FRI BRIEFINGS

Human Safety of Hormone Implants Used to Promote Growth in Cattle

A Review of the Scientific Literature

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INTRODUCTION AND HISTORICAL BACKGROUND

Hormone implants containing estradiol benzoate/progesterone were first approved in 1956 by the U.S. Food and Drug Administration (FDA) for increasing growth, feed efficiency, and carcass leanness of cattle. Later, other implants containing testosterone, zeranol, trenbolone acetate, and combinations of these hormones were developed and approved for use in cattle by the FDA.

Currently, five hormones (progesterone, testosterone, estradiol-17β, zeranol, and trenbolone acetate) are approved for implants in cattle in the U.S.A. But these implants have been officially prohibited in Europe since 1989. Hormone implants are widely used in the U.S.A., Australia, and Canada. According to a review by Preston (*158*), approximately 30 countries have approved one or more of these implants for enhancing the growth of cattle. Use of these implants in cattle according to recommended procedures was declared safe by the following groups:

- FDA, which approved 11 formulations of implants between 1956 and 1996 (*15–21, 158)*
- EEC Scientific Working Group on Anabolic Agents, chaired by Dr. G. E. Lamming in 1987 (*185*)
- Codex Committee on Residues of Veterinary Drugs in Foods, in 1987 (*24*)
- European Agriculture Commission Scientific Conference on Growth Promotion in Meat Production, in 1995 (*79, 158*)
- FAO/WHO (Food and Agriculture Organization/World Health Organization) Joint Expert Committee on Food Additives (JECFA), 1981, 1983, 1988, 1999 (*94–103*)
- Sub-Group of the Veterinary Products Committee from the Ministry of Agriculture, Fisheries, and Food (UK), 1999 (*196*).

Hormone implants in cattle have been the subject of eight significant reviews in the past five years. All except two of these reviews concluded that implants containing the approved doses of hormones and inserted in cattle as recommended would not result in residue levels in meat that would adversely affect human health (*99, 117, 121, 158, 196, 209*). Two dissenting reviews (*2, 184*) expressed concern about the safety of these implants. Another recent review (*105*) considered the implications of hormone pellets that were not properly implanted or discarded at slaughter.

The FDA has established maximum safe tissue residue levels for trenbolone (*19*) and zeranol (*17*) as listed below:*

Hormone	Muscle	Liver	Kidney	Fat
	– – – – – – – (μg/kg) – – – – – – – –			
Trenbolone	50	100	150	200
Zeranol	150	300	450	600

μg/kg = ppb (parts per billion)

*Please refer to note (*) on metric units at the end of the text if these units are unfamiliar.

For the naturally occurring hormones estradiol (*20*), progesterone (*15*), and testosterone (*16*), FDA has set the following allowable incremental increases in hormone levels above those normally present:

Hormone	Muscle	Liver	Kidney	Fat
	– – – – – – – (μg/kg) – – – – – – – –			
Estradiol	0.12	0.48	0.36	0.24
Progesterone	3	12	9	6
Testosterone	0.64	2.6	1.9	1.3

μg/kg = ppb (parts per billion)

JECFA has set lower maximum residue limits for both zeranol (*98*) and trenbolone (*97*) at 2 μg/kg in meat and 10 μg/kg in liver. Acceptable residue levels for estradiol, progesterone, and testosterone in meat have not been recommended by JECFA because this committee concluded that residues from use according to good husbandry practice are unlikely to pose a hazard to human health (*99, 158*). Acceptable daily intake (ADI) maxima of estradiol, progesterone, and testosterone were established by JECFA at 0.05, 30, and 2 μg/kg body weight (bw), respectively, based on reported no- or lowest-observed-effect levels in humans of 5 μg estradiol/kg body weight, 3.3 mg progesterone/kg bw, and 1.7 mg testosterone/kg bw (*99*). ADIs for zeranol and

trenbolone acetate were set at 0.5 and 0.02 μg/kg bw based on feeding experiments with cynomolgus monkeys (*98*) and pigs (*103*).

Safety of hormonal implants has been questioned on the basis that residues from these implants in beef may significantly increase exposure of humans, particularly children, to estrogens and other hormones which may adversely affect health. Estrogens are naturally present in all mammals, with higher concentrations in females during the reproductive phase of their lives. According to evaluations by the International Agency for Research on Cancer (IARC; *215*), there is currently sufficient evidence for the carcinogenicity of estradiol and limited evidence for the carcinogenicity of testosterone to humans. Experiments with some of the other compounds used in implants have shown that, under certain conditions, they may cause adverse effects in experimental animals. Although some recent data indicate that estradiol-17β has genotoxic potential, JECFA scientists pointed out that there are no data demonstrating that concentrations below the no-hormonal-effect level (NHEL) cause adverse effects in animals or humans (*99*).

Whether residues of hormone implants in beef constitute a health risk remains a controversial issue. The 1999 JECFA analysis (*99*) estimated that a person consuming 500 g (about 1.1 lb) of meat from implanted cattle would consume an extra 30–50 ng estradiol per day. This calculation utilized the highest residue levels reported for implanted beef and considered "meat" as a mixture of 300 g muscle, 100 g liver, 50 g kidney, and 50 g fat. (Estradiol levels are higher in organ meats than in muscle.) This additional 50 ng estradiol consumed can be compared to the acceptable daily intake of 50 ng/kg/day or 3000 ng/day for a 60-kg (132-lb) person.

In 1999, the European Commission also issued a report that discussed available data on hormone implants and their potential toxicity and concluded that elevated levels of hormones in meat from implanted cattle might present a hazard, particularly to children (*184*). Invoking the precautionary principle, the Committee decided that beef from implanted cattle should not be allowed in European markets. The precautionary principle is a risk management strategy which "covers cases where scientific evidence is insufficient, inconclusive, or uncertain and preliminary scientific evaluation indicates that there are reasonable grounds for concern ..." (*3*). The concerns expressed by the Committee members include:

- **Sensitivity of hormone assays.** They contend that the routinely used radioimmunoassay is not sensitive enough to accurately measure low hormone levels in blood of children and perhaps in some meat samples. Rather they propose that the very low plasma hormone concentrations measured in children by the recombinant cell yeast bioassay (*109*) are more accurate.
- **Effects of low concentrations of hormones.** They argue that even though excess exposure to hormones in beef from implanted cattle may amount to as little as 1 to several hundred nanograms per person, preadolescent boys and girls and the fetus in utero may be very sensitive to slight increases in hormone levels.
- **Irrelevance of NHEL (no-hormonal-effect level).** They point out that some recent data indicate that estradiol is genotoxic and therefore may have carcinogenic effects at concentrations below the NHEL.

In response, the 1999 review by the UK's Sub-Group of the Veterinary Products Committee (*196*) critically evaluated genotoxicity studies of estradiol and concluded that "None of the publications (research papers) reviewed above provide any substantive evidence that oestradiol is mutagenic/genotoxic." The recombinant yeast cell bioassay was critically evaluated and this Committee was concerned that interfering substances in the crude extracts of serum samples could have caused artificially low serum estradiol measurements. Further validation is required for this assay. Although high concentrations of hormones can certainly disrupt normal metabolic processes, this Sub-group concluded that small increases in hormone intakes from implanted cattle were unlikely to have a significant effect.

In a search of several databases, including Current Contents, Medline, Food Science and

Technology, and Agricola, and of government web sites, a total of 217 references relevant to the issue of safety of hormone implants in cattle were identified and collected. Although the topic of interest is, strictly speaking, whether implants used in cattle leave hormonal residues that present a hazard to human consumers of beef, there are few studies in humans, and therefore regulators and consumers must rely on:

- toxicity data from any human exposures, including effects of natural hormones;
- data from animal and cell culture research to indicate the possible toxicity of these compounds to humans;
- reports of hormone levels in cattle tissues, both endogenous (naturally occurring) hormones and those which originate from implants;
- information on total dietary exposure to compounds with hormonal activity, including hormones in meat from unimplanted as well as implanted cattle and hormones in other foods, in order to put the hormone concentrations in different foods in context of the total diet; and
- reports on the sensitivity of assays used to determine hormone levels and assess potential human exposure.

Information from relevant references on each of these topics is summarized in the following sections. Please refer to note (*) on metric units at the end of the text if these units are unfamiliar.

HORMONE METABOLISM AND TOXICITY IN HUMANS

Estradiol, progesterone, and testosterone are naturally present in humans although the amounts vary with age, sex, diet, exercise, and, in females, with pregnancy and stage of the menstrual cycle. These compounds may be present in serum as unbound "free" compounds or attached to hormone binding proteins. Estradiol 17β is present in high levels in newborn males and females but concentrations drop rapidly after birth so that young children of both sexes have very low concentrations of this compound. In adult males, estradiol concentrations are in the range of 20–40 pg/ml, and most of this is in the bound form (*161, 173*). Estradiol levels in females vary between 40 and 400 pg/ml during the month (*155*). Some women are genetically predisposed to have higher endogenous hormone levels (*67*). After menopause estradiol concentrations decrease to 5–20 pg/ml (*155*).

Children and the fetus in utero have been considered at greater risk from exposure to hormones because their normal physiological hormone levels are low compared to adults. Results from one recent study using a radio-immunoassay indicated that estradiol levels in prepubertal boys and girls were 2.6 and 4.5 pg/ml, respectively (*154*). However, using a recombinant yeast assay, other researchers reported levels of 0.6 pg/ml in girls and 0.08 pg/ml in boys (*109*). The yeast estrogen receptor binding assay appears to be more sensitive than the immunoassay; however, these very low concentrations have been questioned because the solvent system used to extract hormones prior to assay is not very efficient (*11*). Inter- and intra-assay variation at these very low concentrations of estradiol was reported to be 50–60% (*109*).

Oral estrogens generally have poor bioavailability due to extensive metabolism after absorption from the gut. Only 0.1–12% of an oral micronized estradiol dose was found to be bioavailable. Hormone replacement therapy with a daily dose of 0.625 mg conjugated estrogens produces serum estradiol levels of approximately 40 pg/ml in postmenopausal women (*143*). Oral testosterone and progesterone are also reported to have low bioavailability due to gastrointestinal and hepatic inactivation.

Dietary constituents (other than hormones themselves) are well known for influencing serum hormone concentrations. For example, total estradiol levels were significantly lower when women consumed a low fat (20%), high fiber (40 g/day) diet as compared to a more typical high fat (40%), low fiber (12 g/day) diet (*214*). Caloric intake was directly correlated with serum progesterone levels in another

study of premenopausal women (*35*). When male athletes consumed diets high in animal protein, total testosterone levels were significantly higher and estradiol levels were lower than when they consumed an isocaloric vegetarian diet with an equivalent amount of protein (*161*). Serum testosterone concentrations were also directly associated with intake of dietary fat and of saturated fats (*207*).

Although hormones are essential for various physiological processes in the body, excessive amounts may have adverse effects. The most controversial and well documented of these is estradiol. Estradiol stimulates cell division in hormonally sensitive tissues thereby increasing the possibility for accumulation of random errors during DNA duplication. This increased cell proliferation also has the effect of stimulating growth of mutant cells (*67*). Among post-menopausal women using hormone replacement therapy, there appears to be an increased incidence of breast cancer. Two recent references on epidemiological studies are included here as an introduction to the voluminous literature on this subject (*171, 179*). Results from epidemiological studies and experiments with laboratory animals were considered by IARC to be sufficient evidence for the carcinogenicity of estradiol (*215*).

However, only a small proportion of postmenopausal women taking estrogen supplements develop cancer so there are other factors necessary for the development of this disease. A recent review on hormonal carcinogenesis concluded that genetic susceptibility plays a critical role in the development of hormone-related cancers (*67*).

Some research has also investigated the possible influence of consuming estrogens and progesterone in contraceptives or hormone replacement therapy on the incidence or severity of asthma (*48*). Data reported from a variety of small studies was inconsistent: In some cases the hormones appeared to make asthma worse, whereas in other cases taking hormones appeared to improve asthmatic symptoms. Progesterone is also prescribed for some medical conditions and usually has few serious side effects although minor symptoms sometimes occur (*211*).

Some epidemiological studies have also found a link between prostate cancer and higher testosterone levels in males. Prostate cancer is rare before age 40, but the increasing incidence of this cancer with age is much greater for some national populations and ethnic groups with higher serum testosterone concentrations than for others. No environmental or lifestyle risk factors have been identified to explain this difference. Rather, higher endogenous testosterone levels along with a genetic susceptibility appear to determine development of prostate cancer (*67, 215*). Elevated levels of testosterone have not been associated with any other type of cancer in humans.

Since trenbolone and zeranol are not naturally occurring compounds in humans there has been more concern about their possible toxicity. In a single trial, a dose of radioactively labeled trenbolone was given to a human volunteer. Results demonstrated that 63% of the radioactivity was excreted in the urine during the first 72 hours, with over half of the radioactivity present in glucuronides (*191*). This is in contrast to the primarily biliary and subsequent fecal excretion observed in the rat and the cow (*156*).

Some data on human exposure to zeranol are available from a study of workers in a pelletizing plant (*6*). Analyses of blood samples from exposed workers showed no detectable residues of zeranol and no significant elevation or depression of serum concentrations of estradiol or other hormones. It was estimated that workers were exposed to airborne zeranol concentrations of 86–1554 $\mu g/m^3$. Zeranol was also detected in swabs from the lunchroom and on work clothes. Symptoms of breast irritation were reported by some workers but no other acute or chronic effects were evident. Young children of some workers in the plant had also experienced breast symptoms and this was traced to employees wearing their work clothes home.

Zeranol has been used in the past in Europe as an estrogen substitute for postmenopausal women and has been shown to affect the activity of some enzymes in human prostate cell cultures (*198*). Human volunteers given a radioactively labeled dose of zeranol absorbed at least 55% of the dose and then excreted metabolites and glucuronides of this compound primarily in urine whereas dogs, monkeys, and rats excrete radioactive metabolites predominantly in bile and feces (*137*).

Two outbreaks, one of breast enlargement in young school children in Italy (*45*) and another of precocious sexual development in Puerto Rico (*176*), were suggestive of exposure to environmental estrogenic compounds, possibly zeranol. In the case of the Italian children, symptoms disappeared entirely after 8 months and researchers suspected that one consignment of meat or poultry might have contained high levels of some estrogenic compound. In the case of the Puerto Rican children, high levels of estrogenic compounds were said to be present in some local chicken and it was reported that symptoms gradually disappeared after the children stopped consuming local chicken, beef, and milk. However, subsequent investigation of the Puerto Rican outbreak by the Centers for Disease Control (CDC), USDA, and FDA indicated that the outbreak could have been the result of increased awareness and reporting of cases by physicians (*209*).

Summary. Estradiol, progesterone, and testosterone are hormones naturally present in humans at different concentrations depending on age, sex, diet, exercise, and stage in the reproductive cycle. In adult males and premenopausal females, estradiol concentrations are in the range of 20–40 pg/ml and 40 and 400 pg/ml, respectively. After menopause estradiol concentrations decrease to 5–20 pg/ml. Estradiol levels have been measured by radioimmunoassay in prepubertal boys and girls at 2.6 and 4.5 pg/ml, respectively. A recombinant yeast assay detected only 0.6 pg/ml in girls and 0.08 pg/ml in boys.

Pharmacological studies have revealed that oral doses of estradiol have low bioavailability — usually 12% or less. Diets high in fat, calories, and animal protein and low in fiber have been associated with higher estradiol, progesterone, or testosterone levels in several studies.

Epidemiological and experimental data examined by International Agency for Research on Cancer were judged to be sufficient evidence for the carcinogenicity of estradiol to humans. Estradiol stimulates cell division thereby increasing the possibility for random errors during DNA duplication. A small proportion of women taking estrogen supplements develop breast or uterine cancer, indicating that estradiol may be one of several factors important in the development of cancer. Epidemiological studies have also found a link between prostate cancer and higher testosterone levels in males.

Few human studies of the metabolism of trenbolone and zeranol are available. In human trials, radioactive doses of both compounds were primarily excreted in the urine. Zeranol has been used in the past in Europe as an estrogen substitute for postmenopausal women. Occupational exposure to zeranol has produced symptoms of breast irritation but no other acute or chronic effects.

Two outbreaks, one of breast enlargement in young school children in Italy and another of precocious sexual development in Puerto Rico, were suggestive of exposure to environmental estrogenic compounds, possibly zeranol. However, subsequent investigation of these outbreaks revealed no definite evidence of exposure to estrogenic compounds in foods.

TOXICITY STUDIES IN ANIMALS AND CELL CULTURES

Genotoxicity assays

These assays assess damage to DNA and chromosomes which can lead to mutations and heritable changes in genetic information. Not all DNA changes are mutagenic because cells have efficient DNA repair systems. It is generally agreed that both in vivo and in vitro assays should be used to determine the genotoxicity of a compound. Many assays test hormone concentrations that are many-fold higher than normal physiological concentrations, and one must make a judgment as to their relevancy. Also, these hormones are metabolized to different compounds in the body and these metabolites may have greater, lesser, or the same toxicity as the parent compound.

17β-Estradiol

Negative results were obtained for estradiol in the Ames test (bacterial mutagenicity), in the in vitro

hamster cell micronucleus assay for chromosomal damage (*167*), in the in vivo mouse lymphoma forward mutation assay (*89, 147, 167*), and in the in vivo male rat bone marrow germ cell assay (*167*). Estradiol was found to bind covalently to rat liver DNA, but bound residues were removed by repair mechanisms (*10*). Syrian hamsters dosed with estradiol develop kidney tumors, and some changes in microsatellite DNA in kidney cells were observed in treated animals (*74*).

Other in vitro genotoxicity assays have yielded positive results but interpretation was not always clear cut. In some cases changes induced were not stable or occurred at a low frequency so that their significance was questionable. Estradiol induced methotrexate resistance in breast cancer cells in vitro but when methotrexate was withdrawn, resistance was lost, indicating that the change was not inherited (*197*). One report indicating that estradiol caused mutations in an in vitro hamster cell assay actually observed only 5 mutants out of 2,000,000 cells compared to 2 mutants in 2,000,000 cells of the control (*162*). This small difference between experimental and control cultures makes any conclusions questionable. Other reports have demonstrated that estradiol can cause oxidative DNA damage to calf thymus DNA (*138*) and induce transformation and aneuploidy (alteration of chromosome number) in hamster embryo cells in vitro (*201*). Both of these effects occurred at estradiol concentrations several orders of magnitude above normal physiological concentrations of this hormone. Endogenous antioxidants were also observed to drastically decrease oxidative damage to calf thymus DNA. Therefore, these genotoxic effects may not occur under normal physiological conditions in vivo.

In the presence of 25 μg estradiol/ml (but not of 10 μg/ml), chromosomal aberrations and sister chromatid exchanges were observed in human blood peripheral lymphocytes (*1*). (Estradiol levels in human female serum normally range up to 400 pg/ml.)

Results from numerous genotoxicity assays have been summarized and discussed in recent publications (*114, 132, 184, 196*). Overall it appears that estradiol has a weak potential for genotoxic effects.

Progesterone

Negative results were obtained for progesterone in the Ames test (*89*) and in tests for induction of chromosomal aberrations and aneuploidy in cultured Syrian hamster embryo cells (*200*). A high oral dose of 100 mg progesterone/kg body weight did induce the formation of micronuclei in rat liver cells (*123*).

Testosterone

Negative results were obtained for testosterone in the Ames test (*89*), in the in vivo mouse lymphoma forward mutation assay (*167*), and in tests for the induction of chromosomal aberrations and aneuploidy in cultured Syrian hamster embryo cells (*181, 200*). Testosterone was found to bind covalently to rat liver DNA but bound residues were removed by repair mechanisms (*9*). Testosterone exhibited a weak transforming effect on Syrian hamster embryo cells in culture according to some reports (*112, 200*) but not in another (*182*).

Trenbolone

Although one research group reported positive results for trenbolone in the Ames bacterial mutagenicity assay (*119*), another scientist was unable to duplicate these results (*124*) and several other reports indicated that trenbolone is not an effective mutagen in the Ames test (*89, 125, 167, 182*). Negative results were also obtained in the SOS bacterial chromotest, the hamster V79 sister chromatid exchange test, the *rec* bacterial assay (*180*), the test for unscheduled DNA synthesis (*182*), and the test for induction of chromosomal aberrations and aneuploidy in cultured Syrian hamster embryo cells (*167, 200*).

Trenbolone was found to bind covalently to rat liver DNA but bound residues were removed by repair mechanisms (*10, 119, 152, 153*). Trenbolone exhibited a weak to moderate transforming effect on Syrian hamster embryo cells in culture (*112, 125, 182, 200*) and did induce micronucleus formation in Syrian hamster embryo fibroblasts but not in mouse cells (*167, 181*). Richold (*167*), Lone (*117*), the European Commission (*184*), FDA (*18, 21*), and JECFA (*101, 103*) summarized much of the research

done to assess the genotoxicity of trenbolone and concluded that the preponderance of negative tests indicated that this compound does not cause DNA damage or mutations.

Zeranol

Zeranol was found to bind covalently to rat liver DNA at much lower levels than estradiol, and bound residues were removed by repair mechanisms (*10*). Negative results were obtained for zeranol in the Ames test (*89*), in the mouse bone marrow cytogenetic assay, and in the in vivo mouse lymphoma forward mutation assay (*147*). Negative results were also obtained in the SOS chromotest and the V79 sister chromatid exchange test but results were positive in the rec-assay (*180*). Zeranol also did not induce micronuclei in mouse spermatids in vivo or in vitro (*160*). Nearly all the assays listed in the JECFA Toxicology report (*100*) and the review by Lone (*117*) indicate that zeranol is not genotoxic and none were positive for mutagenicity.

Carcinogenicity Tests

The carcinogenicity of estradiol and of testosterone in rodents is related to binding of the hormones to receptors on the surface of cells in reproductive organs and stimulating growth. Testosterone can cause cervical-uterine tumors in female rats and prostate cancer in males but does not appear to be carcinogenic in other organs (*67, 215*). Estradiol is carcinogenic to rodents and primarily affects reproductive organs. However, an increased incidence of bone, pituitary, and lymphoid tumors has also accompanied high doses of estradiol in some rodents (*184, 215*). There have been some reports that high doses of progesterone administered over a long time are correlated with an increase in tumor growth in animals (*117*).

A great deal of research has demonstrated that low estradiol concentrations, where no normal physiological effects of the hormone are observed, do not promote tumor growth. This low hormone concentration has been called the no-hormonal-effect level (NHEL) or the no-observed-effect level (NOEL). In dose–response studies with women, the NOEL was determined to be 5000 nanograms (ng) (*59, 67*).

Estimates of the estrogenic potency of zeranol (oral dose) indicate that it is 150 times less potent than estradiol in rats (*42, 199*) and no estrogenic effects were observed in male cynomolgus monkeys dosed with 5 mg zeranol/kg body weight/day (*100*). The hormone no-effect levels in ovariectomized female rhesus and cynomolgus monkeys were determined to be 0.225 and 0.05 mg/kg/day, respectively (*8, 100, 147*). Zeranol binds to rat hepatic estrogen receptors with about 30% of the affinity exhibited by estradiol (*157*) and is capable of eliciting estrogenic effects in liver in vivo (*127*). When injected at high concentrations (36 mg), zeranol induced hepatic tumors in hamsters; this effect was inhibited by tamoxifen, indicating that estrogen receptors were involved in tumorigenesis (*23*). According to reports of unpublished carcinogenicity studies in rats, dietary zeranol (1.25 or 0.8 mg/kg/day) administered over a 3-year period did not enhance tumor risk at any site although estrogenic effects were observed in the rats (*100, 170*). Long-term carcinogenicity studies involving oral administration of zeranol to rats (2 yr), dogs (7 yr), and rhesus monkeys (10 yr) demonstrated no significant increase in tumors in any of the treated groups (*8*).

Data from unpublished carcinogenicity studies of trenbolone in rodents indicate that 10 or 100 ppm (μg/g) trenbolone in the diet was associated with an increase in liver cancer in mice and 50 ppm trenbolone may have enhanced pancreatic cancer in rats (*170*). The no-hormonal-effect level was estimated to be at or near 0.5 μg/g for both rodent species. Data from castrated rhesus monkeys indicated that the no-hormonal-effect level for oral trenbolone was between 2 and 50 μg/kg/day (*73*).

Other Toxicity Tests/Issues

Steroid hormones are active during the prenatal period and affect the programming and timing of critical events such as birth, puberty, growth rates, and sexual differentiation, including the differentiation of estrogen binding proteins (*2, 56, 184, 189, 196*).

Exposure of pregnant rats to 4 mg zeranol/kg body weight/day depressed maternal weight gain, prolonged gestation period, and decreased number of live births in some animals (*163, 164*). However, zeranol did not appear to be teratogenic (cause birth defects) (*187, 210*). Zeranol administered to mice during pregnancy also decreased fetal size (*150*) and caused some abnormalities in testicular development (*149, 151, 160*).

Estrogens are also known to affect functioning of the immune system. Progesterone and zeranol also affect immune function to an extent related to their estrogenicity (*118*).

Summary. Genotoxicity assays assess damage to DNA and chromosomes which can lead to mutations and heritable changes in genetic information. Both in vivo and in vitro assays are used to determine the genotoxicity of a compound. Negative results were obtained for estradiol, progesterone, testosterone, trenbolone, and zeranol in the Ames test of bacterial mutagenicity and in numerous other in vitro and in vivo tests for genotoxicity. These hormones did bind to DNA molecules but were later removed by repair enzymes, and a few assays with estradiol and trenbolone gave weak positive results. Assay results for the other compounds indicated a lack of genotoxicity. Some scientists believe that the weakly positive results in some tests with estradiol indicate that it is potentially genotoxic to humans while others believe that these results indicate that estradiol is not genotoxic at reasonable concentrations under normal physiological conditions.

The carcinogenicity of estradiol and of testosterone in rodents is related to binding of the hormones to receptors on the surface of cells in reproductive organs. Testosterone and estradiol are carcinogenic to rodents and primarily affect reproductive organs. An increased incidence of bone, pituitary, and lymphoid tumors has also accompanied high doses of estradiol in some rodents. Progesterone has increased carcinogenesis in reproductive tissues in some animal studies. Long-term carcinogenicity studies involving oral administration of zeranol to rats (2 yr), dogs (7 yr), and rhesus monkeys (10 yr) demonstrated no significant increase in tumors in any of the treated groups. Some unpublished carcinogenicity studies of trenbolone in rodents indicate that 10 or 100 ppm (μg/g) trenbolone in the diet was associated with an increase in liver cancer in mice and 50 ppm trenbolone may have enhanced pancreatic cancer in rats.

A great deal of research has demonstrated that low estradiol concentrations, where no normal physiological effects of the hormone are observed, do not promote tumor growth. This low hormone concentration has been called the no-observed-effect level (NOEL). In dose–response studies, the NOEL for estradiol is 5 μg/kg body weight in women, for testosterone is 1.7 mg/kg bw in men, for zeranol is 0.05 mg/kg/day for cynomolgus monkeys, and for trenbolone is between 2 and 50 μg/kg/day for castrated rhesus monkeys.

Steroid hormones are active during the prenatal period and affect the programming and timing of critical events such as birth, puberty, growth rates and sexual differentiation, including the differentiation of estrogen binding proteins. Exposure of pregnant rats to 4 mg zeranol/kg body weight/day depressed maternal weight gain but did not cause birth defects. Zeranol administered to mice during pregnancy caused some abnormalities in testicular development in rats.

Estrogens are also known to affect functioning of the immune system. Progesterone and zeranol also affect immune function to an extent related to their estrogenicity.

HORMONE LEVELS IN CATTLE TISSUES

Numerous research papers have reported data on hormone levels measured in tissues from untreated and implanted cattle. Results are summarized below for cattle with and without hormone implants. When considering these data, one should be mindful of variations in sensitivity of different assays.

Endogenous (naturally occurring) hormone levels in tissues vary with the sex (*61*), age (*5, 26, 50, 141*), and breed (*26*) of cattle and whether the animals have been castrated or are pregnant (*66*). Fat

content also affects the levels of hormones in meat (*52*), and concentrations of some hormones are significantly higher in liver and kidney than in muscle tissue.

Published results of an extensive analysis for chemical residues in beef from cattle slaughtered in the U.S.A. in 1990 revealed that neither zeranol nor trenbolone was present in detectable levels (*190*). Another survey of beef in Ireland also demonstrated that residue levels of zeranol and trenbolone were <0.05 µg/kg in all samples (*144*). However, off-label use of trenbolone has been detected from residues in liver of veal calves in Canada (*105*).

Concentrations of various hormones have been measured in plasma, urine, muscle, kidney, liver, and fat of cattle that have received hormone implants. Most of these papers are included in the bibliography. Several review articles (*121, 158, 178, 209*) have also compiled data from a number of articles on hormone residue levels in cattle. Most of the sources cited in these reviews are included in this report but there are a few articles which could not be obtained.

Further data on hormone levels in tissues other than meat may be found in these references for estradiol (*58, 63, 64, 68, 113, 116, 134, 139, 174, 183*); testosterone (*58, 86, 87, 104, 113, 134, 186*); trenbolone *63, 64, 68, 86, 90, 116, 183*); zeranol (*107, 159)*. Some articles in peer-reviewed journals list hormone concentrations in meat. Additional data from unpublished research have been printed in reports by WHO/FAO (*94–98, 102*) and other regulatory agencies. Some of these reports also discuss metabolites of the hormones which may be present in cattle.

Estradiol

Calves: Natural estradiol levels are low, close to the limits of detection, with maximum reported levels of 5–7 ng/kg in muscle (*94*) and 0.02 ng/ml in serum and 0.2 ng/ml in urine (*5*).

Bulls: Estradiol levels have been measured as 5.8 ng/kg in muscle (*94*), 3.3 pg/ml in plasma (*86*), and 20.8 pg/g in fat (*70*).

Steers: Reported estradiol levels include: (a) 1.8–7.1 pg/ml in plasma (*86, 174*); (b) 2.8–14.4 pg/g in muscle (*49, 70, 71, 94*); (c) 12 pg/g in liver (*70, 72*); and (d) 12.6 pg/g in kidney (*70, 72*).

Heifers: Natural estradiol levels in female cattle vary greatly depending on cycling and pregnancy. Data on heifers (not pregnant or cycling) indicate estradiol levels in the following tissues: plasma, 0.3–2.2 µg/ml (*111, 139, 141*); muscle, 12 pg/g (*70, 71*); liver, 38.3 pg/g (*72*); kidney, 39.8 pg/g (*72*). Estradiol levels in fat vary from 9 pg/g in the luteal phase to 24.2 pg/g in the follicular phase and from 20 to 68 pg/g in pregnant cows (*70, 71*). Estradiol levels in plasma from pregnant cows increase from 52 pg/ml 20 days before giving birth to 277 pg/ml at 5 days before birth (*66*).

Implanted cattle: A study published in 1999 demonstrated that beef from steers implanted with Synovex S contained concentrations of estradiol

Estradiol Concentrations in Muscle (ng/kg) and Plasma (pg/ml) from Implanted and Control Cattle

		Muscle		Plasma		
Cattle	Implant	Control	Implanted	Control	Implanted	Reference
Steer	Synovex-S	2.8	3.0			*49*
Steer	Synovex- S			2.5	9.0	*174*
Steer	Tren/Estra			1.8	18.2	*86*
Bull	Tren/Estra			3.3	23.0	*86*
Heifer	Estradiol 1x			2.2	13.1	*139*
Heifer	Estradiol 2x			2.2	16.8	*139*
Heifer	Tren/Estra			2.2	18.3	*139*
Heifer	Compudose200			0.46	0.95	*111*

Tren/Estra = 120 mg trenbolone + 24 mg estradiol
Estradiol 1× = 19 mg estradiol; 2× = 38 mg estradiol

similar to those detected in meat from unimplanted steers (*49*). Data in the FAO report (*89*) indicated that maximum estradiol levels in meat from implanted steers and heifers were 9.7 pg/g (15 days) and 33 pg/g (30 days). At 61 days after implantation, estradiol concentrations declined to 7.3 and 11 pg/g, respectively. Low residue levels were also reported in other studies (*19, 99*).

Progesterone

Progesterone levels are very low in calves, the concentrations ranging from non-detectable to 1.4 mg/ml in plasma (*5*) and measuring 5.8 mg/kg in fat (*79*).

Progesterone levels in meat (muscle) from steers have been reported to range from 0.21 to 1.2 mg/kg (*49–52, 61*). However, hormone concentrations are much lower in meat with the visible fat removed (0.05 mg/kg) (*52*). Progesterone levels also decrease with age in steers from 0.21 to 0.13 mg/kg (*50*).

Progesterone levels in meat (muscle) from bulls have been reported to range from 0.16 to 0.3 mg/kg (*50–52, 60*). However, hormone concentrations are much lower in meat with the visible fat removed (0.06 mg/kg) (*52*). Progesterone levels also decrease with age in bulls from 0.25 to 0.16 mg/kg (*50*).

Progesterone levels can be much higher in meat from adult heifers, with one report of 18.9 mg/kg (*61*). Hormone levels change during pregnancy, with a plasma concentration of 8.5 ng/ml at 20 days before birth declining to 4.7 ng/ml 5 days before birth and to 0.7 ng/ml 5 days after birth (*66*).

Implanted cattle: A recent study demonstrated that beef from steers implanted with Synovex S contained significantly lower concentrations of progesterone than meat from unimplanted steers (*49*). An FAO report (*95*) stated that progesterone levels in meat from unimplanted steers, calves, and pregnant heifers averaged 0.27, 0.9, and 10.1 mg/kg, respectively. This was compared to concentrations measured in implanted steers at 120 days (0.58 mg/kg) and in implanted calves at 50 days (0.77 mg/kg).

Testosterone

In male calves, testosterone levels have been reported as 0.16 mg/kg in muscle, 1.8 mg/kg in kidney, 3.57 mg/kg in fat, and 0.3–6.3 mg/L in plasma. Corresponding values for female calves are 0.078 mg/kg, 0.57 mg/kg, 0.49 mg/kg, and 0.1 mg/L, respectively (*5, 54*).

Testosterone levels are low in heifers and steers, with concentrations of 0.069 mg/kg in muscle tissue from heifers and 0.01–0.14 mg/kg detected in muscle from steers (*49–52, 54, 61*).

Testosterone levels in bulls vary with breed and age, with serum levels at 15 months of age measured as 5.9 mg/L in Simmental, 2.63 mg/L in Charolais, 1.43 mg/L in Hereford, and 2.54 mg/L in Angus bulls (*26*). At 12 months serum testosterone levels for these 4 breeds ranged from 7.06 to 10.13 mg/L. Testosterone levels in muscle were determined as 0.34–0.73 mg/kg while concentrations in kidney (2.8 mg/kg) and fat (5.3 mg/kg) were much higher (*49–52, 54, 61*).

Implanted cattle: A recent study demonstrated that beef from steers implanted with Synovex S contained similar concentrations of testosterone as detected in meat from unimplanted steers (*49*). Data reported by FAO (*96*) indicated that testosterone levels in muscle of implanted heifers peaked at 30 days at 0.102 mg/kg and then declined to 0.03 mg/kg at 120 days. In female calves, the peak was 0.36 mg/kg at 15 days and declined to 0.225 mg/kg at 50 days. For this hormone, higher levels were detected in kidney and lower levels in liver.

Trenbolone

According to several research reports, trenbolone residues in meat from implanted cattle range from 0.01 to 0.3 mg/kg in most cases (*65, 145, 168, 169, 175*). Higher levels were detected in tissues of calves slaughtered after 50 days (*44*). Data from FAO (*97, 101, 102*) indicated that trenbolone concentrations in muscle from steers were highest on day 15 (after implantation) at 0.25 mg/kg and decreased to 0.07 mg/kg at 75 days. Higher concentrations

were detected in liver and lower levels in kidney. Peak trenbolone levels in muscles of heifers were detected at 30 days (0.65 mg/kg). Concentrations decreased to 0.18 mg/kg at 75 days.

Zeranol

Analyses of tissues from cattle implanted with zeranol (Ralgro) revealed that highest residue levels of zeranol present in muscle, liver, and kidney after 70 days were 0.23, 0.39, and 0.29 mg/kg, respectively. Zeranol was still detectable in some tissues after 120 days but concentrations were very low (*32, 33*). According to other data cited in a FAO report (*98*), zeranol residues in beef peaked at 0.13 mg/kg on day 5 following implantation and then decreased to 0.044 mg/kg after 65 days. When cattle were implanted with more than four times the recommended dose, residue levels were not significantly increased. Data from this report confirmed that much higher concentrations of zeranol were present in liver and kidney tissue.

Recent reports have indicated that zearalenone, a mycotoxin produced on grains and cereals by *Fusarium* spp., can be converted to zeranol in the rumen of cattle (*107*). It appears that some cattle that never had hormone implants do contain traces of zeranol from eating moldy feed.

Summary. Estradiol levels in edible tissues of implanted cattle are usually significantly higher than in controls but the increases are small, in the ng/kg range. The greatest increases reported in an FAO report on estradiol residues were 0.002, 0.0065, 0.005, and 0.0084 mg/kg for implanted bulls, steers, heifers, and calves, respectively. These increases are well below the FDA recommended limits listed in the table on p. 2 and well below estradiol concentrations in muscles of pregnant heifers (0.016 to 0.033 mg/kg).

Progesterone levels in implanted steers are elevated somewhat above background levels, with reports of 0.4 mg/kg in muscle and 3.5 mg/kg in fat as compared to concentrations of 0.2 mg/kg in muscle and 2.5 mg/kg in fat of untreated steers. These increased levels are well below the FDA regulations (*see table on p. 2*). Progesterone levels are 50–100-fold higher in pregnant heifers.

Testosterone levels in implanted heifers peak at approximately 30 days after implantation, with concentrations of 0.1 mg/kg in muscle and 0.34 mg/kg in fat compared to 0.0.02 mg/kg in muscle and 0.026 mg/kg in fat in unimplanted heifers. These increases are well below the permitted levels set by the FDA (*see table on p. 2*).

Trenbolone levels in muscle tissue of treated cattle generally range from 0.01 to 0.3 mg/kg, more than 100-fold lower than the FDA limits. Zeranol residues in muscles of treated cattle were also well below the FDA limits, with highest concentrations reported as 0.23 mg/kg. Residues of both of these growth promoters are higher in liver.

ASSAYS FOR DETERMINATION OF HORMONE LEVELS

A variety of methods have been developed for the determination of hormone concentrations in biological samples. Biological assays and thin layer chromatography procedures were developed some time ago. More recently, in vitro assays with cell lines sensitive to hormones and enzyme-linked and radioactive immunoassays have been used to determine hormone concentrations. Several versions of these assays are commercially available. Gas chromatography–mass spectrometry methods have also been recently refined to detect very low levels of hormones. In addition, yeast cells have been genetically modified to contain genes for the human estrogen receptor linked to reporter genes such as the β-galactosidase gene. These cells have been used to determine levels of 17β-estradiol in human serum samples (*109*) and bovine serum samples (*11*). Although these assays may detect very low levels of estrogenic compounds (0.02 pg/ml according to refernce *109*), interassay variation is approximately 65% at estradiol concentrations of <1 pg/ml (*11*).

Accuracy of all these assays depends on the efficiency of the methods used to extract hormones from plasma or other tissues. Klein (*109*) reported extremely low levels of estradiol in plasma of prepu-

bertal children but Burdge (*11*) stated that their extraction method consistently gave a low recovery of radioactively labeled estradiol. Therefore, Klein's procedure may underestimate the true concentration of plasma estradiol in children. Other scientists are concerned that there are inhibitory substances in the crude extracts which cause inaccurate results (*196*).

Some review papers discuss various factors affecting the accuracy of these assays: liquid chromatography methods (*91*, *92*, *204*); immunoassays (*147*); in vitro assays for estrogenic compounds (*217*); reference materials for trenbolone (*194*) and zeranol (*146*); and affinity columns for extraction (*25*). Since the hormones are present in complex biological matrices, it can be difficult to extract hormones from tissues prior to analyses and there may be interfering substances in the extracts or tissues. McShane et al. (*128*) compared results from radioimmunoassays conducted in two different laboratories for the determination of estradiol, progesterone, and testosterone in human serum samples. Some variability was noted in values reported by the

Methods for determination of hormones in tissues

Hormone	Assay type	Limit of detection**	References
estradiol-17b	TLC	20 ppb	*130, 192*
	GC/MS	1.0–1.1 ppb; 0.5 ppm	*14, 27*
	LC/MS	30 ppb	*38*
	RIA	10 pg/ml; 10 ppb; 0.25 ppb	*5, 40, 131*
	RCBA	1 ppb or less	*11, 109*
	DELFIA	10 ppb	*40*
	ELISA	0.2 ppb	*135*
progesterone	LC/MS	0.1 ppm	*37*
	HPLC/MS/MS	7 pg	*36*
	GC/MS	1.3 ppb; 0.1 ppb	*14, 27, 60*
	DELFIA	314 ppb	*40*
	ELISA	71 ppb; 0.2 ppm	*22, 40*
testosterone	LC/MS	0.1 ppm	*37*
	HPLC	50 ppb	*193*
	HPLC/MS/MS	7 pg	*36*
	GC/MS	0.02 ppb; 0.5 ppm; 0.1 ppb; 0.6 ppb	*7, 14, 27, 60*
	RIA	low ppb	*78*
	DELFIA	225 ppb	*40*
	ELISA	0.2 ppb; 84 ppb	*133, 40*
trenbolone	TLC/HPTLC	0.25–1 ng; 0.5–5.0 ppb	*55, 84, 110, 204*
	LC/HPLC	0.1–5.0 ppb; 0.2 ppb	*91–93, 195, 193, 204,216*
	HPLC/MS	0.5 ppb	*85*
	GC/MS	0.06–4.6 ppb; 0.5 ppm	*7, 14, 27, 28, 85, 122*
	RIA	low ppb; <70 pg	*78, 81, 206*
	ELISA	5 pg; 0.53–12.5 ppm; 0.2–3.0 ppb	*29, 47, 133, 135, 165, 177*
zeranol	HPLC	not reported	*108*
	TLC	10–25 ppb	*88, 129, 130*
	GC/MS	0.15–5. ppb; 0.5 ppm	*14, 27, 122, 172*
	RIA	2.5 ppm; 0.3 ppb	*4, 13, 32, 33, 39*
	ELISA	10 pg; 1.09 ppm	*47, 148*

**For some assays the reported detection limit was indicated as a quantity of the hormone, e.g. pg = picogram (10^{-12} g); in other cases, the detection limit is a concentration: ppm (parts per million) or ppb (parts per billion).

DELFIA = Dissociation Enhanced Lanthanide Fluorescence Immunoassay
ELISA = enzyme linked immunoassay; RIA = radioimmunoassay
GC/MS = gas chromatography/mass spectrometry
HPLC = high performance liquid chromatography; LC = liquid chromatography
RCBA = recombinant cell yeast bioassay; TLC = thin layer chromatography

two laboratories, and it appeared that the assays overestimated the amount of estradiol present in the samples having the lowest concentrations. Fitzgerald et al. (*46*) developed a sensitive electron-capture negative-chemical-ionization gas chromatography–mass spectrometry method for the detection of testosterone and compared it to a nonradioactive immunoassay. There was an excellent correlation between the results of the two procedures when serum samples from males were analyzed but agreement was poor for samples from females. It was suggested that the immunoassay was detecting other cross-reacting compounds (besides testosterone) that caused the discrepancy in the female samples.

A different approach to identify abuse of testosterone in cattle was taken by Mason (*126*) in a recently developed method, gas chromatography–isotope ratio mass spectrometry, which can distinguish between endogenous and external sources of testosterone in cattle. Testosterone produced in cattle will contain a certain ratio of ^{12}C to ^{13}C depending on the diet of the animals. This ratio is likely to be different in manufactured doses of testosterone. Therefore, analyses of the isotope ratio in testosterone from an animal should allow the detection of testosterone abuse.

The table above lists the reported detection limits (sensitivity) and types of assays developed for detection of the five hormones of interest and the research papers reporting these results. The reported detection limits should not be considered strictly comparable because of the variety of research protocols and test materials (muscle, blood serum, urine).

Summary. Assays for hormones in biological tissues have evolved and become more sensitive and accurate during the past 30 years. The most sensitive assays can now reliably detect hormone concentrations in the low parts per billion range. Enzyme-linked- and radio-immunoassays have been the most widely used methods in recent years, and several versions of these assays are commercially available. Methods utilizing gas chromatography–mass spectrometry and a recombinant yeast cell culture are being refined to yield even lower limits of detection. However, there is still significant variability among measurements reported by different laboratories. This variation may be due to differences in the antibody preparations used in immunoassays and to differences in extraction methods which may be more or less efficient in recovering all the hormone molecules in a sample and in removing interfering substances. Controversy over the effects of minute concentrations of hormones and the true concentration of estradiol in prepubertal children emphasizes the importance of repeatable, ultra-sensitive assays for hormones, particularly estradiol, in biological samples.

HORMONE LEVELS IN OTHER FOODS

Estradiol, testosterone, and progesterone are naturally present in many foods of animal origin, and some plant foods also contain these hormones. Hartmann et al. (*59*) present results from their own experiments as well as summarizing much of the previous data from other researchers on hormone levels in foods. A sampling of foods with significant hormone concentrations is presented in the following table. From the analytical results and information on average food consumption in Germany, Hartmann et al. estimate that the average daily intake of estradiol for women, men, prepubertal girls, and prepubertal boys is 0.08, 0.1, 0.07, and 0.08 μg, respectively, with 60–70% supplied by milk products and 15–20% each by eggs and meat/fish. [According to JECFA, the acceptable daily intake of estradiol for a 60-kg adult = 3.0 μg and for a 10-kg child = 0.5 μg (*99*).] Milk products are also a major dietary source of progesterone and testosterone, with eggs and meat also providing significant amounts. Many plant foods contain other compounds which have estrogenic activity and contribute significantly to dietary exposure to estrogenic compounds. More complete data can be found in references *12, 41, 57, 59, 80, 82, 140, 142, 208,* and *213.*

Summary. Estradiol, progesterone, and testosterone are present in many foods of animal origin including beef, pork, poultry, milk, eggs, and fish. Some plant

Hormone concentrations (mg/L or mg/kg; ppb) in foods other than beef

Food	17b-estradiol	progesterone	testosterone
Skim milk	1.4–2.2		
Whole milk	0.01–0.03	9.5–11.8	0.02–0.05
Butter	<0.03	141–300	<0.05
Cheese	0.01–0.03	44.2	0.48–1.41
Eggs	<0.03–0.22	12.5–43.6	0.04–0.49
Chicken meat	<0.03 – 0.02	0.24	<0.02 – 0.03
Boar muscle	0.91	3.71	
Boar fat	0.43	11.96	
Boar liver	9.67	1.2	
Herring	<0.03	0.51	0.07
Potatoes	<0.03	5.07	<0.02
Wheat	<0.07	2.86	0.09
Rice	<0.07	0.38	
Safflower oil	<0.03	0.71	0.21

foods such as potatoes and wheat contain significant levels of progesterone, and other foods, including some oils and wheat, have measurable levels of testosterone. In addition, many plants contain other compounds with estrogenic activity. It has been estimated that milk products provide approximately 80% of the progesterone, 30–40% of testosterone, and 60–70% of estrogens in the diet. Meat and fish provide about 5% of progesterone, 20–30% of testosterone, and 15–20% of estrogens in the diet. Eggs and plant foods are responsible for the remainder of the dietary hormone intake.

*Metric units may not be familiar to some readers.

1 kilogram (kg) = 1,000 grams (g)
= approximately 2.2 lb

For units smaller than grams:

1 gram	=	1,000	milligrams (mg)
	=	1,000,000	micrograms (mg)
	=	1,000,000,000	nanograms (ng)
	=	1,000,000,000,000	picograms (pg)

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