EUROPEAN COMMISSION

XXIV/B3/SC4

DIRECTORATE-GENERAL XXIV
CONSUMER POLICY AND CONSUMER HEALTH PROTECTION
Directorate B - Scientific Health Opinions
Unit B3 - Management of scientific committees II

OPINION OF THE

SCIENTIFIC COMMITTEE ON VETERINARY MEASURES
RELATING TO PUBLIC HEALTH

ASSESSMENT OF POTENTIAL RISKS TO HUMAN HEALTH FROM HORMONE RESIDUES IN BOVINE MEAT AND MEAT PRODUCTS

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30 APRIL 1999

(references completed)

INDEX

1. INTRODUCTION

The Scientific Committee on Veterinary Measures relating to Public Health (SCVPH) was invited to examine the use of hormones for growth promotion purposes in cattle. In particular the SCVPH was requested to answer a number of specific questions.

1.1. Mandate

In the context of the WTO case on *Hormones*, the European Commission intends to evaluate the potential for adverse effects to human health from residues in bovine meat and meat products resulting from the use of the six hormones for growth promotion purposes in cattle and whether the currently available scientific information necessitates the revision of previous risk assessments.

The Commission consequently requests the SCVPH to deliver an opinion on the potential for adverse effects to human health arising from the administration of the six hormones oestradiol-17β, progesterone, testosterone, zeranol, trenbolone acetate and melengestrol acetate used individually or in combinations for animal growth promotion.

1.2. Background

Since 1 January 1989, according to Directive 88/146/EEC replaced later by Directive 96/22/EC, the EC prohibits the administering to a farm animal by any means whatsoever of, *inter alia*, substances having a thyrostatic, oestrogenic or gestagenic action for growth promotion purposes. As a result, the use of the hormones oestradiol-17β, testosterone, progesterone, zeranol, trenbolone acetate and melengestrol acetate alone or in combinations for growth promotion purposes in meat production is prohibited. The prohibition covers both the use of these hormones for domestic production and imports from third countries of meat from animals treated with these hormones for growth promotion purposes. The prohibition reflects the fact that the EC chose a level of sanitary protection of accepting no or "zero" additional risk to human health from the residues in meat and meat products of these hormones when used for growth promotion purposes.

On 1 January 1995, the Agreement on Sanitary and Phytosanitary Measures (SPS Agreement) entered into force, together with the other agreements of the WTO. The SPS Agreement allows Members to choose the level of sanitary protection they wish to achieve in their territory. They can also depart from international standards, guidelines or recommendations on the use of chemical substances, compounds, pesticides, etc. in order to achieve their chosen level of sanitary protection, if there is scientific justification.

Unlike the EC, some countries permit the use of these hormones in cattle for growth promotion purposes. In particular, the United States and Canada allow the use of the above six hormones. The United States has opposed the

EU prohibition on the use of these hormones since its implementation. In 1996, after the entry into force of the SPS Agreement, the United States and Canada formally contested in the WTO the EC prohibition on imports of bovine meat and meat products from animals treated with these six hormones.

1.3. **General considerations and introduction to the report:**

Sanitary measures should be based on risk assessment a task which is a contribution of science to society. Risk assessment was initially confined to a scientific evaluation process of databases that provided the information essential for hazard identification and characterisation, exposure assessment and risk characterisation. Risk assessment however, does not necessarily have to come to "monolithic" conclusions reflecting the mainstream of scientific opinion. In citing the WTO Appellate Body, it has become evident that "*equally responsible and representative governments may act in good faith on the basis of what, at a given time, may be a divergent opinion coming from qualified and respected sources*" (AB-1997-4,WT DS26/AB/R). This implies, that 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 for adverse effects in human health in the real world where people live and work and die*".

The report presented here aims to address equally basic scientific principles and also the emerging concerns related to hormonally active substances in the human environment. Consequently, non-traditional areas of risk assessment such as the growing scientific understanding of the critical role of imprinting in determining a number of subsequent developmental outcomes, the role of hormones in perinatal development, childhood and puberty, as well as the increase in a number of hormonally mediated diseases such as certain forms of cancer (breast cancer and prostate cancer) as well as autoimmune and allergic diseases, have been approached (see section 2.3. and 2.4.). At the same time, a critical evaluation of certain analytical procedures initiated questions towards the reliability of the estimates used for the amounts of each naturally occurring hormone produced by males and females at different ages (see section 3) . This is of importance as these physiological data have been used as cornerstones in hazard and risk characterisation.

Moreover, recent investigations on the bioprocessing of hormones in the mammalian organism confirmed initial studies on metabolic activation resulting in genotoxicity (see 2.5.). Although these biotransformation routes may be considered as minor pathways, the genotoxic nature of the metabolites resulted in the consideration of this distinct toxicological endpoint. In contrast, the majority of risk assessment approaches have considered hormonal no-effect levels. These may be deduced from a linear dose response curve for any given assay endpoint. However, for genotoxic effects as well as developmental, immunological, and neurobiological effects no threshold can be defined, rejecting the possibility to establish a no-effect-level which is a prerequisite in conservative risk assessment procedures.

Consequently the potential risk arising from the use of hormones for growth promoting purposes will be discussed in relation to:

– 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.

It *needs to be noted that the evaluation of the voluntary use of sexual hormones either for therapeutic purposes in individual human patients, contraception, and postmenopausal replacement therapy is beyond the scope of this report. This intentional (voluntary) use is restricted to distinct age groups and is based on an appropriate individual prescription by medical specialists. Thus, these therapeutic applications have to be clearly distinguished from involuntary consumption of residues in meat and meat products, which happens unknowingly and irrespective of age, sex, and individual characteristics of the consumers.*

2. EMERGING CONCERNS RELATED TO HORMONALLY ACTIVE SUBSTANCES

2.1. Definitions

A brief introduction of each of the hormones this report deals with follows. A more comprehensive approach to the subject follows this initial summary.

Oestradiol-17ß

Oestradiol-17ß is the most active of the female sex hormones (oestrogens) synthesized and secreted mainly by the ovary, the adrenals and the testis, which also secrete oestrogens. They affect many functions in organs and systems in humans; the most important relate to the reproductive function. Oestradiol is synthesized and secreted in early stages of embryogenesis and has an active role in the normal development of the female sex accessories during the lifetime of females.

Progesterone

Progesterone is a natural steroid sex hormone synthesized and secreted mainly by the corpus luteum in the ovary of cycling females. The second half of the estrous cycle is when progesterone secretion is most prevalent. Its function in reproduction is linked to the implantation of the egg in the uterus and the growth of the embryo and fetus. As all hormones, progesterone synthesis and secretion is regulated by a series of positive and negative feedback mechanisms in which polypeptidic hormones secreted by the brain (hypothalamus, pituitary) affect circulating progesterone levels.

Testosterone

Testosterone and its more active metabolite, dehydrotestosterone, are the main sex hormones secreted by males. Testosterone is responsible for the early development, and the appearance and maintenance of male secondary sex accessory organs (prostate, secretory glands, penis size, etc) during adulthood. Testosterone secretion is also affected by the complex interaction among all endocrine glands, especially with those in the brain.

As with the above mentioned sex hormones, testosterone is metabolized and as a result, metabolites of different activity are generated. Some of these metabolites play a more active role in certain organs than in others.

Zeranol

Zeranol is a natural mycooestrogen derived from zearalenone, produced by different species of *Fusarium* molds. As with the animal natural oestrogens, zeranol affects oestrogen target organs and therefore disturbs reproductive patterns when introduced accidentally or intentionally in susceptible hosts.

Trenbolone

Trenbolone is a synthetic androgen having anabolic activity several fold above that of testosterone. Trenbolone is metabolized into 17β-trenbolone, its most active derivative. Although data are still incomplete, it is known that trenbolone shares comparable hormonal properties with testosterone .

Melengestrol

Melengestrol is a progestogen, about 30 times as active as progesterone. It interferes with the estrous cycle through the same feedback mechanisms described for progesterone. Its metabolic effect is thought to be due to its ability to increase oestrogen levels in treated heifers.

2.2. Effects of hormones at different stages of life

2.2.1. Effects of sex hormones during the intrauterine and perinatal periods on development

In most animal species, natural hormones regulate the anatomical and functional development of the reproductive tract. Sex hormones exert these important regulatory functions by affecting their targets at different levels of biological complexity (cells, tissues, organs). At the subcellular level, this is done through both specific receptor-mediated and receptor-independent pathways. During organogenesis in intrauterine life, or during the perinatal period, normal patterns of sexual development may be upset by an extemporaneous surge or decline of normal levels of these natural, endogenous, sex hormones. Comparable effects may be elicited by the introduction of man-made chemicals that, while showing very different chemical structures (xenoestrogens), elicit similar overall responses, namely, they disrupt the normal development of the reproductive apparatus in humans and wildlife.

Because of the many tissues that can be considered as targets of these hormones, it is at times difficult to discriminate between direct and indirect effects elicited by natural or synthetic sex steroids. For example, while the end-point of a survey may be the anatomic integrity of the secondary sex organs (uterus, vagina, urethral passages, prostate, penis), these anomalies may be the result of indirect effects originally triggered by hormonal effects on brain target cells (hypothalamus, pituitary, etc). Equally important is the promotional role attributed to oestrogens during the carcinogenic process during adulthood (Soto and Sonnenschein, 1999).

It should be highlighted that the word "oestrogens" was coined to represent compounds of very different chemical structure (from natural oestrogens to xenoestrogens) which have the ability to induce histological changes in the vagina and uterus during the estrous period (Hertz, 1985).

2.2.1.1. Anticipated effects of oestrogens during intrauterine and perinatal periods of development in humans

A comprehensive evaluation of the role of natural and synthetic sex hormones during fetal and perinatal life in humans remains elusive despite the accumulation of data on this subject during the last two decades. Ethical considerations prevent the gathering of experimental data in humans from rigorously controlled experiments; only when accidental exposure occurs can epidemiologic studies help disclose the long-term effect of these encounters. Therefore, conclusions drawn from malformations found in animal populations extemporaneously exposed to actual or suspected oestrogenic compounds become of an inferential nature for humans. Since rigorously controlled experiments cannot be conducted in humans, researchers resort to the use of surrogate models that involve species for which susceptibility and sensitivity to the suspected culprits may resemble that of humans.

Certain organs are more susceptible to the effects of oestrogens, androgens, and anti-androgens during development than during adulthood. These organs include the brain, and the primordial structures responsible for the normal tissue architecture of the primary and secondary sex organs. These organs, in turn, affect each other's physiological roles. For instance, hypothalamic nuclei and the pituitary are affected by sex hormones secreted even at low concentrations by neighbouring brain cells (Guennoun et al., 1997), as well as by distant rudimentary ovaries and testis. Hence, the secretory and responsive roles of these cells, tissues and organs are affected directly and indirectly by sex hormones and polypeptide hormones. A variety of anatomic malformations and dysfunctional imprinting can be anticipated when to this already complex interaction are added the extemporaneous effects of natural and/or synthetic hormones from dietary sources. Given the impossibility of testing every single variation affecting timing and dosage under which experiments could be run, the literature is far from exhaustive.

2.2.1.2. Experimental strategies:

Amphibians, birds, reptiles and mammals (especially, mice and rats) have been used for the purpose of unravelling the role of natural and synthetic sex steroids on organogenesis, with special emphasis on secondary sex organs. Given the vastness of this field of inquiry, many questions await answers. This is mainly due to the different hormone concentration levels at which experimental studies have been conducted, the age at which hormones were administered, and the time length during which the different above mentioned targets were exposed. Clearly, not all experimental possibilities have been covered. Hence, the information available so far falls short of the ideal, or even the sufficient standard to allow observers a well informed judgment when assessing exposure regarding what is acceptable from what is not. This is especially true of the risk of the central nervous system's exposure to sex hormones.

Several laboratories have published evidence which links higher exposure to sex steroids during intrauterine life with anatomo-physiological disruption of normal patterns in the reproductive fitness of adults. These studies followed

two main strategies: The first strategy is the direct exposure of natural and/or xenoooestrogens to hatching eggs in reptiles (Sheehan et al., 1999), fish (Gimeno et al., 1996), and birds (Fry and Toone, 1981), and to pregnant and prepubertal mammals (Bern, 1992; Herbst and Bern, 1988; Newbold, 1999; Zuckerman, 1940; Takasugi, 1979) These hormones may permanently affect the brain (Brawer et al., 1978) and the vagina (Ma et al., 1998). The second strategy is the use of fetal mice positioned in the pregnant uterus between fetuses of the same or opposite sex (see below, and Schmid and Schlatter, 1985).

2.2.1.3. Experimental evidence on the effects of oestrogen exposure during embryogenesis, fetal and perinatal stages.

The administration of DES to laboratory animals during their perinatal age has shown that this synthetic oestrogen is responsible in female mice for the following pathological entities: i) structural malformations of the oviduct, uterus, cervix, and vagina, ii) salpingitis isthmica nodosa; iii) paraovarian cysts (of mesonephric origin); and iv) vaginal adenocarcinoma (Newbold, 1999). In addition, to these anatomic and tissue-based malformations, infertility and sterility were observed in adult mice of both sexes. Regarding mechanisms that these hormones may upset, it has been recently reported that oestrogens down-regulate the expression of mice homeobox genes during development, such as Hoxa-9, -10 and -11 (Ma, 1998).

Whether or not the above mentioned defects are due solely to a direct effect of the hormone on their secondary sex organs, or to the separate and/or simultaneous effect on the brain structures that govern patterns of hormone synthesis and secretion, is only conjectural at this time. Data are unavailable for addressing these issues. Nonetheless, it is likely that a combination of effects are responsible for the symptomatology recorded in young individuals and adults after in utero exposure.

On the one hand, it has also been shown that DES modifies the normal development of the male genital tract by interfering with effects of androgens on the normal Mullerian (paramesonephric) ducts (vom Saal et al 1997); in addition, administration of DES to male mice during the perinatal period results in the appearance of testicular tumors during adulthood (Newbold et al. 1998). It should be noted that this tumor incidence has been recorded in the second and third generations of those males who were originally injected with DES.

Studies have been conducted on the effects of intrauterine positioning of male and female fetuses. Data collected by using the position in the uterine horn of male and female fetuses clearly indicate that the sexual behaviour, genital morphology, timing of puberty, sexual attractiveness, aggressiveness, tissue enzyme activity, and the length of the oestrus cycle of females during adulthood were influenced by the sex of their neighbouring litter mates in the uterine horn (vom Saal, 1989). Also, in addition to the above mentioned characteristics, vom Saal's group has studied the effect of this fetal uterine

positioning on the development of the mouse prostate (vom Saal and Timms, 1999). The outcome of these computer-assisted, three-dimensional reconstructions of the developing prostate show that oestrogen levels during organogenesis affect the anatomic and histopathological phenotype of the prostate during adulthood. These effects seem to be due to the interference by oestrogens of the testosterone and 5α-dehydrotestosterone facilitation process on Mullerian-inhibiting-factor (MIF) (vom Saal et al., 1992). The presence and normal activity of a 5α-reductase in the stroma of the rudiments of 12-day-old male fetuses to 2-day-old newborns is crucial for the normal development of the male genital tract. Interference with the activity of this enzyme, be it through genetic defects or through the presence of drugs or higher oestrogen levels, results in an abnormal pattern of development in the prostate and the external genitalia visible during adolescence and adulthood. A mouse fetus placed between its male siblings has higher testosterone concentrations than mice of the same sex positioned between females. And, a fetus positioned between females has shown oestradiol concentrations higher than a fetus of the same sex positioned between males. By competing with oestradiol, oestrogen antagonists eliminate the effects of intrauterine position, suggesting a receptor-mediated mechanism for this phenomenon. The exposure of slightly higher, but effective, levels of oestrogens and androgens to males and females fetuses, respectively, is due to the anastomosis of capillary beds between neighbouring placentas. The realisation that "physiological" concentrations of androgens and oestrogens affect the development of secondary sex characters and the behaviour of would-be adults represent compelling evidence favouring the notion that no measurable threshold for these developmental effects is available.

Among other effects, vom Saal's group has also concluded that male fetal mice exposed to even low levels of DES show significantly enlarged prostates in adulthood, a significant decrease of the urethra width, and an enlargement of the utriculus, when compared with untreated controls. In addition, adult mice exposed in uterus to xenooestrogens showed a decrease in sperm production, decreased seminal vesicle and epididymal sizes.

2.2.1.4. Specific effects of natural oestrogens administered during the fetal and perinatal periods

It has been acknowledged for decades that oestrogens affect the male reproductive tissues (Zuckerman, 1940). Sexual differentiation in human foetuses takes place between weeks 7 and 12 of gestation (Moore and Persuad, 1993). The female reproductive system develops independently of hormonal regulation, implying that the default state of this function is female (Wilson and Lasnitzki, 1971). With the recent advent of the oestrogen receptor-α and α knockout mice, a more refined knowledge about the role of oestrogens on their target tissues is now being accumulated. Oestrogen and androgen receptors are already present in the rudiments of the Mullerian and Wolffian derivatives during this period. It is therefore likely that even small additional concentrations of sex hormones (natural or synthetic) may deleteriously affect the development of these tissues (hypospadias or undescended testis). Moreover, the affected gonadal rudiments do not complete their full developmental capabilities until puberty. While it is during

adulthood that a disrupted reproductive function may be diagnosed, its origin dates from the exposure of susceptible oestrogen/androgen target cells during the prenatal or even prepubertal period. As mentioned above, data accumulated by vom Saal's group (vom Saal, 1989, vom Saal and Timms, 1999) suggest that no threshold is available for exposure for oestrogens.

The administration of oestradiol to mice, rats, guinea pigs and rabbits during foetal and perinatal life resulted in significant and lasting defects in pituitary-hypothalamic function in males, which in turn, disrupted testicular function during adulthood [involution of epididymal epithelium and seminiferous tubules with the absence of germ cells, reduced testis size and reduced sperm production (Takasugi, 1979; Davies and Danzo, 1981; Orgebin-Crist et al., 1983; Brown-Grant et al., 1975)]. In the case of male hamsters, it is remarkable that the disruption of the reproductive function as a result of exposure to DES during the neonatal period did not affect the levels of circulating androgens (Khan et al., 1998).

2.2.1.5. Effects of synthetic hormones on fetal and perinatal development

On the other hand, few data are available concerning the effects of synthetic hormones such as zeranol, trenbolone and melengestrol, administered during the intrauterine or/and perinatal period on the reproductive function in adults. In a three generation study of rats receiving zeranol at levels up to 0.20 ppm throughout gestation, it has been concluded that the fertility of the offspring is not affected (JECFA, 1988). However, male mice exposed *in utero* to zeranol (150 μg/kg of body weight injected on days 9 and 10 of gestation) show testicular abnormalities (regressive changes in the germinal epithelium and Sertoli cells, and immature morphology of Leydig cells) when testes are examined at 45 days of postnatal life (Perez-Martinez et al., 1997). Moreover, in a multi-generational study, it has been shown that trenbolone acetate, administered to female rats at dietary concentrations of 3 and 18 ppb between 2 weeks before mating and 3 weeks delivery, exert effects on reproductive performance which are more marked in F2 pups than in F1 pups of a comparable age. Indeed, female F1 pups from F1-treated parents show signs of virilization, a delay in the mean vaginal opening and the presence of occlusive strands in the vagina or incomplete vaginal opening. Male pups show a delay in the occurrence of testicular descent and a decrease in weights of seminal vesicle, prostate, testes an epididymis. In addition, in F2 pups from both sexes the adrenal weight was also decreased (JECFA, 1988).

2.2.1.6. The additive effect of exposure to oestrogenic chemicals

Scant published data are available regarding the probable effect of combined oestrogenic compounds on the normal process of organogenesis during fetal, perinatal or pubertal development. It is, however, likely that this is in fact the "real life" situation faced by humans and wildlife. According to recent reports, humans are affected by physiological levels of endogenous ooestrogens and androgens in addition to varying amounts of natural and synthetic sex steroids present in their diet (Colborn et al., 1993).

Among xenoestrogens, bisphenol-A has been shown to leach from plastic coatings applied to the inside of tin cans used in preserves and from non-fully polymerized composites and sealants routinely used by dentists to fill dental cavities (Soto and Sonnenschein, 1996). Also, p-nonylphenol was shown to leach from plastics that may come in contact with foodstuff (Brotons et al., 1994). These are just a small sample of those xenoestrogens whose presence and activity will be properly assessed by measuring the *total oestrogenic burden* of an individual. This assay, now being developed and evaluated by laboratories in Boston, MA, USA, and Granada, Spain, aims at measuring oestrogenic compounds of a variety of sources (natural and synthetic) in oestradiol equivalents present in the plasma and fatty tissues of diverse human and wildlife populations.

Several distinct xenoestrogens operate additively when tested in culture conditions and measured through a proliferative assay using human breast cancer MCF-7 cells (Soto et al., 1997). These data suggest that the combination of natural oestrogens, androgens and xenoestrogens introduced in the diet may result in altered sexual development (Kortenkamp and Altenburger, 1998).

Disrupted patterns of development are most likely generated because synthetic chemicals, regardless of their structure, can play roles similar to those exerted under normal circumstances by natural hormones. Additional sources of disruption can be generated when these so called natural steroids are introduced into their hosts at stages other than those at which they exert their specific regulatory role. This is what has been shown to occur when even low doses of either natural or synthetic steroids are introduced into their host at the wrong developmental stage. Patent teratologic disruption has been documented when even low doses of these hormones were administered during narrow "windows of vulnerability" during either embryonal, fetal or neonatal life. This model has been raised mainly as the result of the unfortunate episode experienced by the administration of diethylstilbestrol (DES) to millions of women during a three-decade period to prevent spontaneous abortion . The research conducted to investigate this iatrogenic event follows what has been called "the DES paradigm" (Herbst and Bern, 1988; McLachlan, 1993).

2.2.1.7. Circulating sex steroid-binding proteins

Another element to be considered of interest in regard to the effects of natural and synthetic sex steroids is represented by the role played by blood-borne steroid-binding proteins during development. Androgen Binding Protein (ABP) is produced by the Sertoli cells of the testis, while Sex Hormone Binding Globulin (SHBG) is produced in the liver. They are products of the same gene, but the affinity of steroids to bind to these proteins differ; in general, androgens bind with slightly higher affinity than oestrogens (Joseph, 1994). Despite attempts to link these proteins as blood carriers of steroids and modulators of androgen action (Nakhla and Rosner,

1996), the role of these binding proteins on the effects of their respective ligands is yet to be clarified. However, studies conducted in cell culture clearly demonstrate that SHBG shifts the dose-response curve of its androgen ligands, thus effectively lowering the availability of these ligands to their target cells (Damassa et al., 1991). This observation suggests that it is likely that androgens and oestrogens not binding to SHBG would be fully available to their target cells, and thus, could be effective even if they are less potent than the natural SHBG ligands during the perinatal and prepubertal periods in humans.

In rodent species, α-fetoprotein (AFP), a plasma-borne protein present during the perinatal period, shows relatively high-binding affinity for oestrogens. It has been speculated that AFP may play a role in buffering the oestrogenic effects of endogenous oestrogens on oestrogen-target cells residing in the brain (pituitary, hypothalamus). In humans, AFP does not seem to have such a role because of the low plasma concentrations of this protein.

2.2.2. *Endocrine effects of sex hormones on growth and puberty in humans*

Reproduction, growth, sexual and social behaviour are under the control of sex hormones in animals and humans. When introduced in the endocrine environment of young individuals or adults, exogenous sex hormones (natural steroids or synthetic substances with steroid-like effects) can exert specific, both direct and indirect effects. Acting directly, sex hormones modulate growth or differentiated functions of target tissues, such as hypothalamus, pituitary, uterus, mammary gland, prostate, seminal vesicles, epididymis, gonads, bone, and muscle which express specific steroid receptors. Acting indirectly, they disrupt the endocrine equilibrium, particularly by modulating the secretion of gonadotropins by pituitary cells, leading to various effects on reproduction. They can also be metabolised in the different tissues and generate metabolites with direct or indirect actions on physiological functions. In this report, the risks of exposure to exogenous sex hormones during childhood or adult life will be evaluated on growth, puberty and reproduction.

2.2.2.1. Physiological levels of steroids in serum during childhood and puberty

Childhood is characterised by extremely low concentrations of steroids in serum. Testosterone and oestradiol levels in prepubertal children are most often below or close to the detection limit of the assays that are used to measure serum levels. Recently, an ultrasensitive assay has been developed for measuring oestradiol in prepubertal children of both sexes (Klein et al., 1994). Results show that the mean serum oestradiol level in prepubertal girls (0.6 pg/ml) is 8-fold greater than the level in prepubertal boys. Besides, the serum concentration of SHBG, which binds testosterone and oestradiol with great affinity, is high in children, by comparison with adults (Andersson et al., 1997). These levels of SHBG regulate both the bioavailability of steroids to target tissues (it is assumed that only the free steroids are directly available for cells), and their metabolic clearing, playing a role of "reservoir" of

steroids. These observations suggest that prepubertal children constitute a high risk population for exogenous sex steroids since endogenous levels are extremely low and the metabolic clearance rate of steroids able to bind SHBG is expected to be lower than in adults (Andersson and Skakkebaek, 1999).

The first endocrine sign of puberty onset is an increase in serum levels of adrenal androgens (particularly dehydroepiandrosterone sulfate), followed later by an increase in gonadal steroids (oestradiol in girls, testosterone and oestradiol in boys), (Klein et al., 1996). Under physiological conditions, oestradiol originates from ovarian follicles in girls and is synthesized by peripheral aromatization of testosterone secreted by testis in boys (Veldhuis et al., 1997). These steroid hormone increases are a metabolic signal for the somatotrope axis to initiate the pubertal growth spurt. Particularly, oestradiol stimulates the secretion of growth hormone (GH) by the pituitary which is followed by an increase in serum level of insulin-like growth factor-I (IGF-I) of hepatic origin through adolescence (Caufriez, 1997; Wilson, 1998). Conversely, GH by acting directly on the gonads, and/or indirectly via enhanced IGF-I hepatic secretion, potentiates the stimulating effect of gonadotropins on production of sex steroids by the gonads (Sharara and Giudice, 1997). It is likely that oestradiol plays a key role in the auto-amplifying loop existing between the somatotrope axis and the gonads, which results in the growth spurt at the onset of puberty. Consequently, it is thought that exogenous sex hormones either with oestrogenic activity (oestradiol itself, phytooestrogens, xenoestrogens) or androgens (testosterone) that by aromatization give rise to oestrogenic compounds may participate in this regulatory loop thereby advancing the onset of the pubertal growth spurt.

2.2.2.2. Actions of sex hormones on growth and bone maturation

Some experimental evidence indicate that oestrogens exert a direct, biphasic effect on bone growth and maturation, with maximal stimulation of epiphyseal growth at low levels in prepubertal boys as well as girls. Administration of low doses of estrodial (4 µg per day administered to 30-40 kg children by a 4 day infusion) resulting in serum oestradiol levels of about 15 pmol/l (4 pg/ml) causes more than a 60% increase over the prepubertal growth rate in both boys and girls (Cutler, 1997). In girls with Turner's syndrome, ethinyl oestradiol doses as low as 25-100 ng/kg/day are associated with increased growth rate, and a 100 ng/kg/day dose is also associated with increased GH release (Ross et al., 1988; Mauras et al., 1989). Conversely, administration of an aromatase inhibitor (testolactone), to boys with familial male-limited precocious puberty produces near normalization of both growth velocity and bone maturation, despite levels of serum testosterone that remain within the adult male range (Cutler, 1997). From this data, researchers concluded that the pubertal growth spurt of both sexes is driven primarily by oestrogens, and that the more rapid epiphyseal maturation of prepubertal girls (vs boys) may be explained by their higher oestradiol levels. These conclusions are reinforced by the observation that oestrogen deficiency due to mutations in the aromatase gene, and oestrogen resistance due to disruptive mutations in the oestrogen receptor gene lead to absence of the pubertal growth spurt, delayed bone maturation, continued growth into

adulthood and unusual tall stature in both sexes (Lee and Witchel, 1997, Mac Gillivray et al., 1998).

2.2.2.3. Actions of sex hormones on secondary sexual characteristics

During adolescence, increased oestradiol serum levels induce breast and uterus development in girls. In boys, increased testosterone serum levels exert direct virilizing and anabolic effects, leading to increases in bone mass and muscle bulk. Contrary to androgens, oestrogens do not alter anabolism in prepubertal boys and girls (Mauras, 1995). However, both androgens and oestrogens affect sexual development. For example, a dominant mutation leading to an aromatase excess in boys is associated with heterosexual precocity and breast development (gynecomastia) (Stratakis et al., 1998). Conversely, aromatase deficiency in girls is associated with increased androgen serum level, pseudohermaphrodism, virilization and polycystic ovaries (Morishima et al., 1995, Mullis et al., 1997). These data provide a baseline from which to interpret conditions where man-driven environmental changes may interfere with anatomophysiological developments in sexual dimorphisms in humans.

2.2.2.4. Potential adverse effects of exogenous sex hormones on growth and puberty upon exposure of prepubertal children

The risks associated with exogenous sex hormone exposure depend on both the dose and the duration of exposure. However, increasing evidence blur the notion of a fixed threshold of sensitivity for each tissue and/or physiological function. Moreover, this previously perceived fixed threshold can vary with the developmental stage and the sex of the exposed individuals. For example, the sensitivity of the hypothalamus/pituitary system to steroids is much higher in childhood than during adolescence and adult age.

What are the long-term consequences of prepubertal, extemporaneous exposure to steroids on the reproductive activity in adults? In female rats, postnatal androgenization by a single injection of testosterone alters permanently the response of uterine cells to oestrogen action by a mechanism called "hormonal imprinting" (Mena et al., 1992). Similarly, in the male hamster, neonatal administration of DES disrupts reproductive function in adults, and alters permanently androgen responsiveness in target tissues, despite apparently normal pubertal development (Khan et al, 1998). In the rat, hormonal imprinting caused by allylestrenol (a steroid used in prevention of abortion), when administered perinatally, produces changes in sexual behaviour of adults and modifications in testosterone and progesterone levels in males and females respectively (Pap and Csaba, 1995). In addition, this treatment reduces the concentration of thymus glucocorticoid receptors and increases the concentration of uterus oestrogen receptors at adult age (Csaba and Inczefi-Gonda, 1998). Interestingly, imprintability persists only to the 4th postnatal day in the rat, indicating that this process occurs only through a critical period or window period of vulnerability.

Examples of oestrogenic environmental contamination leading to anomalies in growth, sexual development and puberty have been reported in humans. In

Puerto Rico, over 10 000 documented cases of anomalous sexual development, including premature breast development (thelarche), premature sexual hair (pubarche), pseudoprecocious puberty, gynecomastia, ovarian stimulation syndrome, virginal hyperplasia of the breasts and polycystic ovaries, have been described in the past 28 years (Perez-Comas, 1988). These anomalies were associated with high serum total oestrogens and accelerated bone age in 85% and 26% of the studied cases, respectively. The clinical signs receded or diminished in a significant number of patients after diet control; however, the nature of the oestrogenic contaminant was not firmly identified. Other examples of breast enlargement and pseudoprecocious puberty were observed in Italy where accidental oestrogen intake from contaminated food was also suspected in these cases (Fara et al., 1979, Pasquino et al., 1982). In 1980, analysis of Italian baby food made with homogenized veal revealed the presence of DES, likely originating from implants which were not removed before slaughtering of animals (Loizzo et al., 1984). In most reported cases of accidental contamination, anomalies were transient and reversible but the long-term effects of exposure to oestrogenic compounds are yet unknown. When the reproductive function is concerned, at a mean follow up of 10 years there is no evidence that pharmacological doses of sex hormones administered during puberty in the management of constitutionally tall stature have a long term effect, since menstrual cycles, fertility and pregnancy are comparable with adult controls (De Waal et al., 1995). However, the consequences seen in adults for prior exposure during prepuberty in populations with a high genetical polymorphism are presently unknown.

Beside these cases of accidental contamination, continued environmental exposure of healthy children to very low oestrogen levels might also have important consequences. This is likely given the absence of a threshold concentration below which an observer could safely claim lack of effect. Conservatively, one might suspect a slight increase in height and/or a decrease in the age when final height is achieved, and a younger age of pubertal onset. There has been an increase in final height and a decrease in the age of pubertal development in Western countries during this century. Presently, this trend is generally attributed to the improved nutritional status in these societies. Interestingly, in a study including 17,000 girls between 3 and 12 years of age, signs of oestrogenic stimulation such as breast and/or pubic hair development have been observed in 27.2% of African-American girls and 6.7% white American girls at 7 years of age (Herman-Giddens et al., 1997). This indicates that precocious puberty is somewhat common in USA. Tiwary (1994) suggested that cosmetic (hair) products, containing oestrogen or placenta extracts, may be related to some of the increased prevalence of early puberty in African-American girls. However, the importance of environmental oestrogenic compounds present in plastics, insecticides, and meat from animals treated with sex hormones, while suggestive, remains as only a possibility in affecting an early onset of puberty.

2.2.3. Effects of sex hormones on reproduction

2.2.3.1. Physiological actions of steroid hormones

Under physiological conditions, synthesis and secretion of the sex steroids by gonads are regulated by two pituitary gonadotropins, the folliculo-stimulating hormone (FSH) and the luteinizing hormone (LH). Testosterone in men and progesterone and oestradiol in women exert negative feedback effects on the secretion of FSH and LH, acting on the hypothalamo-pituitary system. Moreover, in adult non-pregnant females, high serum levels of oestradiol (about 300 pg/ml in humans) induce, by positive feedback effects on the hypothalamus, a discharge of gonadotropins which triggers ovulation at each ovarian cycle. In the female, progesterone secreted by the corpus luteum after ovulation, and oestradiol secreted by the ovarian follicles after the corpus luteum demise, drive the ovarian cycles.

In both sexes, steroids are absolutely necessary for fertility. Testosterone sustains spermatogenesis, acquisition of sperm maturation and motility in the epididymis, and supports the function of accessory glands (prostate and seminal vesicles). Oestradiol and progesterone regulate folliculogenesis and oocyte maturation in ovarian follicles, prepare uterus for implantation of the embryo, support gestation and induce mammogenesis.

2.2.3.2. Potential adverse effects of exogenous sex hormones on reproduction

It is established that a sustained increase in serum levels of steroids induces a decrease in secretion of gonadotropins (negative feed-back effect), leading to arrest of spermatogenesis and azoospermia in the male, and interruption of ovarian cycles and blocking of ovulation in the female. The contraceptive pill for women and the contraceptive treatments for men presently being tested are derived from our knowledge of this regulatory process.

Clearly, the doses sufficient to induce sterility in adults are higher than the doses able to affect growth and puberty in young individuals (Cutler, 1997). In the male rat, doses as low as 10 ng oestradiol/rat or of the catechol oestrogens 2-OHE$_2$ and 4-OHE$_2$/rat induce, after 21 daily exposures, morphological lesions and spermatogenesis arrest without affecting gonadotropin release; a direct effect of these oestrogens on developing spermatids has been proposed (Seegers et al., 1991). In men, infertility has been associated with an increase in intratesticular aromatase, leading to an increase in intratesticular and seminal oestradiol levels (Bujan et al., 1993; Ichikawa, 1995).

Experiments in rats, mice, hamsters, dogs, pigs, cattle, sheep or monkeys have shown that exogenous sex hormones, including natural steroids as well as growth promoters (such as trenbolone acetate, zeranol or melengestrol acetate), administered by ingestion, injections or implants, induce dose-dependent deleterious effects on reproduction in males and females (JECFA, 1988).

A link between environmental sex steroids and infertility in adult humans has been proposed. It has been suggested that the increase in fibrocystic disease

of the breasts, polycystic ovaries, menstrual irregularities and infertility observed in adult women from Puerto Rico is associated with the presence of an oestrogenic contamination (Perez-Comas, 1988). Moreover, use of anabolic steroids for body-building is associated with oligospermia and azoospermia (Turek et al., 1995; Sørensen and Ingerslev, 1995). Furthermore, male reproductive health appears to be deteriorating in many countries during the last few decades (Skakkebaek and Keiding, 1994). Presently, it is thought that exposure to oestrogenic or environmental antiandrogenic chemicals during fetal and childhood development can result in reproductive defects, particularly in declining semen quality (Toppari et al., 1996).

2.3. Human exposure to oestrogens and its relationship to cancer

No study has assessed the effects of hormones as growth promoters in farm animals on cancer occurrence in humans. Arguments to be considered when evaluating the hypothesis of a potential link between use of food promoters in farm animals and cancer in humans come both from descriptive epidemiology, including studies in migrants and etiologic epidemiology on diet and cancer as well as on hormones and cancer.

Given the nature of the substances being examined, most of the attention is directed toward hormone-dependent cancers: cancer of the breast in women and prostate cancer in men. Other cancers of interest include testicular cancer in men and colon cancer in both sexes.

2.3.1. Descriptive epidemiology

The geography of diseases has long been one of the first clues leading to the development of etiologic hypotheses. Cancer patterns differ across countries and continents, as well as over time.

The incidence rate of breast cancer, standardised on the age structure of the world population (a procedure allowing comparison of populations with different age composition directly regarding the occurence of a given disease) varies greatly across the world, from a minimum of 7.1 new cases per 100 000 women per year in Kwangwha, Korea to 103.7 among non-Hispanic Caucasians in Los Angeles, United States. As shown in table A4, the highest rates of breast cancer are observed in North America, where hormone-treated meat consumption is highest in the world, followed in decreasing order by the European countries, Oceania (except the American territories of Hawaii), Central and South America and well behind, by Asia and Africa (Parkin et al, 1997). Currently one cannot confirm nor refute the association between high rates of breast cancer and high hormone-treated meat consumption in North-America. This should be urgently studied.

Prostate cancer shows similar variations, with incidence rates ranging from 0.5 new cases per 100 000 men per year in Qidong, People's Republic of China to 142.3 among black men in Atlanta, USA. In fact, its geographic distribution (table A5) is comparable to that of breast cancer (Parkin et al, 1997).

It should also be noted that the incidences of breast cancer and prostate cancer have greatly increased between the 1970s and the 1990s (Coleman et al, 1996) and are still following an upward trend (Dinse et al, *in press*).

The pattern of testicular cancer is less contrasted with a much smaller range of values for the incidence rates from 0.2 new cases per 100 000 men per year in Setif, Algeria, or Qidong, People's Republic of China to 10.3 in Graubunden, Switzerland. Highest rates are found in Europe and lowest in Africa and Asia (table A6).

For colon cancer, either in men (table A7) or in women (table A8), patterns are broadly similar to breast and prostate cancers with high rates in North American whites, Oceania and at a slightly lower level in Europe. In men, rates range from 0.4 new cases per 100 000 men per year in Setif, Algeria to 35.0 for the black of Detroit, USA. In women, the extremes are 0.4 new cases per 100 000 women per year in Barshi, Paranda and Bhum, India to 29.6 in non-Maori of New Zealand.

The results of descriptive epidemiology are further reinforced by migrant population epidemiology. Studying cancer risk modification as a function of migration history, whether from one continent to another, or from one country to another, or even within the same country, can give useful information on the respective impact of genetic susceptibility and lifestyle or environmental factors. This is true especially when the occurrence of the cancer of interest is subject to large geographical variations (Iscovich and Parkin, 1998; Bouchardy et al., 1998; Geddes et al., 1993; Steinitz et al., 1989). Many studies have shown that the level of breast cancer risk in migrant populations gradually converges towards that prevailing in the host country after several generations. One of the most classical example is the study of breast cancer risk among successive generations of Chinese women migrating from the People's Republic of China to Hong Kong and further on to the USA (Sasco, 1989). The case of Italian migrants in Australia provides a striking example, whereby new environmental conditions and recently adopted lifestyles affect the level of risk of breast cancer (McMichael and Gilles, 1988). Similar findings have been noted for gastrointestinal cancer (McMichael et al., 1980). These results suggest that lifestyle habits, including diet, play a role in the occurrence of cancers, and especially during the childhood period and around puberty.

2.3.2. *Etiologic epidemiology*

Several types of information are available regarding the role of diet, and also that of hormonal agents.

2.3.2.1. Cancer in relation to the consumption of meat and meat products

Meat consumption is possibly associated with increased risks of breast and prostate cancers. Results deriving from studies conducted on vegetarian populations are consistent with this finding. The overall cancer risk, and in particular breast cancer, is generally lower among non-meat consumers. However, it must be underlined that while the evidence is particularly striking for America, less marked results have been reported for Europe (World

Cancer Research Fund, 1997). Again the link if any with consumption of hormone-treated meat cannot, at present, be confirmed nor refuted.

In this review of the World Cancer Research Fund published in 1997, it was concluded that diets high in meat possibly increase the risk of breast cancer. This statement was based on the fact that three of eight prospective studies have reported an increased risk of breast cancer with higher meat intake, and that a meta-analysis of seven cohort and case-control studies has also shown an increased risk at 1.5, confirmed in several case-control studies not included in this combined analysis. In contrast, it was also concluded that diets high in poultry possibly have no relationship with the risk of breast cancer (World Cancer Research Fund, 1997). A recently published study evaluating diet during adolescence and risk of breast cancer among young women found increases of borderline significance in risk of breast cancer for intake of chicken or high-fat meat (Potischman et al, 1998). The role of N-acetyltransferase genetic polymorphisms in mediating risk of breast cancer in relation to meat consumption was not supported by the results of a traditional case-control study (Ambrosone et al., 1998) nor of a nested case-control study (Gertig et al., 1999).

The most recent and exhaustive review published in 1998 shows even clearer trends. Out of 20 case-control studies carried out in countries with different meat consumption levels, 17 observed higher risks associated with higher total meat, red meat or processed meat consumption, although only 11 studies were statistically significant. One study found a non-significantly lower risk associated with higher meat consumption, one found no association and all others had increased relative risks ranging from 1.1 to 3.5. Among ten cohort studies, one found no association and the other nine had estimates above one but statistically significant in only five. Values of relative risks ranged from 1.2 to 2.4. In four cohort studies, a grading effect associated with the number of meat servings per week was described. The conclusion of that review was that there is moderately consistent evidence that higher meat consumption, particularly red or fried/ browned meat is associated with higher risk of breast cancer (Department of Health, UK, 1998).

The weight of the evidence in favour of the role of meat consumption in prostate cancer is somewhat similar. Among populations with a high overall meat intake, three of four cohort studies and four of five case-control studies show an increased risk in association with higher consumption. Associations were not seen in the Japanese cohorts in Japan or Hawaii or among Seventh-day Adventist men. It was therefore concluded that diets high in meat possibly increase the risk of prostate cancer (World Cancer Research Fund, 1997).

In the 1998 review, for prostate cancer, all case-control studies found higher risks associated with higher meat consumption and in five studies out of six, the results were statistically significant, with a relative risk around 2 for highest compared to lowest consumption. Eight cohort studies also examined this association. Three found significantly higher risk of prostate cancer associated with higher meat or red meat consumption, two found non

statistically higher risk for total meat consumption, one found non statistically lower risk for total meat, one found non significantly lower risk for red meat and one found no difference for fried meat. The conclusion was that there is a weakly consistent evidence that total meat consumption is associated with the risk of prostate cancer (Giovannucci, 1998). For red meat, the evidence is moderately consistent (Department of Health, UK, 1998).

For milk consumption, a conservative judgement was that diets high in milk and dairy products possibly increase the risk of prostate cancer. It should be noted that most of the evidence came from case-control studies with only modest support from the cohort data (World Cancer Research Fund, 1997).

2.3.2.2. Hormones and cancer

Another important contribution of etiologic epidemiology is the evaluation of the carcinogenicity of certain hormones. In the IARC Monograph of June 1998, the potential carcinogenicity of hormonal replacement therapy and oral contraceptives was assessed. Post-menopausal oestrogen therapy and combined oral contraceptives have been classified as human carcinogens (IARC Monographs Group 1), whereas post menopausal oestrogen-progestogen therapy and progestins only contraceptives are considered as possibly carcinogenic to humans (Group 2B) (IARC, 1999, see tabel A9). In previous IARC monographs of 1987 (IARC, 1987) and 1996 (IARC, 1996), other hormones and hormone-derived products have also been evaluated or re-evaluated regarding their potential carcinogenicity. DES, (the first recognised transplacental carcinogen for humans), non-steroidal and steroidal oestrogens, oestrogens, sequential oral contraceptives and tamoxifen were then classified in Group 1 of recognised human carcinogens. Group 2A included androgenic (anabolic) steroids. Group 2B included medroxyprogesterone acetate as well as progestins. Finally clomiphene acetate, oestradiol mustard, droloxifene and toremifene were classified as group 3 (table A9). Detailed results are given in the appendix (see table A10)

Recent data implicate a polymorphism in catechol-O-methyltransferase (COMT) in breast cancer risk. Methylation by COMT is an important pathway for inactivation of catechol-oestrogens (see also 2.5.). The allele encoding low activity COMT may be a contributor in particular to post menopausal breast cancer (Lavigne et al, 1997), although this has not been confirmed by a more recent study (Millikan et al, 1998). In fact menopausal status, as well as weight may modify this association (Thompson et al, 1998). Even physical exercise and training have an impact on 4-hydroxycatechol oestrogen metabolism (De Cree et al, 1997).

In conclusion, when examining the available data on the three endogenously produced human hormones, 17ß-oestradiol, testosterone and progesterone, it became evident that the fact that these compounds are natural does not automatically make them fully safe. In fact, the hormonal profile is of substantial importance for development of breast and endometrial cancer. A higher risk of breast cancer is associated with specific aspects of the reproductive life of women such as early menarche, nulliparity or late first birth, and late menopause. These features reflect the endogenous hormonal

milieu. Women with high levels of oestrogens in particular free oestrogens, not linked to the sex hormone binding globulin, have a higher risk of breast cancer development (Henderson et al, 1988).

Since the years when preceeding reports were written, such as the FAO/WHO or JECFA monographs knowledge has greatly increased, in particular on oestrogens. Different types of hormonal receptors (α and β) have been identified and their functions better defined. Also, steroid metabolism has been better studied. Genotoxic effects, independent from the presence of hormonal receptors, have been recognized for metabolites of the parent compounds. These concern essentially catechol-oestrogens and corresponding quinones, in particular 4 hydroxylated derivatives (Service, 1998). In addition, activation reactions during oestrogen metabolism contribute by oxidative stress to genotoxic effects. The possibility of synergism between the genotoxic activity of selected oestrogen derivatives and the classical promotional effect of steroïds cannot be excluded.

2.3.2.3. The issue of dose

Most of our current knowledge of the carcinogenicity of oestrogens relates to their use as human medicines, mostly for oral contraception, hormonal replacement therapy for menopause and treatment of pregnancy related complications. These indications entail exposure to relatively high doses and for prolonged periods of time.

Evidence of a dose-response relationship based on number of years of exposure exists for post menopausal oestrogen therapy and endometrial cancer and may be discussed for breast cancer in certain conditions of exposure.

No data are currently available on the genotoxic effects of exogenous low-dose oestrogens. Yet, there is no recognised threshold for genotoxicity. This lack of threshold for genotoxicity is comparable to the lack of threshold for the effect of natural sex steroids on fetuses. This conclusion is based on the work of vom Saal's group whereby the presence of slightly higher, but physiological, levels of oestrogens and androgens among male and female fetuses, respectively, affected histological and behavioral parameters during adulthood (Vom Saal and Timms, 1999)

The difficulty of evaluating health effects at low dose is here compounded by the fact that the data on exposures of human populations are exceedingly limited. No large data are available on representative samples of foods collected in countries allowing or banning growth promoters in farm animals. Most often, published levels concern measurements realized by the producers of the substances themselves under experimental conditions. However, data on the concentration of hormones and their metabolites present in edible tissues of treated animals are lacking. In addition, the methods used for measurements require a critical reappraisal. Data on the nature and amount of metabolites produced by the target animal are missing. Some of these needed data will come from on-going studies initiated recently by the EC. The concerns on the obsolete character of determinations of hormonal

products in meat also apply to determinations of physiological levels of steroid hormones in humans at various stages in life. This should lead to a reconsideration of the levels considered as safe and permissible in the preceding reports.

Whereas it is recognized that some physiological states in animals, such as gravidity, will be associated with endogenous hormonal levels which may be quite high, one should note that meat consumption from pregnant heifers is exceptional as usually these animals are not slaughtered. Finally, the effects of cumulative exposure even to low levels of oestrogenic compounds are unknown.

2.3.2.4. Susceptible populations

As described previously depending on gender and age, hormones exhibit a range of endogenous concentrations of oestrogen, testosterone and progesterone. Excess exposure to these hormones through consumption of meats from hormone-treated cattle is low but significant (see section 3). The most sensitive humans will be those with the lowest base levels of these hormones. Thus, the concerns about products with hormonal action apply in particular to foetuses as well as prepubertal children, and finally to specific sexes (exposure of women to testosterone; exposure of men to oestrogens).

The effects of in utero exposure to DES are the best documented in particular in terms of cancer risk (clear-cell adenocarcinoma of the vagina; breast cancer and testicular cancer). Effects of premature thelarche have also been described for DES (Loizzo et al, 1984) and possibly for zeranol contaminated poultry (Perez-Comas, 1988).

Some critical susceptibility phases have been described as "windows of vulnerability" corresponding in particular to the time period from adrenarche to first ovulatory cycles and possibly up to first pregnancy, as well as later on in perimenopause.

2.3.3.   Other hormonal effects relevant to cancer

Evidence that steroidal oestrogens are associated with cancer in humans, as well as evidence that oestradiol is carcinogenic in experimental animals led IARC to classify steroidal oestrogens as recognised human carcinogens (Group 1 of IARC Monographs, Table A9) (IARC, 1987; Lipschutz and Vargas, 1994). Animal studies indicate for example, that oestradiol increases the incidence in mice of mammary, pituitary, uterine, cervical, vaginal, testicular, lymphoid and bone tumours (Huseby, 1980; Highman et al., 1980; Highman et al., 1987; Nagasawa et al., 1980).

The role of oestrogens (17β-oestradiol) in breast cancer occurrence is suggested by the influence of reproductive life events, which are in part indicators of specific hormonal profiles. Breast cancer risk is increased among women who had an early menarche, nulliparity or late first birth or late menopause. Several studies have indicated that women who had higher endogenous levels of oestrogens, in particular not bound to SHBG have a higher risk of breast cancer (Toniolo et al., 1995; Berrino et al., 1996;

Bernstein et al., 1990a and b; Shimizu et al., 1990; Pike et al., 1992). Indication of a potential role of exposure at specific time periods come from the observation that women born with a high birth weight, reflective of higher levels of hormones in the uterine milieu, may have a slightly increased risk of breast cancer in later life. This would tend to indicate an influence of within physiological variations in hormonal exposure as well as enhanced susceptibility of the foetus to such exposure (see section 2.2.).

2.4. Sexual hormones and the immune system

It is noteworthy to remember that it was the effect of administration of testosterone and other male hormone derivatives in developing chicken that led to the discovery of the crucial role of the Bursa of Fabricius in the early differentiation of the lymphocytes involved in antibody secretion, that were named B-lymphocytes for this reason (Glick et al. 1956; Warner 1965). This discovery was the cornerstone of the considerable development of modern immunology (Cooper et al. 1966).

Sexual hormones are clearly involved in the multifactorial immune equilibrium that takes place at the first stages of pregnancy to protect the foetus against rejection as well as insuring his (her) growth and development (Voisin et al., 1990).

Moreover, recent studies have emphasized the role of sexual hormones among a variety of hormonal compounds and neurotransmitters in the regulation of immune responses in circumstances of life such as stressing events. The role in the initiation and course of immunologically-related disorders like autoimmune diseases has been identified for years, and possible influence on the emergence of allergic diseases is a quite new field of concern that deserves attention.

2.4.1. Sexual hormones and autoimmunity

The relationship between sexual hormones, and especially oestrogens, and autoimmune diseases has received considerable attention. Indeed most of the autoimmune diseases, including Systemic Lupus Erythematosus (SLE), Rheumatoid Arthritis, autoimmune thyroiditis, are observed almost exclusively in women (Meyer et al., 1991; 1995; Hahn, 1998; Lipsky, 1998; Wartofsky, 1998). Numerous studies in NZB and NZB/W mice, an animal model of SLE, have focused on the role of sexual hormones in the development of the disease and the onset of symptoms (Andrews et al., 1978; Milich and Geshwin, 1980; Theofilopoulos and Dixon, 1981; Steinberg et al.,1984). Oestrogens have been closely shown to be, besides genetic factors, a key-factor for the early onset of the disease in experimental mice and for the rapid kidney dysfunction, related to the autoimmune process, which is responsible for the fatal outcome (Roubinian et al.,1977; Eissensberg and Cohen, 1982; Dixon, 1982). In women with autoimmune diseases, a relationship between pregnancy and the evolutive course of the disease is well known, as well as the influence of menopause and therapeutic administration of female sexual hormones (Meyer et Kahn, 1991; Hahn, 1998; Khamashta et al. 1997). No sound epidemiological data are currently available to establish a

link between nutrition, especially meat consumption, and the occurrence of (and apparent current increase in) autoimmune diseases. However, in addition to genetic factors and possible infectious (viral) triggering agents, oestrogenic status, as well as modifications at other steps of the subtle hormonal balance that influence sexual and reproductive life (such as increase in prolactin) have clearly shown to be related to the immunological imbalance that leads to autoimmune diseases (Lavalie et al., 1987; Walker et al., 1995; Whitacre et al., 1994; Elenkof et al., 1997; Vuitton et al., 1999).

2.4.2. *Sexual hormones and tolerance to the foetus in pregnancy; orientation of the immune response and allergic diseases*

The role of progesterone has been particularly established: receptors for progesterone are present in subtypes of T-lymphocytes known to modulate the immune response towards tolerance rather than cytotoxicity, allowing the presence of the "foreign" foetus in the uterus, while exerting mild immunosuppressive effects at the total body level in the pregnant female (Voisin et al., 1991; Lin et al., 1993; Szekeres-Bartho et al., 1996; Piccinni and Romagnani, 1996; Segerson et al., 1997). This can eventually result in the occurrence of particular infections, such as Cytomegalovirus infection in pregnancy (Hirsch, 1998). This particular orientation of the immune response, currently known as the "Th2 profile" is the result of a complex cascade of events that involves cytokines, steroidal and non-steroidal hormones, including sexual hormones, and neurotransmitters, as well as the exposure to particular environmental antigens (Romagnani, 1992; Vuitton et al., 1999). This profile has been shown to be associated with non-cytotoxic, albeit potentially deleterious, IgE dependent allergic reactions (Brown et al., 1997; Romagnani, 1992).

The major increase in allergic diseases in all developed countries, especially the USA, in the last 3 decades is a growing source of concern for public health in these countries (Schlumberger, 1987; Wütrich , 1987; Weiss et al., 1992; Bonini et al., 1994; European Community Respiratory Health Survey, 1996). Although the relationship between meat consumption, and especially meat from hormone-treated cattle, and these epidemiological changes in the incidence of allergic diseases has not been specifically addressed, it must be noted that they actually mimic those evoked above, in the report, about breast and prostate cancer: world distribution is nearly identical, changes observed when subjects from low-prevalence countries move to high-prevalence countries are similar, and nutritional factors linked to family incomes, educational level of mothers and "Western style" cultural habits, including moving from a vegetarian diet towards meat consumption and an early diversification of diet in babies, cannot be excluded (Martinez, 1994; Woolcock, 1995; Matricardi, 1997; Strachan, 1997; Black and Sharp, 1997; Nowak et al., 1998).

Large cohorts of children are currently studied, comparing life-style, number of siblings, socio-economic parameters, infections in childhood and infancy, in children born in Western-style of living countries such as ex-Western Germany or Sweden, and children from neighbour countries with different life styles such as ex-Eastern Germany and Estonia (von Mutius et al., 1994; Jögi

et al., 1998; Nowak et al., 1998). Recommendations should be given to the researchers involved in the follow-up of these cohorts to take "meat consumption" into account and similar studies should be done in similarly comparable cohorts of North American subjects with different meat consumption levels and/or consumption of hormone treated *vs.* non-hormone treated meat.

This potential link between sexual hormone status and development and/or course of allergic diseases is further reinforced by the common observation of the influence of the pre-pubertal period on the improvement of atopic dermatitis as well as occurrence of asthma with the onset of puberty and in the premenstrual and perimenopausal period (Chandler et al., 1997) as well as recent findings on a relationship between fetal growth and maturation and occurrence of allergic diseases (Strachan, 1994; Brown et al., 1997; Shaheen, 1997).

2.5. OESTROGEN METABOLITES AS DNA-REACTIVE AND GENOTOXIC SUBSTANCES

2.5.1. *DNA damage by oestrogen-3,4-quinones*

In the last few years, research into the effects of oestrogens has expanded into considering the ability of oestrogen metabolites to damage DNA and produce mutations (Service, 1998). Such mutations may play a role in the initiation of cancer. The oestrogens 17β-oestradiol (E_2) and estrone (E_1) are metabolized via two major pathways: 16α− hydroxylation and formation of catechol oestrogens (CE, see Fig. 1) (Ball and Knuppen, 1980). The CE formed are the 2-hydroxy (2-OH) and 4-hydroxy (4-OH) derivatives. Generally, these two CE are mainly inactivated by O-methylation catalyzed by catechol-O-methyltransferases (COMT). Other possible conjugations are glucuronidation and sulfation of the CE. If these conjugations are insufficient, there is the possibility that CE are oxidized to semiquinones (not shown) and quinones (CE-Q, Fig. 1). The CE-Q can conjugate with glutathione (GSH). Another inactivating process occurs by reduction of the CE-Q to CE, catalyzed by quinone reductase. If these two inactivating processes are insufficient, oestrogen-2,3-quinones can react with DNA to form stable adducts that remain in DNA unless repaired (Stack, et al., 1996; Dwivedy, et al., 1992). E-3,4-quinone, if not inactivated, reacts with DNA to form depurinating adducts, more specifically two rotational conformers bound to the N-7 of guanine or N-3 of adenine (Stack, et al., 1996; Li, et al., 1998); these adducts are lost from DNA by cleavage of the glycosidic bond, leaving apurinic sites in DNA (Stack, et al., 1996; Cavalieri, et al., 1997).

One current hypothesis is that formation of N7-guanidine and N3-adenine-adducts by reaction of E-3,4-quinone with DNA is the tumor-initiating event in several human cancers, which include breast, endometrial, ovarian, prostate and, possibly, brain tumors (Cavalieri, et al., 1997). In fact, when tested in male Syrian golden hamsters, the 4-OHE are carcinogenic in the kidney, whereas the 2-OHE are not (Liehr, et al., 1986; Li and Li, 1987). Evidence collected thus far on the formation of catechol-oestrogen-N7-guanidine

adducts *in vitro* and *in vivo* (see below) supports a possible role of oestrogens as tumor initiators.

2.5.2. *Properties of 4-Catechol-Oestrogens: Formation of Depurinating Adducts and Tumor Initiation*

Reaction of CE-Q with the nucleoside deoxyguanosine and the purine base adenosine was conducted to gain insight into the behavior of these electrophiles with DNA bases (Stack, et al., 1996; Li, et al., 1998). In addition, the synthetic adducts serve as standard compounds for *in vitro* and *in vivo* studies aimed at understanding the genotoxic effects of CE-Q. When $E_1(E_2)$-3,4-Q was reacted with deoxyguanosine or adenine, 4-OHE$_1$(E$_2$)-1(α,β)-N7-guanidine or 4-OHE$_1$(E$_2$)-1(α,β)-N3-adenosine (Fig. 1) was formed, respectively (Stack, et al., 1996; Li, et al., 1998). These adducts are mixtures of two conformational isomers resulting from the restricted rotation of the guanidine moiety about the N7-guanidine-C1(E) bond or the adenosin moiety about the N3(adenosine)-C1(E) bond. Attack of the CE-Q on deoxyguanosine at the N-7 position or on deoxyadenosine at the N-3 position results in loss of the deoxyribose moiety to form the N7-guanidine or N3-adenosine depurinating adduct.

Reaction of E_1-2,3-Q with deoxyguanosine or deoxyadenine resulted in the formation of 2-OHE$_1$-6-N^{2}dG or 2-OHE$_1$-6-N^{6}dA, respectively (Stack, et al., 1996). These are stable adducts that would remain in DNA, unless repaired.

The reaction of CE-Q with DNA demonstrates the type of DNA damage that occurs when these electrophiles bind to DNA (Cavalieri, et al., 1997). Reaction of E_1-2,3-Q and E_2-2,3-Q with calf thymus DNA produced 8.59 and 1.13 µmol stable adducts/mol of DNA-phosphate, respectively (Dwivedy, et al., 1992). Reaction of E_1-3,4-Q and E_2-3,4-Q with DNA generated 0.11 and 0.07 µmol of stable adducts per mol of DNA-P and the depurinating adducts 4-OHE$_1$(E$_2$)-1(α,β)-N7-guanidine at 59 and 213 µmol/mol DNA-P, respectively (Cavalieri, et al., 1997). Thus, when both stable and depurinating adducts are considered, the E-3,4-Q produce a much higher level of binding with DNA. Binding of CE-Q via enzymic activation of the CE precursors by horseradish peroxidase, lactoperoxidase and cytochrome P450 has also been demonstrated to give similar results (Cavalieri, et al., 1997).

Formation of the 4-OHE$_1$-1(α,β)-N7-guanidine depurinating adduct (1-2 µmol/mol DNA-P) was also detected *in vivo* by treatment of the mammary glands of female Sprague-Dawley rats with 200 nmol of E$_2$-3,4-Q or 4-OHE$_2$ at each of four glands (Cavalieri, et al., 1997).

In summary, the CE-Q derived from 4-OHE$_1$(E$_2$) and 2-OHE$_1$(E$_2$) are DNA reactive and display different chemical properties in their reaction with nucleosides and DNA. Consistent with the hypothesis of a role of CE metabolites in tumor initiation is the finding that E-2,3-Q bind to form stable DNA adducts (Stack, et al., 1996), and the corresponding CE do not induce kidney tumors in Syrian golden hamsters (Liehr, et al., 1986; Li and Li, 1987). In contrast, the E-3,4-Q form predominantly depurinating adducts with generation of the 4-OHE$_1$(E$_2$)-1(α,β)-N7Gua and 4-OHE$_1$(E$_2$)-1(α,β)-

N3Ade (Stack, et al., 1996; Li et al., 1998), and the 4-CE are carcinogenic in Syrian golden hamsters (Liehr, et al., 1986; Li and Li, 1987). The ability of CE metabolites to damage DNA indicates that the oestrogens are genotoxic and, thus, have no dose threshold for their possible hazardous effects.

2.5.3. Redox Cycling of Catechol-Oestrogens, Their Semiquinones and Quinones

Redox cycling generated by reduction of CE-Q to semiquinones by cytochrome P450 reductase and subsequent oxidation to CE-Q by molecular oxygen can form reactive oxygen species (ROS) superoxide radicals and then hydroxyl radicals. In turn, hydroxyl radicals can produce lipid hydroperoxides (Wang and Liehr, 1995) and DNA damage (Liehr, et al., 1986; Roy and Liehr, 1988; Liehr and Roy, 1990; Liehr, 1994; Nutter, et al., 1991; Nutter, et al., 1994). The DNA damage includes formation of 8-hydroxy-dA and 8-hydroxy-dG and has been found to be abundant in mammary DNA from breast cancer patients (Malins, et al., 1993) and prostate DNA from prostate cancer patients (Malins, et al., 1997). There are well over 30 different types of oxidized bases that can be formed in DNA; their levels exceed those of the stable carcinogen-induced adducts with DNA bases by about two orders of magnitude, being on average $1/10^5$ *versus* $1/10^7$ normal DNA bases, respectively (Dizdaroglu, 1991; Frenkel, 1992). Some of these oxidized DNA bases are mutagenic (Shirname, et al., 1987; Tchou and Grollman, 1993; Faig, et al., 1994).

Formation of either 8-hydroxy-dG or hydroxymethyldeoxyuridine in the target tissues of oestrogen-mediated carcinogenesis has been shown repeatedly. The animal models often utilized are the Syrian hamster kidney (Liehr, 1997) and dorsolateral prostate in the Noble rat (Han, et al., 1995). In fact, increased levels of oxidized bases in human DNA may precede the development of cancer and, thus, may serve as biomarkers of cancer risk (Djuric, et al., 1996; Malins, et al., 1998; Frenkel, et al., 1998).

Androgens, in particular testosterone, have been found to increase the incidence of prostate tumors in rats (Pollard and Luckert, 1986a and b). This observation has led to the idea that these hormones are tumor promoters. In Noble rats, treatment with both oestrogen and androgen induces prostate tumors (Drago, et al., 1980; Drago, 1984; Bosland, et al., 1995), suggesting that oestrogens could initiate the tumors and androgens promote them.

In addition, redox cycling of oestrogen quinones/semiquinones/quinones can contribute to the initiating process by (1) enhancing oxidation of CE to CE-Q, thus rendering less competitive the process of protection by conjugating enzymes (Fig. 1), and (2) forming excessive 8-hydroxy-dG and 8-hydroxy-dA that can increase the critical mutagenic events. If CE metabolites play a role in the induction of cancer, following tumor initiation by genotoxic events, hormone receptor-mediated events would play a major role in the promotion and progression phases of tumor development.

2.5.4. *Testing the genotoxicity of oestrogens*

Oestrogens are at most weak carcinogens. Model systems for testing their carcinogenicity include the kidney of male Syrian golden hamsters and the mammary gland of female ACI rats. The standard tests of genotoxicity are not appropriate to demonstrate the activity of these substances. This lack of mutagenicity may be related to the use of liver extracts for enzymatic activation, since oestrogen 4-hydroxylase activity is low in this organ. Additionally, the reactive intermediates may not be able to travel into target cells, and the types of DNA damage assayed may not be sufficient. The genotoxicity of oestrogen metabolites may best be assayed in mammalian cells with appropriate metabolizing enzymes (for example the human breast epithelial MCF-7 cell line), and the DNA damage analyzed should include not only point and frameshift mutations, but also chromosomal changes.

In summary, over the last few years the genotoxic effects of oestrogens have been investigated in greater detail. The DNA adducts formed by quinone metabolites of catechol oestrogens [and the nonsteroidal synthetic oestrogen hexestrol (Jan, et al., 1998)] have been identified in a number of biological systems. Mutagenicity and carcinogenicity tests of oestrogens and catechol oestrogens have shown activity, but more widely applicable tests need to be developed.

3. GENERAL CONSIDERATIONS RELATING TO EXPOSURE ASSESSMENT

3.1. Quantification of hormones in edible tissues

Implants containing 17β-oestradiol (E2) (or oestradiol-benzoate [E2-b]) alone or in combination with either testosterone (or testosterone propionate), progesterone or trenbolone acetate (TBA), a synthetic anabolic steroid, are used to promote growth and enhance the efficiency of food utilization in beef cattle. Other implants containing TBA alone, zeranol alone, and TBA plus zeranol are also used. The various implants, their composition, and target animal are shown in Tables A1 and A2 of the Annex. All of these hormones with the exception of the synthetic compound, trenbolone acetate, and the mycooestrogen, zeranol, are endogenous in animals. Thus humans consuming beef from untreated cattle are exposed to residues of E2, testosterone and progesterone and their metabolites at levels naturally present in the meat. Zeranol is naturally occurring and it is likely that some background level is present. On the other hand, TBA and another growth promoting hormone, melengestrol acetate (MGA), which is added to cattle feed, are synthetic and thus, the background level in humans should be zero.

A crucial question is whether consumption of meat from cattle treated with hormones under conditions approved in the USA *vs* non hormone-treated cattle, causes increased exposure to these hormones. The first step in this determination is a calculation of the theoretical increased daily hormone intake when consuming beef from treated as opposed to untreated animals. The recent JECFA report (of February, 1999) presented calculations of a theoretical excess maximum intake (μg/person/day) of oestrogen (E_2 +

estrone [E_1]), testosterone and progesterone (see table A3). From this overview, referring to products presently licensed in the USA, it is obvious that, with the exception of pregnant heifers, the use of these growth-promoting hormones will result in an additional excess daily intake of oestrogens of 1.1 to 83.9 ng/person (E2 + E1), of progesterone of 64-467 ng/person, and of testosterone of 5-189 ng/person. It is worthwhile to indicate that these data refer to the parent compounds only and do not include contributions from metabolites. It should also be noted that these hormone implant-induced increases in hormone levels result in oestrogen and testosterone exposures below that which would occur upon consumption of beef from pregnant heifers. However, meat from pregnant heifers accounts for only a relatively small amount of the beef consumed, as these animals ar slaughtered only incidentally.

3.2. Exposure in relation to endogenous hormone production in humans at different stages of life

A second question to be addressed concerns whether daily exposure to the excess hormone present in beef from hormone-treated cattle provides a significantly increased exposure to individuals consuming the meat. Table 1 shows ranges of blood hormone concentrations in women and men.

Table 1: Endogenous Steroid Hormone Levels in Females and Males*

Hormone	Women Prepubertal	Women Follicular-Luteal	Women Postmenopausal	Men Prepubertal	Men Adult
Oestradiol pg/ml	8-23	10-375	0-28	5-14	6-44
Estrone pg/ml	19	15-250	15-55	16	15-65
Progesterone ng/ml	0.1-0.4	0.2-24		0.1-0.3	0.3-0.5
Testosterone ng/ml	0.1-0.2	0.4-0.8	0.4-0.8	0.1-0.2	3-9

* *Values from Jones, 1991; Goodman, 1996; Dorgan et al., 1996; Zeleniuch-Jacquotte et al., 1997; Szymczak et al., 1998; Toniolo et al., 1995; Klein et al., 1994; Lippe et al., 1974; Angsusingha et al., 1974; and hospital clinical pathology lab normal ranges.*

The data show that premenopausal women have the highest levels of endogenous oestrogen (oestradiol and estrone) and progesterone. Oestradiol and progesterone production rates in premenopausal women during the follicular phase have been determined to be approximately 445 µg/day and 418 µg/day, respectively (JECFA, 1987). During pregnancy, oestradiol levels rise dramatically to approximate values of 18,000 pg/ml (Goodman, 1996). Oestradiol and progesterone production rates during late pregnancy have been determined to be approximately 13,800 µg/day and 94,000 µg/day, respectively (JECFA, 1987). In men, daily production rates for oestradiol and progesterone are approximately 48 µg/day and 416 µg/day, respectively (JECFA, 1987). In prepubertal boys, oestradiol and progesterone production rates have been reported as being 6 µg/day and 150 µg/day, respectively

(JECFA, 1987). Thus, prepubertal and postmenopausal women and prepubertal and adult men have the lowest levels of endogenous oestrogens and progesterone and thus would represent the individuals most likely to be at increased risk for adverse health effects that might be associated with exposure to exogenous sources of oestrogens.

As expected, men have the highest levels of blood testosterone and the daily production rate has been determined to be approximately 6,500 μg/day (JECFA, 1987). Testosterone levels are much lower and similar in women and prepubertal men. It has been reported that daily production rates of testosterone are between 140 to 240 μg/day in adult women and 32 and 65 μg/day in prepubescent girls and boys, respectively (JECFA, 1987). These data suggest that all women and prepubertal men represent the individuals at greatest risk for adverse health effects that might be associated with exposure to exogenous sources of testosterone.

In human plasma, E_2, E_1 and sulfated forms of E_2 and E_1 are found bound to proteins. Only 1 to 3 percent of E_2 is detected as free hormone. In contrast, 40-50% of E_2 is bound to SHBG, while another 40-50% is bound to albumin. For instance, Toniolo et al., (1995) reported that in the sera of postmenopausal women containing 28 pg/ml total oestradiol, approximately 45% was bound by SHBG and 54% by albumin, with 1% remaining as free hormone. Although the percent bound to each of these proteins is almost equal, the biologically active steroid is the fraction of E_2 which is either unbound or weakly bound to albumin; the binding affinity of E_2 for SHBG is more than 10 000x that of albumin (10^{-8} vs 10^{-4} moles/L) (Fentiman et al., 1988; Lipsett, 1971). This suggests two things: First, it is likely that the binding capacity of SHBG is saturated since if it were not, given its much greater binding affinity one would not expect to detect oestradiol bound to albumin. This implies that SHBG does not serve as a "buffer" to hold free hormone levels constant. However, the function of E2 binding to SHBG is not well understood. While cells have specific membrane receptors for SHBG (Germain et al., 1997), SHBG appears to function to inhibit the effects of added free oestrogen, at least as determined in vitro in an MCF-7 cell proliferation assay (Damassa et al., 1991; Fortunati et al., 1998). In addition, unliganded SHBG can bind to its receptor and induce intracellular cAMP levels (Fortunati et al., 1996). SHBG levels can be affected by exposure to exogenous agents. For example, high intakes of caffeinated beverages increased SHBG (Nagata et al., 1998). In addition, SHBG levels are elevated in prepubertal children compared to adults (Andersson and Skakkebaek, 1999). Furthermore, increased levels of SHBG have been shown to occur upon oestrogen exposure and increased SHBG levels have the effect of decreasing oestrogen clearance in adults (Andersson and Skakkebaek, 1999). Second, given the abundance of albumin and its low hormone binding affinity, it is possible that albumin represents a high capacity-low affinity "sink" for a variety of lipophilic compounds including steroid hormones and phytochemicals (Baker, 1998).

The hormone levels presented above (Table 1) were determined by radio-immunoassays (RIA). Use of these assays has frequently been associated with production of variable results, particularly when used to detect low levels of

endogenous hormones (Carlstrom, 1996; Potischman et al., 1994, Klein et al., 1994; documents prepared for the EC by Andersson and Skakkebaek, February 1999). Klein et al. (1994) developed and carefully characterized an ultrasensitive assay (100 fold more sensitive than RIAs) consisting of yeast cells genetically engineered to express the oestrogen receptor and a reporter gene under the control of a tandem repeat of the vitellogenin oestrogen response element. This assay is highly specific for detection of oestradiol. Using this assay Klein et al. (1994) report detecting serum oestrogen concentrations of 0.6 pg/ml and 0.08 pg/ml in prepubertal girls (n = 21, 7.7 years old) and prepubertal boys (n = 23, 9.4 years old), respectively. These values are considerably lower than the range of oestradiol levels shown in Table 1 that are typically reported for female (8-23 pg/ml) and male (5-14 pg/ml) prepubertal children. A corollary is that perhaps the hormone residues in beef, which are also low and which have also been determined by RIA are equally variable and over representative of the actual hormone concentrations. This is a critical area requiring additional study.

3.3. Exposure considerations upon misuse

The guidelines for implantation in cattle of growth promoting hormones are set by the requirements of Good Veterinary Practice, however, in most countries hormone containing implants are available "over the counter". Given this widespread availability of the implants and the incentive provided by the enhanced growth and feed utilisation efficiency resulting from hormone use, it is likely that some degree of misuse will occur. Types of misuse possible include improper placement of the implants (i.e. in tissue used for consumption rather than in the ears which should be discarded), off-label use, including use in non-approved animals (e.g. veal calves and pigs), overdosing, disease treatment, and use of black-market non-approved hormones. Each of these possibilities for misuse could result in over exposure of humans consuming beef from the affected cattle.

The likelihood of abusive use and difficulties of control of the use of growth promoters in cattle is addressed elsewhere (see Draft Report on Assessment of Risks of Hormonal Growth Promoters in Cattle with Respect to Risks arising from Abusive Use and Difficulties to Control, 1999).

3.3.1. Misplaced implants

It is generally appreciated that the highest concentration of hormone residues will be at the implantation sites. In a study of the residues of TBA, zeranol, and DES remaining in the ears of cattle at slaughter, from 6% to 30% of the original dose remained in the ears from 65 to 150 days after implantation. The range of hormone amounts was approximately 6 to 54 mg even after these prolonged "withdrawal" times. These data indicate that consumption of tissue from implantation sites would result in substantial excess exposure.

Model calculations provide estimates of the extent of excess exposure that could result from consumption of meat containing implantation sites. The model calculations were based on implants containing 200 mg TBA. It was indicated that consumption of food containing bovine meat contaminated by

TBA-containing implants could result in exposure to hormone residues at levels greater than 30 fold (baby food, babies) and 3 to 67 fold (sausages, children and adults) above their respective ADIs (Draft Report on Assessment of Risks of Hormonal Growth Promoters in Cattle with Respect to Risks Arising from Abusive Use and Difficulties of Control, 1999).

3.3.2. Off-label use

Off-label use includes use of implants and feed premixes in species for which they were not approved. For example, use of growth promoting hormones in veal calves is not approved in the US or Canada. However, the Canadian Food Inspection Agency, in two surveys to evaluate misuse of TBA in veal calves uncovered violations. In one (1987), 33% of 281 samples and in the second (1997-98), 40% of 210 samples of liver from veal calves revealed the presence of TBA residues.

Furthermore, preliminary results from a study commissioned by the EC intended to determine the amount of hormone residues in US meat and offal samples detected the presence of off-label synthetic hormonal growth promoters in calf liver (Stephany and André, First Interim Report, 1999).

In an ongoing EC commissioned study of hormone residues in edible tissue from beef of cattle designated to be hormone-free from the USA, measurable levels of trenbolone, zeranol and melengestrol were detected in approximately 12% of 258 samples of bovine meat (Stephany and André 1999, interim report). Various examples of overdosing by simultaneous and/or repeated implantation of various implants in USA feedlots have also been documented by EC inspection missions (Draft Report, 1999, see above).

Finally, hormones might be used in other species including pigs and poultry. The potential residue burden originating from these illegal treatments are currently under investigation. In these studies, the stability of hormones and their metabolites in manure and the possible risk to the environment will be addressed as well.

3.3.3. Black-market drugs

The possibility that non-authorized pharmaceutical formulations of hormones will be used in animals can not be excluded. These black-market drugs may comprise an even higher risk to public health, as their quality is often inadequate.

Finally, misplaced implants and/or illegal drugs may impair animal health and welfare and animal husbandry (due to induction of behavioral abnormalities). These possible effects to animals health and welfare have not been discussed in detail , as this report is focussing on public health.

3.3.4. Secondary risks

It is well recognized that the expression of drug-metabolizing enzymes (DMEs) is regulated by hormones and sex differences in the disposition and metabolism of veterinary drugs have been described in various animal species, including ruminants (Witkamp et al., 1991, Witkamp et al., 1993a and b, van't Klooster et al., 1993, Motesissa et al., 1996). More specifically, the effect of trenbolone and testosterone on the plasma elimination rate of different veterinary drugs, including sulfamethazine and trimethoprim were studied in goats. It could be demonstrated that these anabolic steroids prolong the elimination rate of drugs (van Miert et al., 1988). In consideration of these findings it has to be assumed that the use of hormones for growth promoting pupuses will increase the prevalence of undesirable residues (in particular of antibiotics which are frequently used in bovine therapy) in edible tissues of treated animals.

In conclusion, it has to be noted that misplaced implants and black market drugs comprise the risk that extremely high levels of residues of hormones remain in edible tissues of animals. In addition, it has to be noted that the contemporaneous use of growth promoting hormones and veterinary therapeutics drugs increases the prevalence of undesirable risidues in edible tissues of bovines.

4. CHARACTERISTICS OF THE INDIVIDUAL COMPOUNDS UNDER CONSIDERATION

4.1.   17 β-oestradiol

17β-Oestradiol (E_2), estra-1,3,5 (10)-triene-3,17β-diol, is an 18-carbon steroid hormone and the most potent of the naturally occurring oestrogens. This hormone is produced primarily by the developing follicle of the ovary in adult females. E_2 exerts its pleotropic biological effects on cell growth and differentiation largely through receptor-mediated mechanisms. E_2 binds with high affinity and high specificity to intracellular proteins know as oestrogen receptors (JECFA, 1988; JECFA, 1999; Anstead et a., 1997). Two subtypes of oestrogen receptor (ER) are known, ER-α and ER-β.   It is known that these proteins can form both homo- and heterodimer complexes, yet current information about the ER-β subtype is limited. The value of the dissociation constant of E2 for the ER-α is in the 0.1-1.0 nM range (Anstead et al., 1997; Giguere et al., 1998). The aromatic A-ring and 3-OH group of E_2 are known to be important components of the ligand binding activity and receptor activation activities of E_2.

While most oestrogen metabolites are known to have greatly diminished relative binding affinities for ER-α, the catechol metabolites, 2-OH-E_2 and 4-OH-E_2 are know to bind with approximately 100 and 150 percent of the relative binding affinity of oestradiol, respectively. Further methylation of these catechol oestrogens results in greatly diminished relative binding affinities (Anstead et al., 1997; Giguere et al., 1998; Katzenellenbogen et al., 1996; Jenster, 1998).

4.1.1.   *Pharmacokinetics and biotransformation of 17β-oestradiol in animals*

In cattle, oestradiol is administered as either E_2 or oestradiol benzoate by sub-cutaneous implantation in the ear. The ear, along with any residual drug, is discarded at slaughter. Oestradiol benzoate is rapidly hydrolysed in the target animal to E_2. In the circulatory system of the animal, E_2 derived from the implant is indistinguishable from endogenous E_2 (JECFA, 1988). The dosage of oestradiol varies with the manufacturer of the implant and ranges between 10 and 45 mg per animal (JECFA, 1988). It is assumed that the improvement in weight gain and feed efficiency observed following the administration of 17ß-ooestradiol is that the pituitary is stimulated to produce increased quantities of growth hormone (JECFA, 1988; JECFA, 1999).

After administration of radio-labelled E_2 to calves, radioactivity in urine consisted primarily of 17α-oestradiol with much lesser amounts of estrone. Conjugates of both E_2 and E_1 were also detected. Radioactivity in faeces

included primarily 17α-oestradiol, as well as E_2 and E_1 each in the non-conjugated form (JECFA, 1988; Ivie et al., 1986).

4.1.2. Oestradiol disposition in the target animal

After administration of oestradiol benzoate, the major metabolites found in muscle were 17α-oestradiol (38-70% of extracted radioactivity) and E_1 (17-45%). The pattern of metabolites in fat was similar to that in muscle. The highest residues were found in kidney and liver. The major oestrogenic metabolites in kidney were 17α-oestradiol, 17α-oestradiol-glucuronide, E_2 and E_1. In liver, the major metabolite could not be identified (40% of the extracted radioactivity). E_2, E_1, estriol, and glucuronides accounted for the remaining radioactivity (JECFA, 1988; Dunn et al., 1977). The nature of the unidentified polar metabolites from livers of steers was investigated in another study using radiolabeled E_2. The major polar metabolite was the β-D-glucopyranoside of 17α-oestradiol. The 3-β-D-glucuronate of 17α-oestradiol, and other 17-glycosides of oestradiol were also characterized (JECFA, 1988; Rao et al., 1979). Recent studies have begun to consider the formation in cattle of the major oestrogen metabolites found in humans, i.e. the 2-OH, 4-OH and 16α-OH-oestrogens. While it is likely that these routes of metabolism are present in cattle, quantitative measures are not yet published.

Table 2: Mean tissue concentrations of oestradiol in untreated and treated animals in ng/kg:

	Muscle		Liver		Kidney		Fat	
	E2	E1	E2	E1	E2	E1	E2	E1
Heifer	7.1	5.9	8.2	7.5	8.5	6.4	5.6	5.3
Treated Heifers (84 days)	5.8	6.4	7.3	6.7	26.3	10.2	9.3	9.9
Heifer	5.54	2.51	1.54	1.7	2.89	1.42	13.4	10.9
Treated Heifers (30 days)	33.2	6.43	23.1	3.69	23.5	5.88	86.7	54.4
Heifer	5	Ne	32	ne	17	ne	15	ne
Treated Heifers (84 days)	106	Ne	512	ne	259	ne	75	ne
Veal calve	6.8	10.8	14.7	9.1	10.6	7.8	11.2	13.9
Treated Veal (56 days)	18.4	11.7	32.0	22.3	57.0	34.7	38.5	24.6
Bull	6.3	7.7	8.5	6.0	10.0	8.7	9.1	15.3
Treated Bulls (63 days)	8.5	6.8	16.6	6.9	19.9	15.1	20.2	29.6
Steer	0.84	1.60	0.91	0.66	1.57	1.02	1.82	8.47
Treated Steers (15 days)	9.7	2.12	5.36	2.04	13.7	4.44	41.4	20.3
Steer	7	Ne	32	ne	13	ne	12	ne
Treated Steers (50 days)	60	Ne	193	ne	134	ne	96	ne
Pregnant heifers (240 days gestation)	32.7	523	1027	145	274	142	76.5	2786

E2 = 17ß-oestradiol; E1 = estrone; ne = not estimated

4.1.3. Interactions

In a 1997 study, Henricks et al. evaluated the effects in heifers of the growth promoting steroids E_2, trenbolone acetate (TBA), and melengestrol acetate (MGA) on serum concentrations of E_2 and trenbolone-17β (TBOH). Serum concentrations of E_2 and TBOH were measured on day 0, 1, 3, 5, 7, 13, 21, 28, 42, 56, 84, 112, and 140 in animals administered the following treatments: 1) control; 2) MGA, .0.5mg/day; 3) Revalor-H (14 mg E2+140 mg TBA); 4) Revalor-H + MGA; 5) Finaplix-H (200mg TBA); and 6) Finaplix-H + MGA. Treatments 3 and 4 increased ($p<.05$) serum E_2 concentrations; peak concentrations (62.7 to117.0 pg/mL) occurred between day 21 and day 56. Feeding MGA (treatment 4) had no effect on this increase in serum E_2 concentration (70.4 to 82.5 pg/mL). From day 84 until day 140, serum E_2 was greater for the Revalor-H treatment (19 pg/mL) than for control (7 pg/mL) or Finaplix-H treatment (6.5 pg/mL). Serum E_2 concentrations increased 2- 3-fold from day 56 to 140 in controls fed MGA, compared with controls not fed MGA (13). The relationship between the removal of the implant and drug elimination was not investigated in this study.

4.1.4. Pharmacokinetics and biotransformation of 17β-oestradiol in humans.

The oxidative metabolism of endogenous oestrogens is known to occur at several positions including carbons C-1, C-2, C-4, C-6, C-7, C-11, C-14, C-15, C-16, and C-18. The major oestrogens detected in serum and urine are the 2-hydroxylated metabolites. The liver is the primary site of oestrogen metabolism, where rates of 2- and 16α-hydroxylation, catalysed by P4501A2, P4503A3 and P4503A4, greatly exceed that of 4-hydroxylation. Because 4-hydroxylated metabolites represent only a small percentage of the total amount of oestrogens detected in the urine, 4-hydroxylation has been considered a minor metabolic route of metabolism. However, it is now understood that extrahepatic tissue 4-hydroxylation of E_2 may play a significant role in oestrogen homeostasis. In several organs which are sites of oestrogen-induced tumours, the rate of E_2 4-hydroxylation equals or exceed the rate of 2-hydroxylation, and in comparison to normal tissue, elevated E_2 4-hydroxylase activity has been observed in samples prepared from breast and uterine tumours. In humans, cytochrome P4501B1 has been identified as the most significant E_2 4-hydroxylase. This enzyme is expressed primarily in extra-hepatic tissues (reviewed in Zhu and Conney, 1998, Martucci and Fishman, 1993).

Specific information about the absorption, biotransformation and elimination of E_2, E_1 and 17α-oestradiol from meat and meat-product is not available. The effects of cooking and other processing on the bioavailability of such compounds is also lacking. Based on the lipophilicity of oestradiol, there is no reason to assume that such compounds will be poorly absorbed. Metabolic studies of orally administered 17ß-oestradiol indicate that as much as 20 percent of a 2 mg dose of micronized E_2 is absorbed, with a serum half-life in the range of 2 to 16 hours (Zimmermann et al., 1998; Vree and Timmer, 1988: Ginsburg et al., 1998). In a 1998 study (Lippert et al., 1998) of oestradiol metabolism in postmenopausal woman orally administered oestradiol valeriate, 2 mg/day for 2 weeks, it was shown that along with the increased serum concentrations of oestradiol, there was a proportionate increase in the level of estrone, 2-hydroxyestrone and 16α-hydroxyestrone. Thus exposure to exogenous oestrogens leads to increased levels of the parent oestrogen compounds and their metabolites.

4.1.5. Assessment of excess exposure to oestrogens from consumption of hormone-treated beef

The data in Table A3 (Annex) show that consumption of beef from hormone-treated non-pregnant cattle can result in excess exposure to oestrogens (1 to 84 ng/person/day. For comparison of excess intake with the endogenous levels of these hormones in humans, the median value (6.8 ng/person/day, excluding the pregnant heifer data) of excess oestrogen exposure determined from Table A3 (Annex) was used, (recognising that the range of excess

exposure could be from 1 to 84 ng/person/day, depending on the implant used).

At its February, 1999 meeting, JECFA established the ADI for 17β-oestradiol as 0-50 ng/kg bw/day. This value is based on a study in postmenopausal women where conjugated equine oestrogens at doses of 0.3, 0.62. 1.2 and 2.5 mg were administered for two weeks followed by no treatment for three weeks. This regimen was repeated four times after which serum levels of corticosteroid binding protein (CBG) were determined. No increase in CBG levels was detected at the 0.3 mg dose (equivalent to 5 µg/kg bw/day) which was thus considered to represent the no-observed-effect level (NOEL). In another analysis (it is not clear if this was part of the same study or a different one), the dose of 0.3 mg of conjugated equine oestrogen was determined to be the NOEL for induction of serum concentrations of follicle-stimulating hormone, angiotensinogen, SHBG and CBG. It was stated that fine-particle 17ß-oestradiol and the conjugated equine oestrogens were equipotent for all four hormone-dependent end points. In a separate study, the bioavailability of fine-particle 17β-oestradiol administered orally was determined to be 5% compared to a dose administered intravenously. Sixty percent of the fine-particle 17β-oestradiol dose was determined to appear in the serum as estrone and estrone sulfate. While the results of these studies would appear to indicate that the maximum excess exposure level (84 ng/person/day) for oestrogen derived from hormone-treated beef is below the NOEL, there are several concerns. First, neither the actual data nor references to peer-reviewed publication of this data were available. Second, it is uncertain whether the use of fine-particle 17β-oestradiol, and in particular conjugated equine oestrogens, represents appropriate surrogates for consumption of oestrogens in association with beef. The equine oestrogens consist predominately of equilin and equilenin, which are chemically different from oestradiol.

In the USA, the FDA has established an acceptable level of exposure for oestradiol (Table 3). These values represent parent hormone residue levels in uncooked meat that are considered unlikely to produce any physiological effects in individuals chronically ingesting animal tissues.

Table 3: Acceptable levels of oestradiol levels in beef (Ref.: Code of Federal Regulations (CFR) 21, Part 556, Tolerances for residues of new animals drugs in food)

Tissues	Oestradiol (ng/kg)
Muscle	120
Liver	240
Kidney	360
Fat	480

The FDA guidelines state that: "…no physiological effect will occur in individuals chronically ingesting animal tissues that contain an increase of endogenous steroid equal to 1% or less of the amount in micrograms produced by daily synthesis in the segment of the population with the lowest daily production. In the case of oestradiol and progesterone, prepubertal boys

synthesize the least, in the case of testosterone, prepubertal girls synthesize the least" (taken from Andersson and Skakkebaek, 1999). The convention used by JECFA as the basis for determination of daily consumption of hormones is based on eating 500 g of meat per day (300g muscle, 100g liver, 50g kidney and 50 g fat). Based on this and the acceptable oestradiol levels in beef shown in Table 3, total daily consumption of currently acceptable levels of oestradiol would be 102 ng. This value represents approximately 1-2% of the currently used calculated daily production rates for oestradiol in prepubescent children.

As mentioned previously in the *Exposure Considerations Section*, the daily production rate for oestradiol was estimated to be 6 µg/day oestradiol in boys. These daily production rate (PR) values are determined by the formula:

PR (µg/day) = plasma concentration (µg/ml) x metabolic clearance rate (MCR, ml/day)

However, there are two potential problems with these values. First, as mentioned previously (*Exposure Considerations Section)*, determination of plasma concentrations of oestradiol is subject to considerable variability, relative insensitivity given its low levels in children, and interference. A new, highly specific, more sensitive assay for oestradiol indicated that blood oestradiol levels in girls may be as much a 13 fold less and in boys 100 fold less than previous determinations using RIAs indicate. Second, it does not appear that MCRs have ever been determined directly in children. Rather, it appears as if MCR values from adult women were used in the calculations of the PRs for children (Anderson and Skakkebaek, 1999). This approach may or may not be valid given the known differences in levels of SHBG (higher in children, which would reduce clearance), and likely differences in uptake and metabolism, etc. Given these issues, it is possible that the safety margin for oestradiol exposure used by the FDA may be in error and that acceptable levels of hormone residues in beef could be much lower. (Similar concerns apply to progesterone and testosterone).

The median level of excess exposure to oestradiol from consuming meat from hormone-treated cattle is 6.8 ng/person/day (calculated from Table A3, Annex, range 1 to 84 ng/person/day). For comparative purposes, assuming 100% absorption and a whole blood volume of 78ml/kg body weight, for a 40 kg child, based on the median value for excess oestrogen exposure, the blood concentration calculates to be 2.2 pg/ml (1 to 26 pg/ml).

If the blood oestrogen levels are 100 fold lower than previously determined and the MCR too high by a factor of 10, the oestradiol daily production rate could be as low as 6 ng, and 1% of this would be 60pg. Thus, the FDAs acceptable daily intake (102 ng/person/day, see above) could exceed the daily production rate of oestradiol by 1,700 fold. While there is some experimental evidence in support of the currently used blood levels of oestradiol being 100 fold too high (Klein et al., 1994), the other assumptions used in coming to this conclusion may be too conservative. Thus, if absorption is reduced to 10% and the MCR for children is only 1/2 that of adults, the FDA acceptable daily intake could still be 85 fold too high. Given all of the uncertainties in

these estimates, it appears that the data are insufficient to form the basis of a sound risk assessment. Clearly, this is an important area for additional research.

The toxicological issues of concern arising from the indicated excess exposure include endocrine, developmental, immunological, neurobiological, immunotoxic, genotoxic and carcinogenic effects. Specific hazardous effects are detailed below.

4.1.6. Genotoxicity

Oestradiol is an endogenous oestrogen that undergoes extensive tissue specific oxidative metabolism discussed above (see chapter 2.4.). The predominant metabolites are the prooxidant 2-OHE and 4-OHE, which can undergo further biotransformation to semiquinones and quinones. Redox cycling of the semiquinones and quinones can produce superoxide and the quinones can form DNA base adducts (see Fig. 1). Thus, oestrogen oxidative metabolites can be directly or indirectly genotoxic (Cavalieri et al., 1997; Frenkel, 1992).

The mutagenicity/genotoxicity of oestradiol and its catechol and 16α-hydroxy metabolites has been investigated in several recent studies which are summarised below.

Table 4: Summary of genotoxity and mutagenicity assays

Assay	Results	(refs)
Ames Assay ± s-9	Negative	(Richold, 1988)
Mutation ± s-9 Mouse L5178Y TK +/- cells	Negative	(Richold, 1988)
Micronucleus SHE cells	Negative	(Schiffmann et al., 1988)

In vivo chromosomal aberrations
    Rat bone marrow
    Rat spermatogonial cells
	Negative
	Negative
		(Richold, 1988)
		(Richold, 1988)

Cell transformation SHE cells	Negative	(Schiffmann et al., 1988)

Methyltrexate resistance MCF-7 cella
    E$_2$
    2-OHE$_2$
    4-OHE$_2$
    16α-OHE2
	Weak
	Moderate
	
	Strong
	
	Strong
		(Thibedeau et al., 1998)
		(Thibedeau et al., 1998)
		
		(Thibedeau et al., 1998)
		
		(Thibedeau et al., 1998)

Microsatellite instability in transformed 10T1/2 cells E$_2$	Positive	(Paquette, 1996)

Microsatellite instability in Syrian hamster kidney in vivo E_2	Positive	(Hodgson et al., 1998)
Mutagenicity V79 cells HPRT E_2 (low conc., 10^{-10} M only)	Positive	(Rajah and Pento, 1995)
Chromosomal aberrations V79 cells E_2	Positive	(Eckert and Stopper, 1996)
Aneuploidy E_2 Syrian hamster fibroblasts Human fibroblasts	Positive Weakly positivie	(Tsutsui et al., 1990) (Tsutsui et al., 1990)

A couple of very recent studies stand out as being of potential significance, especially if reproduced and extended. One is that of Thibedeau et al. (1998) where it was observed that E_2, 2-OH- and 4-OH-E_2, and 16α-OH-E_1 induced methyltrexate (MTX) resistance. This was a well-done study where, using the Luria-Delbruck fluctuation test, they demonstrated that MTX-resistant MCF-7 human breast carcinoma cells did not pre-exist in the population. Subsequent exposure to the MCF-7 cells to the oestrogens for 12 population doublings resulted in the enhancement of MTX resistance. Compared to spontaneously arising MTX resistance, E_2, 2-OHE$_2$, 4-OHE$_2$ and 16α-OHE$_1$, all at 10^{-8} M, enhanced MTX resistance 3.2, 33, 88 and 88 fold, respectively. This was unrelated to their "oestrogenicity". It was not determined whether this resulted from gene amplification, effects on expression, or mutation. However, MTX resistance generally arises through gene amplification. If this turns out to be the mechanism of enhancement by the oestrogens, it would support the notion that oestrogens have direct, destabilizing effects on the genome. Consistent with this is the finding of microsatellite instability in oestrogen-induced tumors (Hodgson et al., 1998). Loeb and co-workers (Jackson et al., 1998) have hypothesized that the mutation rate in normal cells is insufficient to account for the large numbers of mutations observed in cancer cells and that therefore, tumor cells must acquire a mutator phenotype. They also demonstrated that microsatellite instability can be caused by oxidative DNA damage. The detection of microsatellite instability in oestrogen-induced tumors suggests that oestrogen treatment can cause oxidative DNA damage leading to this phenotype.

A second significant finding is that E_2, at 10^{-10} M caused a 2.4 fold increase in mutation at the hprt locus in V79 cells (Rajah and Pento, 1995). Increased mutation was not observed at lower concentrations. Liehr and coworkers have reproduced these findings (personal communication). The mutagenicity of E_2 at low, but not high, concentrations is consistent with the observations of Markides et al. (1998) that E_2 exhibits prooxidant properties at low concentrations and antioxidant properties at high concentrations. These findings demonstrate the importance of conducting future studies on the

mutagenicity of oestrogens over a broad range of concentrations ranging from 10^{-12} to 10^{-6} M.

In summary, E_2 has genotoxic potential. Evidence is building that oestrogens, most likely through oxidative metabolism to catechols and beyond to semiquinones and quinones, are DNA reactive and mutagenic.

4.1.7. DNA adducts and DNA damage

Treatment of cultured Syrian hamster embryo cells with 2-OHE$_2$ or 4-OHE$_2$ induced the formation of stable DNA adducts, as well as cell transformation (Hayashi et al., 1996).

Treatment of Sprague-Dawley rat mammary gland with 4-OHE$_2$ or E$_2$-3,4-Q results in the formation of DNA adducts, including 4-OHE$_2$-1-N7Gua (Cavalieri et al., 1997). The presence of DNA and GSH adducts, which are also formed in vitro (Cavalieri, et al., 1997; Cao, et al., 1998), indicates that E$_2$ is converted in vivo to genotoxic metabolites, namely the CE-3,4-Q (Cavalieri et al., 1997) The 6-hydroxyoestrogen-6-sulfates also form DNA adducts in vitro (Itoh, et al., 1998). These DNA-damaging effects indicate that no threshold exists for the risk from oestrogen metabolites.

Aneuploidy and altered chromosomal structure were observed in the kidney of E$_2$-treated Syrian hamsters (Tsutsui et al., 1990; Li and Li, 1996; Banerjee, et al., 1994; Li, et al., 1993). The frequency of micronuclei also was elevated three-fold in the kidney of hamsters treated with E$_2$ for three months (Banerjee et al., 1991). Aneuploidy and altered chromosome structure were induced by E$_2$ in Syrian hamster embryo cells (Tsutsui et al., 1987), which are subsequently transformed to tumor cells (McLachlan et al., 1982). Micronuclei were also induced in ovine seminal vesicle cells, (Schnitzler et al., 1994) and human chorionic villi cells (Schuler et al., 1996).

E$_2$ disrupts cytoplasmic microtubules in Chinese hamster V79 cells (Aizu-Yokota et al., 1994) and in human breast cancer MCF-7 and MDA-MB-231 cells (Sato et al., 1992). Since the MCF-7 cells are oestrogen receptor positive and the MDA-MB-231 cells are oestrogen receptor negative, the disruption of microtubules by E$_2$ is not a receptor-mediated process.

The E$_2$ metabolites 2-methoxyE$_2$ and 2-OHE$_2$ cause chromosome damage in MCF-7 cells, including abnormal spindle formation, fragmented spindle poles and uneven chromosome distribution (Seegers et al., 1989). Some of the hydroxy and methoxy metabolites of E$_1$ and E$_2$ disrupt cytoplasmic microtubules in V79 cells Sato and Aizu-Yokota, 1996).

Single-strand breaks in DNA are induced by E$_1$-3,4-Q in MCF-7 cells treated with 4-OHE$_1$ (Nutter et al., 1991, 1994) and in the kidney of Syrian hamsters treated with E$_2$ or 4-OHE$_2$, prior to the development of neoplasms in this organ (Han and Liehr, 1994a). The level of 8-OHdG in the DNA of Syrian hamsters treated with 4-OHE$_2$ (Han and Liehr, 1994b). These effects may arise, at least in part, from oestrogen-induced generation of free radicals and lipid hydroperoxides.

4.1.8.   Carcinogenicity

Most of our current knowledge of the carcinogenicity of oestrogens relates to their use as human medicines, mostly for oral contraception, hormonal replacement therapy for menopause and treatment of pregnancy related complications. These indications entail exposure to relatively high doses and for prolonged periods of time.

No data are currently available on the effects of exogenous low-dose oestrogens. However, genotoxic effects independent from the presence of hormonal receptors have been recognised for metabolites of certain oestrogens, as indicated above.

4.1.8.1. Carcinogenicity in Animals

Combined with human data, evidence that oestradiol (E_2) is carcinogenic in animals was sufficient for IACR to classify oestradiol as a carcinogen (IARC, 1987; Lipschutz and Vargas, 1994). For example, increases the incidence in mice of mammary, pituitary, uterine, cervical, vaginal, testicular, lymphoid and bone tumors (Huseby, 1980; Highman, et al., 1980; Highman, et al., 1987; Nagasawa, et al., 1980). In rats, treatment with oestratiol or oestrone increased the incidence of mammary and/or pituitary tumors (Inoh, et al., 1985; Noble, et al., 1975; Shull, et al., 1997). In hamsters, E_2 induces kidney tumors in males and in ovariectomized females (Kirkman, 1959). In addition, the catecholoestrogens 4-OHE$_2$ and 4-OHE$_1$ induce kidney tumors, although 2-OHE$_2$ and 2-OHE$_1$ do not (Liehr, et al., 1986; Li and Li, 1987).

Transgenic mice overexpressing the *Wnt-1* gene have a high incidence of mammary tumors within a few months after birth (Bocchinfuso, et al., 1998). In addition, when Wnt-1 mice were crossbred with oestrogen receptor-α knockout (ERKO) mice, the offspring also have a high incidence of mammary tumors (Bocchinfuso, et al., 1998). Ovariectomized crossbred mice have lower production of E_2 and the incidence of mammary tumors is significantly reduced (Bocchinfuso, et al., 1998). These results indicate that induction of mammary tumors relies on the presence of E_2, but not that of the major oestrogen receptor, suggesting a genotoxic role of E_2 in the induction of these mammary tumors.

4.1.8.2. Carcinogenicity in Humans

In humans, oestrogen administration is considered a risk factor for human endometrial adenocarcinoma (Greenwald, et al., 1977; Siiteri, et al., 1980), with the risk increasing with larger doses of oestrogen and longer periods of treatment (Key, et al., 1988). Increasing evidence also indicates that oestrogen is a risk factor for breast cancer (Feigelson and Henderson, 1996; Bernstein, 1998; Toniolo, et al., 1995; Adlercreutz, et al., 1994; Henderson, et al., 1993). Epidemiological studies have demonstrated strong relationships between the levels of endogenous oestrogen and risk of breast cancer (Toniolo, et al., 1995; Berrino, et al., 1996; Bernstein, et al., 1990a and b; Shimizu, et al., 1990; Pike, et al., 1992).

In conclusion, whereas it is clear that exogenous oestrogens, present in oral contraceptives or used in hormonal replacement therapy in women, are responsible for an increased risk of endometrial cancer and to lesser extent some increased risk of breast cancer, there is no direct evidence on the consequences of the contribution of exogenous 17β-oestradiol originating from the consumption of treated meat. Yet we know from the data derived from human populations within the ranges of physiological values of hormones in blood, that high levels are associated with an increased risk of breast cancer. Also known are the carcinogenic effects of 17β-oestradiol in experimental animals as well as the deleterious effects in pre- and perinatal development (see section 2). Finally, in consideration of the recent data on the formation of genotoxic metabolites of oestradiol, suggesting that 17ß-oestradiol acts as complete carcinogen, by exerting tumour initiating and promoting effects, it has to be concluded, that no quantitative estimate of the risk related to residues in meat could be presented.

4.1.9. *Effects on growth and reproduction*

17β–oestradiol is the major oestrogen secreted by the ovaries in women of adult age. Plasma levels of 17β-oestradiol are low in men and very low to undetectable in children. In adolescent girls and boys, puberty is associated with an increasing plasma level of 17β–oestradiol which exerts a direct, biphasic effect on bone growth and maturation, with maximal stimulation of epiphyseal growth at low levels in prepubertal boys as well as girls. Administration of low doses of oestradiol (4 μg per day administered to 30-40 kg children by a 4 day infusion) corresponding to a serum oestradiol level of about 15 pmol/l (4 pg/ml) causes more than a 60% increase over the prepubertal growth rate in both boys and girls (Cutler, 1997). In girls with Turner's syndrome, oestradiol doses as low as 25-100 ng/kg per day are associated with increased growth rate (Ross et al., 1988; Mauras et al., 1989). Oestradiol deficiency due to mutations in the aromatase gene and oestradiol resistance due to disruptive mutations in the oestrogen receptor gene lead to the absence of pubertal growth spurt, delayed bone maturation, continued growth into adulthood and very tall stature in both sexes (Lee and Witchel, 1997, MacGillivray et al., 1998). It is concluded that the pubertal growth spurt of both sexes is driven primarily by oestradiol, and that the more rapid epiphyseal maturation of prepubertal girls (vs boys) may be explained by their higher oestradiol levels. These observationsstrongly suggeste that environmental 17β–oestradiol can, even when administered at very low doses, modulate growth of chidren of both sexes and decrease the age when final height is achieved and puberty is reached.

17β–oestradiol exerts a feminizing effect on secondary sexual characters. During adolescence, the increasing serum level of oestradiol induces breast and uterus development in girls. In boys with a dominant mutation leading to an aromatase excess, enhanced oestradiol levels in plasma are associated with heterosexual precocity and breast development (gynecomastia) (Stratakis et al., 1998). At puberty, breast development occurs only after the onset of the pubertal growth spurt in girls and is uncommon in boys. Consequently, it is

suggested that the dose at which environmental 17β oestradiol could exert feminizing effects in human populations is higher than the dose able to stimulate growth in prepubertal children.

Under physiological conditions, endogenous 17β–oestradiol secreted by the ovary in the female exerts negative feedback effects on the secretion of the gonadotropins FSH and LH, acting on the hypothalamo-pituitary system. Moreover, in adult non-pregnant females only, high serum levels of 17β–oestradiol (about 300 pg/ml in humans) induce, by positive feedback effect on the hypothalamus, a discharge of gonadotropins which triggers ovulation at each ovarian cycle. It is established that administration of exogenous oestradiol leading to a sustained increase in serum levels induces a decrease in secretion of gonadotropins (negative feedback effect), and thereby arrest of spermatogenesis and azoospermia in the male, and interruption of ovarian cycles and blocking of ovulation in the female. In experimental animals, the doses of exogenous oestradiol sufficient to induce sterility in adults are generally high. However in the male rat, doses as low as 10 ng/rat of oestradiol or of the catecholoestrogens 2-OHE2 and 4-OHE2 induce, after 21 daily exposures, morphological lesions and spermatogenesis arrest without affecting gonadotropin release, so that a direct effect of these oestrogens on developing spermatids has been proposed (Seegers et al., 1991). It can be noticed that in men, infertility has been associated with an increase in intratesticular aromatase, leading to increase in intratesticular and seminal oestradiol levels (Bujan et al., 1993; Ichikawa, 1995).

From these observations, it is suggested that environmental 17β–oestradiol could exert deleterious effects on fertility in men and women, by acting through various, direct and indirect, mechanisms.

4.1.10. Effects on the immune system

Oestrogen induced immuno modulation is a subject of rapidly growing interest. (Cutolo et al., 1995). Oestrogens appear to play a central role in the immune response and in immune mediated diseases (see also 2.4.). Recent reports have demonstrated the presence of oestrogen receptors on various cells involved in the immune response, including thymocytes, macrophages and endothelial cells. Indicators of a possible important role of oestrogens have been proposed to be the effects of pregnancy and the oral contraceptive pill on infection susceptibility and allergy (Wjst and Dold, 1997). Animal studies indicate the potential of oestrogens to cause immune depression and enhance infection susceptibility but give little indication of the dose response relationship.

Bovines with high oestrogen levels showed an increased number of leucocytes and neutrophil granulocytes in blood. However, stimulation or no effects of oestrogens have been reported for a number of other parameters of immune system functioning (e.g. phagocytes, reactive metabolite generation, cytotoxicity). (Wessendorf et al., 1998).

In broiler chicks, administration of 1 µg/kg body weight daily for 50 days caused a decrease in the number of leucocytes and reduced macrophage activity (Al Afaleg and Homeida, 1998).

A slight, but non statistically significant immuno depression was seen in male calves given s.c. implants of 17β oestradiol (20mg). In combination with trenbolone acetate (140 mg), a statistically significant change was observed which was not seen with trenbolone acetate alone. No such effects were seen in female calves (Gropp et al., 1975).

In female mice, previously primed with β oestradiol which were challenged with *N. gonorrhoeae*, the survival in the vagina of the bacterium was significantly increased compared with control animals (Arko et al., 1997). In vitro studies using hybridoma clones have demonstrated that 17β oestradiol, introduced in polyelectrolyte complexes containing bovine serum albumin, invokes considerable increases in a steroid specific immune response. (Balsalp et al., 1996).

These data indicate that oestrogens modulate the immune system in many species. Direct human data at near physiological levels of oestradiol are lacking. Vingerhoets et al., (1998) have conducted a self-reporting questionnaire study of DES daughters. A statistically significant difference in the incidence of infections was identified compared with control. This may be considered to be linked to imprinting by DES in utero.

In conclusion, at relatively high doses oestradiol does produce a number of adverse effects on the immune system in humans e.g. allergy to topical oestradiol (Boehnke and Gall, 1996). The above findings while indicating a possible concern are insufficient to identify whether immune effects could occur in consumers from the ingestion of meat or meat products containing oestradiol residues.

4.2.   Testosterone

Testosterone, 17β-hydroxyandrost-4-en-3-one or α-4-androsten-17β-ol-3-one, is a 19-carbon steroid hormone that has potent androgenic properties including maintenance of testicular function and the development of secondary sex characteristics in males. Early in life, testosterone influences the development of external genitalia and is important in the differentiation of the Wolffian ducts. At puberty and thereafter, androgens affect the testes and are essential for spermatogenesis. Androgens have strong anabolic effects that include increased protein synthesis in muscle and bone (Rosenfield, 1972; Chang et al., 1995). In organs such as prostate, 5α-reductase can convert testosterone into 5α-dihydrotestosterone (DHT), a more potent androgen. The actions of both testosterone and DHT are mediated through their high affinity and high specificity binding and activation of an intracellular protein, the androgen receptor (AR). This AR protein is a member of the steroid hormone superfamily. The ligand-activated androgen receptor mediates its effects on cell growth and differentiation through the activation and/or suppression of specific gene transcription in target organs. Androgen receptors are detected in tissues of females, as well as males. The presence of

this receptor in organs such as the ovary indicates significant activity of androgens in both sexes. Furthermore, the androgen receptor is thought to be involved in ovarian tumorigenesis, as it has been detected in 67 percent of ovarian tumors. Although current information indicates the presence of only a single androgen receptor, it is known that different subsets of genes may be activated by either testosterone or DHT (Chang et al., 1995). Thus, as for other steroid hormone receptor pathways, the mechanism of androgen activity is only partially understood. It is also of importance to note that testosterone can be aromatized to oestradiol in tissues containing CYP450 aromatase (CYP19).

In animals, testosterone or testosterone propionate, alone or in combination with other hormonally active substances, is used primarily to improve the rate of weight gain and feed efficiency. This effect is most likely a consequence of the anabolic action of androgens.

4.2.1. Pharmacokinetics and Biotransformation of Testosterone in animals

Testosterone or testosterone propionate is administered by subcutaneous implantation in the ear. The ear, along with any residual drug, is discarded at slaughter. The dosage of testosterone varies with the manufacturer of the implant, but is most often 200 mg per animal (JECFA, 1988). In the circulatory system of the animal, testosterone derived from the implant is indistinguishable from endogenous testosterone, i.e. enzymatic transformation of the biologically active molecule into less active metabolites. Excretion is predominately via the biliary route, and to a lesser extent via the urine. In general, the fraction of the hormone eliminated in the urine is in the conjugated form, while the fraction found in the feces is in the free form. For testosterone propionate, enzymatic cleavage of the ester produces testosterone which, is again metabolized as the endogenous compound (Hoffmann and Karg, 1976; Hoffmann and Evers, 1986).

Little information is available about the specific metabolic routes and elimination rates for testosterone in cattle. In rats, seven pathways of testosterone oxidation, 2α-, 2β-, 6β-, 15β-, 16α-, and 18-hydroxylation of testosterone and 17-oxidation of androstenedione, have been sexually differentiated in mature animals (male/female = 7-200 fold) but not in immature animals. Furthermore, specific cytochrome P450 enzymes were shown to metabolize testosterone selectively at these various positions (Sonderfan et al., 1987).

4.2.2. Testosterone disposition in the target animal

When heifers and female calves were treated with implants containing 200 mg of testosterone or testosterone propionate an increase of testosterone concentrations in muscle, liver, kidney and fat was determined, Table 5 .

Table 5: Levels of testosterone in tissues of treated and untreated animals in ng/kg:

	Muscle	Liver	Kidney	Fat
Heifer	19.6	12.9	189	25.5
Heifer treated (30 days)	102	34.1	451	339
Pregnant heifer (240 days gestation)	418	274	4014	694
(240 days gestation)				
Female calf	6	108	96	22
Treated calf (30 days)	360	196	588	1027
Bull	535	749	2783	10950

4.2.3. Pharmacokinetics and Biotransformation (in humans).

In humans, the oxidative metabolism of testosterone occurs predominantely in the liver at the 6β-position, and to a lesser extent at the 15α-, 15β-, and 2β-positions. Cytochrome P4503A4 has been shown to be the major testosterone 6β-hydroxylase in human liver, catalyzing testosterone hydroxylation at the 15α-, 15β-, and 2β-positions, as well (25). Human liver cytochromes P4502C9 and P4502C19 also have been shown to possess significant testosterone hydroxylase activity (Yamazaki and Shimada, 1997).

Orally administered testosterone is readily absorbed when given by mouth, but has limited bioavailability because most of the hormone is metabolized on first pass through the liver before reaching the systemic circulation (Baird et al., 1969). Inactivation of testosterone occurs primarily in liver, where it is metabolized to androstanedione and ultimately to androsterone and other compounds that are excreted as glucuronides and sulfate conjugates (Fotherby and James, 1972). In men and women, the metabolic clearance rate of testosterone was reported to be 516 and 304 liters/meter-squared/day, respectively. In men, the production of dihydrotestosterone was 0.39 mg/day, 50 % being derived from the transformation of plasma testosterone (Saetz et al., 1972).

4.2.4. Assessment of Excess Exposure to testosterone from Consumption of Hormone-treated Beef

Table A3 (Annex) shows that consumption of beef from hormone treated vs non-treated cattle results in exposure to excess levels of testosterone ranging from 5 to 189 ng/person/day, depending upon the implant used.

At its February 1999 Meeting, the JECFA established for testosterone an ADI of 0.2 ug/kg bw (14 µg/70 kg person) on the basis of a study in eunuchs. This value includes a safety factor of 1000 to protect more sensitive populations and because of the small number of subjects in the study used to determine the NOEL. In that study, oral administration of a dose of 100 mg/day (equivalent to 1.7 mg/kg bw/day) of fine-particle testosterone to five eunuchs had no effect on sexual function indexes while a dose of 400 mg/day

restored full sexual function. The dose of 100 mg/day was taken as the NOEL in this study. In another study in postmenopausal women, treatment with 10mg/day methyltestosterone was found to induce signs of virilisation. The ADI for testosterone established by the JECFA (14 µg/person) is greater than the highest excess exposure to testosterone (189 ng/person) that could occur from ingesting hormone-treated beef. However, there are concerns regarding the strength of the study that provided the data for determination of the ADI. First, neither the actual data nor reference to a peer-reviewed publication was provided. Second, the dose-response was limited to two doses and the ADI was estimated from just a single dose where no effect was observed, rather than a curve derived from all the data available.

The tolerance levels for testosterone levels in uncooked tissues of steers and calves established by the FDA (Ref.: Code of Federal Regulations (CFR) 21, Part 556, Tolerances for residues of new animals drugs in food) are:

Tissue	Testosterone (µg/kg)
Muscle	0.64
Liver	1.3
Kidney	1.9
Fat	2.6

Based on these levels, consumption of 500 g/day of beef (300 g muscle, 100 g liver, 50 g each of kidney and fat) would result in exposure to approximately 0.6 µg/person/day. The maximum excess exposure to testosterone estimated to occur upon consumption of meat from hormone-treated cattle, 189 ng/person/day (Table A3, Annex) represents 33% of the acceptable level established by the FDA (0.6 µg/person/day) which also represents approximately 1-2% of the daily production rate for testosterone of 32 µg/day estimated for prepubertal girls. However, there is considerable uncertainty associated with the validity of the daily production rate data. It is possible that this value has been over estimated by one to two orders of magnitude, in which case excess testosterone intake from hormone-treated beef could at best exceed the 1% FDA safety margin and at worst be greater than that naturally present.

Testosterone Levels in Human Blood: As expected, men have the highest levels of blood testosterone (Table 1, section 4.1) and the daily production rate has been determined to be approximately 6,500 µg/day (JECFA, 1987). Testosterone levels are much lower and similar in females and prepubertal males. It has been reported that daily production rates of testosterone are between 140 to 240 µg/day in adult women and 32 and 65 µg/day in prepubescent girls and boys, respectively (JECFA, 1987). These data suggest that all females and prepubertal males represent the individuals are greatest risk for adverse health effects that might be associated with exposure to exogenous sources of testosterone.

The toxicological issues of concern include endocrine, developmental, immunological, neurobiological, immunotoxic, genotoxic and carcinogenic effects. Specific hazardous effects are detailed below.

4.2.5. *Mutagenicity and genotoxicity of testosterone*

The mutagenicity/genotoxicity of testosterone has been investigated in several studies which are summarised below:

Table. 6: Summary on the studies on mutagenicity and genotoxicity of testosterone

Assay	Results	[ref]
Mutation $\pm$ s-9 Mouse L5178Y TK+/- cells	Negative	(Richold, 1988)

In vivo chromosomal aberrations
     Rat bone marrow
     Rat spermatogonial cells
	Negative
	Negative
		(Richold, 1988)
		(Richold, 1988)

Thus, no genotoxicity has been demonstrated with the limited testing to date.

4.2.6. *DNA adducts and DNA damage*

A low level of covalent binding of testosterone to rat liver DNA has been reported (Barraud et al., 1984).

No information is available on DNA damage induced by testosterone or its metabolites. Testosterone is, however, aromatized to oestradiol, which is metabolized to reactive forms that damage DNA and induce mutations (see section 4.1 above).

4.2.7. *Carcinogenicity*

Feeding of testosterone has been reported to induce uterine tumours in mice and prostate tumors in rats (Van Nie et al., 1961; Noble, 1977). Whereas the evidence in favour of carcinogenicty was considered sufficient for testosterone in experimental animals, data in humans are limited.

In conclusion, although endogenous testosterone may play a role in the occurrence of prostate cancer, the evidence is currently weak. However, in consideration of the limited data on genotoxicity and taking into account that testosterone might be aromatized to oestradiol, which had found to be genotoxic, no conclusive quantitative estimate of the risk arising from the excess intake with meat and meat products from treated animals can be made. Based on the above mentioned epidemiological data androgenic anabolic steroids including testosterone were considered as probable carcinogenic to humans (IARC group 2 A)

4.2.8. Effects of testosterone on growth and reproduction

Testosterone is the major androgen secreted by testes in men of adult age. Plasma levels of testosterone are low in women and very low to undetectable in children.

In adolescent boys, puberty is associated with an increasing plasma level of testosterone which exerts direct virilizing and anabolic effects, leading to increases in bone mass and muscle bulk. In adolescent girls with aromatase deficiency, enhanced testosterone levels are associated with pseudohermaphrodism, virilization and polycystic ovaries (Morishima et al., 1995; Mullis et al., 1997).

Continuous administration of exogenous testosterone can induce deleterious effects in the reproduction of male and female mammals. Particularly in the adult male, a sustained increase in plasma level of testosterone administered by ingestion, injections or implants, induces a decrease in secretion of gonadotropins, leading to alterations or arrest of spermatogenesis and azoospermia.

Hormonal imprinting effects of testosterone have been reported in rodents. In the female rat, postnatal androgenization with a single injection of testosterone alters permanently by imprinting the response of uterine cells to oestrogen action (Mena et al., 1992). Moreover, imprinting of female offspring with testosterone results in insulin resistance and changes in body fat distribution at adult age in rats (Nilsson et al., 1998). These changes are similar to those observed in adult female rats or women receiving testosterone. No dose response estimate can be given for this effect yet.

4.2.9. Effects of testosterone on the immune system

There are limited experimental data on the effects of testosterone on immuno response but none on the dose response aspects.

Testosterone (0.9 mg twice weekly for 3 weeks) has been claimed to increase the susceptibility of mice to Plasmodium Chabaudi malaria by imposing restrictions on the genes controlling resistance. This enhanced susceptibility continued for at least 12 weeks after testosterone withdrawal. In contrast the reduction in spleen cell number had completely reversed by this time. The mechanism is considered to involve hormone imprinting (Benter et al., 1997).

It has been shown that female mice, subsequently treated with testosterone, have reduced burdens of worms compared with non testosterone treated controls. No such effect was observed if testosterone was given after an infective dose of worms was administered (Nakazawa et al., 1997)

In broiler chicks, given 0.1 mg per kg body weight of testosterone, a significant decrease of total leucocytes, lymphocytes and weight of the bursa of Fabricious was noted indicating an immuno supressant effect of testosterone (Al Afaleg and Homeida, 1998).

In conclusion, these studies are inadequate to enable a judgement to be made on whether meat containing testosterone residues could have an adverse effect on the immune system of consumers.

4.3. Progesterone

Progesterone, pregn-4-ene-3, 20-dione, is a C-21 steroid hormone and the most potent endogenous progestogen. This hormone is present in all steroid-producing organs, and its production rate varies widely as a function of the phase of a woman's menstrual cycle and pregnancy. The major physiologic function of progesterone is to prepare the uterus for implantation and to maintain pregnancy. The production of progesterone in the corpus luteum of the ovary in adult females is controlled by pituitary luteinizing hormone. Progesterone is essential for uterine development, implantation, blastocyst development and maintenance of the fetus and the uterus during pregnancy. Progesterone opposes some of the effects of oestrogens, and in non-pregnant females, this hormone inhibits the cyclic release of luteinizing hormone and follicle stimulating hormone. The actions of progesterone require prior stimulation with oestrogens, perhaps to increase expression of progesterone receptor (PR). The PR is a member of the steroid hormone superfamily of receptor proteins and mediates the biologic activity of progesterone through gene regulatory mechanisms (Mahesh et al., 1996; Katzenellenbogen, 1996).

In animals, progesterone is used primarily in combination with oestrogenic compounds in order to improve their rate of weight gain and feed efficiency, and to suppress oestrus in feedlot heifers.

4.3.1. Pharmacokinetics and biotransformation of progesterone in animals

Progesterone is administered by subcutaneous implantation in the ear. The ear, along with any residual drug, is discarded at slaughter. The dosage of progesterone is 200 mg per animal (JECFA, 1988). In the circulatory system of the animal, progesterone derived from the implant is indistinguishable from endogenous progesterone (Baird et al., 1969). The metabolism of progesterone in cattle has been investigated using radiolabeled compound (Estergreen et al., 1977; Purdy et al., 1980; Lin et al., 1978). Animals were administered progesterone, $50\mu g/kg$ twice daily for 15 days. Each of the last three injections contained 0.9 mCi [^{14}C]-progesterone and the animals were killed 2-3 hours after the final treatment. Most of the radioactivity in all extracts corresponded to the parent compound (54% of the free radioactivity in muscle and 69 and 73% of the free and conjugated radioactivity, respectively in fat), (Lin et al., 1978). The major metabolites detected in muscle (16% of total radioactivity) included: 5α-pregnane-3, 20-dione (9%); 20-β-hydroxy-4-pregnen-3-one (8%); 3α-hydroxy-5β-pregnan-20-one (13%); and 3α-hydroxy-5α-pregnan-20-one (3%). The major metabolite detected in fat (62% of the total radioactivity) was 20-β-hydroxy-4-pregnen-3-one (11%), (Estergreen et al., 1977). Little is known about the specific enzymes in cattle that metabolize progesterone, although hepatic cytochrome P450 enzymes are likely involved in the metabolic clearance of this hormone.

4.3.2. Progesterone disposition in the target animal

In all studies 200mg of progesterone was administered by implantation in the ear. An increase in progesterone concentration was only estimated in fat tissue of male calves with a change from 1.6 to 8.7 µg/kg (50 days after implantation). The levels of progesterone in tissues of treated and untreated animals are presented in table 7.

Table 7: Progesterone tissue levels in µg/kg:

	Muscle	Liver	Kidney	Fat
Steer	0.27	0.26	0.17	2.48
Steer treated (61 days)	0.41	0.35	0.20	3.40
Male calf	0.90	0.75	4.07	1.6
Treated calf (50 days)	0.77	0.77	1.41	8.7
Pregnant heifer	10.1	3.42	6.19	239

4.3.3. Pharmacokinetics and biotransformation of progesterone in humans

In humans, the oxidative metabolism of progesterone occurs in the liver at the 6β-position and to a lesser extent at the 16α- and 2β-positions. Cytochrome P4503A4 has been shown to be the major progesterone hydroxylase, catalyzing the formation of each of these products (Yamazaki and Shimada, 1997). Significant extrahepatic metabolism and clearance also has been observed and is believed to proceed subsequent to initial 5α-reduction via non-cytochrome P450 routes (Chantilis et al., 1996).

Absorption of exogenous progesterone in humans is rapid, irrespective of the route of administration, reaching peak plasma concentrations within 4 hours of oral administration (de Lingnieres et al., 1980). However, this hormone undergoes extensive metabolism in the gut, intestinal wall and liver (Adlercreutz and Martin, 1980). Only a small fraction of the administered dose is detected as progesterone in serum, while most of the steroid circulates as inactive 5β-pregnane-3α-ol-20α-diol-glucuronide (de Lingnieres et al., 1980; Adlercreutz and Martin, 1980).

4.3.4. Assessment of excess exposure to progesterone from consumption of hormone-treated beef

Table A3 (Annex) shows that consumption of beef from hormone treated vs non-treated non-pregnant cattle results in exposure to excess levels of progesterone ranging from 64 to 467 ng/person/day, depending upon the implant used.

At its February 1999 Meeting, the JECFA established for progesterone an ADI of 0-30 µg/kg bw (0-2,100 µg/70 kg person). This value was based on studies where a lowest-observed-effect level (LOEL) of 200 mg fine-particle progesterone (equivalent to 3.3 mg/kg bw) was determined and includes a safety factor of 100 to allow for extrapolation from the LOEL to a NOEL. In one study, designed to explore anti-proliferative and secretory endpoints in

the endometrium, women were treated with 300 or 600 mg/day of fine-particle progesterone for two weeks following a thirty day pretreatment with oestrogen. The group treated with the 300 mg dose showed incomplete conversion of the uterus to full secretory activity whilst the group receiving the 600 mg dose did. In an additional studies using 200 or 300 mg oral doses of progesterone for one or five years, there was no evidence of endometrial hyperplasia or carcinoma. In addition, it was stated that a single oral dose of 200 mg fine-particle progesterone produced concentrations of progesterone in blood similar to those found during the luteal phase of the ovulatory cycle. While these data indicate that the daily exposure from consuming hormone-treated beef is well below the ADI, there is some concern regarding determination of the ADI. First, neither the actual data nor reference to a peer-reviewed publication was provided. Second, the dose-response was limited to two doses and the ADI was estimated from just a single dose rather than a curve derived from all the data available.

The tolerance levels for progesterone levels in uncooked tissues of steers and calves established by the FDA (Ref.: *Code of Federal Regulations (CFR) 21, Part 556, Tolerances for residues of new animals drugs in food*) are:

Tissue	Progesterone (μg/kg)
Muscle	3
Liver	6
Kidney	9
Fat	12

Based on these levels, consumption of 500 g/day of beef (300g muscle, 100g liver, 50g each of kidney and fat) would result in exposure to approximately 2.6 μg/person/day. This amount represents approximately 1-2% of the daily production rate for progesterone of 150 μg/day estimated for prepubertal boys, and approximately 0.3% of the maximum excess exposure to progesterone estimated to occur upon consumption of meat from hormone-treated cattle (Table A3, Annex). However, there is considerable uncertainty associated with the validity of the daily production rate data. It is possible that this value has been over estimated by one to two orders of magnitude, in which case excess progesterone intake from hormone-treated beef could at best exceed the 1% FDA safety margin and at worst be greater than that naturally present.

Progesterone Levels in Human Blood: The data show that premenopausal women have the highest levels of endogenous progesterone (Table 1, section 3.1). Progesterone production rates in premenopausal women during the follicular phase have been determined to be approximately 418 μg/day (JECFA, 1987 monograph). During pregnancy, progesterone production rates during late pregnancy have been determined to be approximately 94,000 ug/day (JECFA, 1987 monograph). In men, the daily production rate for progesterone is approximately 416 μg/day, respectively (JECFA, 1987). In prepubertal boys, the progesterone production rate has been reported to be

150 µg/day (JECFA, 1987). Thus, prepubertal and postmenopausal females and prepubertal and adult males have the lowest levels of endogenous progesterone and thus would represent the individuals most likely to be at increased risk for adverse health effects that might be associated with exposure to exogenous sources of oestrogens.

The toxicological issues of concern include endocrine, developmental, immunological, neurobiological, immunotoxic, genotoxic and carcinogenic effects. Specific hazardous effects are detailed below.

4.3.5. *Mutagenicity and genotoxicity of progesterone*

No information is available.

4.3.6. *DNA adducts and DNA damage*

No information is available, although the 1999 JEFCA report refers without data or reference to induction of single-strand DNA breaks and DNA adducts from in vivo and in vitro studies.

4.3.7. *Carcinogenicity*

Progesterone has been shown to increase the incidence of tumours in laboratory animals in the following target tissues: mammary gland, ovary, uterus and vagina (Jones and Bern, 1977; Frank, et al., 1979). .

The evidence for carcinogenicity in humans is considered inadequate. Most of the human evidence on the effect of progesterone on cancer risk comes from studies on progestogen-only contraceptives, available for the last 20 years, either as progestogen–only mini-pills or intramuscular depot injections and subcutaneous implants. Studies have been conducted on breast, endometrial, cervical, ovarian and liver cancer, as well as cutaneous melanoma.

In conclusion, as the evidence is considered sufficient in experimental animals for the carcinogenicity of medroxy progesterone acetate, (and inadequate for levorgestrel), the overall evaluation of IARC resulted in the allocation of progestogen-only contraceptives in group 2B, possibly carcinogenic to humans. At present the data are insufficient to make any quantitative estimate of the risk arising from the exposure to residues in meat and meat products originating from treated animals.

4.3.8. *Effect of progesterone on growth and reproduction*

Progesterone is the major progestogen secreted by the corpus luteum during the luteal phase of the ovarian cycle in women of adult age. Plasma levels of progesterone are low in men, in children and in women during the follicular phase of the ovarian cycle.

In physiological conditions, endogenous progesterone secreted by the ovary in the female exerts negative feedback effects on the secretion of the luteinizing hormone (LH), acting on the hypothalamo-pituitary system. During the luteal phase of the ovarian cycle, LH pulsatility is low and

ovulation is prevented until the demise of the corpus luteum. Continuous administration of exogenous progesterone to an adult female leads to interruption of ovarian cycles and blocking of ovulation, when the doses administered allow to obtain plasma levels similar to those of a normal luteal phase. In cattle, implants delivering lower doses of progesterone corresponding to subluteal plasma levels induce an increase in LH pulsatility, the lengthening of the estrous cycle and the presence of large persistent follicles on ovaries (Stock and Fortune, 1993). In the male, alterations of spermatogenesis can be induced by progesterone treatments. No assessment of the dose response relationship has been presented yet.

4.3.9. Effects of progesterone on the immune system

A limited number of investigations have been made. Progesterone has been shown to reduce the ability of cows to combat bacteria introduced into the uterus (Rowson et al., 1963).

Progesterone when administered at high levels has also been shown to depress the production of antibodies to experimental infections of candida albicans. At low doses immuno stimulation was claimed (Malhur, et al., 1978)

In sheep progesterone was found to regulate immune function, resulting in its inhibition at the utero placental interface without systemic immuno suppression (Hansen, 1998).

In conclusion, these data indicate that progesterone can cause immuno depression; however, they are insufficient to make any realistic assessment of the dose response relationship.

4.4. Trenbolone

Trenbolone acetate (TBA), 17β-hydroxyestra-4,9,11-triene-3-one, is a synthetic steroid with anabolic properties. It is 8 to 10 times as potent as testosterone (Bouffault and Willemart, 1983). In animals, TBA, alone or in combination with 17ß-ooestradiol, is used to improbe weight gain and feed efficiency. This effect is most likely a consequence of the anabolic acion of this androgen. The various TBA-containing implants, their composition, and target animal are shown in Tables A1 and A2.

4.4.1. Pharmacokinetics and biotransformation of trenbolone in animals

TBA is administered by subcutaneous implantation in the ear. The ear, along with any residual drug, is discarded after slaughter. The dosage of TBA varies with manufacturer of the implant, ranging between 40 and 300 mg per animal (JECFA, 1988). TBA upon entering the circulatory system is rapidly hydrolyzed to its active free form, 17β-trenbolone (TBOH). In the bovine species, the 17α-epimer is the major metabolite occurring in the excreta, bile and liver; the 17β-epimer is the major metabolite occurring in muscle (Jouquey et al., 1983). Elimination in the bile and urine occurs following conjugation, predominately to glucuronic acid (Pottier et al., 1979; Pottier et al., 1981). Also in blood plasma, conjugated TBOH has been determined;

concentrations were 13% of those of free TBOH. In addition a number of other metabolites have been identified in bile. However, only trendion seems to occur in some qualitative amounts. In 1978, Ryan and Hoffman (Ryan and Hoffman, 1978) reported remarkable discrepancies in residue concentrations as determined by radiotracer studies and RIA; they concluded that the much lower values obtained by RIA were due to the formation of covalently bound nonextractable residues. This observation was further substantiated (Evrard and Maghuin-Rogister, 1988), and in vitro studies (43) have demonstrated the involvement of hepatic cytochromes P450 in the formation of these type of residues. The metabolism of TBA appears complex and species dependent. Further investigations of both the metabolic fate and the chemical nature of the covalently bound residues is warranted (Metzler, 1999).

4.4.2. Trenbolone disposition in the target animal

TBA is rapidly metabolised to its free active form, alpha and beta TBOH. In cattle, the β-epimer is the major metabolites in muscle. The concentrations of α- and β-TBOH, free and conjugated have been measured in muscle, liver, kidney and fat of treated cattle at various times after implantation. Table 8 shows the residue values for these tissues at the time after implantation where the highest level of ß-TBOH was detected in muscle.

Table 8: Residue Levels (ng/kg) of α- and β-TBOH (free + conjugated) in tissues of treated cattle

Implant	Animal	Free/ conjugate	Muscle		Liver		Kidney		Fat	
			17α-	17β-	17α-	17β-	17α-	17β-	17α-	17β-
Torelor*	Steer	Free	272	9	323	226	67	76	293	62
		conjugate	43	10	772	1708	36	210	31	36
Finaplix *	Heifer	Free	645	102	440	286	445	155	1021	113
		conjugate	75	59	972	2920	167	309	31	60
Revalor*	Calves	Free	228[+]	105	908	1078	586	196		
		conjugate			366	754	207	221		

*30 days post implant; + free + conjugate; Data from JECFA, 1987

4.4.3. Pharmacokinetics and biotransformation of trenbolone in humans

The metabolism of trenbolone in humans has not been extensively studied. In one study, Spranger and Metzler (Spranger and Metzler, 1991) examined the disposition of 17-β-trenbolone in a single human subject administered 0.04 mg/kg body weight. Urine was collected in fractions for 72 hours after ingestion. The fraction of the first 3-h urine contained the highest concentration of radioactivity and was used for the analysis of metabolites. Of the urinary material, 54 % was present as glucuronides, which contained mostly 17α-trenbolone, 17β-trenbolone and trendione. At least five other polar metabolites, presumably hydroxylated products, were detected in smaller amounts. A total of 54% of the administered radioactivity was found in the urine after 26 hours, and 63% after 72 hours (Spranger and Metzler,

1991). Further analyses of the formation of polar metabolites of trenbolone is essential to the assessment of the risk of repeated dietary exposure of humans to this compound.

4.4.4. Assessment of exposure to trenbolone from consumption of hormone-treated beef

Since TBA does not occur naturally, by definition endogenous levels in humans should be zero. Thus, any residues detected in the meat of treated cattle represent excess exposure to individuals consuming the meat.

The ADI for trenbolone acetate recommended by the JECFA(1987) for humans was 0-0.1 µg/kg body weight based on a non-hormonal effect level of 2 µg TBA/kg in a study in pigs. In 1988 the Expert Committee on Food Additives (JECFA), using this same non-effect level established a temporary ADI of 0.01 µg TBA/kg body weight (0.7 µg/70 kg person), and recommended a temporary Acceptable Residue Level of 1.4 µg/kg bovine meat for β-TBOH on the basis of consumption of 500 g meat by a 70 kg person.

The FDA (*CFR 21, Part 556, Tolerances for residues of new animals drugs in food*) has set tolerance limits for TBA levels in uncooked tissues of cattle.

Tissues	TBA (µg/kg)
Muscle	50
Liver	100
Kidney	150
Fat	200

Based on these levels, consumption of 500g of meat/person/day (comprised of 300g muscle, 100g liver, 50g kidney and 50g fat) the acceptable daily consumption of TBA could reach 43 µg/person/day, an amount considerably greater than recommended by the JECFA. This value greatly exceeds the recommended ADI.

The toxicological issues of concern include endocrine, developmental, immunological, neurobiological, immunotoxic, genotoxic and carcinogenic effects. . Specific hazardous effects are detailed below.

4.4.5. Mutagenicity and genotoxicity

17β-Trenbolone (β-TBOH) is a synthetic androgen. 17α-trenbolone (α-TBOH) is a metabolite formed in cattle. Both, the parent compound and its metabolite (α-TBOH), have been extensively tested for their mutagenic/genotoxic potential. The results are summarized as follows (table 9).

Table 9: Mutagenicity testing of trenbolone and its metabolite

Mutagenicity/genotoxicity of α-TBOH		
Assay	Result	[ref]
Ames Assay ± s-9	Negative	(Richold, 1988)
In vitro cytogenetics ± s-9		
Human lymphocytes	Negative	(Richold, 1988)
In vivo bone marrow ± s-9	Negative	(Richold, 1988)
DNA repair syn. HeLa cells ± s-9	Negative	(Richold, 1988)
Mutation ± s-9		
Mouse L5178Y TK +/- cells	Negative	(Richold, 1988)
CHO cells HPRT	Negative	(Richold, 1988)
Micronucleus		
SHE cells – s-9	Positive	(Schiffmann et al., 1988)
C3H10T1/2 cells – s-9	Negative	(Schiffmann et al., 1988)
Cell transformation – s-9		
SHE cells	Positive	(Schiffmann et al., 1988)
C3H10T1/2 cells	Negative	(Schiffmann et al., 1988)

Mutagenicity/genotoxicity of β-TBOH		
Assay	Result	[ref]
Ames Assay TA 1535,1538, 98, 100 ± s-9	Negative	(Richold, 1988)
Ames Assay TA100 – s-9 but not – s-9	Positive	
In vitro cytogenetics ± s-9		
Human lymphocytes	Negative	(Richold, 1988)
CHO	Negative	(Richold, 1988)
In vivo chromosomal aberrations		
Rat bone marrow	Negative	(Richold, 1988)
Rat spermatogonial cells	Negative	(Richold, 1988)
DNA repair syn. HeLa cells ± s-9	Negative	(Richold, 1988)
Covalent binding		
Rat liver	Negative	(Richold, 1988)
Rat liver	Weak	(Lutz et al., 1988)
Calf thymus *in vitro* – s-9	Positive	(Lutz et al., 1988)
Salmonella DNA	Positive	(Lutz et al., 1988)
Mutation ± s-9		
Mouse L5178Y TK +/- cells	Equivocal	(Richold, 1988)
CHO cells HPRT	Negative	(Richold, 1988)
V-79 HPRT	Negative	(Richold, 1988)
Micronucleus		
Mouse *in vivo*	Negative	(Richold, 1988)
CHO cells ± s-9	Negative	(Richold, 1988)
SHE cells – s-9	Positive	(Schiffmann et al., 1988)
C3H10T1/2 cells – s-9	Negative	(Schiffmann et al., 1988)
Sister chromatid exchange ± s-9		
V79 cells	Negative	(Scheutwinkel et al., 1986)
SOS chromotest ± s-9	Negative	(Scheutwinkel et al., 1986)
REC assay	Negative	(Scheutwinkel et al., 1986)
Cell Transformation – s-9		
SHE cells	Positive	(Schiffmann et al., 1988)
C3H10T1/2 cells	Negative	(Schiffmann et al., 1988)

Both 17α- and 17β- TBOH gave the same results. As the 17α-metabolite is more weakly androgenic, it might be concluded that the genotoxic effects of TBOH are not related to their hormonal activity. The ability of 17β-TBOH to transform Syrian hamster embryo cells has to be noted (Lasne, et al., 1990), although another laboratory found negative results at all concentrations tested (Tsutsui, et al., 1995).

4.4.6. DNA adducts and DNA damage

Covalent binding of [^{3}H]17β-TBOH to DNA was observed after incubation with rat liver postmitochondrial supernatant *in vitro* and in rats *in vivo* after oral or i.p. administration (Lutz et al., 1988). Binding of TBOH to rat liver DNA was also observed by Barraud et al. (1984) and Petit et al. (1989). Formation of DNA adducts is also observed in rat hepatocytes cultured with 30 μM β-TBOH (Metzler, 1999).

4.4.7. Carcinogenicity

Feeding of high doses of Trenbolone acetate (TBA) to mice induced a significant amount of liver hyperplasia and tumors; in rats a slight increase in islet-cell tumors of the pancreas was observed (WHO Technical Report No. 696, 1983). A 2-year carcinogenesis bioassay in male and female rats and mice did not provide definitive results on the carcinogenicity of β-TBOH (mentioned in Schiffman et al., 1988).

In humans, no data are currently available to assess the carcinogenicity of trenbolone.

In conclusion, in consideration of the lack of in vitro short-term assays on mutagenicity and genotoxicity of other TBOH metabolites other than α-TBOH, and in consideration of the equivocal results of cell transformation assays and the in vivo studies, the available information is insuficient to complete a quantitative risk assessment. It has also to be noted that a considerable fraction of TBOH residues seems to be covalently bound to tissues.

4.4.8. Effect of trenbolone on growth and reproduction

Deleterious effects of trenbolone acetate exposure were reported in the reproduction of both male and female mammals of various species (JECFA, 1988). In the adult male, trenbolone acetate administered by ingestion, injections or implants induces a decrease in testis, seminal vesicle and prostate weights and alterations in spermatogenesis. In the adult female, such treatments induce virilization and alteration or suppression of ovarian cycles. In a study involving women volunteers given i.m. doses of 10 mg trenbolone acetate every-other-day during 14 days, disturbances of the menstruation cycle have been reported.

Some data in rodents indicate that administration of trenbolone acetate during the intrauterine or/and perinatal period alters the reproductive function in adults. In a multi-generation study, it has been shown that trenbolone acetate, administered to female rats at dietary concentrations of 3 and 18 ppm

between 2 weeks before mating and 3 weeks after birth of youngs exerts effects on reproductive performance which are more marked in F2 pups than in F1 pups of a comparable age. Indeed, female F1 pups from F1-treated parents show signs of virilization, a delay in the mean vaginal opening and the presence of occlusive strands in the vagina or incomplete vaginal opening. Male pups show a delay in the occurrence of testicular descent and a decrease in weights of seminal vesicles, prostate, testes and epididymis. In addition in F2 from both sexes, the adrenal weight is also decreased (JECFA, 1988). These data do not allow a realistic assessment of a dose response relationship.

4.4.9. Effects of trenbolone on the immune system

Investigations on the effects of trenbolone on the immune system are very limited.

A slight, but non statistically significant, immuno depression was seen in male calves given SC implants of trenbolone acetate (140 mg). A statistically significant change was observed when a combination of trenbolone acetate and oestradiol (20 mg) was used. No such change was seen with oestradiol alone. In female calves no such effects were observed (Gropp et al., 1975).

In conclusion, this information is insufficient to assess the possible impacts of low levels of trenbolone in meat and meat products on consumers.

4.5. Zeranol

Zeranol (α-zearalanol) is an oestrogenic derivative of the mycooestrogen zearalenone. This oestrogen depresses the endogenous gonadotropins, luteinizing hormone and follicle stimulating hormone. Zeranol binds to the oestrogen receptor in swine, rats and chickens with a binding affinity similar to that of DES, which is much greater than that of oestradiol (Fitzpatrick, et al., 1989). In rat liver, zeranol was shown to bind to the oestrogen receptor and to DNA in a manner similar to that of oestradiol (Mastri, et al., 1986). Dietary administration of castrate female Rhesus monkeys for two consecutive days provided a no-effect level of 1 mg/kg/day (Fuller, et al., 1982). The zeranol-containing implants and target animals are summarised in Table A2 of the Annex.

4.5.1. Pharmacokinetics and biotransformation of zeranol in animals

The half-life of zeranol plus its metabolites in the blood was 26 h in New Zealand rabbits and 18 h in Rhesus monkeys (Migdalof, et al., 1983). Glucuronide and sulfate conjugates were found in the urine. Both zearalenone and taleranol (isomeric β-zearalanol) have been found as metabolites of zeranol in cattle (Sharp and Dyer, 1972; Duchatel and Maghuin-Rogister, 1985; Jansen, et al., 1986; Kim, et al., 1986).

When zeranol was metabolized by uninduced or Arochlor-induced rat liver microsomes, five new metabolites, tentatively identified as monohydroxylated derivatives, and small amounts of taleranol and zearalenone were observed

(Metzler, 1999). Three of the five monohydroxylated derivatives of zeranol were also observed with bovine liver microsomes.

4.5.2. *Zeranol disposition in the target animal*

In a study of cattle implanted in the ear with 30 mg of tritium-labeled zeranol, the tissue residues peaked at 5-15 days and then slowly decreased (Tarr, et al., 1984). At 65 days, approximately 60% of the initial dose remained at the implant site. The maximum residue level occurred in the liver and never exceeded 10 µg/kg, whereas the residue level in muscle did not exceed 0.13 µg/kg.

In cows implanted with Ralgro (36 mg) and slaughtered 70 days later, the average values of zeranol determined by a radioimmunoassay were 0.127 µg/kg in muscle, 0.184 µg/kg in fat, 0.299 µg/kg in liver and 0.157 µg/kg in kidney (Dixon and Mallinson, 1986).

In steers implanted with Ralgro (36 mg) and slaughtered 70 days later, the levels of zeranol in liver, kidney, muscle and fat were 0.200, 0.126, 0.725 and 0.073 µg/kg (Dixon, et al., 1986).

In a study of cattle receiving 1 to 6 implants of Ralgro (36 mg/dose), separated by 65 days, and slaughtered 65 days after the last implant, the livers of calves receiving 4, 5 or 6 doses contained an average of 0.73, 1.52 and 1.10 µg zeranol/kg, respectively, (IMC, undated).

When steers were implanted with 36 mg of zeranol and slaughtered 67 days later, only liver and kidney had detectable residues of zeranol, measuring 0.349 and 0.076 µg/kg, respectively (O'Keefe, 1984).

A residue study was conducted in which zeranol was administered to cattle under "abusive conditions of use". Steers were implanted with 24-168 mg of zeranol and slaughtered on the fifth day after implanting. Additional steers were injected with zeranol (in DMSO/saline) twice daily for three consecutive days and slaughtered on the third day after the last injection. These steers received 552-4128 mg of zeranol. In the implanted steers, the residue of zeranol plus metabolites ranged from 0.18 to 0.25 µg/kg muscle and 1.0 to 2.9 µg/kg liver. In the injected steers, zeranol plus metabolites ranged from 0.17 to 0.72 µg/kg muscle and 20 to 130 µg/kg liver (Cross and Byers, 1987). A second analysis of these tissues by selective ion monitoring capillary gas chromatography/mass spectrometry resulted in very similar data, with addition of data on residues in kidney: less than 0.25 to 1.08 µg/kg in the implanted animals and 5.14 to 30.71 µg/kg in the injected steers (Chichila, et al., 1988).

Zeranol is mainly metabolised in animals to zeralenone and taleranol. Only a few studies were available and discussed by the FAO/WHO [2, 4] and it was summarized that the mean residue levels calculated as zeranol equivalents did not exceed 0.2 µg/kg in muscle, 10 µg/kg in liver, 2 µg/kg in kidney and 0.3 µg/kg in fat.

Table 10 a: Total residue of ^{3}H-zeranol (µg/kg): Eighteen beef cattle were implanted with 30 mg of ^{3}H-zeranol and two animals were slaughtered at various times after implantation.

Withholding period (days)	Liver	Kidney	Muscle	Fat	Bile
2	2.5	0.74	0.099	0.10	80
5	8.2	1.7	0.13	0.30	270
15	7.3	1.3	0.10	0.25	230
30	4.2	0.97	0.054	0.26	140
45	3.4	0.89	0.047	0.14	120
65	1.5	0.75	0.044	0.098	56

Table 10 b: Data obtained from a residue study with steers implanted with 24 to 168 mg of zeranol and slaughtered on the fifth day after implanting: Residue evels of zeranol and its metabolites by implant dosing in µg/kg:

Dose	Muscle			Liver	
	Zeranol	Zearalenone	Taleranol	Zeranol	Taleranol
24 mg	0.13	0.05	< 0.02	1.0	-
48 mg	0.21	0.10	< 0.02	Ne	-
72 mg	0.16	0.20	< 0.02	Ne	-
120 mg	0.16	0.09	< 0.02	Ne	-
168 mg	0.13	0.09	< 0.02	2.9	-

ne = not estimated

4.5.3. Pharmacokinetics and biotransformation of zeranol in humans

In humans, the half-life of zeranol plus its metabolites in the blood was 22 h (Migdalof, et al, 1983). Urinary excretion was predominant and included glucuronide and sulfate conjugates. A substantial portion of the dose was not accounted for (23%) and is assumed to be unknown metabolites, possibly including hydroxylated zeranol or ring-opened zearalenone (Metzler, 1999). The half-life of zeranol suggests that this compound plus its derivatives can accumulate in humans consuming zeranol-containing food on a regular basis.

4.5.4. Assessment of exposure to zeranol from consumption of hormone-treated beef

Zeranol is naturally occurring (as derivative of the mycotoxin zearalenone) and, as such, it is likely that there are "endogenous" levels in the meat of untreated cattle.

In a study using ovariectomized female cynomologus monkeys, a non-hormonal effect level was determined to be 0.05 mg/kg body weight for zeranol. Based on these data, JECFA established an ADI of 0 – 0.5 µg/kg body weight, which corresponds to a daily intake of zeranol of 35 µg for a 70

kg person consuming 500 g meat. This set the Acceptable Residue Level for zeranol at 10 µg/kg in bovine lever and 2 µg/kg in bovine muscle.

The FDA (*Ref.: CFR 21, Part 556, Tolerances for residues of new animal drugs in food*) has set tolerance limits for zeranol levels in uncooked tissues of cattle.

Tissues	Zeranol (µg/kg)
Muscle	150
Liver	300
Kidney	450
Fat	600

For consumption of 500g of meat/person/day (comprised of 300g muscle, 100g liver, 50g kidney and 50g fat) the acceptable daily consumption of zeranol could reach 128 ug/person/day. This value exceeds the ADI by almost 4 fold.

The toxicological issues of concern include endocrine, developmental, immunological, neurobiological, immunotoxic, genotoxic and carcinogenic effects. Specific hazardous effects are detailed below.

4.5.5. Mutagenicity and genotoxicity of zeranol

The mutagenicity/genotoxicity was investigated in only a few tests, as presented in the following table.

Table 11: Summary of the data on mutagenicity and genotoxicity of zeranol

Assay	Result	[ref]
Sister chromatid exchange ± s-9 V79 cells	Negative	(Scheutwinkel et al., 1986)
SOS chromotest ± s-9	Negative	(Scheutwinkel et al., 1986)
REC assay in *Bacillus subtilis*	Positive	(Scheutwinkel et al., 1986) (Ueno and Kubota, 1976)

These few assays with equivocal results are insufficient for an evaluation of the mutagenic/genotoxic properties of zeranol.

4.5.6. DNA adducts and DNA damage

Tritium-labeled zeranol was found to bind to rat liver DNA after treatment with a single dose of 60 µg/kg (Barraud et al., 1984).

DNA adducts of zearalenone (of which zeranol is a metabolite) have been observed in the kidney and liver of female mice treated with a single dose of zearalenone (2 mg/kg, i.p or orally) (Pfohl-Leskowicz et al., 1995). After

repeated doses of zearalenone, DNA adducts were recovered in mouse ovaries.

Complete inhibition of DNA synthesis was produced in human peripheral blood lymphocyte cultures at a concentration of 30 µg/mL (Cooray, 1984).

4.5.7. Carcinogenicity

A two-year oral carcinogenicity study with Fisher 344/N rats given zeranol in the diet at 25 or 50 ppm (1 or 2 mg/kg) showed no treatment-related neoplastic effects (*National Tox. Prog. Report no. 235, 1982*). With B6C3F1 mice fed zeranol in the diet at doses up to 100 ppm (about 17 mg/kg), no effects were observed with males, but oestrogen-related effects were seen in females in several tissues, as well as myelofibrosis in the bone marrow.

Dose-dependent induction of adenomas and carcinomas of the liver were found in zeranol-treated male and female Armenian hamsters, reaching 100% for adenomas and 75% for carcinomas at the highest dose (Coe et al., 1992). In comparison with DES, zeranol was much more carcinogenic for the liver than expected based on its relative oestrogenic activity.

Zeranol also induced a low incidence of renal tumors of Syrian hamsters (Li and Li, 1987).

No data are currently available on cancer risk for humans linked to meat with zeranol residues.

In conclusion, in consideration of the limited data on mutagenicity/gentoxicity and the clear evidence for an induction of liver adenomas and carcinomas in one animal species (Armenian hamsters) no assessment of the possible carcinogenicity of zeranol can be made.

4.5.8. Effects of zeranol and growth and reproduction

Deleterious effects of zeranol exposure have been reported in reproduction of both male and female mammals of various species (JECFA, 1988). In the adult male, a sustained increase in the plasma level of zeranol administered by ingestion, injections or implants induces a decrease in testis, seminal vesicle and prostate weights and alterations or arrest of spermatogenesis. In the adult female, such treatments induce alteration or suppression of ovarian cycles and endometrial hyperplasia. In rodents, a decrease in ovulation rate and litter size has been reported.

In a three generation study of rats receiving zeranol at levels up to 0.20 ppm throughout gestation, it has been concluded that fertility of the offspring is not affected (JECFA, 1988). However, male mice exposed in utero to zeranol (150 µg/kg of body weight injected on days 9 and 10 of gestation) show testicular abnormalities (regressive changes in the germinal epithelium and Sertoli cells, and immature morphology of Leydig cells) when testes are examined at 45 days of postnatal life (Perez-Martinez et al., 1997). No estimate of the dose-response relationship for these effects can be made.

4.5.9. *Effects of zeranol on the immune system*

No relevant data on the effects of zeranol on the immune system were found.

In conclusion, the available data do not allow a quantitative estimate of the risk arising from exposure to zeranol residues. In addition, further data are needed on the nature of the metabolites formed in bovines.

4.6. Melengestrol

Melengestrol acetate (17-acetoxy-6-methyl-16-methylenepregna-4,6-diene-3,20-dione, MGA), a progestogen, is a synthetic anabolic steroid used for improved efficiency in feed utilization, growth stimulation and estrus suppression of feedlot heifers (Federal Register, 1968 and 1969). It is also effective as an estrus synchronization agent for cattle (Zimbelman, et al., 1970). In intact heifers, treatment with MGA causes significant increases in pituitary luteinizing hormone (Fed. Regist., 1968). It appears that MGA blocks the cyclic surge in luteinizing hormone that produces ovulation, but allows the tonic release of luteinizing hormone that synergizes with follicle stimulating hormone for oestrogen production. The progestogen activity of MGA is approximately 30 times greater than that of progesterone, as measured in the rat (Duncan, et al., 1964). MGA had no androgenic activity and very little oestrogenic activity (Duncan, et al., 1964).

In humans, the onset of menses is delayed by a dose of 7.5 or 10 mg MGA/day in normal ovulating women, but not by a dose of 5 mg MGA/day (Duncan, et al., 1964). Based on these and other data from human studies, a minimal effective daily dose of 0.7 mg MGA and a no-effect daily dose of 0.4 mg MGA was derived for MGA in women (Lauderdale, et al., 1977). Using the maximum residue levels of MGA in beef muscle and fat (less than 10 ppb after 48h withdrawal), it was further calculated that daily consumption of 100 pounds of MGA-fed heifer meat would not exceed the no-effect dose.

4.6.1. *Pharmacokinetics and biotransformation of melengestrol in animals*

MGA metabolites formed in cattle have been detected, but not identified (Krzeminski, et al., 1981). Cattle fed radiolabeled MGA excreted MGA and its metabolites mostly in feces (87%), with 13% in urine. Approximately 15% of the MGA was excreted intact (Lauderdale, et al., 1977). In fat, 86% of the MGA was intact, but only 48% was not metabolized in muscle and 29% in liver and kidney (Krzeminski, et al., 1981).

In vitro metabolism of MGA by Arochlor-induced rat liver microsomes yielded seven monohydroxylated and five dihydroxylated metabolites separated by HPLC and identified by HPLC/MS (Metzler, 1999). The major metabolites produced by the rat liver microsomes were also observed with bovine liver microsomes.

4.6.2. Melengestrol disposition in the target animal

The highest concentration of MGA and its derivatives was found in the liver, although fat was found to be the actual target tissue for MGA. In studies of pregnant heifers fed MGA at 0.4 mg/head daily, MGA concentrations of 10-20 ppb were found in the fat, but the levels decreased to less than 10 ppb at 48 h after withdrawal (Lauderdale, et al., 1977).

4.6.3. Pharmacokinetics and biotransformation of melengestrol in humans

The half-life of orally administered MGA to women was estimated to be 3.5 days for doses of 3 to 5 mg (Cooper, et al., 1967). MGA is extensively metabolized in women to more than 20 compounds, with 74% excreted in urine and feces. Intact MGA, as well as its glucuronide and sulfate conjugates, were identified in urine. Two of the metabolites have been tentatively identified as the 2α-hydroxy and 6-hydroxymethyl derivatives (Cooper, et al., 1967).

4.6.4. Assessment of exposure to melengestrol from consumption of hormone-treated beef

Only limited data are available concerning MGA residues in treated cattle. It is an orally active progestogen that is mixed in animal feed to provide a daily consumprion level of 0.2 to 0.8 mg. MGA is reported to be located mainly in fat at concentrations $\leq$ 25 μg/kg. The FDA (CFR 21, Part 556, Tolerances for residues of new animal drugs in food) has set the tolerance level for MGA at 25 μg/kg in the fat tissue of treated cattle.

The toxicological issues of concern include endocrine, developmental, immunological, neurobiological, immunotoxic, genotoxic and carcinogenic effects. Specific hazardous effects are considered below.

4.6.5. Mutagenicity and genotoxicity of melengestrol

No information is available.

4.6.6. DNA adducts and DNA damage

No information is available.

4.6.7. Carcinogenicity

In female SHN mice, MGA (10 mg pellets implanted subcutaneously every two months) slightly increased the incidence of mammary tumors, but not the incidence of preneoplastic hyperplastic alveolar nodules (Nagasawa, et al., 1988).

In conclusion, in view of the lack of data on mutagenicity / carcinogenicity and on DNA interactions and in consideration of carcinogenicity studies conducted only in one animal species, these data are inadequate to assess the carcinogenetic potential of MGA.

4.6.8. Effect of melengestrol on growth and reproduction

Melengestrol acetate can block ovulation and the menstrual or estrous cycle in females from various species. In addition, it is capable of increasing the serum prolactin concentration of mice fed MGA at a daily rate of 0.2-0.8 mg (Lauderdale et al., 1977). Heifers implanted with MGA for improving growth performance show two- to three-fold increase in serum oestradiol concentrations (Henricks et al., 1997). This effect is likely due to the presence of large persistent follicles on ovaries occurring in response to the increase in LH pulsatility induced by MGA treatment (Anderson and Day, 1994; Imwalle et al., 1998). These data do not allow an estimate of the dose response relationship.

4.6.9. Effect of melengestrol on the immune systeme

Available data are very limited. The only study uncovered is an investigation of the effects of MGA on the ability of cows to remove *E. coli* that have been introduced into the uterus. The results of this study were inconclusive (Zimbelman RG. et al., 1970)

The information is insufficient to make a scientific judgement on whether MGA may cause effects on the immune system at levels which could occur in meat treated with MGA growth promoters.

In conclusion, Melengestrol is an orally active progestogen. The toxicological issues of concern include endocrine, developmental, immunological, neurobiological, immunotoxic, genotoxic and carcinogenic effects. As indicated above, the data available on the different issues does not allow a conclusive assessment either of the amounts of residues in edible tissues (in particular no quantitative data are available on the concentration of metabolites in edible tissues) or the nature of toxicological effects. This implies that the available information is insufficient for a quantitative estimate of the risk to the consumer of meat and meat products derived from treated animals.

5. EXECUTIVE SUMMARY

In humans and animals, sex hormones including oestradiol, progesterone and testosterone are involved directly and indirectly in growth and development. Due to the obvious ability to improve weight gain and feed efficiency in meat producing animals, natural hormones and/or the synthetic surrogates (zeranol, melengestrol, trenbolone) have been used in agricultural practice for several decades. This extensive use in certain parts of the world has intensified the discussion on possible adverse effects in human health. Concerns are based on the accumulating evidence on the fragility of the endocrine equilibrium in all stages of life as well as the potential genotoxicity of these compounds and their metabolites. Exogenous hormone exposure may disrupt this delicate equilibrium as is evidenced by the pronounced effects of oestrogens and testosterone on functional imprinting. Thus even exposure to residual amounts of hormonally active compounds as present in meat and meat products needs to be evaluated in terms of potentially adverse effects to public health.

Previous risk assessments were based on the following assumptions:

- A formal risk assessment of the natural hormones oestradiol, progesterone and testosterone was considered to be unnecessary as these hormones are produced endogenously in human beings and considerable inter-individual differences in blood levels of these hormones had been observed. However, very recently Acceptable Daily Intake (ADI) values for these substances have been proposed for the first time (JECFA, 1999)

- In view of the average amount of endogenous hormones produced daily in men and women, the exposure arising from the consumption of meat from treated animals was considered to be negligible.

- The epidemiological evidence on the association between the exposure to 17β-oestradiol, progesterone, and testosterone, and certain forms of cancer, in particular breast cancer, prostate and testis cancer was considered to originate from the hormonal activity of these compounds. It was assumed that these pathologies can occur only at intake levels higher than those required for physiological response (see hormone replacement-therapy).

Based on these assumptions made for the natural hormones, the possible effects of synthetic hormones were described as follows:

- For the synthetic hormones, an ADI can been identified on the basis of a no-hormonal-effect-level and subsequently ADIs have been established for zeranol and trenbolone.

- Toxicological effects in animals such as liver hyperplasia and tumours in mice following feeding of trenbolone acetate, and pituitary gland tumours in mice following feeding of zeranol, were considered to be due to the compound's hormonal activity.

These assumptions disregard a number of important factors which have been discussed in detail in the report presented here. Particular reference is made to:

- The growing scientific understanding of the critical role of hormonal imprinting in early stages of life determining a number of subsequent developmental outcomes.

- The increasing awareness of the delicate endocrine equilibrium at different stages of life, making it necessary to distinguish between individual age groups and sexes in the assessment of hormone exposure.

- The reliability of estimates used in previous risk assessments for the amounts of each natural occurring hormone produced in men and women at different ages and especially in prepubertal children, which has been questioned in recent publications.

- The fact, that adverse effects of hormones may be mediated not only by mechanisms involving hormone receptors, but may be related directly to metabolite formation, as recently published data indicate the existence of metabolism-mediated genotoxic effects of certain hormones, particularly oestradiol.

- The specific distribution and observed increase of hormone associated diseases, such as breast cancer and prostate cancer in many countries of the world initiating the question on the contribution of residues of growth promoting agents in meat to the total load of hormones and hormone-like substances in the human diet. In this evaluation of the possible exposure to residues, the accidental and/or deliberate misuse (in bovines and other species) of the compounds under consideration also needs to be considered, together with the effects of combinations of different compounds in the same implant.

Finally, it should be emphasised that an overall risk assessment on the deliberate use of hormones in animal production should also address the potential environmental impact taking into account the effects of individual hormones, combinations of different hormones as well as the combination with other industrial substances exerting hormonal effects. Secondary risks have to be considered as treatment with hormones changes the distribution and excretion of xenobiotic substances in bovines. This implies that meat and meat products derived from treated animals may contain higher levels of undesirable residues than those from untreated animals. These aspects have not been addressed in detail in the report, which focuses on human exposure to hormone residues via the consumption of meat and meat products from treated animals.

Conventionally, risk assessment is structured to address independently the intrinsic properties of the compound under consideration (hazard identification), the evaluation of the nature of effects in terms of a dose-response relationship (hazard characterisation), the estimate of the dose/concentration of a compound in the daily diet (exposure assessment) resulting in the assessment of the incidence and severity of potential adverse effects.

Taking into account the data provided in the report the following conclusions can be drawn:

Intrinsic properties: Steroid hormones fulfil an important role at different stages of mammalian development comprising prenatal development, growth, reproduction and sexual and social behaviour. (Section 2.2). The importance of individual hormones varies between sexes and age and a disruption of the endocrine equilibrium may result in multiple biological effects (Section 2.2 - 2.4). There is an association between steroid hormones and certain cancers and an indication that meat consumption is possibly associated with increased risks of breast cancer and prostate cancer (Section 2.3.) The fact that these

compounds are natural does not automatically make them fully safe. The intrinsic properties (hazards) of steroid hormones indicate the essentiality to subject any additional amount of hormonal active substances, which might be present in the daily diet (Section 3), to a careful risk assessment.

Endogenous hormone levels at different stages of life- prepubertal children: Each phase of life, from embryogenesis onwards, is characterised by a well defined sex-dependent hormonal balance. The biological/endocrine effects of hormones are mediated by specific hormone receptors during each of these phases. Receptor-binding is influenced by sequestration involving protein-binding, which includes a specific binding to albumin, binding to sex hormone binding globuline (SHBG) and to androgen binding protein (ABP) (see 2.2.1.7.). The levels of these binding proteins vary during the human life cycle and regulate both, the bioavailability of steroids to target tissues (it is assumed that only the free steroids are ligands for hormone receptors) and the metabolic transformation and rate of excretion. These observations suggest that prepubertal children constitute a high risk population for exogenous sex steroids since the endocrine equilibrium at that age is based on the low production rate (PR) and the considerable binding to proteins (circulating SHBG), compensating for a low metabolic clearance rate (MCR) (see 2.2.2.4. and 3.2.). All previous risk assessments have been based on estimates of PR and MCR values which have been deduced from measurements of hormone levels in plasma by direct immuno-assays. Comparing these data with recent findings obtained with much more sensitive and specific bioassays which have been recently developed for oestradiol, it has to be concluded that both the actual hormone production rate as well as the clearance rate has been overestimated (up to 100 fold) in the past (see 3.2.). Comparable assays also need to be developed for progesterone and testosterone. Thus, the evident increase in the amount of hormones ingested with the daily diet must be re-evaluated in the light of uncertainties relating to endogenous hormone production and metabolic clearance capacity.

Hormone levels in animals and human exposure: Endogenous hormones and their metabolites are present in measurable amounts in various animal tissues including meat (Section 3.1. and 4.1.5., 4.2.4., 4.3.4., 4.4.4., 4.5.4., 4.6.4.). The concentrations found, reflect different stages of the animals life cycle as exemplified by the high levels of testosterone in tissues of male cattle (bulls) or oestrogen and progesterone levels in tissues of young females (heifers) at a late stage of pregnancy (240 days gestation). Heifers are slaughtered and enter the food chain only exceptionally. It is therefore questionable whether levels in such animals should be included in estimates of the upper range of hormonal levels in meat and edible tissues.

In contrast, for pharmaceutical products containing one or more of the three natural hormones, it is estimated that the use of these growth promoting agents will result in an additional excess daily intake of oestrogens in the range of 1 to 84 ng/person (17 β oestradiol + estrone), of progesterone of 64 to 467 ng/person, and of testosterone of 5 to 189 ng/person. As the levels of the synthetic compounds used as growth promoting agents are virtually zero in untreated animals, any residual amount in edible tissues must be regarded as excess exposure (see section 3.1). No validated data exists on the bioavailability of hormones and their metabolites after oral ingestion with meat.

However, it needs to be emphasized that, as part of the risk management, adequate residue control programs for each of the six hormones are essential to monitor legal and illegal use.

Susceptible populations: It is evident that when identifying the most sensitive sub-group of the human population, attention must be given to those which have the lowest physiological hormone levels. Prepubertal children are of the greatest concern. For women, some critical phases have been described as "windows of vulnerability" corresponding in particular to the time period from adrenarch to first ovulatory cycles and possibly up to first pregnancy, as well as later on in menopause (see 2.3.2.4.). Correspondingly for males, the most critical phase occurs in prepubertal boys as mentioned above. Finally, emerging concerns that already *in utero* exposure to unbalanced hormone levels might influence functional imprinting, determining a number of subsequent developmental outcomes (see 2.2) need to be considered.

In conclusion, it can be stated that the demonstrated excess exposure to hormonal active substances (referring to both the natural hormones as well as their synthetic equivalents) requires a detailed evaluation of their potential hormonal and toxicological effects. In consideration of the obvious differences in sensitivity of sex and age groups to hormones no threshold level can be established.

Genotoxicity of the individual hormones and their metabolites: The recently described evidence for biotransformation mediated genotoxicity of oestrogens (see 2.5.) deserves attention as no threshold can be defined for genotoxic substances. Quinone metabolites of catechol oestrogens induced DNA adduct formation in a number of biological systems. It has been suggested that if catechol-oestrogen metabolites play a role in the induction of cancer, following tumour initiation by genotoxic events, hormone receptor-mediated effects would play a major role in the promotion and progression phase of tumour development. These recent mechanism based findings are in agreement with the epidemiological findings. No qualified data are available covering all aspects of the biotransformation of progesterone and testosterone. However, it should be noted that testosterone can be aromatized to oestradiol and thus may contribute to the formation of DNA-reactive metabolites (see 4.2.6.). Progesterone might induce single strand breaks and DNA adducts, but detailed information has not been published yet (see 4.3.6.). Trenbolone and its metabolites have been extensively tested for their mutagenic/genotoxic potential (see 4.4.5.). It can be concluded from the majority of these experiments that 17 β-TBOH and its metabolite 17 α-TBOH lack significant mutagenic/genotoxic activity. However, the finding that at least 17β-TBOH binds covalently to DNA resulting in the formation DNA adducts (in rat hepatocytes) do not allow a conclusive evaluation (see 4.4.6). DNA-binding has been described also for zeranol (see 4.5.6). In addition, a dose dependent induction of adenomas and carcinomas of the liver have been found in zeranol treated male and female Armenian hamsters. In these studies, zeranol was much more carcinogenic for the liver than expected based on its oestrogenic activity (see 4.5.7.). For melengestrol no data has been published on its potential mutagenicity and genotoxicity (see 4.6.5.).

In summary

Taking into account both the hormonal and non-hormonal toxicological effects as mentioned above, and described in more detail in the report, it has to be concluded that the issues of concern include neurobiological, developmental, reproductive and immunological effects, as well as immunotoxicity, genotoxicity and carcinogenicity. In consideration of the recent concerns relating to the lack of understanding of critical developmental periods in human life as well as the uncertainties in the estimates of endogenous hormone production rates and metabolic clearance capacity, particularly in

prepubertal children, no threshold level and therefore no ADI can be established for any of the 6 hormones.

Major conclusions

- 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.

6. ANSWERS TO THE QUESTIONS IN THE MANDATE.

The deadline set by the WTO Arbitrator for the EC to bring its measure into conformity with SPS agreements is 13 May 1999. The SCVPH is, therefore, requested to deliver an opinion before the end of April 1999, responding as far as possible to the question stated below. If this opinion is not conclusive, the Commission will request the SCVPH to subsequently complete its opinion as soon as possible in the light of the studies initiated by the Commission

QUESTIONS:

In light of the mandate and taking into account:

- the use of the six hormones, individually or in combinations, for growth promotion purposes in cattle,

- the current scientific information with respect to health-related biological actions such as genotoxicity, carcinogenicity, embryotoxicity (including teratogenicity), endocrine and reproductive effects of the hormones in question and their metabolites,

- possible long-term exposure and potential synergistic effects,

- the current techniques and standards for the evaluation of the safety of substances leaving residues in food,

- possible effects on the most vulnerable parts of the population, in particular young children.

the following detailed questions should be addressed:

Question 1

(a)   What are the potential adverse effects on human health from residues in bovine meat and meat products resulting from the use of the six hormones in question for growth promotion in cattle?

In answering this question, the SCVPH is asked to include an assessment in terms of carcinogenicity, genotoxicity, embryotoxicity (including teratogenicity) and endocrine, reproductive or other effects, taking into account long-term and synergistic effects from exposure to residues of these hormones and their metabolites in meat and meat products.

(b)   To what extent is the currently available information (clinical and epidemiological evidence included) sufficient to allow the SCVPH to complete its assessment, in particular for melengestrol acetate (MGA)?

Answer

ad 1 (a): The potential adverse effects on human health from residues in bovine meat and meat products include endocrine, developmental and neurobiological, immunological as

well as carcinogenic, genotoxic and immunotoxicological effects as described in the report and addressed in the executive summary.

These effects can be attributed to either the parent compound or the metabolites. Residue analysis has focussed in the past on the quantification of the amounts of residues of the parent compounds and those metabolites exerting hormonal activity. Recent data indicate that other metabolites occur additionally which have genotoxic activity. For example, 17-ß oestradiol can be metabolized to 2-OH, 4-OH and 16α-OH oestrogens. Particular the 2-OH, and 4-OH oestrogens have been found to be directly or indirectly genotoxic. This implies that 17-ß oestradiol may act as tumor initiator as well as tumor promoter. These findings are in agreement with epidemiological data and resulted in the classification of 17-ß oestradiol as human carcinogen (Group 1 according to the IARC classification). This implies that any excess exposure towards 17-ß oestradiol and its metabolites resulting from the consumption of meat and meat products presents a potential risk to public health in particular to those groups of the population which have been identified as particularly sensitive such as prepubertal children.

It should be noted that for these genotoxic metabolites in bovine tissues, no threshold level can be established. In addition, no threshold level can be established for any of the hormonally active compounds and metabolites which might exert endocrinal, developmental and neurobiological, immunological or immunotoxicological effects.

_ad 1 (b):_ With the exception of 17-ß oestradiol, the currently available information for testosterone, progesterone and the synthetic hormones zeranol, trenbolone and particularly MGA has been considered inadequate to complete an assessment. This conclusion is based upon:

- incomplete data on the biotranformation pathways of these compounds and the possible biological activity of the metabolites formed in bovine tissues as, for example, testosterone might be aromatized to oestradiol.

- lack of data on the potential genotoxicity of these metabolites in consideration of the current state of the art for genotoxicity testing as indicated in the answer to question 2 (a).

- insufficient data on immunological and immunotoxical effects.

Based on experimental and epidemiological data, testosterone and progesterone have been classified by IARC as Group 2 substances - probable/possible carcinogens in humans. No epidemiological data are available for zeranol, trenbolone and MGA (melengesterol acetate) although residues of hormonally active compounds in (poultry) meat have been shown to exert an oestrogenic response in prepubertal children in certain countries.

Thus, no final conclusions can be drawn with respect to the safety of at least five out of the six substances under consideration, until the above described issues have been clarified. For oestradiol genotoxicity has already been demonstrated explicitly.

Question 2

The main criticism of the WTO Panel and Appellate Body reports of the scientific evidence presented by the EC was that this evidence "constitute general studies which do indeed show the existence of a general risk of cancer, but they do not focus on and do not address the particular kind of risk here at stake - carcinogenic or genotoxic potential of the residues of those hormones found in meat derived from cattle to which the hormones had been administered for growth promotion purposes".

In relation to the reply to question 1, please specify:

(a)   which are the appropriate systems (in vivo and/or in vitro) to test for potential carcinogenic or genotoxic effects?

(b)   if it has been scientifically established that these hormones or their metabolites are carcinogenic or genotoxic in general in vivo and/or in vitro studies, is it still necessary to establish whether the residues in bovine meat are carcinogenic or genotoxic?

(c)   whether the risk assessments of the five hormones by JECFA in 1987/1989 and of MGA by the US and Canadian authorities were based on evidence focusing specifically on the lack of carcinogenic, genotoxic, or endocrine effects of the residues of these hormones in bovine meat and meat products?

Answer

ad 2a: Appropriate systems to test for potential carcinogenic and genotoxic effects are all cells, tissues or tissue cultures or any other model resembling bovine and human metabolism (pronounced species differences in biotransformation are well documented in the literature). This implies that a battery of tests is needed, first to establish bovine biotransformation of the compounds under consideration (usually bovine microsomes or bovine hepatocytes and, with respect to oestrogens, bovine cells/tissues expressing CYP1B1) and, secondly, in the assessment of human bioprocessing of the bovine metabolites, systems resembling human biotransformation reactions (generally microsomes, human cell lines or genetically engineered cells) carrying at the same time a reporter gene to assess genotoxic events. However, it should be noted that standard tests of genotoxicity are not appropriate as the lack of a response in these assays may be related to the use of liver extracts for enzymatic activation, since oestrogen 4-hydroxylase activity is low in this organ. Additionally, the reactive intermediates may not be able to travel into target cells, and the types of DNA damage assayed may not be sufficient. The genotoxicity of oestrogen metabolites may best be assayed in mammalian cells with appropriate biotransformation enzymes (for example the human breast epithelial MCF-7 cell line), and the DNA damage analysis should include not only point mutations and frameshift mutations, but also chromosomal damage.

ad 2b: If there is convincing evidence, as in the case of 17-ß oestradiol that metabolites of a hormone are genotoxic the answer is no. In the present state of the art, compounds that are used intentionally in food production and food processing should not yield genotoxic residues because no threshold can be established for this toxicological effect.

In addition, no threshold level can be established for other effects as cited in the answer to question 1a.

ad 2c: Since risk assessment was based at that time on hormonal effects only and no excess exposure was envisaged, the genotoxic and carcinogenic potential of residues in meat and meat products was not considered.

However, in the 1999 report of JECFA, more recent work on biotransformation mediated genotoxicity was cited.

As detailed in the report presented here evidence is provided that for hormones, no threshold can be defined either for the endocrine, developmental, immunological and neurobiological effects or for their potential immunotoxicity and carcinogenicity. This statement is also made in the light of the emerging concerns of the effects of hormones at different stages of life and the accumulating epidemiological findings on tumor incidence as recently summarized by IARC.

Question 3

The basis for the risk assessments of the five hormones by JECFA in 1987/1989 and of MGA by the competent US and Canadian authorities was the assumption that their carcinogenic potential is exclusively related to their hormonal activity. Is this assumption still valid in light of the currently available scientific information, e.g. on carcinogenicity, genotoxicity, endocrine and synergistic effects from long-term exposure to residues of one or more of these hormones?

Answer

In acknowledging the recent findings on the metabolism based genotoxicity of 17-ß oestradiol (see chapter 2.5 of the report) it has to be stated that the assumption that the carcinogenic potential is exclusively related to the hormonal activity is no longer valid.

In addition it is worthwhile mentioning recent improvements in analytical techniques applied in the measurement of physiological hormone levels. The introduction of more sensitive and specific bioassays/oestrogen receptor assays (as outlined in detail in the text of the report) indicated that a critical reappraisal of the endogenous hormone levels in certain segments of the human population, such as prepubertal boys and girls is required.

Question 4

(a)   What are the potential adverse effects if the approved conditions of use stated in the label instruction are not respected, for instance due to other routes of administration, higher and more frequent doses, different or unauthorised combinations or non-respect of prescribed withdrawal periods?

(b)   Are there particular risks associated with the practical administration of these substances?

(c)   Are adequate analytical methods available to control that the approved conditions of use for the six hormones and, in particular, the three natural ones have been adhered to?

Answer

ad 4a: Unauthorised and inappropriate use, higher doses and more frequent applications will obviously result in higher residue concentrations in tissues of treated animals comprising bovines and non-bovine species. Concerns have been expressed for the potential risk of extremely high residue levels if implants are misplaced, which includes the possibility that the total content of an implant will be present in a portion of minced meat or in a batch of meat products.

These extreme concentrations might exert acute hormonal effects and increase the likelihood that genotoxic metabolites are formed.

ad 4b: No direct risk for any person correctly handling the implants in daily agricultural practice can be envisaged. In the case of MGA, which is administered as a feed additive, data on the pharmaceutical quality of individual products should be reviewed, to assess risks arising from involuntary inhalation of dust particles containing the active ingredient. No information in this respect has been published to the knowledge of the SCVPH.

Appropriate training in the handling and the administration of these products is essential.

ad 4c: Sensitive analytical methods are available for all 6 compounds. However, in case of the natural hormones these methods can not discriminate between the endogenous and the administered hormones. Furthermore, appropriate methods to analyze the potentially toxic metabolites are lacking at present.

It is recommended that in the establishment of advanced and validated analytical techniques, the determination of species-specific bovine metabolites of the 6 hormones is adequately addressed. The patterns of the metabolites might allow discrimination between endogenous hormone levels and hormone levels acquired as a consequence of treatment. It must be emphasized that adequate residue control programs for each of the six hormones are essential to monitor legal and illegal use.

Figure 1
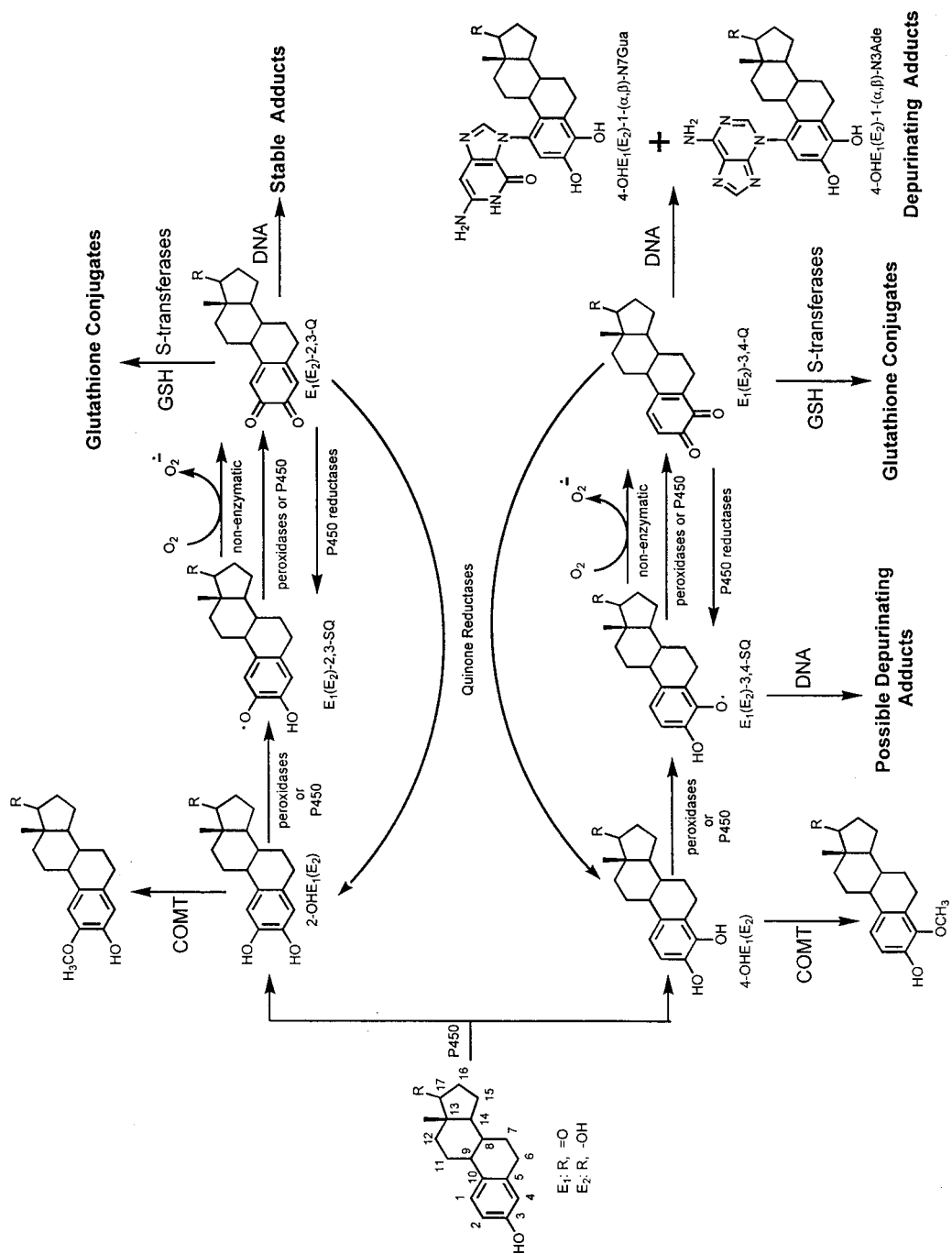
7. ANNEX

Table A1: Composition of Implants Containing Endogenous Steroids that are used for Growth Promotion (mg/implant)

Product	E2	E2-b	T	T-P	P	Animal
Compudose	24/45					Cattle
Synovex S		20			200	Steers
Synovex H		20		200		Heifers
Synovex C		10		100		Calves
Steeroid	20				200	Steers
Heiferoid	20			200		Heifers
Implix BM	20				200	Steers
Implix BF	20		200			Heifers

Table A2: Composition of Implants Containing Synthetic Steroids and a mycooestrogen that are used for Growth Promotion (mg/implant)

Product	E2	TBA	Zeranol	MGA	Animal
Torelor	40	200			Steers
Revalor lactose	20	140			Calves
Revalor G	8	40			Steers
Revalor S	24	120			Steers
Revalor H	14	140			heifers
Finaplix-S		140			Steers
Finaplix		300			Steers
Ralgro			36		Cattle
Forplix		140	36		
Premix[+]				0.25-0.5 mg/day	

Use of Torelor and concomitant or sequential use of Implix/Revalor is not approved

E$_2$= oestradiol, T = Testosterone, T-p = Testosterone propionate, P = Progesterone

TBA = Trenbolone Acetate

Tables from Report of the Joint FAO/WHO Expert Committee on Food Additives (JECFA) Fifty-second Meeting, February 1999; Thirty-second Meeting, 1987;

+ Supplied as a powder to be mixed into the feed.

Table A3: Theoretical Maximum Daily Intake (ng/person/day) (From JECFA, 1999)

Product	Animals	Footnote #	Treatment*	Excess E2 + E1	Excess P	Excess T
Synovex-S (E2-b + P)	Steers	1	15	6.8	64	
Synovex-H (E2-b + T-p)	Heifers	1	15	16		53
Synovex-C (E2-b + T)/ Synovex-H	Calves (Female)		119	3.3	467	
Synovex-C/ (E2-b + T) Synovex-S (E2-b + P)	Calves (Castrated males)	1	Implanted on days 0, 118, 240, Slaughtered on day 301	13	119	
None	Pregnant heifers	Data included for comparison	240 days pregnant	465 (E2 + E1)		
Synovex-H (E2-b + T-p)	Pregnant Heifers	2	240 days pregnant, 61 days implanted	465-39 = 426		377
Steer-oid (E2 + P)	Steers		15	4	76	
Heifer-oid (E2 + T-p)	Heifers	3	15	2		5
Compudose (E2)	Steers	4	70-180	4.2		
Compudose	Heifers	4	84	1.1		
Compudose	Bull calves	4	-	15		
	Bulls	4	-	3.8		
	ZEBU steer	4	-	-1.9		
FINAPLIX (TBA)	Heifers	5	30	34.2		
TORELOR (TBA + E2)	Steers	5	Implanted on days 0, 60; slaughtered on day 90	74.2		
Revalor (TBA + E2)	Heifers	6	30	5.6		
Revalor	Steers	6	15	1.4		
Revalor	Calves (male)	7	80	55.3		
Revalor + Implix (E2 + P)	Calves (male)	7	100**	82.7	174	
Revalor	Calves (female)	7	80	52.4		
Revalor + Implix BF (E2 + T)	Calves (female)	7	15[+]	83.9		189

*   Time of slaughter (days) after implantation; ** Control slaughtered on day 80; [+]Controls slaughtered on day 30.

Legend to table A3:

(1)   Calculations based on free hormone concentrations in muscle, liver, kidney, and fat. The conjugated hormones were not determined. In a separate experiment using ^{14}C-labeled hormones the fractions of total hormone residues present as conjugates was determined. These results indicated that the estimates of the Theoretical Maximum Daily Intakes (TMDI) of oestrogens for consumption of tissues from steers and heifers implanted with Synovexfi S/H should be multiplied by a factor of two (2).

(2)   Calculations based on determinations of the free hormones in muscle, liver, kidney and fat; the fractions of conjugated hormones were not determined.

(3)   Calculations based on determinations of free hormones in muscle and fat and thus likely underestimate the "true" TMDIs.

(4)   The method of analysis included deconjugation and thus the TMDIs likely reflect the total parent compound available.

(5)   The free and conjugated fractions of E2 were determined in all tissues while free estrone was determined only in liver and fat.

(6)   The method used likely did not include deconjugation.

(7)   No methods were provided. The values for E2 are based on concentrations found only in the liver.

Table A4 Incidence of breast cancer in women. Geographic variations (Parkin *et al*, 1997)

Region	Registry	Incidence rate*	Region	Registry	Incidence rate*
Africa	Algeria, Setif	9.5	Asia	China, Qidong	11.2
	Mali, Bamako	10.2		India, Barshi, Paranda and Bhum	8.7
	Uganda, Kyadondo	20.7		Japan, Osaka	24.3
				Korea, Kangwha	7.1
Central and South America	Argentina, Concordia	60.2		Viet-Nam, Hanoi	18.2
	Ecuador, Quito	26.8			
	Peru, Lima	32.3	Europe	Austria, Tyrol	64.9
				Czech Republic	45.1
North America	Canada, British Columbia	84.3		Denmark	73.3
	Canada, Ontario	78.1		France, Doubs	67.3
	USA, SEER, whites	90.7		France, Isère	85.9
	USA ,SEER, blacks	79.3		Italy, Ragusa	44.1
	USA, Los Angeles, non-Hispanics, whites	103.7		Italy, Varese	73.5
	USA, Los Angeles, Hispanics, whites	57.4		Netherlands	79.6
	USA, Los Angeles, blacks	80.9		Spain, Basque Country	45.8
	USA, Los Angeles, Chinese	36.8		Sweden	72.9
	USA, Los Angeles, Philippinos	69.3		Switzerland, Geneva	77.8
	USA, Los Angeles, Koreans	21.4		United Kingdom, Oxford	80.9
	USA, Los Angeles, Japanese	63.0			
			Oceania	Australia, Victoria	66.7
				New Zealand, non Maoris	77.2
				New Zealand, Maoris	77.1

* expressed in number of new cases per 100 000 women per year, after standardisation on age structure of the world population.

Table A5 Incidence of prostate cancer. Geographic variations (Parkin *et al*, 1997)

Region	Registry	Incidence rate*	Region	Registry	Incidence rate*
Africa	Algeria, Setif	1.8	Europe	Austria, Tyrol	51.6
	Mali, Bamako	5.4			24.1
	Uganda, Kyadondo	27.7		Czech Republic	31.0
				Denmark	44.0
Central and South America	Argentina, Concordia	16.2		France, Doubs	48.0
	Ecuador, Quito	22.4		France, Isère	12.0
	Peru, Lima	19.4		Italy, Ragusa	28.2
				Italy, Varese	39.6
North America	Canada, British Columbia	84.9		Netherlands	21.0
	Canada, Ontario	63.0		Spain, Basque Country	55.3
	USA, SEER, whites	100.8		Sweden	49.0
	USA, SEER, blacks	137.0		Switzerland, Geneva	32.5
	USA, Los Angeles, non Hispanics, whites	91.9		United Kingdom, Oxford	
	USA, Los Angeles, Hispanics, whites	60.4			47.6
	USA, Los Angeles, blacks	130.6	Oceania	Australia, Victoria	37.8
	USA, Los Angeles, Chinese	20.2		New Zealand, non Maoris	44.4
	USA, Los Angeles, Philippinos	46.1		New Zealand, Maoris	
	USA, Los Angeles, Koreans	10.2			
	USA, Los Angeles, Japanese	47.2			
Asia	China, Qidong	0.5			
	India, Barshi, Paranda et Bhum	1.4			
	Japan, Osaka	6.8			
	Korea, Kangwha	0.9			
	Viet-Nam, Hanoi	1.2			

* expressed in number of new cases per 100 000 men per year, after standardisation on the age structure of the world populati

Table A6. Incidence of testis cancer. Geographic variations (Parkin et al., 1997)

Region	Registry	Incidence Rate*	Region	Registry	Incidence Rate*
Africa	Algeria, Setif	0,24	Asia	China, Qidong (1988-1992)	0,24
	Mali, Bamako	0,42		India, Barshi, Paranda and Bhum (1988-1992)	0,33
	Uganda, Kyadondo	0,74		Japan, Osaka (1988-1992)	1,40
				Korea, Kangwha (1986-1992)	0,31
Central and South America	Argentina, Concordia	2,87		Viet Nam, Hanoi (1991-1993)	0,73
	Ecuador, Quito	3,48	Europe	Austria, Tyrol (1988-1992)	6,29
	Peru, Lima	2,88		Czech Republic (1988-1992)	5,20
North America	Canada, British Columbia	4,20		Denmark (1988-1992)	9,18
	Canada, Ontario	4,06		France, Doubs (1988-1992)	5,40
	US, Los Angeles: Non-Hispanic White	5,77		France, Isere (1988-1992)	3,68
	US, Los Angeles: Hispanic White	3,01		Italy, Ragusa (1988-1992)	0,95
	US, Los Angeles: Black	1,11		Italy, Varese (1988-1992)	3,84

US, Los Angeles: Chinese	1,00	The Netherlands (1989-1992)	3,98
US, Los Angeles: Filipino	1,12	Spain, Basque Country (1988-1991)	2,13
US, Los Angeles: Korean	0,82	Sweden (1988-1992)	4,75
US, Los Angeles: Japanese	0,87	Switzerland, Geneva (1988-1992)	7,42
US, SEER: White	5,36	UK, Oxford (1988-1992)	5,73
US, SEER: Black	0,72	Oceania	
		Australia, Victoria (1988-1992)	4,48
		New Zealand: Non-Maori (1988-1992)	5,63
		New Zealand: Maori (1988-1992)	7,07

* expressed in number of cases per 100 000 men per year, after standardisation on the age structure of the world population

Table A7 Incidence of colon cancer in men. Geographic variations (Parkin et al., 1997)

Region	Registry	Incidence Rate*	Region	Registry	Incidence Rate*
Africa	Algeria, Setif	0,45	Asia	China, Qidong (1988-1992)	2,10
	Mali, Bamako	3,12		India, Barshi, Paranda and Bhum (1988-1992)	0,69
	Uganda, Kyadondo	3,15		Japan, Osaka (1988-1992)	20,73
				Korea, Kangwha (1986-1992)	4,47
Central and South America	Argentina, Concordia	17,22		Viet Nam, Hanoi (1991-1993)	5,22
	Ecuador, Quito	3,89	Europe	Austria, Tyrol (1988-1992)	20,52
	Peru, Lima	5,57		Czech Republic (1988-1992)	24,01
North America	Canada, British Columbia	20,69		Denmark (1988-1992)	20,62
	Canada, Ontario	30,10		France, Doubs (1988-1992)	19,47
	US, Los Angeles: Non-Hispanic White	28,89		France, Isere (1988-1992)	22,26
	US, Los Angeles: Hispanic White	17,11		Italy, Ragusa (1988-1992)	9,83
	US, Los Angeles: Black	34,79		Italy, Varese (1988-1992)	27,43
	US, Los Angeles: Chinese	18,28		The Netherlands (1989-1992)	21,89

US, Los Angeles: Filipino	14,43	Spain, Basque Country (1988-1991)	17,09
US, Los Angeles: Korean	14,12	Sweden (1988-1992)	17,72
US, Los Angeles: Japanese	26,73	Switzerland, Geneva (1988-1992)	25,20
US, SEER: White	28,13	UK, Oxford (1988-1992)	20,62
US, SEER: Black	33,57		
		Oceania	
		Australia, Victoria (1988-1992)	27,47
		New Zealand: Non-Maori (1988-1992)	31,16
		New Zealand: Maori (1988-1992)	21,46

* expressed in number of cases per 100 000 men per year, after standardisation on the age structure of the world population

Table A8. Incidence of colon cancer in women. Geographic variations (Parkin et al., 1997)

Region	Registry	Incidence Rate*	Region	Registry	Incidence Rate*
Africa	Algeria, Setif (1990-1993)	0,58	Asia	China, Qidong (1988-1992)	2,00
	Mali, Bamako (1988-1992)	1,40		India, Barshi, Paranda and Bhum (1988-1992)	0,44
	Uganda, Kyadondo (1991-1993)	3,36		Japan, Osaka (1988-1992)	13,10
Central and South America	Argentina, Concordia (1990-1994)	15,26		Korea, Kangwha (1986-1992)	2,30
	Ecuador, Quito (1988-1992)	5,66		Viet Nam, Hanoi (1991-1993)	2,91
	Peru, Lima (1990-1991)	5,29	Europe	Austria, Tyrol (1988-1992)	16,65
North America	Canada, British Columbia (1988-1992)	17,65		Czech Republic (1988-1992)	15,71
	Canada, Ontario (1988-1992)	22,81		Denmark (1988-1992)	19,85
	US, Los Angeles: Non-Hispanic White (1988-1992)	20,62		France, Doubs (1988-1992)	13,72
	US, Los Angeles: Hispanic White (1988-1992)	11,83		France, Isere (1988-1992)	15,54
	US, Los Angeles: Black (1988-1992)	26,46		Italy, Ragusa (1988-1992)	10,48
				Italy, Varese (1988-1992)	18,70

US, Los Angeles: Chinese (1988-1992)	12,34	The Netherlands (1989-1992)	18,93
US, Los Angeles: Filipino (1988-1992)	8,12	Spain, Basque Country (1988-1991)	10,56
US, Los Angeles: Korean (1988-1992)	8,16	Sweden (1988-1992)	15,85
US, Los Angeles: Japanese (1988-1992)	20,60	Switzerland, Geneva (1988-1992)	15,42
US, SEER: White (1988-1992)	20,80	UK, Oxford (1988-1992)	17,07
US, SEER: Black (1988-1992)	26,85	Oceania	
		Australia, Victoria (1988-1992)	22,90
		New Zealand: Non-Maori (1988-1992)	29,60
		New Zealand: Maori (1988-1992)	16,03

* expressed in number of cases per 100 000 women per year, after standardisation on the age structure of the world population

Table A9: Hormonal agents evaluated in IARC monographs

Group 1 :   Carcinogenic to humans

- diethylstilbœstrol (DES)
- post menopausal oestrogen therapy
- non steroidal oestrogens
- steroidal oestrogens
- combined oral contraceptives
- sequential oral contraceptives
- tamoxifen

Group 2A: Probably carcinogenic to humans

- androgenic (anabolic) steroids

Group 2B: Possibly carcinogenic to humans

- medroxyprogesterone acetate
- progestins
- post menopausal oestrogen-progestogen
- oral contraceptives, progestins only

Group 3:   Unclassifiable as to carcinogenicity to humans

- clomiphene citrate
- œstradiol mustard
- toremifene
- droloxifene

Table A10.1: Evaluation of carcinogenicity of hormonal compounds in IARC Monographs

Detailed results. Group 1

Compound	Evidence for animal carcinogenicity	Evidence for human carcinogenicity	Overall evaluation	Volume	Year of publication
Diethylstilboestrol	Sufficient	Sufficient	Group 1	21 suppl. 7	1979 1987
Oestrogen therapy, Postmenopausal	= sufficient for oestradiol 17β and oestrone = limited for conjugated equine oestrogens, equilin and oestriol = inadequate for d-equilenin	Sufficient	Group 1	72	1999
Oestrogens, non steroidal Diethylstilboestrol Dienoestrol Hexoestrol Chlorotrianese	 Sufficient Limited Sufficient Inadequate	 Sufficient	Group 1 Group 1	21 suppl. 7	1979 1987
Oestrogens, steroidal Oestrogen replacement therapy Conjugated oestrogens Oestradiol 17β and esters Oestriol Oestrone Ethinyloestrodiol	 Limited Sufficient Limited Sufficient Sufficient	 Sufficient	Group 1	21 suppl. 7	1979 1987
Compound	Evidence for animal carcinogenicity	Evidence for human carcinogenicity	Overall evaluation	Volume	Year of publication

Compound	Evidence for animal carcinogenicity	Evidence for human carcinogenicity	Overall evaluation	Volume	Year of publication
Oral contraceptives combined	= sufficient for • ethinyloestradiol plus ethynodiol • diacetate and mestranol plus norethynodrel = limited for • ethinyloestradiol plus megestrol acetate • mestranol or ethinyloestradiol plus chlormadinone acetate • mestranol plus ethynodiol diacetate • mestranol plus lynestrol • mestranol or ethyloestradiol plus norethisterone • ethinyloestradiol plus norgestrel • quinestrol plus quigestranol = sufficient for norethynodrel plus lynoestrol = limited for • chlormadinone acetate • cyproterone acetate • ethynoldiol diacetate • megestrol acetate • norethisterone acetate • norethisterone = inadequate for norgestrel = sufficient for ethynyloestradiol and mestranol	Sufficient	Group 1	72	1999

Compound	Evidence for animal carcinogenicity	Evidence for human carcinogenicity	Overall evaluation	Volume	Year of publication
oral contraceptives, sequential	Inadequate for dimethisterone in combination with ethinyloestradiol	Sufficient	Group 1	21 suppl. 7	1979 1987
Tamoxifen	Sufficient	= sufficient for endometrial cancer = inadequate for other cancer sites	Group 1	66	1996

Table A10.2: Evaluation of carcinogenicity of hormonal compounds in IARC Monographs

Detailed results. Group 2A and 2B

Compound	Evidence for animal carcinogenicity	Evidence for human carcinogenicity	Overall evaluation	Volume	Year of publication
Androgenic (anabolic) steroids	Sufficient for testosterone	Limited	Group 2A	suppl. 7	1987
Medroxyprogesterone acetate	Sufficient	Inadequate	Group 2B	21 suppl. 7	1979 1987
Oestrogen-progestogen therapy, post menopausal	Inadequate for conjugated equine oestrogens plus progestogen	Limited	Group 2B	72	1999
Progestins Medroxyprogesterone Chlormadinone acetate Dimethisterone Ethynodiol acetate 17α hydroxyprogesterone caproate lynoestrol megestrol acetate norethisterone norethynodrel nogestrel progesterone	Sufficient Limited Inadequate Limited Inadequate Inadequate Limited Sufficient Limited Inadequate Sufficient	Inadequate Inadequate	Group 2B	21 suppl. 7	1979 1987
Progestogen-only contraceptives	Sufficient for medroxyprogesterone acetate Inadequate for levonorgestrel	Inadequate	Group 2B	72	1999

Table A10.3: Evaluation of carcinogenicity of hormonal compounds in IARC Monographs

Detailed results. Group 3

Compound	Evidence for animal carcinogenicity	Evidence for human carcinogenicity	Overall evaluation	Volume	Year of publication
Clomiphene citrate	Inadequate	Inadequate	Group 3	21 suppl. 7	1979 1987
Droloxifene	Inadequate	Inadequate	Group 3	66	1996
Oestradiol mustard	Sufficient	Inadequate	Group 3	9	1975
Toremifene	Inadequate	Inadequate	Group 3	66	1996

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