically active lipoidal esters and the possibility of accumulation (Larner *et al*., 1985) might contribute significantly to an undesirable exposure to oestrogenic substances. The impact of residual protein bound non-extractable oestrogen remains to be elucidated.

In conclusion, it has to be stated that lipoidal esters of oestradiol add to the oestrogen exposure, as mentioned above. While the oral bioavailability of these metabolites was high in animal experiments, no information is available on the oral bioavailability in humans following dietary exposure via contaminated meat products.

4.1.6. Alteration of gene expression by oestrogenic compounds

The aim of the study was to assess to what extent human breast cancer MCF7 cells are induced to express endogenous oestrogen-regulated genes when challenged with natural and synthetic hormones (**study 17**).

A comparison of the inductive effects of oestradiol, DES, zeranol and genistein was made on the expression pattern (through a competitive PCR method) of six genes (PS2, TGF-beta3, mono-amino oxidase, MRG1, ATB0 and GSTμ3). The sensitivity of these markers varied significantly depending on the oestrogen used and the concentrations applied.

Of particular interest is the finding that oestrogens down-regulate the expression of GSTμ3, a member of the phase-two biotransformation enzymes that is involved also in the protection of cells against DNA damage by free oxygen radicals.

4.2. Recent findings on the mutagenicity and genotoxicity of 17β-oestradiol

The pro-carcinogenic effect of 17β-oestradiol was initially considered to be receptor-mediated. Recently evidence is accumulating that 17β-oestradiol also possesses mutagenic and genotoxic activities. Already in the previous Opinion a variety of studies were cited indicating that 17- β oestradiol (E_2) as well as oestrone (E_1) can be converted into catechol oestrogens. These catechol oestrogens may be inactivated by COMT-mediated O-methylation, or be conjugated to glucuronic acid of sulfate conjugates. However, in an alternative pathway, catechol oestrogens might be oxidized to form semiquinones and quinones. E-3,4-quinone, if not inactivated, reacts with DNA to form depurinating adducts (N7-guanidine and N3-adenine adducts). Particularly the N7-guanidine adducts are considered to play a crucial role in the initiation of oestrogen-dependent tumours (Cavalieri *et al*., 2001; Devanesan *et al.*, 2001; Lavigne *et al.*, 2001; Sasco, 2001; Cavalieri *et al*., 2002,).

While the oestrogenic activity of 17α-E_2 is weaker compared to 17β-E_2, metabolic activation to genotoxic metabolites occurs to a similar extent for both. Catechol metabolites of both, 17β-E_2 and 17α-E_2, form stable DNA adducts *in vitro* as assayed by (^{32}P-post-labelling DNA adduct analysis. However, in a microbial mutagenicity assay and an assay detecting DNA damage in mammalian cells (Comet assay) no genotoxic activity was observed for 17β-E_2, 17α-E_2 or the respective catechol derivatives **(study 3)** (Hoogenboom *et al*., 2001).

More recently, Terashima *et al.* (2001) reported that by conducting site-specific mutagenesis with oligodeoxyribonucleotides containing either the 2-hydroxyoestrogen-6-yl-N^2–deoxyguanosine or 2-hydroxyoestrogen-6-yl-N^6–deoxyadenosine, mutations were induced in simian kidney (COS-7) cells. With the deoxyguanosine adducts, G → T transversions were detected. With the deoxyadenosine adducts, A → T transversions and a few A → G transitions were detected.

Somatic mutations were also induced in Syrian hamster embryo cells at the Na^+/K^+ ATPase and/or *hprt* loci following treatment of the cells with 4-hydroxyoestradiol, 4-hydroxyoestrone or 2-methoxyoestrone for 48 h. Some of the oestrogen metabolites induced chromosomal aberrations in the cells following treatment for 24 h (Tsutsui *et al.*, 2000a).

In addition, treatment of Syrian hamster embryo cells with 0.1 or 0.3 μg/mL 2-methoxyoestradiol for 48 h induced a statistically significant increase in the frequency of somatic mutations at the Na^+/K^+ ATPase and/or *hprt* loci. Treatment of the cells with 2-methoxyoestradiol for 24h, induced chromosomal aberrations (Tsutsui *et al.*, 2000b).

The ability of oestradiol, oestrone and their catechol oestrogens to form DNA adducts in Syrian hamster embryo fibroblasts was studied by using the ^{32}P-postlabeling method. (Yagi *et al.*, 2001). DNA adducts were detected in cells treated with each of the catechol oestrogens at concentrations of 10 μg/mL for 1 h or more. The 2- and 4-hydroxyoestradiol each formed a single adduct, whereas 2- and 4-hydroxyoestrone each formed two adducts (Yagi *et al.*, 2001).

Furthermore, oestradiol significantly increased the mutation frequency of the *hprt* gene in Chinese hamster V79 cells by 2.57-8.78- fold compared to controls, at both, physiological and pharmacological concentrations (10^{-11}, 10^{-10}, 10^{-7}, and 10^{-6} M) (Kong *et al.*, 2000). Polymerase chain reaction (PCR) amplification of the DNA and molecular analysis of the *hprt* coding sequence identified genetic lesions in 15 of 21 colonies selected after treatment of the cells with 10^{-10} M oestradiol. Base substitutions, such as T $\rightarrow$ G or T $\rightarrow$ A transversions were the most common mutations (38%) and frequently occurred at 122 bp or 407 bp of the *hprt* coding sequence. Deletion mutations were detected in 29% of the clones. Transition mutations and a four-base insertion were also identified, each in one mutant clone. The inability of the anti-oestrogen ICI 182,780 to inhibit the induction of mutations by oestradiol demonstrates that the mutagenic effects of oestradiol in V79 cells were not mediated through oestrogen receptor-α.

Chakravarti *et al.*, (2001) examined whether an abundance of abasic sites induced by depurinating DNA adducts causes DNA repair infidelity. The dorsal skin of female SENCAR mice was treated with 200 nmole of oestradiol-3,4-quinone, and the DNA adducts formed after 1 h were analysed. In addition the spectra of mutations in the Harvey (H)-*ras* oncogene were determined between 6 h and 3 days after treatment. Oestradiol-3,4-quinone formed more than 99% depurinating adducts, approximately equal amounts of the rapidly-depurinating 4-hydroxyoestradiol-1-N3adenine and the slower-depurinating 4-hydroxyoestradiol-1-N7guanine. Between 6 h and 3 days, oestradiol-3,4-quinone induced abundant A $\rightarrow$ G transition mutations in H-*ras* DNA, frequently in the context of a 3'-G residue. Since G-specific mutations were infrequently found, it appears that the slow rate of depurination of the N7guanine adducts during active repair may not generate a threshold level of G-specific abasic sites to affect repair fidelity.

In summary, additional and conclusive data have now been published in the scientific literature to demonstrate that 17β-oestradiol is genotoxic. 17β-oestradiol induces mutations in various cultured mammalian cells. The reactive metabolite, oestradiol-3,4-quinone, also induces mutations in mouse skin *in vivo*. The catechol oestrogen-quinones form DNA adducts in cultured cells and in mouse skin.

4.3. Recent findings on the biological effects of testosterone and progesterone

The biological effects of testosterone and progesterone have recently been reviewed (WHO Food Additives Series 43). The primary scope of this report

was to summarise the data referring to the individual use of these compounds in veterinary medicinal products. However, in formulations used for growth promotion, testosterone and progesterone are only present in formulations also containing 17β-oestradiol. Subsequently, this report included the description of several assays in which genotoxic effects of 17β-oestradiol were demonstrated (see also chapter 4.2. above). The endpoints of these assays included mutations in mammalian cells, DNA damage and DNA adduct formation in animals and mammalian cells, as well as micronucleus formation and sister chromatid exchange in animals. However, there is no evidence that progesterone or testosterone have genotoxic potential.

4.4. Recent findings on the biological effects of zeranol and trenbolone

4.4.1. Biotransformation

The biotransformation of trenbolone acetate and zeranol were studied in *in vitro* systems including liver microsomes from humans, untreated bovines, and untreated and arochlor-induced rats, as well as bovine and rat liver slices (**study 4**).

With respect to trenbolone, the metabolism of both α- and β-trenbolone was studied and the metabolites were analysed by gas chromatography/mass spectrometry. Seven monohydroxylated products were identified, but α-trenbolone was less readily metabolised than β-trenbolone. The oxidative metabolites of trenbolone were chemically stable. However, differences were observed in the extent of conjugation of the various metabolites. Human liver microsomes significantly converted the weakly androgenic α-trenbolone to the strong androgen β-trenbolone.

Microsomal metabolism of zeranol gave rise to zearalanone and β-zearalanol, as well as five novel monohydroxylated products, identified by gas chromatography/mass spectrometry. Some of these metabolites were very susceptible to auto-oxidation.

In conclusion, this study demonstrated that the oxidative and conjugative metabolism of the hormonally active growth promoters trenbolone and zeranol by liver microsomes and/or slices was more complex than previously recognised. Investigation of the *in vivo* metabolism of these growth promoters in bovines and humans would be required in order to exclude possible adverse effects.

4.4.2. Binding to Sex Hormone Binding Globulin/Steroid Binding Protein

It was the aim of the study (**study 10**) to determine whether anabolics and their metabolites compete with natural sex hormones for binding to sex hormone binding globulin (SHBG / SBP). Theoretically, if this were indeed the case, tissues would be deprived of natural hormones that affect the development of sex hormone target organs during diverse stages of development.

The data collected, shows a pattern of binding to SHBG and competition with ^{3}H-testosterone by ethynyl oestradiol, zearanolol alpha and beta, 19-

nortestosterone, trenbolone acetate and 17β - trenbolone, and other natural androgens, not much different from those reported by others. The synthetic compounds did not bind to SHBG in blood plasma with high affinity.

In conclusion, the lack of significant binding of zeranol and its metabolites to SHBG suggest that when present in plasma their effect in brain and other oestrogen target organs is not neutralised by their weak binding to this plasma-borne protein.

4.4.3. Mutagenicity and genotoxicity

A comparative study was designed to determine the potential of the xenobiotic growth promoters zeranol (α-zearalanol), 17β-trenbolone and melengestrol acetate to cause genetic damage in various *in vitro* systems (**study 2**). In this study zeranol did not induce genotoxicity or mutagenicity, as determined by three endpoints: formation of DNA adducts in rat hepatocytes, induction of *lac*I mutations in *E. coli* and induction of *hprt* mutations in cultured V79 cells. At high concentrations, it showed borderline induction of micronuclei in V79 cells. The zeranol metabolites zearanone and β-zearalanol also induced low levels of micronuclei in V79 cells.

17β-Trenbolone and trenbolone acetate formed very low levels of DNA adducts in cultured rat hepatocytes. 17β-Trenbolone did not induce *lac*I mutations in *E. coli* or *hprt* mutations in V79 cells, but both 17β-trenbolone and its metabolite trendione induced a very low level of micronuclei in V79 cells. 17β-Trenbolone was found to inhibit cell proliferation, probably arresting cells in the G2 phase. This inhibition prevents adequate testing of 17β-trenbolone by these assays (Metzler and Pfeiffer, 2001).

4.5. Recent findings in the biological effects of melengestrol acetate

In the previous Opinions in 1999/2000 it was stated that the information available to the public on melengestrol acetate (MGA) is scarce. Recently, with the WHO Food Additives Series 45 (corresponding to the 54th meeting in 2000 of the JECFA, a detailed report on MGA was published. It provided a comprehensive review of the pharmacokinetic/toxicokinetic parameters (adsorption, distribution, metabolism and excretion) and toxicological properties of MGA in various species. No original data are presented in this review and the majority of the references are to reports that have not been published in the peer-reviewed scientific literature.

Recent investigations adding to this report can be summarised as follows:

4.5.1. Recent data on the biotransformation of MGA

Commercial MGA consistently contains several chemical impurities at an estimated level of about 5%. These represent decomposition products, apparently reflecting an inherent instability of the parent compound (WHO Food Additive Series 45). In a recent study on the metabolism of MGA in rat, bovine and human liver microsomes **(study 4)** using a HPLC-based detection method, incubations with microsomes from arochlor-induced rats revealed 12 distinct metabolites. No indication was given as to whether this

was a reproducible finding. When using HPLC/MS, peaks 1-5 were identified as dioxygenated MGA and peaks 6-12 as monooxygenated MGA.

Hepatic microsomes from untreated rats, bovines and humans biotransformed MGA into three major metabolites (corresponding to peaks 6, 7 and 10) and several minor metabolites. The major metabolites were identical in all three species, but formed in different proportions by each microsome preparation. These major metabolites were identified as monooxygenated MGA. Their subsequent incubation with the arochlor-induced rat microsomes resulted in their conversion to metabolites corresponding to the peaks observed in the first study (peak 6 was metabolised to peaks 1, 2 and 5, peak 7 to peaks 2 and 3; peak 10 was not metabolised to detectable products).

MGA metabolism was also studied in rat and bovine liver slices, although no original data were shown **(study 4).** The authors stated that in both *in vitro* systems the same spectrum of metabolites could be observed, with the rat liver slices being more active than the bovine preparations. Metabolites represented by peaks 1, 3, 6 and 7 but not 10 observed in the microsome studies were detected.

In summary, these preliminary data indicated that the metabolism of MGA is more complex that previously assumed, but further experiments should verify the specific metabolite pattern in target animal species as well as man.

4.5.2. MGA-binding to Sex Hormone Binding Globulin/Steroid Binding Protein and the human androgen and progesterone receptors

The results from two studies have become available and have been published on the binding of MGA to sex hormone binding globulin (**study 10**). Bauer *et al.* (2000) reported that a 2,500-fold excess of MGA displaced approximately 75% of radioactively labeled dehydrotestosterone (DHT) from human sex hormone binding globulin. To put this into perspective, the relative displacement of radioactive DHT was 100% by DHT, 99.5% by testosterone, 54% by progesterone, and 70% by 17β-trenbolone. These results show that MGA has some sex hormone binding globulin potential. However, no indication of the reproducibility was provided, nor were concentration response curves developed.

Additional results on the binding of various growth promoters to trout steroid binding protein (SBP) and to human SBP were provided by **study 10**. The data were expressed as relative inhibition of testosterone binding at concentrations of 10^{-5} M and as ED_{50} values in M/L. The ED_{50} for MGA binding to the trout SBP was $5x10^{-7}$, and for human SBP, $1x10^{-5}$ M/L. For binding to the trout SBP, this compared with ED_{50} values of 10^{-5} for DES, $4x10^{-9}$ for 17β-oestradiol and $6x10^{-9}$ for testosterone. For the human SBP, this corresponds to ED_{50} values of $>10^{-5}$ for DES, $2x10^{-7}$ for 17β-oestradiol, and $1x10^{-8}$ for testosterone. However, as no indication was provided regarding the reproducibility of these results, data have to be considered as preliminary.

Bauer *et al.* (2000) determined the relative binding affinity (RBA) to the recombinant human androgen receptor (rhAR) and to the bovine progesterone receptor (bPR) of various compounds **(study 5)**. The RBA to rhAR for MGA was 0.31% and that of the metabolites 6, 7 and 10 (provided by contractors of study 4) were each <1.30%. This compared with RBAs to rhAR of 100%, 109%, 31% and 4% for DHT, 17β-trenbolone, testosterone and progesterone, respectively. These findings suggest that MGA has weak AR binding potential. The RBA for binding to the bPR for MGA was 526% of that of progesterone itself. MGA metabolites 6, 7 and 10 had bPR RBAs of 85, 46, and 28%, respectively. This compared with bPR RBAs of 1.4%, 137%, and 1.2% for DHT, 17β-trenbolone, and testosterone, respectively.

In summary, these results demonstrate that MGA has a very strong potential to bind to bovine progesterone receptors, although these data need further verification.

4.5.3. Mutagenicity and genotoxicity of MGA

The genotoxicity of MGA was investigated, by assaying V79 cells for the induction of mutations at the HPRT locus and the induction of micronuclei, and by measuring the induction of LacI mutations in *E. coli*. (**study 4**). The results were negative in several experiments using concentrations of either 50-125 μM for HPRT mutations, 20-100 μM for micronuclei induction, and 400 μM for LacI mutations. In preliminary experiments with rat liver slices *in vitro* MGA was shown to induce covalent DNA adducts by means of ^{32}P-postlabelling analysis (Kranz *et al.*, 2002). Using V79 cells, it was observed that MGA caused the appearance of apoptotic cells in a concentration- and time-dependent manner. However, HPLC analysis of MGA revealed that commercial MGA contained a number of impurities. In subsequent studies it was found that pure MGA did not induce apoptosis whereas a mixture of the impurities was active (**study 4**; Metzler and Pfeiffer, 2001). These results indicate that pure MGA is not pro-apoptotic.

5. RECENT DATA ON ENDOCRINE AND DEVELOPMENTAL EFFECTS OF HORMONES

Additional studies were conducted aimed at elucidating the effects of hormones and hormonally active substances on the development of the reproductive system.

5.1. Experimental studies in rabbits

The effects of zeranol (ZER), trenbolone acetate (TBA) and melengestrol acetate (MGA) on the development of the reproductive system and differentiation of testicular germ cells in the rabbit were investigated (**study 11**). This study provides an animal model for the effect of prenatal and later exposure to hormones on cryptorchism and testicular dysgenesis, which in humans are strong risk factors for testicular germ cell cancer and more generally for testicular dysgenesis syndrome. Rabbits were chosen because of a long juvenile development, ease of collecting samples (blood and semen) and natural propensity to developing testicular tumours.

The exposure protocols comprised an unexposed control group, a zeranol treated group (gestational and lactational exposure by sub-cutaneous implants at 0.25 mg/kg b.w. once a month). 6 additional groups were treated with either trenbolone acetate or melengestrol acetate, differentiating between (a) gestational and lactational exposure, (b) adolescent exposure, and (c) adult exposure, respectively. Trenbolone acetate was administered by subcutaneous injection in safflower oil weekly at 0.5 mg/kg b.w., and melengestrol acetate was dissolved in corn syrup and given daily *per os* at 0.5 mg/kg b.w.).

The authors summarised the results as follows:

- Cryptorchism occurred only in isolated cases, after exposure to TBA or ZER.

- An adult exposure to MGA increased mean total body weight, including the weight of liver and kidney, whereas early post-natal treatment with ZER resulted in a transient decrease in the body weight, with later catch-up gain.

- Testicular weight was slightly affected after pre- and peri-natal treatment: MGA caused a decrease in the weight, whereas TBA resulted in a slight increase.

- Spermatogenesis, as assessed by testicular histology and a score for the relative degree of germinal epithelial loss (DGEL score) was only slightly affected, primarily in the MGA treated adults. The average DGEL scores in other groups were increased because of the presence of isolated cases of cryptorchid testes.

- No testicular tumours or adenomas were observed. However in the foetal testes in animals that were exposed *in utero* to any of the three synthetic hormones, the proportion of gonocytes expressing a germ cell marker, PG-2 was increased. The structure and function of this antigen is not known, thus the biological significance of this observation is not clear, but chronological changes in germ cell differentiation are suspected.

- Exposure to MGA or ZER during early adolescence caused a transient decrease in serum gonadotrophin levels with a significant increase in oestrone.

- Animals exposed to any of the three hormones during gestation had lower serum concentrations of testosterone later in life, however their sperm concentrations were not significantly affected.

The authors conclude that: "the results of the investigations indicate that prenatal exposure to low doses of synthetic hormones (MGA, TBA and ZER) may affect the function of male reproductive tract in rabbits, although the effects are not as severe as those observed after exposure to the high doses. The effects are most pronounced if the exposure to these synthetic hormones has occurred early in life. All three compounds readily cross the placental barrier and accumulate to a variable degree in foetal tissues. The

effects of ZER and TBA are more severe than the effects of MGA in animals exposed during development, however MGA has marked effects on spermatogenesis when administered in adults. An exposure to synthetic hormones, primarily during development, but also later in life can influence the reproductive hormones, although this does not significantly affect spermatogenesis. Although it is not known to what extent these observations can be extrapolated to humans, a caution concerning the use of these compounds is advised".

5.2. *In utero* exposure and breast cancer: a study of opposite sexed twins

The hypothesis investigated in this study is that having a male co-twin leads to increased *in utero* exposure to oestrogens, the latter being also reflected in birth weight. (Kaijser *et al.*, 2001).

The study was based on the Swedish Twin registry for twins born between 1926 and 1967 (**study 13**). It is therefore population-based and unbiased. Linkage is done with the cancer registry, for breast cancer occurring between 1972 and 1995. The inclusion criteria were therefore such that some early breast cancer arising in the older cohorts may have been missed. The study was also restricted to twins with a reported gestational age of at least 33 weeks. The main result of this study is a very strong effect of birth weight on the incidence of breast cancer with an odds ratio of up to 12 for a birth weight of more than 3500 g compared to a birth weight of less than 2000 g.

The authors conclude, "that among female twins with male co-twins, high birth weight constitutes a strong independent risk factor for breast cancer. This study gives further credence to the hypothesis that breast cancer risk is influenced by hormonal exposures *in utero*." (Kaijser *et al.*, 2001).

In addition, a retrospective study on long-term effects in children following suspected exposure to oestrogen-contaminated meat has been presented (**study 12**). The study is based on an incident in a school in Milan, Italy, where an outbreak of gynaecomastia had occurred for unknown reasons. Exposed subjects tended to have reached an earlier puberty and less often had children. One case of testicular seminoma was found in an exposed subject. The clinical exams revealed more virilisation among exposed women. Oestradiol and progesterone levels were lower in the exposed individuals. Among men, those exposed had slightly lower testicular volume. Other parameters, including socio-economic factors and incidence of chronic illnesses were equivalent. As the reason for this incident remains unknown, the relevance of these data remains unclear.

In conclusion, these three studies suggest that *in utero* or pre- and peripubertal exposure to hormones (including animal evidence on synthetic products) may affect pubertal development. The epidemiological studies with opposite sexed twins indicate that prenatal exposure to hormones may be linked to adult cancer risk.

6. IMPACT OF THE EXTENSIVE USE OF HORMONALLY ACTIVE COMPOUNDS ON THE ENVIRONMENT

The potential endocrine disrupting activity of anabolic steroids used in beef production has not been addressed in the previous Opinions of the SCVPH. The emerging concerns, related to compounds generally referred to as Endocrine Disruptors, stimulated investigations towards the potential hazards related to the extensive use of hormones in beef production. Thus, the results obtained in the frame of the 17 studies will be presented briefly in Annex 1.

It has to be acknowledged that the Scientific Committee on Toxicity, Ecotoxicity and the Environment (CSTEE) has issued an Opinion in 1999 on "Human and wildlife health effects of endocrine disrupting chemicals, with emphasis on wildlife and on ecotoxicology test methods".

7. GENERAL CONCLUSIONS

The review of the 17 studies launched by the European Commission and a recent scientific literature allows the following conclusions:

- Ultra-sensitive methods to detect residues of hormones in animal tissues have become available, but need further validation.

- Studies on the metabolism of 17β-oestradiol in bovine species indicate the formation of lipoidal esters, disposed particularly in body fat. These lipoidal esters show a high oral bioavailability in rodent experiments. Thus, the consequence of their consumption needs to be considered in a risk assessment.

- Experiments with heifers, one of the major target animal groups for the use of hormones, indicated a dose-dependent increase in residue levels of all hormones, particularly at the implantation sites. Misplaced implants and repeated implanting, which seem to occur frequently, represent a considerable risk that highly contaminated meats could enter the food chain. There is also a dose-dependent increase in residue levels following the oral administration of melengestrol acetate at doses exceeding approved levels, with a corresponding increased risk that contaminated meats could enter the food chain.

- Convincing data have been published confirming the mutagenic and genotoxic potential of 17β-oestradiol as a consequence of metabolic activation to reactive quinones. *In vitro* experiments indicated that oestrogenic compounds *might* alter the expression of an array of genes. Considering that endogenous oestrogens also exert these effects, the data highlight the diverse biological effects of this class of hormones.

- No new data regarding testosterone and progesterone relevant to bovine meat or meat products are available. However, it should be emphasized that these natural hormones are used only in combination with 17β-oestradiol or other oestrogenic compounds in commercial preparations.

- Experiments with zeranol and trenbolone suggested a more complex oxidative metabolism than previously assumed. These data need further clarification as

they might influence a risk assessment related to tissue residues of these compounds.

- Zeranol and trenbolone have been tested for their mutagenic and genotoxic potential in various systems with different endpoints. Both compounds exhibited only very weak effects.

- Data on the genotoxicity of melengestrol acetate indicate only weak effects. However, pro-apoptotic effects were noted in some cell-based assays, which were attributed to the impurities in commercial formulation. Further experiments should clarify the toxicological significance of these impurities.

- Model experiments with rabbits treated with zeranol, trenbolone or melengestrol acetate, mirroring their use in bovines, were designed to study the consequences of pre- and perinatal exposure to exogenous hormones. All compounds crossed the placental barrier easily and influenced to varying degrees the development of the foetus, at the doses used in the experiments.

- Epidemiological studies with opposite-sexed twins, suggest that the exposure of the female co-twin *in utero* to hormones results in an increased birth weight and consequently an increased adult breast cancer risk.

- Several studies were devoted to the potential impact of the extensive use of hormones on the environment. Convincing data were presented indicating the high stability of trenbolone and melengestrol acetate in the environment, whereas preliminary data were provided on the potential detrimental effects of hormonal compounds in surface water.

In conclusion, after re-appraisal of the data from the 17 studies and recent scientific literature, the SCVPH confirms the validity of its previous O*pinions (in 1999 and 2000) on the Assessment of Potential Risks to Human Health from Hormone Residues in Bovine Meat and Meat Products*, and that no amendments to those opinions are justified.

8. ACKNOWLEDGEMENTS

This opinion of the Scientific Committee on Veterinary Measures relating to Public Health is substantially based on the work of an ad hoc working group of the Committee. The working group included members of the Committees and external experts.

The working group was chaired by

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- Dr Wolfgang PFAU (rapporteur)
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9. ANNEX 1

Anabolic hormones such as oestrogen, androgen, and progesterone are produced in marine and terrestrial vertebrate animals. Animals of the aquatic ecosystems are most sensitive to endocrine disrupters due to the potential for tissue accumulation of lipophilic compounds via water passing the brachia as well as cutaneous exposure. To study the fate of the anabolic steroids and their potential endocrine disrupting activity, one must consider multiple levels of the fate of such compounds regarding both degradation and residual activity. These levels include:

- Metabolism and excretion by the target animal,
- Degradation kinetics during storage of the manure,
- Adsorption to the soil, and
- Chemical and/or microbial degradation.
- Effects on the hormone sensitive fauna of the aquatic ecosystem, and
- Potential oral activity in terrestrial vertebrates including humans.

9.1. Stability of anabolic agents and their metabolites in manure and soils

In a series of experiments **(study 14)** the degradation kinetics of excreted anabolic agents under different manure storage conditions and the fate of such steroids in different soils was studied.

Trenbolone in liquid manure: Following treatment of heifers (these animals were the same as used in the above described study on experimental implanting (see 4.1.4) the concentration of trenbolone in manure canals (liquid manure: mixture of urine, faeces and waste water) was studied. Excreted trenbolone was measured by solid phase extraction, HPLC, and quantification by EIA. In a typical manure sample the concentration of trenbolone-17α (TbOH-17α) was greater than TbOH-17β, and greater than trendione. The limit of detection and of determination was reported for each compound. The actual concentration was determined for TbOH-17α, and the concentrations of the other two residues were then calculated based on the determined recovery rates and cross-reactivity in the EIA assay.

All three trenbolone derivatives showed a parallel course of concentration as a function of collection dates and the total concentration of each hormone paralleled the number of hormone applications/animal/number of animals contributing to the manure mass. The amount of TbOH-17α (4000 pg/g manure) was 22 times the concentration of TbOH-17β (180 pg/g) and 49 times the concentration of trendione (80 pg/g). Analysis of trenbolone residues during the storage of liquid manure were used to calculate half-lives of TbOH-17α (267 days) and TbOH-17β (257 days).

Trenbolone in dung disposals and soil: The concentration of TbOH-17α in solid fresh dung (faeces only; before storage) was 10-15 times greater than in liquid manure. This was even more pronounced for TbOH-17β, with factors of 10-100 fold. The concentration of residues decreased over 4.5 months of storage, yet stored samples still contained amounts up to 10ng/g of TbOH-17α. Small amounts of residues were detected in dung hill effluents. Trenbolone residues were detectable in soil, although at lesser concentrations relative to liquid manure or solid dung. Nonetheless, in soil fertilised with stored manure, trenbolone residues were detected 8 days after spreading on fields. Solubility of TbOH-17β in water was determined to be 21 mg/L. Determination of soil adsorption isotherms indicates a strong accumulation in soil.

These studies demonstrate the presence of trenbolone residues in manure, the concentrations in liquid manure representing 12 percent of the administered dose and in solid dung, 20 percent. The behaviour of the excreted anabolic steroids in soil can be compared to well-documented agricultural or industrial pollutants. In fact, the properties of these residues are similar to those of chlorinated organic compounds and aromatic hydrocarbons. The initial concentration of TbOH-17α in stored manure was about 1.8 ng/g, and reached values up to 14 ng/g in solid dung. Trenbolone residues are washed from the dunghill by rainwater and appear in the dunghill effluent. Melengestrol acetate is detectable in faeces with a recovery rate in faeces of 12 % of the applied dose.

Faecal excretion of melengestrol acetate (MGA) and stability in dung and soil: In faeces, MGA was not detectable by LC-MS in any control animal. MGA was detected in the faeces of MGA-fed animals, with concentrations increasing over time and with dose. At 24 hours post feeding, the concentration in faeces ranged from 2.5 ng/g faeces (1x dose) to 18.5 ng/g (10x dose). In solid dung the range of MGA concentrations was 0.26 to 7.8 ng/g (mean = 2.6). During storage for 135 days, this concentration decreased by 50%. In soil, MGA was detected up to 6 months, with values up to 8 pg/g soil. Assessment of the solubility of MGA revealed a value of 0.09 mg/l. Determination of soil absorption isotherms indicated a strong accumulation in soil.

The mean concentration of MGA in solid dung was 2.6ng/g and after 4.5 months of storage values had decreased only by 50% (1.3 ng/g). In soil fertilised with solid dung, MGA was detected more than 194 days later.

In conclusion, the previous Opinions of the SCVPH did not consider the potential environmental impact of the use of anabolic steroids in beef cattle. The results presented in this report have not yet been published, but appear to be rigorous and appropriately controlled. The presented results indicate that the environmental impact of anabolic steroids is potentially great and further studies to determine the biological and chemical stability of such steroids in soil and water are warranted. In addition, little information is available on the endocrine disrupting potential of the metabolites of MGA.

9.2. Contamination of surface water with effluents from feed lots

Following the results on faecal excretion and stability of MGA and Trenbolone in manure and soil, it is likely that such agents will be present in surface water downstream of cattle farms. This hypothesis is based on evidence that natural oestrogens from human urine and xenooestrogens from detergents and plasticizers are present in river water downstream of sewage outlets. Thus, a study was designed to test the hypothesis that the use of anabolic steroids in beef cattle results in the release of biologically active metabolites into the environment **(study 15**). To assess the potential risk to aquatic ecosystems, water was collected upstream and downstream of feedlots, with the aim to (1) measure the concentration of anabolic agent and natural hormones, and, (2) to assess the hormonal activity of the mixture of chemicals present in the water and (3) the endocrine disrupting potential of such chemical present in water (**study 16**).

9.2.1. Bioassays applying MCF-7 cells

The measurement of biological active contaminants focussed on compounds with oestrogenic and androgenic activity. The E-screen assay, which is based on the proliferation of MCF-7 cells in response to oestrogens was applied. Measurement of androgens was made using the A-screen assay, based on the androgen-mediated decrease in proliferation rates in MCF7-AR1 cells. Both assays are based on the Relative Proliferative Potencies (RPPs) of standard anabolic hormones. The M_{50} value is defined as the concentration that produces an oestrogenic (or androgenic) effect that is 50 percent of the maximal response.

The M_{50} values were established for α-zearalanol (0.13 nM), β-zearalanol (0.6 nM), zearalenone (0.94 nM), zearalanone (0.43 nM). The M_{50} values for androgens were reported to be: α-trenbolone (2.35 nM), β-trenbolone (0.15 nM). Melengestrol acetate (MGA) had neither oestrogenic nor androgenic activity.

Subsequently, water samples from different sites were extracted and analysed.

The results obtained indicate that extracts of water samples from the retention pond and the contaminated site, and the intermediate site had the highest androgenic and oestrogenic activities. Tap water samples and the blank had undetectable levels of activity.

None of the standard anabolic compounds were detected by conventional analytical measures, with the exception of oestrone. The concentration measured by analytical procedures represented only 0.001% of the relative activity as found by the bioassay, pointing toward obvious differences in sensitivity of the biological assays *versus* the analytical methods.

In summary, the presented data show that surface water downstream from a feedlot is contaminated with compounds that have oestrogenic and androgenic activities. The levels of androgens in field water samples exceed the levels of oestrogenic compounds by 3 to 4 fold. A direct link to the use of

anabolic steroids in beef cattle production cannot be established from these data, as herbicides, pesticides, phthalates and other chemicals were found at the reference sites and downstream of the feedlot, which might be equally responsible for the positive results of the bioassays.

9.2.2. Bioassays with aquatic sentinel species

In a complementary study, the **fathead minnow**, a small fish found frequently in river waters, served as an indicator organism. Two affected sites related to cattle feedlots in Nebraska, USA, and designated as Contaminated Site and Intermediate Exposure Site and Far Reference Site, located upstream were studied. Morphometric analysis revealed that in males and females minnows, no significant difference could be noted in fork length and mass among the 3 sites. However, in females, interocular distance was significantly different from contaminated or intermediate exposure sites as compared to the reference site. Males from the reference site had significantly larger testes than those from the other sites and the interocular distance was reduced in male minnows originating from the Contaminated and Intermediate Exposure sites. Histopathological examination revealed no apparent pathology of the ovaries or testes. Assessment of the gonadal steroidogenesis showed no significant differences in gonadal oestradiol or testosterone production. However, when the data from females were expressed as an oestrogen/androgen ratio, an alteration of this index was found as females at the contamination site exhibited a “masculine” sex hormone ratio. In testes of male minnows, the *in vitro* testosterone synthesis in animals originating from the contaminated and intermediate exposure sites was decreased. Hepatic vitellogenin induction was not observed in animals from any site.

In conclusion, this report presents data resulting from a study of a native fish population (fathead minnows), which according to its typical morphology may serve as an indicator organism to detect water contamination. The authors report significant alterations in gonadal steroidogenesis and testes size. Signs of de-masculinisation (lower testosterone synthesis, altered head morphogenesis and smaller testes size) were observed in males, and signs of masculinisation of females (decreased oestrogen/androgen ratio). Yet no specific compounds were identified explaining these alterations.

10. ANNEX 2: OVERVIEW OF THE STUDIES AND PUBLICATIONS IN RELATION TO THEM

Name of all studies and publications in relation to them*.

Title of the study	publications
Presence of estrogen in meat (delivery of samples)	no publications to be done
Hormones as growth promoters: genotoxicity and mutagenicity of Zeranol &Trenbolone	"Genotoxic potential of xenobiotic growth promoters and their metabolites" *(APMIS 109:89-95, 2001)*
Metabolic pathways of estrogens as steroidal growth promoting agents	"Estrogenic activity of estradiol and its metabolites in the ER-CALUX assay with human T47D breast cells" *(APMIS 109: 101-107, 2001)*
Metabolites of melengestrol acetate, trenbolone acetate & zeranol in bovine & humans	"Metabolism of melengestrol acetate and trenbolone"; *(publication foreseen)*
Application of anabolic agents to food producing animals- health risks through disregard of requirements of good veterinary practice	1)"Detection of melengestrol acetate residues in plasma and edible tissues of heifers" *(The Veterinary Quarterly 21: 154-158, 1999*) 2) "Detection of anabolic residues in misplaced implantation sites in cattle" (*Journal of AOAC International 83(4); 809-819, 2000)* 3) "Suppression of androstenone in entire male pigs by anabolic preparations" (*Livestock Production Science- 69: 139-144, 2001*) 4) "A sensitive enzyme immunoassay (EIA) for the determination of Melengestrol acetate (MGA) in adipose and muscle tissues" (*Food Additives and Contaminants 18(4):285-291, 2001)* 5) "Characterisation of the affinity of different anabolics and synthetic hormones to the human androgen receptor, human sex hormone binding globulin and to the bovine progestin receptor" (*APMIS 108: 838-846, 2000)* 6) "Dose-dependent effects of melengestrol acetate (MGA) on plasma levels of estradiol, progesterone and luteinizing hormone in cycling heifers and influences on oestrogen residues in edible tissues" (*APMIS 108: 847-854, 2000*) 7) "Hormone contents in peripheral tissues after correct and off-label use of growth promoting hormones in cattle: Effect of the implant preparations Finaplix-H®, Ralgro®, Synovex-H® and Synovex Plus®" (*APMIS 109: 53-65, 2001)* 8) "Tissue-specific expression pattern of estrogen receptors (ER): Quantification of ERα and ERβ mRNA with real-time RT-PCR" *(APMIS 109: 345-355, 2001)*
Analysis of 500 samples for the presence of growth promoters	"Hormones found in meat samples from regular controls within the EU and from US imports" (*Chemical awareness; issue 9, July5th 2000*)
Analysis of 500 samples for the presence	1)"Ultra trace detection of a wide range of anabolic

*published on the Internet (http://europa.eu.int/comm/dgs/health_consumer/library/press/press57_en.pdf)

of growth promoters	steroids in meat by gas chromatography coupled to mass spectrometry" (*Journal of Chromatography A, 867: 219-233, 2000)* 2)"Le contrôle des anabolisants dans la viande" (The survey of anabolic agents in meat.) (*Annales de Toxicologie Analytique, vol.XII, no.1,2000*
Comparison of assay methods	1)"Frequency and molecular analysis of *hprt* mutations induced by estradiol in Chinese hamster V79 cells" *(International Journal of Oncology 17:1141-1149, 2000)* 2)"Estrogens as endogenous genotoxic agents-DNA adducts and mutations" *(Monographs, JNCI 27: 75-93, 2000)* 3)"Tissue-specific synthesis and oxidative metabolism of estrogens" *(Monographs, JNCI 27: 95-112, 2000)* 4)"Induction of uterine adenocarcinoma in CD-1 mice by catechol estrogens" *(Cancer Research 60: 235-237, January 15, 2000)* 5)"Genotoxicity of the steroidal estrogens estrone and estradiol: possible mechanism of uterine and mammary cancer development" *(Human Reproduction Update, 7 (3): 273-281,2001)*
Bioassay of estrogenic/anti- estrogenic compounds	"Assessment of oestrogenic potency of chemicals used as growth promoter by in-vitro methods" *(Human Reproduction 16: 1030-1036, 2001)*
Interaction of xenobiotics with sex hormone binding globulin;impact on endogenous steroid transport, bioavailability, mechanism of action	scientist has not yet indicated name of journal and publication date
Reproductive sequelae of developmental exposure of rabbits to trenbolone, zeranol & MGA; emphasis on differential & neoplastic transformation of germ cells	Publication foreseen by the end 2001
Long term effects in children to estrogenized meat	"Accidental gynecomastia in children" *(APMIS 109(suppl.103): 203-209, 2001)*
Androgen exposures in utero, risk of breast cancer	"A study of opposite-sexed twins" (*Journal of The National Cancer Institute, JNCI, volume 93, issue 1;60-62, 3.1.2001)*
Endocrine disrupting activity of anabolic steroids used in cattle	1)"Characterisation of the affinity of different anabolics and synthetic hormones to the human androgen receptor, human sex hormone binding globulin and the bovine progestin receptor" (*APMIS 108: 838-846, 2000)* 2)"The fate of trenbolone acetate and melengestrol acetate after application as growth promotants in cattle - environmental studies" *(Environmental Health Perspectives- in preparation*
Screening water samples for estrogenic &androgenic anabolic chemicals	*scientist has not yet indicated name of journal and publication date.* *some results can be found in APMIS 109(suppl.103): 551-556, 2001* General discussion on "Existing guidelines for the use of meat hormones and other food additives in Europe and USA"
Endocrine disrupting effects of cattle farm effluent on environmental sentinel species	"A reexamination of variation associated with environmentally stressed organisms" *(Human Reproduction update vol.7 (no.3): 265-272, 2001)*
Human cells exposed to the estrogenic compound zeranol	"Oestrogenic potencies of zeranol, oestradiol, diethylstilboestrol, Bishpenol-A, and genistein:

	Implications for exposure assessment of potential endocrine disrupters" *(Human Reproduction 16: 1037-1045, 2001)*
APMIS - Acta Pathologica, Microbiologica et Immunologica Scandinavia AOAC - Scientific Association Dedicated To Analytical Excellence JNCI - Journal of the National Cancer Institute	

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