A REVIEW TO UPDATE AUSTRALIA'S POSITION ON THE HUMAN SAFETY OF RESIDUES OF HORMONE GROWTH PROMOTANTS (HGPs) USED IN CATTLE

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ABBREVIATIONS

Time		**Weight**	
d	Day	**bw**	Body weight
h	Hour	**g**	Gram
min	Minute	**kg**	Kilogram
mo	Month	**mg**	Milligram
wk	Week	**µg**	Microgram
s	Second	**ng**	Nanogram
yr	Year	**wt**	Weight
Length		**Dosing**	
cm	Centimetre	**id**	Intradermal
m	Metre	**im**	Intramuscular
µm	Micrometre	**inh**	Inhalation
mm	Millimetre	**ip**	Intraperitoneal
nm	Nanometre	**iv**	Intravenous
		po	Oral
		sc	Subcutaneous
		mg/kg bw/d	mg/kg bodyweight/day
Volume		**Concentration**	
L	Litre	**M**	Molar
mL	Millilitre	**ppb**	Parts per billion
µL	Microlitre	**ppm**	Parts per million

Chemistry

bPR	bovine uterine progestin receptor
BSO	L-buthionine(S,R)sulfoximine
COMT	catechol-*O*-methyltransferase
CYP1A1	oestrogen-2-hydroxylase
CYP1β1	17β-oestradiol-4-hydroxylase
dA	deoxyadenosine
dG	deoxyguanosine
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
EIA	enzyme immunoassay
ER	oestrogen receptor
ERα	oestrogen receptor alpha
ERβ	oestrogen receptor beta
hER	human oestrogen receptor
GC-MS	gas chromatography-mass spectrometry
hprt	hypoxanthine-guanine phosphoribosyltransferase
hER	human oestrogen receptor
hSHBG	human sex-hormone binding globulin
HPLC	high performance liquid chromatography
LC-DAD	liquid chromatography with diode array detection
LC-MS	liquid chromatography-mass spectrometry
mRNA	messenger ribonucleic acid
MNNG	*N*-methyl-*N*-nitro-*N*-nitroso-guanidine
8-oxo-dG	8-hydroxy-2'-deoxyguanosine
PCR	polymerase chain reaction
rhAR	recombinant human androgen receptor
RSA	relative stimulatory activity (for oestrogenicity)
rtER	rainbow trout oestrogen receptor
RIA	radioimmunoassay
RNA	ribonucleic acid

ROS	reactive oxygen species
rtER	rainbow trout oestrogen receptor
RT-PCR	real-time polymerase chain reaction
TBME	*tert* butyl-methyl-ether
TCDD	2,3,7,8-tetrachlorodibenzo -p-dioxin
TGFβ3	transforming growth factor beta 3
TG^r	6-thioguanine resistant
VTG	vitellogenin

Hormones

DES	diethylstilboestrol
DHT	dihydrotestosterone
E_2	17β-oestradiol
FSH	follicular stimulating hormone
LH	luteinising hormone
MGA	melengestrol acetate
TBA	trenbolone acetate
TBO	trenbolone
17α-TBO	17-alpha-trenbolone
17β-TBO	17-beta-trenbolone

Oestrogen metabolites, conjugates and adducts

E_1	oestrone
2-OHE_1	2-hydroxyoestrone
4-OHE_1	4-hydroxyoestrone
16α-OHE_1	16α-hydroxyoestrone
2-OCH_3E_1	2-methoxyoestrone
4-OCH_3E_1	4-methoxyoestrone
4-OHE_1-2-SG	4-hydroxyoestrone glutathione
4-OHE_1-2-Cys	4-hydroxyoestrone cysteine
4-OHE_1-2-NAcCys	4-hydroxyoestrone N-acetylcysteine
E_2-2,3-Q	17β-oestradiol-2,3-quinone
E_2-3,4-Q	17β-oestradiol-3,4-quinone
EE	17α-ethinyl oestradiol
E_2S	17β-oestradiol-17-stearate
2-OHE_2	2-hydroxy-17β-oestradiol
4-OHE_2	4-hydroxy-17β-oestradiol
16α-OHE_2	16α-hydroxy-17β-oestradiol
2-OCH_3E_2	2-methoxy-17β-oestradiol
4-OCH_3E_2	4-methoxy-17β-oestradiol
4-OHE_2-2-SG	4-hydroxy-17β-oestradiol glutathione
4-OHE_2-2-Cys	4-hydroxy-17β-oestradiol cysteine
4-OHE_2-2-NAcCys	4-hydroxy-17β-oestradiol N-acetylcysteine
2-OHE-N^2-dG	N^2-(2-hydroxyoestrogen-6-yl)-2'-deoxyguanosine
2-OHE-N^6-dA	N^6-(2-hydroxyoestrogen-6-yl)-2'-deoxyadenosine
E_3	oestriol
2-OHE_3	2-hydroxyoestriol

Terminology

ADI	acceptable daily intake
agvet	agricultural and veterinary
ANOVA	analysis of variance
ERKO	oestrogen receptor-α knockout
GD	gestational day
GAP	good agricultural practice
GVP	good veterinary practice
HGP	hormone growth promotant
LOD	limit of detection

LOEL	low observable effect level
LOQ	limit of quantitation
MANOVA	multivariate analysis of variance
MRL	maximum residue level
NHEL	no hormonal effect level
NOEL	no observable effect level
OR	odds ratio
SHE	Syrian hamster embryo

Organisations and Publications

AFFA	Agriculture, Fisheries and Forestry-Australia
APVMA	Australian Pesticides and Veterinary Medicines Authority
CVM	US Centre for Veterinary Medicine
DPSC	Drugs and Poisons Schedule Committee
EU	European Union
EC	European Commission
FAO	Food and Agriculture Organisation of the United Nations
JECFA	Joint WHO/FAO Expert Committee on Food Additives
IARC	International Agency for Research on Cancer
NDPSC	National Drugs and Poisons Schedule Committee
NHMRC	National Health and Medical Research Council
PACC	Pesticides and Agricultural Chemicals Committee
PACSC	Pesticides and Agricultural Chemicals Sub Committee
PHAC	Public Health Advisory Committee
PSC	Poisons Schedule Committee
SCA	Standing Committee on Agriculture
TCVD	Technical Committee on Veterinary Drugs
TGA	Therapeutic Goods Administration
USDA	United States Department of Agriculture
US FDA	United States Food and Drug Administration
VPHC	Veterinary Public Health Committee
WHO	World Health Organisation
WTO	World Trade Organisation

EXECUTIVE SUMMARY

In beef-exporting countries such as Australia, the USA, Canada and New Zealand, certain natural and synthetic hormones are authorised for use as growth promoting agents in cattle. These hormone growth promotants (HGPs) include naturally occurring steroids such as 17β-oestradiol, progesterone and testosterone, and their synthetic counterparts zeranol, melengestrol acetate and trenbolone acetate.

The human safety and toxicology of HGPs have been extensively assessed by regulatory authorities in each country, in addition to expert scientific committees of the World Health Organisation. There is also a plethora of published scientific studies relating to the biochemistry and physiology of these compounds. Based on this extensive database, all international bodies and national regulatory agencies, except the European Commission (EC), have concluded that residues of registered HGPs do not pose a threat to consumers when HGPs are used according to Good Veterinary Practice (GVP). The use of HGPs on farm animals was banned by the EC in 1988. This ban was extended in 1989 to include the importation of beef and beef products from countries allowing the use of HGPs.

In 1999, the EC released a risk assessment in response to a 1997 World Trade Organisation ruling that the European ban was not based on scientific evidence or a proper risk assessment. As part of its risk management framework, the EC had adopted a precautionary approach and had considered social, economic and political factors as part of its risk assessment. It concluded that 17β-oestradiol was a "complete" carcinogen and that progesterone, testosterone, zeranol, melengestrol acetate and trenbolone acetate should all be viewed as having potentially endocrine-disrupting, developmental, immunological, neurobiological, immunotoxic, genotoxic and carcinogenic effects due to the lack of data to support an alternative view. Prior to the release of this 1999 risk assessment, the EC commissioned 17 new studies to fill data gaps that it had identified. In April 2002, the EC released a second risk assessment, which considered data from these commissioned studies in addition to other studies that had become available in the peer-reviewed scientific literature since its 1999 review. This second risk assessment conducted by the EC reaffirmed the conclusions the EC had reached in 1999.

In light of the potential health concerns attributed to the use of HGPs by the EC, and the ongoing polarisation in scientific and regulatory opinion regarding their safety, the current scoping study was undertaken to independently evaluate those studies commissioned or cited by the EC in their April 2002 risk assessment. The objective was to determine whether there is any new scientific evidence, beyond that already evaluated nationally and internationally, to indicate a need for a review of HGPs by the Australian Pesticides and Veterinary Medicines Authority (APVMA).

Forty-two published papers were evaluated covering residue analyses in cattle, *in vitro* and *in vivo* oestrogenicity and genotoxicity assays, environmental studies, metabolism studies, studies examining the mutagenicity and genotoxicity of 17β-oestradiol, and several review articles.

Only limited new information was presented in the EC-commissioned studies and other cited literature regarding the metabolism, endocrine-disrupting potential, genotoxicity or carcinogenicity of progesterone, testosterone, melengestrol acetate, trenbolone acetate and

zeranol. Studies on the potential environmental impact of HGPs were considered to have limited value in assessing dietary risk of HGP residues to humans.

There was no adequate evidence to suggest that 17β-oestradiol is mutagenic.
Certain catechol oestrogen metabolites were genotoxic at high concentrations when administered directly to cells or animals. However, biochemical mechanisms exist to control the generation of potentially genotoxic metabolites *in vivo* and to eliminate DNA adducts that might be formed.

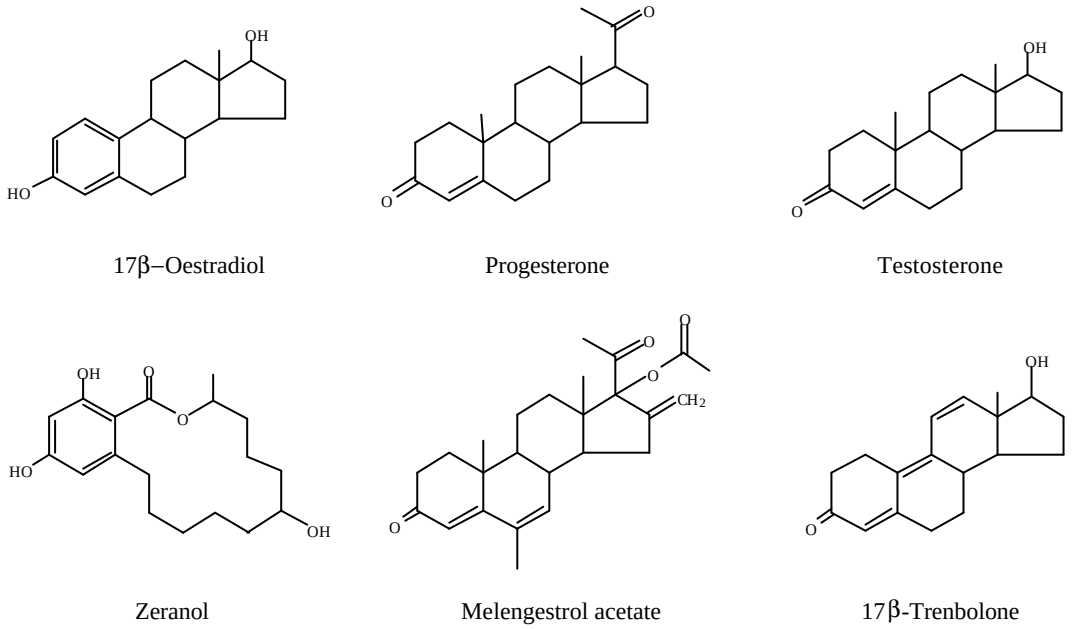
Several studies confirmed that the use of HGPs according to Good Veterinary Practice (GVP) does not generate violative levels of residues in cattle tissues. The highest residue levels were found in the remaining implanted pellet, thus supporting the current implantation and management protocol for HGPs in countries where they are registered for use. This protocol involves injecting the implant subcutaneously into an ear, which is subsequently discarded at slaughter, thereby preventing pellets from inadvertently entering the food supply. The assumption by the EC that registration of HGPs will inevitably lead to misuse was considered to be unsubstantiated. In Australia, it is considered that control-of-use provisions prevent significant misuse of agricultural and veterinary (agvet) chemicals (ie. application of implants to other areas of animals where there may contaminate the carcass).

There was no data to associate the consumption of residues of HGPs currently registered for use in non-European countries with adverse health effects in humans, including cancer risk. To adequately determine the incremental risk associated with very low levels of HGP residues in meat, the total dietary intake of hormones from all sources would need to be evaluated.

Overall, the following conclusions can be made: (1) a review of the new data does not indicate any grounds for amending Australia's current regulatory position with respect to HGPs - ie. that there is unlikely to be any appreciable health risk to consumers from eating meat from cattle that have been treated with HGPs according to GVP; (2) there is no new scientific evidence to indicate a need for the reconsideration by the Australian Pesticides and Veterinary Medicines Authority (APVMA) of the present use of HGPs under GVP conditions; and (3) in view of the complexity of assessments of total dietary hormone intakes and the contentious views expressed in various international forums, the use of HGPs in meat producing animals should be kept under ongoing consideration by the Advisory Committee on Pesticides and Health.

INTRODUCTION

Hormone growth promotants (HGP) are used to increase the growth, feed conversion efficiency and carcass leanness of cattle, and are typically administered via subcutaneous implants in the ears. HGPs include certain naturally-occurring steroids such as 17β-oestradiol, progesterone and testosterone, and their synthetic counterparts zeranol, melengestrol acetate (MGA)[1] and trenbolone acetate (TBA) (see Figure below). Zeranol (α-zearalanol) is a resorcyclic acid lactone and an oestrogenic derivative of the mycoestrogen zearalenone, which is produced by *Fusarium* moulds. Although hormonal implants are widely used in beef-exporting countries such as the USA, Canada, New Zealand and Australia, they have been officially banned in Europe on safety concerns since 1988. The consequence of the European ban has been an ongoing protest from the USA and Canada, which has reinforced the polarisation in scientific and regulatory opinion regarding the safety of HGPs.



17β–Oestradiol | Progesterone | Testosterone

Zeranol | Melengestrol acetate | 17β-Trenbolone

Structures of natural and synthetic HGPs

Health concerns associated with HGPs

The health concerns associated with hormonal compounds used as growth promotants (and also as therapeutic agents) are their carcinogenic and endocrine-disrupting potentials. By virtue of their normal biochemical action, low concentrations of steroid hormones (nM) bind to and activate their intracellular receptors, which interact with hormone response elements in DNA, leading to the transcription of genes that induce cell proliferation and growth. Therefore a hormonal substance could promote carcinogenicity in hormone-sensitive tissues through such a proliferative mechanism. Though receiving less attention, the other health concern is the endocrine-disrupting effects of HGPs, notably their potential to perturb normal development in sensitive subpopulations like prepubertal children and in developing foetuses.

[1] MGA, unlike the other HGPs, is administered via the diet as a feed additive

The issue with regard to dietary HGP residues is whether or not the low levels that may be found in the meat of HGP-treated cattle, can cause adverse health effects in humans. Any risk to human health from the dietary intake of HGP residues needs to be balanced against the total dietary intake of hormones (occurring naturally in meat, milk, eggs, offal, fish etc.) and hormone-like compounds (occurring as phytoestrogens etc in plants, fungi etc).

Difficulties associated with assessing the safety of HGPs

Humans and cattle synthesise, metabolise and eliminate a range of steroid hormones as part of their normal biology and in addition ingest hormonally active compounds as part of their diet. Any consequences on human health following the treatment of cattle with HGPs are complicated by the difficulty in assessing the risk associated with the ingestion of low levels of HGP residues when humans are constantly exposed to relatively high and fluctuating levels of endogenously-produced and other exogenously-derived hormones.

Compounds such as 17β-oestradiol, progesterone and testosterone are naturally occurring constituents of the diet (eg. meat, fish, milk, eggs). Additionally there are numerous plant-derived oestrogenic compounds that have potent biological activity (eg. isoflavones, coumestans, lignans) that are also consumed as part of the diet. Humans have been exposed to low levels of compounds like zearalenone (which is metabolised to zeranol) for thousands of years given its natural occurrence as a fungal contaminant (mainly *Fusarium spp*) on a number of plant products (eg. wheat, barley, maize). The potential for these naturally-occurring substances to elicit hormonal effects in humans is likely to be just as significant as any potential effect of HGP residues received via the ingestion of meat. Studies have shown that there is a large variation in endogenous hormone levels in cattle and therefore residues in meat from HGP-treated animals are likely to make a small contribution to overall hormone intake or exposure from natural sources. Therefore, previous scientific assessments had concluded that the incremental risk from HGPs would be quite small.

Technically, it has been difficult to detect any significant fluctuations in oestradiol, progesterone and testosterone residues in treated *versus* untreated cattle, given their high natural background concentrations in animal tissue. A range of assays have been developed to measure the six HGPs (reviewed by Doyle 2000)[2] and these show varying degrees of sensitivity and reliability. Indeed, one of the ongoing problems, which is not particularly unique to these compounds, has been the variability in measurements performed by different laboratories.

Risk Assessments of HGPs

Various international bodies and national regulatory agencies have evaluated the toxicology of, and dietary risk associated with, HGP residues. There is also a plethora of published scientific studies relating to the biochemistry and physiology of HGPs, particularly the natural steroid hormones. While the weight-of-evidence indicates that registered HGPs do not pose a threat to human safety when used according to good veterinary practice (GVP), this view is not held unanimously throughout the world. In particular, the EC has completed two risk assessments that have concluded that the use of HGPs is potentially unsafe for consumers. Consequently the EC has maintained its ban on the use of HGPs, including the importation of meat and meat products from countries that utilise them. The only mechanism

[2] Doyle E (2000) Human safety of hormone implants used to promote growth in cattle. FRI Briefings.

available for non-European countries to access the European market is via a HGP-free accreditation program.

Purpose of the current review

In light of the potential health concerns attributed to the use of HGPs by the EC, and the ongoing polarisation in scientific and regulatory opinion regarding their safety, the current review was undertaken to:

(1) examine the international status of considerations of the human safety of HGP residues in cattle; and

(2) independently evaluate the EC's opinion on the risk to human health from consuming HGP residues, with particular emphasis on their most recent risk assessment (April 2002) and those studies commissioned or cited by them to substantiate this opinion.

INTERNATIONAL SCIENTIFIC ASSESSMENTS OF HGPs

The European Commission (EC)

The current polarisation in scientific and regulatory opinion surrounding the use of HGPs stems from the 1988 EC ban, which prohibited the use of HGPs on farm animals. This ban was extended in 1989 to include the importation of beef and beef products from countries allowing the use of growth promotants. The catalyst for the ban was not the use of the currently registered HGPs but rather the synthetic hormone diethylstilboestrol (DES). (It should be noted that DES is not registered for use in food producing animals in Australia). DES was linked to abnormal sexual development in Italian babies due to the consumption of tinned baby food made from French veal contaminated with traces of DES. In 1981, the EC banned the use of DES and recommended that HGPs be reviewed. The conclusion of the subsequent review[3] was that the three natural hormones (17-β-oestradiol, progesterone, testosterone) would not present any harmful effects to the health of the consumer when used under the appropriate conditions as growth promoters in farm animals, but zeranol and trenbolone acetate required more data to support their use. Subsequently, a ban on all HGPs was instigated and remains in place.

Following a protest by the US and Canada, The World Trade Organisation (WTO) ruled against the EC ban in 1997[4] concluding that it was not based on scientific evidence or an adequate risk assessment. The EC was allowed 15 months to conduct a risk assessment of hormone-treated meat.

The subsequent EC risk assessment on hormone residues in bovine meat and meat products[5] arrived at the following conclusions:

- There is a substantial body of evidence suggesting that 17β-oestradiol is a complete carcinogen (tumour initiator and promoter), but the available data does not allow a quantitative risk estimate;
- Data pertaining to the 5 other growth-promoting hormones (progesterone, testosterone, zeranol, TBA & MGA) does not allow a quantitative risk assessment;
- Potential developmental, immunobiological, neurobiological, immunotoxic, genotoxic and carcinogenic effects can be envisaged for all 6 hormones, with prepubertal children considered to be a high risk group, however the available data do not allow a quantitative risk assessment;
- In view of the intrinsic properties of the hormones, and taking into account epidemiological findings, no threshold levels or acceptable daily intakes[6] (ADIs) can be established for any of the 6 hormones.

Following their 1999 risk assessment, the EC moved to impose a definite ban on the use of 17β-oestradiol and its ester-like derivatives in farm animals except for therapeutic purposes. A provisional ban was also placed on progesterone, testosterone, zeranol, TBA and MGA,

[3] EEC Scientific Working Group on Anabolic Agents, chaired by Dr GE Lamming. Scientific report on anabolic agents in animal production. Vet. Rec. 1987: 389-392.
[4] EC measures concerning meat and meat products (hormones). AB-1997-4. Report of the Appellate BodyWT/DS26/AB/R. WT/DS48/AB/R. 16 January 1998.
[5] Opinion of the Scientific Committee on Veterinary Measures Relating to Public Health (SCVPH): Assessment of potential risks to human health from hormone residues of bovine meat and meat products; 30th April 1999.
[6] The acceptable daily intake (ADI) for humans is considered to be a level of intake of a chemical that can be ingested daily over an entire lifetime without any appreciable risk to health.

pending further scientific evidence. Internationally there appears to be very little support for the EC position either on the basis of their scientific assessments or the resulting risk management procedures.

Later in 1999, the Sub-Group of the Veterinary Products Committee from the UK Ministry of Agriculture, Fisheries and Food, critically evaluated the scientific reasoning and methods of argument adopted in the EC's risk assessment[7]. The group were unable to support the conclusion that risks associated with the consumption of meat from hormone treated cattle may be greater than previously thought. Additionally, they had concerns regarding the scientific reasoning in a number of key areas, throwing significant doubt on the conclusions of the EC. In response, the EC reviewed this report and considered whether there was any recent scientific information that would lead the SCVPH to revise its opinion[8].

Prior to the release of its 1999 risk assessment, the EC had commissioned 17 new studies (see Annex 1) to fill a number of data gaps that it had identified. Consequently, a second risk assessment[9] was released in April 2002, which considered findings from these commissioned studies, in addition to other studies that had become available in the peer-reviewed scientific literature since its 1999 review. Overall, the EC confirmed the validity of its previous opinion and made the following conclusions:

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

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

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

- Convincing data have been published confirming the mutagenic and genotoxic potential of 17β-oestradiol as a consequence of metabolic activation to reactive quinones. *In vitro* experiments indicated that oestrogenic compounds alter the expression of an array

[7] Sub-Group of the Veterinary Products Committee, Ministry of Agriculture, Fisheries, and Food. Executive summary and critical evaluation of the 1999 opinion of the scientific committee on veterinary measures relating to public health. 1999. http://www.vmd.gov.uk/old_vmd_web_pages/finalrep.htm

[8] Review of specific documents relating to the SCVPH opinion of 30 April 99 on the potential risks to human health form hormone residues in bovine meat and meat products; adopted on 3 May 2000. http://europa.eu.int/comm/food/fs/sc/scv/outcomeen.html

[9] Opinion of the Scientific Committee on Veterinary Measures Relating to Public Health (SCVPH) on review of previous SCVPH opinions of 30 April 1999 and 3 May 2000 on the potential risks to human health from hormone residues in bovine meat and meat products; 10th April 2002.

of genes. Considering that endogenous oestrogens exert these effects, the data highlight the diverse biological effects of this class of hormones.

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

- Experiments with zeranol and trenbolone suggested a more complex oxidative metabolism than previously assumed. These data need further clarification as they might influence a risk assessment related to tissue residues of these compounds.

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

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

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

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

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

WHO/FAO Joint Expert Committee on Food Additives (JECFA)

The Joint FAO[10]/WHO[11] Expert Committee on Food Additives (JECFA) is an international expert scientific committee that evaluates the safety of food additives, contaminants, naturally occurring toxicants and residues of veterinary drugs in food. It has been meeting since 1956 and provides advice to the FAO, WHO, to FAO and WHO member governments, and to the Codex Alimentarius Commission.

JECFA has considered the toxicology and human safety of HGPs at a number of meetings (see Table below). Complete details of the scientific evaluations performed by JECFA can be obtained in the original meeting reports, and the toxicological and residue monographs, which are listed in Annex 2 of this document.

JECFA considerations of HGPs

Compound	Meeting (year)	Outcome
17β-Oestradiol Progesterone Testosterone	25th (1981)	Unlikely to be of concern when used properly
	32nd (1987)	ADI considered unnecessary. Residues unlikely to pose a hazard to human health when used according to GVP. Residue monographs prepared. MRLs considered unnecessary.
	52nd (1999)	Combined toxicological monograph. ADIs established. MRLs "not specified" for the edible tissues of cattle when the products are used according to GVP – ie. residues do not present a health concern.
Melengestrol acetate	54th (2000)	Toxicological monograph. ADI established. Residue monograph. Temporary MRLs set for liver and fat - expressed as the parent compound.
Trenbolone acetate	26th (1982)	Unlikely to pose an endocrinal or toxicological hazard when used properly.
	27th (1983)	Provisional acceptance of use. Residues below the threshold concentration for producing endocrinal effects.
	32nd (1987)	Toxicological and residue monographs prepared. Establishment of temporary ADI. Temporary MRLs set for α-trenbolone (liver & kidney) and β-trenbolone (bovine meat).
	34th (1989)	Toxicological and residue monographs prepared. ADI established. MRLs set for α-trenbolone (liver) and β-trenbolone (muscle).
Zeranol	26th (1982)	Unlikely to pose an endocrinal or toxicological hazard when used properly.
	27th (1983)	Provisional acceptance of use. Residues below the threshold concentration for producing endocrinal effects.
	32nd (1987)	Toxicological and residue monographs prepared. ADI established. MRLs set for bovine liver and muscle.

GVP = Good Veterinary Practice; ADI = Acceptable Daily Intake; MRL = Maximum Residue Level

17b-oestradiol, progesterone and testosterone

17β-oestradiol, progesterone and testosterone were first considered by JECFA in 1981 (25th Meeting). The Committee examined evidence indicating that residues of 17β-oestradiol would contribute very little to the overall intake of oestrogenic substances in a normal diet. The report from this meeting[12] also indicated that endogenous oestrogen production is at least

[10] Joint Food and Agriculture Organisation of the United Nations
[11] World Health Organisation
[12] WHO Technical Report Series No. 669.

1000-fold greater than the intake of residues of 17β-oestradiol. JECFA concluded that it seemed unlikely that there was any cause for concern when 17β-oestradiol, progesterone and testosterone are properly used in animal production. However, the report outlined some concerns with synthetic anabolic agents, chemically modified hormones and hormonally-active substances in plants, including their "extreme potency", tumorigenic potential and metabolites that might have endocrinological or toxicological effects.

At their 32nd Meeting (1987), JECFA considered a range of studies relating to the biological activity, carcinogenicity, embryotoxicity and mutagenicity of 17β-oestradiol, progesterone and testosterone. In experimental animals, all 3 compounds were found to cause tumours in various hormone-sensitive tissues (eg. mammary tissue, uterus, ovary and prostate). These tumours occurred at doses that caused obvious hormonal effects, and therefore they were attributed to the hormonal activity of each compound. It was recognised that all 3 compounds occur naturally in mammals and are present in a variety of foodstuffs such as dairy products, plant material and tissues from untreated cattle. Furthermore, JECFA noted that the levels of 17β-oestradiol in treated cattle fall within the normal range found in untreated cattle. After comparing the intake of 17β-oestradiol, progesterone and testosterone that would result from the ingestion of meat from treated cattle, with the normal daily production of these compounds in humans, JECFA concluded that the amount of exogenous 17β-oestradiol, progesterone and testosterone ingested from meat from treated cattle would be incapable of exerting a hormonal or a toxic effect in humans. JECFA did not consider that an ADI was necessary for a hormone that is produced endogenously in human beings. JECFA concluded that residues arising from the use of 17β-oestradiol, progesterone and testosterone as growth promoters in accordance with good animal husbandry practice are unlikely to pose a hazard to human health. Therefore JECFA considered that it was unnecessary to establish MRLs[13] for these 3 naturally-occurring steroid hormones.

JECFA undertook an extensive evaluation of the 3 natural HGPs at their 52nd Meeting (1999), including a quantitative estimation of the levels that can be safely consumed. The databases for 17β-oestradiol, progesterone and testosterone were extensive and included published oral bioavailability, metabolism and toxicological data. For 17β-oestradiol, epidemiological studies in women taking oral contraceptives, and mechanistic studies conducted in laboratory animals, were also evaluated. Numerous reports of studies in humans that had been treated with progesterone or testosterone were also examined.

The overall conclusions of the 52nd Meeting were that:

- 17β-oestradiol, progesterone and testosterone have low oral bioavailability due to their inactivation in the GIT and by the liver;
- 17β-oestradiol, progesterone and testosterone have low acute oral toxicity;
- Adverse effects occurring in laboratory animals following repeated dosing were attributable to the hormonal activity of each compound. Hormonal effects were considered to be appropriate for evaluating human safety because they occurred at doses below which other forms of toxicity were manifest;
- 17β-oestradiol, but not progesterone or testosterone, has genotoxic potential;
- The carcinogenicity of 17β-oestradiol was probably a result of its interaction with hormone receptors. Available data suggested that the increased incidence of cancers of

[13] The maximum residue level (MRL) is the maximum concentration of a residue resulting from registered use of an agricultural or veterinary chemical. The MRL is set at a level which is not likely to be exceeded if the agricultural or veterinary chemical is used according to good agricultural or veterinary practice.

the breast and endometrium observed among women receiving postmenopausal oestrogen replacement therapy was due to the hormonal effects of oestrogens;
- Progesterone is not carcinogenic;
- The increased incidence of prostatic cancer in testosterone-treated rats was attributable to the hormonal activity of this compound;
- 17β-oestradiol and progesterone are not teratogenic, while testosterone is embryotoxic;

An ADI of 0-0.00005 mg/kg bw/d was established for 17β-oestradiol, calculated using a NOEL of 0.005 mg/kg bw/d for changes in hormone-dependent parameters in postmenopausal women. A safety factor of 10 was applied to account for normal variation between individuals, with an additional 10-fold safety factor added to protect populations of various sensitivities. For progesterone, an ADI of 0-0.03 mg/kg bw/d was established using the Low Observable Effect Level (LOEL) of 3.3 mg/kg bw/d for uterine changes in women. A 10-fold safety factor was applied to account for normal variation between individuals, with an additional safety factor of 10 used because of the use of a LOEL in the absence of a NOEL. An ADI of 0-0.002 mg/kg bw/d was established for testosterone, calculated using the NOEL of 1.7 mg/kg bw/d for restoration of sexual function in eunuchs. A safety factor of 10 was used to account for normal variation between individuals, with an additional 10-fold safety factor applied to protect sensitive populations and another 10-fold safety factor applied because of the low group size in the pivotal study (n=5).

At its 52nd Meeting, JECFA also examined residue data (of administered products) and undertook calculations to obtain conservative theoretical estimates of possible excess of dietary intake in persons who consume large amounts of meat (eg. 500 g/d) obtained from treated cattle. For total oestrogens, the highest excess intake was 30-50 ng/person/d, which is less than 2% of the ADI for 17β-oestradiol. For progesterone, the highest intake was approximately 500 ng/person/day, which is approximately 0.03% of the ADI. For testosterone, the highest intake was approximately 60 ng/person/d, which is approximately 0.05% of the ADI. JECFA concluded that there was no need to specify MRLs for 17β-oestradiol, progesterone and testosterone for the edible tissues of cattle when products are used according to GVP. The rationale for this conclusion was that average hormone concentrations in treated cattle are within the normal physiological concentration range of untreated cattle.

Melengestrol acetate, trenbolone acetate and zeranol

Trenbolone acetate (TBA) and zeranol were first considered by JECFA in 1982 (26th Meeting) in light of a recommendation from a WHO Working Group[14] that the available data on these compounds should be evaluated. JECFA considered that residues of TBA and zeranol were unlikely to pose any significant toxicological hazard of a non-endocrinal nature. However, it was recommended that both compounds be evaluated at a future meeting due to the absence of documentation on: residue levels; GVP in relation to the use of these compounds; and details of methods of analysis. In addition, the Committee had some "slight reservations" about the adequacy of the toxicology data.

At the 27th Meeting (1983), JECFA re-examined TBA and zeranol in light of likely residue levels. There was no evidence that zeranol was carcinogenic in rats, dogs and monkeys; the

[14] Health aspects of residues of anabolics in meat. Copenhagen, WHO Regional Office for Europe, 1982 (EURO Reports and Studies 59).

NOEL for oestrogenic activity in dogs was 0.025 mg/kg bw/d. Long-term feeding studies in mice revealed liver hyperplasia and tumours at 0.9-9 mg/kg bw/d TBA, and a slight increase in pancreatic islet-cell tumours in rats at 1.85 mg/kg bw/d. JECFA attributed these tumours to the hormonal action of TBA; the NOEL for hormonal activity in mice was 0.05 mg/kg bw/d, while the LOEL in rats was 0.025 mg/kg bw/d. The available data indicated that residues of zeranol and its metabolites would not exceed 1 µg/kg in animal tissue. For TBA, residue levels would not exceed 3 µg of α-TBO per kg of liver and 0.5 µg β-TBO per kg of other tissues. It was concluded that human exposure to TBA or zeranol would be "far below" the hormonally-active levels in animal models. JECFA provisionally accepted the use of TBA and zeranol as anabolic agents for meat production when used in accordance to GVP.

At the 32nd Meeting (1987), toxicological monographs were prepared for TBA and zeranol. JECFA considered TBA had no effect on reproduction at 0.03 mg/kg bw/d and was not teratogenic. A "comprehensive range" of *in vitro* and *in vivo* genotoxicity tests revealed that TBA, α- and β-TBO were not genotoxic. JECFA reiterated its previous conclusion that liver hyperplasia and tumours in mice, and pancreatic islet cell tumours in rats, were consequent to the hormonal activity of TBA. A temporary ADI of 0-0.00001 mg/kg bw/d was set, based on a No Hormonal Effect Level (NHEL) of 0.002 mg/kg bw/d for histological changes in the seminal vesicles of castrated male monkeys that had been dose orally with β-TBO. Although not explicit in the report[15], a 200-fold safety factor was used in this calculation. A separate NHEL for α-TBO was unable to be set due to the lack of suitable toxicological data. A temporary MRL of 0.0014 mg/kg for β-TBO in bovine meat was set on the basis of a daily intake by a 70 kg person of 500 g meat. JECFA noted that the hormonal activity of α-TBO is approximately 10% of β-TBO and therefore a temporary MRL was set at 0.014 mg/kg.

Zeranol was also evaluated at the 32nd Meeting. Administration of zeranol to cattle according to good animal husbandry practice resulted in levels (calculated as zeranol equivalents) not exceeding 0.0002 mg/kg in muscle, 0.01 mg/kg in liver, 0.002 mg/kg in kidney and 0.0003 mg/kg in fat. Zeranol had no effect on reproduction and was not teratogenic. It was weakly oestrogenic in long-term studies in laboratory animals. In rats, doses up to 1.25 mg/kg bw/d were oestrogenic but not carcinogenic. In male mice, oestrogenic effects occurred at the highest dose tested of 2.25 mg/kg bw/d. At this dose, an increased incidence of anterior lobe tumours of the pituitary gland occurred. However, JECFA concluded that the tumorigenic effect of zeranol was attributable to its oestrogenicity and that a NHEL would allow a safe level of exposure to be made. An ADI of 0-0.0005 mg/kg bw/d was set, based on the NHEL level of 0.05 mg/kg bw/d (maturation index of vaginal epithelial cells) in a 13 week study conducted on ovariectomised female monkeys. A 100-fold safety factor was apparently chosen to calculate this ADI. MRLs for zeranol were set at 0.01 mg/kg for bovine liver and 0.002 mg/kg for bovine muscle.

At its 34th Meeting (1989), JECFA examined a range of published genotoxicity studies and concluded that it was unlikely that TBA was genotoxic. An ADI of 0-0.00002 mg/kg bw/d was set by applying a 100-fold safety factor to the marginal effect level of 0.002 mg/kg bw/d in a 14-week pig study. This marginal effect level was supported by the NHEL in monkeys of 0.002 mg/kg bw/d for β-TBO. An MRL of 0.002 mg/kg was recommended for β-TBO in muscle, and an MRL of 0.01 mg/kg was recommended for α-TBA in liver. JECFA agreed that it would be unlikely that these MRLs would be exceeded when TBA is used according to

[15] WHO Technical Report Series No. 763

GVP. Conservative estimates indicated that the ADI would not be exceeded at any time following the implantation of TBA pellets at the recommended dose rate.

JECFA evaluated MGA in 2000 (54th Meeting). The database was extensive and included pharmacokinetic and biotransformation studies, as well as a range of toxicology studies conducted in laboratory animals. Results from human studies were also evaluated. The main findings of the JECFA evaluation are summarised as follows:

- There are only limited data on the bioavailability and pharmacokinetics of MGA following oral dosing;
- There is limited information on the biotransformation of MGA;
- MGA has low acute oral toxicity;
- Progestational and corticosteroidal effects were the most sensitive endpoints in short-term tests conducted in laboratory animals;
- MGA indirectly modulates mammary tumorigenesis in mice, possibly by stimulating the secretion of prolactin;
- MGA was not genotoxic;
- MGA suppressed oestrus in rats, dogs and cattle. There were significant effects on fertility and pregnancy. There were no adverse reproductive effects in males;
- MGA was embryotoxic, foetotoxic and teratogenic in rats and rabbits. JECFA attributed these effects to the corticosteroid activity of MGA.

The most appropriate endpoint for evaluating the safety of residues of MGA was the progestational effect in non-human primates. An ADI of 0-0.00003 mg/kg bw/d was established by applying a 200-fold safety factor to the LOEL of 0.005 mg/kg bw/d for effects on the menstrual cycle of monkeys. An additional 2-fold safety factor was chosen because a LOEL rather than a NOEL was used as the basis for the ADI.

Temporary MRLs were set for liver and fat pending information on the analytical methods for quantifying MGA in these tissues. MRLs for liver and fat were 0.002 mg/kg and 0.005 mg/kg, respectively, expressed as the parent compound. No MRLs were recommended for muscle and kidney because residues in these tissues are generally low or below the LOQ.

Summary

JECFA's current position on the human safety of HGPs is that when used according to GVP, residues do not pose a hazard to human health. A summary of JECFA ADIs and MRLs for the various HGPs is provided in the Table overleaf.

ADIs and MRLs set by JECFA

Compound	ADI (mg/kg bw/d)	MRL (mg/kg)
17β-Oestradiol	0-0.00005	Not specified for muscle, liver, kidney & fat (cattle)*
Progesterone	0-0.03	Not specified for muscle, liver, kidney & fat (cattle)*
Testosterone	0-0.002	Not specified for muscle, liver, kidney & fat (cattle)*
Melengestrol acetate	0-0.00003	0.002 liver (temporary) 0.005 fat (temporary)
Trenbolone acetate	0-0.00002[#]	0.002 muscle (β-Trenbolone) 0.01 liver (α-Trenbolone)
Zeranol	0-0.0005	0.002 bovine muscle 0.01 bovine liver

* Residues generated following uses according to GVP were considered unlikely to adversely effect human health; [#] ADI covers both the parent molecule (trenbolone acetate) and the two main metabolites (α and β trenbolone).

International Agency for Research on Cancer (IARC)

The International Agency for Research on Cancer (IARC) is part of the WHO, and coordinates and conducts epidemiological and laboratory research into the causes of cancer, including mechanisms of carcinogenesis, and the development of scientific strategies for cancer control. In assessing the carcinogenicity of a compound, IARC considers evidence arising from human and experimental animal data as well as any other relevant scientific data (eg. *in vitro* studies). IARC assigns a carcinogenic rating to a compound based on this data, according to the broad classification scheme outlined in the following Table.

IARC Classification Scheme for carcinogenic potential

Classification	Description
Group 1	The agent (mixture) is carcinogenic to humans. The exposure circumstance entails exposures that are carcinogenic to humans.
Group 2A	The agent (mixture) is probably carcinogenic to humans. The exposure circumstance entails exposures that are probably carcinogenic to humans.
Group 2B	The agent (mixture) is possibly carcinogenic to humans. The exposure circumstance entails exposures that are possibly carcinogenic to humans.
Group 3	The agent (mixture or exposure circumstances) is not classifiable as to its carcinogenicity to humans.
Group 4	The agent (mixture) is probably not carcinogenic to humans.

The evaluation of the carcinogenic potential of steroid hormones is summarised in the Tables below. Complete details of these evaluations are to be found in the appropriate monographs as indicated. IARC evaluated the carcinogenicity of 17β-oestradiol, progesterone and testosterone in 1974, 1979 and 1987. Zeranol (zearalenone) was evaluated in 1993, while MGA and TBA have not been evaluated. 17β-oestradiol and testosterone were classified in Group 2A, progesterone in Group 2B and Zeranol in Group 3. Oral contraceptives and post-menopausal hormone replace therapies have been evaluated on a number of occasions: combined oral contraceptives and post-menopausal oestrogen-progestogen therapy were evaluated in 1987 and 1999 (Group 1); progestogen-only contraceptives and post-menopausal oestrogen therapy were evaluated in 1999 (Group 2B).

IARC have made the following general conclusions on the carcinogenic potential of sex hormones (Monograph Vol 6 and 21):

- In humans, endogenous hormones are important in the initiation and progression of tumours. The incidence of tumours in humans could be altered by exposure to various exogenous hormones, singly or in combination.
- For an exogenous oestrogen to seriously perturb the normal hormonal environment, intake must be of the same order as, or greater than, the amounts of oestrogens produced endogenously.
- The majority of experimental animal studies with oestrogens, which have resulted in carcinogenesis, have been at very high levels.
- The mechanism(s) by which hormones induce cancer is not understood. Hormones may stimulate carcinogenesis in several ways and provide a background for subsequent tumorigenesis by chemical, physical or viral agents and promote the growth and metastasis of tumours once they have been initiated.

Summary of IARC evaluations of steroid hormones

Compound	Year	IARC Monograph	Classification	Conclusions
17b-Oestradiol	1974	6	-	Following sc injection or implantation, mammary, pituitary, uterine, cervical, vaginal and lymphoid tumours and interstitial-cell tumours of the testis occurred in mice. Mammary and pituitary tumours observed in rats. Malignant kidney tumours observed in male hamsters. Neonatal sc exposure in mice resulted in pre-cancerous and vaginal lesions later in life. Increased incidence of mammary and pituitary tumours in strains of mice having a spontaneous incidence of these tumours. Studies in monkeys were inadequate. No human case reports or epidemiology studies.
	1979	21	-	Sufficient evidence for the carcinogenicity of 17β-oestradiol in experimental animals [Subcutaneous administration increased the incidences of mammary, pituitary, uterine, cervical, vaginal and lymphoid and interstitial-cell tumours of the testis in mice. Mammary and/or pituitary tumours were increased in rats. Malignant kidney tumours occurred in intact and castrated male hamsters, and ovariectomised but not intact females. Uterine and abdominal lesions occurred in guinea pigs. A sc injection to neonatal mice resulted in pre-cancerous and vaginal lesions later in life. Teratogenic actions on the genital tract and possibly other organs, and impairment of fertility] No adequate human data, but reasonable to regard 17β-oestradiol as a carcinogenic risk to humans.
	1987	Suppl 7	Group 2A	Sufficient evidence for carcinogenicity to experimental animals [Increased incidences of mammary, pituitary, uterine, cervical, vaginal, testicular, lymphoid and bone tumours in mice. Increased incidence of mammary and/or pituitary tumours in rats. Malignant kidney tumours in males and in ovariectomised female hamsters. Fibromyomatous uterine and abdominal lesions were observed in guinea pigs.] No chromosomal aberrations in bone marrow cells of mice treated *in vivo*. Micronuclei but no aneuploidy, chromosomal aberrations or sister chromatid exchange in human cells *in vitro*. Aneuploidy and unscheduled DNA synthesis in rodent cells *in vitro*, but no mutagenicity, DNA strand breaks or sister chromatid exchange. Not mutagenic in bacteria. No data available on the genetic and related effects in humans.
Progesterone	1974	6	-	Increased incidence of ovarian, uterine or mammary tumours in mice. Progesterone increased the incidence of tumours produced by known carcinogens when given after their administration. No case reports or studies available in humans.
	1979	21	-	Limited evidence for the carcinogenicity of progesterone in experimental animals [Increased incidence of ovarian, uterine and mammary tumours in mice. Insufficient dog data. Neonatal exposure enhanced the occurrence of precancerous and cancerous lesions of the genital tract. Increased mammary tumourigenisis in female mice. No case reports or epidemiological studies available. No evaluation of the carcinogenicity of progesterone to humans.

Compound	Year	IARC Monograph	Classification	Conclusions
Progesterone	1987	Suppl 7	Group 2B	Sufficient evidence for carcinogenicity in experimental animals [Increased incidence of ovarian, uterine and mammary tumours in mice. Neonatal exposure enhanced the occurrence of precancerous and cancerous lesions of the genital tract and increased mammary tumorigenesis in female mice. Mammary gland nodules in dogs.] No human data on genetic and related effects. No evidence of genotoxicity.
Testosterone	1974	6	-	Uterine tumours in mice following sc implantation with testosterone. No adequate rat studies. Tumours in hamsters following combined oestrogen/testosterone treatment. No adequate epidemiological data.
	1979	21	-	Sufficient evidence for carcinogenicity in experimental animals [Cervical-uterine tumours in mice following sc implantation of testosterone propionate to mice. Prostatic adenocarcinoma in male rats. Neonatal treatment of female mice with testosterone (sc) induced lesions of the genital tract and increased mammary tumours in adults. Testosterone was embryolethal and caused virilisation in female offspring]. No adequate human data, but reasonable to regard testosterone as a carcinogenic risk to humans.
	1987	Suppl 7	Group 2A	Sufficient evidence for carcinogenicity in experimental animals [Same evidence cited as in 1979]. No sperm head abnormalities or micronuclei in mice. Not mutagenic in bacteria. No data on genetic and related effects in humans. Probably carcinogenic in humans [association between testosterone levels and prostate cancer risk].
Zearalenone (zeranol)	1993	56	Group 3	There is limited evidence in experimental animals for the carcinogenicity of zearalenone [Increased incidence of hepatocellular adenomas in female mice and of pituitary tumours in both sexes. No increased tumour incidence in rats. Oestrogenic effects in pigs and experimental animals. Chromosomal anomalies in cultured rodent cells, but no recombination in yeast or gene mutation or DNA damage in bacteria.] There is either inadequate or no evidence available in humans for the carcinogenicity of toxins derived from *Fusarium sp* (including zearalenone).

Summary of IARC evaluations of oral contraceptives and post-menopausal hormone replacement therapy

Compound	Year	IARC Monograph	Classification	Conclusions
Combined oral contraceptives	1987[1]	Suppl 7	Group 1	There is sufficient evidence that combined oral contraceptives cause benign and malignant liver tumours in humans. There is also conclusive evidence that these agents protect against cancers of the ovary and endometrium in humans. There is sufficient evidence for carcinogenicity to animals from progesterone in combination with 17β-oestradiol (neonatal exposure of mice to progesterone plus 17β-oestradiol resulted in an increased incidence of mammary tumours)
	1999[2]	Vol 72	Group 1	There is sufficient evidence in humans for the carcinogenicity of combined oral contraceptives, based on an increased risk of hepatocellular carcinoma (in the absence of hepatitis viruses). There is conclusive evidence that these agents protect against cancers of the ovary and endometrium in humans. There is sufficient evidence in experimental animals for the carcinogenicity of ethinyloestradiol + enthynodiol, mestranol + norethynodrel, ethinyloestradiol, mestranol, norethynodrel and lynoestrenol. There is limited evidence in experimental animals for the carcinogenicity of chlormadinone acetate, cyproterone acetate, ethynodiol diacetate, megestrol acetate, norethisterone acetate and norethisterone. There is inadequate evidence in experimental animals for the carcinogenicity of levonorgestrel and noregestrel.
Hormonal contraceptives, progestogens only[3]	1999	Vol 72	Group 2B	Inadequate evidence in humans for the carcinogenicity of progestogen-only contraceptives [No evidence of an increased risk of breast, cervical or liver cancer in women. No evidence of an increased risk of cutaneous malignant melanoma. Some evidence of a reduced risk of endometrial cancer. No data on genetic activity in humans.] There is sufficient evidence in experimental animals for the carcinogenicity of medroxyprogesterone acetate. There is inadequate evidence in experimental animals for the carcinogenicity of levonorgestrel.

Compound	Year	IARC Monograph	Classification	Conclusions
Post-menopausal oestrogen therapy[4]	1999	72	Group 1	There is sufficient evidence in humans for the carcinogenicity of post-menopausal oestrogen therapy [small increase in breast and endometrial cancer risk; no association with cervical, ovarian, liver and gall bladder, colorectal and thyroid cancer or cutaneous malignant melanoma]. There is sufficient evidence in experimental animals for the carcinogenicity of 17β-oestradiol and oestrone [Increased incidence of mammary, pituitary, uterine, cervical, vaginal, lymphoid and interstitial-cell tumours of the testis in mice following po dosing with oestradiol. Pituitary tumours in rats treated with oestradiol dipropionate. Malignant kidney tumours in castrated male rats and ovariectomised female rats treated with oestradiol. 4-hydroxy metabolites of oestradiol induced renal cell carcinomas in castrated male hamsters. Increased mammary tumours and pituitary tumours in mice following po or sc administration of oestrone. Implantation of oestrone resulted in malignant kidney tumours in male hamsters.] There is limited evidence in experimental animals for the carcinogenicity of conjugated equine oestrogens, equilin and oestriol. There is inadequate evidence in experimental animals for the carcinogenicity of d-equilenin.
Post-menopausal oestrogen-progestogen therapy	1987	Suppl 7	Group 3	Evidence in humans of a protective effect against endometrial cancer. Equivocal evidence for an effect on the incidence of breast cancer. No other relevant data available.
	1999	72	Group 2B	There is limited evidence in humans for the carcinogenicity of post-menopausal oestrogen-progestogen therapy. No real increase in cancer risk compared to oestrogen-only therapy. There is inadequate evidence in experimental animals for the carcinogenicity of conjugated equine oestrogens plus progestogen. Only one study available (equine oestrogens + medroxyprogesterone acetate) in female rats showed an increase in mammary tumours in the presence of a known mammary carcinogen. In ovariectomised cynomolgus monkeys, oestrogen-progestogen combination increased the incidence of mammary gland hyperplasia. No information available on the genotoxic effects post-menopausal oestrogen-progestogen therapy

1 = includes chlormadinone acetate + mestranol, chlormadinone acetate + ethinyloestradiol, ethynodiol + mestranol, ethynodiol + ethinyloestradiol, lynoestrenol + mestranol, megestrol acetate + ethinyloestradiol, norethisterone acetate + ethinyloestradiol, norethisterone + mestranol, norethynodrel + mestranol, norgestrel + ethinyloestradiol, progesterone + 17β-oestradiol; 2 = the oestrogen component is either ethinyloestradiol or mestranol, and the progestogens are either cyproterone acetate, desogestrol, ethynodiol diacetate, gestodene, levonorgestrel, lynoestrenol, megestrol, norethisterone acetate, norethynodrel, norgestimate and norgestrel; 3 = includes medroxyprogesterone acetate, levonorgestrel and progesterone; 4 = includes oestradiol, oestrone, conjugated equine oestrogens, equilin, oestriol and d-equilenin.

United States Food and Drug Administration (FDA)

The Centre for Veterinary Medicine (CVM) within the US Food and Drug Administration (FDA) is responsible for ensuring that veterinary drugs and medicated feeds are safe and efficacious for animals, and that foodstuffs derived from treated animals are safe for the consumer.

17β-oestradiol, progesterone, testosterone, MGA, TBA and zeranol are all registered in the USA for use as growth promotants in beef cattle[16]. Zeranol is also registered for use in sheep. With the exception of MGA, which is administered as a feed additive, all these hormones are formulated as implantable pellets that are injected subcutaneously under the skin of the animal's ear.

With regard to the 3 natural HGPs, the FDA has determined that when 17β-oestradiol, progesterone and testosterone are used in accordance with their approved conditions of use, concentrations in edible tissues remain within the normal physiological range determined for untreated age-and sex-matched cattle. Therefore the risk to the consumer from natural hormone residues in meat from treated cattle is considered to be negligible. The FDA has set allowable incremental residue levels for 17β-oestradiol, progesterone and testosterone in edible tissues from cattle and lamb (see Table below). These levels were set based on the conclusion that no harmful effects will occur in individuals chronically ingesting animal tissues that contain an incremental increase in endogenous steroid equal to 1% or less of the amount produced daily by the segment of the population with the lowest daily production. No residues resulting from the use of these compounds are permitted in excess of these increments above the concentrations naturally present in untreated animals.

Allowable incremental residue levels for 17b-oestradiol, progesterone and testosterone

Tissue	Residue (mg/kg tissue)		
	17b-oestradiol[1]	**progesterone**	**testosterone[2]**
In uncooked edible tissues of heifers, steers and calves			
Muscle	0.00012	0.003	0.00064
Fat	0.00048	0.012	0.0026
Kidney	0.00036	0.009	0.0019
Liver	0.00024	0.006	0.0013
In uncooked edible tissues of lambs			
Muscle	0.00012	0.003	-
Fat	0.00006	0.015	-
Kidney	0.00006	0.015	-
Liver	0.00006	0.015	-

1 = oestradiol and related esters; 2 = testosterone propionate; - = no tolerance set

The synthetic HGPs have been scientifically evaluated and ADIs and/or tolerances[17] set. The relevant FDA guideline for establishing a tolerance (now called a "safe concentration") can be found at http://www.fda.gov/cvm/guidance/guidline3pt4.html. A tolerance of 0.025 mg/kg has been established for MGA in the fat of cattle because it was concluded that residues of

[16] Code of Federal Regulations (CFR), Title 21, Part 522, 556 & 558.

[17] The tolerance or safe concentration of total residues is calculated based on the ADI and a food factor constant according to the following formula: Tolerance = [ADI (mg/kg bw/d) x 60 kg] ÷ (Food factor x 1.5 kg/d)], where 60 kg is the average adult human bw, 1.5 kg is the estimated daily food intake and the food factor is the proportion of daily food intake comprising of the particular tissue.

parent MGA at or below this level would not elicit a hormonal response. The tolerance for MGA corresponds to the limit of sensitivity of the analytical method.

The ADI for total TBO (presumably α– and β–trenbolone) is 0.0004 mg/kg bw/d, based on the NHEL of 0.04 mg/kg bw in a monkey study, and using a 100-fold safety factor. The ADI for total residues of zeranol is 0.00125 mg/kg bw/d, based on a NOEL of 0.125 mg/kg bw/d and a 100-fold safety factor. The safe residue levels of TBO and zeranol in uncooked edible cattle tissues are summarised in the Table below. It should be noted that zeranol residues are not permitted in uncooked edible tissues of sheep as determined by a specific method of analysis (see 21 CFR 556.760).

Safe residue levels (mg/kg) of TBA and zeranol in uncooked edible cattle tissues.

Compound	Muscle	Liver	Kidney	Fat
Trenbolone (α & β)	0.05	0.1	0.15	0.2
Zeranol	0.15	0.3	0.45	0.6

The Australian position

Introduction

HGPs have been approved for use in Australia since the mid 1970s. Implants containing 17β-oestradiol, progesterone, testosterone, TBA or zeranol, or various combinations thereof, are registered for use in cattle in all states and territories (it should be noted that MGA is not registered for use as a growth promotant in Australia). However, the Tasmanian Government moved to prohibit the use of HGPs in 2000 purely as a marketing strategy to gain access to niche markets such as Japan and Europe. This move has been criticised by other states because Australia already has an accreditation program to enable access to these HGP-free markets.

HGP Accreditation Program

As Australia does not ban the use of HGPs, it invests large sums of money into operating a hormone-free cattle program to accommodate the European market. This program controls and monitors the distribution and use of HGPs in meat production. The *HGP Free Accreditation Scheme*[18] is a national system that allows the full traceability of all animals within the scheme through the use of the National Livestock Identification Scheme (NLIS). Cattle within the HGP Free Accreditation Scheme must not have been treated with HGPs at any time in their lives. The seriousness of Australia's commitment to protecting European trade is evidenced from a recent case where a Victorian Company was fined by the APVMA for keeping inaccurate records regarding the supply of HGPs. The EC, as a consequence of its policy on HGPs, audits suppliers such as Australia, Canada and New Zealand. European auditors have inspected Australia in 1999[19] and 2000[20] and are due for another visit in 2003.

Public health considerations of HGPs by regulatory committees in Australia

A detailed history of the public health considerations of HGPs by regulatory committees in Australia, since 1969, is provided in Annex 3.

More recently, the Office of Chemical Safety's (OCS) Advisory Committee on Pesticides and Health (ACPH) has considered the human safety of HGP residues at its 21st to 24th meetings (May 2001 to October 2002; see Annex 4 for the extract minutes of these meetings). These considerations culminated in a position paper, which provided an overview of the issue and made some general conclusions on the human safety of HGPs (see Annex 5). The ACPH concluded that there is no appreciable health risk to consumers from eating meat from animals treated with registered HGPs according to GVP. They recognised that this conclusion was incompatible with the EC's position and supported the continuation of the current HGP Free Accreditation Program, as an interim or ongoing measure, to accommodate the European market. They decided to keep HGPs as a continuing agenda item so as to monitor international developments and scientific assessments. If there was any new evidence to

[18] HGP Free Accreditation Scheme Rules (July 2000) Australian Quarantine and Inspection Service (AQIS). Agriculture, Fisheries and Forestry Australia (AFFA).
[19] Final report of a mission carried out in Australia from 11 to 23 November 1999 relating to the production of fresh bovine meat for export to the European Union. DG/SANCO)/1170/1999 – MR final
[20] Final report of a mission carried out in Australia from 9 to 21 November 2000 in order to evaluate the operation of controls over residues in products of animal origin and over the production of fresh meat, game meat, and milk and milk products. DG(SANCO)/1217/2000 – MR final.

suggest that HGP residues were a health concern, there would be a recommendation that this issue be considered within the APVMA's Chemical Review Program.

Toxicological Assessments of HGPs

Toxicological assessments of MGA, TBA and zeranol have been conducted by OCS of the Therapeutic Goods Administration (TGA) within the Department of Health and Ageing as part of the approval process for product registration, administered by the Australian Pesticides and Veterinary Medicines Authority (APVMA). However, it should be noted that there are currently no MGA products registered for use in Australia; APVMA considered a registration application for a liquid pre-mix but it was withdrawn and the product never registered. Toxicological assessments of 17β-oestradiol, progesterone and testosterone for the purposes of growth promotion in food-producing animals have not previously been conducted by Australian regulatory agencies.

Australian ADIs and/or MRLs for HGPs are summarised in the Tables below and are compared with corresponding JECFA values. The absence of Australian ADIs and MRLs for 17β-oestradiol, progesterone and testosterone is due to their exemption from the MRL standard because any residues are identical to, or indistinguishable from, natural food components.

Summary of Australian ADIs (mg/kg bw/d) for HGPs

Compound	TGA	JECFA
17β-oestradiol	-	0-0.00005
Progesterone	-	0-0.03
Testosterone	-	0-0.002
MGA	0.00005	0-0.00003
TBA	0.0001 (α-trenbolone) 0.00001 (β-trenbolone)	0-0.00002[1]
Zeranol	0.0002	0-0.0005

1 = The JECFA ADI covers both the parent molecule (trenbolone acetate) and the two main metabolites (α- and β-trenbolone).

Summary of Australian MRLs (mg/kg) for HGPs

Compound	APVMA	JECFA
17β-oestradiol	-	#
Progesterone	-	#
Testosterone	-	#
MGA	*	0.002 bovine liver (temporary) 0.005 bovine fat (temporary)
TBA	0.002 cattle meat 0.01 cattle, edible offal of	0.002 muscle (β-trenbolone) 0.01 liver (α-trenbolone)
Zeranol	0.005 cattle meat 0.02 cattle, edible offal of	0.002 bovine meat 0.01 bovine liver

= Residues generated according to GVP were considered unlikely to adversely effect human health; * = There are no registered products in Australia containing MGA, hence no need for any MRLs; the APVMA did consider a registration application but it was withdrawn and the product never registered. The ADI was established during the evaluation of that application.

MGA was evaluated by the OCS in 1999. An ADI of 0.0005 mg/kg bw/d was set based on the hormonal NOEL of 0.005 mg/kg bw/d for hormonal and menstrual cycle variables

[reduced luteinising hormone (LH) and peak 17β-oestradiol; increased menstrual cycle length) at higher doses in a monkeys over 3 successive menstrual cycles, and using a 100-fold safety factor.

TBA was evaluated by the OCS in 1984, 1988, 1990, 1991, 1992 and 1993. ADIs for α-trenbolone and β–trenbolone of 0.0001 and 0.00001 mg/kg bw/d were set in 1988 based on NOELs of 0.01 and 0.001 mg/kg bw/d, respectively for hormonal effects in pigs, and using a 100-fold safety factor (the β-epimer has approximately 10 times more hormonal activity than the α–epimer). TBA was determined to have low acute oral toxicity (LD_{50}s >1000 mg/kg bw). Hormonal effects were typical in subchronic, chronic and reproduction studies conducted in laboratory animals. There was no evidence that TBA was genotoxic.

Zeranol was evaluated by the OCS in 1985, with supplementary data considered in 1986 and 1988. The ADI of 0.0002 mg/kg bw/d was set in 1988 based on a hormonal NOEL of 0.015 mg/kg bw/d in a 2-year rat study for the presence of cervical stratified squamous epithelium at the next highest dose, and using a 100-fold safety factor. This NOEL was comparable to that established in a 3-month monkey study (0.05 mg/kg bw/d for perineal skin changes and withdrawal bleeding in ovariectomised monkeys) and a 2-year mouse study (0.02 mg/kg bw/d for bw effects at the next highest dose)

Monitoring of HGP residues in Australian animal commodities - National Residue Survey

The Australian National Residue Survey (NRS)[21] is a program conducted by Agriculture, Fisheries and Forestry-Australia (AFFA) and was established in the early 1960s. The NRS conducts monitoring surveys in addition to surveillance, compliance testing and residue prevention projects. A range of commodities is covered by NRS monitoring surveys including animal, plant and selected fisheries and aquaculture products. The primary function of the NRS is to monitor chemical residues and environmental contaminants in the products of participating industries.

A range of compounds is analysed in random samples from food-producing animals including hormones, non-steroidal anti-inflammatories, β-agonists, pesticides, antimicrobials, antihelminthics and metals. While the majority of samples are obtained from cattle, sheep and pigs, samples from poultry, horse, goat, deer, ostrich, emu, game pig, kangaroo, possum, camel and buffalo are also analysed.

The annual results of the NRS from 1998 to 2002 revealed no evidence of the illegal use of hormones for growth promoting purposes, including MGA, TBA and zeranol. (annual NRS survey results can be found at the AFFA website listed in the footnotes). Illegal use covers the use of an unregistered compound, the off-label use in other animal or an exceedance of the MRL.

No MGA residues were detected in fat samples from cattle. TBA was detected in 8/310 liver samples from cattle during the 2001-2002 survey, but at unspecified levels below the MRL. The detection of TBA residues in cattle during previous surveys was restricted to a small number of faecal and urine samples (1/216 faecal and 141 urine samples in 1998-1999 and 2/73 urine samples in 1999-2000). Zeranol has been detected in cattle, sheep and poultry, predominantly in faecal and urine samples. However, in all cases residues were detected in

[21] http://www.affa.gov.au/nrs

association with metabolites of zearalenone and were therefore attributable to the natural intake of *Fusarium* spp.-infected pasture, grain or plant material containing zearalenone. During 1998-1999, 25/301 cattle liver samples tested positive for zeranol/zearalenone but at levels below the MRL (unspecified). A new analytical method was apparently utilised during the 1999-2000 survey that differentiated various zearalenone metabolites; 6/159 liver samples tested positive for zearalenone, 0/159 for α–zearalanol (zeranol), 0/59 for β-zearalanol (taleranol), 9/159 for α-zearalenol and 16/159 for β-zearalenol. Only 1/324 liver samples tested positive for zeranol during 2000-2001, while no residues were detected during 2001-2002.

DISCUSSION

Introduction

The current review was undertaken to examine the international status of considerations of the human safety of HGP residues in cattle, and to independently evaluate the EC's opinion on the risk to human health from consuming HGP residues, with particular emphasis on their most recent risk assessment (April 2002). The intent was not to undertake a detailed toxicological evaluation of 17β-oestradiol, progesterone, testosterone, MGA, TBA and zeranol, but rather to independently review and critique those studies commissioned or cited by the EC in their latest risk assessment. The objective was to determine whether there is any scientific evidence, beyond that already evaluated nationally and internationally, to indicate a need for a review of HGPs by Australian regulatory agencies via the APVMA's Chemical Review Program. It should be noted that Australia has a long history of regulatory considerations of HGPs (see Annex 3).

All of the studies evaluated as part of this review were accessible only as published scientific papers, the main limitation of which was the general lack of reporting detail. Consequently, a truly independent evaluation of the original data was not possible. Detailed evaluations of the EC-commissioned and EC-cited studies are contained in Annex 1.

The genotoxic and carcinogenic potential of HGPs

One of the main health concerns associated with the use of HGPs is their genotoxic and carcinogenic potential. While the ability of certain hormones to promote carcinogenesis via epigenetic mechanisms is scientifically accepted, the idea that hormones like 17β-oestradiol should be considered as complete carcinogens[22] is a new concept to arise from the EC's risk assessments.

There was very little new evidence presented by the EC regarding the genotoxic or carcinogenic potential of progesterone, testosterone, MGA, TBA or zeranol. *In vitro* studies indicated that MGA, TBA, zeranol and their metabolites were not mutagenic in bacterial and mammalian cells (up to 150 or 400 µM and 125 or 1500 µM, respectively), while only marginally positive results were obtained in an *in vitro* micronucleus test at near cytotoxic concentrations (Metzler & Pfeiffer 2001). 17β-Trenbolone was marginally positive in a ^{32}P-post labelling assay at 30 µM. Marginal evidence of genotoxicity was determined for metabolites of TBA and zeranol generated by liver microsomes, but only at cytotoxic concentrations (Metzler & Pfeiffer 2001). A conference abstract (Kranz et al 2002) reported the formation of DNA adducts in rat liver slices following MGA treatment, but in the absence of any data, this finding was considered to have limited value. An unpublished EC study (no. 4), which was not evaluated due to the unavailability of the data, reportedly found that MGA was not genotoxic up to 400 µM. EC-commissioned scientists (Metzler & Pfeiffer 2001) concluded that there is presently no evidence that MGA, TBA or zeranol residues in meat of treated animals pose a genotoxic risk to the consumer.

Most of the focus of the EC has been on 17β-oestradiol, which it considers to be a complete carcinogen. The EC stated that "additional and conclusive data have now been published in the scientific literature to demonstrate that 17β-oestradiol is genotoxic". This data included:

[22] A complete carcinogen is a substance capable of both initiating and promoting cancer

(1) a hypothetical model to explain the genotoxicity of 17β-oestradiol via the formation of genotoxic metabolites and reactive oxygen species (ROS); (2) tissue levels of potentially genotoxic metabolites; and (3) *in vitro* and *in vivo* evidence of the genotoxicity of 17β-oestradiol and some of its catechol metabolites.

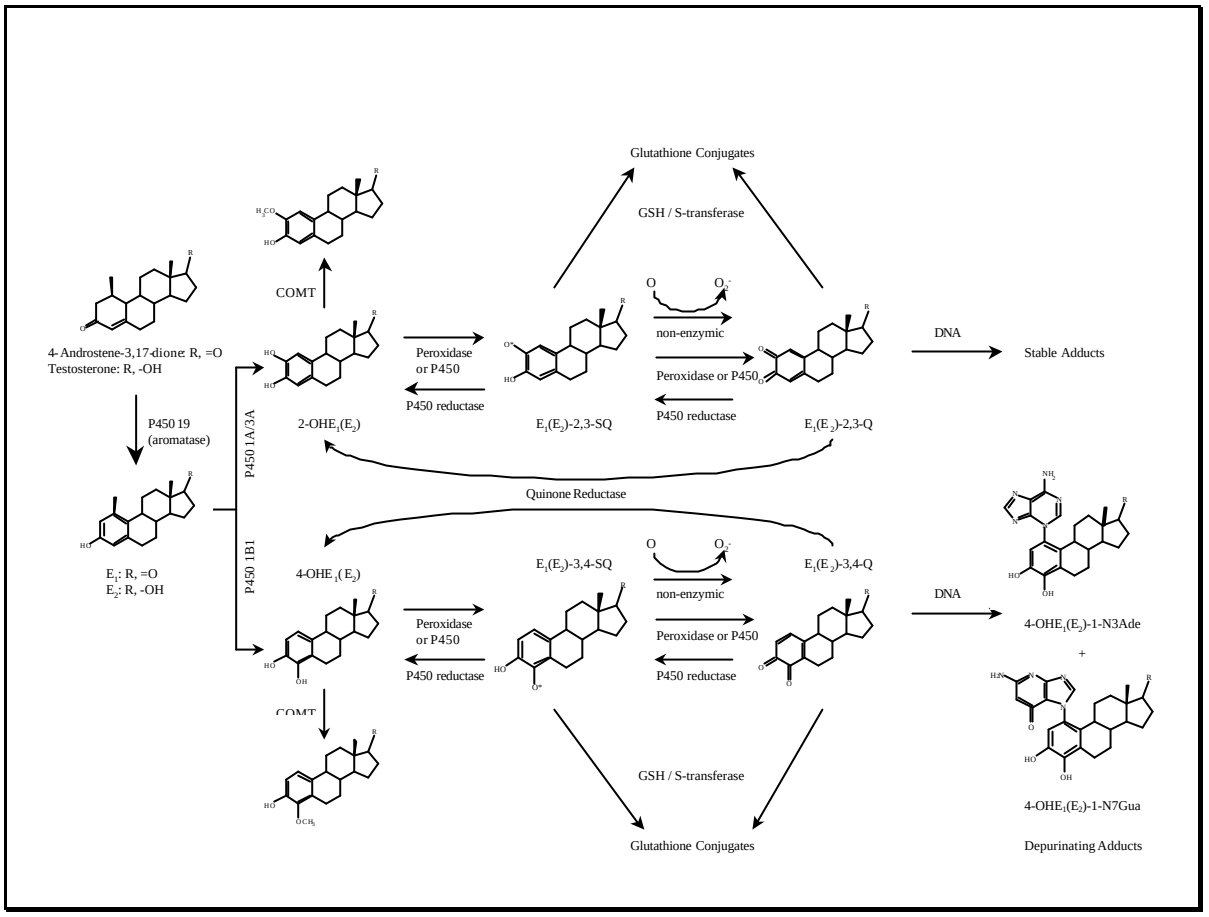
(1) Model for the genotoxicity of 17b-oestradiol

Cavalieri et al (2000) hypothesised that the mechanism of 17β-oestradiol-induced carcinogenicity is via the generation of genotoxic catechol oestrogen metabolites (see Figure overleaf). Briefly, endogenous 17β-oestradiol and oestrone are metabolised to 2- and 4-catechol oestrogens (ie. 2- and 4-hydroxy-17β-oestradiol/oestrone). If the 4-catechol oestrogens are further oxidised to catechol oestrogen-3,4-quinones, they can react with DNA to form depurinating adducts. These adducts can generate apurinic sites, which may initiate cancer. The metabolism of catechol oestrogens also generates ROS that may also damage genetic material. While this model is plausible, its main weakness is the lack of supporting experimental evidence that 17β-oestradiol is actually mutagenic. However, it was argued that previous bacterial and mammalian cell mutation assays were insufficiently sensitive to detect the weak mutagenic activity of oestrogens (Cavalieri et al 2000). Subsequently, Liehr (2001) suggested that new assays might be required to detect weak mutagens. There is some evidence (see below) that catechol oestrogen quinones can generate DNA adducts *in vitro*, but there is no evidence that these occur *in vivo* at levels that would outstrip normal DNA repair mechanisms. Furthermore, there is no evidence that catechol oestrogen quinones are generated at sufficiently high enough levels *in vivo* to cause genotoxicity.

An extension of the above model is the role that various endogenous cytochrome P450s [eg. aromatase, 17β-oestradiol-4-hydroxylase (CYP1β1)] might play in oestrogen-induced carcinogenesis. Jefcoate et al (2000) argued that overexpression of aromatase in combination with the presence of 4-hydroxylases could generate physiologically and pathologically significant levels of oestrogen metabolites. While there is an apparent link between increased aromatase expression and breast cancer development, no data were provided to show that increased aromatase expression or CYP1β1 actually leads to increased tissue levels of catechol oestrogen metabolites or ROS.

(2) Metabolite profiles in tissues

Various oestrogen metabolites have been detected in the rat prostate following ip administration of 4-hydroxyoestradiol or 17β-oestradiol-3,4-quinone (6 µmol/100 g bw), but the role they might play in prostate cancer development was undetermined (Cavalieri et al 2002). The metabolite profile of mammary tumour and hyperplastic tissues from oestrogen receptor-α knockout mice included various levels (pmol/g tissue) of catechol oestrogen metabolites and their glutathione and cysteine conjugates (Devanesan et al 2001). However, these levels were variable and further studies would need to be undertaken to determine the possible role oestradiol metabolites might play in mammary carcinogenesis in mice.



Proposed model for the genotoxicity of 17b-oestradiol (Cavalieri et al 2000) [E_1 = oestrone, E_2 = 17β-oestradiol, E_3 = oestriol; 4-OHE_1 = 4-hydroxyoestrone; 4-OHE_2 = 4-hydroxy-17β-oestradiol; 2-OHE_1 = 2-hydroxyoestrone; 2-OHE_2 = 2-hydroxy-17β-oestradiol; 4-OCH_3E_1 = 4-methoxyoestrone; 4-OCH_3E_2 = 4-methoxy-17β-oestradiol; 2-OCH_3E_1 = 2-methoxyoestrone; 2-OCH_3E_2 = 2-methoxy-17β-oestradiol; SQ = semiquinone, Q = quinone; ROS = reactive oxygen species]

(3) In vitro and in vivo evidence for the genotoxicity of 17b-oestradiol and its metabolites

Based on the model proposed by Cavalieri et al (2001), the genotoxic potential of 17β-oestradiol appears to depend, to a large extent, on the formation of catechol oestrogen metabolites, such as 4-hydroxyoestradiol. An incorrect assumption would be that evidence of genotoxicity of a particular metabolite indicates that the parent compound is genotoxic.

A number of *in vitro* studies suggested that certain oestradiol metabolites have genotoxic potential. Kong et al (2000) concluded that 17β-oestradiol was weakly mutagenic based on a marginal increase in the frequency of *hprt* mutations in mammalian cells at 1 µM. In view of the observations that there was no effect at lower concentrations, that the mutation frequency was markedly lower than the positive control, and the absence of any evidence that this was a reproducible effect, the significance of this finding was unclear. The EC cited Hoogenboom et al (2001) as evidence that oestrogen metabolites are genotoxic. However, this study only examined the oestrogenic activity of 17β-oestradiol and oestrone, and therefore can not be used to conclude that oestradiol metabolites are genotoxic.

Experiments conducted by Tsutsui et al (2000a) on Syrian hamster embryo cells (SHE) indicated that 2-hydroxyoestrone, 4-hydroxyoestrone and 4-hydroxyoestradiol inhibited cell

growth, caused cytotoxicity and induced cellular transformation and chromosomal aberrations, at 3-10 µg/mL (~10-30 µM). 17β-Oestradiol, oestrone and their catechol metabolites were found to induce aneuploidy in a concentration-dependent manner over a concentration range of approximately 1-10 µg/mL (~4-30 µM). With the exception of 4-hydroxyoestradiol, which caused a significant increase in mutations at the Na/K ATPase locus, 17β-oestradiol and its metabolites were not mutagenic. While this study suggested that some metabolites of E_2 have genotoxic potential, the toxicological significance of these findings was unclear due to the concomitant occurrence of cytotoxicity, and the lack of a positive control. In a second study by Tsutsui et al (2000b), cell transformation, chromosomal aberrations and aneuploidy were induced in SHE cells treated with 2-methoxyoestradiol[23] at cytotoxic concentrations [$\geq$ 0.3 µg/mL (~1 µM)].

The *in vitro* and *in vivo* formation of DNA adducts has been considered as evidence for the genotoxic potential of 17β-oestradiol. The problem with this view is that studies using high concentrations of presumed metabolites are not in themselves evidence that the parent compound is or could be genotoxic.

N3ade and N7gua adducts of 4-hydroxyoestradiol have been detected in mouse skin following direct application of 200 nmol/50 µL 17β-oestradiol-3,4-quinone (Chakravarti et al 2001). However, the relevance of this finding to the dietary intake of 17β-oestradiol in meat is unclear. A study reporting mutations in simian kidney cells following site-specific mutagenesis with N2gua or N6ade adducts of 2-hydroxyoestradiol or 2-hydroxyoestrone (Terashima 2001) did not indicate that such mutations could be generated *in vivo* or following treatment with 17β-oestradiol. DNA adducts were formed in SHE cells following treatment with cytotoxic concentrations of catechol oestrogen metabolites [$\geq$1 µg/mL (~ 3 µM)], however, neither 17β-oestradiol or oestrone were cytotoxic or generated adducts at concentrations up to 10 µg/mL (~30 µM)(Yagi et al 2001).

The incidence of uterine adenocarcinomas in mice was increased following neonatal exposure to 4-hydroxyoestradiol (66%), while in comparison, the incidence following exposure to 17β-oestradiol was relatively low (7%) (Newbold & Liehr 2000). The control incidence was 0%. While the possible mechanism of this increase with 4-hydroxyoestradiol was not defined, it was associated with oestrogenicity (increased uterine weight) and therefore could well have been due to an epigenetic mechanism. Other studies have also suggested that the genotoxic potential of 4-hydroxyoestradiol is related to its oestrogenic activity (Hoogenboom et al 2001), while Kong et al (2000) suggested that mutagenic activity is not associated with binding to the ERα .

All of these findings need to be balanced against the fact that there are various biochemical mechanisms to prevent the formation of potentially genotoxic oestrogen metabolites, and to remove DNA adducts that might be formed. It would only be when these mechanisms are overloaded that any adverse effects might occur. The formation of glutathione, cysteine and N-acetyl-cysteine conjugates of 2- and 4-hydroxyoestradiol/oestrone, and subsequent excretion, is an important part of this mechanism; glutathione depletion was found to generate N7gua adducts of 17β-oestradiol/oestrone-3,4-quinone in the kidney but not the liver of male Syrian golden hamsters following treatment with 17β-oestradiol (Cavalieri et al

[23] 2-methoxy-17β-oestradiol is generated from the conversion of 2-hydroxy-17β-oestradiol by catechol-*O*-methyltransferase (COMT)

2001). Inhibition of catechol-*O*-methyltransferase (COMT) (which catalyses the conversion of 2- and 4-hydroxyoestradiol/oestone to the water soluble, more readily excretable 2- and 4-methoxyoestradiol/oestrone) caused an increase in oxidative DNA damage in MCF-7 cells that had been incubated with 17β-oestradiol (Lavigne et al 2001). This DNA damage appeared to be attributable to the increased formation of 2-hydroxyoestradiol. It should be noted that in the absence of COMT inhibition, no oxidative DNA damage was detected in MCF-7 cells treated with up to 10 μM 17β-oestradiol (Lavigne et al 2001). These findings indicate that only significant disruption of normal oestrogen metabolism may generate potentially genotoxic metabolites.

Endocrine disrupting potential of HGPs

Besides carcinogenicity, the other significant health concern associated with the use of HGPs is their ability to cause imbalances in hormone-mediated biological events such as the development of the foetus or sexual development in prepubertal children. However, exogenous hormones would need to reach a level equal to or greater than endogenous hormones in order to perturb homeostasis (IARC Monograph 6 & 21).

In vitro studies

Measurement of the oestrogenic potency of HGPs has been performed using ligand binding (Bauer et al 2001) or gene expression assays (Le Guevel & Pakdel 2000; Hoogenboom et al 2001, Feffers et al 2001). One of the difficulties in interpreting the overall result of these assays is that the magnitude of oestrogenicity relative to 17β-oestradiol appeared to be dependent on the assay type (Le Guevel & Pakdel 2000). Furthermore, these types of *in vitro* assay do not allow the determination of the biological consequence of the binding event or the modulation of gene expression (eg. cell transformation).

TBA, MGA and a variety of natural and synthetic steroid hormones were found to bind to the androgen receptor (AR), human sex-hormone binding globulin (hSHBG) and the bovine uterine progestin receptor (bPR) (Bauer et al 2000). 17β-TBO was found to have the same or slightly higher affinity than dihydrotestosterone (DHT) and progesterone for the AR and PR, respectively, while the affinity of MGA for the PR was approximately 5-fold higher than progesterone. However, Bauer et al (2000) concluded that TBA residues with significant binding capacity and potential endocrine-disrupting activity would actually be excreted from cattle. *In vitro* analysis of the oestrogenic activity of HGPs revealed that 17β-oestradiol was the most potent of zeranol, testosterone, TBO, TBA, MGA and zearalenone (Le Guevel & Pakdel 2001). 17β-oestradiol was found to be generally more potent than zeranol at inducing oestrogen responsive genes in MCF-7 cells (Leffers et al 2001). The exception to this finding was in relation to the expression of *the MRG1/p35srj* gene, which appeared to be more sensitive to zeranol than to 17β-oestradiol.

In vivo studies

Treatment of cattle with the recommended dose of MGA caused elevations in plasma and tissue levels of 17β-oestradiol and plasma levels of luteinising hormone (LH), while higher (off-label) doses actually reduced 17β-oestradiol and LH levels (Hageleit et al 2000). The elevation in 17β-oestradiol levels had no effect on the number or size of ovarian follicles or the presence of the corpus luteum. Zeranol was shown to have relatively little effect on the

tissue specific expression pattern of ERα and ERβ mRNA in cattle at up to 10-fold the recommended dose, despite obvious oestrogenic effects (decreased uterine weight and effects on the oestrus cycle) (Pfaffl 2001).

In order for HGP residues to potentially interfere with the developing foetus, they must be able to cross the placenta. A study conducted in rabbits found that MGA, TBA and zeranol can indeed cross the placenta and were detected in plasma and tissues (Lange et al 2002). The levels of these hormones in foetuses were lower than in dams. An unpublished part of EC study 11 concluded that prenatal exposure to low doses of MGA, TBA or zeranol may affect the function of the male reproductive tract in rabbits. In the absence of data, giving details about the findings, no comment can be made regarding this conclusion.

Human data

The EC reported that 3 studies suggested that *in utero*, pre- and perinatal exposure to hormones might affect pubertal development. It should be noted that only 2 'studies' had been conducted and one of these was actually a review. In this review (Chiumello et al 2001), it was argued that hormones ingested in meat can cause gynecomastia, however no data was actually provided and there was no evidence cited to suggest that currently registered HGPs cause adverse health effects in humans. The majority of the paper was focussed on the human health effects of the banned hormone DES. Furthermore, a range of other causative factors were also considered, including genetics, diet, age and environment contaminants.

A Swedish study concluded that among female twins with male co-twins, high birthweight constitutes an independent risk factor for breast cancer (Kaijser et al 2001). The manner in which this finding relates to hormone exposure is that unspecified previous evidence had suggested an association between birthweight and endogenous antenatal oestrogen exposure. However, the authors' conclusion that *in utero* exposure to hormones influences breast cancer risk was considered to be unsubstantiated because the study did not directly test an association between oestrogen exposure and cancer risk and no data were provided or cited to substantiate any link between oestrogen exposure and high birthweight.

Oestrogenicity of lipoidal esters of 17b-oestradiol

The EC concluded that lipoidal esters of 17β-oestradiol show a high oral bioavailability in rodent experiments and are more potent than 17β-oestradiol. No studies were commissioned or cited by the EC to support the claim that lipoidal esters of 17β-oestradiol have high oral bioavailability. Furthermore, only marginal differences in oestrogenic activity (measured as an increase in uterine weight) were observed in rats following po, sc or iv dosing with 17β-oestradiol or 17β-oestradiol-17-stearate (Larner et al 1985; Paris et al 2001). Marginal differences in uterine weight and the occurrence of histopathological uterine and vaginal abnormalities occurred following oral dosing at and above 250 nmol/kg bw/d for 6 days (Paris et al 2001). The kinetics of distribution of both compounds to the uterus was also different, with 17β-oestradiol-17-stearate having a more prolonged oestrogenic action than 17β-oestradiol (Larner et al 1985). Another study (Mills et al 2001) revealed differential effects on cell proliferation, with 17β-oestradiol causing greater stimulation of uterine endometrial cell proliferation than oestradiol 17β-oestradiol-17-stearate, and 17β-oestradiol-17-stearate inducing greater mammary glandular cell proliferation than 17β-oestradiol.

The EC concluded that lipoidal esters of oestradiol should be taken into account as part of the risk assessment of HGPs because they may contribute to a "sizeable" portion of the total residue level and therefore add to oestrogen exposure. It should be noted that lipoidal esters of oestradiol are found naturally in untreated cattle, and that levels measured in treated cattle were found to be variable (Maume et al 2001). Therefore, further studies using greater numbers of animals would need to be conducted in order to determine the possible contribution of lipoidal esters to total hormone exposure. Furthermore, studies remain to be conducted on the bioavailability of lipoidal esters in humans and whether they can in fact accumulate in tissue.

Residue levels in treated *versus* untreated cattle

The analysis of residues in treated *versus* untreated cattle is an obvious requirement for the human risk assessment of HGPs. An important component of this analysis is the development of suitable test methods, a number of which have recently been developed (Le Bizec 2000; Marchand et al 2000; Stephany 2000 & 2001; unpublished EC study 1). However, the EC reported that these as yet have not been validated.

The EC drew attention to the importance of measuring oestradiol metabolites as well as parent 17β-oestradiol/oestrone. They indicated that previous analytical techniques, which only focussed on major readily extractable metabolites, might have under measured oestrogen exposure. Available results from Hoogenboom et al (2001) and unspecified earlier studies found that 17β-oestradiol and oestrone were the main metabolites and that no catechol metabolites were detectable.

The exceedance of an MRL is the only satisfactory criterion for assessing whether HGPs have been misused and whether there may be a risk of adverse health effects in consumers. However, the exceedance of an MRL is not in itself an indication of a threat to human health; this can only be concluded following the determination of the dietary intake of a HGP residue and comparing it to the ADI. The detection of residues of registered HGPs at levels below the MRL indicates that they are being used properly and would not pose a threat to human safety. In Australia, the NRS conducted annually from 1998 to 2002 did not find any evidence of the illegal use of MGA, TBA or zeranol for growth promoting purposes.

The EC commissioned a number of studies to analyse residues in various cattle tissues following implantation with commercial anabolic agents. The underlying assumption of the EC was that registration of HGPs would inevitably lead to misuse. As a consequence, most of the studies adopted scenarios of misuse or off-label use such as implantation at sites other than the ears, or overdosing. The EC concluded that there is the risk of excessive exposure from misplaced or off-label implants and incorrect dosing regimes (EC study 5; Daxenberger et al 1999; Hageleit et al 2000 & 2001).

The proper placement (ie. in the ear) of certain commercial anabolic preparations resulted in apparent dose-related increases in 17β-oestradiol, its metabolites or conjugates in liver, kidney and fat (Lange et al 2001; Maume et al 2001). Levels in these tissues appeared to be higher than the controls at and above the recommended dose. In contrast, such an effect was not apparent in muscle, where the level of 17β-oestradiol was similar to the controls following administration of the labelled dose. The EC concluded that tissue levels of 17β-oestradiol may be higher in treated *versus* untreated cattle and that the level depends on the treatment regime and hormone level in the implants. A major deficiency in the majority of

residue studies, which limited their value, was the low group sizes (1-2 animals/group) and the high level of variability between animals. Therefore, most studies would need to be repeated to confirm their findings.

In cattle, the highest tissue levels of 17β-oestradiol, TBA, testosterone and zeranol following implantation with various commercial anabolic preparations were generally in the liver, then the kidney, fat and muscle (Lange et al 2001; Maume et al 2001). MGA appears to preferentially distribute to fat in cattle (Daxenberger et al 1999) and rabbits (Lange et al 2002). While it is plausible that MGA could accumulate in fat as suggested by some scientists (Daxenberger et al 1999; Lange et al 2002), studies examining the kinetics of tissue distribution and elimination are necessary before this conclusion can be justified.

The major finding from residue analyses in cattle is that proper use of registered HGPs (as per label directions) does not generate violative levels of residues (ie. above MRLs) (Daxenberger et al 2000; Daxenberger et al 2001; Lange et al 2001). In fact, off-label use of zeranol and testosterone at up to 10-fold the label dose did not lead to violations of Codex MRLs or FDA allowable residue levels (Lange et al 2001).

The importance of GVP in the use of HGPs was evidenced from a study where approximately 30% of implants could not be found following implantation at off-label sites in cattle (Daxenberger et al 2000). In addition, significant levels of hormone residues remained in the implants at slaughter (8 weeks), which could potentially pose a risk to consumers if inadvertently processed in to food. A similar finding occurred in a study conducted on boars (Daxenberger et al 2001). These findings support the current implantation and management protocol for HGPs in countries where they are registered for use. This protocol involves the implantation in to the ear, which is discarded at slaughter, thereby preventing hormone residues in the remaining pellet from entering the food supply.

Biotransformation of HGPs

The biotransformation of 17β-oestradiol or oestrone to catechol oestrogens and catechol oestrogen quinones has been previously discussed. The EC reported an unpublished study on the oxidative and conjugative metabolism of MGA, TBO and zeranol by liver microsomes and/or slices. The EC concluded that the metabolism of these compounds is more complex than previously recognised and that metabolite profiles in humans should be established. In the absence of an evaluation of the data, no comment can be made regarding this conclusion. It is agreed that studies examining the *in vivo* metabolism of HGPs would provide useful information on the types and concentrations of metabolites generated. However, the analysis of HGP metabolism in its own right would not indicate that these metabolites cause adverse health effects.

Dietary intake of hormones

One of the difficulties in assessing the risk to consumers from ingesting exogenous hormones in meat is the fact that humans consume a diet already rich in hormonally-active compounds. For example, the level of 17β-oestradiol in an egg is greater than that in meat from both treated and untreated cattle (median levels of 6.5 ng/50 g egg, 2.5 ng/250 g untreated cattle meat and 5 ng/250 g treated cattle meat) (Stephany 2000 & 2001). Studies have also confirmed that zeranol residues occur naturally in untreated cattle (Lange et al 2001; Pfaffl 2001) due to the ingestion of *Fusarium*-contaminated feed (eg. grain, pasture). Therefore the

incremental risk associated with the consumption of meat from HGP-treated cattle can only be evaluated by placing it in the context of the total dietary intake of hormones from all sources. Stephany (2000 & 2001) recommended that hormone intake in meat and meat products, poultry, milk, dairy products, eggs, fish and fish products should be determined to assess this total dietary risk from hormones. This approach was reiterated by Jegou et al (2001).

In terms of actual dietary intake of HGPs, EC scientists have calculated that the theoretical intake of oestradiol in a 500 g portion of meat from both treated (implanted with pellet containing 17β-oestradiol + TBA) and untreated steers was well below the JECFA ADI for 17β-oestradiol (0.2 and 1.3%, respectively), while even overdosing cattle with 4 implants of Revalor®-S resulted in 17β-oestradiol levels that only reached 5% of the ADI (Maume et al 2001). At its 52nd Meeting (1999), JECFA examined residue data and performed dietary intake calculations to obtain conservative theoretical estimates of possible excess of dietary intake in persons who consume large amounts of meat (eg. 500 g/d) obtained from treated cattle. For total oestrogens, the highest excess intake was 30-50 ng/person/d, which is less than 2% of the ADI for 17β-oestradiol. For progesterone, the highest intake was approximately 500 ng/person/day, which is approximately 0.03% of the ADI. For testosterone, the highest intake was approximately 60 ng/person/d, which is approximately 0.05% of the ADI.

An important factor in considering the dietary risk associated with HGP residues is their oral bioavailability. At their 52nd Meeting, JECFA (1999) concluded that 17β-oestradiol, progesterone and testosterone have low oral bioavailability due to gastrointestinal and hepatic inactivation. The oral bioavailability of 17β-oestradiol was approximately 5% in young women (Kuhnz et al 1993), while JECFA concluded that the bioavailability of progesterone in humans was less than 10%. The bioavailability of testosterone was approximately 4% in young women (Tauber et al 1986). There is relatively little data on the bioavailability of the synthetic HGPs, although Lone (1997) reported that TBA is inactive via the oral route. The low oral bioavailability, particularly of the natural HGPs, indicates that it can not be assumed that HGP residues in food will actually be available *per se*.

Environmental effects of HGPs

An EC-commissioned study provided information on the stability and possible persistence of TBA and MGA in the environment following excretion from cattle (Schiffer et al 2001). A largely unpublished study, which was mentioned in two journal articles (Jegou et al 2001; Orlando & Guillette 2001), alleged that there were population effects in fathead minnow exposed to cattle ranch effluent. While these types of studies may assist the assessment of the environmental impact of hormones, they do not have any direct value in assessing the dietary risk to consumers from hormones in meat.

The precautionary principle

A new issue to arise from the EC's assessments from a scientific perspective is that while HGP exposure may be quite small, the excess or incremental risk associated with carcinogenicity cannot be addressed because of the possibility that such substances may initiate carcinogenesis via a genotoxic mechanism. Due to insufficient information available

to make an assessment of that incremental risk, the EC had adopted the *precautionary principle*[24].

This precautionary approach is reflected by the authors in several of the studies commissioned or cited by the EC, despite the absence of any findings that would suggest that HGPs pose a hazard to human health. Metzler and Pfeiffer (2001) concluded that there is presently no evidence that MGA, TBA or zeranol residues in meat of treated animals pose a genotoxic risk for the consumer, however, they maintained that the lack of information on the metabolites does not necessarily mean that the parent compounds are safe. Sasco (2001) recommended the adoption of the precautionary principle until a better understanding of the endocrine events involved in puberty and fertility is obtained in order to determine the role of hormone pathways in the occurrence and development of cancer. Although there is no evidence that significant levels of oestradiol metabolites are found in meat or are generated in humans, some have argued that even small amounts may trigger a "chain reaction" via the generation of DNA reactive species in a futile redox cycle even without any experimental evidence to support this proposal (Cavalieri et al 2002).

The illegal use of HGPs

A factor that has at times exacerbated the HGP debate is the issue of the misuse of existing registered products, combinations of registered products that were never intended to be combined, and also the use of unregistered agents or those that have been withdrawn from the market, such as DES. It is sometimes the perception of consumers that unregistered products are used widely, or that unapproved doses and combinations are used. This perception has periodically caused regulatory bodies a degree of concern and at the same time reinforced the adverse view of some consumers regarding the safety of agricultural products.

Published EC considerations have not clearly acknowledged the potential impact of the black market trade in growth promoting agents, including the illegal use of DES. Many of the issues referred to in the EC documents as implicating hormones in currently agricultural use were associated with the alleged illegal use of DES. One of the underlying assumptions of the EC is that approval of registered use of HGPs necessarily leads to misuse. The problem with this viewpoint is that it could be applied to literally any agricultural or veterinary chemical. In addition, the incentive to misuse HGPs is negated by findings indicating that the recommended labelled dose results in the optimal effects (Hageleit et al 2001). However, the EC maintains that multiple applications (ie. overdosing) are unavoidable under "practical conditions".

Summary

With the exception of the assessment conducted by the EC, all scientific assessments undertaken in the international arena have reached the same conclusion - when registered HGPs are used properly (ie. according to GVP), residues do not pose an unacceptable health risk to consumers.

Following a review of the published studies commissioned or cited by the EC in their April 2002 risk assessment, the following statements can be made:

[24] http://europa.eu.int/comm/food/fs/pp/pp_index_en.html

- 17β-oestradiol is an endogenously produced hormone necessary for normal biological function. There is still no adequate evidence that 17β-oestradiol is mutagenic in bacterial or mammalian cell test systems.

- There is evidence that certain catechol oestrogen metabolites are genotoxic at high concentrations when administered directly to cells or animals. However, biochemical mechanisms exist to control the generation of potentially genotoxic metabolites and to eliminate DNA adducts that might be formed.

- Residues in cattle implanted with commercial anabolic preparations according to GVP do not violate MRLs or tolerances established as acceptable on the basis of consideration of toxicological data.

- More refined risk estimates would be assisted by estimation of the total dietary intake of hormones from all sources.

CONCLUSIONS

(1) A review of the new data does not indicate any grounds for amending Australia's current regulatory position with respect to HGPs - ie. that there is unlikely to be any appreciable health risk to consumers from eating meat from cattle that have been treated with HGPs according to GVP;

(2) There is no new scientific evidence to indicate a need for the reconsideration by the APVMA of the present use of HGPs under GVP conditions; and

(3) In view of the complexity of assessments of total dietary hormone intakes and the contentious views expressed in various international forums, the use of HGPs in meat producing animals should be kept under ongoing consideration by the Advisory Committee on Pesticides and Health.

ANNEX 1: EVALUATION OF STUDIES COMMISSIONED OR CITED BY THE EC

In 1998, the EC launched 17 new studies to address certain data gaps it had previously identified following the ruling of the WTO (see Table below). The majority of these studies have been published in peer reviewed scientific journals and were evaluated as part of the current review. A number of these 17 EC-commissioned studies have not been published to date, and were therefore unable to be evaluated. Furthermore, the original study data was not available from the Commission.

Details of the 17 EC-commissioned studies

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

Title of the study publications	Publications
	mass spectrometry" (*Journal of Chromatography A, 867: 219-233, 2000)* 2)"Le contrôle des anabolisants dans la viande" (The survey of anabolic agents in meat.) (*Annales de Toxicologie Analytique, vol.XII, no.1,2000*
Comparison of assay methods	1)"Frequency and molecular analysis of *hprt* mutations induced by estradiol in Chinese hamster V79 cells" *(International Journal of Oncology 17:1141-1149, 2000)* 2)"Estrogens as endogenous genotoxic agents-DNA adducts and mutations" *(Monographs, JNCI 27: 75-93, 2000)* 3)"Tissue-specific synthesis and oxidative metabolism of estrogens" *(Monographs, JNCI 27: 95-112, 2000)* 4)"Induction of uterine adenocarcinoma in CD-1 mice by catechol estrogens" *(Cancer Research 60: 235-237, January 15, 2000)* 5)"Genotoxicity of the steroidal estrogens estrone and estradiol: possible mechanism of uterine and mammary cancer development" *(Human Reproduction Update, 7 (3): 273-281,2001)*
Bioassay of estrogenic/anti- estrogenic compounds	"Assessment of oestrogenic potency of chemicals used as growth promoter by in-vitro methods" *(Human Reproduction 16: 1030-1036, 2001)*
Interaction of xenobiotics with sex hormone binding globulin; impact on endogenous steroid transport, bioavailability, mechanism of action	scientist has not yet indicated name of journal and publication date
Reproductive sequelae of developmental exposure of rabbits to trenbolone, zeranol & MGA; emphasis on differential & neoplastic transformation of germ cells	Publication foreseen by the end 2001
Long term effects in children to estrogenized meat	"Accidental gynecomastia in children" *(APMIS 109(suppl.103): 203-209, 2001)*
Androgen exposures in utero, risk of breast cancer	"A study of opposite-sexed twins" (*Journal of The National Cancer Institute, JNCI, volume 93, issue 1;60-62, 3.1.2001)*
Endocrine disrupting activity of anabolic steroids used in cattle	1)"Characterisation of the affinity of different anabolics and synthetic hormones to the human androgen receptor, human sex hormone binding globulin and the bovine progestin receptor" (*APMIS 108: 838-846, 2000)* 2)"The fate of trenbolone acetate and melengestrol acetate after application as growth promotants in cattle - environmental studies" *(Environmental Health Perspectives- in preparation*
Screening water samples for estrogenic & androgenic anabolic chemicals	*scientist has not yet indicated name of journal and publication date. Some results can be found in APMIS 109(suppl.103): 551-556, 2001* General discussion on "Existing guidelines for the use of meat hormones and other food additives in Europe and USA"
Endocrine disrupting effects of cattle farm effluent on environmental sentinel species	"A reexamination of variation associated with environmentally stressed organisms" *(Human Reproduction update vol.7 (no.3): 265-272, 2001)*
Human cells exposed to the estrogenic compound zeranol	"Oestrogenic potencies of zeranol, oestradiol, diethylstilboestrol, Bishpenol-A, and genistein: Implications for exposure assessment of potential endocrine disrupters" *(Human Reproduction 16: 1037-1045, 2001)*

SUMMARIES OF 17 EC-COMMISSIONED STUDIES

Study 1: Presence of oestrogens in meat

No publication.

Study 2: Hormones as growth promoters: genotoxicity and mutagenicity of zeranol and trenbolone

Metzler and Pfeiffer (2001) reviewed the *in vitro* metabolism and genotoxicity of 17β-trenbolone (17β-TBO), melengestrol acetate (MGA) and zeranol, including experiments conducted in their laboratory. A number of mono- and/or dihydroxylated metabolites of 17β-TBO (14), MGA (12) and zeranol (5) were generated by rat, human or bovine liver microsomes. Forward mutation assays conducted in V79 cells and *E.coli* were negative. Marginally positive results were generated with 17β-TBO and zeranol in the micronucleus test using V79 cells at near-cytotoxic concentrations. A marginally positive result was obtained for 17β-TBO in the ^{32}P-postlabelling assay. The weight-of-evidence indicated that these compounds were not genotoxic. Commercial MGA was found to contain impurities that induced apoptosis in V79 cells. The study authors acknowledged that there is presently no evidence that residues in meat from treated animals pose a genotoxic risk to the consumer, however they concluded that the lack of information on the biological activity of the metabolites does not necessarily mean that they are safe. This report lacked detail and transparency in both the experimental methods and results.

Study 3: Metabolic pathways of oestrogens as steroidal growth promoting agents

The *in vitro* oestrogenic activity of 17β-oestradiol (E_2) and a number of its metabolites was determined using the ER-CALUX assay, which employed human T47D breast cancer cells. E_2 and oestrone (E_1) had a similar level of activity, with most other compounds several orders of magnitude less oestrogenic. 4-hydroxy-17β-oestradiol (4-OHE_2) was the most potent of the metabolites (approximately half the activity of E_2), while 17β-oestradiolbenzoate was the most potent of the synthetic 17-hydroxy esters (4 times less active than E_2). *In vitro* metabolism studies confirmed the formation of E_1 from E_2, and E_2 from E_1, in wild-type T47D cells. The authors speculated on the likely risk to consumers from eating meat containing metabolites of E_2 and concluded that the naturally-occurring esters of E_2 should be taken into account when evaluating the amount of oestrogens present in meat from treated and untreated animals. (Hoogenboom et al 2001)

Study 4: Metabolites of MGA, TBA and zeranol in bovine and humans

Publication pending.

Study 5: Application of anabolic agents to food producing animals – health risks through disregard of requirements of good veterinary practice

In a study aimed at measuring the concentration of MGA in various cattle tissues, 2 heifers/group received 0, 0.5, 1.5 or 5 mg/kg bw/d MGA in the diet for 56 days. An additional group of 2 heifers was fed 0.5 mg/kg bw/d MGA for the same duration, but were subjected to a 48-hour withdrawal period prior to slaughter. There was a dose-related increase in the concentration of plasma MGA, with the concentration approximately 10-40% lower

over days 30-56 compared to days 3.5-29.5. In animals withdrawn from treatment 48 hours before slaughter, plasma levels were unchanged 24 hours after withdrawal and had fallen to undetectable levels in only one of the heifers at slaughter. The highest tissue residues occurred in perirenal fat, followed by the liver, then the kidney or muscle. There was approximately 5-fold more MGA in muscle and kidney relative to the concentration in plasma, 20-40-fold more in liver and 200-fold more in fat; the authors concluded that this was evidence of tissue accumulation (in fat). While it is feasible that MGA could accumulate in fat due its lipophilic nature, this conclusion was not considered to be justified because the kinetics of tissue distribution and elimination had not been determined. Withdrawal of MGA 48 hours prior to slaughter had no effect on residue levels in fat and muscle, but the concentration in the liver fell to below the limit of detection (LOD). Significant limitations to this study included the small group sizes and consequent lack of statistical analysis. (Daxenberger et al 1999)

A number of experiments were undertaken to measure residue levels in cattle following implantation at various "off-label" sites with Revalor-H®, Finaplix-H®, Synovex-plus®, Synovex-H®, Compudose®, Component EC®, Implus-S® or Ralgro®. Eight weeks after implantation, 77% of the implantation sites were identifiable, while approximately 67% of the actual pellets were found. In 7/31 animals, none of the pellets could be located. At slaughter, the highest residue levels (mg) were found in the remaining pellet, which led the study authors to conclude that the risk assessment of hormone residues in meat should be focussed on the remaining pellets. Residue levels in the surrounding 2.5 cm of tissue were 2-3 orders of magnitude lower than in the implant, while residues in the next 1.5 cm of tissue were a further 2 orders of magnitude lower. The study authors speculated that the misplacement of implant preparations or the failure to discard ears following slaughter could allow mg amounts of hormone residues to enter the food supply and cause acute toxicity in consumers. They concluded that on economic grounds, the use of HGPs could not simply be rejected; the important issue was that the implantation and management of their use should aim to eliminate the risk of leaving significant hormone residues. A limitation to this study was the considerable variability in residue levels detected in implants and the surrounding tissue. Several of the experiments should be viewed as preliminary as only single animals were treated. Acknowledged by the study authors was the problem of damaged or cut pellets contaminating surrounding tissue during the actual excision of the implant and preparation of tissue samples. (Daxenberger et al 2000)

Daxenberger et al (2001) examined the effect of commercial anabolic preparations on the concentration of androstenone in the fat of intact boars and measured hormone residues at implantation sites. Four boars/group were implanted in the neck (to simulate off-label use) with 2 doses of Synovex-H® (total dose of 400 mg testosterone propionate + 40 mg oestradiol benzoate) or Synovex Plus® (total dose of 400 mg TBA + 56 mg oestradiol benzoate). The control group consisted of 4 untreated animals. Synovex Plus® caused a reduction in androstenone in adipose tissue and a concomitant reduction in plasma testosterone, while such an effect did not occur with Synovex-H®. Both preparations caused a slight reduction in testis weight, which was not statistically significant. Analysis of residues remaining at the implantation site (implant plus surrounding tissue) following slaughter revealed the highest residues in the pellets. Besides the parent compounds, traces of hydrolysed testosterone or 17β-TBO were detected in the surrounding tissue. The authors argued that potentially, the industrial processing of an implantation site along with the useable carcass could contaminate meat with residues above the international MRLs for TBO and E_2. The limitation to this argument is that in those countries where anabolic implants are

approved for use in cattle, the prescribed implantation sites are the ears, which are discarded at slaughter and therefore do not enter the food supply. The authors, however, maintained that the availability of anabolic agents used in cattle might lead to misuse in other species. Statistical analysis was not performed on all the results.

Hageleit et al (2001) developed an enzyme immunoassay (EIA) for the determination of MGA in bovine adipose and muscle tissues. The detection limit was 0.4 ng/g in fat and 0.05 ng/g in muscle. Negligible cross-reactivity to natural steroid hormones, their precursors or metabolites occurred, while cross-reactivity to megestrol acetate, medroxyprogesterone acetate, 6α-methyl-17α-OH-progesterone acetate, chlormadinone acetate or 17α-acetoxyprogesterone was below 10%. Measurement of MGA concentrations in fat and muscle samples from a previous study (Daxenberger et al 1999) yielded results that were comparable with those obtained using GC-MS or LC-MS. It was suggested that for confirmatory or forensic purposes, the necessary mass-spectrophotometric-based methods should be employed.

The affinity of 17β-TBO, MGA and a variety of natural and synthetic steroids for the recombinant human androgen receptor (rhAR), human sex-hormone binding globulin (hSHBG) and the bovine uterine progestin receptor (bPR) were evaluated using competitive radioligand binding assays. 17β-TBO was found to have a relatively high affinity for both the rhAR and bPR (the same or slightly higher than DHT and progesterone, respectively), while a number of its metabolites [17α-trenbolone (17α-TBO) and triendione)] had relatively low affinities. 17β-TBO and its metabolites had relatively low to negligible affinity for the hSHBG. MGA exhibited a high affinity to the bPR (approximately 5-fold higher than progesterone) but a low affinity to the rhAR. The 3 main metabolites of MGA showed low affinity to both the bPR and rhAR, however the affinity for the bPR reportedly fell within the range of natural progesterone levels. The study authors concluded that residues of 17β-TBO with significant binding capacity and potentially endocrine-disrupting activity would be excreted after treatment of cattle. However, they provided no comment on the possible health risk to consumers from eating meat containing TBO residues. Furthermore, they speculated that PR active substances might be excreted from cattle treated with MGA. Their final comment on the safety of these compounds was that the biodegradation, distribution and bioefficacy of the breakdown products of 17β-TBO and MGA are necessary before considering them safe. This report lacked methodological detail and no statistical analysis was performed on the data. (Bauer et al 2000)

Hageleit et al (2000) examined the dose-dependent effects of MGA on plasma levels of E_2, progesterone and luteinising hormone (LH) in cycling heifers and the effect on oestrogen residues in edible tissues. This study had previously been published (Daxenberger et al 1999), with some additional data presented here. MGA caused dose-related perturbations in the plasma concentration of progesterone, E_2 and LH, in addition to tissue levels of E_2 and E_1. Treatment with MGA (0.5 mg/kg bw/d) resulted in elevated levels of tissue and plasma E_2, and plasma LH. Higher doses of MGA reduced plasma and tissue levels of E_2, and plasma LH. These effects occurred in the absence of any impact on the number and size of ovarian follicles, or on the presence of corpus luteum. Limitations to this study were the low group sizes, a consequent lack of statistical analysis and the lack of individual animal data.

Lange et al (2001) examined two scenarios of possible misuse of commercial anabolic preparations in cattle, namely, overdosing in heifers and off-label use in veal calves. In the first study, 2 Holstein Friesian heifers/group were treated with a 1-, 3- or 10-fold dose of

Finaplix-H® (200 mg TBA), Ralgro® (36 mg zeranol) or Synovex-H® (200 mg testosterone propionate + 20 mg oestradiol benzoate). In the second study, 2 Holstein Friesian calves/group were implanted with Synovex-H® or Synovex Plus® (200 mg TBA + 28 mg oestradiol benzoate). Animals were slaughtered after 8 weeks and hormone residues quantified in muscle, liver, kidney and fat by HPLC/EIA. There was a dose-related increase in hormone residues in all tissues with the exception of testosterone levels in liver, which were unaffected by treatment. Generally, the highest residues were detected in the liver, then the kidney, fat and muscle. The proportion of oestrogen and TBA metabolites varied between tissues. The use of Finaplix-H®, Ralgro® or Synovex-H® in heifers at the recommended dose, and the off-label use of Synovex-H® or Synovex-Plus® in veal calves, did not lead to violations of Codex MRLs or FDA-allowable residue levels. Off-label use of zeranol and testosterone propionate at up to 10-fold the labelled dose in heifers also did not violate permissible residue levels. However, overdosing heifers with TBA or oestradiol benzoate at 3- or 10-fold the labelled dose resulted in violations of Codex MRLs or FDA-allowable residue levels. In some instances, the interpretation and significance of the results was difficult to ascertain due to the variation between the 2 animals in each group and the lack of statistical analysis. This study indicated that HGP use is acceptable when animals are treated according to approved directions.

Pfaffl et al (2001) examined the effect of zeranol on the tissue-specific expression pattern of oestrogen receptor (ER) mRNA using real time polymerase chain reaction (RT-PCR). Two Holstein-Friesian heifers/group were implanted with a 1-, 3- or 10-fold dose of Ralgro® (36 mg zeranol) and slaughtered 8 weeks later. There was a dose-related increase in the plasma concentration of zeranol above the background level in the controls. Treatment-related effects on the oestrus cycle and a decreased uterine weight occurred. There was relatively little effect on the tissue-specific expression pattern of ERα and ERβ, with the exception of the kidney and jejunum, where ERα mRNA was significantly reduced. The highest expression of ERα occurred in the uterus, mammary gland, liver, neck muscles and hind leg muscles, while the lowest levels were measured in the jejunum, kidney, abomasum and lung. The expression of ERβ was markedly lower than that of ERα, with the highest levels measured in the uterus and kidney medulla, and the lowest levels measured in the abomasum and lung. The low group sizes were a limitation to this study.

Study 6: Analysis of 500 samples for the presence of growth promoters

Stephany (2000 & 2001) reviewed the different regulatory approaches to HGPs in Europe and the US, and summarised the results of residue analyses of European Union (EU) and US beef. In 1994, an analysis of 1183 beef entrecote samples from the EU detected traces of 17 androgens and 2 gestagens (1.6% of samples), while an analysis of 936 liver samples found that 10% contained clenbuterol. Based on residue testing programs, the author estimated that the overall level of misuse in the EU could be 5-15% (of samples). However, the probability of consuming a "highly" contaminated 250 g steak in the EU was estimated to be less than one in 70,000. Analysis of meat imported from the US found that 75% of samples contained traces of MGA (actual amount unspecified), while TBA was detected in 20% of samples. Evidence was cited to indicate that the dietary intake of E_2 in an egg is greater than that in meat from both treated and untreated cattle; the median level in untreated cattle, treated cattle and in a egg is less than 2.5 ng/250 g steak, 5 ng/250 g steak and 6.5 ng/50 g egg, respectively. The author's main recommendations were that: it is important that the risk to human health from the consumption of meat from HGP-treated cattle be evaluated in relation to the total dietary intake of hormones from meat and meat products, poultry, milk, dairy

products, eggs, fish and fish products; and an international database should be established providing information on the actual levels of natural and synthetic hormones in food commodities.

Study 7: Analysis of 500 samples for the presence of growth promoters

An analytical method was developed to measure trace amounts of a wide range of anabolic agents and their metabolites in tissue samples from treated and untreated cattle. An unspecified number of European meat and liver samples were found to contain residues of anabolic compounds. Residues of chlormadinone (2.5 µg/kg) or MGA (1.5 µg/kg) were found in meat samples (unspecified number). 17α-TBO (9.0 µg/kg) and 17β-TBO (1.5 µg/kg) were detected in one liver sample, while zeranol (1.2 µg/kg) and taleranol (0.09 µg/kg) were detected in another (total number of samples analysed unspecified). These positive residue samples were reportedly obtained from cattle that had been treated with the parent compounds (ie. chlormadinone, MGA, TBA or zeranol). It was reported that 23 steroids were detected in the 5-100 ng/kg range but no data were provided to substantiate this finding. The study authors indicated that 600 routine samples were also analysed but the results of these were not reported. This study lacked detail on the source and number of the samples analysed, including the incidence of violative levels of HGPs residues. (Marchand et al 2000).

A method was developed in the National Reference Laboratory for hormones (French Ministry of Agriculture) in order to simultaneously assay various anabolic agents according to the requirements of the EC 96/23 Directive for meat or liver control. Meat samples were lyophilised and extracted with organic solvents. Phase II metabolites were hydrolysed and the esters saponified. Free compounds were re-extracted by liquid-liquid and solid-phase extraction. Twenty-three anabolic agents were detected by GC-MS, with the detection limit between 5-100 ng/kg. An English version of the abstract was only available for assessment. Le Bizec et al (2000).

Study 8: Comparison of assay methods

The mutation frequency and molecular alterations of the hypoxanthine-guanine phosphoribosyltransferase (*hprt*) gene were examined in Chinese hamster V79 cells following exposure to physiological and pharmacological concentrations of E_2.
In a single experiment, a relatively high concentration of E_2 (1 µM) caused a marginal increase in the frequency of *hprt* mutations in V79 cells. In the absence of any evidence that this was a reproducible effect, that there were no effects at lower concentrations and the fact that the mutation frequency was markedly lower than the positive control, the study authors conclusion that E_2 is weakly mutagenic and a complete carcinogen was considered to be unsubstantiated. PCR and molecular analysis of the *hprt* gene in mutants revealed a variety of simple base substitutions and deletion mutations. However deletion mutations were also identified in mutant clones obtained from the untreated and solvent controls. Qualitative evidence indicated that V79 cells express ERα. Mutagenicity assays performed in the presence of ICI 182,780 (an ERα antagonist) failed to inhibit E_2-induced mutations in the *hprt* gene. The study authors concluded that these findings suggested that the weakly mutagenic activity of E_2 does not involve the ERα. (Kong et al 2000)

Cavalieri et al (2000) reviewed the potential of oestrogen and oestrogen metabolites to induce DNA damage and gene mutations both *in vitro* and *in vivo*. The overall mechanism of DNA

damage (leading to cancer formation) was considered to be due to the oxidation of catechol oestrogens to catechol oestrogen quinones, which may react with DNA to form depurinating adducts; these generate apurinic sites, which may initiate cancer. The metabolism of catechol oestrogens also generates reactive oxygen species (ROS) that may also cause genetic damage. Oestrogens were also considered to generate ROS indirectly via the modulation of immune responses and via the stimulation and proliferation of inflammatory cells. While cells have a variety of inactivation systems to remove these potentially DNA-damaging compounds, excessive metabolite formation (particularly 4-OHE_2) and their oxidation to DNA-reactive quinones was proposed as the pathway leading to the genotoxic effects responsible for tumour formation. The authors maintained that previous bacterial and mammalian cell mutation assays were insufficiently sensitive to detect the weak mutagenic activity of oestrogens. However, the reviewing toxicologist considered that the authors' conclusion that oestrogens are genotoxic carcinogens was unjustified because the weight-of-evidence indicated that oestrogens and oestrogen metabolites are not mutagenic.

Jefcoate et al (2000) reviewed the contribution of oxidative metabolites of oestrogen to breast carcinogenesis, with particular emphasis on the roles of various cytochrome P450s [aromatase, 17β-oestradiol-4-hydroxylase (CYP1β1)] and catechol oestrogens (namely 4-OHE_2). The central hypothesis was that the carcinogenic effect of oestrogens is due to the oxidation of E_2 to 4-OHE_2 by CYP1β1; this metabolite is genotoxic and can undergo redox cycling to form ROS, which are themselves genotoxic. The main limitation to this hypothesis was the uncertainty that tissue levels of E_2 would be high enough to generate biologically active levels of 4-OHE_2. It was argued that overexpression of aromatase in combination with the presence 4-hydroxylases could generate physiologically and pathologically significant levels of oestrogen metabolites. While data were presented that showed the link between increased aromatase or CYP1β1 expression and breast cancer, no data were provided to show that increased aromatase expression or CYP1β1 actually leads to increased tissue levels of carcinogenic catechol oestrogen metabolites or ROS.

An experimental mouse model was used to investigate the carcinogenic potential of a number of catechol oestrogens. Mice were exposed neonatally to 2- or 4-OHE_2, E_2 or 17α-ethinyl oestradiol (EE) at 2 µg/pup/day for 5 days, then sacrificed at 12 or 18 months of age. There was a significant increase ($p<0.01$-0.05) in the total incidence of uterine adenocarcinomas in mice treated with 4-OHE_2 (incidence of 66%), EE (43%), 2-hydroxyoestradiol (2-OHE_2) (12%) or E_2 (7%). No uterine adenocarcinomas were detected in the control group, while the historical control incidence is 0.86-4%. The oestrogenic potency of each of the above compounds was determined using an *in vivo* neonatal mouse assay. There was an approximately 2-fold increase in the uterine weight of mice treated with 4-OHE_2, a result that was statistically significant ($p<0.05$). Increased uterine weights were also detected following treatment with EE or E_2 but neither of these findings was statistically significant. 2-OHE_2 showed minimal oestrogenic activity. These results indicated the metabolite, 4-OHE_2, was the most carcinogenic of the test compounds, which appeared to correlate with its relatively high oestrogenic potency. The metabolite, 2-OHE_2, and the synthetic oestrogen, EE, showed lower carcinogenic and oestrogenic activity than 4-OHE_2. Uneven group sizes were used in this study and no other toxicological endpoints were examined besides the incidence of uterine adenocarcinomas. In addition, only one dose level was tested and the group sizes in the oestrogenicity assay were not specified. (Newbold & Liehr 2000)

Liehr (2001) reviewed the evidence pertaining to the genotoxicity and carcinogenicity of steroidal oestrogens and proposed a mechanism for oestrogen-induced tumorigenesis. The

overall conclusions of the author were that: natural oestrogens are weak mutagens and carcinogens relative to "more powerful carcinogens" such as polycyclic hydrocarbons; catechol oestrogen metabolites are the precursors of DNA damaging reactive intermediates; and that oestrogens should be considered as complete carcinogens because of their dual action as compounds capable of inducing genotoxicity and cell proliferation.

Study 9: Bioassay of oestrogenic/anti oestrogenic compounds

The oestrogenic potencies of E_2, zeranol (α-zearalanol), testosterone, TBO, TBA, MGA and the mycotoxin zearalenone were determined utilising 3 *in vitro* bioassays. These assays involved the measurement of the expression of certain oestrogen-inducible genes. Various metabolites of the above compounds were also assessed including 17α-oestradiol, E_1, 17α-epitestosterone, 19-nortestosterone, androstendione, zearalanone, β-zearalanol, α-zearalenol and β-zearalenol. The highest oestrogenicity was predominantly confined to E_2, DES and EE. The potency of several oestrogenic compounds such as zeranol varied depending on the assay that was used. This variability suggested that certain assays may over- or underestimate the oestrogenicity of a compound. (Le Guevel & Pakdel 2001)

Study 10: Interaction of xenobiotics with sex hormone binding globulin; impact on endogenous steroid transport, bioavailability and mechanism of action

Publication pending.

Study 11: Reproductive sequelae of developmental exposure of rabbits to trenbolone, zeranol and melengestrol acetate; emphasis on differential and neoplastic transformation of germ cells.

The extent of transplacental passage and residue formation in foetal tissue of MGA, TBA and zeranol and their metabolites following maternal exposure was determined (Lange et al 2002). MGA, TBA and zeranol were detected in dams and found to cross the placenta following dietary or subcutaneous administration. The 3 compounds were detected in all corresponding maternal and foetal plasma samples, and the majority of tissue samples, with the concentrations generally lower in foetuses than in dams. TBA was extensively metabolised in dams and foetuses, however there appeared to be a different metabolic pathway operating in both. There was some suggestion that MGA accumulated in maternal and foetal fat and liver. While MGA, TBA and zeranol were shown to cross the placenta, there was no indication of any adverse effect in dams or foetuses. The significance of some of the results (eg. comparisons between male and female foetuses, comparisons of residue levels between tissue and between dams and foetuses) were unclear due to the absence of statistical analyses.

An unpublished study, described in the EC's latest risk assessment, reported that prenatal exposure to MGA, TBA or zeranol may affect the function of the reproductive tract of male rabbits. In the absence of a critical review of the data supporting this study, and the unavailability of the study report, no comment can be provided on this finding.

Study 12: Long term effects in children to oestrogenised meat

Chiumello et al (2001) discussed the possible causes of abnormal palpable breast tissue in men (gynecomastia) including endocrine disorders, exposure to hormonally active

compounds in drugs, food and the environment, or unknown causes. No data was provided, however a number of studies were cited in an attempt to support the authors' conclusion that hormones ingested in meat can cause breast enlargement and other deleterious immediate or long-term effects. The majority of study citations related to the adverse health effects of the banned substance DES, while there was no mention of the effects of hormones currently registered for use as growth promotants in non-European countries. Therefore, the value of this 'review' was unclear. The authors made a number of unsubstantiated claims regarding the adverse health effects of hormones and the incidence of the illegal use of HGPs in countries where they are banned. While the authors argued that environmental and dietary contaminants are the cause of the increased incidence of advanced pubertal development, they also made the point that it could be caused by genetic factors, obesity or altered socioeconomic status. There are other dietary sources of oestrogenic compounds besides meat (eg. dairy products, fungal oestrogens, phytoestrogens in food) but there was no discussion of how these could contribute to overall hormone intake and possibly impact on pubertal development. This paper had limited value due to the absence of data and the lack of a critical review of the studies cited.

Study 13: Androgen exposures *in utero*, risk of breast cancer

A Swedish study was undertaken to investigate the association between birthweight and breast cancer among female twins of opposite-sexed twin pairs. The rationale for the study was previous unspecified evidence linking antenatal oestrogen exposure with high birthweight. Study subjects were identified through record linkage between the Swedish Twin Registry and the Swedish Cancer Registry. Aged-matched control subjects were drawn from the same population. There was a statistically significant trend of increased breast cancer risk with increasing birthweight. When birthweight was analysed as a continuous variable, the OR for each kg increase in birthweight was 2.3-2.4. It was reported that the association between birthweight and breast cancer risk was even more pronounced for those women diagnosed with breast cancer before 50 years of age (ORs of 4-5). There was no significant relationship when stratifying the analysis on male co-twin birthweight and the duration of gestation. No adjustment was made for known risk factors for breast cancer, however these were not considered by the study authors to confound the results as they were unlikely to be associated with female birthweight. The authors concluded that among female twins with male co-twins, high birthweight constitutes an independent risk factor for breast cancer. Furthermore, they suggested that these findings support the hypothesis that *in utero* exposure to hormones influences breast cancer risk. The reviewing toxicologist did not consider that this conclusion was justified because the study did not directly test an association between oestrogen exposure and cancer risk, and no data were provided or cited to substantiate any link between oestrogen exposure and high birthweight. (Kaijser et al 2001)

Study 14: Endocrine-disrupting activity of anabolic steroids used in cattle

The fate of TBA and MGA after administration to cattle was examined by Schiffer et al (2001). TBA was implanted via commercially available anabolic preparations (unspecified), while MGA was administered in the feedmix at 65 mg/animal. Liquid manure, solid dung, and soil that had been fertilised with manure from treated animals were sampled at various times. Measurement of all samples included extraction, clean-up (solid phase extraction), separation of metabolites and interfering substances by HPLC, and quantitation using an EIA. Measurements were validated by GC-MS or LC-MS. Metabolites of TBA (ie. 17α-TBO, 17β-TBO and trendione) were detected in liquid manure at levels up to 1700, 160 and 120

pg/g, respectively. The half-life of 17α-TBO and 17β-TBO was approximately 250 days. Levels in solid dung were up to 75400, 4265 and 4700 pg/g, for 17α-TBO, 17β-TBO and trendione, respectively. The stability of trenbolone metabolites was greater in solid dung than in liquid manure. MGA was detected in liquid manure up to approximately 20 ng/g, while in solid dung the highest level was 7760 pg/g. Levels of TBO and MGA were highly variable due possibly to the heterogeneity of the dung. Metabolites of TBA were detected in soil up to 248 pg/g and were not detectable at 93 days after fertilisation. MGA was measured in soil up to 34 pg/g, and was still detectable 194 days after fertilisation. The study authors concluded that MGA and TBA should be investigated further for their potential endocrine-disrupting activity in agricultural ecosystems. The relevance of these findings to the human health assessment of TBA and MGA was unclear.

Study 15: Screening water samples for oestrogenic and androgenic anabolic chemicals

A transcript of a discussion from an international workshop on hormones and endocrine disruptors in food and water speculated on the possible risk to human health from hormone contaminants in the environment, particularly those generated from the use of HGPs in cattle. Some findings were reported indicating that hormones are detectable in waste water from cattle feedlots and in effluent from sewage plants. Microbial activity was also considered to contribute to the concentration of hormonally active compounds in the environment. The participants main conclusions were that: (1) it is difficult to accurately assess the contribution of hormones from any single food source to overall risk, without knowledge of total hormone intake; (2) measurement of hormone levels in humans is important to determine actual risk; and (3) JECFA should compile a new database of endocrine disruptors. (Jegou et al 2001).

Study 16: Endocrine disrupting effects of cattle farm effluent on environmental sentinel species

Orlando & Guillette (2001) reviewed data supporting the premise that exposure to sublethal levels of endocrine-disrupting contaminants results in phenotypic variation within a population of organisms. The only example relevant to HGPs was an unpublished study conducted by the authors, on fathead minnow exposed to cattle ranch effluent. This study, conducted in Nebraska, USA, was described briefly in a previous evaluation (Jegou et al 2001). There was no significant difference in the mean fork length, inter-ocular distance and head width between male fathead minnows from contaminated and reference streams. However, the variance of the 3 parameters was significantly increased ($p<0.0001$-0.0013) in minnows from the contaminated stream. The authors concluded that increased phenotypic variance may be an important early sign of population disturbance and as such could prove to be an early indicator of exposure to "subtle toxicants" such as endocrine disruptors. This study had limited value in assessing the risk to human health from the consumption of meat from HGP-treated cattle.

Study 17: Human cells exposed to the oestrogenic compound zeranol

The oestrogenic potencies of zeranol (and 5 of its metabolites), E_2, DES, genistein and bisphenol A were compared by analysing differences in expression levels of 6 oestrogen-regulated genes in human MCF7 cells. While the study authors concluded that zeranol, E_2 and DES were approximately equipotent, overall, E_2 had the highest potency (albeit marginal in some cases). Genistein and bisphenol A were found to have relatively low potencies in this particular assay. Of the 6 genes, the *MRG1/p35srj* gene appeared to be more sensitive to

zeranol than any of the other compounds. Metabolites of zeranol were reported to induce 5 of the 6 oestrogen-sensitive genes, the most potent being zeranol and α-zearalenol. The study authors speculated that the relatively high potency of zeranol suggested that zeranol intake from beef products would have an impact on the health of consumers. However, it was acknowledged that it was not possible to undertake a proper risk assessment because of the absence of data regarding zeranol in human serum after ingestion of meat products from treated animals. No statistical analyses was conducted, and for some findings, no data were actually provided. (Leffers et al 2001)

SUMMARIES OF RECENT STUDIES CITED BY THE EC

In their April 2002 risk assessment, the EC cited a number of recent scientific publications to support their opinions regarding the human safety of HGP residues. These scientific publications have been evaluated as part of the current review.

Metabolism of 17b-oestradiol and quantitative analysis of oestrogen residues in edible tissues from treated steers

Five Hereford steers/group were implanted with 0, 1, 2 or 4 pellets of Revalor®-S (140 mg TBA + 28 mg E_2) and slaughtered 90 days later. E_2 metabolites were isolated from muscle, fat, liver and kidney using a multi-step extraction procedure, and then quantified by GC-MS. There was a high level of variability in metabolite levels within each treatment group, however, concentrations appeared to be dependent on the tissue type and number of implants administered. The highest levels were detected in liver followed by kidney, fat and muscle. Although an apparent dose-related increase in certain metabolites occurred in some tissues, the significance of the results were difficult to ascertain as only concentration ranges were given and no statistical analysis had been performed. The theoretical daily intake of E_2 residues in a 500g portion of meat from untreated steers, and from steers in the 1-, 2- and 4-implant groups, was calculated to be 0.2, 1.3, 3.9 and 4.7% of the JECFA ADI for E_2. (Maume et al 2001)

Oestrogenic potency of residues

Paris et al (2001) examined the uterotrophic effect of 17β-oestradiol-17-stearate (E_2S), a saturated fatty acid 17-ester of 17β-oestradiol, in juvenile female rats. Six rats/group were dosed by oral gavage with 0, 2.5, 25, 250 or 2500 nmol/kg bw/d E_2 or E_2S in corn oil for 6 consecutive days. Positive control animals received EE at 34 nmol/kg bw/d. Rats were sacrificed 24 hours after the last treatment. There was a significant increase in relative uterine weight at and above 250 nmol/kg bw/d E_2 or E_2S, but there was no apparent difference between the 2 compounds. The magnitude of the effect at 250 nmol/kg bw/d E_2 was reportedly of the same magnitude as EE. Various histopathological abnormalities in the uterus occurred at and above 250 nmol/kg bw/d E_2 or E_2S (endometrial cell hypertrophy/hyperplasia, loose endometrial stroma and myometrial hypertrophy). At these same doses, microscopic vaginal abnormalities also occurred (epithelial cell hyperplasia, hyperkeratosis, epithelial cell hyperplasia and hyperkeratosis). While these abnormalities appeared to be somewhat greater in rats treated with E_2S, the magnitude of this difference was unclear. While the overall difference in oestrogenic potency between E_2 and E_2S appeared to be marginal, multi-dimensional statistical analyses suggested that E_2S was approximately 10-fold more oestrogenic than E_2. The NOEL for uterotrophic effects

(increased uterine weight, histopathological abnormalities in the uterus and vagina) was 25 nmol/kg bw/d E_2S and E_2. This publication lacked reporting detail.

Mills et (2001) compared the activity of E_2S with that of E_2 for stimulating the growth of mammary glandular cells and uterine endometrial cells in ovariectomised female rats. In the first experiment, 5-6 rats/group were surgically implanted under the skin of the back with an osmotic mini-pump containing 0 or 0.83 mM E_2 or E_2S (estimated to release 5 nmol/d) in 70% ethanol and 30% DMSO. Rats were sacrificed 10 days later. In a second experiment, 7-8 rats/group were implanted with an osmotic mini pump containing 0, 0.083 or 0.83 mM E_2 or E_2S (estimated to release 0.5 and 5 nmol/day, respectively). Rats were sacrificed 23 days later. There was a clear differential effect of E_2 or E_2S on cell proliferation; E_2 was more potent than E_2S at inducing uterine endometrial cell proliferation and E_2S was more potent than E_2 at inducing mammary glandular cell proliferation. Other treatment-related effects included increased uterine weights and plasma prolactin, and reduced bw gain and plasma LH. The magnitude of these effects was generally comparable between the 2 compounds. The authors speculated on the possible role of fatty acid esters of endogenous oestrogens and their bioactive metabolites in tumour formation but acknowledged that more studies are necessary to test this hypothesis.

Larner et al (1985) compared the oestrogenicity of E_2 and E_2S following a single sc or iv injection to juvenile female, or ovariectomised adult, rats. Various doses of either compound were given at up to 50 nmol/rat and oestrogenic activity determined via the measurement of wet uterine weight. The kinetics of radiolabelled E_2 or E_2S distribution to the uterus was also examined in juvenile rats. The results of various experiments revealed that E_2S had a more prolonged oestrogenic action on the uterus compared to free E_2. The authors hypothesised that one of the roles of the fatty acid on E_2S is to protect it from metabolism and therefore prolong the life of E_2. The dose routes used in this study (sc, iv) were not directly applicable to the dietary risk assessment of hormones. This publication lacked experimental detail.

Mutagenicity and genotoxicity of 17b-oestradiol

Oestrogens and 4-catechol oestrogens have previously been found to induce kidney but not liver tumours in male Syrian golden hamsters. This particular animal model was used to study the activation and deactivation of oestrogen metabolites. Groups of 4 male hamsters were given a single injection of E_2 (8 µmol E_2/100 g bw, ip) in trioctanoin/DMSO (9:1) and then sacrificed at 1, 2, or 4 hours. In a separate experiment, hamsters were pretreated with the glutathione-depleting agent, L-buthionine(S,R)sulfoximine (BSO) (0.6 mmol/100 g bw, sc), 2.5 hours prior to the administration of E_2. Kidney and liver levels of various oestrogen metabolites, conjugates and adducts were analysed by HPLC. E_2 was present at the highest level followed by E_1, 2-OHE_2 or 2-hydroxy-oestrone (2-OHE_1) and 2-methoxyoestradiol (2-OCH_3E_2). The increase in E_2 and its metabolites following incubation with β-glucuronidase/sulfatase suggested that a proportion of these compounds were conjugated with glucuronic or sulfuric acid. Glutathione conjugates were generally higher in the kidney, but in the absence of statistical analysis or adequate reporting detail it was unclear whether these differences were significant. The most notable effect of glutathione depletion was the generation of the depurinating DNA adduct, 4-OHE_2/E_1-1-N7Gua, in the kidney but not the liver. A limitation to this study was the absence of control data. (Cavalieri et al 2001)

To examine the possible role of 4-catechol oestrogens in the initiation of prostate cancer, 10 male rats were given a single ip injection of 4-OHE_2 or 17β-oestradiol-3,4-quinone (E_2-3,4-

Q) at 6 µmol/100 g bw in 200 µL of trioctanoin/DMSO. After 90 minutes, rats were sacrificed and the levels of various oestrogen metabolites and conjugates analysed in 4 regions of the prostate using HPLC. While the metabolic profiles of rats treated with 4-OHE_2 or E_2-3,4-Q was somewhat different, the highest metabolite levels were detected in the ventral or anterior prostate, followed by the dorsolateral prostate and periuetheral prostate and urethra. Following treatment with 4-OHE_2, 4-OHE_2, 4-OHE_1, 4-methoxy-17β-oestradiol (4-OCH_3E_2), 4-methoxy-oestrone (4-OCH_3E_1) and 4-hydroxy-17β-oestradiol glutathione (4-OHE_2-2-SG) were detected. Administration of E_2-3,4-Q resulted in the formation of 2 additional conjugates, namely 4-hydroxy-17β-oestradiol cysteine (4-OHE_2-2-Cys) and 4-hydroxy-17β-oestradiol N-acetylcysteine (4-OHE_2-2-NAcCys). This study did not address the role of catechol oestrogen metabolites in prostate cancer development. (Cavalieri et al 2002)

Direct application of 200 nmol E_2-3,4-Q to the skin of SENCAR mice resulted in the formation of DNA adducts in the epidermis after 1 hour, as measured by ^{32}P-post-labelling or HPLC. The level of depurinating adducts was approximately 6000-fold higher than the level of stable adducts. Epidermal DNA from skin samples was isolated and a 500 base-pair H-*ras* exon amplified by PCR. The amplification product was cloned into the pUC18 plasmid and transformed in to *E. coli*. H-*ras* inserts in the clones were sequenced to determine mutations. While no mutations in the H-*ras* gene were detectable after 1 hour, longer treatment times (up to 2 days) resulted in mutations that were predominantly A/T→G/C transitions. The value of this study was limited by the dosing route (dermal), the use of only one (and a very high) test concentration and the absence of a solvent control. Furthermore, there was no evidence that the DNA damage caused by E_2-3,4-Q could actually occur *in vivo* to initiate carcinogenesis. (Chakravarti et al 2001)

A preliminary study conducted by Devanesan et al (2001) analysed the levels of catechol oestrogen metabolites in tumour and hyperplastic tissue from oestrogen receptor-α knockout (ERKO) mice, using HPLC. The underlying hypothesis was that these mice metabolise oestrogens abnormally leading to the generation of potentially mutagenic metabolites in the mammary epithelium. Various metabolites and conjugates of E_2 or E_1 were detectable in both hyperplastic and tumour mammary tissue, however, there was some variability in the levels and types of metabolites detected between mice, and following treatment of tissue with β-glucuronidase or sulfatase. Additional studies would need to be completed with more animals to ascertain the significance of these findings.

Lavigne et al (2001) examined the effects of altered catechol-*O*-methyltransferase (COMT) activity on cellular levels of catechol oestrogen metabolites and oxidative DNA damage. MCF-7 cells were pretreated with 10 nM 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) for 72 hours to induce CYP4501A1 and CYP4501B1. These two cytochrome P450s generate catechol oestrogens by hydroxylating E_2 or E_1 at the 2- or 4- positions. Cells were incubated with 0, 3 or 10 µM Ro 41-0960 (a COMT inhibitor) for 30 minutes before the addition of 0, 0.1, 0.3, 1 or 10 µM E_2. After 9 or 15 hours, the levels of oestrogen metabolites in tissue culture media were analysed by HPLC and the levels of oxidative DNA damage analysed by measuring 8-hydroxy-2'-deoxyguanosine (8-oxo-dG) in cellular DNA. Inhibition of COMT with Ro 41-0960 caused a significant increase in oxidative DNA damage in TCDD-pretreated MCF7 cells that had been incubated with E_2. This DNA damage appeared to be associated with increased 2-OHE_2 formation. In the absence of COMT inhibition, no oxidative DNA damage was observed (above background) at E_2 concentrations up to 10 µM. These findings

suggested that COMT is protective against oxidative DNA damage caused by catechol oestrogen metabolites.

Sasco (2001) briefly reviewed the epidemiology of breast cancer and discussed some of the risk factors associated with its development including genetics and family history, reproductive life, diet, exercise and exposure to specific agents (eg. radiation, magnetic fields, chemicals, viruses, hormones and endocrine disruptors). There was some speculation on the potential association between HGP residues in meat and increased breast cancer risk, but no evidence was cited to support this association. It was concluded that a better understanding of the endocrine events involved in puberty and fertility is necessary in order to ascertain the role of hormone pathways in the occurrence and development of cancer. The author also advocated the enactment of the precautionary principle whenever possible to prevent exposures during specific periods of vulnerability. The value of this review was limited, as by the author's own admission it was not intended to be fully comprehensive but simply to highlight "potentially controversial conditions, which could in the future be recognised as new risk factors".

To examine the mutagenic properties of 2-hydroxyoestrogen-derived DNA adducts in mammalian cells, the COS-7 simian kidney cell line was transfected with circular ss DNA (100 fmol) containing either N^2-(2-hydroxyoestrogen-6-yl)-2'-deoxyguanosine (2-OHE-N^2-dG) or N^6-(2-hydroxyoestrogen-6-yl)-2'-deoxyadenosine (2-OHE-N^6-dA). Adducts had been prepared by reacting unmodified 15-mer oligodeoxynucleotides containing a single deoxyguanosine (dG) or deoxyadenosine (dA), with 2-OHE_1, 2-OHE_2 or 2-hydroxyoestriol. Progeny plasmids were recovered and used to transform *E. coli* DH10B cells. Transformants were analysed for mutations by oligodeoxynucleotide hybridisation. The 2-OHE-N^2-dG adducts produced only G $\rightarrow$ T mutations, while the 2-OHE-N^6-dA adducts produced both A $\rightarrow$ T and A $\rightarrow$ G mutations, with the A $\rightarrow$ T mutations predominating. The mutational frequency was dependent on the type of 2-hydroxyoestrogen-derived adduct. (Terashima 2001).

A variety of experiments were conducted using Syrian hamster embryo (SHE) cells to examine the ability of E_2, E_1, 2-OHE_1, 4-OHE_1, 2-methoxyoestrone (2-OCH_3E_1), 16α-hydroxyoestrone (16α-OHE_1), 2-OHE_2 and 4-OHE_2 to induce cell transformation, gene mutations, chromosomal aberrations and aneuploidy. These compounds varied in their abilities to induce these genotoxic endpoints. With the exception of 4-OHE_2, which caused a significant increase in mutations at the Na/K ATPase locus, E_2 and its metabolites were not mutagenic. While this study suggested that some metabolites of E_2 have genotoxic potential at high and/or cytotoxic concentrations, the findings should be considered as equivocal due to the absence of a positive control in the majority of experiments and any evidence that the findings were reproducible. (Tsutsui et al 2000a)

Another study by Tsutsui et al (2000b) examined the ability of 2-OCH_3E_2 to induce cell transformation, gene mutations, chromosomal aberrations and aneuploidy in SHE cells. 2-OCH_3E_2 caused an initial elevation in the mitotic index of SHE cells, which was followed by decreased cell growth and increased multinucleation. Cell transformation, mutations at the *hprt* locus, chromosomal aberrations and aneuploidy were all induced by the test compound. However, due to the occurrence of frank cytotoxicity and/or the lack of a positive control, these findings were considered to be equivocal.

Yagi et al (2001) examined the ability of four catechol estrogens of E_2 and oestrone to induce DNA adducts in SHE cells, using the ^{32}P-post-labelling assay. At and above 1 µg/mL, 2-OHE_2, 4-OHE_2, 2-OHE_1 and 4-OHE_1 were cytotoxic. Four different DNA adducts were generated following treatment with the 4 catechol oestrogen metabolites, with 4-OHE_1 causing the greatest level of adduct formation, followed by 2-OHE_1, 4-OHE_2 and 2-OHE_2. Neither E_2 nor E_1 were cytotoxic or generated adducts. This study indicated that catechol oestrogen metabolites can generate DNA adducts in SHE cells at cytotoxic concentrations and that catechol metabolites of E_1 having a greater potential than E_2 at generating adducts.

The following Table summarises the genotoxicity and carcinogenicity findings for 17β-oestradiol from both the EC-commissioned and cited studies.

Summary of genotoxicity and carcinogenicity findings for 17b-oestradiol

Study description	Findings	Conclusions	Reference
In vitro genotoxicity assays			
Mutation assay in V79 cells (*hprt* locus) on E_2	Marginal increase in mutants at 1 µM in one experiment. Same types of mutations seen in controls and E_2-treated cells	±	Kong et el (2000)
Dioxin-treated MCF-7 cells treated with E_2	Various metabolites/conjugates formed. Oxidative DNA damage only occurred when COMT was inhibited.	+ but only with COMT inhib	Lavigne et al (2001)
Transfection of COS-7 cells with N2 or N6 adducts of 2-OHE_1, 2-OHE_2	Mutations caused by these adducts	+	Terashima et al (2001)
Cell transformation, mutations, aberrations & aneuploidy in SHE cells treated with oestrogen metabolites	E_2 and 8 of its metabolites varied in their abilities to induce these genotoxic endpoints. No positive control used in the majority of experiments.	±	Tsutsui et al (2000a)
Cell transformation, mutations, aberrations & aneuploidy in SHE cells treated with 2-OCH_3E_2	Effects in the presence of frank cytotoxicity.	±	Tsutsui et al (2000b)
Adduct formation in SHE cells treated E_2 and E_2 metabolites	Adducts formed at cytotoxic concentrations	±	Yagi et al (2001)
In vivo assays			
Induction of uterine adenocarcinoma in CD-1 mice with E_2 and E_2 metabolites	4-OHE_2 was carcinogenic while E_2 was not. Carcinogenicity due to oestrogenic activity.	+ for 4-OHE_2 - for E_2	Newbold & Liehr (2000)
Metabolite profile in male Syrian golden hamsters treated with E_2	Various metabolites and conjugates formed in liver and kidney. Depletion of glutathione generated N7 DNA adducts of E_1/E_2-3,4-Q	+ but in the absence of glutathione	Cavalieri et al (2001)
Metabolite profile in rat prostate following injection of 4-OHE_2 or E_2-3,4-quinone	Various metabolites generated. Role in carcinogenesis unclear	NA	Cavalieri et al (2001)
Dermal application of E_2-3,4-Q to mice	N3 and N7 adduct formed in skin	NA	Chakravarti et al (2001)

Study description	Findings	Conclusions	Reference
Metabolite profile in ERKO mice	Various catechol oestrogen metabolites and conjugates formed. Preliminary study.	NA	Devanesan et al (2001b)
Review articles			
Model of oestrogen-induced carcinogenicity via the formation of genotoxic catechol oestrogen metabolites and ROS		NA	Cavalieri et al (2000)
Essentially a repeat of Cavalieri et al (2000) with emphasis on the role of aromotase and CYP1β1 in breast cancer		NA	Jefcoate et al (2000)
Also repeated many of the concepts outlined by of Cavalieri et al (2000) and Jefcoate et al (2000)		NA	Liehr (2001)
Brief 'brainstorming' review of breast cancer and its environmental causes		NA	Sasco (2001)

E_2 = 17β-oestradiol; 4-OHE_2 = 4-hydroxy-17β-oestradiol; 2-OCH_3E_2 = 2-methoxy-17β-oestradiol; + = positive finding; - = negative finding; ± = equivocal finding; COMT = catechol-*O*-methyltransferase; ROS = reactive oxygen species; NA = not applicable for the assessment of the genotoxic and carcinogenic potential of 17β-oestradiol.

Mutagenicity and genotoxicity of MGA

A conference abstract by Kranz et al (2002) briefly described the analysis of DNA adduct formation in rat liver slices following exposure to MGA and other unspecified steroidal compounds with progestational activity. All compounds were reported to give rise to the formation of DNA adducts, however, the relative levels of adducts and the pattern of adduct formation were dependent on the compound, the enrichment procedure and the sex of the animal. It was reported that total adduct levels induced by MGA increased by up to 50-fold using female animals but amounted to 20% or less of the levels obtained with cyproterone acetate (positive control). In the absence of adequate experimental detail and publication, this abstract was considered to have limited value in assessing the genotoxicity of MGA.

EVALUATION OF 17 EC-COMMISSIONED STUDIES

Study 1: Presence of oestrogens in meat

No publication.

Study 2: Hormones as growth promoters: genotoxicity and mutagenicity of zeranol and trenbolone

Metzler M & Pfeiffer E (2001) Genotoxic potential of xenobiotic growth promoters and their metabolites. APMIS 109: 89-95.

Experimental: This paper reviewed data reported in the scientific literature, as well as published and unpublished data generated in the authors' laboratory on the *in vitro* metabolism and genotoxicity of 17β-TBO, MGA and zeranol. Only data emanating from the study authors' laboratory was evaluated here.

The oxidative *in vitro* metabolites of 17β-TBO, MGA and zeranol generated from rat, human and bovine liver microsomes were analysed by HPLC and GC-MS. No further experimental details were provided. The genotoxicity of 17β-TBO, MGA and zeranol was assessed using 4 different *in vitro* assays. The induction of forward mutation in the *hypoxanthine phosphoribosyltransferase* (*hprt*) gene in cultured male Chinese hamster V79 lung fibroblasts, and the induction of micronuclei in the same cell line, were determined as previously described (Kulling & Metzler 1997). The induction of forward mutations in the *lacI* gene of E. coli strains CM6114 and NR6113 was determined according to the method of Miller (1992). The formation of DNA adducts in cultured primary rat hepatocytes was determined by the ^{32}P-postlabelling technique according to the method of Topinka (1993). The concentration range tested for each compound and the number of experiments performed are given in the Table below. No further experimental details were given including the rationale for the concentration range, the identity of the vehicle and the positive/negative controls.

Findings: 'Typical' HPLC profiles for 17β-TBO following metabolism by human and rat liver microsomes were provided (no profile was provided for bovine microsomes). Hepatic microsomes from all 3 species were reported to generate triendione as well as 8 monohydroxylation products of 17β-TBO and 5 of trendione. It was reported that microsomes from bovine liver generated 17α-TBO and 2 additional monohydroxylated metabolites of 17α-TBO.

Forward mutation assays in V79 cells and *E.coli* were negative, while the authors reported that marginally positive results were obtained using the micronucleus test in V79 cells and ^{32}P-postlabelling in primary rat hepatocytes (see Table below). In the micronucleus test, it was reported that up to a 3-fold increase in micronucleated cells occurred at the highest dose (125 μM) relative to the controls, however 17β-TBO was reported to be cytotoxic at 150 μM. The metabolite trendione was reported to be weakly positive and caused a doubling in micronucleated cells at 50 μM, with cytotoxicity occurring at 100 μM. In the ^{32}P-

postlabelling assay, 75 $\pm$ 5 adducts per 10^9 nucleotides were reported at 30 µM 17β-TBO compared to 0.5 per 10^9 nucleotides in untreated hepatocytes.

Results of genotoxicity testing of 17b-TBO, MGA and zeranol

Assay	Concentration range	No. of experiments	Result
Forward mutation (V79 cells)			
17β-TBO	50-125 µM	7	negative
MGA	50-125 µM	5	negative
Zeranol	50-150 µM	4	negative
Forward mutation (E coli)			
17β-TBO	1500 µM	5	negative
MGA	400 µM	5	negative
Zeranol	400 µM	5	negative
Micronucleus test (V79 cells)			
17β-TBO	50-125 µM	5	marginally positive
MGA	20-100 µM	5	negative
Zeranol	50-150 µM	3	marginally positive
^{32}P-postlabelling (primary rat hepatocytes)			
17β-TBO	3-30 µM	2	marginally positive
MGA	not tested	not tested	not tested
Zeranol	3-30 µM	2	negative

A HPLC profile of MGA metabolites generated by rat hepatic microsomes was provided, while no data was given for human or bovine microsomes. It was reported that all 3 species generated 7 mono- and 5 dihydroxylated metabolites. Forward mutation assays in V79 cells and *E.coli* were negative, while no micronuclei were generated in V79 cells (see Table above). Apoptosis was reported to occur in V79 cells at 75 and 100 µM MGA based on the observations of deformation of the nuclei and shortening of the metaphase chromosome. Graphically presented data illustrated that the number of apoptotic V79 cells increased over time following 3 h of treatment with 100 µM MGA. The same time course occurred with the positive control (staurosporin). Furthermore, DNA laddering was visualised following gel electrophoresis of MGA-treated cells. Additional experiments by the study authors using HPLC revealed that the apparent apoptotic activity of MGA was attributable to the presence of several impurities, not to pure MGA.

A HPLC profile illustrated that 5 novel metabolites and small amounts of β-zearalanol and zearalanone were generated following the metabolism of zeranol by hepatic microsomes from rats. It was reported that GC-MS revealed that the 5 novel metabolites were monohydroxylated derivatives of zeranol. It was reported that 3 of these were also formed by bovine liver microsomes in addition to β-zearalanol. Forward mutation assays in V79 cells and *E.coli* were negative, while no DNA adducts were generated in primary rat hepatocytes (see Table above). There was an approximate doubling in the number of micronucleated cells in the presence of 150 µM zeranol, β-zearalanol and zearalanone, which the authors described as a "borderline induction".

Conclusions: A number of mono- and/or dihydroxylated metabolites of 17β-TBO, MGA and zeranol were generated by rat, bovine and human liver microsomes. The majority of genotoxicity assays conducted on 17β-trenbolone, MGA and zeranol were negative. A number of weakly positive or equivocal results in the micronucleus test occurred at near-cytotoxic concentrations. The weight-of-evidence indicates that 17β-TBO, MGA and zeranol

were not genotoxic. Undefined impurities in MGA were shown to induce apoptosis. The study authors acknowledged that there is presently no evidence that residues in the meat of treated animals pose a genotoxic risk for the consumer, however they concluded that the lack of information on the biological activity of the metabolites does not necessarily mean that they are safe. This report lacked detail and transparency in both the experimental methods and results.

Study 3: Metabolic pathways of oestrogens as steroidal growth promoting agents

Hoogenboom LAP, De Haan L, Hooijerink D, Bor G, Murk AJ & Brouwer A (2001) Oestrogenic activity of oestradiol and its metabolites in the ER-CALUX assay with human T47D breast cells. APMIS 109: 101-107.

Experimental: The oestrogenic activity of E_2, 17α-oestradiol, E_1, the synthetic 17-hydroxy benzoate, palmitate and oleate esters of E_2, and the 2- and 4-hydroxy metabolites of E_1 and E_2 was measured using the ER-CALUX assay. This assay is based on the increased expression of a luciferase reporter gene in T47D breast cancer cells following exposure to oestrogens. The ER-CALUX assay was performed as previously described (Legler et al 1999). All compounds were dissolved in DMSO and prepared just prior to use due to their possible instability. Experiments were performed in triplicate. Dose-response curves were generated using a computer software package (SlideWrite Plus 6.00) and a one ligand curve fitting. Following subtraction of the vehicle control, the EC_{50} was determined for each compound. Oestrogenic potency, expressed as the relative equivalency factor, was calculated using the formula $EC_{50}(x)/EC_{50}(E_2)$.

Metabolism experiments were performed using wild-type T47D cells that had been incubated with 0.35 μM [^{14}C]E_2 or [^{14}C]E_1. Following incubation for 4 or 24 hours, metabolites were extracted twice with diethyl ether and analysed by HPLC using UV and radioactive detection. To confirm the identity of the metabolites, cells were incubated with 10 μM unlabelled E_2 or E_1, presumably for 4 or 24 hours, and analysed by GC-MS. The purity of all compounds was also confirmed using GC-MS.

Findings: The relative oestrogenicity of each compound (compared to E_2) is summarised in the Table below. While representative dose-response curves were shown for each compound, no EC_{50} data were provided except for E_2 and 17α- (EC_{50} values of between 2-30 pM and 300-1000 pM, respectively). E_2 and E_1 had a similar level of activity, which was reportedly confirmed in 3 independent experiments. Most other compounds were several orders of magnitude less oestrogenic than E_2/E_1; 4-OHE_2 had a relatively high level of oestrogenic activity compared to the other hydroxy metabolites (~half the potency of E_2). Of the synthetic esters, 17β-benzoate was the most oestrogenic but was still 4 times less active than the parent compound.

GC-MS indicated that no detectable levels of oestrone were present in any of the standards, however, several compounds were found to contain traces of E_2. In particular, 2- and 4-methoxy contained 2.2% and 1.1% of the parent compound, respectively. In light of the fact that low levels of contaminating E_2 could interfere with the assay, the study authors omitted the results for 2- and 4-OHE_2 from the study.

Oestrogenic potency (oestradiol equivalency factor) of 17b-oestradiol and various metabolites and synthetic esters of 17b-oestradiol

Compound	Oestradiol Equivalency Factor[1]
E_2	1.0
E_1	1.0
17α-oestradiol	0.03
2-OHE_2	0.00015
4-OHE_2	0.45
2-OHE_1	0.00065
4-OHE_1	0.0054
2-OCH_3E_1	0.0015
4-OCH_3E_1	0.0014
17β-oestradiolbenzoate	0.25
17β-oestradiolpalmitate	0.05
17β-oestradiololeate	0.01

1 = calculated using the formula $EC_{50}(x)/EC_{50}(E_2)$.

There was a time-related increase in the metabolism of E_2 and E_1 by wild-type T47D cells (see Table below). Incubation of cells with [^{14}C]E_2 resulted in a decrease in E_2 and a concomitant increase in E_1 over 24 hours. In contrast, incubation of cells with radiolabelled [^{14}C]E_1 resulted in an increase in E_2 and decrease in E_1. It was reported that GC-MS confirmed that E_1 was formed from E_2 following incubation of cells with unlabelled E_2; the reverse was also confirmed.

Results of the metabolism of radiolabelled E_2 and E_1 by wild-type T47D cells

Compound	Time (h)	% 17b-oestradiol	% oestrone
[^{14}C]E_1	4	80 ± 4	14 ± 2
	24	59 ± 2	39 ± 1
[^{14}C]E_1	4	11 ± 1	85 ± 2
	24	43 ± 3	51 ± 2

Results are expressed as the % of total radioactivity in the radiochromatogram. It was not specified whether these values represented means ± SEM.

Conclusions: In this particular *in vitro* assay, E_2 was shown to be the most oestrogenic of a range of metabolites and synthetic derivatives of E_2. E_1 was found to have a similar oestrogenic activity as E_2, while 17α-oestradiol was approximately 30-fold less active. 4-OHE_2 was the most potent of the metabolites, while 17β-oestradiolbenzoate was the most potent of the synthetic 17-hydroxy esters. The authors speculated on the likely risk to consumers from eating meat containing metabolites of E_2 and concluded that the natural esters of E_2 should be taken into account when evaluating the amount of oestrogens present in meat from treated and untreated animals.

Study 4: Metabolites of melengestrol acetate, trenbolone acetate and zeranol in bovine and humans

Publication pending.

Study 5: Application of anabolic agents to food producing animals – health risks through disregard of requirements of good veterinary practice

Daxenberger A, Meyer K, Hageleit M & Meyer HHD (1999) Detection of melengestrol acetate residues in plasma and edible tissue of heifers. Vet Quart 21: 154-8.

Experimental: Two Holstein-Friesian Heifers per group (51-72 weeks old, mean bw of 320 kg; source unspecified) received 0, 0.5, 1.5 or 5 mg/kg bw/d MGA in the diet for 56 days and were then slaughtered at an "EU official slaughterhouse" by an unspecified means. The dose selection was based on the recommended dose of 0.5 mg/kg bw/d. An additional group of 2 heifers were fed 0.5 mg/kg bw/d MGA for the same duration, but were subjected to a 48-hour withdrawal period prior to slaughter. Diets were reportedly prepared at the authors research site, with the composition of the diet and concentration of MGA verified by GC-MS and HPLC, respectively (results not provided). MGA-containing feed was given to each animal in the morning and when completely consumed, was followed by regular feed. Details of housing conditions were unspecified. Every 84 hours, blood samples were collected by venipuncture from the jugular vein. Following slaughter, tissue samples were taken from neck muscle, liver, kidney and perirenal fat.

Plasma concentrations of MGA were measured using an EIA (Meyer & Hoffmann 1987), which was stated as having a detection limit of 22 ng/L and a recovery rate of 93%. The intra- and inter-assay coefficients of variation were 5 and 13%, respectively. Following extraction and clean-up of kidney, liver, muscle and fat samples, quantification of MGA was performed, with internal standards, by LC-MS (kidney, liver and muscle), or after derivatisation, by GC-MS (fat). The assay used to measure MGA in edible tissues reportedly complied with the requirements of the Commission Decision (93/256/EEC) of 14th April 1993 relating to the measurement of residues of substances having a hormonal or thyreostatic action. It was stated that the entire study was quality assured. No statistical analysis was performed.

Findings: While plasma samples were taken every 3.5 days, mean plasma concentrations were only given for 2 time intervals; day 3.5-29.5 and day 30-56. There was a dose-related increase in plasma MGA concentrations, with the concentration approximately 10-40% lower over days 30-56 compared to days 3.5-29.5 (see Table below). In the 2 heifers withdrawn from treatment 48 hours before slaughter, plasma levels were unchanged 24 hours after withdrawal and had fallen to undetectable levels in only one of the heifers at slaughter.

The concentrations of MGA in edible tissues for each animal are summarised in the Table below. The highest levels were found in perirenal fat, followed by the liver, then the kidney or muscle. There was a dose-related increased in MGA in each tissue. Withdrawal of MGA treatment 48 hours prior to slaughter had no effect on the concentration of MGA in fat and muscle, but the concentration in the liver fell to below the LOD. At the highest dose (5.0 mg/kg bw/d), one of the heifers had an unusually low concentration of MGA in the liver (6.3 µg/kg *versus* 60.9 µg/kg in the other animal). The authors calculated that there was approximately 5-fold more MGA in muscle and kidney relative to the concentration in plasma, 20-40-fold more in liver and 200-fold more in fat; it was concluded that this was evidence of tissue accumulation (in fat). While it is feasible that MGA could accumulate in fat due its lipophilic nature, it would have been informative to measure tissue levels over time to determine the kinetics of distribution and elimination.

Plasma MGA concentrations in heifers

Treatment (mg/kg bw/d MGA)	MGA concentration (ng/L)		
	Day 3.5-29.5	Day 30-56	Day 3.5-56
0 (control)	nd nd	nd nd	nd nd
0.5 (48 h withdrawal)	44.7 ± 12 51.7 ± 9	31.4 ± 15 47.3 ± 11	38.1 ± 15 49.5 ± 10
0.5	40.3 ± 13 36.5 ± 8	36.5 ± 7 28.2 ± 5	38.4 ± 10 32.3 ± 8
1.5	148 ± 34 130 ± 18	116 ± 26 119 ± 14	132 ± 34 124 ± 16
5.0	321 ± 61 361 ± 86	207 ± 30 217 ± 45	264 ± 76 289 ± 100

Results are expressed as the mean ± 1 SD of the 8 measurements during each interval for each animal; nd = not detected

MGA concentrations in edible tissue (µg/kg tissue)

Treatment (mg/kg bw/d MGA)	Perirenal fat	Liver	Kidney	Muscle
0 (control)	<2 <2	<0.5 <0.5	<0.5 <0.5	<0.2 <0.2
0.5 (48 h withdrawal)	7.0 7.9	<0.5 <0.5	<0.5 <0.5	0.3 0.4
0.5	6.5 8.4	1.0 0.8	<0.5 <0.5	0.2 0.2
1.5	24.1 33.9	2.3 7.7	0.5 0.4	0.5 0.9
5.0	6.3* 60.9*	5.1 7.6	2.0 1.7	1.3 1.0

Data are the absolute concentrations for each animal; * while the average of these 2 values is 33.6, the authors indicated in a later summary table that the average of these values was 59.

The authors concluded that their results showed that treatment with a 3-fold higher labelled dose of 0.5 mg/kg bw/d can exceed the tolerance of 25 ppb in fat of cattle set by the FDA. However, the important finding was that the recommended dose did not exceed this tolerance. Furthermore, consumers are more likely to eat muscle rather than fat, which had a relatively low level of MGA. It was argued that "it cannot be assumed that the labelled application conditions will be applied" and therefore the use of MGA would need to be controlled. While the hazard to consumers was not considered by the authors to be "severe", they concluded that MGA should be toxicologically evaluated by the FAO/WHO (Note: a toxicological evaluation of MGA was conducted by JECFA in 2000).

Conclusion: A dose-related increase in plasma and tissue concentrations of MGA occurred in heifers over a 56-day dietary treatment period. MGA appears to preferentially distribute to fat followed by the liver. Withdrawal of MGA 48 hours prior to slaughter had no effect on fat and muscle residue levels, while residues in liver were reduced. Significant limitations to this study included the small group sizes and consequent lack of statistical analysis.

Daxenberger A, Lange IG, Meyer K and Meyer HHD (2000) Detection of anabolic residues in misplaced implantation sites in cattle. Journal of AOAC International 83(4): 809-819.

Experimental: A number of experiments were undertaken to determine the residue levels of hormones in cattle following implantation at various "off-label" sites with commercial anabolic preparations.

(1) Five Holstein-Friesian heifers (77-99 weeks old, 367 kg average bw) were injected in the neck (*M. splenius, M. serratus*), midway between the shoulder and the head and approximately 6 cm below the cervical spine, with a single dose (7 pellets) of Revalor-H® (140 mg TBA + 14 mg E_2), 8 weeks before slaughter. Two untreated heifers served as controls.

(2) A group of 12 heifers was implanted 8 weeks before slaughter at different sites with Revalor H®. Single animals were injected in the shoulder (*M. deltoides*), leg *(M. semitendinosus*), external ear (pinna; the authorised location for implantation), earground a (*M. cervicoauricolaris*), earground b (*M. cervicoscutularis*), earground c (*M. cervicoauricolaris*), base of the tail (*M. coccygeus*), breast muscle (*M. pectoralis desc.*) dewlap, neck, scutulum or crown (*M. frontalis*).

(3) A group of 7 heifers was implanted in the neck, 8 weeks before slaughter, with either a single dose of Finaplix-H® (200 mg TBA; 10 pellets), Synovex plus® (200 mg TBA + 28 mg oestradiol-benzoate; 8 pellets), Synovex-H® (200 mg testosterone propionate + 20 mg oestradiol-benzoate; 8 pellets), Compudose® (25.7 mg E_2; 1 pellet), Component EC® (100 mg progesterone + 10 mg oestradiol-benzoate; 4 pellets), Implus-S® (200 mg progesterone + 20 mg oestradiol-benzoate; 8 pellets) or Ralgro® (36 mg zeranol; 3 pellets).

(4) A group of 3 heifers was implanted in the neck with Revalor-H® for different lengths of time. One animal was implanted with a single dose at 4 or 12 weeks, while the third animal was implanted 3 times at 4, 12 and 20 weeks prior to slaughter.

(5) One steer (389 kg bw, 102-weeks old), one male and one female calf (140 and 105 kg bw, respectively; 13 and 14-weeks old, respectively) were treated as in (1). No control steers or calves were used.

All animals were obtained from an approved livestock provider and deemed to be healthy following a veterinary inspection. Heifers were cycling and gynaecologically intact. Gynaecological examination and analysis of TBO, zeranol and MGA in initial blood samples, confirmed that none of the animals had previously been treated with anabolic steroids. Animals were fed 2 kg hay/d, and head and corn silage *ad libitum*.

Animals were sacrificed at an EU-official slaughterhouse, and the implantation site and surrounding tissue (1.5-3 kg) excised and systematically cut into pieces having a maximum diameter of 5 mm. All detectable pellets from each implantation site were extracted with disposable scalpels to prevent contamination of samples, then pooled and analysed together. Residue analysis was performed on the visible remainder of the pellet, the inner 2.5 cm area of tissue around the implant, and the outer area (ie. the next 1.5 cm of tissue). Approximately 50-150 g of tissue was collected with each pellet. For pellets in ears, a 3 cm area of the pinna

surrounding the injection site, including cartilage and skin, served as the inner area, while the earground was the outer area. No tissue samples were taken from the crown as the pellets adhered to the bone.

Hormones were twice extracted from homogenised tissue samples by *tert* butyl-methyl-ether (TBME). Following clean-up on an octadecyl-silica cartridge, residue analysis was performed by liquid chromatography with diode array detection (LC-DAD). Pellets were treated in a similar manner as tissue samples but were extracted 3 times with TBME. Recovery of all hormones after standardisation ranged from 88.3 to 108.2% (mean = 99%), while the coefficient of variation (CV) ranged from 0.25 to 8.1% (mean = 3.2%), depending on the hormone.

The content of the pellets was verified by LC-DAD and the identity of the active constituents confirmed by GC-MS of heptafluorobutyryl derivatives, as previously described (Daeseleire et al 1991; Casademont et al 1996). It was stated that the entire study was quality assured and the QA status of the laboratory was inspected by the EC Reference Laboratory (CRL, Bilthoven, The Netherlands).

Findings

Detection of implantation sites and pellets: Of the 31 animals implanted (excluding the animal implanted in the ear), 77% of the implantation sites were detectable by visual inspection (see Table below). The lowest level of detection (60%) occurred in experiment 4, where animals were implanted for different lengths of time. Of the 210 pellets implanted, 67% were recovered, with the lowest recovery (40%) also occurring in experiment 4. No pellets were found in 7 of the 31 animals. It was reported that pellets were mainly embedded in connective tissue sheaths and had generally decreased in size. At an unspecified number of implantation sites, some pellets were found to have migrated, as they were located within pockets of 3-4 cm in diameter. Absent pellets were presumed to have dissolved.

Detection of implantation sites and pellets in cattle

Experiment	No. implantation sites	% implantation sites detected	No. pellets implanted	% pellets detected
Experiment 1	5	80	35	66
Experiment 2	11	82	77	81
Experiment 3	7	86	42	69
Experiment 4	5	60	35	40
Experiment 5	3	67	21	62
Total	31	77	210	67

Residues remaining in pellets after 8 weeks: The average amounts of TBA and E_2 remaining in Revalor-H® pellets (experiment 1 and 2) were 42.9 mg (range 19.8-57.5 mg) and 4.6 mg (range 1.96-6.45 mg), respectively (calculated by the study authors to be equal to 30 and 32.7% of the applied dose, respectively). When Revalor-H® was implanted into the pinna of the ear (ie. the authorised implantation site), the amounts of TBA and E_2 were 74.3 and 7.91 mg, respectively (~50% recovery). No implants were found in 3 animals implanted with Revalor-H®; one implanted in the neck (experiment 1), one implanted at the base of the tail and one implanted in the scutulum (experiment 2).

Residue levels remaining in pellets from the 2 calves (experiment 5) were similar to heifers, while no implants could be found in the steer. Implantation of a heifer, 4 weeks before slaughter (as opposed to 8 weeks), resulted, not surprisingly, in higher levels of TBA and E_2 in the recovered pellets (59.0 and 6.06 mg, respectively, or 42.2 and 43.3 %; experiment 4). No pellets could be found in the heifer that was implanted 12 weeks before slaughter. No meaningful information was obtained from the animal that was implanted at 4, 12 and 20 weeks before slaughter as the identity of each pellet could not be determined.
Residues levels remaining in various commercial anabolic preparations (experiment 3) are summarised as followings: Finaplix-H® = 33.3 mg TBA (16.7% recovery); Synovex plus® = 4.76 mg TBA, 0.68 mg E_2 (2.4% recovery); Synovex-H® = 31 mg testosterone propionate, 3.26 mg E_2 (15.5 and 16.3% recovery, respectively); Compudose® = 15 mg E_2 (58.4% recovery); Component EC® = 32.9 mg progesterone, 3.82 mg E_2 (32.9 and 38.2 % recovery, respectively); Implus-S® = 52.6 mg progesterone, 7.10 mg oestradiol-benzoate (26.3 and 35.5% recovery, respectively). Ralgro® was not evaluated as the pellets were either completely dissolved or could not be found. The above results were considered to be preliminary as the sample size for each implantation was only one animal. It was reported that a small faction (0.1-0.3% of the applied dose) of TBA was hydrolysed to 17β-TBO in those implants that contained this hormone. Furthermore, there appeared to be no difference in the level of 17β-TBO between the various commercial preparations. Hydrolysis of oestradiol benzoate and testosterone propionate did not occur.

Inner tissue residues: Implantation of heifers with Revalor-H® (experiment 1 and 2; n=13) resulted in highly variable concentrations of TBA (0.47-118 µg/g; 22.02-12557.51 µg total), 17β-TBO (0.58-19 µg/g; 41.72-1447.19 µg total) and E_2 (0.05-37.03 g/g; 11.09-3931.19 µg total) in the 2.5 cm of tissue immediately surrounding the pellets. The study authors attributed the relatively high residue levels in some animals to the method of tissue preparation. In some animals the pellets could not be located and thus it was necessary to dissect the tissue. During this process, the scalpel may have cut a pellet and contaminated the surrounding tissue.

Residue levels in the male/female calves (experiment 5) were 3.49/0.21 µg/g TBA, 2.34/1.01 µg/g 17β-TBO and 5.09/0.65 µg/g E_2. The total amounts detected in the complete tissue mass were 202.35/14.79, 135.5/71.65 and 5.09/0.65 µg, respectively.

Residue levels in the inner tissue from heifers treated with various commercial anabolic preparations (experiment 3) were considered to be preliminary as the sample size for each implantation was only one animal. However, there was evidence that 84% of the TBA in Synovex Plus® was hydrolysed, 17% of the testosterone propionate in Synovex H® was hydrolysed and approximately 10% of the oestradiol benzoate in Component EC® and Implus S® was hydrolysed. No E_2 was detected around the Compudose® pellets. Synovex plus® resulted in very low tissue residue levels (0.01, 0.03 and 0.04 µg/g for TBA, 17β-TBO and E_2, respectively).

Implantation of a single heifer with Revalor-H®, 12 weeks before slaughter (experiment 4), resulted in negligible levels of TBA, 17β-TBO and E_2. In contrast, implantation for a shorter period of time (ie. 4 weeks) resulted in concentrations of 2.61, 2.11 and 3.93 µg/g for TBA, 17β-TBO and E_2, respectively.

Outer tissue residues: Outer tissue areas (ie. the next 1.5 cm of tissue from the inner tissue area) surrounding Revalor-H® pellets were reported to contain <10 ng/g TBA (2 µg total)

except for samples from the neck, earground and breast muscle which were higher (50-220 ng/g; 5.3, 20.3 and 8.9 µg total, respectively). The heifer implanted for 4 weeks (experiment 4) also had a relatively high amount of TBA (58.5 µg). The study authors reported that 17β-TBO was detected in 15/24 samples (mean = 37 ng/g, total = 6 µg) and only 6/24 Revalor-H® implantation sites contained residues of E_2 above the detection limit (mean of 140 ng/g, 26 µg total).

Analysis of outer tissue areas in animals treated with commercial anabolic preparations (experiment 3) revealed that Finaplix-H® generated relatively high levels of TBA (13.8 µg/g, 677 µg total) and 17β-TBO compared to the inner area and other preparations. Synovex Plus® was reported to generate 2.5 µg total TBO (39% 17β-trenbolone) and 2.8 µg E_2. Finaplix-H® was reported to yield 10.8 µg testosterone propionate, 3.6 µg free testosterone and 5.6 µg oestradiol benzoate. No detectable residues were found for Compudose®, Component EC® and Implus®.

Conclusions: Approximately 30% of anabolic implants were not recovered 8 weeks after implantation at off-label sites in cattle. At slaughter, the highest residue levels were found in the remaining pellet, which led the authors to conclude that the risk assessment of hormone residues in meat should be focussed on the remaining pellets. The authors speculated that the misplacement of implant preparations or the failure to discard ears following slaughter could allow mg of hormone residues to enter the food supply and cause acute toxicity in consumers. They emphasised the importance of GVP. Their final conclusion was that because of the 'enormous economic benefits', the use of HGPs cannot simply be rejected and that the important issue was that the implantation and management of their use should aim to eliminate the risk of leaving significant hormone residues. A limitation to this study was the considerable variability in residue levels detected in implants and the surrounding tissue. Several of the experiments should be viewed as preliminary as only single animals were treated. Acknowledged by the study authors was the problem of pellets being damage during their excision, and subsequently contaminating the surrounding tissue.

Daxenberger A, Hageleit M, Kraetzl W-D, Lange IG, Claus R, Le Bizec B & Meyer HHD (2001) Suppression of androstenone in entire male pigs by anabolic preparations. Livestock Production Science 69: 139-144.

Experimental: Androstenone is a sex pheromone that causes an unpleasant odour in carcasses of postpubertal male pigs. This experiment was undertaken to determine the effect of commercial anabolic preparations on the concentration of androstenone in the fat of intact boars. Hormone residues in implantation sites were also examined.

Four boars/group (Landrace X Pietrain crossbreeds, 19-weeks old, average bw of 80.5 kg; source unspecified) were implanted in the neck (to simulate off-label use) with 2 doses of Synovex-H® (total dose of 400 mg testosterone propionate + 40 mg oestradiol benzoate) or Synovex Plus® (total dose of 400 mg TBA + 56 mg oestradiol benzoate). A control group consisted of 4 untreated animals. Boars had been fed *ad libitum* with a commercial diet until 12 weeks of age, followed by a finishing diet until the end of the experiment. Few details on housing conditions were provided except that boars were exposed to a 10/14 h light/dark cycle. Blood samples were collected by venipuncture of the jugular vein before treatment, then at 7, 21 and 31 days after implantation. Five weeks after implantation, animals were slaughtered by an unspecified means. Immediately after slaughter, the complete implantation

site, testes and perirenal fat were collected. The testes were weighed and measured, while the implantation site was prepared further in the laboratory to separate the implant from surrounding tissue.

Plasma concentrations of testosterone and 17β-TBO were measured by EIA as previously described (Meyer & Hoffman 1987; Blottner et al 1996), while residues in recovered implants were quantified by HPLC and diode array detection (Daxenberger et al 2000). Boar taint steroids in adipose tissue were quantified by EIA (Claus et al 1997), with the concentration of androstenone confirmed by GC-MS (Le Bizec et al 1996). No details of statistical analyses were provided.

Findings: The authors reported that there was no significant difference in average daily weight gain or food consumption between the treatment and control groups. The authors drew attention to graphically presented data illustrating that plasma testosterone concentrations decreased from day 0 (pretreatment) to day 7 in all four boars implanted with Synovex Plus® (containing TBA and E_2). No such effect occurred in boars implanted with Synovex-H® (containing testosterone and E_2). However, 3 of the 4 control boars also showed a decrease in plasma testosterone from day 0 to 7. Examination of testosterone data from the entire implantation period (days 7, 21 and 31) revealed an approximately 4-fold decrease in plasma testosterone in boars treated with Synovex Plus® compared to the controls (see Table below), however it was not specified whether this result was statistically significant. The slight increase in plasma testosterone in boars implanted with Synovex-H® was attributable to a single animal (#500) with a high level of testosterone.

Effect of Synovex-HÒ or Synovex PlusÒ on plasma testosterone and androstenone in adipose tissue and the testis weight of boars

Group	Testosterone (ng/mL)[a]	Androstenone (ng/g)	Testis weight (g)		
			Left	Right	Total
Control	1.4 ± 0.42 (0.68-2.35)	688 ± 238 (312-1312)	227 ± 5.4	244 ± 13	470 ± 18
Synovex-HÒ	2.2 ± 0.91 0.60-4.82)	401 ± 298 38-1291)	202 ± 43	197 ± 50	400 ± 92
Synovex PlusÒ	0.30 ± 0.19 (0.08-8.87)	50 ± 14 (16-73)	188 ± 33	184 ± 38	372 ± 71

Results are expressed as means ± 1 SEM (n=4), with the range shown in parentheses; a = results given as the mean during the entire implantation period (days 7, 21 and 31).

Synovex Plus® reduced androstenone in adipose tissue by approximately an order of magnitude, but it was not specified whether this effect was statistically significant (see Table above). Synovex-H® implantation appeared to have a marginal effect on androstenone concentrations (see Table above), however analysis of individual animal data revealed that 3/4 boars actually had markedly lower androstenone concentrations than the control. The lack of a clear effect of Synovex-H® may have been due to a single outlying animal (#500) that had a highly elevated level of androstenone (ie. the same animal with elevated testosterone).

There was a slight reduction in testis weight in boars implanted with Synovex-H® or Synovex Plus® although neither of these findings were statistically significant (see Table above). The authors reported that there was a slight (r=0.679) but significant (p=0.015) linear regression between total testis weight and the concentration of androstenone in adipose tissue.

Analysis of residues remaining at the implantation site (implant plus surrounding tissue) following slaughter revealed that the highest residues were in the pellets (see Table below). Besides the parent compounds, traces of hydrolysed testosterone (0.08 ± 0.03 mg) or 17β-TBO (1.4 ± 0.4 mg) were detected in the surrounding tissue. No evidence of hydrolysis of oestradiol benzoate was observed. The relative effective dose as calculated by the authors was 2.32 mg/kg bw testosterone propionate and 0.22 mg/kg bw oestradiol benzoate for Synovex-H®, and 3.33 mg/kg bw TBA and 0.43 mg/kg bw oestradiol benzoate for Synovex Plus®.

Hormone residues detected at implantation sites (implant plus surrounding tissue)

Group	Testosterone propionate or TBA (mg)		Oestradiol benzoate (mg)	
	Pellet	Tissue	Pellet	Tissue
Synovex-H®	175 ± 16 (136-206)	4.6 ± 2.5 (2-12.1)	18.8 ± 1.4 (14.6-20.9)	0.58 ± 0.21 (0.3-1.2)
Synovex Plus®	63 ± 16 (31.9-106)	20.7 ± 10 (1.4-48.6)	9.4 ± 2.5 (4.8-16.4)	5.5 ± 1.5 (2.5-9.6)

Results expressed as the mean ± 1 SEM, with the range in parentheses

In considering that the implantation sites contained an average of 179 mg testosterone propionate or 85 mg TBA, and 17 mg oestradiol benzoate, the authors argued that hypothetically, the industrial processing of an implantation site along with the useable carcass could contaminate meat with residues above the MRL for trenbolone and E_2. The limitation to this argument is that in those countries where anabolic implants are approved for use in cattle, the prescribed implantation site is the ear, which are discarded at slaughter and therefore do not enter the food supply. The authors suggested that the availability of anabolic agents used in cattle might lead to misuse in other species.

Conclusions: Implantation of boars with an anabolic preparation containing TBA and oestradiol benzoate (Synovex Plus®) caused a reduction in androstenone in adipose tissue and a concomitant reduction in plasma testosterone. Implantation with a different preparation containing testosterone propionate and oestradiol benzoate did not have such an effect. Both preparations caused a small reduction in testis weight, which was not statistically significant.

Hageleit M, Daxenberger A & Meyer HHD (2001) A sensitive enzyme immunoassay (EIA) for the determination of melengestrol acetate (MGA) in adipose and muscle tissues. Food Additives and Contaminants 18(4): 285-291.

This paper described the development and validation of a screening method for the quantitation of MGA residues in bovine perirenal fat and muscle based on a competitive EIA. Negligible cross-reactivity to natural steroid hormones, their precursors or metabolites occurred. Cross-reactivity to megestrol acetate (10%), medroxyprogesterone acetate (6.6%), 6α-methyl-17α-OH-progesterone acetate (5.2%), chlormadinone acetate (4.8%) and 17α-acetoxyprogesterone (3.9%) was reported. A detection limit of 0.38 ng/g in fat and 0.05 ng/g in muscle was attained. The mean accuracy in fat and muscle was 71.6 and 75.5%, the mean intra-assay variation was 7.6 and 5.6%, and the mean inter-assay variation was 14 and 11%, respectively. Measurement of MGA concentrations in fat and muscle samples from a previous study (Daxenberger et al 1999; see evaluation above) yielded results that were compatible with those obtained using GC-MS or LC-MS. It was stated that this new method complied with requirements for screening methods of the European Commission Decision

93/256/EC. The authors suggested that for confirmatory or forensic purposes, the necessary MS-based methods should be employed.

Bauer ER, Daxenberger A, Petri T, Sauerwein H & Meyer HHD (2000) Characterisation of the affinity of different anabolics and synthetic hormones to the androgen receptor, human sex hormone binding globulin and to the bovine progestin receptor. APMIS 108: 838-46.

Experimental: The affinity of a variety of natural and synthetic steroids (see Table below) to the recombinant human androgen receptor (rhAR), human sex-hormone binding globulin (hSHBG) and the bovine uterine progestin receptor (bPR) were evaluated using competitive radioligand binding assays. The majority of compounds were obtained commercially or were provided by other laboratories. The 3 main MGA metabolites (M6, M7 and M10) were produced *in vitro* by incubation of bovine liver microsomes with the parent compound and separated by HPLC. It was reported that M10 was unstable, with its concentration decreasing by 50% over the course of the study.

The rhAR assay was performed as previously described (Bauer et al 1998). The baculovirus expression system was used for the production of the rhAR according to the methods of Chang et al (1992) and Janne et al (1993). Aliquots of rhAR were incubated with 0.4 nM [^{3}H]dihydrotestosterone ([^{3}H]DHT) in the presence or absence of increasing concentrations of one of the test compounds. The exact concentrations of the test compounds and the identity of the vehicle were unspecified. Dihydrotestosterone (DHT) was used in all experiments as the positive control. Following incubation at 0-4°C for 16 h, dextran-coated charcoal was used to separate bound and free ligand. Following centrifugation, the amount of radioactivity in the supernatant was measured by scintillation counting. Specific binding was determined as the relative difference in total binding and non-specific binding observed in the presence of a 250-fold surplus of unlabelled DHT. All measurements were performed in triplicate.

The bPR assay was performed as previously described (Meyer et al 1988) using uterine cytosols prepared according to the method of Sauerwein & Meyer (1989). Cytosol was incubated with 1.5 nM [^{3}H]ORG 2058 (16α-ethyl-21-hydroxy-19-nor[6,7-^{3}H]pregn-4-ene-3,20-dione) in the presence or absence of increasing concentrations of one of the test compounds. Progesterone was used in all experiments as the positive control. The remaining assay conditions were the same as those described for the rhAR assay.

The SHBG-binding assay was performed by incubating hSHBG with 0.4 nM [^{3}H]DHT and 0-, 250-, 2500- and 25000-fold concentrations of the test compounds compared to [^{3}H]DHT. Assay conditions were the same as those described for the rhAR assay. Specific binding was determined as the relative difference in total binding and non-specific binding (1 µM DHT).

All binding data were analysed by non-linear, exponential regression with 4 parameters. The equation used was $y = y_0 + A/[1 + \exp(-(x/x_0)/B]$ where y_0 is the baseline of the curve, A the plateau value of the curve, B the slope and x_0 the point of inflection of the displacement curve. Inhibition constants (K_I) were calculated using the equation $K_I = IC_{50}/(1 + S/K_D)$ where IC_{50} is the concentration of the unlabelled compound which causes 50% displacement of the labelled compound, which is different from x_0 and must be calculated separately. S represents the concentration of the labelled ligand and K_D the dissociation constant. K_D values were determined by Scatchard analysis of DHT binding to the hAR and of progesterone binding to the bPR.

Findings: Graphically represented data illustrated the competitive binding of ^{3}H-DHT and DHT to the rhAR, and ^{3}H-ORG 2058 and progesterone to the bPR. Scatchard plots revealed that the K_D for DHT was 2.66 x 10^{-9} M (r^2=0.9932) while for progesterone it was 2.33 x 10^{-9} M (r^2=0.9593). Saturation plots revealed K_D values of 2.24 x 10^{-9} M (r^2=0.9950) and 2.26 x 10^{-9} M (r^2=0.9942), respectively. K_I values were calculated as the mean of both measurements. It was reported that displacement curves were sigmoidal, with a plateau at the maximum and a baseline always below 5%, indicating that all compounds were able to inhibit binding of the labelled ligand almost completely.

The relative binding affinities for each of the test compounds compared to the controls are summarised in the Table below. 17β-TBO was the most effective compound at competing with DHT for binding to the rhAR, and appeared to have a slightly higher affinity than DHT itself. It was unclear whether this finding was statistically significant. MGA and its metabolites had relatively weak affinities for the rhAR.

Relative binding affinities (RBA)* of a variety of natural and synthetic steroids for rhAR, bPR and hSHBG

Compound	RBA (%)		hSHBG†
	rhAR DHT = 100%	bPR Progesterone = 100%	
DHT	100	1.38	0.00
17β-TBO	108.86	137.40	29.39
Allyltrienolone	75.42	1082.74	19.20
19-nortestosterone	75.22	19.51	5.61
Boldenone	48.76	24	0.74
Medroxyprogesterone acetate	48.61	222.89	81.96
Methyltestosterone	35.93	7.79	1.37
Testosterone	31.31	1.	0.56
Chlormadinone acetate	14.61	1080.53	32.85
E_2	4.88	2.34	Data not evaluated
17α-TBO	4.49	2.04	94.76
Progesterone	3.83	100.00	46.57
Epitestosterone	1.63	0.06	10.69
Triendione	0.36	1.03	87.96
MGA	0.31	526.31	25.24
MGA metabolite 6	<1.30	84.75	Data not evaluated
MGA metabolite 7	<1.30	45.53	Data not evaluated
MGA metabolite 10	<1.30	28.26	Data not evaluated

* = calculated according to the equation RBA(x) = Ki(DHT)*100/Ki(x), to the rhAR, bPR and hSHBG; † = residual specific 3H-DHT binding (%) in the presence of a 2500-fold concentration of the test compound

Allyltrienolone, medroxyprogesterone acetate, chlormadinone acetate (ie. the therapeutically used synthetic progestagens) had high relative binding affinities (RBAs) for the bPR compared to progesterone. MGA showed an approximately 5-fold higher RBA than progesterone, while the RBA of 17β-TBO was approximately 1.4-fold higher (than progesterone). The 3 metabolites of MGA (6, 7 and 10) exhibited RBAs of between 28 and 85% indicating that they possessed some potential to bind to the bPR.

DHT, boldenone, testosterone and methyltestosterone (ie. the compounds used as doping agents) had relatively high affinities for hSHBG and could completely block specific ^{3}H-DHT binding at concentrations 250-fold higher than DHT. In contrast, MGA, 17α- and β-

TBO, and triendione had much lower affinities; graphically presented data illustrated that a 25000-fold concentration of these compounds could not completely block specific ^{3}H-DHT binding to hSHBG.

Conclusions: Based on the relatively high affinity of 17β-TBO to both the rhAR and bPR, the authors concluded that residues with significant binding capacity and potentially endocrine-disrupting activity would be excreted after trenbolone treatment (of cattle). However, they provided no comment on the possible health risk to consumers from eating meat containing TBO residues. The authors also speculated that, given the relatively high affinity of MGA to the bPR and the low affinity of the 3 main metabolites (which apparently fell within the range of natural progesterone levels), PR active substances might be excreted from cattle treated with MGA. Their final comment on the safety of these compounds was that the biodegradation, distribution and bioefficacy of the breakdown products of MGA and TBA are necessary before considering them safe. While this study presented some interesting comparative *in vitro* data on the binding of MGA and TBO to hormone receptors, it remains unclear whether consumption of tissue residues would generate high-enough plasma concentrations of MGA and TBA in humans to competitively bind to the AR/PR and exert a significant effect. This report lacked methodological detail and no statistical analysis was performed on the data.

Hageleit M, Daxenberger A, Kraetzl W-D, Kettler A and Meyer HHD (2000) Dose-dependent effects of melengestrol acetate (MGA) on plasma levels of oestradiol, progesterone and luteinising hormone in cycling heifers and influences on oestrogen residues in edible tissues. APMIS 108: 847-54.

Experimental: This study has previously been published (Daxenberger et al 1999); additional data was presented relating to the effects of MGA on plasma levels of progesterone, E_2 and LH, and tissue levels of E_2. Cycling Holstein Friesian heifers were treated with MGA and samples of plasma were collected at various times, as described in the above evaluation of Daxenberger et al (1999). To examine the pulsatile secretion of LH in each heifer, 2 days before treatment and at 2 and 7 weeks post-treatment, 6-hour plasma profiles were generated in 20-minute intervals using a jugular vein catheter. Animals were slaughtered by an unspecified means after 56 days and perirenal fat and loin (muscle) samples taken. The apparent stages of the oestrous cycle were estimated according to the criteria of Ireland et al (1980), with ovarian follicles categorised as "big" or "small" (diameters of >10 mm and 3-10 mm, respectively). Plasma concentrations of MGA and progesterone were determined using EIAs according to the methods of Daxenberger et al (1999) and Prakash et al (1987), respectively. Plasma LH was quantified by RIA (Schams et al 1972), while plasma 17β-oestradiol was quantified by EIA after HPLC purification. Following extraction and HPLC separation, tissue concentrations of oestrone and E_2 were quantified by EIA (Lange et al 2001). LH data were statistically analysed using a Student-Newman-Keuls test. No other statistical analysis was performed on any other data.

Findings: One control heifer had a cystic corpus luteum, while no corpora lutea were detected in any other animals. All heifers appeared to be in the late follicular phase of the oestrus cycle (day 18-21), with the exception of the above control animal, which was not cycling. There was no treatment-related effect on the number or diameter of follicles
Graphically presented data of plasma MGA concentrations in each animal every 3.5 days illustrated a clear dose-related increase. At 0.5 mg/kg bw/d, mean plasma levels were reportedly between 25-50 pg/mL, while they were approximately 3- and 9-fold higher at 1.5

and 5 mg/kg bw/d, respectively. No MGA was detected in the plasma of control heifers. There was a time-related decrease in plasma MGA at 5 mg/kg bw/d, which was not evident at the lower doses.

It was reported that prior to treatment, all heifers had progesterone levels "typical for the luteal phase". Graphically-presented data of plasma progesterone in each animal every 3.5 days illustrated a difference in cycling between the 2 control heifers; one showed evidence of a persistent corpus luteum (stable level of progesterone), while the other showed evidence of cycling (fluctuations between <0.2 and 5.7 ng/mL). There was a dose-related decrease in plasma progesterone levels, with levels falling to below 0.3 ng/mL at 0.5 mg/kg bw/d MGA, and to below the LOD at the two higher doses (ie. <0.2 ng/mL).

Results of plasma E_2 levels were also presented graphically for each animal. The concentration of plasma E_2 measured in the 2 control heifers followed a similar pattern as the concentration of progesterone; a constant level of E_2 (0.8 pg/mL) was measured in one heifer, while the concentration fluctuated between 0.5 and 11.7 pg/mL in the other. At 0.5 mg/kg bw/d, MGA caused "frequent acyclic pulses" of E_2, with an average concentration of 4.9 $\pm$ 3.3 pg/mL. There was a dose-related decrease in the frequency of these pulses, with an average concentration at 1.5 and 5 mg/kg bw/d of 1.9 $\pm$ 0.9 and 1.2 $\pm$ 0.4 mg/kg bw/d, respectively.

Analysis of E_2 and E_1 levels in fat and muscle are summarised in the Table below. At 0.5 mg/kg bw/d MGA, there was an approximately 7-fold elevation in E_2 in muscle relative to the control. At 1.5 mg/kg bw, the concentration of muscle E_2 was similar to the control, while no E_2 was detectable at 5 mg/kg bw/d. A similar trend was observed in fat, however, the concentration of E_2 was markedly higher than that in muscle. No detectable levels of E_1 were found in muscle. Fat E_1 was elevated at 0.5 mg/kg bw/d MGA relative to the control, but fell to below control levels at 1.5 mg/kg bw/d, and was undetectable at 5 mg/kg bw/d. Withdrawal of MGA 48 hours prior to slaughter reduced tissue levels of E_2 and E_1, however, levels of both remained higher than the control.

Tissue residue levels (pg/g tissue) of E_2 and E_1 in heifers following MGA treatment

MGA concentration (mg/kg bw/d)	Muscle (loin)	Fat	
	E_2	E_2	E_1
0	0.2	8.0	8.1
	<0.15	11.9	7.8
0.5 (48 h withdrawal)	0.3	18.9	16.7
	0.3	19.5	9.1
0.5	1.3	30.4	20.6
	1.8	34.0	10.9
1.5	<0.15	6.9	5.3
	0.2	3.3	<2.0
5	<0.15	<2.0	<2.0
	<0.15	4.7	<2.0

Results are expressed as the absolute value for each animal

MGA was found to affect the plasma concentration of LH plus the number of pulses/h during the 6-hour profiles. At 0.5 mg/kg bw/d, MGA caused a significant ($p<0.05$) increase in plasma LH compared to the control (1.23 $\pm$ 0.04 *versus* 1.02 $\pm$ 0.03 ng/mL), while plasma LH was significantly decreased ($p < 0.05$) at 1.5 and 5 mg/kg bw/d (0.81 $\pm$ 0.05 and 0.75 $\pm$ 0.02 ng/mL, respectively). Mean pulses ($\pm$ 1 SEM) per hour were also significantly increased

($p<0.05$) at 0.5 mg/kg bw/d MGA (0.75 ± 0.09 pulses/h). While there was an increase in pulses/h at 1.5 mg/kg bw/d (0.42 ± 0.05 pulses/h) and a decrease at 5 mg/kg bw/d (0.08 ± 0.05 pulses/h), neither result was significantly different to the control (0.25 ± 0.08 pulses/h)

Conclusion: MGA caused dose-related perturbations in the plasma concentration of progesterone, E_2 and LH, in addition to tissue levels of E_2 and E_1. Treatment with the labelled dose of MGA (0.5 mg/kg bw/d) resulted in elevated levels of tissue and plasma E_2, and plasma LH. Higher doses of MGA reduced plasma and tissue levels of E_2, and plasma LH. These effects occurred in the absence of any consequence on the number and size of ovarian follicles, or on the presence of corpus luteum. Limitations to this study were the low group sizes (n=2) consequent with a lack of statistical analysis, and the lack of individual animal data.

Lange IG, Daxenberger A & Meyer HHD (2001) Hormone contents in peripheral tissues after correct and off-label use of growth promoting hormones in cattle: Effect of the implant preparations Finaplix-Hâ, Ralgroâ, Synovex-Hâ and Synovex Plusâ. APMIS 109: 53-65.

Experimental: This study examined two scenarios of possible misuse of commercial anabolic preparations in cattle, namely, overdosing in heifers and off-label use in veal calves. In the first study, 2 Holstein-Friesian heifers/group (unspecified age, bw & source) were treated with a 1-, 3- or 10-fold doses of Finaplix-H® (200 mg TBA), Ralgro® (36 mg zeranol) or Synovex-H® (200 mg testosterone propionate + 20 mg oestradiol benzoate). Two untreated heifers served as the control group. Implants were administered into the right ear according to the manufacturer's instructions. In those heifers treated with a 10-fold dose, 5 implants were injected into each ear. In the second study, 2 Holstein Friesian calves/group (unspecified age, bw & source) were implanted with Synovex-H® (200 mg testosterone propionate + 20 mg oestradiol benzoate) or Synovex Plus® (200 mg TBA + 28 mg oestradiol benzoate). Two untreated calves served as the control group.

No details of housing or feeding conditions were provided. Animals were slaughtered after 8 weeks by an unspecified means at an experimental EU-official abattoir. Approximately 500 g samples of muscle (loin), liver, kidney and perirenal fat were collected and stored at –20°C prior to residue analysis. Following liquid-liquid extraction and solid phase clean-up, hormone levels were measured in tissue samples by HPLC/EIA. The detection limit for the HPLC/EIA analysis ranged from 1-13 pg/g depending on the analyte, and the method was validated according to the guidelines of the European Commission decision 93/256/EEC. The HPLC analysis also followed a quality assurance procedure. EIAs for TBA, oestrogens and testosterone were performed as previously described (Meyer et al 1987; Blottner et al 1996). The authors developed an EIA for zeranol that was based on the same "scheme" as the EIAs for TBA, oestrogens and testosterone. There was some cross-reactivity with α–zearalenol (6.5%) and β-zearalanol (3.0%), minimal cross reactivity with other zeranol metabolites (<1%), and no cross reactivity with a range of other hormones.

No statistical analyses were performed.

Findings: Tissue residue levels (pg/g) were tabulated for each animal, however, no means or standard deviations/errors were provided. In some instances, the interpretation and significance of the results was difficult to ascertain due to the variation between the 2 animals in each group and the lack of statistical analysis.

Heifers implanted with Synovex-H® (200 mg testosterone propionate + 20 mg oestradiol benzoate) showed dose-related increases in tissue testosterone and oestrogen (17α-oestradiol, E_2 and E_1). The exception to this trend was in the liver, where the concentration of testosterone was unaffected by treatment. The highest concentrations of testosterone were measured in the kidney, followed by the fat, muscle and liver (maximum levels of 627, 628, 102 and 63 pg/g respectively). However, the largest increase relative to the control was seen in fat (maximum levels of 89, 313 and 628 ng/g at 1-, 3- and 10-fold implant doses, respectively, compared to the maximum level in the control of 14 ng/g). Dose-related increases in 17α-oestradiol, E_2 and E_1 occurred in liver kidney and fat, with all treated heifers having residue levels higher than the controls (see Table below). The highest residues were detected in the kidney and liver, followed by the fat and muscle. The proportion of oestrogen metabolites varied between tissues.

E_1, E_2 and 17α-oestradiol levels (pg/g) in tissues from heifers implanted with 1-, 3- and 10-fold doses of Synovex-H (200 mg testosterone propionate + 20 mg oestradiol benzoate)

Tissue	Control	1-fold	3-fold	10-fold
Loin (muscle)				
E_2	3.9/3.3	3.5/1.7	4.4/4.2	20/16
17α-oestradiol	4.4/<1.5	1.5/<1.5	1.5/<1.5	1.5/<1.5
E_1	<1.1/<1.1	<1.1/<1.1	<1.1/<1.1	<1.1/<1.1
Liver				
E_2	12/<8.3	23/37	55/56	164/122
17α-oestradiol	96/28	148/351	573/360	2206/931
E_1	<6.7/<6.7	19/27	34/30	169/70
Kidney				
E_2	<8.1/28	113/31	78/136	225/102
17α-oestradiol	67/37	267/227	426/408	2267/926
E_1	<6.6/8.8	91/9.2	36/44	223/92
Fat				
E_2	<4.2/<4.2	18/25	67/65	211/184
17α-oestradiol	<4.9/<4.9	<4.9/<4.9	<4.9/<4.9	<4.9/<4.9
E_1	<3.4/<3.4	5.4/8.3	18/9.1	82/44

Levels shown for both animals

There was a dose-related increase in the tissue concentrations of 17β-TBO, 17α-TBO and trendione in heifers implanted with Finaplix-H® (200 mg TBA). The highest levels were found in the liver, then the fat, kidney and muscle. All 3 metabolites were detected in each tissue to varying degrees. 17β-TBO was the main residue found in fat and muscle (maximum levels of 2503 and 317 pg/g, respectively), while 17α-TBO predominated in liver and kidney (maximum levels of 33801 and 1404, respectively).

There was a dose-related increase in the tissue concentrations of zeranol in heifers implanted with Ralgro® (36 mg zeranol). The highest levels were detected in the liver, followed by the kidney, fat and muscle (maximum levels of 8132, 2981, 291 and 57 pg/g respectively). Zeranol was detected in the liver samples from both control animals (60/70 pg/g) and also in the kidney of one of these same animals(12 pg/g). However, these concentrations were up to several orders of magnitude lower than in treated heifers. Zeranol occurs naturally as a fungal contaminant (mainly *Fusarium spp*) on a number of plant products (eg. wheat, barley and maize), and therefore its detection in control animals was not unusual.

Off-label use of Synovex-H® or Synovex Plus® in veal calves at the recommended dose for use in cattle resulted in elevations in tissue oestrogens, testosterone or TBA, which were lower than the levels measured in heifers at the same dose. The levels of oestrogen metabolites in calves was somewhat difficult to interpret due to the large variability between the 2 animals in each group; one often showing an elevation relative to the control and the other showing no effect. On average, the highest residues of oestrogen were detected in the kidney and liver, followed by fat and muscle. The proportion of oestrogen metabolites also varied between tissues and generally followed a pattern similar to that observed in heifers. The exception was 17α-oestradiol, which was detectable in the muscle of calves but not heifers. Testosterone levels were elevated only in the muscle and fat of calves implanted with Synovex-H® (maximum levels of 125 and 11 pg/g, respectively, compared to the control values of 7.7 and <1.7 pg/g, respectively). TBO residues were elevated in calves treated with Synovex Plus®, but again there was considerable variability (~10-fold) in residues between the 2 animals in each group. The highest TBA residues were detected in the liver followed by the kidney, fat and muscle. 17α-TBO was the main metabolite in liver and kidney (maximum levels of 1877 and 678 pg/g, respectively), while equal levels of 17β-TBO and trendione were detected in fat (maximum levels of approximately 200 pg/g). 17β-TBO was the main metabolite in muscle (maximum level of 23 pg/g).

The authors reported that the Codex MRL for 17α-TBO in liver (10 ng/g) was exceeded in both heifers implanted with a 10-fold dose of Finaplix-H® (34 and 27 ng/g), with a slight exceedance (11 ng/g) also occurring in one heifer treated with a 3-fold dose of Finaplix-H®. No other exceedances for TBO residues occurred in any other tissues. The Codex MRLs for zeranol in muscle (2 ng/g) and liver (10 ng/g) were not exceeded following implantation with up to a 10-fold dose of Ralgro®. It was reported that the FDA-allowable levels of E_2 in liver and kidney (0.24 and 0.36 ng/g, respectively) were exceeded following implantation with a 3- and 10-fold dose of Synovex-H® (approximately 0.4 and 1.5 ng/g after 3- and 10-fold doses, respectively). No such exceedances occurred in fat or muscle. The FDA-allowable levels of testosterone were not exceeded in any tissue following implantation with up to a 10-fold dose of Synovex-H®. The off-label use of Synovex-H® or Synovex-Plus® in calves, at the labelled dose for use in cattle, did not exceed the FDA-allowable levels of testosterone or E_2.

Conclusions: The use of Finaplix-H®, Ralgro® or Synovex-H® in heifers at the recommended labelled dose, and the off-label use of Synovex-H® or Synovex-Plus® in veal calves, did not lead to a violation in allowable residue levels. Off-label use of zeranol and testosterone propionate at up to 10-fold the labelled dose in heifers also did not violate allowable residue levels. However, overdosing heifers with TBA or oestradiol benzoate at 3- or 10-fold the labelled dose resulted in a violation of the allowable residue levels. While the value of this study was limited by the small group sizes and consequent lack of statistical analysis, it indicated that HGP use is acceptable when animals are treated according to approved directions.

Pfaffl MW, Lange IG, Daxenberger A & Meyer HHD (2001) Tissue-specific expression pattern of oestrogen receptors (ER): Quantification of ERa and ERb mRNA with real-time RT-PCR. APMIS 109: 345-55.

Experimental: Two Holstein Friesian heifers/group (unspecified age, bw & source) were implanted with a 1-, 3- or 10-fold dose of Ralgro® (36 mg zeranol) and slaughtered 8 weeks later by an unspecified means. The implantation site was presumably the ear. Housing and

feeding conditions were unspecified. A group of 2 heifers served as the control group. Blood samples were collected on days 0, 1, 8, 22, 36, 49 and 56 after implantation, an anticoagulant added, and plasma prepared by centrifugation and decantation. Samples were stored at –20°C prior to analysis.

After slaughter, all heifers were examined gynaecologically. The genital tract was removed and macroscopically examined for the condition of the uterus (size, existence and appearance of mucosa), the corpus luteum (number of cysts, post- or pre-ovulatory status, any abnormalities) and the number of small and large follicles (3-10 and > 10 mm, respectively). The oestrus cycle day was estimated according to the criteria of Ireland et al (1980). The concentration of zeranol in plasma was measured by an EIA as previously described (Lange et al 2001), with the detection limit reportedly 12 pg/mL. ERα and ERβ mRNA were quantified in the following tissues using a RT-PCR method: uterus, mammary gland, liver, lung, spleen, heart muscle, kidney medulla, kidney cortex, rumen, abomasum, jejunum, *longissimus dorsi*, hind leg muscle, shoulder muscles and neck muscles.

Findings: Control heifers were reported to be in day 14 of the oestrus cycle and showed no uterine or ovarian abnormalities. Heifers treated with a 1-fold dose of Ralgro® were found to be in a later stage of the oestrous cycle (day 18-20) and the ovaries of both animals had one dominant and up to 10 small follicles. One animal also had a corpus luteum. At the 3- and 10-fold doses, heifers were reported to have a "very small" uterus and a limit of 6 ovarian follicles. The oestrus cycle day of heifers treated with the 3- or 10-fold doses were unreported.

Graphically presented data illustrated a dose-related increase in the plasma concentration of zeranol in heifers implanted with Ralgro®. At the 1-, 3- and 10-fold doses, plasma concentrations ranged from approximately 7-25, 10-50 and 10-1000 pg/mL, respectively. At the 1- and 3-fold doses, plasma concentrations tended to increase over time, while at the 10-fold dose, concentrations were elevated over the first half of the treatment period and then decreased to below 30 pg/mL at slaughter. Zeranol was detected in the 2 untreated control heifers at a mean concentration of 6.9 pg/mL. As outlined in the previous evaluation, the detection of zeranol in untreated heifers is not unusual due to its natural occurrence as a fungal contaminant in certain grains.

Graphically presented data illustrated a significant treatment-related decrease in ERα expression in the kidney medulla and jejunum ($r=0.561$, $p<0.05$; $r=0.925$ $p<0.001$, respectively). No details were provided on the method of statistical analysis. There were no other effects on ERα and ERβ expression in any other tissues. As Ralgro® had no effect on ER expression in 13/15 tissues, the authors pooled the results of ER expression within one tissue (ie. across all treatment groups) to ascertain the mean expression levels of ERα and ERβ. The highest expression of ERα (expressed as the number of molecules in 25 ng RNA) was determined in the uterus (980,000), mammary gland (205,000), liver (200,000), neck muscles (145,000) and hind leg muscles (100,000), while the lowest levels were measured in the jejunum (1,550), kidney cortex, kidney medulla and abomasum (~4,000) and lung (5400). The expression of ERβ was markedly lower, with the highest levels measured in the uterus (80,000), kidney medulla (35,100) and kidney cortex (29,300). The lowest levels of ERβ were measured in the abomasum (650) and lung (900).

Conclusions: Implantation of heifers with Ralgro® at doses at and above the labelled dose resulted in effects on the oestrus cycle and a decreased uterine weight. At these same doses there was relatively little effect on the tissue-specific expression pattern of ERα and ERβ, with the exception of the kidney and jejunum where ERα was significantly reduced. The main limitation to this study was the low group size.

Study 6: Analysis of 500 samples for the presence of growth promoters

Stephany RW (2000) Hormones found in meat samples for regular controls within the European Union and from US imports. Chemical Awareness 9: 13-14.

Stephany RW (2001) Hormones in meat: different approaches in the EU and in the USA. APMIS 109(Suppl 103): S357-64.

This review discussed the different regulatory approaches to HGPs in Europe and the USA, and summarised some results of residue analyses in beef. While the use of HGPs in Europe is a regulatory offence, their use in the USA is legal; 5 hormones are authorised for use in solid ear implants (E_2, progesterone, testosterone, TBA & zeranol) and one is authorised as a feed additive (MGA). In the US, regulation of HGPs is via compliance with MRLs but there are no approved laboratories for analysing HGP residues. In contrast, the EU controls illegal use by HGP inspection and residue monitoring programs coordinated by approved National and Community Reference Laboratories. The types of tissues examined by the EU include urine and faeces if animals are still on the farm, and bile, blood, eyes, liver and kidneys if animals are slaughtered. Residue analysis of actual meat samples is rare.

In 1994, an analysis of 1183 European entrecote samples detected traces of 17 androgens and 2 gestagens (1.6% of samples), while an analysis of 936 liver samples found that 10% contained clenbuterol. There have been a range of substances detected by National Reference Laboratories including those with oestrogenic, androgenic, progestagenic, glucocorticoid, thyreostatic and beta-adrenergic activities. It was indicated that the level of misuse varies dramatically across the EU, with some member states showing no misuse and others showing extensive misuse. The author estimated that the overall level of misuse based on the residue testing programs could be 5-15%.

The author reported that the hormone content in 250 grams of meat from illegally treated animals is typically less than 250 ng/steak, while it can be in the mg range at the actual implantation site. It was estimated that the probability of consuming a “highly” contaminated 250 g steak in the EU is less than one in 70,000.

A random study conducted in 1999 on meat imported from the USA to the EU (Stephany & Andre 1999) reportedly found that 75% (n=103) of bovine meat samples contained traces of MGA (amounts unspecified). TBA was detected in 20% of samples, while no zeranol or any other hormones were found. Evidence was cited to indicate that the dietary intake of E_2 in an egg is greater than that in meat from treated and untreated cattle. Random studies conducted in 1998 and 1999 on US meat (Stephany & Andre 1999, 2000) estimated that the median dietary intake of E_2 in a 250 g steak from untreated cattle was less than 2.5 ng (mean of 1 ng/250 g), while it was 5 ng (mean of 7.5 ng/250 g) in treated cattle. In contrast, the median dietary intake of E_2 in a 50 g egg was found to be 6.5 ng (mean of 6.8 ng) (Rossum et al 2000).

The author made the following conclusions:

(1) The risk to human health from the consumption of meat from HGP-treated cattle should be evaluated in relation to the total dietary intake of hormones from meat and meat products, poultry, milk, dairy products, eggs, fish and fish products. The method of food preparation in the household also needs to be taken into consideration.
(2) While the EU has a large and generally adequate network of residue laboratories to regulate the illegal use of HGPs, residue analysis is performed on non-edible sample material.
(3) In the EU at present there are a limited number of laboratories operational for the residue analysis of HGPs in edible products such as muscle meat. In the US, there are no adequate testing laboratories to perform residue analysis in farm animals or products.
(4) There is no adequate database in the world with current information about the levels natural and xenobiotic hormones in common food commodities of animal origin.
(5) Governments should launch large-scale inspection programs to generate sufficient data on the actual levels of registered and unregistered hormones in various commodities.
(6) An international database should be established under the auspices and guidance of the WHO/FAO CODEX Alimentarius.

Study 7: Analysis of 500 samples for the presence of growth promoters

Marchand P, le Bizek B, Gade C, Monteau F & Andre F (2000) Ultra trace detection of a wide range of anabolic steroids in meat by gas chromatography coupled to mass spectrometry. Journal of Chromatography A 867: 219-233.

This paper described the development of an analytical method to measure trace amounts of a variety of anabolic agents and their metabolites in tissue samples. The method was developed predominantly to facilitate monitoring for the illicit use of hormones in Europe. Control liver and muscle samples (15 g) were obtained from untreated cattle (unspecified number). These same samples were fortified at different levels (unspecified), depending on the analytes being measured, and used as quality control samples. Commercial samples were collected by the Veterinary Inspection Services (unspecified location) or were from cattle treated with anabolic steroids or related compounds. The number of cattle sampled was unspecified. All samples were stored at –18°C prior to analysis.

Samples were lyophilised then underwent liquid-liquid extraction followed by deconjugation via enzymatic hydrolysis with *Helix pomatia*. Solid-phase extraction on a polymeric stationary phase was performed prior to hydrolysis of ester residues under alkaline conditions. Liquid-liquid partitioning was used to separate the analytes into phenolic (stilbenes, resorcylic acid lactones and phenolic steroids) and ?4-3-one containing molecules (androgens and progestagens). Phenolic and ?4-3-one containing molecules were purified by solid-phase extraction on silica columns. Each compound subgroup (?4-3-one compounds except TBO metabolites; resorcylic acid lactones and phenolic steroids; stilbenes) underwent specific derivatisation prior to analysis by GC-MS.

The Table below lists the analytes screened. Chromatograms illustrated that *Helix pomatia* deconjugation increased the yield of 17α-oestradiol by approximately 50%, while only a 5% increase was obtained for E_2. This finding led the study authors to conclude that 17α-oestradiol was more conjugated than E_2. Chromatograms were provided for meat samples

fortified with 200 ng/kg *trans*-DES and 500 ng/kg *trans*-[$^2H_6$]DES, as well as samples fortified with 200 ng/kg 17α- and 17β-nandrolone, and 500 ng/kg [$^2H_3$]17β-nandrolone.

List of screened analytes

Group	Analytes
Stilbenes	*cis*-DES, hexestrol, dienestrol, *trans*-DES
RALs	zeranol, taleranol
Oestrogens	17α-oestradiol, E_2
Androgens	17α-TBO, 17β-TBO, 17α-nandrolone, 17α-testosterone, 17β-nandrolone, 17β-testosterone, norethandrone, bolasterone, methyltestosterone, ethinyltestosterone, norethandolone
Gestagens	progesterone, megestrol, chlormadinone, medroxyprogesterone, MGA

RALs = resorcylic acid lactones

An unspecified number of meat and liver samples were found to contain residues of anabolic compounds; chromatograms were provided to substantiate these findings. Residues found in meat included chlormadinone[25] (2.5 µg/kg) or MGA (1.5 µg/kg). 17α-TBO (9.0 µg/kg) and 17β-TBO (1.5 µg/kg) were detected in one liver sample, while zeranol (1.2 µg/kg) and taleranol (0.09 µg/kg) were detected in another. These positive residue samples were reportedly obtained from cattle that had been treated with the parent compound. It was reported that 23 steroids were detected in the 5-100 ng/kg range but no data were provided to substantiate this finding.

Confirmatory analysis was reportedly performed using low resolution MS but in most cases a high resolution mass filter instrument was required to detect the low levels of residues. The study authors indicated that 600 routine samples were also analysed but the results of these were not reported. This study lacked detail on the source and number of the samples analysed, including the incidence of violative levels of HGPs residues.

Le Bizec, Marchand P & André F (2000) Le controle des anabolisants dans la viande (The survey of anabolic agents in meat). Annales de Toxicologie Analytique 7(1): pages unspecified.

Only an abstract (English version) was available for review.

A method was developed in the National Reference Laboratory for hormones (French Ministry of Agriculture) in order to simultaneously assay various anabolic agents according to the requirements of the EC 96/23 Directive for meat or liver control. Meat samples were lyophilised and extracted with organic solvents. Phase II metabolites were hydrolysed and the esters saponified. Free compounds were re-extracted by liquid-liquid and solid-phase extraction. Twenty-three anabolic agents were detected by GC-MS, with the detection limit between 5-100 ng/kg.

[25] Chlormadinone is a synthetic progesterone analogue

Study 8: Comparison of assay methods

Kong L-Y, Szaniszlo P, Albrecht T & Liehr JG (2000) Frequency and molecular analysis of hprt mutations induced by oestradiol in Chinese hamster V79 cells. International Journal of Oncology 17: 1141-1149.

Experimental: The mutation frequency and molecular alterations of the *hprt* gene were examined in Chinese hamster V79 cells following exposure to physiological and pharmacological concentrations of E_2. A clonogenic assay was initially performed to determine the effect of E_2 on cell survival. V79 cells (unspecified density) were treated with 10^{-4}, 3×10^{-5}, 10^{-6}, 3×10^{-6}, 10^{-7} or 3×10^{-7} M E_2 in 0.1% v/v ethanol and polyethylene glycol 400 for 48 hours. Control cells were treated with solvent only. Cells were dissociated with trypsin, reseeded (n=3) and cultured for 7 days. Surviving colonies were formalin fixed, stained with methylene blue and counted.

To examine the mutagenic activity of E_2 on the *hprt* gene, V79 were incubated with 10^{-13}, 10^{-12}, 10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7} or 10^{-6} M E_2 dissolved in 0.1% v/v ethanol and polyethylene glycol 400 (sample sizes unspecified). These concentrations were selected based on the results of the above clonogenic assay. The study authors indicated that the above concentration range was only tested once, while 3 separate assays tested concentrations of 10^{-10}, 10^{-9} or 10^{-8} M. Untreated cells and solvent-treated cells served as the negative controls, while cells treated with 1 µg/mL of N-methyl-N-nitro-N-nitroso-guanidine (MNNG) served as the positive control. Following 96 hours of treatment, cells were dissociated and replated in selection medium containing 6 µg/mL 6-thioguanine. Mutants without a functional *hprt* enzyme can survive in the presence of 6-thioguanine, unlike non-mutant cells where the conversion of 6-thioguanine to nucleotide and incorporation into DNA is fatal. The plating efficiency was measured by culturing cells in non-selective medium. Colonies of 6-thioguanine resistant (TG^r) cells were cultured for 7 days then either subcultured or fixed and stained. The mutation frequency was calculated by determining the number of TG^r colonies relative to the number of cells seeded in the selection medium, after correcting for the plating efficiency.

TG^r clones were selected from widely separate colonies or colonies on different culture plates. Genomic DNA and total RNA were isolated from wild type and mutant V79 cells using a commercial kit. First-strand cDNA was synthesised by reverse transcription using a commercial pre-amplification system. PCR amplification of *hprt* cDNA and genomic DNA was performed as previously described, with slight modification (Bradley et al 1981). *Hprt* cDNA derived from TG^r mutants and wild-type V79 cells by RT-PCR amplification was purified using a commercial column and sequenced. Sequence data were analysed using computer software.

The expression of ERα in V79 cells was determined by PCR as previously described (Bhat & Vadgama 2000). To investigate the possible involvement of ERα in mutagenesis, V79 cells were treated with the ERα antagonist, ICI 182,780, at 10^{-10}, 10^{-9}, 10^{-8} or 10^{-7} M for an unspecified period of time. Control cells were treated with medium alone, solvent or MNNG. In 2 subsequent experiments, 10^{-10} or 10^{-9} M E_2 was added to cells 2 hours after incubation with equal or 10-fold higher concentrations of ICI 182,780. Control cells were treated only with E_2. The frequency of *hprt* mutations was determined as described above.

Statistical differences between groups were determined using a student's t-test. Differences were considered to be significant when $p<0.05$.

Findings: E_2 did not affect the survival of V79 cells at and below 3×10^{-5} M. Survival at the next highest dose (10^{-4} M) was approximately 12%. There was an approximately 9-fold increase in the number of *hprt* mutants at the highest concentration of 10^{-6} M E_2 (see Table below), but it was unclear whether this result was statistically significant. In contrast, the positive control (MNNG) caused an approximately 200-fold enhancement in *hprt* mutants compared to the solvent control. Marginal increases in the number of mutants above control levels occurred at lower doses, however, there was no dose-response effect nor any evidence of statistical significance. The authors did, however, state that the result obtained at 10^{-10} M was significant ($p=0.003$), but in the absence of an effect at the next 2 doses, the reviewing toxicologist did not consider that this result was biologically significant. Furthermore, the interpretation of these results was made difficult by the fact that only 'representative results' were provided from 4 different assays. In light of the low mutation frequency relative to the positive control, that an effect (albeit small) was seen only at the highest dose, that results from all experiments were not provided, and that there was no evidence of a reproducible effect, the mutagenicity of E_2 should be considered as equivocal.

Mutagenicity of E_2 at the *hprt* locus of V79 cells

Treatment	Mutations/10^6 cells	Enhancement[1]
Untreated control	0.74	-
Solvent control	0.65	0
MNNG (1 mg/mL)	136.42	209
E_2		
10^{-6} M	6.36	8.78
10^{-7} M	2.36	2.63
10^{-8} M	0.48	0.026
10^{-9} M	1.26	0.93
10^{-10} M	2.89	3.45
10^{-11} M	2.32	2.57
10^{-12} M	1.08	0.66
10^{-13} M	0.82	0.26

1 = Ratio of induced mutation frequencies (treatment/solvent control) corrected for the solvent control values

The Table below summarises the types of lesions detected in the *hprt* gene of TG^r mutants, with similar types of lesions detected in both control and treated clones. A total of 27 mutant clones were examined, including 6 derived from the blank and solvent controls, and the remainder from those cells treated with 10^{-6}, 10^{-7}, 10^{-10} or 10^{-11} M E_2. Of the 21 TG^r mutants derived following treatment with E_2, 9 (43%) had base changes in the coding region of the *hprt* gene, with T→G or T→A transversions being the most common base change (6/21, 29%). Deletion of *hprt* exons occurred in 6/21 (29%) of TG^r mutants. As the same T→G and T→A transversions occurred in two groups of 3 clones, respectively, the study authors concluded that there are 2 potential 'hot spots' for the induction of point mutations by E_2.

Types of lesions in the *hprt* gene of TG^r mutants

Treatment	No mutant clones isolated	Deletions of *hprt* exons	Base change		
			Transition	Transversion	Insertion
Untreated control	3	1	1	0	0
Solvent control	3	1	1	1	0
10^{-6} M E_2	7	2	0	3	1
10^{-7} M E_2	4	3	1	0	0
10^{-10} M E_2	6	1	0	3	0
10^{-11} M E_2	4	0	1	0	0

Photographic evidence was provided to illustrate that V79 cells express ERα, however the study author's conclusion that V79 cells express abundant levels of ERα was not substantiated by any quantitative data. Mutagenicity assays performed in the presence of ICI 182,780 (an ERα antagonist) failed to inhibit E_2-induced mutations in the *hprt* gene. In fact, ICI 182,780 was found to act in an additive manner with E_2 to increase the number of *hprt* mutants. At 10^{-7} or 10^{-10} M, ICI 182,780 caused an approximately 10-fold increase in *hprt* mutants compared to the solvent control. E_2 (10^{-9} M) alone or in combination with equal or a 10-fold higher concentration of ICI 182,780 resulted in an approximately 2-, 3- and 5-fold, respectively, increase in mutants. At 10^{-10} M, E_2 alone or in combination with equal or a 10-fold higher concentration of ICI 182,780 resulted an approximately 9-, 7- and 19-fold, respectively, increase in mutants. The study authors concluded that these findings suggested that the weakly mutagenic activity of E_2 does not involve the ERα.

Conclusions: In a single experiment, a relatively high concentration of E_2 (1 μM) caused a marginal increase in the frequency of *hprt* mutations in V79 cells. In the absence of any evidence that this was a reproducible effect, that there were effects at lower concentrations and that the mutation frequency was markedly lower than the positive control, the study authors conclusion that E_2 is weakly mutagenic and a complete carcinogen was considered to be unsubstantiated.

Cavalieri E, Frenkel K, Liehr JG, Rogan E & Roy D (2000) Chapter 4: Estrogens as endogenous genotoxic agents – DNA adducts and mutations. JNCI Monograph 27: 75-93.

This review considered the evidence relating to the potential of oestrogen and oestrogen metabolites to induce DNA damage and gene mutations both *in vitro* and *in vivo*. Polycyclic aromatic hydrocarbons (PAH) had been studied by two of the authors as model carcinogenic compounds over a 25-year period and were considered to be a "good model" for understanding the process of chemically-induced carcinogenesis. It was suggested that PAHs were also useful surrogates for studying oestrogen-induced mutations because of their geometric resemblance to oestrogens and the fact that both classes of compounds contain aromatic rings. The mechanism of tumour initiation by PAH was discussed, including the role of DNA adducts in this process. Studies conducted on the role of PAH-DNA adducts in tumour initiation apparently instigated investigations into the formation of similar adducts by oestrogen metabolites.

Evidence relating to the role of catechol oestrogen quinones and apurinic sites in cancer initiation was discussed. The overall hypothesis was that catechol oestrogens (the major metabolites of E_2 and E_1) are oxidised to catechol oestrogen quinones, which may react with DNA to form depurinating adducts. These adducts generate apurinic sites, which may initiate cancer. A variety of *in vitro* and *in vivo* studies illustrated that catechol oestrogens are

enzymatically oxidised to catechol oestrogen quinones, which bind to DNA. It was noted that there are a variety of inactivation mechanisms (*O*-methylation, glucuronidation and sulfation) that protect cells from catechol oestrogen toxicity. However, if there is excessive formation of metabolites such as 4-OHE_2, and inactivation of catechol oestrogens is incomplete, catalytic oxidation to catechol oestrogen semiquinones and quinones can occur. If the subsequent inactivation of catechol oestrogen semiquinones and quinones via glutathione conjugation is incomplete, they may react with DNA to yield stable and depurinating adducts.

The authors concluded that the combination of the increased formation of 4-hydroxylated catechol oestrogens and their oxidation to DNA-reactive quinones is the pathway leading to the genotoxic effects that cause oestrogen-induced tumour formation. This conclusion was supported by evidence indicating that the formation of 4-hydroxyoestrogen predominates in those organs that are susceptible to oestrogen-induced tumours (eg. hamster kidney, rat pituitary gland and mouse uterus) but not in those tissues that are resistant (eg. liver).

The formation of reactive oxygen species (ROS) via endogenous oestrogens, and their proposed role in hormone-induced cancer, was discussed. The authors reviewed data relating to the ability of ROS to modify DNA and the types of oxidative base derivatives that can be formed (eg. 8-hydroxy-2'-deoxyguanosine and 5-hydroxymethyl-2'-deoxyuridine). Much like the existence of mechanisms for the inactivation of catechol oestrogens and catechol oestrogen quinones, there are a number of enzyme-based repair systems that remove oxidised bases from genetic material. The concept that DNA damage occurs when the capacity of these inactivation systems is exceeded was reiterated. The authors argued that elevated levels of oxidised DNA (relative to the background level) might be indicative of an oestrogen-induced carcinogenic process. While examples from animal models (including unpublished data), cellular models and humans were given to illustrate the possible link between the presence of oxidised DNA bases and carcinogenesis or cell transformation, no evidence was provided to show any link with oestrogen treatment.

Following on from the apparent link between oestrogen exposure and the generation of ROS and carcinogenesis, the authors considered that oestrogens could generate oxidants both directly and indirectly. The most reasoned evidence was in relation to the generation of superoxide anion (O_2^-) from the metabolism of oestrogens. The generation of catechol oestrogen semiquinones and quinones concomitantly generates O_2^-, which may react further to produce H_2O_2. It was argued that semiquinones and quinones are capable of redox cycling and therefore even small amounts of E_2 could generate significant ROS. Any ROS generated in this manner could then oxidise DNA or cause cellular damage. It was noted that the same inactivation mechanisms that mitigate the toxicity of catechol oestrogens and catechol oestrogen semi/quinones also mitigate the generation of ROS during oestrogen metabolism.

Besides any damaging effects on DNA, ROS were also considered to affect cellular function via the oxidation of other macromolecules, such as proteins and lipids. The consequent generation of additional ROS was considered to amplify cellular damage and instigate a 'chain reaction' of oxidant formation. It was also argued that oestrogens can generate ROS via the modulation of immune responses (eg. cytokine production) and via the stimulation and proliferation of inflammatory cells (eg. phagocytes, macrophages, polymorphonuclear leukocytes, neutrophils and granulocytes), which produce large quantities of oxidants.

The last section of this review involved the discussion of whether E_2 was a genotoxic or epigenetic carcinogen. While E_2 had been implicated in certain types of cancers, the lack of

mutagenic activity in previous bacterial and mammalian cell mutation assays had led to the conclusion that oestrogens were non-genotoxic and non-mutagenic. The authors noted that these findings were inconsistent with the ability of catechol oestrogen metabolites to covalently bind to DNA and to generate ROS that could oxidise DNA bases. Rather than considering that these latter effects do not lead to a mutagenic outcome (due possibly to endogenous repair mechanisms or that they are not the sole determinant of mutagenicity), it was proposed that previous mutation assays were not sensitive enough to detect the weakly mutagenic activity of oestrogens. A variety of studies were cited (including unpublished data from the authors' laboratory) to support the hypothesis that oestrogens can cause multiple types of "genetic insults" that contribute to genomic instability. It was implicit in the authors' argument that this genomic instability leads to mutations and cancer.

The overall conclusion of this review was that oestrogens are genotoxic carcinogens. However, the reviewing toxicologist considered that this conclusion was unjustified because the weight-of-evidence indicated that oestrogens and oestrogen metabolites are not mutagenic.

Jefcoate CR, Liehr JG, Santen RJ, Sutter TR, Yager JD, Yue W, Santner SJ, Tekmal R, Demers L, Pauley R, Naftolin F, Mor G & Berstein L (2000) Chapter 5: Tissue-specific synthesis and oxidative metabolism of estrogens. JNCI Monographs 27: 95-112.

This review examined the contribution of oxidative metabolites of oestrogen to breast carcinogenesis, with particular emphasis on the roles of various cytochrome P450s [aromatase (CYP19), 17β-oestradiol-4-hydroxylase (CYP1β1)] and catechol oestrogens (namely 4-OHE_2). The central hypothesis was that the carcinogenic effect of oestrogens is due to the oxidation of E_2 to 4-OHE_2 by CYP1β1; this metabolite is genotoxic and can undergo redox cycling to form ROS, which are themselves genotoxic. The main limitation to this hypothesis was the uncertainty that tissue levels of E_2 would be high enough to generate biologically active levels of 4-OHE_2. It was argued that overexpression of aromatase in combination with the presence 4-hydroxylases could generate physiologically and pathologically significant levels of carcinogenic metabolites.

Evidence to substantiate the link between oestrogen exposure and breast carcinogenesis included studies conducted in rodents, epidemiological studies and a variety of studies conducted in women receiving oestrogen-replacement therapy. The authors drew attention to the lack of suitable randomised, controlled, double-blind studies to demonstrate conclusively that oestrogen replacement therapy during menopause increases breast cancer risk. Additionally, the absolute increase in breast cancer risk is actually small (one extra case per 100 women receiving oestrogen for at least 10 years). It was indicated that the relationship between oestrogen levels and subsequent breast cancer risk had been difficult to establish. Earlier studies were negative, which the authors attributed to a lack of sensitivity or specificity of the test methods. Conflicting results have also been reported and some findings have been difficult to replicate. Other "compelling evidence" included results from studies conducted in women who had undergone bilateral oophorectomy, where there was a 75% reduction in the incidence of breast carcinoma, and the demonstration that a reduction in oestrogen production in women reduces the incidence of breast cancer.

Oestrogens can be synthesised in several tissues, with aromatase catalysing the rate-limiting step from androgens to oestrogens. The role of aromatase in the development of breast cancer is evidenced from studies showing that aromatase inhibitors prevent spontaneous breast

tumours in rats. Additionally, aromatase inhibitors are currently used to treat breast cancer in women. The hypothesis that *in situ* synthesis of E_2 plays a more important role in breast physiology than the delivery of E_2 synthetised in other tissues, was supported by the finding that aromatase is present in breast tissue, albeit at lower concentrations than that found in the placenta and the ovaries. Furthermore, immunohistochemical staining of breast cancer tissue revealed high levels of aromatase in individual cells, however it remains unclear whether aromatase activity predominates in stromal or epithelial cells.

Four animal models were described to support the hypothesis that aromatase overexpression may be a cause of breast cancer. Three involved the hyperplastic alveolar nodule model, while the 4th was a transgenic mouse model. The statement that several laboratories had obtained evidence that malignant breast tissues contain aromatase mRNA and enzyme activity was unsubstantiated. Studies were cited to illustrate that benign breast tissue expresses aromatase (epithelial and stromal cells), while aromatase activity has also been detected in macrophages within breast tissue. It was suggested that any one of these 3 cell types could overexpress aromatase leading to an increase in E_2 metabolism. There was some speculation on the mechanism of overexpression, which included transcription, stimulation of mRNA or protein, activating mutations or via prostaglandin stimulation. While the link between breast cancer development and aromatase was reasonably clear, no evidence was provided that an increase in tissue aromatase actually leads to an increase in E_2 or catechol oestrogen metabolites.

Two theories of oestrogen-induced carcinogenesis were discussed. One theory involves the hormonal activity of oestrogen causing an increase in cell proliferation. The second theory, as described in the above evaluation of the review by Cavalieri et al (2000), involves the generation of genotoxic metabolites and ROS due to the metabolic oxidation of E_2 and E_1. It was argued that these 2 pathways could act together; DNA damage caused by catecholoestrogen quinones or ROS could be actualised by increased cell proliferation. The authors noted that the main weakness to the second theory was that oestrogen levels in breast tissue are not high enough to generate biologically-active levels of metabolites, however, they indicated that this criticism has yet to be substantiated.

Following a discussion of oxidative metabolism of oestrogens (covered in the previous evaluation) the authors concluded that 4-OHE_2 was a carcinogenic metabolite that could be formed: (1) as a minor by-product of NADPH-dependent 2-hydroxylation of either E_1 or E_2 due to a lack of specificity of oestrogen-2-hydroxylases; (2) by specific NADH-dependent 4-hydroxylation of E_1 or E_2 catalysed by CYP1β1 or other 4-hydroxylases; (3) by organic hydroperoxide-dependent oestrogen-2 and 4-hydroxylase activity; and (4) by deconjugation of 4-methoxyoestrogens and other catechol oestrogen metabolites.

The second section of this review examined the enzymes responsible for generating catechol oestrogen metabolites and the role they might play in oestrogen-induced tumorigenesis. It was recognised that 4-hydroxylated metabolites are a relatively small proportion of the total oestrogen metabolites. However, evidence was cited to support a role for 4-hydroxylation in oestrogen carcinogenesis and included: (1) the rate of 4-hydroxylation is equal to or exceeds the rate of 2-hydroxylation in human and mouse uteri, rat pituitary and hamster kidney, organs that are susceptible to oestrogen-induced tumours; (2) elevated CYP1β1 has been measured in human breast tissue and uterine tumours; (3) 4- but not 2-OHE_2 is carcinogenic in male hamster kidneys; and (4) several laboratories have reportedly demonstrated that oestrogen-3,4-quinones can form DNA adducts.

The authors reviewed data relating to the identification and molecular characterisation of CYP1β1. It was suggested that further studies on the translation, processing and subcellular localisation of CYP1β1 are warranted due to data gaps. The primary site of oestrogen metabolism is the liver, where the rate of 2-hydroxylation "greatly exceeds" the rate of 4-hydroxylation. This preference for the 2-hydroxylation pathway is attributable to the relatively high level of oestrogen-2-hydroxylases (CYP1A1). However, because CYP1β1 "has the highest catalytic efficiency (turnover/K_m) of any reported oestrogen hydroxylase", it was suggested that CYP1β1 could play a role in oestrogen homeostasis in extrahepatic organs that have relatively low levels of CYP1A1.

It was reasoned that the relative expression of CYP1β1 and CYP1A1 would determine the level of conversion of E_2 to 2- or 4-hydroxylation products. The expression of CYP1A1 is apparently undetectable in breast tissue suggesting that little 2-OHE_2 would be produced *in vivo*. In contrast, CYP1β1 is detectable in breast tissue and is likely to be the major source of 4-OHE_2 formation. No data were provided to confirm that the presence of CYP1β1 actually generates 4-OHE_2 in breast tissue. The authors also noted that it was unclear whether the low formation of 4-OHE_2 is physiologically or pathologically significant.

The expression of CYP1β1 has been detected in a variety of tissues, with mRNA levels reportedly highest in the kidney, prostate, uterus and mammary tissue, while lower levels have been detected in a range of other organs/tissues. However, few studies have actually measured CYP1β1 protein levels or enzyme activity, and no data were provided to indicate that increased CYP1β1 actually leads to increased tissue levels of 4-OHE_2. While acknowledging the data gaps, the authors concluded that expression of CYP1β1 and formation of catechol oestrogens have been independently associated with oestrogen-related tumours in multiple tissues and species. They contend that this data "strongly" supports the hypothesis of the role of catechol oestrogens in oestrogen-related carcinogenesis.

Newbold RR & Liehr JG (2000) Induction of uterine adenocarcinoma in CD-1 mice by catechol estrogens. Cancer Research 60: 235-237.

Experimental: The carcinogenic potential of catechol oestrogens was investigated using an experimental mouse model of hormonal carcinogenesis (Newbold et al 1990). Pups from outbred female CD-1 [Crl:CD-1 (ICR) BR] mice (NIEHS, Research Triangle Park, NC, USA) were injected subcutaneously with 2- or 4-OHE_2, E_2 or EE in corn oil at a dose of 2 µg/pup/d, on days 1-5 of neonatal life. Control mice received corn oil alone. Group sizes were unspecified. The dose selection was based on a previously published study (Newbold et al 1990). Mice were weaned at 21 days of age and housed 4/cage under standard laboratory conditions. Animals were sacrificed by cervical dislocation at 12 or 18 months of age. An unspecified number of tissue samples were taken from the reproductive tract of each animal and these were fixed, sectioned, stained with haematoxylin and eosin, then examined by light microscopy. If any pathological abnormalities were evident, additional sections were made and examined. The number of mice with reproductive tract tumours was scored, with the incidence of uterine adenocarcinomas statistically analysed using a Fisher's exact test.

In a separate experiment, the oestrogenic potency of each compound was determined by administering each compound in corn oil to a minimum of 10 pups/group by subcutaneous injection on days 1-4 of neonatal life. Control pups received corn oil alone. Mice were

sacrificed on neonatal day 5 and their bodyweights and uterine weights recorded. Uterine weights were statistically analysed using a Duncan's multiple range test.

Findings: There was a significant increase ($p<0.01$-0.05) in the incidence of uterine adenocarcinomas in mice treated with 2-OHE_2, 4-OHE_2 and EE compared to the control (see Table below). The most carcinogenic compound was 4-OHE_2 (total incidence of 66%), followed by EE (43%), 2-OHE_2 (12%) and E_2 (7%). No uterine adenocarcinomas were detected in the control group. The historical control incidence of uterine adenocarcinomas in this particular strain of mouse is 0.86-4% (Giknis & Clifford 2000). Light micrographs illustrated well-differentiated invasive uterine adenocarcinoma and carcinoma in an 18-month old mouse treated neonatally with 4-OHE_2. The study authors stated that the type of tumour induced was in all cases similar to those previously produced by DES or E_2 (Newbold et al 1990).

Incidence of uterine adenocarcinomas in mice treated neonatally with oestrogens at 2 mg/pup/day (sc) for 5 days

Compound	Incidence of uterine adenocarcinomas		
	12 months	18 months	Total
2-OHE_2	3/21 (14)	2/19 (11)	5/40 (12)*
4-OHE_2	14/19 (74)	9/16 (56)	23/35 (66)**
E_2	0/5 (0)	1/10 (10)	1/15 (7)
EE	9/24 (38)	9/18 (50)	18/42 (43)**
Corn oil (control)	0/12 (0)	0/22 (0)	0/34 (0)

Results are expressed as the number of mice with uterine adenocarcinomas/total number of animals. The % incidence of tumours is shown in parentheses. * $p < 0.05$ compared to the control; ** $p < 0.01$ compared to the control

The *in vivo* oestrogenic potency of each test compound (judged as an increase in uterine weight) is summarised in the Table below. There was an approximately 2-fold increase in the uterine weight of mice treated with 4-OHE_2, a result that was statistically significant ($p<0.05$). Increased uterine weights were also detected in mice treated with EE or E_2 but neither of these findings were statistically significant. 2-OHE_2 showed minimal oestrogenic potency.

Oestrogenic potency of various oestrogens in neonatal mice

Compound	Uterine wet wt/bw ratio x 100[1]	Oestrogenic potency (% uterine wet wt gain)[2]
Corn oil (control)	0.199 $\pm$ 0.03	100
2-OHE_2	0.213 $\pm$ 0.03	107
4-OHE_2	0.424 $\pm$ 0.06	213
E_2	0.265 $\pm$ 0.03	133
EE	0.291 $\pm$ 0.04	146

1 = Data expressed as the mean $\pm$ SEM; 2 = data expressed as the % increase over controls; $p < 0.05$ compared to the control.

Conclusions: 2- or 4-OHE_2 or EE were carcinogenic in mice that had been neonatally exposed for 5 days. The oestrogen metabolite, 4-OHE_2, was the most carcinogenic of the test compounds, which appeared to correlate with its relatively high oestrogenic activity. Uneven group sizes were used in this study and no other toxicological endpoints were examined besides the incidence of uterine adenocarcinomas. In addition, only one dose level was tested and the group sizes in the oestrogenicity assay were not specified.

Liehr JG (2001) Genotoxicity of the steroidal oestrogens oestrone and oestradiol: possible mechanism of uterine and mammary cancer development. Human Reproduction Update 7(3): 273-283.

This review examined the evidence pertaining to the genotoxicity and carcinogenicity of steroidal oestrogens and proposed a mechanism for oestrogen-induced tumorigenesis. Evidence was cited that oestrogens induce tumours in laboratory animals, while elevated plasma concentrations, due to hormone therapy or oral contraceptives, have been linked to an increased risk of breast and uterine cancer in humans (albeit modest). IARC have also classified oestrogens as carcinogenic in humans. A common theme throughout the review was that oestrogens are relatively weak carcinogens compared to "other more powerful carcinogens used in laboratory tests" such as benzo[a]pyrene or 7,12-dimethylbenzanthracene.

A variety of data relating to the genotoxicity of oestrogens was reviewed. Evidence was cited that oestrogens can induce DNA damage both *in vitro* and *in vivo*, manifesting as single strand breaks, 8-hydroxylation of guanine bases or by the formation of DNA adducts. The author concluded that oestrogen-induced DNA damage most likely arises from catechol oestrogen metabolites because they, unlike their parent molecules, are capable of covalently binding to DNA and *in vivo* DNA damage frequently occurs in organs that have high rates of catechol oestrogen formation. While oestrogens appear to damage DNA, summary information from previous reports showed the failure of oestrogens and oestrogen metabolites to induce mutations in conventional *in vitro* gene mutation assays. The author surmised that this negative mutagenicity had led to the proposal that oestrogens are non-genotoxic carcinogens. Therefore the existing basis of hormone-dependent cancer was considered to be due to the hormonal activity of oestrogens as mediated by oestrogen receptors.

The author summarised evidence that was inconsistent with the premise that oestrogen-induced cancer occurs via ER-mediated processes or by numerical chromosomal changes. Such evidence was that the presence of ERs were not necessary for cell transformation or aneuploidy to occur, hormonal activity may not be necessary for tumour induction, catechol oestrogen metabolites may play a crucial role in oestrogen-initiated tumour formation, the onset of mammary tumorigenesis was delayed but not eliminated in ER knockout mice and that aneuploidy by itself is not necessary for the development of cancer. In light of this inconsistent evidence, the author concluded that the mechanism of oestrogen-induced cancer might combine endocrinal and non-genotoxic events.

While previous studies had indicated that oestrogens were not mutagenic, the author re-examined the mutagenicity of oestrogens and oestrogen metabolites due to the possibility that conventional *in vitro* mutation assays may have overlooked other important genetic lesions. In the current review, a broader definition of 'genetic lesions' was accepted because of the recent identification of a mixture of various chromosomal and gene modifications that form the genetic basis of human cancer (Lengauer et al 1998). This broader definition of 'genetic lesions' includes numerical or structural chromosomal aberrations, gene amplification, microsatellite alterations and small changes of specific genes. A summary of recent reports illustrated that these types of genetic lesions were detected in various types of mammalian cells that had been treated with E_2 or E_2 metabolites. Based on these recent findings, the author concluded that oestrogens are mutagenic and that previous assays had failed to detect any mutagenic activity because they were designed to detect only one or a few specific point

mutations in one specific gene. It was suggested that it may be necessary to redesign mutations assay systems to optimise them for the detection of weak mutagens, such as E_2.

In light of this evidence regarding the broader mutagenic effects of oestrogens, the author proposed a mechanism of oestrogen-induced carcinogenesis. E_2 may be converted to 4-OHE_2 or E_1, which may undergo metabolic redox cycling. Oestrogen quinone reactive intermediates and free radicals generated by the redox cycle may induce various types of DNA damage. This genotoxicity may result in gene mutation and cell transformation. Tumours may develop from cells transformed by the genotoxic action of oestrogens, which proliferate in response to an oestrogen-receptor-mediated stimulus.

The overall conclusions of the author were that: natural oestrogens are weak mutagens and carcinogens relative to "more powerful carcinogens" such as polycyclic hydrocarbons; catechol oestrogen metabolites are the precursors of DNA damaging reactive intermediates; and that oestrogens must be considered as complete carcinogens because of their dual action as compounds capable of being genotoxic and inducing cell proliferation.

Study 9: Bioassay of oestrogenic/anti-oestrogenic compounds

Le Guevel R & Pakdel F (2001) Assessment of oestrogenic potency of chemicals used as growth promoter by in-vitro methods. Human Reproduction (5): 1030-1036.

Experimental: The oestrogenic potencies E_2, zeranol (α-zearalanol), testosterone, TBO, TBA, MGA and the mycotoxin zearalenone were determined utilising 3 *in vitro* bioassays. Various metabolites of the above compounds were also assessed including 17α-oestradiol, E_1, 17α-epitestosterone, 19-nortestosterone, androstendione, zearalanone, β-zearalanol, α-zearalenol and β-zearalenol. EE and DES were used as standards. All compounds were stored in dimethylsulphoxide (DMSO) at -20°C.

Bioassay 1 was performed as previously described (Wrenn & Katzenellenbogen 1993; Petit et al 1995; Petit et al 1997) utilising a recombinant BJ-ECZ yeast strain. This assay reportedly has a detection limit of 0.1-1 nmol/L. The BJ-ECZ yeast strain expresses human or rainbow trout oestrogen receptor (hER and rtER, respectively) and contains a reporter gene with 2 oestrogen-responsive elements. The activation of the reporter gene is therefore dependent on the presence of exogenous oestrogens and results in the production of β-galactosidase. Three independent clones were incubated with one of the above test compounds (vehicle unspecified) at 0.1, 1 and 10 µmol/L for 4 h at 30°C. The positive control was E_2 (0.01, 0.1 and 1 µmol/L) while the negative control was undefined. No rationale was given for the selection of the test concentrations. The level of β-galactosidase was then quantified via a colorimetric reaction and expressed as the mean OD_{405}/mg protein/min $\pm$ 1 SEM. In addition, dose-response experiments were performed for both hER and rtER, using a 1/2 serial dilution series. Results were expressed as the mean $\pm$ 1 SEM of 3-5 independent experiments. Dose-response relationships were analysed by non-linear regression using the sigmoidal dose-response curve as a model. EC_{50} values and the % relative stimulatory activity (RSA) to EE were determined from the dose-response curves. EE was used to calculate the RSA as it is not metabolised and to avoid misinterpretation of oestrogenic potency as E_2 is metabolised in Ishikawa cells (see bioassay 3).

Bioassay 2 was based on the induction of the vitellogenin (*VTG*) gene in rainbow trout hepatocyte aggregates, and reportedly has a detection limit of approximately 10 nmol/L. Hepatocyte aggregates were formed as previously described and cultured under standard laboratory conditions for this cell type (Flouriot et al 1993). Three independent culture plates containing 5-day old aggregates were treated for 48 h with one of the above test compounds (vehicle unspecified) at 1 and 10 µmol/L. The positive control was E_2 (0.01, 0.1 and 1 µmol/L) while the negative control was undefined. Total RNA was extracted using a commercial kit and the expression of the *VTG* gene quantified by slot blot hybridisation. Results were expressed as the mean % induction relative to E_2 $\pm$ 1 SEM.

Bioassay 3 involved a human endometrial carcinoma cell line (Ishikawa cells), which has an oestrogen-inducible alkaline phosphatase (AP). This assay was performed as previously described (Littlefield et al 1990) and reportedly has a detection limit of 1 pmol/L. Test compounds were dissolved in ethanol and a dose response experiment performed using 1/5 serial dilutions. After 48 h, the level of AP (OD_{405}) was quantified via a colorimetric reaction. Results were expressed as the mean $\pm$ 1 SEM of 6 independent experiments. Dose-response relationships were analysed by non-linear regression using the sigmoidal dose-response curve as a model. EC_{50} values and the % relative stimulatory activity (RSA) to EE were determined from the dose-response curves.

Findings: Graphically presented data illustrated the level of induction of the *Lac Z* reporter gene in hER and rtER recombinant BJ-ECZ yeast. At all concentrations tested (0.1, 1 & 10 µmol/L), 17α-oestradiol, E_2, E_1, DES, EE, zearalenone, α-zearalenol, α-zearalanol (zeranol) and β-zearalanol exhibited relatively high potency. MGA showed some oestrogenic activity at the highest concentration (10 mol/L), while the remaining compounds were relatively inactive. The results of dose-response experiments performed on those compounds classified as oestrogenic are summarised in the Table below. Compared to the positive control (EE), E_2 and DES were the most active compounds. For some compounds (DES, α-zearalenol, 17α-oestradiol, α-zearalanol, zearalanone) there appeared to be a considerable difference in their oestrogenicity depending on whether the assay was conducted using the hER or rtER recombinants.

RSA* values of oestrogenic compounds obtained from dose-response curves with human oestrogen receptor (hER) and rainbow trout oestrogen receptor (rtER recombinant BJ-ECZ yeast.

Compound	Recombinant	n	RSA (%)
EE	rtER	10	100
	hER	8	100
E_2	rtER	11	68
	hER	9	83
DES	rtER	9	82
	hER	9	21
E_1	rtER	8	16
	hER	9	29
α-zearalenol	rtER	6	29
	hER	8	2.2
17α-oestradiol	rtER	6	3
	hER	6	12
α-zearalanol	rtER	12	11
	hER	9	1.4
zearalanone	rtER	12	11

Compound	Recombinant	n	RSA (%)
zearalanone	hER	9	0.6
zearalenone	rtER	12	6
	hER	9	0.5
β-zearalanol	rtER	6	3
	hER	6	0.4
β-zearalenol	rtER	6	>0.01
	hER	6	0

* Relative stimulatory activity relative to EE

Graphically presented data illustrated the level of induction of the *VTG* gene in rainbow trout hepatocyte aggregate cultures. Compounds exhibiting the highest oestrogenic activity included E_2, E_1, DES, EE and α-zearalenol. Compounds exhibiting a moderate level of induction included zearalenone, α-zearalanol (zeranol) and β-zearalanol. TBO showed a relatively low level of activity, while the remaining compounds were inactive.

The results of dose-response experiments in Ishikawa cells on those compounds classified as oestrogenic are summarised in the Table below. EE and DES had the highest potency, while E_2 showed much lower activity compared to that determined in the 2 previous bioassays. The low potency of E_2 can be attributed to its metabolism in this particular cell line. Some other compounds (eg. E_1 and 17α-oestradiol) also showed lower activity, while α-zearalenol showed greater potency compared to that detected using the other assays.

RSA* values of oestrogenic compounds obtained from dose-response curves in Ishikawa cells

Compound	n	RSA (%)
EE	5	100
DES	6	125
α-zearalenol	6	75
zeranol (α-zearalanol)	5	19
zearalenone	7	9
zearalanone	7	7
E_2	7	3
β-zearalanol	7	2
E_1	4	0.1
17α-oestradiol	7	0.1
β-zearalenol	4	0.02

* Relative stimulatory activity relative to EE

Conclusions: E_2, DES and EE exhibited the highest potency in 3 *in vitro* bioassays. The potency of several oestrogenic compounds such as zeranol (α-zearalanol) varied depending on the assay that was used. This variability suggested that certain assays may over- or under-estimate the oestrogenicity of a compound.

Study 10: Interaction of xenobiotics with sex hormone binding globulin; impact on endogenous steroid transport, bioavailability and mechanism of action

Publication pending.

Study 11: Reproductive sequelae of developmental exposure of rabbits to trenbolone, zeranol and melengestrol acetate; emphasis on differential and neoplastic transformation of germ cells.

Only part of this EC-commissioned study has been published and therefore evaluated, namely data relating to the transplacental passage of MGA, TBO and zeranol. The biological effects of these compounds on the developing rabbit remain to be published.

Lange IG, Daxenberger A, Meyer HHD, Rajpert-De Meyts E, Skakkebaek NE & Veeramachaneni (2002) Quantitative assessment of foetal exposure to TBA, zeranol and MGA, following maternal dosing in rabbits. Xenobiotica. 32(8): 641-651.

Experimental: Two pregnant Dutch-Belted rabbits (unspecified age, bw, source & mating/fertilisation details) were fed 0.5 mg/kg bw/d MGA in corn syrup between gestation days (GD) 14 and 17, while 2 other rabbits were injected subcutaneously with 0.5 mg/kg bw/d TBA in safflower oil. One rabbit was implanted subcutaneously with 0.5 mg/kg bw zeranol (unspecified source) on GD 14. Two untreated animals served as controls. Housing and feeding conditions were unspecified. Blood samples were taken (unspecified site and method of sampling) from the dams prior to and 2 hours after treatment, on GD 14, 21 and 27. Blood samples were taken from foetuses following euthanasia (unspecified method) on GD 27. Samples of skeletal muscle, liver, kidney and fat were collected from all dams and 2 foetuses/sex/group on GD 27. Placentas were also collected. All samples were stored at -20°C prior to analysis.

Following liquid extraction, plasma concentrations of TBA (17β-TBO & 17α-TBO), MGA and zeranol were determined by ELISAs as previously described (Meyer & Hoffman 1987; Hageleit et al 2001; Lange et al 2001). Analytes were extracted from homogenised tissue samples, separated by HPLC and analysed by an ELISA, as previously described (Lange et al 2001). The efficacy of the extraction and specificity of the HPLC separation had been confirmed previously (Daxenberger et al 2000). The authors indicated that internal standardisation was applied to fulfil the requirements of the EC concerning methods for detecting residues of substances having a hormonal or thyrostatic action (Commission of the European Communities 1993). The detection limit for each ELISA was calculated as the difference of the mean relative binding of all blank samples and its 3-fold standard deviation. It was stated that the sensitivity of the ELISAs was in the pg/g range. No statistical analyses were performed.

Results: MGA, TBA and zeranol were detected in all corresponding maternal and foetal plasma samples, and the majority of tissue samples.

The detection limit for TBA in plasma was 0.048 ng/mL. The concentrations of TBA in plasma samples from the 2 dams were 16/8.3, 14/4.5 and 12/9.8 ng/mL at GD 14, 21 and 27, respectively. Pretreatment concentrations were <0.048 ng/mL. The mean concentration of TBA in foetal plasma was 5 ng/mL, with female foetuses having lower plasma concentrations than male foetuses (6.0 and 5.2 ng/mL *versus* 4.8 and 4.4 ng/mL). Tissue levels of 17β-TBO, 17α-TBO and TBA are summarised in the Table below. In dams, 17β-TBO was the main metabolite in fat and muscle, while 17α-TBO was the main metabolite in liver and kidney. Residues were generally lower in foetal tissue than maternal tissue, with the exception of 17α-TBO, which was higher in muscle and liver than in the dams. 17α-TBO was the main metabolite in all foetal tissue, indicating a different metabolic profile than in dams. Relatively

little TBA was detected in both maternal and foetal tissue, indicating extensive metabolism of the parent compound.

Tissue levels of 17b-TBO, 17a-TBO and TBA in dams and foetuses

Tissue	17b-TBO (pg/g)	17a-TBO (pg/g)	TBA (pg/g)
Muscle			
Dams †	8200/8100 (74%)	2800/2500 (24%)	220/170 (1.8%)
Foetuses ‡	990/1100/1100/580 (18%)	5300/5600/4500/2100 (82%)	54/13/<6.4/<5.9 (0.3%)
Liver			
Dams †	390/370 (3.5%)	12000/8700 (96%)	57/79 (0.6)
Foetuses ‡	51/43/110/33 (0.5%)	9100/11000/18000/9700 (99%)	27/53/66/25 (0.4)
Kidney			
Dams †	5900/4800 (4.8%)	0000/48000 (95%)	1000/500 (0.7%)
Foetuses ‡	43/370/260/- (2.0%)	1800/15000/16000/- (98%)	43/<19/<15/- (0.1%)
Fat			
Dams †	40000/26000 (63%)	22000/12000 (33%)	2500/1700 (4.1%)
Foetuses ‡	7400/8100/4600/2600 (36%)	13000/9900/10000/7100 (63%)	170/230/183/240 (1.3%)
Placenta			
Foetuses ‡	6300/5400/7600/7600 (42%)	7500/7900/10000/9400 (57%)	150/110/240/19 (1.1%)

All results are expressed as absolute values with the % of each metabolite included in parentheses; † n=2; ‡ n=4

The concentration of zeranol in the plasma from the single treated dam was 190, 99 and 120 pg/mL on GD 14, 21 and 27, respectively. Zeranol levels in the 2 female foetuses were higher than in the 2 males (210/160 pg/mL *versus* 130/140 pg/mL, respectively). In the dam, the concentration of zeranol was greatest in the kidney (860 pg/g), followed by the liver (180 pg/g), fat (55 pg/g) and muscle (24 pg/g). No zeranol was detected in foetal fat, while zeranol was only detectable in the liver from one female foetus (65 pg/g). In foetuses, zeranol levels were highest in female kidney (240/210 pg/g) and liver (140/200 pg/g), while lower levels were detected in males (36/60 pg/g for kidney and 97/76 pg/g for liver). Zeranol levels in placenta were 140/140 pg/g for males and 97/90 pg/g for females.

Plasma levels of MGA in the 2 treated dams were 1.80/0.45, 1.19/0.82 and 0.85/0.42 ng/mL on GD 14, 21 and 27, respectively. Residue levels in the 2 female foetuses were higher than in the 2 males (0.48/0.31 ng/mL *versus* 0.39/0.27 pg/mL, respectively). The levels of MGA in maternal and foetal tissue are summarised in the Table below, with the highest levels detected in liver and fat. This led the study authors to conclude that MGA accumulates in these tissues, however these results only suggested that MGA is preferentially distributed to these tissues. Levels in foetal liver and fat were approximately 20-fold lower than in dams. The concentration of MGA in foetal muscle was marginally higher than in dams, while the concentration in placenta was relatively low.

MGA levels (pg/mL) in maternal and foetal tissues

Tissue	Dams (n=2)	Foetuses (n=4)
Muscle	290/700	990/950/1000/880
Liver	190000/160000	5300/7700/5100/6200
Kidney	2800/2500	1100/1000/600/1000
Fat	28000/72000	3100/4700/5600/7000
Placenta	-	750/690/950/810

Conclusions: MGA, TBA and zeranol were detectable in dams and shown to cross the placenta in rabbits. However, there was no indication that this resulted in any adverse effects

in dams or foetuses. The significance of some of the results (eg. comparisons between male and female foetuses, comparisons of residue levels between tissue and between dams and foetuses) were unclear due to the absence of statistical analyses. This study should be viewed as preliminary, due to the small group sizes.

NOTE: An unpublished study, described in the EC's latest risk assessment, reported that prenatal exposure to MGA, TBA or zeranol may affect the function of the reproductive tract of male rabbits. In the absence of a critical review of the data supporting this study, and the unavailability of the study report, no comment can be provided on this finding.

Study 12: Long term effects in children to oestrogenised meat

Chiumello G, Guarneri MP, Russo G, Stroppa L & Sgaramella P (2001) Accidental gynecomastia in children. APMIS 109(Suppl 103): S203-9.

This paper discussed the possible causes of abnormal palpable breast tissue in men (gynecomastia), which are endocrine disorders, exposure to hormonally active compounds in drugs, food and the environment, or unknown causes. No data was provided, however a number of studies were cited to support the authors' conclusion that hormones ingested in meat can cause breast enlargement and other deleterious immediate or long-term effects. The majority of study citations related to the adverse health effects of the banned substance DES, while there was no mention of the effects of hormones currently registered for use as growth promotants in non-European countries. Therefore, the relevance of this 'review' was unclear. The Table below summarises the evidence cited by the authors to support their explanation for the possible causes of accidental gynecomastia in children.

Evidence for the possible cause of accidental gynecomastia in children

Cause of gynecomastia	Evidence
Endocrine disorders	No studies cited
Drugs	(1) Gynecomastia in children exposed to oestrogenic cream used by their mothers; (2) Gynecomastia in children of workers in a DES manufacturing plant due to absorption of DES from the father's clothing; (3) Gynecomastia in adolescents taking anabolic steroids to enhance athletic performance; (4) Breast enlargement caused by phytoestrogens in marijuana; (5) Side effects due to growth hormone therapy.

Cause of gynecomastia	Evidence
Hormones in food	(1) Oestrogens and androgens influence breast cancer risk; (2) Observations in the 1970s of the high incidence of vaginal adenocarcinoma in women treated with DES during pregnancy; (3) Studies proving the toxicity of DES; (4) In the 1980s, malformations in the reproductive system and seminoma occurred in a young many following *in utero* exposure to DES; (5) Toxic effects of DES on the male reproductive system; (6) Up until the 1980s, DES was found in baby food in Italy; (7) An epidemic of gynecomastia in Italy in 1979 where hormone contaminated meat was suspected as the causative agent; (8) In the 1980s in Italy, isolated cases of pseudoprecocious puberty linked to contaminated meat ingestion; (9) A Puerto Rican study reporting over 10,000 cases of abnormal sexual development linked to the consumption of poultry contaminated with oestrogens as well as environmental contamination caused by pharmaceutical industries producing oestroprogestinics.
Hormones in the environment	No studies cited

The authors made a number of unsubstantiated claims regarding the adverse health effects of hormones and the incidence of the illegal use of HGPs in countries where they are banned. While the authors argued that environmental and dietary contaminants are the cause of the increased incidence of advanced pubertal development, they also made the point that it could be caused by genetic factors, obesity and altered socioeconomic status. Although it was mentioned that there are other dietary sources of oestrogenic compounds besides meat (eg. dairy products, fungal oestrogens, phytoestrogens in food), there was no discussion of how these foods could contribute to overall hormone intake and possibly impact on pubertal development. This paper had limited value due to the absence of data and the lack of a critical review of the studies cited.

Study 13: Androgen exposures *in utero*, risk of breast cancer

Kaijser M, Lichtenstein P, Granath F, Erlandsson G, Cnattingius S & Ekbom A (2001) In utero exposures and breast cancer: a study of opposite-sexed twins. Journal of the National Cancer Institute 93(1): 60-62.

Experimental: This study was undertaken to investigate the association between birthweight and breast cancer among female twins of opposite-sexed twin pairs. The rationale for the study was previous evidence indicating a "pronounced association between birthweight and antenatal oestrogen exposure". However, no data were provided or cited to substantiate this apparent association. Dizygotic twins were considered a suitable study group to investigate this association due to the broad range and higher levels of pregnancy oestrogens compared with both single pregnancies and monozygotic twin pregnancies. It was stated that the study was approved by the Research Ethics Committee at the Karolinska Institute, Stockholm, Sweden.

Study subjects were identified through record linkage between the Swedish Twin Registry on twins born between 1926 and 1967, and the Swedish Cancer Registry. Eligible case subjects were female twins with male co-twins, who developed breast cancer between 1972 and 1995. Aged-matched control subjects were drawn from the same population, with 2 control subjects selected for each case subject. Birth records were initially obtained for 104 case and 127 control subjects. The study was restricted to twins with a reported gestational age of ≥ 33

weeks, which left 90 pairs of case and control subjects. Odds ratios (ORs) were modelled by conditional logistic regression; adjustment variables were male sibling birthweight and gestational duration. All *P* values were 2-sided. Birthweight was categorised in groups of 500 g as the study authors wanted to obtain a broad range of birthweight categories. Equidistant scores for the categories were used for trend tests and a likelihood ratio test was used for the analysis of interaction.

Findings: There was a trend of increased breast cancer risk with increasing birthweight which was statistically significant for both the crude and adjusted models (p=0.007 and 0.03, respectively) (see Table below). Women with a birthweight of 3500 g reportedly had a 12-fold increase in breast cancer risk compared to women with a birthweight of 2000 g. However, this finding was probably not as marked as appears as the very high OR at 3500 g may be attributable to the use of only one control subject. When birthweight was analysed as a continuous variable, the OR for breast cancer risk increased 2.4 for each kg increase in birthweight (by 2.3 after adjustment). It was reported that the association between birthweight and breast cancer risk was even more pronounced for those women diagnosed with breast cancer before 50 years of age [crude and adjusted ORs of 5.0 and 4.3, respectively (95% confidence intervals of 1.63-15.1 and 1.24-14.7, respectively)]. There was no significant relationship when stratifying the analysis on male co-twin birthweight and the duration of gestation, however the study authors stated that "the power of this analysis was limited". Furthermore, no adjustment was made for known risk factors for breast cancer. These undefined risk factors were not considered by the study authors to confound the results as they were unlikely to be associated with female birthweight.

Crude and adjusted ORs* and 95% confidence intervals (CIs) for breast cancer according to female birthweight category

Adjustment variables	Crude OR (95% CI)	Adjusted OR (95% CI)
Female birthweight category (g)		
1310-2000	1.0 (referent)	1.0 (referent)
2001-2500	3.2 (0.79-12.6)	3.3 (0.81-13.6)
2501-3000	3.5 (0.96-13.0)	3.1 (0.79-12.5)
3001-3500	5.8 (1.30-25.7)	5.6 (1.10-27.9)
3501-3980	12.1 (1.06-138.8)	11.8 (0.94-147.8)
P for trend	0.007	0.03
Female birthweight (kg) continuous	2.4 (1.28-4.6)	

* adjusted for male co-twin birthweight

Conclusions: The study authors concluded that among female twins with male co-twins, high birthweight constitutes an independent risk factor for breast cancer. They also suggested that these findings support the hypothesis that *in utero* exposure to hormones influences breast cancer risk. However, the reviewing toxicologist did not consider that this conclusion was justified because the study did not directly test an association between oestrogen exposure and cancer risk, and no data were provided or cited to substantiate any link between oestrogen exposure and high birthweight.

Study 14: Endocrine disrupting activity of anabolic steroids used in cattle

Schiffer B, Daxenberger A, Meyer K & Meyer HDD (2001) The fate of TBA and MGA after application as growth promoters in cattle: environmental studies. Environmental Health Perspectives 109(11): 1145-1151.

Experimental: Forty-one Holstein Friesian cattle (unspecified source, age, bw) were implanted at an unspecified site with commercially available anabolic preparations (source and identity unspecified) containing TBA. The total amount administered equalled 3340 mg (presumed to be per 41 cattle; ie. 82 mg/animal). The authors stated that all animals were treated according to the *Code of Ethics of the World Medical Association* (Declaration of Helsinki) and the guiding principles in the *Guide for the Care and Use of Laboratory Animals* (NIH, USA). Manure was collected via a collection canal and pumped into an open cylindrical storage pit every 2 weeks. While the manure in the collection canal was heterogenous, a "stirring propeller" in the storage pit was claimed to achieve "good" homogeneity before sampling. The manure was stored under anaerobic conditions and contained all animal excrement, the stable cleaning water and atmospheric precipitation that also reached the storage pit. The estimated volume of the manure was approximately 170 m^3 and the excrement weighed approximately 100 tons. Liquid manure samples were taken every 2 weeks from the collection canal, every 2 or 4 weeks from the storage pit, and prior to spreading on the fields. An unspecified "small fraction" was spread on an experimental field in November following completion of the animal experiments, while the majority was used for fertilisation after 4.5 months of storage. Samples of the stored manure were collected monthly.

Half lives of 17α- and 17β-TBO were calculated according to the formula $c(t) = c(0) \times e^{\lambda \cdot t}$, where t is the time, c(t) is concentration at time t, c(0) is concentration at the beginning and λ is the constant of decay. The half-life was calculated using the formula $t_{1/2} = -\ln (1/2)/\lambda$.

In a separate experiment, 12 cattle were implanted at an unspecified site with TBA, the total amount administered equalling 5600 mg (presumed to be per 12 cattle; ie. 466 mg/animal). Dung was collected from 31 days before to 56 after treatment in "a traditional procedure with the help of straw", and placed onto a dung heap. The total volume of the dung heap was approximately 40 m^3, with an estimated mass of excrement of 20 tons. Samples (unspecified number) were taken from the top, middle and bottom of the dung heap. Samples of liquid effluent were also collected. In November, the dung was transferred to a sealed "storage ground", and after 4.5 months, samples were taken. The authors indicated that the mixing of the dung heap was inevitable during transportation to the storage ground.

Fresh liquid manure containing TBA was spread over one section (unspecified area) of a field in November, while stored liquid manure was spread over another section in March. The field was located at an experimental farm where maize was apparently cultivated according to good agricultural practice. Soil samples were collected from 3 representative locations of each experimental section of the fields immediately after fertilisation, monthly for the first 3 months, then every second month until October (ie. the end of the cultivation period).
In another experiment, 13 cattle were treated with MGA via a feed premix that was prepared from reference material at the Institute of Animal Nutrition, Technical University of Munich-Weihenstephan, Germany. The total amount of MGA applied was 840 mg (presumed to be per 13 cattle; ie. 65 mg/animal). Faecal samples were taken twice per week from each animal.

Dung was collected over an unspecified period of time, and stored and sampled as described above.

All samples were stored at -25°C. Steroid extraction and purification were performed as previously described (Meyer & Hoffman 1987). TBA was separated from its metabolites by HPLC and then quantified by EIA according to published procedures of (Meyer & Hoffman 1987; Meyer 1989). For soil samples, analytical enrichment was performed due to the expected low concentrations of TBO and its metabolites. The identity of TBA residues was confirmed by GC-MS. LC-MS was used to quantify MGA in faeces. Quantitation of MGA in solid dung was also performed by LC-MS but with some modifications to the extraction process. An enzyme immunoassay was used to quantify MGA in soil as the sensitivity using LC-MS was insufficient. The Table below summarises the accuracy, precision and LOD for the measurement of TBO and MGA by EIA or LC-MS.

Validation parameters for the measurement of 17a-TBA and MGA in liquid manure, solid dung, soil and faeces

Parameter	Liquid manure	Solid Dung		Soil		Faeces
	17a-TBA[a]	17a-TBA[a]	MGA[b]	17a-TBA[a]	MGA[a]	MGA[b]
Detection limit (pg/g)	4	5	200	0.4	0.2	200
Spikes (pg/g)	100/450/1800 (n=3)	1000/4750/18500 (n=3)	5000 (n=5)	3/45/240 (n=3)	4/20/40 (n=4)	1000/5000/10000 (n=3)
Mean recovery (%)	42[c]	32[c]	102.6[d]	30[c]	25[c]	100.8[d]
Mean precision (%)	30	10	2.8	12	12	5.0

a = performed by EIA; b = performed by LC-MS; c = not corrected by standardisation; d = corrected by internal standardisation

Findings: Manure from the collection canal was reportedly heterogenous, with graphically-presented data illustrating variation of up to 70 pg/g for trendione and 17β-TBO between two samples collected from different areas of the canal. While it was stated that 17α-TBO was the main metabolite followed by 17β-TBO and trendione (22 and 49 times higher, respectively), graphically-presented data was reported only for 17β-TBO and trendione. Graphically-presented data illustrated the stability of the 3 metabolites in liquid manure over 160 days, however the data for 17α-TBO was considered to be equivocal due the absence of any data points on the line graph. It was stated that the level of 17α-TBO decreased from 1700 pg/g to 1100 pg/g over 160 days, while 17β-TBO fell from 160 to 100 pg/g. A half-life of 267 and 257 days was calculated for 17α- and 17β-TBO, respectively. No decline in the concentration of trendione in liquid manure occurred, which the study authors suggested might have been due to the possible oxidation of 17α- and 17β-TBO.

Prior to storage, 17α-TBO was the main metabolite in solid dung (see Table below), with the level of 17β-TBO and trendione at least an order of magnitude lower. The highest levels occurred in the middle of the dung heap, with the lowest levels detected in the effluent. 17α-TBO was also the main metabolite in solid dung after storage (ie. before spreading on the fields) and the middle of the heap also had the highest residue levels. The high variability between the two samples of stored dung suggested that more samples should have been taken

even though the authors attributed this to the heterogeneity of the dung heap caused by transportation.

Residue levels of 17a-TBO, 17b-TBO and trendione in solid dung

Sample (position in dung heap)	17a-TBO pg/g	17b-TBO pg/g	Trendione pg/g
Solid dung before storage			
Fresh (~1 m below top)	13820	1000	1225
Medium (height 2.5 m)	75400	4265	4700
Old (height 0.5 m)	4726	484	405
Effluent	227	19	10
Solid dung before spreading on field (n=2)			
Top of heap	ND/ND	11/ND	ND/ND
Middle of heap	10100/ND	292/ND	842/ND
Bottom of heap	100/318	60/14	70/ND

All values were corrected by the recovery rate; ND = below the limit of detection.

17α-TBO, 17β-TBO and trendione were detected in soil that had been fertilised with liquid and solid manure (see Table below), but at levels much lower (~2 orders of magnitude) than in solid dung. This was attributable to the dilution effect of spreading the dung over the fields. Fertilisation of soil in autumn with fresh liquid manure resulted in residues that were below the limit of detection after 31 days. In soil fertilised in spring with stored liquid manure, levels of 17α-TBO, 17β-TBO and trendione decreased over time; by day 40, no detectable residues were present (see Table below). A similar pattern occurred for soil fertilised in spring with stored solid dung, with no detectable residues measured at day 93. It was suggested by the authors that the apparently greater stability in solid dung was due to absorption of TBO to straw material which "possibly protected the substances from degradation or leaching".

Following administration to cattle via the feed, the concentration of MGA in faeces increased with dose; mean (± SD) levels 12 hours after dosing with 0.5, 1.5 and 5 mg/kg bw/d were 1.6 ± 0.1, 5.3 ± 0.6 and 13.8 ± 1.8 ng/g, respectively. Higher concentrations were detected 24 hours after dosing (2.5 ± 0.2, 6.5 ± 0.1 and 18.5 ± 0.1 ng/g, respectively) which probably reflected the passage through the GIT. The concentration of MGA in solid dung was highly variable ranging from 260 to 7760 pg/g before storage and 374-6028 ng/g after 4.5 months (see Table below). The authors attributed this variation to the heterogeneity in the dung heap due to transportation. It appeared that MGA was relatively stable in the dung heap over the storage period. MGA was also detected in soil, which had been fertilised with solid dung (see Table above). In soil fertilised in spring with stored solid dung, MGA was detected for up to 194 days after application, suggesting that it is more stable than TBO.

Levels of TBO and MGA residues in soil

Sample (Days after fertilisation)	17a-TBO (pg/g)	17b-TBO (pg/g)	Trendione (pg/g)	MGA (pg/g)
Soil fertilised in spring with stored liquid manure				
A-B (1) n=2	248/164	8.1/5.1	21/18	-
C-E (8) n=3	11/8.6/48	ND/ND/2.4	2.2/1.0/15	-
F (40)	ND	ND	ND	-
Soils fertilised in spring with stored solid dung				
A-C (26) n=3	5.8/3.3/11	0.7/0.4/1.0	2.6/1.3/4.1	34/11/17
D-F (58) n=3	3.4/1.2/ND	1.0/0.5/ND	1.2/0.9/ND	1.5/1.4/3.8

Sample (Days after fertilisation)	17a-TBO (pg/g)	17b-TBO (pg/g)	Trendione (pg/g)	MGA (pg/g)
G-I (93) n=3	ND	ND	ND	1.7/4.9/7.3
J-L (159) n=3	-	-		0.6/ND/0.5
M-O (194) n=3	-	-		2.4/ND/6.2

ND = below the limit of detection; Capital letters represent samples taken from different locations of the same field.

Concentrations of MGA in solid dung

Sample (position within dung heap)	MGA (pg/g)
Solid dung before storage	
Top	380/658/3419/731
Higher middle	260/4200/5104/3037
Lower middle	7760/417/1351/741
Bottom	6524/2619/4076
Solid dung before spreading on fields (after 4.5 months storage)	
Upper middle	3470/1600
Middle level	374/6028
Lower level	421/1493

Values corrected by internal standardisation

Sample calculations performed by the authors indicated that the estimated recovery of TBO in liquid manure was 8%, while in solid dung it ranged from 3 to 42%. A previous study (Daxenberger et al 2000) indicated that approximately one third of the TBO remained at the implantation site. The recovery of MGA was calculated to be approximately 12% in faeces and 0.6 to 18% in solid dung. The reasons given for these low recoveries included the binding of the compounds to dung and the degradation by microorganisms.

Conclusions: Metabolites of TBA were detected in liquid and solid manure from cattle that had been implanted with commercial anabolic preparations, and in soil that had been fertilised with dung from these same animals. MGA was also detected in manure and fertilised soil following administration to cattle via the feed. The relevance of these findings to the human health assessment of TBA and MGA was unclear. The study authors concluded that MGA and TBO should be investigated further for their potential endocrine-disrupting activity in agricultural ecosystems. The high variability in residue levels suggested that greater sampling should have occurred. No statistical analysis was performed. There was no information on the metabolism of these compounds in cattle and it was unclear whether the metabolites of TBA were generated directly by the cattle or by environmental micro-organism. Any comparisons between levels of TBA and MGA were unclear given the different administration routes (implant *versus* diet).

Study 15: Screening water samples for oestrogenic and androgenic anabolic chemicals

Jegou B, Soto A, Sundlof S, Stephany R, Meyer H & Leffers H (2001) General discussion on the existing guidelines for the use of meat hormones and other food additives in Europe and USA. APMIS 109(Suppl. 103): S551-6.

This article was a transcript of a discussion between the authors and a number of other scientists at an international workshop on hormones and endocrine disruptors in food and water, held at University Hospital Rigshospitalet, Copenhagen, Denmark, from the 27th-30th

May 2000. Very little data was provided and the article speculated on the possible risk to human health from hormone contaminants in the environment, particularly those generated from the use of HGPs in cattle.

Data were described from a US study that examined whether feedlots contaminate water courses with anabolic hormones. Total oestrogenic and androgenic activities were measured in various water samples from a feedlot retaining pond, the confluence of a stream and river, a stream indirectly draining a complex of feedlots and various upstream reference sites. Fish (Fat Head Minnows) were collected at each site and 2 tap water samples were also collected for comparison. The assays used to measure oestrogenic and androgenic activities were the E-SCREEN and A-SCREEN bioassays, respectively (no details provided). The results of these assays are summarised in the Table below and show that higher levels of androgens and oestrogens were detected downstream of feedlots than at the upstream reference site. The identity of these androgenic and oestrogenic compounds was not determined. Hormonal activity was also detected at the reference site, which suggested that there were other sources of hormones other than those emanating from the feedlots. It was reported (pers comm) that male fish were feminised and female fish were androgenised at these downstream sites. This study was considered to have limited value due to the absence of experimental detail, the lack of statistical analysis and that it had not been published (no study citation given). There was no indication of the source or possible nature of the hormonal contamination, particularly as hormonal activity was also detected at the reference site at levels not dissimilar to some of the 'contaminated' sites.

Oestrogen and androgen levels in water samples draining from feedlots

Sample	Androgen eq. (pmol/L)	Oestrogen eq. (pmoles/L)
Retaining pond	9.62 ± 1.5%	1.73 ± 6.2%
Confluence of stream/river	3.83 ± 13.6%	2.23 ± 16.7%
Stream	4.58 ± 1%	0.65 ± 1.5%
Upstream reference site	2.45 ± 15.7%	1.15 ± 18.6%
Tap water	nd	nd
Blank	nd	nd

nd = not detected

In addition to excreta from farm animals, a number of other sources of oestrogenic and androgenic contaminants were considered important sources of environmental hormones, including sewage effluent and microbial conversion/activation of inactive compounds into hormonally-active ones. A German study had reportedly detected testosterone, dihydrotestosterone and 5 major metabolites in effluent from sewage treatment plants.

The authors considered that it was important to measure hormone levels in the environment (eg. in water, effluent from feedlots and sewage treatment facilities) to ascertain possible environmental exposure of the population. However, the measurement of actual hormone levels in humans (so-called internal exposure) is necessary in order to determine the real risk to human health. Unfortunately this type of data is currently lacking.

Results from an uncited/unpublished US FDA study indicated that the concentration of E_2 in muscle from hormone-treated steers and heifers is 35 and 40-43 pg/kg, respectively. In contrast, the concentration of E_2 in untreated pregnant heifers is 2460 pg/kg. The fact that the concentration of E_2 in treated cattle falls with the normal physiological range was used as evidence of the safe use of hormones

The main conclusions of the discussion were that it is difficult to accurately assess the contribution of hormones from any single food source without knowledge of total oestrogen intake, and that JECFA should re-examine HGPs and compile a new database of endocrine disruptors.

Study 16: Endocrine disrupting effects of cattle farm effluent on environmental sentinel species

Orlando EF & Guillette Jr LJ (2001) A re-examination of variation associated with environmentally stressed organisms. Human Reproduction Update 7(3): 265-272.

This ecotoxicological review discussed the premise that exposure to sublethal levels of endocrine-disrupting contaminants results in phenotypic variation within a population of organisms. Various examples were cited and/or described to illustrate this apparent phenomenon, however, the only example relevant to HGPs was an unpublished study conducted by the authors, on fathead minnow exposed to cattle ranch effluent. This study, conducted in Nebraska in the USA, was described briefly in a previous evaluation (see above evaluation of Jegou et al 2001). Additional information was as follows. Fish were randomly sampled using 6 mm^2 seine and minnow traps, with traps checked daily at each site. Immature fish were rejected from the study as determined by gross anatomical inspection of the gonad. Fork length, inter-ocular distance (IO) and head width were recorded and data from all adult fish were retained. Statistical differences between means were analysed using an unpaired t-test and variances were analysed using an F-test for homogeneity of variance. No further details were provided.

There was no significant difference in the mean fork length, IO and head width between male fathead minnows from contaminated and reference streams (see Table below). However, the variance of the 3 parameters was significantly increased ($p<0.0001$-0.0013) in minnows from the contaminated stream (see Table below).

Fork length, inter-ocular distance (IO) and head width of male fathead minnow exposed to cattle effluent

Site	Length (cm)	Length – s^2	IO (mm)	IO – s^2	Head (mm)	Head – s^2
Contaminated	6.246	1.463**	5.579	2.784*	8.30	3.465**
Reference	6.847	0.085	6.771	0.389	9.34	0.255

s^2 = variance; * $p_F<0.0013$; ** = $p_F<0.0001$

The authors concluded that increased phenotypic variance may be an important early sign of population disturbance and as such could prove to be an early indicator of exposure to "subtle toxicants" such as endocrine disruptors. This study had limited value in assessing the risk to human health due to the consumption of meat from HGP-treated cattle.

Study 17: Human cells exposed to the oestrogenic compound zeranol

Leffers H, Naesby M, Vendelbo B, Skakkebaek NE & Jorgensen M (2001) Oestrogenic potencies of zeranol, oestradiol, diethylstilboestrol, bisphenol-A and genistein: implications for exposure assessment of potential endocrine disruptors. Human Reproduction 16(5): 1037-1045.

Experimental: The oestrogenic potencies of zeranol, E_2, DES, genistein and bisphenol A were compared by analysing differences in expression levels of 6 oestrogen-regulated genes in human MCF7 cells (an oestrogen-dependent cell line) according to the method of Jorgensen et al (2000). Cells were cultured for 6 days, under the standard laboratory conditions for this cell line, prior to the addition of the test samples. Four replicate samples of E_2, DES, zeranol (α-zearalanol), genistein and bisphenol A, and duplicate samples of 5 zeranol metabolites (α-zearalenol, β-zearalanol, β-zearalenol, zearalenone and zearalanone) were prepared in ethanol. Controls consisted of cells exposed to the vehicle alone (ethanol) or to 100 nmol/L of the anti-oestrogen, ICI 182.780. The concentration selection was based on the oestrogenic potency of each compound, which was reportedly determined in pilot experiments. E_2, DES and zeranol were tested at 1 fmol/L to 10 pmol/L, genistein at 0.1 nmol/L to 10 µmol/L, and bisphenol A at 1 nmol/L to 10 µmol/L.

The expression of the *PS2*, *ATB0+*, *transforming growth factor b3* (*TGFb3*), *monoamine oxidase A*, *MRG1/p35srj* and *GST mu3* genes were analysed by a competitive polymerase chain reaction (PCR) method. The resulting PCR products were visualised by polyacrylamide gel electrophoresis and the intensity of the bands analysed using a phosphor imager following normalisation for the background. All values were calculated as the fold induction or reduction compared with the mean of 4 samples of ICI 182.780. Results for E_2, DES, zeranol, genistein and bisphenol A were expressed as the mean of 3-4 independent samples $\pm$ the standard deviation. Results for the zeranol metabolites represented the mean of 2 samples.

Findings: The relative potencies of E_2, DES, zeranol, genistein and bisphenol A are summarised in the Table below. The majority of the data were depicted graphically and no statistical analyses were performed.

E_2, DES and zeranol were approximately equipotent at inducing the *PS2* gene (~1 pmol/L) while much higher molar concentrations of genistein and bisphenol A were required to cause similar levels of induction (10 nmol/L and 100 nmol/L, respectively). The study authors reported that the level of induction of the *PS2* genes was proportional to the concentration of the test material. The level of induction of the *ATB0+* gene was approximately an order of magnitude less than the *PS2* gene for all 6 oestrogens. There was approximately an order of magnitude difference in the relative potencies of E_2, DES and zeranol (~1, 10 and 100 pmol/L, respectively), while for genistein and bisphenol A, the relative potencies were similar to the *PS2* gene.

The study authors reported (no data provided) that E_2, DES and zeranol were equipotent at inducing the *TGFb3* gene, while expression was less sensitive to genistein and bisphenol A. It was reported that genistein and bisphenol A caused a down-regulation of the *TGFb3* gene (at 0.01-0.1 and 0.1-1 µmol/L, respectively). The *monoamine oxidase A* gene was induced by E_2, zeranol and DES, while genistein and bisphenol A were much less affective (no data provided).

Relative potencies† of E_2, DES, zeranol genistein and bisphenol A

Gene	Relative potency
PS2	E_2= DES = zeranol >>>>genistein > bisphenol A
ATB0+	E_2 > DES > zeranol >>>> genistein > bisphenol A
TGFb3	E_2 = DES = zeranol >>>> genistein > bisphenol A
monoamine oxidase A	E_2 > zeranol > DES >>>> genistein > bisphenol A
MRG1/p35srj	Zeranol > DES >> E_2 >>>> genistein > bisphenol A
GST mu3	E_2 >>> zeranol > DES

† = fold-induction compared with ICI 182.780. Each > represents an approximate order of magnitude.

The *GST mu3* gene was down regulated by E_2, DES and zeranol. The study authors reported that it required approximately 16 hours of treatment to cause this down-regulation, which contrasts to 2-8 hours of treatment for the other tested genes. It was clear that E_2 was the most potent of the 3 compounds at down-regulating this gene (at 1-10 fmol/L). The study authors concluded that zeranol was an order of magnitude more potent than DES, however any difference between zeranol and DES was unclear from the graph (similar levels of down-regulation and overlapping standard deviations). Statistical analysis would have assisted in delineating any difference between zeranol and DES. It was reported that the positive control compound (ICI 182.780) sometimes caused a significant (no p value) down-regulation in the *GST mu3* gene. The *MRG1/p35srj* gene was down regulated by E_2, DES and zeranol. It appeared that zeranol was marginally more potent than DES at down regulating this gene, while E_2 was approximately 3 orders of magnitude less potent than zeranol. No data were shown for genistein and bisphenol A.

The relative oestrogenic potencies of 5 zeranol metabolites are summarised in the Table below. Results were shown only for the induction of the *ATB0+* gene. Zeranol and α-zearalenol were reported to have similar potencies and to be more oestrogenic than the other compounds. There was no indication by the study authors that any of the metabolites caused the down-regulation of the *MRG1/p35srj*, while the effect on the *GST mu3* did not appear to have been tested.

Relative potencies† of zeranol and its metabolites

Gene	Relative potency
PS2	α-zearalenol > Zeranol > zearalanone > zearalenone > β-zearalanol > β-zearalenol
ATB0+	Zeranol > α-zearalenol > zearalanone > zearalenone > β-zearalanol > β-zearalenol
TGFb3	α-zearalenol > Zeranol > zearalanone > zearalenone > β-zearalanol > β-zearalenol
monoamine oxidase A	α-zearalenol > Zeranol > zearalanone > zearalenone > β-zearalanol > β-zearalenol
MRG1/p35srj	α-zearalenol = Zeranol > zearalanone > zearalenone > β-zearalanol > β-zearalenol

† = fold-induction compared with ICI 182.780. Each > represents an approximate order of magnitude.

Conclusions: The study authors concluded that the potency of zeranol was similar to that of E_2 and DES, and that one of the 6 genes tested (ie. *MRG1/p35srj*) appeared to be more sensitive to zeranol than E_2. However, E_2 was more potent overall than zeranol and DES, and genistein and bisphenol A were up to 5-6 orders of magnitude less potent than E_2. Metabolites of zeranol were reported to induce 5 of the 6 oestrogen-sensitive genes, the most potent being zeranol and α-zearalenol. The study authors speculated that the relatively high potency of zeranol suggested that zeranol intake from beef products could have an impact on the health of consumers. However, it was acknowledged that it was not possible to undertake a proper risk assessment because of the absence of data regarding zeranol in human serum

after ingestion of meat products from treated animals. No statistical analyses were conducted, and for some findings no data were actually provided.

EVALUATION OF RECENT STUDIES CITED BY THE EC

Metabolism of 17b-oestradiol and quantitative analysis of oestrogen residues in edible tissues from treated steers

Maume D, Deceuninck Y, Pouponneau K, Paris A, Le Bizec B & Andre F (2001) Assessment of oestradiol and its metabolites in meat. APMIS 109: 32-8.

Experimental: Five Hereford steers/group (200 kg mean bw; age and source unspecified) were injected with 1, 2 or 4 implants of Revalor®-S (containing 140 mg TBA + 28 mg E_2). The steers treated with 2 implants were injected with one at day 0 and the other at day 45. It was presumed that the implantation site was one or both of the ears, as per the manufacturer's directions. A group of 5 untreated steers served as the control group. Housing and feeding conditions were unspecified. Steers were slaughtered 90 days later "according to the usual breeding plan". Samples of meat (unspecified site), liver, kidney and peri-renal fat were collected and stored frozen at -18°C until analysis.

All forms of non-covalently bound oestrogens were extracted from tissue samples using a method derived from Folch et al (1957). Briefly, tissues were lyophilised, delipidated and subjected to liquid-liquid extraction. Enzymatic hydrolysis of the conjugated glucuronidates proceeded, followed by saponification and purification on a SPE C18 column. An alkaline liquid-liquid clean-up was performed followed by further purification on a DIOL SPE column. Acid hydrolysis of glycoside forms was performed and samples purified on a G60 silica gel column. Finally, samples were derivatised prior to quantitation by GC-MS.

Findings: The authors reported that the analytical technique did not allow the quantification of E_1, and therefore results were only shown for 17α-oestradiol and E_2. Various levels of free, glucuronidates, diconjugates, glycosides and lipoidal esters of 17α-oestradiol and E_2 were detected in muscle, fat, liver and kidney (see Table below). There was a high level of variability in metabolite concentrations within each treatment group, however, concentrations appeared to be dependent on the tissue type and number of implants administered. The highest tissue levels were detected in liver followed by kidney, fat and muscle. The authors drew particular attention to the level of free and lipoidal esters of E_2 in fat, which were proportionally increased with the number of administered implants. Their conclusion that E_2 is accumulated in fat was not considered to be unjustified as no temporal measurements of tissue distribution and elimination had been made. Although an apparent dose-related increase in certain metabolite concentrations occurred in some tissues, the significance of the results were somewhat difficult to ascertain as only concentration ranges were given (no means ± SD) and no statistical analysis had been performed.

Concentrations of 17b- and 17a-oestradiol in muscle, fat, liver and kidney from steers implanted with Revalor â-S

Tissue	E_2			
	Free	*Glucuronides*	*Diconjugates*	*Lipoidal esters*
Muscle				
0	3-8	0	0-2	0-18
1	0-32	0	0-3	0-8
2	30-482	0-3	1-3	3-30
4 implants	32-97	0-10	0-4	4-9

Tissue	E_2			
	Free	*Glucuronides*	*Diconjugates*	*Lipoidal esters*
Fat				
0	0	nd	nd	0-26
1	0-69	nd	nd	0-48
2	93-172	nd	nd	38-90
4 implants	91-161	nd	nd	80-140
Liver				
0	1-2	0-1	5-19	12-15
1	3-103	0-16	5-7	8-37
2	13-82	3-49	0-1	4-21
4 implants	29-192	14-76	0-4	12-35
Kidney				
0	1-4	1	nd	1-15
1	2-55	0-22	nd	0-4
2	45-86	36-62	nd	5-10
4 implants	64-132	31-101	nd	5-15
	17 a -oestradiol			
	Free	*Glucuronides*	*Glycosides*	*Lipoidal esters*
Muscle				
0	0	0	nd	nd
1	0	0	nd	nd
2	3-6	0-2	nd	nd
4 implants	3-6	0-2	nd	nd
Fat				
0	0	0	nd	nd
1	0-3	0	nd	nd
2	12-51	0-10	nd	nd
4 implants	8-31	0	nd	nd
Liver				
0	0-2	0-1	0-2	0
1	12-546	0-20	0-19	0-28
2	119-328	10-68	13-53	4-18
4 implants	212-790	40-169	27-106	12-57
Kidney				
0	0-2	1	nd	0
1	3-150	2-79	nd	0-4
2	116-232	12-51	nd	5-12
4 implants	137-264	8-31	nd	9-17

Results are expressed as the range of concentrations within each group; nd = not detected

The authors calculated the theoretical daily intake of oestradiol residues in a 500 g portion of meat, consisting of 300 g muscle, 100 g liver, 50 g fat and 50 g kidney obtained from the 3 treatment groups (see Table below). While there appeared to be a dose-related increase in the intake of oestradiol (statistical significance undetermined), intake fell well below the JECFA ADI of 50 ng/kg (3 µg/day); mean daily intakes were 0.2, 1.3, 3.9 and 4.7% of this ADI for the control, 1, 2 and 4-implant groups, respectively.

Total oestradiol[1] in a theoretical daily intake of 500 g[2] meat

Treatment	Range (ng)	Mean (ng)
Untreated	4-10	6
1 implant	3-110	40
2 implants	78-201	119
4 implants	80-211	142

1 = free + conjugated derivatives of 17α- and 17β-oestradiol; 2 = 300 g muscle + 100 g liver + 50 g fat + 50 g kidney.

Conclusions: The implantation of steers with Revalor®-S (containing 140 mg TBA + 28 mg E_2) caused an increase in the levels of oestradiol metabolites in muscle, fat, liver and kidney, which was dependent on the number of implants administered. Intake calculations indicated that the consumption of a theoretical portion of meat obtained from treated cattle was well below the JECFA ADI for 17β-oestradiol.

Oestrogenic potency of residues

Paris A, Goutal I, Richard J, Becret A & Gueraud F (2001) Uterotrophic effect of a saturated fatty acid 17-ester of estradiol-17b administered orally to juvenile rats. APMIS 109:365-75.

This study was stated to have been conducted in accordance with OECD principles of GLP.

Experimental: Juvenile female SD rats (bw unspecified; 14-days old; Charles River, St Aubin-les-Elbeuf, France), supplied with their mothers, were acclimatised for a period of 8 days prior to treatment. Rats were weaned at age 21 days and allocated to treatment groups according to bw and clinical condition. Six rats/group (mean bw of 58 g; range of 48-66) were dosed by po gavage with 0 (control), 2.5, 25, 250 or 2500 nmol/kg bw/d E_2 or 17β-E_2S in corn oil for 6 consecutive days. The dose volume was 5 mL/kg bw/d. Positive control animals received EE at 34 nmol/kg bw/d. Rats were deprived of food 4 hours prior to dosing. Groups were housed in the same cage and fed *ad libitum* a standard diet (UAR, Villemoisson-sur-Orge, France). Animals were sacrificed by CO_2 asphyxiation and exanguinated 24 hours after the last treatment. Blood was collected from the abdominal artery and stored at -20°C prior to analysis of E_2 and FSH by RIA.

Body weights were recorded prior to sacrifice. All rats were subjected to a post-mortem examination of the abdominal cavity, with particular attention given to the reproductive tract. Wet uterine weights were recorded. Following the determination of the volume of fluid in the uterine horns and the removal of any uterine fluid, empty uterine weights were also recorded. The uterus and vagina from all rats were histopathologically examined following staining with haematoxylin-eosin. The following parameters were recorded and scored according to severity: immaturity of the uterus and vagina; loose endometrial stroma, dilated lumen and myometrial hypertrophy; epithelial cell hypertrophy/hyperplasia and evidence of mitosis in the uterus; epithelial cell hyperplasia and hyperkeratosis in the vagina; and granulocyte infiltration in epithelial cells.

Uterine weights and histopathological findings (ie. grades) were statistically analysed by ANOVA. The means of different parameters were compared using a Student-Newman-Keuls test. These parameters included uterus weight gain, uterus and vagina immaturity, epithelial cell hypertrophy/hyperplasia in the uterine endometrium and vagina, loose endometrial stroma, myometrial hypertrophy, epithelial cell hyperkeratosis in vagina, granulocyte infiltration in vaginal epithelium and the concentration of plasma FSH. A multivariate analysis of variance (MANOVA) and a factorial discriminant analysis were used to assess the comparative effects of treatments, taking into account all the variables. All statistical analyses were performed using SAS.

Findings: There was a significant increase in relative uterine weights ($p<0.05$) at and above 250 nmol/kg bw/d E_2 or E_2S, which was reportedly the same magnitude as the positive

control (see table below). At 250 nmol/kg bw/d the effect of E_2S was statistically higher ($p=0.009$) than the effect of E_2, but a similar difference was not detected at the highest dose. Overall, these data did not indicate that there was any difference in oestrogenic potency between E_2 and E_2S. The authors reported that there was no significant treatment-related effect on body weight gain.

Effect of E_2 and E_2S on relative uterine weights in juvenile female rats

Dose (mg/kg bw/d)	E_2	E_2S
0	0.088 ± 0.025^a	0.088 ± 0.025^a
2.5	0.076 ± 0.023^a	0.065 ± 0.005^a
25	0.094 ± 0.027^a	0.100 ± 0.024^a
250	0.164 ± 0.031^b	0.223 ± 0.033^b
2500	0.210 ± 0.032^b	0.239 ± 0.025^b

Results expressed as the mean ± standard deviation/error (unspecified) % of bw; a, b = significant differences in mean uterine weight between doses ($p< 0.05$)

Macroscopic examination revealed immaturity of the uterus and the vagina at 0, 2.5 and 25 nmol/kg bw/d E_2 or E_2S. Results at higher doses were not reported.

Various histopathological abnormalities were observed in the uterus or vagina at and above 250 nmol/kg bw/d E_2 or E_2S. Photographic evidence illustrated: the presence of myometrial hypertrophy, loose endometrial stroma and luminal epithelial cell hypertrophy/hyperplasia in the uterus at 2500 nmol/kg bw/d of E_2S; and hyperkeratosis and epithelial cell hyperplasia in the vagina at 250 nmol/kg bw/d E_2S. It was reported that at 250 nmol/kg bw/d E_2, all 6 rats had endometrial cell hypertrophy/hyperplasia (1/6 minimal, 4/6 slight and 1/6 moderate) in addition to slight to moderate loose endometrial stroma and myometrial hypertrophy. All 6 rats treated with an equivalent dose of E_2S had moderate endometrial cell hypertrophy/hyperplasia, loose endometrial stroma and myometrial hypertrophy. Similar findings were recorded at 2500 nmol/kg bw/d but were reportedly of a higher intensity for E_2 and E_2S (moderate and marked, respectively). A single rat at 250 nmol/kg bw/d E_2, and most (unspecified) rats at 250 and 2500 nmol/kg bw/d E_2S reportedly showed evidence of mitotic activity. At these same doses, single animals reportedly showed slightly dilated lumen.

Slight (1/6), moderate (2/6) or marked (3/6) epithelial cell hyperplasia of the vagina was reported at 250 nmol/kg bw/d E_2 in addition to moderate hyperkeratosis in (4/6 animals). At 2500 nmol/kg bw/d E_2, moderate (1/6) to marked (5/6) epithelial cell hyperplasia was reported, the later associate with slight hyperkeratosis. All rats dosed with 250 and 2500 nmol/kg bw/d E_2S showed marked epithelial cell hyperplasia in addition to hyperkeratosis, which was reportedly minimal to marked at 250 nmol/kg bw/d and slight to moderate at 2500 nmol/kg bw/d.

Based on the descriptions and limited histopathological data provided, it appeared that E_2S had relatively greater effect than E_2 at 250 and 2500 nmol/kg bw/d. The magnitude of this difference was not quantifiable based on the information provided. Observations for the positive control were not reported, however the authors indicated that the effects seen with E_2 and E_2S were greater than those of the positive control. Extensive statistical analyses were undertaken to compare the uterotrophic effects of E_2 and E_2S. Analysis of 10 experimental parameters (listed above) by a MANOVA revealed a highly significant dose-related effect with either compound ($p<0.0001$). More elaborate analyses (discriminant analysis and calculation of Mahalanobis distances) confirmed that there were no significant uterotrophic

effects in rats treated with 2.5 and 25 nmol/kg bw/d E_2 or E_2S. Results at higher doses and in the positive control were all determined to be statistically different to the control. These higher doses were grouped according to statistical similar oestrogenic response: EE and 250 nmol/kg bw/d E_2; 2500 nmol/kg bw/d E_2 and 250 nmol/kg bw/d E_2S; and 2500 E_2S. The authors reported that the later gave the greatest statistical effect. Based on these statistical groupings, the authors concluded that E_2S was 10-fold more oestrogenically potent than E_2.

Results of plasma E_2 and FSH levels in each group were largely unreported. Significantly higher levels (p value unspecified) of E_2 were reportedly detected 24 hours after the last dose of 2500 nmol/kg bw/d E_2S (range 24.1-168.2 pmol/L). Plasma FSH concentrations were reportedly unaffected by treatment. These findings (ie. for plasma E_2 and FSH) should be viewed as inconclusive due to the lack of reporting detail.

Conclusions: This study indicated that very high doses of E_2 or E_2S (above 250 nmol/kg bw/d) induced uterotrophic effects in juvenile female rats, manifesting as increased uterine weight and histopathological abnormalities in the uterus and vagina. The NOEL for these effects was 25 nmol. Biologically there was a marginal difference in the effects seen at high doses between E_2 and E_2S, while multi-dimensional statistical analyses suggested that E_2S was significantly more potent than E_2. Limitations to this study were that: bodyweight and absolute uterine weight data were not provided; a proportion of the histopathological data was unreported; macroscopic observations were incompletely reported; and while the histopathological findings were apparently statistically analysed, these results were not reported.

Mills LH, Lee AJ, Parlow AF & Zhu BT (2001) Preferential growth stimulation of mammary glands over uterine endometrium in female rats by a naturally occurring estradiol-17-fatty acid ester. Cancer Research 61: 5764-5770.

Experimental: Ovariectomised female SD rats (bw unspecified; mean age of 6.5 weeks; Harlan Sprague Dawley Laboratories, Houston, TX, USA) were acclimatised for one week prior to treatment. Animals were housed under standard laboratory conditions and provided with food and water (unspecified sources) *ad libitum* throughout the acclimatisation and experimental periods. In the first experiment, 5-6 rats/group were surgically implanted under the skin of the back with an osmotic mini-pump (model 1002, Alzet, location unspecified; release rate of 0.25 µL/h for up to 14 days) containing 0 or 0.83 mM E_2 or E_2S in 70% ethanol and 30% DMSO. These pumps were estimated to release 5 nmol/day of compound and were prepared according to the manufacturers instructions. Rats were sacrificed 10 days later by an unspecified means. In a second experiment, 7-8 rats/group were implanted with an osmotic mini pump (model 2004, Alzet, location unspecified; release rate of 0.25 µL/h for up to 28 days) containing 0, 0.083 or 0.83 mM E_2 or E_2S (estimated to release 0.5 and 5 nmol/day, respectively). Rats were sacrificed 23 days later by an unspecified means. Wet uterine weights were recorded for each rat. The proliferation of cells in the breast and uterus was determined by bromodeoxyuridine (BrdUrd) labelling as previously described (Green et al 1992). Blood samples were collected from each animal and the concentrations of E_2, LH, follicular stimulating hormone (FSH) and prolactin determined by RIAs. Statistical analysis was performed by an unspecified test.

Findings: Graphically presented data illustrated the effect on uterine weight, and cell proliferation in the breast and uterus, following treatment with 5 nmol/d E_2 or E_2S for 10 days. Uterine weights were comparable in E_2- and E_2S-treated rats (~0.3 g), with both

approximately 6 to 7-fold higher than the controls. The proliferation of uterine endometrial cells was elevated by 2.6-fold ($p<0.02$) in E_2S-treated rats and 16.3-fold ($p<0.001$) in E_2-treated rats, relative to the control. This differential effect of each compound was reversed in mammary glandular cells, where treatment with E_2S or E_2 increased the level of proliferation by 5.2-fold ($p<0.001$) and 1.6-fold ($p<0.01$), respectively, relative to the controls. Examples of BrdUrd-stained endometrial and mammary tissues were shown, which illustrated the increased cellular proliferation associated with E_2 and E_2S treatment.

Graphically presented data illustrated the effect on uterine weight, and cell proliferation in the breast and uterus, following treatment with 0.5 or 5 nmol/d E_2 or E_2S for 23 days. Uterine weights in both treatment groups and at both doses were significantly higher (no p values given) than the controls, with the magnitude of this increase being dose dependent. At 5 nmol/d, there was no difference in uterine weights between E_2- and E_2S-treated rats, confirming the result of the previous experiment conducted over 10 days. However, at the lower dose (0.5 nmol/d) uterine weights in the E_2-treated rats were significantly higher ($p<0.001$) than the E_2S-treated rats.

The proliferation of uterine endometrial cells was elevated in both treatment groups and at both doses, with the magnitude of this increase being dose dependent. E_2 was approximately 5-fold more potent than E_2S at inducing endometrial cell proliferation at 0.5 nmol/d, a result that was statistically significant ($p<0.0001$). While E_2 was also more potent than E_2S at 5 nmol/d, the magnitude of this difference was less than at the lower dose (~1.3-fold) and was not reported to be statistically significant.

At 0.5 nmol/d, the proliferation of mammary glandular cells appeared to be slightly elevated with E_2 and E_2S compared to the controls, however, this result was determined to be significant ($p<0.001$ and $p<0.01$, respectively). At 5 nmol/d, a more pronounced treatment-related increase in proliferation occurred (10.7- and 4.3-fold increases compared to the control for E_2S and E_2, respectively). At 5 nmol/d, E_2S induced an approximately 2-3-fold greater level of proliferation than E_2, but the statistical significance of this difference was not determined.

Graphically presented data showed that E_2- or E_2S-treated rats had a lower bw gain than the controls over the 23 day treatment period. The bw gain of E_2-treated rats was marginally lower than that of E_2S-treated rats. The statistical significance of these findings was not reported.

The effect of E_2 or E_2S on plasma E_2, LH, FSH and prolactin is summarised in the Table below. There was an apparent dose-related increase in plasma E_2 in rats treated with E_2, but this result was not statistically different to either the controls or the E_2S-treated rats. There was a significant dose-related decrease ($p<0.01$-0.05) in LH in E_2-treated rats, and a significant decrease ($p<0.05$) in E_2S-treated rats at the top dose (5 nmol/d). There was no treatment related effect on FSH, while prolactin was significantly elevated ($p<0.05$) at 5 nmol/d E_2 and E_2S. There was no significant difference in plasma prolactin levels between E_2 and E_2S-treated rats.

Plasma concentrations of E_2, LH, FSH and prolactin in E_2- or E_2S-treated rats

Treatment	E_2 (pg/mL)	LH (ng/mL)	FSH (ng/mL)	Prolactin (ng/mL)
Control	4.2 ± 1.6	18 ± 1.1	39 ± 2.1	5 ± 1.4
E_2S 0.5 nmol/d	8.6 ± 0.5	21 ± 1.3	47 ± 2.9	4 ± 0.3
E_2S 5 nmol/d	8.2 ± 0.4	3 ± 0.7**	35 ± 1.8	17 ± 4.3*
E_2 0.5 nmol/d	6.8 ± 1.3	10 ± 1.2*	48 ± 2.0	5 ± 0.7
E_2 5 nmol/d	10.6 ± 1.5	2 ± 0.7**	37 ± 2.8	12 ± 1.8*

Results expressed as means ± 1 SEM (n=6-8)

Conclusions: This study illustrated the differential effect of E_2 and E_2S on cell proliferation in ovariectomised female rats treated with either compound at 0.5 or 5 nmol/kg bw/d for 10 or 23 days; E_2 was more potent than E_2S at inducing uterine endometrial cell proliferation and E_2S was more potent than E_2 at inducing mammary glandular cell proliferation. Other treatment-related effects included increased uterine weights and plasma prolactin, and reduced bw gain and plasma LH. The magnitude of these effects was generally comparable between the 2 compounds. The authors speculated on the possible role of fatty acid esters of endogenous oestrogens and their bioactive metabolites in tumour formation but acknowledged that more studies are necessary to test this hypothesis.

Larner JM, MacLusky NJ & Hochberg RB (1985) The naturally occurring C-17 fatty acid esters of estradiol are long-acting estrogens. J Steroid Biochem 22(3): 407-413.

Experimental: Juvenile female SD rats (22 days old, 35-40 g bw; Charles River Laboratories, Wilmington, MA) were injected subcutaneously with 0, 5, 25 or 50 nmol/rat E_2 or E_2S in 0.1 mL sesame oil over 3 days (one third of the total dose was administered daily over the 3 days). Control rats were injected with 0.3 mL sesame oil. There were 3 or 6 rats/group. Rats were sacrificed by cervical dislocation at 1, 3 or 4 days after the last injection. In a separate experiment, 5 rats/group were injected intravenously with E_2 or E_2S in saline at 5 unspecified doses (~0.25, 1, 2, 5 or 16 nmol). A vehicle control group consisted of 10 rats. Rats were sacrificed after 24 hours. To determine the kinetics of oestrogenic activity following E_2 or E_2S treatment, juvenile rats were given a single iv injection of 5.3 nmol/rat E_2 or E_2S ($n \geq 4$) and sacrificed at 0, 6, 12, 24, 36, 48, 60, 72, 84, or 96 hours later. In a separate experiment, ovariectomised adult rats (age and bw unspecified) were given a single iv injection of 132 nmol/kg bw E_2 or E_2S (n=4-26) and sacrificed at 0, 6, 9, 12, 24, 36, 48, 60, 72, 96 or 120 hours later.

To examine the kinetics of E_2 and E_2S distribution to the uterus, 3 juvenile rats/group were injected intravenously with [6,7-^{3}H]oestradiol (^{3}H-E_2) or [6,7-^{3}H]oestradiol-17-stearate (^{3}H-E_2S) at 5.3 nmol/rat and sacrificed by decapitation at 1, 4, 8, 12, 24, 36 or 48 hours. The level of radioactivity in the uterus was measured by scintillation counting (following tissue homogenisation and extraction) or HPLC.

In all experiments, rats were maintained under standard laboratory conditions and provided with food (unspecified) and water *ad libitum* throughout the study. In all experiments, wet uterine weights were recorded for each rat. Where necessary, data were subjected to logarithmic transformation, and statistically analysis using a 1-way ANOVA and Duncan's multiple range test.

Findings: Graphically presented data illustrated that absolute uterine weights were significantly elevated ($p<0.01$-0.05) in juvenile rats following subcutaneous administration of

E_2 or E_2S for 3 days; the exception to this finding was at the lowest dose of E_2S, which was not significantly different to the control. The magnitude of the increase was dependent on the compound, dose and time after dosing. E_2 caused an initial increase in uterine weight (ie. at day 1 after the last injection), which was of a greater magnitude than that occurring with E_2S. The uterine weight of E_2-treated rats declined to control levels over 4 days, while uterine weights increased markedly in E_2S-treated rats.

Graphically presented data illustrated that relative uterine weight was significantly increased ($p<0.05$) in juvenile rats, 24 hours after a single iv injection of E_2 or E_2S. Relative uterine weight was approximately 2 to 3-fold higher in E_2S- *versus* E_2-treated over a dose range of 1-16 nmoles. At the only lower dose of 0.25 nmol, this difference was only marginal. For E_2 and E_2S, uterine weight increased dose-relatedly and reached and maximum at 1 and 5 nmol, respectively.

Graphically presented data showed that following a single iv injection of 5.3 nmol E_2 or E_2S, the maximum increase in absolute uterine weight was reached at 12 hours post injection, falling to control levels by 48 hours. Over the first 12 hours post-injection, absolute uterine weights of E_2S-treated rats were comparable to E_2-treated rats; they then increased to their maximum level at 36 hours and were maintained for up to 96 hours. Vaginal opening was reported in 7/8 E_2S-treated rats at approximately 90 hours post-injection.

In adult ovariectomised rats there was a slightly different pattern of increase in uterine weight following a single iv injection of 134 nmol/kg bw E_2 or E_2S. Up to 24 hours after injection, E_2 and E_2S caused a similar pattern of increase, with a maximal effect reached at 12 hours. In E_2-treated rats, uterine weight then fell to control levels by 60 hours post-injection. In contrast, uterine weight in E_2S-treated rats plateaued from 24-36 hours and was then re-elevated to the maximum level at 48 hours. Uterine weight then steadily declined over time, reaching control levels by 120 hours post-injection.

Following injection of juvenile rats with ^3H-E_2 or ^3H-E_2S, maximum levels of total radioactivity or ^3H-E_2 in the uterus occurred at 1 and 12 hours, respectively. Maximum levels of radioactivity and ^3H-E_2 in E_2-treated rats were approximately twice that detected in E_2S-treated rats. However, the level of unconjugated radioactivity was greater in the uterus of E_2S- *versus* E_2-treated rats (range of 49-86% versus 14-57%, respectively).

Conclusions: This study revealed that E_2S has a more prolonged oestrogenic action on the uterus compared to free E_2. The authors hypothesised that one of the roles of the fatty acid on E_2S is to protect it from metabolism and therefore prolong the life of E_2. The dose routes used in this study (sc, iv) were not directly applicable to the dietary risk assessment of hormones. This publication lacked experimental detail.

Recent findings on the mutagenicity and genotoxicity of 17b-oestradiol

Cavalieri EL, Kumar S, Todorovic R, Higginbotham S, Badawi AF & Rogan EG (2001) Imbalance of estrogen homeostasis in kidney and liver of hamsters treated with estradiol: Implications for estrogen-induced initiation of renal tumours. Chem Res Toxicol 14: 1041-1050.

Experimental: Groups of 4 male Syrian golden hamsters (6-weeks old; bw unspecified; Eppley colony, unspecified location) were given a single ip injection of 8 µmol E_2/100 g bw in 300 µL of trioctanoin/DMSO (9:1) and sacrificed by an unspecified means after 1, 2, or 4 hours. Pre/experimental housing and feeding conditions were unspecified. E_2 had been synthesised according to the method of Dwivedy et al (1992) and the dose-selection was based on a previous study (Devanesan et al 2001b). In a separate experiment, hamsters were pretreated with a single sc injection of 0.6 mmol/100 g bw L-buthionine(S,R)sulfoximine (BSO) in 2 mL saline, 2.5 hours prior to the administration of E_2. BSO is a glutathione-depleting agent. Hamsters were then maintained for 2 hours. An untreated or vehicle control group was also included in each experiment. Following sacrifice by an unspecified means, kidney and liver samples were collected and stored at -80°C. Tissue samples were homogenised and divided into 3 portions, with one portion left untreated, one incubated for 6 hours at 37°C and the third incubated with β-glucuronidase/sulfatase for 6 hours at 37°C. Following liquid-liquid extraction, metabolites, conjugates and adducts were analysed by HPLC, with their identification achieved by comparison with 32 "authentic" standards that had been synthesised by the authors or obtained commercially. Data were analysed using computer software, with the results expressed as the average of 2 experiments. It was reported that the level of variation in individual analyte levels was 10-30%; it was unclear whether this variation occurred between experiments or within experiments.

Findings: Graphically-presented data illustrated the metabolite/conjugate profiles in the kidney and liver of hamsters treated with E_2 for 1, 2 or 4 hours, and the effect of β-glucuronidase/sulfatase treatment on these profiles. The major metabolites were 2-OHE_2/E_1 and 2-OCH_3E_2/E_1 in both the kidney and liver. The levels of some analytes were affected by time, notably the glutathione conjugates in the kidney, which were only evident at 2 hours. Treatment of samples with β-glucuronidase/sulfatase increased the analytical levels of 2-OHE_2/E_1 and 2-OCH_3E_2/E_1, suggesting that the phenolic groups of these metabolites are conjugated with either glucuronic or sulfuric acid.

The results of a subsequent experiment, in which hamsters were treated with 8 µmol E_2 for 2 hours, are summarised in the Table below. The parent compound (E_2) was present at the highest level, followed by E_1, 2-OHE_2/E_1 and 2-OCH_3E_2. The increase in E_2 and its metabolites following incubation with β-glucuronidase/sulfatase confirmed the previous finding that a proportion of these compounds are conjugated with glucuronic of sulfuric acid. Glutathione conjugates were generally higher in the kidney, but in the absence of statistical analysis or the provision of the range or SD/SEM, it was unclear whether these differences were significant.

Levels[1] of oestrogen metabolites and conjugates in hamsters (nmol/g tissues) administered a single dose of E_2 (8 mmol/100 g bw, ip)

Metabolite/conjugate	Not incubated		Incubated		Incubated with b-glucuronidase/sulfatase	
	Kidney	Liver	Kidney	Liver	Kidney	Liver
E_2	3.80	0.40	2.05	1.17	14.8	17.0
E_1	0.57	0.10	1.72	0.61	1.28	0.58
2-OHE_2	0.08	0.42	0.82	2.01	1.78	3.05
2-OHE_1	0.08	0.32	0.56	0.50	0.84	1.36
4-OHE_2	<0.01	0.11	<0.01	0.05	0.01	0.07
4-OHE_1	0.04	0.07	0.18	0.41	0.26	0.31
16α-OHE_2	0.01	0.01	0.08	0.01	0.07	0.03
16α-OHE_1	<0.01	0.01	0.03	0.14	0.23	0.10
2-OCH_3E_2	0.04	0.07	0.21	0.34	1.11	2.78
2-OCH_3E_1	<0.01	0.04	0.11	0.54	0.10	0.72
2-OHE_1-1(+4)-SG	<0.01	0.03	<0.01	0.03	<0.01	0.01
2-OHE_2-1(and 4)-Cys	<0.01	<0.01	0.30	0.32	0.49	0.29
2-OHE_1-1(+4)-Cys	<0.01	<0.01	0.22	<0.01	0.22	<0.01
2-OHE_2-4-NAcCys	<0.01	0.01	0.49	0.15	0.39	0.19
2-OHE_1-1(+4)-NAcCys	<0.01	<0.01	0.04	0.04	0.11	<0.01
4-OHE_1-2-SG	<0.01	<0.01	<0.01	0.02	<0.01	<0.01
4-OHE_2-2-Cys	<0.01	<0.01	<0.01	<0.01	0.03	0.04
4-OHE_1-2-Cys	<0.01	<0.01	<0.01	<0.01	0.05	<0.01
4-OHE_2-2-NAcCys	<0.01	<0.01	0.14	<0.01	0.21	0.06
4-OHE_1-2-NAcCys	<0.01	<0.01	<0.01	0.05	<0.01	<0.01

1 = data are expressed as the mean of 2 experiments.

The effect of glutathione depletion following pretreatment of hamsters with BSO is summarised in the Table below. 2- and 4-OHE_2/E_1 were decreased in the kidney but increased in the liver. A reduction was seen in the level of 2-OCH_3E_2/E_1 in the kidney but this did not occur in the liver. Glutathione depletion, not surprisingly, resulted in a decreased level of conjugates in kidney and liver, while the depurinating DNA adduct, 4-hydroxyoestradiol/ostrone-1-N7Gua, was detected in the kidney.

Effect of glutathione depletion (ie. BSO) on the formation of selected oestrogen metabolites, conjugates and adducts (nmol/g tissue) in hamsters treated with E_2

Analyte	Kidney		Liver	
	E_2	E_2 + BSO	E_2	E_2 + BSO
2-OHE_2/E_1	2.66	1.02	4.75	10.27
4-OHE_2/E_1	0.29	0.14	0.44	1.04
2-OCH_3E_2/E_1	1.13	0.42	4.16	4.46
E_2/E_1-2,3-Q-conjugates[a]	1.36	0.21	0.63	0.13
E_2/E_1-3,4-Q-conjugates[a]	0.30	0.09	0.06	0.01
E_2/E_1-3,4-Q N7Gua adducts	<0.01	0.27	<0.01	<0.01

Tissue samples were treated with β-glucuronidase/sulfatase; a = includes all compounds produced by reaction of catechol oestrogen quinones with glutathione and detected with a -SG, -Cys or –NAcCys moiety.

Conclusion: A variety of catechol oestrogen metabolites and conjugates were detectable in the liver and kidney of male hamsters following a single injection of a very high dose of E_2 (8 μmol). A greater level of catechol oestrogen conjugates appeared to be formed in the kidney, with depletion of glutathione leading to the generation of a DNA adduct. This study had a number of limitations including the lack of statistical analysis, the absence of any results or

statements relating to metabolite levels in the controls and the lack of reporting detail such as the range or SD/SEM.

Cavalieri EL, Devanesan P, Bosland MC, Badawi AF & Rogan EG (2002) Catechol estrogen metabolites and conjugates in different regions of the prostate of Noble rats treated with 4-hydroxyestradiol: implications for estrogen-induced initiation of prostate cancer. Carcinogenesis 23(2): 329-333.

Experimental: Groups of 10 male NBL/Cr rats (11-12 weeks old; approximately 280-300 g bw; Charles River, Raleigh NC, USA) were given a single ip injection of 4-OHE_2 or 17β-oestradiol-3,4-quinone (E_2-3,4-Q) at 6 µmol/100 g bw in 200 µL of trioctanoin/DMSO (4:1). A group of control animals was treated with the vehicle only. After 90 minutes, rats were sacrificed by an unspecified means and the ventral prostate, anterior prostate, dorsolateral prostate and periuethral prostate and urethra, dissected out and frozen at -80°C. These 4 regions of the prostate were pooled for each group, and following tissue homogenisation and liquid-liquid extraction, the level of oestrogen metabolites and conjugates were analysed by HPLC. No statistical analysis was performed.

Findings: The levels of oestrogen metabolites and conjugates in the 4 regions of the prostate are summarised in the Table below. The authors reported that no E_1 or E_2 or their metabolites or conjugates were detected in prostate tissue from control rats.

Prostate levels of oestrogen metabolites and conjugates (pg/g) in rats, 90 min after administration of 4-OHE_2 or E_2-3,4-Q (6 mmol/100 bw, ip)

Metabolite/conjugate	Ventral prostate	Anterior prostate	Dorsolateral prostate	Periurethral prostate and urethra
4-OHE_2				
4-OHE_2	352 (22%)	277 (22%)	554 (44%)	172 (31%)
4-OHE_1	9 (1%)	30 (2%)	8 (1%)	nd
4-OCH_3E_2	389 (25%)	627 (51%)	485 (39%)	242 (44%)
4-OCH_3E_1	786 (50%)	230 (19%)	194 (16%)	129 (23%)
4-OHE_2-2-SG	32 (2)	70 (6%)	8 (1%)	12 (2%)
E_2-3,4-Q				
4-OHE_2	98 (10%)	211 (20%)	281 (44%)	78 (20%)
4-OHE_1	29 (3%)	22 (2%)	27 (4%)	21 (6%)
4-OCH_3E_2	240 (24%)	465 (45%)	190 (29%)	161 (42%)
4-OCH_3E_1	515 (51%)	100 (10%)	63 (10%)	70 (18%)
4-OHE_2-2-SG	48 (5%)	124 (12%)	35 (5%)	20 (5%)
4-OHE_2-2-Cys	35 (3%)	64 (6%)	30 (5%)	20 (5%)
4-OHE_2-2-NAcCys	40 (4%)	55 (5%)	20 (3%)	15 (4%)

Results are the mean of 2 experiments, with a 10-30% level of variability. The % of each metabolite is shown in parentheses. nd = not detected.

Following a single ip injection of 4-OHE_2 the highest metabolite levels were found in the ventral prostate, followed by the anterior prostate and dorsolateral prostate, with the periuethral prostate and urethra having the lowest levels. The levels of 4-OHE_1 and 4-hydroxy-17β-oestradiol glutathione (4-OHE_2-2-SG) were relative low throughout all 4 regions of the prostate. The levels of 4-OHE_2, 4-methoxy-17β-oestradiol (4-OCH_3E_2) and 4-methoxy-oestrone (4-OCH_3E_1) varied depending on the region; the ventral prostate had the

highest level of 4-OCH_3E_1, the anterior prostate the highest level of 4-OCH_3E_2 and the dorsolateral prostate the highest level of the 'parent' 4-OHE_2.

The metabolite profile in the prostate of rats treated with E_2-3,4-Q was somewhat different to that of rats treated with 4-OHE_2 and included the detection of 2 additional conjugates, namely, 4-hydroxy-17β-oestradiol cysteine (4-OHE_2-2-Cys) and 4-hydroxy-17β-oestradiol N-acetylcysteine (4-OHE_2-2-NAcCys). The highest metabolite levels were found in the ventral and anterior prostate, followed by the dorsolateral prostate and periuethral prostate and urethra. All 4 regions had similar proportions of 4-OHE_1, 4-OHE_2-2-Cys and 4-OHE_2-2-NacCys. The dorsolateral prostate had the highest proportion of 4-OHE_2, while the ventral prostate had the highest level (and proportion) of 4-OCH_3E_1. The anterior prostate and periuethral prostate and urethra had relatively high proportions of 4-OCH_3E_2, while there appeared to be a greater proportion of 4-OHE_2-2-SG in the anterior prostate. In the absence of statistical analysis the significance of these differences was unclear.

Conclusions: Various oestrogen metabolites were detectable in 4 regions of the rat prostate following a single ip injection of 4-OHE_2 or E_2-3,4-Q. While the study provided a qualitative analysis of the types of oestrogen metabolites that may be generated in the rat prostate, it had a number of deficiencies that limited its value in determining the role of oestrogen metabolites/conjugates in the development of prostate cancer. There was no evidence that metabolism of 4-OHE_2 or E_2-3,4-Q actually occurred in the prostate. The dose route was not relevant to the assessment of dietary exposure to hormones. The administration of 4-OHE_2 or E_2-3,4-Q rather than parent E_2 could have short-circuited normal metabolic pathways. Only one time point (eg. 90 minutes) was sampled following administration; it remains to be determined what the kinetics of metabolism and elimination might be for these metabolites in the prostate.

Chakravarti D, Mailander PC, Li K-M, Higginbotham S, Zhang HL, Gross ML, Meza JL, Cavalieri EL & Rogan EG (2001) Evidence that a burst of DNA depurination in SENCAR mouse skin induces error-prone repair and forms mutations in the H-ras gene. Oncogene 20: 7945-7953.

Experimental: The dorsal skin of female SENCAR mice (8-weeks old; bw unspecified; NCI, Fredrick, MD, USA) were shaved and 1 day later treated with 200 nmol of E_2-3,4-Q in 50 µL acetone:DMSO (9:1) for 1 hour. Two independent experiments were conducted (n=6 & 7). No solvent control group was tested. E_2-3,4-Q had been synthesised as previously described (Dwivedy et al 1992). The dorsal skin of each mouse was prepared and the level of stable DNA adducts formed in epidermal chromosomal DNA analysed by ^{32}P-post-labelling (Chen et al 1996). A positive control DNA sample consisted of calf thymus DNA (1.25 mg) that had been incubated with E_2-2,3-Q (3.5 µmol) as described by Dwivedy et al (1992). Depurinating DNA adducts were solvent extracted and then analysed by HPLC. Confirmation of DNA adducts was performed by MS.

The levels and types of mutations in the H-*ras* gene in 12 mice treated dermally with 200 nmol E_2-3,4-Q or 600 nmol 17β-oestradiol-2,3-quinone (E_2-2,3-Q) in 100 µL acetone:DMSO (70:30) were determined as previously described (Chakravarti et al 1998b & 2000). Three untreated shaved mice served as controls. Briefly, mice were sacrificed at 1, 6 or 12 hours, or 1 or 3 days after treatment. Epidermal DNA from skin samples was isolated and a 500 base-pair H-*ras* exon amplified by PCR. The amplification product was cloned into the pUC18

plasmid and transformed in to E coli. H-*ras* inserts in the clones were sequenced to determine mutations.

Findings: The level of stable DNA adducts in mouse skin epidermis was 0.004 µmol/mol DNA phosphate (average of 2 experiments), while the level of depurinating adducts was 12.5 and 12.1 µmol/mol DNA phosphate for the N3ade and N7gua adducts of 4-OHE_2, respectively. It was reported that the levels of depurinating and stable adducts varied by =10% and 30%, respectively. The positive control generated 1.78 µmol/mol DNA phosphate of stable adducts.

Treatment of mouse skin with 200 nmol E_2-3,4-Q for one hour failed to generate any mutations in 32 plasmids, while 1 mutation was generated in 36 plasmids derived from untreated skin. In contrast, treatment of mouse skin with 600 nmol E_2-2,3-Q generated 4 mutations/35 plasmids. Treatment of mouse skin with E_2-3,4-Q for 6 h, 12 h, 1 day or 2 days generated 7/29, 6/30, 11/50 and 4/40 mutations/plasmid, respectively. The majority of mutations were A/T→G/C transitions (5/7, 4/6, 7/11 and 3/4 at 6 h, 12 h, 1d and 2 d, respectively).

Conclusion: Direct application of 200 nmol E_2-3,4-Q to the skin of SENCAR mice resulted in the formation of DNA adducts in the epidermis after 1 hour, with the level of depurinating adducts approximately 6000-fold higher than the level of stable adducts. While no mutations in the H-*ras* gene were detectable at this time, longer treatment times (up to 2 days) resulted in mutations that were predominantly A/T→G/C transitions. The value of this study was limited by the dosing route (dermal), the use of only one (and a very high) test concentration and the absence of a solvent control. Furthermore, there was no evidence that the DNA damage caused by E_2-3,4-Q could actually occur *in vivo* and generate mutations.

Devanesan P, Santen RJ, Bocchinfuso WP, Korach KS, Rogan EG & Cavalieri E (2001b) Catechol estrogen metabolites and conjugates in mammary tumors and hyperplastic tissue from estrogen receptor-α knockout (ERKO)/Wnt-1 mice: implications for initiation of mammary tumors. Carcinogenesis 22(9): 1573-1576.

This preliminary study examined the levels of catechol oestrogen metabolites in tumour and hyperplastic tissue from oestrogen receptor-α knockout (ERKO) mice. The underlying hypothesis of the study authors was that these mice metabolise oestrogens abnormally leading to the generation of potentially mutagenic metabolites in the mammary epithelium. Mammary hyperplastic and/or tumour tissue was obtained from one female ERKO/Wnt-1 mouse or one ovariectomised ERKO/Wnt-1 mouse. Alternatively, tissue from 2 mice were combined to give approximately 1 g tissue. Following tissue homogenisation and liquid-liquid extraction, levels of catechol oestrogen metabolites were quantified by HPLC.

Results of the metabolite analysis are given in the Table below. The 4-hydroxy metabolites of E_2 and E_1 predominated in hyperplastic mammary tissue, while a number of conjugated metabolites were also detected, namely the glutathione conjugates of 4-hydroxy E_2/ E_1, and the cysteine and N-acetyl-cysteine conjugates of 4-OHE_2. Hyperplastic tissue from a single ovariectomised mouse had a higher proportion of 4-OHE_2-2-NAcCys than tissue from non-ovariectomised mice. Catechol metabolites were detected only in tumour tissue from the unovariectomoised mouse; the metabolite profile was similar to that of the hyperplastic tissue, except that 2-OH glutathione and cysteine metabolites of E_2 were not detected.

Levels of catechol oestrogen metabolites and conjugates in mammary tissue from ERKO/Wnt-1 mice (pmol/g tissue)

Metabolite/conjugate	Hyperplastic mammary gland (n=2)	Hyperplastic tissue from ovariectomised mouse (n=1)	Tumour tissue (n=2)	Tumour tissue from ovariectomised mouse (n=1)
2-OHE_1	-	-	-	-
2-OHE_2	-	-	-	-
2-OCH_3E_1	-	-	-	-
2-OCH_3E_2	-	-	-	-
4-OHE_1	2.4	0.5	1.9	-
4-OHE_2	8.5	1.8	3.0	-
4-OCH_3E_1	-	-	-	-
4-OCH_3E_2	-	-	-	-
4-OHE_1-2-SG	0.6	1.2	2.8	-
4-OHE_2-2-SG	0.4	-	-	-
4-OHE_1-2-Cys	-	-	-	-
4-OHE_2-2-Cys	0.2	-	-	-
4-OHE_1-2-NAcCys	-	-	-	-
4-OHE_2-2-NAcCys	1.1	3.9	1.6	-

- = below the LOD

In an additional experiment, hyperplastic mammary tissue was enzymatically treated with β-glucuronidase or sulfatase for 6 h at 37°C. The metabolite profile was somewhat different to that in the above Table. No E_2 or E_1 was detected, while neither oestrogen was analysed in the first experiment. Metabolites detected in untreated hyperplastic mammary tissue (ie. a repeat of the above experiment) included 2-OHE_1 (1.1 pmol/g), 4-OHE_2 (9.8 pmol/g), the 4-OHE_2 glutathione conjugate (1.3 pmol/g), the 4-OHE_1 cysteine conjugate (0.4 pmol/g) and the 4-OHE_2 N-acetylcysteine conjugate (1.0 pmol/g). In hyperplastic mammary tissue treated with β-glucuronidase or sulfatase the following metabolites were detected: 2-OHE_1 (0.8 pmol/g), 2-OHE_2 (1.9 pmol/g), 4-OHE_1 (0.9 pmol/g), 4-OHE_2 (10.9 pmol/g), the 4-OHE_1 glutathione conjugate (3.3 pmol/g), the 4-OHE_1 cysteine conjugate (0.7 pmol/g) and the 4-OHE_2 N-acetylcysteine conjugate (0.7 pmol/g)

In conclusion, various oestrogen metabolites are detectable in both hyperplastic and tumour mammary tissue from ERKO/Wnt-1 mice. There appears to be some variability in the levels and types of metabolites detected between mice, and following treatment of tissue with β-glucuronidase or sulfatase. Additional studies would need to be completed with more animals to ascertain the significance of these findings.

Lavigne JA, Goodman JE, Fonong T, Odwin S, He P, Roberts DW & Yager JD (2001) The effects of catechol-O-methyltransferase inhibition on estrogen metabolite and oxidative DNA damage levels in estradiol-treated MCF-7 cells. Cancer Research 61: 7488-74894.

Experimental: MCF-7 cells (American Type Culture Collection, Manassas, VA, USA) at 40% confluence were incubated with 10 nM 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in DMSO for 72 hours to induce cytochrome 450 1A1 and 1B1 (CYP4501A1 and CYP4501B1, respectively). These two cytochrome P450s generate catechol oestrogens by hydroxylating E_2 or E_1 at the 2- or 4- positions. Control cultures were incubated with DMSO. TCDD-containing medium was removed, replaced with maintenance medium (phenol red-free IMEM + 5% foetal bovine serum) and incubated overnight. Cells were then incubated with fresh medium $\pm$ 3 or 10 μM Ro 41-0960 [an inhibitor of catechol-*O*-methyltransferase

(COMT)] for 30 min before the addition of 0, 0.1, 0.3, 1 or 10 μM E_2 in 95% ethanol (n=3). Control cultures were treated with 95% ethanol. After 9 or 15 hours, cells were either frozen at -80°C for protein determination or scraped in to high potassium medium for the analysis of oxidative DNA damage. In addition, tissue culture medium was collected for the analysis of oestrogen metabolites.

Cellular protein was quantified spectrophotometrically using a commercially available fluorescamine dye. COMT activity was determined using a modification to a previously published method (Nissinen & Mannisto 1985). The level of oxidative DNA damage was analysed by measuring 8-hydroxy-2'-deoxyguanosine (8-oxo-dG) in cellular DNA. Frozen cells were lysed, their DNA extracted and subjected to enzymatic hydrolysis according to a modified method of Shigenaga et al (1994). Analysis of DNA digests was performed by HPLC and the results expressed as the ratio of 8-oxo-dG:dG x 10^6 calculated from peak areas based on calibration curves of 8-oxo-dG and dG standards. The concentrations of oestrogen and oestrogen metabolites in cell culture media were measured by HPLC, following treatment of samples with β-glucuronidase and solid phase extraction. Identification and quantification of metabolites was achieved by comparison with commercial standards (Steraloids Inc., Newport, RI, USA).

Results were statistically analysed by linear regression or a Student's t-test, with differences considered to be significant when $p<0.05$.

Findings: It was reported that treatment of MCF-7 cells with 10 μM Ro 41-0960 for 48 hours was not cytotoxic and inhibited COMT activity by 87 and 90% at 6 and 24 hours, respectively. The effect of inhibiting COMT activity with 10 μM Ro 41-0960 on the levels of oestrogen metabolites in TCDD-pretreated MCF-7 cells exposed to 1 μM E_2 for 15 hours is summarised in the Table below. It was reported that the level of oestrogen metabolism in control cells was limited, with 80% of the oestrogens recovered as E_2, 18% as E_1 and the <2% as other metabolites. In TCDD-pretreated cells, E_2, 2-OHE_2 and 4-OCH_3E_2 accounted for approximately 90% of the total recovered oestrogens. Inhibition of COMT activity with Ro 41-0960 approximately halved the level of E_2, while there was almost complete inhibition of 2-methoxyoestradiol formation and complete inhibition of 4-OCH_3E_2 formation. There was a marked increase in the level of 2-OHE_2. No statistical analysis was performed on these results.

Effect of COMT inhibition on the levels of oestrogen metabolites in MCF-7 cells

Oestrogen/metabolite	TCDD pretreated	TCDD pretreated + Ro 41-0960
E_2	191.4 ± 39.2 (28.6)	100.8 ± 20.1 (13.8)
2-OH E_2	225.3 ± 23.9 (33.6)	594.3 ± 46.2 (81.5)
4-OH E_2	11.0 ± 9.8 (1.6)	8.1 ± 2.4 (1.1)
2-MeO E_2	203.3 ± 23.1 (30.3)	2.4 ± 2.7 (0.3)
4-MeO E_2	20.7 ± 2.3 (3.1)	-
E_1	3.2 ± 1.4 (0.5)	5.7 ± 2.7 (0.8)
2-OH E_1	nd	nd
4-OH E_1	4.0 ± 1.0 (0.6)	14.3 ± 0.6 (2.0)
2-MeO E_1	10.2 ± 0.2 (0.5)	3.9 ± 3.2 (0.5)
4-MeO E_1	nd	nd

Results are expressed as the mean pmol/mg cell protein ± the range of 2 determinations. The % of the total recovered oestrogens is in parentheses; nd = not detected

Graphically-presented data illustrated the effect of COMT inhibition on E_2-induced oxidative damage as determined by 8-oxo-dG analysis. There was no significant effect on the level of oxidative DNA damage following treatment of cells with Ro 41-0960, TCDD, 10 µM E_2, TCDD + 10 µM E_2 or TCDD + Ro 41-0960. In contrast, inhibition of COMT with 3 µM Ro 41-0960 caused a significant ($p<0.001$) increase in the level of 8-oxo-dG when TCDD-pretreated cells were incubated with 1 or 10 µM E_2 for 9 hours. The level of increase relative to the control was approximately 3-fold and 5-fold at 1 and 10 µM E_2, respectively.

To further investigate the relationship between oxidative DNA damage and oestrogen metabolism, TCDD-pretreated cells were incubated with 3 µM Ro 41-0960 and 0.1, 0.3 or 1 µM E_2 for 9 hours. As shown in the Table below, there was a significant concentration-related increase ($p<0.001$) in the level of oxidative DNA damage, which was concomitant with an increase in 2-OHE_2. At a lower concentration of Ro 41-0960 (0.3 µM), treatment of cells with 1 µM E_2 resulted in significantly less ($p=0.012$) catechol oestrogen metabolites (2- and 4-OHE_2) and 8-oxo-dG than at 3.0 µM Ro 41-0960 (90.9 *versus* 158.2 pmol/mg protein and 56 ± 12 versus 116 ± 21 8-oxo-dG/106 dG, respectively).

Effect of COMT inhibition on the level of E_2-induced oxidative damage in MCF7 cells

Oestrogen	TCDD	TCDD + 0.1 µM E_2	TCDD + 0.3 µM E_2	TCDD + 1 µM E_2
E_2	-	129.6 ± 37.6 (79.9)	264.5 ± 38.4 (83.9)	694.8 ± 210.6 (79.7)
2-OH E_2	-	25. 7 ± 7.6 (15.9)	42.9 ± 5.9 (13.6)	152.3 ± 5.7 (17.5)
4-OH E_2	-	<1	<1	5.9 ± 1.8 (0.7)
2-MeO E_2	-	<1	<1	<1
4-MeO E_2	-	<1	<1	<1
8-oxo -dG/dG x 10^6	8 ± 1	36 ± 5*	70 ± 18*	116 ± 21*

Results are expressed as the mean pmol/mg cell protein ± 1 SD (n=3); values in parentheses represent the % of the total recovered oestrogens; * $p<0.001$ compared to the control

Conclusions: Inhibition of COMT with Ro 41-0960 caused an increase in oxidative DNA damage in TCDD-pretreated MCF7 cells that had been incubated with E_2. This appeared to be associated with an increase in 2-OHE_2. This finding suggested that COMT is protective against oxidative DNA damage caused by catechol oestrogen metabolites. In the absence of COMT inhibition, no oxidative DNA damage was observed (above background) at E_2 concentrations up to 10 µM.

Sasco AJ (2001) Epidemiology of breast cancer: an environmental disease. APMIS 109:321-32.

This review briefly covered the epidemiology of breast cancer and discussed some of the risk factors associated with its development including genetics and family history, reproductive life, diet, exercise and exposure to specific agents (eg. radiation, magnetic fields, chemicals, viruses, hormones and endocrine disruptors). An overview of the association between exposure to exogenous hormones and breast cancer was given. A number of IARC monographs were cited as evidence for the association between the use of combined oral contraceptives or post-menopausal oestrogen replacement therapy and a small increase in relative breast cancer risk. There was some speculation on the potential association between HGP residues in meat and increased breast cancer risk, but no evidence was cited to support this association. It was indicated that risk assessors had to rely on indirect evidence as no study would ever likely be available comparing exposed *versus* unexposed populations. The lower incidence of breast and other cancers in non-meat eaters was used as support for the

idea that the consumption of meat was somehow risky. The author suggested that increased knowledge of hormone receptors would be helpful (in assessing risk from hormones in meat) and cited a few studies relating to the genotoxicity of oestrogen metabolites (see Cavalieri et al 2000; Jefcoate et al 2000). It was concluded that a better understanding of the endocrine events involved in puberty and fertility is necessary in order to ascertain the role of hormone pathways in the occurrence and development of cancer. The author also advocated the enactment of the precautionary principle whenever possible to prevent exposures during specific periods of vulnerability. The value of this review was limited, as by the author's own admission it was not intended to be fully comprehensive but simply to highlight "potentially controversial conditions, which could in the future be recognised as new risk factors".

Terashima I, Suzuki N & Shibutani S (2001) Mutagenic properties of estrogen quinone-derived DNA adducts in simian kidney cells. Biochemistry 40: 166-172.

The SV40-transformed simian kidney cell line, COS-7 (State University of New York, Stony Brook, USA) containing the single stranded pMS2 plasmid vector that confers neomycin and ampicillin resistance, was transfected with circular ss DNA (100 fmol) containing a single DNA adduct, according to a previously established procedure (Moriya 1993). The adduct was either N^2-(2-hydroxyoestrogen-6-yl)-2'-deoxyguanosine (2-OHE-N^2-dG) or N^6-(2-hydroxyoestrogen-6-yl)-2'-deoxyadenosine (2-OHE-N^6-dA). Adducts had been prepared by reacting unmodified 15-mer oligodeoxynucleotides containing a single deoxyguanosine (dG) or deoxyadenosine (dA), with 2-OHE_1, 2-OHE_2 or 2-hydroxyoestriol (2-OHE_3). Controls were transfected with the unmodified 15-mer oligodeoxynucleotides containing a single dG or dA. Progeny plasmids were recovered according to the method of Hirt (1967) and used to transform *E. coli* DH10B cells. Transformants were analysed for mutations by oligodeoxynucleotide hybridisation using previously established methods (Inouye & Inouye 1987; Moriya et al 1988).

2-OHE-N^2-dG and 2-OHE-N^6-dA adducts reduced the transformation efficiency of COS-7 cells to 50-72% and 68-72% of the controls, respectively. The mutational specificity of 2-hydroxyoestrogen quinone-derived DNA adducts in COS-7 cells are summarised in the Table below. The 2-OHE-N^2-dG adducts produced only G → T mutations, with the E_2 adduct generating the highest level of mutants (18.2%). In contrast, the 2-OHE-N^6-dA adducts produced both A → T and A → G mutations, with the A → T mutations predominating. The E_1 and E_3 adducts generated more A → T mutations than the E_2 adduct, while the E_1 and E_2 adducts only generated the A → G mutations.

Mutational specificity of 2-OHE-N^2-dG and 2-OHE-N^6-dA adducts in COS cells

Lesion	Targeted mutations (%)			
	G	T	A	C
dG	100	0	0	0
2-OHE_1-N^2-dG	95.6 (94.5-96.7)	4.4 (3.3-5.5)	0	0
2-OHE_2-N^2-dG	81.8 (81-82.4)	18.2 (17.6-19)	0	0
2-OHE_3-N^2-dG	98.7 (98.5-98.8)	1.3 (1.2-1.5)	0	0
dA	0	0	100	0
2-OHE_1-N^6-dA	3.6 (3.3-4.3)	10.9 (7.3-17.1)	85.5 (78.6-89.4)	0
2-OHE_2-N^6-dA	2.7 (1.3-4.2)	3.3 (2.6-4.2)	94.0 (91.6-96.2)	0
2-OHE_3-N^6-dA	0	14.1 (14.1-14.2)	85.9 (85.8-85.9)	0

Results are the mean of 2 experiments using individual progeny plasmids, with the range given in parentheses.

In conclusion, the *in vitro* generation of DNA adducts by 2-hydroxyoestrogens were found to produce mutations following transfection of DNA containing the adducts into the SV40-transformed simian kidney cell line, COS-7. The mutational specificity and frequency was dependent on the type of 2-hydroxyoestrogen.

Tsutsui T, Tamura Y, Yagi E & Barrett JC (2000a) Involvement of genotoxic effects in the initiation of estrogen-induced cellular transformation: studies using syrian hamster embryo cells treated with 17b-estradiol and eight of its metabolites. Int J Cancer 86: 8-14.

Experimental: To examine the effect on cell growth, SHE cells in logarithmic growth phase were cultured overnight and then incubated with E_2, E_1, 2-OHE_1, 4-OHE_1, 2-OCH_3E_1, 16α-OHE_2, 2-OHE_2, 4-OHE_2 and oestriol (E_3) at 0, 0.3, 1, 3 or 10 µg/mL for 1, 2 or 3 days. All compounds had been obtained commercially from Sigma (St Louis, MO, USA), dissolved in DMSO to a concentration of 3 mg/mL and diluted as appropriate. Cells were trypsinised and counted by an unspecified method.

To examine the potential to induce cell transformation and somatic mutations, SHE were cultured overnight, and incubated with the above compounds at 0, 0.3, 1, 3 or 10 µg/mL for 48 hours. Bens[α]-pyrene (1 µg/mL) was used as a positive control in the mutation assay only. Cells were trypsinised, divided and replated. For the examination of morphological transformation, cells were incubated for 7 days to form colonies. Cells were fixed and stained, and the number of surviving colonies with >50 cells, and morphologically-transformed colonies, were scored according to the criteria of Barrett et al (1978). For the examination of somatic mutations, cells were cultured for 4 days, replated (n=10) in selection medium (18 µM 6-thioguanine or 1.1 mM ouabain), cultured for 7 days and the mutation frequency at the Na/K ATPase and *hprt* loci determined as previously described (Barrett et al 1978).

To examine the induction of chromosomal aberrations and perturbations in chromosome number, SHE cells were incubated overnight, then treated with the above compounds at 0, 0.3, 1, 3 or 10 µg/mL for 24, 48 or 72 hours. The 24 hour group was used for scoring chromosome aberrations, while the 48 and 72 hour groups were used for scoring chromosome number. Cells were treated with 0.2 µg/mL colcemid 3 hours prior to the end of treatment, and metaphase chromosomes prepared. One hundred metaphases/group were scored for the determination of chromosome aberrations and chromosome number.

Cell transformation, mutation and chromosomal aberration data were statistically analysed using a χ^2-test.

Findings: Graphically-presented data illustrated a dose-dependent decrease in cell growth following incubation of SHE cells with E_2, E_1 or their metabolites. Cytotoxicity, (evidenced as a decrease in cell number) occurred at 3 µg/mL 2- and 4-OHE_1, and 2-OHE_2, and at 10 µg/mL E_2, 2-OCH_3E_1 and 4-OHE_2. No cytotoxicity was evident following treatment with E_1, 16α-OHE_1 or E_3.

The results of the cell transformation assay are summarised in the Table below. With the exception of E_3, there was a significant concentration-related increase ($p<0.05$-0.01) in morphological transformation and a concomitant decrease in survival following incubation of cells with E_2, E_1 or their metabolites. Overall, 2- and 4-OHE_1 caused the greatest level of

transformation but only in the presence of frank cytotoxicity. The authors stated that transformed colonies were indistinguishable from those generated by treatment with bens[α]-pyrene or DES, although no data were provided to substantiate this statement. Overall, these findings were considered to be equivocal because no positive control compound was tested (eg. benzo[α]-pyrene); the level of transformation was small (most values were below the upper limit of spontaneous transformations of 0.6%) and only occurred at cytotoxic concentrations; data were statistically analysed using a χ^2-test rather than a 1-sided Fisher's Exact Test; and the absence of any transformed colonies in the control group meant that the occurrence of any transformants in the treatment groups could be seen as a positive result. This experiment would need to be repeated to confirm these findings.

Effect of E_2, E_1 and their metabolites on cell survival and cell transformation

Compound	**Concentration** (μg/mL)	**% cell survival**	**% cell transformation**[1]
Control (DMSO)	0	100	0
E_2	1, 3, 10	104, 90, 36	**0.15, 0.25, 0.48**
E_1	1, 3, 10	103, 108, 102	**0.12, 0.30, 0.33**
2-OHE_1	0.3, 1, 3	107, 95, 29	**0.12, 0.32, 0.67**
4-OHE_1	0.3, 1, 3	91, 65, 43	**0.25, 0.92, 1.42**
2-OCH_3E_1	1, 3, 10	79, 85, 8	**0.09, 0.12, 0.25**
16α-OHE_1	1, 3, 10	104, 113, 89	0.10, **0.13, 0.25**
2-OHE_2	0.3, 1, 3	101, 77, 26	*0.14*, **0.19, 0.32**
4-OHE_2	0.3, 1, 3	103, 95, 94	**0.20, 0.28, 0.35**
E_3	1, 3, 10	88, 98, 96	0.05, 0, 0.04

1 = no. transformed colonies ÷ total no colonies x 100; italicised values, $p<0.05$; bolded values, $p < 0.01$

There was a significant increase ($p<0.01$-0.05) in the frequency of mutations at the Na/K ATPase and/or *hprt* loci following treatment with 4-OHE_1, 2-OCH_3E_1, 2-OHE_2, 4-OHE_2 or benzo[α]-pyrene (the positive control) (see Table below). None of the other test compounds (including E_2) had an effect on mutation frequencies. Increases in the mutation frequency at the Na/K ATPase locus following treatment with 4-OHE_1, 2-OCH_3E_1 or 2-OHE_2 were discounted because they occurred at cytotoxic concentrations (4-OHE_1) or did not follow a dose-response relationship (2-OCH_3E_1 and 2-OHE_2). Only the effect of 4-OHE_2 on the Na/K ATPase locus followed a dose-response relationship, with the increase in mutants occurring at non-cytotoxic concentrations. With regard to the *hprt* locus, only 4-OHE_1 appeared to cause an increase in mutations to a level similar to the positive control, at both cytotoxic and non-cytotoxic concentrations. The incidental increase in *hprt* mutants at 3 μg/mL 2-OCH_3E_1 was not considered to be treatment-related due to the lack of a dose-response relationship.

Induction of mutations in the Na/K ATPase and *hprt* loci by oestrogen metabolites

Compound	**Concentration** (μg/mL)	**Mutation frequency**	
		Na/K ATPase	***hprt***
Control	0	3.3×10^{-7}	1.2×10^{-6}
E_2	1	$<3.0 \times 10^{-6}$	$<3.0 \times 10^{-6}$
	3	$<3.0 \times 10^{-6}$	$<3.0 \times 10^{-6}$
	10	$<3.0 \times 10^{-6}$	$<3.0 \times 10^{-6}$
4-OHE_1	0.3	$<2.0 \times 10^{-6}$	5.0×10^{-6}
	1	$<2.0 \times 10^{-6}$	2.5×10^{-5} **
	3	2.8×10^{-6} *	2.5×10^{-5} **
2-OCH_3E_1	1	5.6×10^{-6} **	1.7×10^{-6}
	3	$<4.0 \times 10^{-6}$	1.2×10^{-5} **
	10	2.1×10^{-5} **	5.0×10^{-6}

Compound	Concentration (μg/mL)	Mutation frequency	
		Na/K ATPase	*hprt*
2-OHE_2	0.3	1.9×10^{-6}	3.3 x 10-6
	1	5.6×10^{-6} **	$<4.0 \times 10^{-6}$
	3	$<4.0 \times 10^{-6}$	$<4.0 \times 10^{-6}$
4-OHE_2	0.3	$<3.0 \times 10^{-6}$	3.3×10^{-6}
	1	3.7×10^{-6} **	6.7×10^{-6}
	3	1.5×10^{-5} **	$<4.0 \times 10^{-6}$
benzo[α]-pyrene	1	3.6×10^{-5} **	2.8×10^{-5} **

* p<0.05; ** p<0.01

There was a concentration-related increase (p<0.01-0.05) in chromosomal aberrations in cells treated with E_1, 2-OHE_1, 4-OHE_1, 2-OHE_2 or 4-OHE_2, with only the highest concentrations tested significantly different to the control (p<0.05-0.01) (see Table below). None of the other test compounds (including E_2) induced chromosomal aberrations. Significant levels of aberrant metaphases induced by 2- and 4-OHE_1, and 2-OHE_2 only occurred at cytotoxic concentrations. The majority of aberrations were breaks or gaps (~80-96%), with lower levels of exchanges and dicentric chromosomes also scored. In the absence of any evidence that the occurrence of aberrations was a reproducible effect, and due to the lack of a positive control, these findings were considered to be equivocal.

Induction of chromosomal aberrations by oestrogen metabolites

Compound	Concentration (μg/mL)	% Aberrant metaphases
Control	0	1.0
E_2	1, 3, 10	2.0, 3.0, 1.0
E_1	1, 3, 10	1.0, 5.0, 10.0*
2-OHE_1	0.3, 1, 3	2.0, 5.0, 22.0**
4-OHE_1	0.3, 1, 3	4.0, 7.0, 36.0**
2-OHE_2	0.3, 1, 3	6.0, 7.0, 17.0**
4-OHE_2	0.3, 1, 3	2.0, 6.0, 21.0**

* p<0.05; ** p<0.01

With the exception of E_3, all test compounds induced aneuploidy (see Table below) evidenced as an increase in the % tetraploid and near tetraploid cells and/or an increase in near diploid cells. The occurrence of aneuploidy was concomitant with cytotoxicity. Those compounds that appeared to cause the greatest effect were E_2, 2-OHE_1, 2- and 4-OHE_2. In the absence of any evidence that the induction of aneuploidy was a reproducible effect and due to the lack of a positive control, these findings were considered to be equivocal.

Effect of oestrogen metabolites on the occurrence of aneuploidy

Compound	Concentration (μg/mL)	% Diploid cell	% Aneuploid cells	
			Tetraploid & near tetraploid	Near diploid
Control[1]	0	92	7	1
E_2[1]	1, 10	84, 45	6, 18*	10*, 37***
E_1[1]	1, 3, 10, 30	88, 91, 83, 79	8, 7, 10, 13	4, 2, 7, 8*
2-OHE_1[1]	0.3, 1, 3	89, 71, -	9, 25**, -	2, 4, -
2-OHE_1[2]	0.3, 1, 3	72, 67, -	21**, 25***, -	7, 8*, -
4-OHE_1[1]	0.3, 1, 3	87, 83, 74	10, 13, 22**	3, 4, 4
4-OHE_1[2]	0.3, 1, 3	82, 76, -	12, 18*, -	6, 6, -
2-OCH_3E_1[1]	1, 3, 10	94, 92, 45	4, 4, 28***	2, 4, 27***
16α-OHE_1[1]	1, 3, 10	92, 91, 87	6, 6, 5	2, 3, 8*
2-OHE_2	0.3, 1, 3	93, 68, -	4, 20*, -	3, 12**, -

Compound	Concentration (µg/mL)	% Diploid cell	% Aneuploid cells	
			Tetraploid & near tetraploid	Near diploid
4-OHE_2	0.3, 1, 3	91, 79, 72	8, 11, 18*	1, 10*, 10*

1= 48 h treatment time; 2 = 72 h treatment time; * $p<0.05$; ** $p<0.01$; *** $p<0.001$; - = few metaphases

Conclusions: Oestrogen metabolites varied in their abilities to inhibit cell growth, induce cell transformation and gene mutation, and cause chromosomal aberrations and aneuploidy. With the exception of 4-OHE_2, which caused a significant increase in mutations at the Na/K ATPase locus, E_2 and its metabolites were not mutagenic. While this study suggested that some metabolites of E_2 have genotoxic potential at high and/or cytotoxic concentrations, the findings should be considered as equivocal due to the absence of a positive control in the majority of experiments and any evidence that the findings were reproducible.

Tsutsui T, Tamura Y, Hagiwara M, Miyachi T, Hikiba H, Kubo C & Barrett JC (2000b) Induction of mammalian cell transformation and genotoxicity by 2-methoxyestradiol, an endogenous metabolite of estrogen. Carcinogenesis 21(4): 735-740.

Experimental: To examine the effect on cell growth, log-phase SHE cells were cultured overnight, then incubated with 2-OCH_3E_2 at 0, 0.1, 0.3 or 1 µg/mL for 24, 48 or 72 hours (n=4). The test compound had been obtained commercially from Sigma (St Louis, MO, USA), dissolved in DMSO to a concentration of 10 mM, and diluted as appropriate. Cells were trypsinised and counted by an unspecified method. To examine the effect on mitosis and the occurrence of multinucleation, SHE cells were cultured overnight then incubated with 0.1, 0.3 or 1 µg/mL 2-OCH_3E_2 or 20 µg/mL E_2 for 2, 4, 6, 8, 10, 12, 24 and 48 hours. Following trypsinisation, cells were prepared as previously described (Tsutsui et al 1990) and scored for mitotic index and multinucleation. Greater-than 1000 cells were scored for each treatment group.

The induction of cell transformation and somatic mutation at the Na/K ATPase and *hprt* loci were examined as described previously (see evaluation of Tsutsui et al 2000a), except that cells were incubated with 0, 0.03, 0.1, 0.3 or 1.0 µg/mL 2-OCH_3E_2 for 48 hours (n=20). Benzo[α]-pyrene (1 µg/mL) was used as the positive control. The ability of 2- OCH_3E_2 to induce chromosomal aberrations and to affect chromosome number were examined by culturing SHE cells overnight, followed by incubation with the test compound at 0, 0.3, 1, 3 or 10 µg/mL for 24 or 48 hours. No positive control was used. In some experiments, cells were co-treated with 1.0 µg/mL 2- OCH_3E_2 and 30 µM α–naphthoflavone (an inhibitor of 2-hydroxylase that converts 2-OCH_3E_2 to 2-OHE_2) or 1.0 µg/mL 2-OCH_3E_2 and 0.5 mM L-ascorbic acid for 24 hours.

Cells were treated with 0.2 µg/mL colcemid 3 hours prior to the end of treatment, and metaphase chromosomes prepared as previously described (Tsutsui et al 1983). One hundred metaphases/group were scored for the determination of chromosome aberrations and chromosome number.

Cell transformation, mutation and chromosomal aberration data were statistically analysed using a χ^2-test.

Findings: Graphically presented data illustrated that there was a concentration-related decrease in cell growth following treatment with 2- OCH_3E_2. Cell growth was decreased at 0.3 µg/mL, while 1.0 µg/mL 2- OCH_3E_2 was cytotoxic. Graphically presented data also

illustrated that there was a concentration- and time-related increase in the mitotic index and % of multinucleated cells following treatment with 2- OCH_3E_2. There was no effect on either parameter at the lowest concentration. At 0.3 and 1.0 µg/mL, the % of nucleated cells increased over 48 hours (maximums of approximately 40 and 90%, respectively). The mitotic index was elevated from 2 hours post-treatment, reaching a maximum at 8 hours (~10 and 45% at 0.3 and 1.0 µg/mL, respectively), and then declining to 0% over the remainder of the experimental period. Treatment of cells with 20 µg/mL E_2 resulted in a similar time-related increase in the mitotic index (maximum of approximately 20% at 8 hours) and the % of nucleated cells (maximum at 48 hours of approximately 70%).

There was a concentration-related increase in the level of morphological transformation, which was statistically significant at and above 0.1 µg/mL 2- OCH_3E_2 (see Table below). However, transformation was associated with frank cytotoxicity, evidenced as a decrease in cell survival. While the highest concentration of 2- OCH_3E_2 induced a significant increase in transformation that was approximately 15-fold higher than the positive control (benzo[α]-pyrene), this finding was considered to be equivocal due to the very high level of cytotoxicity and the fact that a total of only 29 colonies were scored (the minimum should be 1000). There was no justification given for the use of a χ^2-test test to analyse the results rather than a 1-sided Fishers Exact Test.

There was an incidental significant increase ($p<0.05$) in the mutation frequency at the Na/K ATPase locus following treatment with 0.1 µg/mL 2- OCH_3E_2, but in the absence of an effect at the next highest dose, this was unlikely to be treatment-related (see Table below). The mutation frequency at the *hprt* locus was significantly elevated at 0.3 µg/mL 2- OCH_3E_2, but given the occurrence of cytotoxicity at and above this concentration, the lack of any data at the next highest dose, and the fact that the mutation frequency was an order of magnitude lower than with benzo[α]-pyrene, this finding was considered to be equivocal.

Effect of 2-OCH_3E_2 on cell transformation and mutation in the Na/K ATPase and *hprt* loci

Compound	Concentration (µg/mL)	% Survival	% Transformation	Mutation frequency	
				Na/K ATPase	*hprt*
2-OCH_3E_2	0	100	0.01	$<7.0 \times 10^{-6}$	$<7.0 \times 10^{-6}$
	0.03	97.7	0.03	$<4.0 \times 10^{-6}$	$<4.0 \times 10^{-6}$
	0.1	81.9	0.14*	8.0×10^{-6}**	$<7.0 \times 10^{-6}$
	0.3	19.9	0.23**	8.0×10^{-7}	5.7×10^{-6}**
	1.0	0.3	3.45**	nd	nd
benzo[α]-pyrene	1	60.0	0.22**	3.4×10^{-5}**	2.5×10^{-5}**

* $p<0.05$; ** $p<0.001$; nd = not detected

There was a concentration-related increase in the % of aberrant metaphases and the induction of aneuploidy following treatment with 2-OCH_3E_2, with the effect at 0.3 and 1.0 µg/mL statistically different from the control ($p<0.05$-0.01) (see Table below). This effect was concomitant with cytotoxicity. The induction of chromosomal aberrations with 1 µg/mL 2-OCH_3E_2 was unaffected by co-treatment with 30 µM α-naphthoflavone but was increased with 0.5 mM L-ascorbic acid; this suggested that aberrations were probably attributable to 2-OCH_3E_2 rather than its quinone derivatives. The main type of aberration was chromosomal pulverisation, followed by breaks and exchanges. The apparent treatment-related effects on

the occurrence of aberrations and aneuploidy were only seen at cytotoxic concentrations. In the absence of a positive control, these findings should be viewed as equivocal.

Effect of 2-OCH_3E_2 on the occurrence of chromosomal aberrations and aneuploidy

Compound	Concentration (mg/mL)	% Aberrant metaphases	% Diploid cells	% Aneuploid cells	
				Near diploid	Tetraploid & near tetraploid
2-OCH_3E_2	0	1	95	0	5
	0.1	2	88	2	10
	0.3	10*	19	9**	72**
	1.0	32**	1	0	99**
α-naphthoflavone	30 µM	1	-	-	-
2-OCH_3E_2 α-naphthoflavone	1.0 30 µM	29**	-	-	-
L-ascorbic acid	0.5 mM	0	-	-	-
2-OCH_3E_2 L-ascorbic acid	1.0 0.5 mM	48**	-	-	-

* $p<0.05$; ** $p<0.01$

Conclusions: 2-OCH_3E_2 caused an initial elevation in the mitotic index of SHE cells, which was followed by decreased cell growth and increased multinucleation. Cell transformation, mutations at the *hprt* locus, chromosomal aberrations and aneuploidy were all induced by the test compound. However, due to the occurrence of frank cytotoxicity or the lack of a positive control, these findings were considered to be equivocal.

Yagi E, Barrett JC & Tsutsui T (2001) The ability of four catechol estrogens of 17b-oestradiol and oestrone to induce DNA adducts in Syrian hamster embryo fibroblasts. Carcinogenesis 22(9): 1505-1510.

Experimental: To determine the cytotoxicity of E_2, E_1, 2-OCH_3E_2, 4-OCH_3E_2, 2-OHE_1 and 4-OHE_1, SHE cells were cultured for 2 days and then incubated with 0, 1, 3 or 10 µg/mL of the test compounds for 6 hours. Cells were then trypsinised, replated (n=3) and incubated for 7 days. The numbers of surviving colonies (>50 cells) were scored following fixation and staining with Giesma. All test compounds had been obtained commercially from Sigma (St Louis, MO, USA), dissolved in DMSO at a concentration of 10 mg/mL and then diluted in tissue culture media as appropriate. No positive control was used. Data were statistically analysed using a Student's t-test.

The ability of the above test compounds to cause DNA adduct formation was examined by culturing SHE cells for 2 days, then incubating them with 0, 1, 3 or 10 µg/mL of the test compounds for 6 hours. DNA adducts were then analysed by the ^{32}P-post-labelling assay (Hayashi et al 1996; Reddy et al 1986). Benzo[α]-pyrene (1 µg/mL) was used as the positive control. Quantitative estimates of the number of adducts/10^8 nucleotides were determined by Cerenkov counting. It was stated that all experiments were performed 2-3 times.

Findings: Graphically-presented data showed that there was a significant concentration-related decrease in cell survival following treatment of SHE cells with 2-OCH_3E_2, 4-OCH_3E_2, 2-OCH_3E_1 or 4-OCH_3E_1, at and above 1 µg/mL. Cell survival was unaffected by treatment with E_2 or E_1 up to 10 µg/mL.

The results of DNA adduct formation following treatment with E_2, E_1 or their catechol metabolites, are summarised in the Table below. Examples of autoradiograms for each compound were provided by the authors to illustrate the occurrence of the 4 adduct spots. A single autoradiogram showed that a number of extra spots were formed following treatment with 1 µg/mL benzo[α]-pyrene, but no quantitative measurement of these spots was given. Of the catechol oestrogen metabolites, 4-OHE_1 caused the greatest level of adduct formation, followed by 2-OHE_1, 4-OHE_2 and 2-OHE_2. Neither E_2 nor E_1 caused adduct formation.

Number of DNA adducts/10^8 nucleotides formed in SHE cells following treatment with catechol oestrogen metabolites

Compound	Concentration (µg/mL)	Adduct spot				Total
		a	b	c	d	
E_2	10, 30	nd	nd	nd	nd	0, 0
E_1	10, 30	nd	nd	nd	nd	0, 0
2-OHE_2	10	0.31	nd	nd	nd	0.31
4-OHE_2	10	nd	0.56	nd	nd	0.56
2-OHE_1	0.3, 1, 3, 10	nd	nd	nd, nd, 0.32, nd	nd, nd, 1.95, 0.37	0, 0, 0.32, 2.32
4-OHE_1	0.3, 1, 3, 10	nd	nd	0.33, 0.38, 0.84, 3.52	0.62, 0.66, 0.70, 0.81	0.95, 1.04, 1.54, 4.33

nd = not detected

Conclusions: Catechol oestrogen metabolites caused DNA adducts in SHE cells at cytotoxic concentrations. The catechol metabolites of E_1 appeared to have a greater potential to generate adducts compared to those of E_2. Neither E_2 nor E_1 had any effect on cell survival or adduct formation.

Mutagenicity and genotoxicity of MGA

Kranz S, Pfeiffer E & Metzler M (2002) Formation of DNA adducts of melengestrol acetate in precision-cut rat liver slices. Naunyn-Schm Arch Pharmacol. 365(suppl 1): R140. ABSTRACT from the 43rd Spring Meeting of the German Society for Experimental and Clinical Pharmacology and Toxicology, Mainz, Germany, March 12-14, 2002.

Liver slices from male and female SD rats were incubated with MGA, megestrol acetate and other unspecified steroidal compounds with progestational activity, for 24 hours and then analysed for the formation of DNA adducts by ^{32}P-postlabelling. Cyproterone acetate and chlormadinone acetate were used as positive controls. All compounds were reported to give rise to the formation of DNA adducts, however, the relative levels of adducts and the pattern of adduct formation were dependent on the compound, the enrichment procedure and the sex of the animal. It was reported that total adduct levels induced by MGA increased by up to 50-fold using female animals but amounted to 20% or less of the levels obtained with cyproterone acetate. In the absence of adequate experimental detail and publication, this abstract was considered to have little value in assessing the genotoxicity of MGA.

ANNEX 2: LIST OF JECFA EVALUATIONS

JECFA Meeting Reports relating to the consideration of HGPs

Evaluation of certain food additives (Twenty-fifth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 669, 1981.

Evaluation of certain food additives and contaminants (Twenty-sixth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 683, 1982.

Evaluation of certain food additives and contaminants (Twenty-seventh report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 696, 1983.

Evaluation of certain veterinary drug residues in food (Thirty-second report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 763, 1988.

Evaluation of certain veterinary drug residues in food (Thirty-fourth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 788, 1989.

Evaluation of certain veterinary drug residues in food (Fifty-second report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 893, 2000.

Evaluation of certain veterinary drug residues in food (Fifty-fourth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 900, 2001.

JECFA toxicology monographs

Toxicological evaluation of certain veterinary drug residues in food. WHO Food Additives Series, No. 23. Cambridge University Press, 1988. Trenbolone acetate & zeranol.

Toxicological evaluation of certain veterinary drug residues in food. WHO Food Additives Series, No. 25, 1990. Trenbolone acetate.

Toxicological evaluation of certain veterinary drug residues in food. WHO Food Additive Series, No. 43, 2000. 17β-oestradiol, progesterone & testosterone.

Toxicological evaluation of certain veterinary drug residues in food. WHO Food Additives Series, No. 45, 2000. Melengestrol acetate.

JECFA residue monographs

Residues of some veterinary drugs in animals and foods. FAO Food and Nutrition paper, No. 41, 1988. 17β-oestradiol, progesterone, testosterone, melengestrol acetate, trenbolone acetate and zeranol.

Residues of some veterinary drugs in animals and foods. FAO Food and Nutrition Paper, No. 41/2, 1990. Trenbolone acetate.

Residues of some veterinary drugs in animals and foods. FAO Food and Nutrition Paper, No. 41/12, 2000. 17β-oestradiol, progesterone & testosterone

Residues of some veterinary drugs in animals and foods. FAO Food and Nutrition Paper, No. 41/13, 2000. Melengestrol acetate

ANNEX 3: PUBLIC HEALTH CONSIDERATIONS OF HGPS BY REGULATORY COMMITTEES IN AUSTRALIA

Australian public health standards for agricultural and veterinary chemicals that may enter the food chain include the Poisons Schedule, First Aid and Safety Directions, the human acceptable daily intake (ADI) and the Acute Reference Dose (ARfD). A further regulatory standard called the maximum residue level (MRL) is a legal upper limit for residues in unprocessed food (eg. grain, meat etc.) and is used as an indicator of good agricultural practice (GAP).

From the mid 1950s until 1992, Australian public health standards were set by committee process under the auspices of the NHMRC. 'Pesticide Tolerances' in food were first set in 1956 by the Food Additives Committee. Between 1962 and 1966, the Food Additives Committee maintained a Sub-Committee on Pesticides and Agricultural Chemical Residues in or on Foods (later re-named the Pesticide Residues in Food Sub-Committee), which adopted the then existing Canadian scheme as a basis for establishing tolerances. From 1967 onwards, Australian MRLs and ADIs for pesticides were established by the Pesticide and Agricultural Chemicals Committee (PACC), until the Department of Health and Ageing became directly responsible in November 1992. Responsibility for pesticide and veterinary chemical MRLs in food was transferred to the APVMA in June 1994, after which the PACC was removed from the control of the NHMRC and re-constituted as the Advisory Committee on Pesticides and Health (ACPH). The ACPH provides the Department of Health and Ageing, the TGA and the NRA with advice on issues of policy and practice having possible implications for public health and the proper use of chemicals in agriculture and elsewhere.

Poisons Schedules for agricultural and veterinary chemicals, drugs and some other hazardous substances are set by the National Drugs and Poisons Schedule Committee (NDPSC). Originally known as the Committee on Poisons Scheduling, the NDPSC was established in 1955 as a sub-committee of the NHMRC Public Health Committee. The NDPSC publishes its decisions in the Standard for the Uniform Scheduling of Drugs and Poisons, which recommends controls on availability, labelling, packaging and advertising. These are incorporated into and enforced by the various Australian State and Territory legislative systems. In 1994, the NDPSC was transferred from the NHMRC to the Australian Health Ministers' Advisory Council, and was re-constituted again in 1999 as a Statutory Committee of the Therapeutic Goods Administration.

A third committee formerly involved in chemicals management was the NHMRC Standing Committee on Toxicity (SCOT), which was active between 1985 and 1994. SCOT was responsible for providing specialised advice on complex toxicological matters to all the NHMRC Public Health Committee subordinate committees, including the PACC and NDPSC. In response to referrals from these committees, SCOT undertook evaluation of some drugs, pesticides, food additives, poisons, consumer products, chemicals and other hazardous substances relevant to public health.

A detailed history of the public health considerations of HGPs by regulatory committees in Australia is detailed below.

Month/Year	Regulatory Activity
9/69	PSC considered a submission from William Coopers & Nephews for the registration of zeranol. VPHC had recommended to PSC that zeranol should not be permitted for use as a growth promotant due to its oestrogenic activity and the absence of evidence on the undesirable effects of oestrogens. Registration of zeranol did not proceed.
5/74	PSC considered an application from Cooper Australia for to scheduling of zeranol for use as a growth promotant. PSC believed that zeranol should be exempt from scheduling on toxicity grounds and referred it to the VPHC for advice concerning its open sale and use.
8/74	PSC of the opinion that zeranol should be exempt from scheduling on toxicity grounds. PSC awaiting information from VPHC regarding advice for the open sale and use of zeranol.
11/74	NHMRC agreed that zeranol has low oestrogenic activity and would not be a public health hazard if used according to directions. Use should conform with FDA recommendations, which specify a withdrawal period of 65 days before slaughter. PSC agreed that it is necessary to develop an affective method of policing use, and because of restrictions that would need to be observed, it should be available on prescription only and classified under S4 of the Uniform Poisons Standard.
	PACC received an application from Cooper Australia Ltd for a MRL for zeranol. In view of the oestrogenic activity of zeranol, PACC did not recommend a MRL.
2/75	PACC concluded that zeranol residues were still detectable in carcases 65 days after application and were above the level that could be recommended, particularly in view of the compound being a suspected carcinogen. PACC was unable to set a MRL.
5/75	PACC noted the PHAC recommendation that the NHMRC, in light of further information received since October 1974, rescind its recommendation that zeranol would not be a public health hazard if used according to label directions.
	The NHMRC directed that approval of use be withdrawn and that zeranol not be included in the Uniform Poisons Standard. PSC agreed that approval be withdrawn but did not agree that zeranol be removed from the Uniform Poisons Standard. Zeranol was placed on the prohibited substance list.
8/75	PACC indicated that the use of any substance having oestrogenic activity for the purpose of promoting weight gain in livestock was contrary to existing State Legislation and to current recommendation of both the Australian Agricultural Council and the NHMRC.
11/76	PSC was requested to give further consideration to the scheduling of zeranol, and was informed that the Coordinating Committee in Agriculture was considering the matter. PSC agreed to defer the item until the Coordinating Committee in Agricultural Chemicals presented a submission.
2/77	PACC reviewed its position on zeranol and maintained that it is oestrogenic. PACC agreed not to make a recommendation until the NHMRC reviewed its policy on low potency oestrogens and similar substances with oestrogenic activity used in animal production.
11/77	PSC noted that the VPHC (November 1977) had again discussed the public health implications of the use of zeranol in livestock.
5/78	PSC noted an extract of the November 1977 meeting of the VPHC concerning zeranol and low potency oestrogens. The latest information was deferred for consideration until the August 1978 meeting.
8/78	PSC agreed to keep oestrogens under review for the next meeting.
11/78	PSC noted that zeranol had been discussed for almost 5 years and that a firm recommendation needed to be made to PHAC. PSC had considered that the toxicity of zeranol (rat acute oral LD_{50} = 40 g/kg bw) justified an exemption from scheduling. However, undesirable oestrogenic activity put the compound in the category of a hormone and as such was precluded from use by a recommendation from the 53rd Session of NHMRC (1962). PSC recommended that NHMRC deletes zeranol from the prohibited substance list and adds it to S4.
12/78	PACC considered a submission from the DPI for the establishment of an MRL for zeranol. A provisional MRL for zeranol in cattle meat of 0.02 mg/kg, with a 65-day withdrawal period was set.
5/79	PSC noted that PHAC accepted their recommendation that zeranol no longer be a prohibited substance and reclassified to S4.

5/79	PACC agreed to finalise the toxicological assessment and establish a NOEL for zeranol, even though PHAC had approved its use in cattle. PACC requested additional data from Wellcome Australia Ltd (4-year dog study, 4-year study in the rhesus monkey, oestrogen assay in female rhesus monkeys and a 3-year generation reproduction and teratology study).
8/79	PSC considered an application from Elanco for the scheduling of Compudose®, containing oestradiol-17ß. PSC recommended a S6 entry on toxicity grounds. PACC referred the issue of hormone use in animal production to the PHAC.
11/79	PSC rejected an application from Wellcome Australia to rescheduled zeranol from S4 to S6.
	PACC noted that PHAC had directed the question of hormone use for growth promotion be referred to the SCA for consideration.
2/80	Wellcome Australia Ltd provided the PACC with a 3-generation rat reproduction study on zeranol. The study failed to establish an effect level as the dose selection was too low to produce compound related effects.
3/80	PSC noted that the SCA had established a Working Party to review the use of hormones in cattle production.
5/80	PACC received a letter from Wellcome Australia Ltd commenting on the zeranol rat study, which had been reviewed at the February Meeting. The PACC reiterated its previous request for additional data (5/79) that the company had indicated was available.
	PACC considered an application from Wellcome Australia Ltd, for the establishment of a MRL for zeranol in sheep. Residue data was considered adequate and radiolabel studies indicated that the residue level was 4 ppb. A provisional MRL for zeranol in sheep meat was established at 0.02 mg/kg, with a 40-day withdrawal period.
5/80	PACC considered oestrogenic substances should be considered on their merits and that a blanket restriction on their use was unwarranted, taking into consideration the fact that residues resulting from the use of such products were not considered to pose a hazard to human health..
6/80	PSC consider a request from Wellcome Australia to reschedule zeranol from S4 to S6. The matter was, however, still under consideration by the NHMRC pending a review by the SCA on the use of hormones as growth promotants in animal production.
8/80	Additional data confirmed the oestrogenic activity of zeranol. PACC agreed that Wellcome should be requested to provide studies, which produced a clear cut NOEL for oestrogenic activity. PACC considered that this could be satisfactorily achieved in a 90-day rat feeding study. The provisional MRL in cattle meat of 0.02 mg/kg, with a 65-day withdrawal period, was confirmed.
11/80	PACC considered an application from Wellcome Australia Ltd's for the use of zeranol in goats. No recommendation was proposed until further toxicological data had been received.
5/81	PSC could not accommodate the request to consider rescheduling zeranol until PHAC had indicated a change of policy regarding the use of hormones as growth promotants
	PACC considered that further data was unlikely and therefore agreed to establish the NOEL for zeranol at 0.02 mg/kg/day based on a 6-month dog study and a 3-generation rat reproduction study. Applying a 100-fold safety factor, the ADI was established at 0.0002 mg/kg/day.
11/81	PSC noted that the PHAC had considered the use of oestrogenic substances for growth promotion in cattle. The EC had not come to a definite decision regarding 17β-oestradiol, progesterone, testosterone, trenbolone and zeranol, which made it difficult for the PHAC to recommend rescission of the NHMRC's policy not to allow the use of these substances for growth promotion in view of Australia's meat export trade with Europe. PHAC therefore agreed to defer a final decision pending resolution of the situation.
	PSC received a submission from Hoechst for the scheduling of trenbolone acetate when used alone or in conjunction with 17ß-oestradiol as a growth promotant in cattle. No NOEL had been demonstrated and therefore scheduling was deferred until the MRLs had been finalised by the PACC.
8/82	PSC revised the S6 entry for 17β-oestradiol by adding the word 'bovine' so that the entry reads "… for use as a growth promotant in bovine cattle", and amended the S6 zeranol entry to read "… in implants for use as a growth promotant in steer cattle".

8/82	PACC considered a submission from Elanco Products Company for the establishment of a MRL for 17β-oestradiol (Compudose®). It was recommended that implants be removed prior to slaughter to prevent them from inadvertantly being included in processed meat products. A new Appendix 2, Part 1 entry was recommended as follows: Oestradiol 17 beta – growth promotion implant in cattle subject to removal prior to slaughter.
11/82	PSC considered a submission from Syntex Australia for the clearance of Synovex®, (containing oestradiol + testoterone). PSC agreed that no Withholding Period or MRL were necessary due to the difficulty in distinguishing the level of hormones released from the implant from levels occurring naturally. PSC recommended that 17β-oestradiol be deleted from S6 and that the following new S6 entry be added: OESTRADIOL-17-BETA (a) in ear implants for growth promotion in bovine cattle (b) in combination with progesterone or testosterone in ear implants for growth promotion in bovine cattle.
	PSC considered an application from Wellcome Australia Ltd for the inclusion of testosterone cypionate in S6 when used as a growth promotant. PSC deferred a decision until information was received on the effect of testosterone cypionate on the background level of testosterone, and original data relating to the 21 day withholding period.
	PACC considered a request from Syntex Animal Health that MRLs be established for their products Synovex-S® (oestradiol benzoate + progesterone) and Synovex-H® (oestradiol benzoate + testosterone propionate), used as growth promotants in cattle. Residues were similar to naturally occurring levels in animals, were lower than those in humans and contributed less to the diet than foods such as milk and meat from untreated cattle. PACC recommended that the NHMRC adopt the following Appendix 2, Part 1 entry: Extend current entry for oestradiol benzoate, progesterone and testosterone propionate to include: "when implanted in the ear for growth promotion purposes in cattle".
11/82	PACC considered a submission from Elanco Products Company regarding the NHMRC's requirement for the silicon rubber implant of 17β-oestradiol to be removed prior to slaughter. Taking into account generally accepted slaughter house practices, the Committee agreed to reverse its decision and not require the implants removal. PACC recommended the current Appendix 2, Part 1 oestradiol –17 beta entry be deleted and replaced with "when implanted in the ear for growth promotion purposes in cattle."
2/83	PACC considered an application from Wellcome Australia Ltd seeking the establishment of a MRL and withholding period for testosterone cypionate when used as a growth promotant. A recommendation was not proposed until further information was received.
8/83	PACC considered a submission from Hoechst Australia Ltd seeking the establishment of MRLs for trenbolone acetate, and trenbolone acetate in combination with 17ß oestradiol, when used as growth promotants in cattle. PACC noted that no NOEL had been determined and that there was evidence that trenbolone might be a carcinogenic promoter - therefore the application was rejected.
11/83	Following receipt of a toxicologist's report, PSC recommended a new S6 entry as follows: trenbolone (a) in ear implants for growth promotion in bovine cattle; (b) in combination with progesterone or testosterone in ear implants for growth promotion in bovine cattle.
	PSC considered Wellcome Australia's application for an extension of the scheduled use of testosterone cypionate (BANROT) to cover its use as a growth promotant. PSC agreed that no recommendation could be made until previously requested information had been received from the company.
	PSC considered a scheduling request from Syntex Australia to include testosterone cyclopentylpropionate noting that testosterone cyclopentylpropionate was the chemical name for testosterone cypionate, and recommended that testosterone cypionate be included S6.
2/84	PSC recommended that the following amendments: the testosterone entry to include (b) in combination with oestradiol-17-beta or trenbolone in ear implants for growth promotion in bovine cattle; and the zeranol entry to read: ZERANOL in ear implants for use as a growth promotant in steer cattle.

2/84	NDPSC recommended a new S6 entry for trenbolone and amendments to the S6 entries for 17ß-oestradiol and progesterone as follows: TRENBOLONE (a) in ear implants for growth promotion in bovine cattle; (b) in combination with oestradiol-17-ß in ear implants for growth promotion in bovine cattle. OESTRADIOL-17-beta (a) in ear implants for growth promotion in bovine cattle; (b) in combination with progesterone, testosterone or trenbolone in ear implants for growth promotion in bovine cattle. PROGESTERONE (a) in a silicone rubber elastomer when used as a controlled-release implant for synchronisation of oestrus in cattle; (b) in combination with oestradiol-17-beta or trenbolone in ear implants for growth promotion in bovine cattle.
	Wellcome Australia Ltd provided the PACC with additional information relating to testosterone cypionate. It was agreed that studies should include actual values rather than just geometric means. As there was only data for sheep available, no recommendation for cattle could be proposed. PACSC agreed to recommend the addition to Appendix 2 Part I[26] as follows: Testosterone cypionate – for uses as a growth promotant for sheep when injected subcutaneously – for control of posthitis and balanitis in sheep
	PACC considered additional information from Hoechst Australia Ltd for the establishment of a MRL for trenbolone acetate. PACC agreed that clarification was needed on the overseas registration status, including registered uses, and relevant data should be provided to establish a NOEL for androgenic effects. PACC also noted the JECFA document on zeranol and that no carcinogenic effects were observed.
5/84	PSC recommended the following new S4 entry: ZERANOL except when included in S6 (ie. Zeranol in ear implants for use as a growth promotant in steer cattle).
8/84	PSC noted an EEC statement that the toxicological data for trenbolone and zeranol were inadequate, therefore recommending that those substances be prohibited for use in food producing animals. The PSC agreed that the toxicological basis of this statement needed to be determined prior to any further action.
	PACC received supplementary data from Hoechst Australia Ltd to assist in the establishment of a MRL for trenbolone acetate. A male monkey study was determined to be inadequate for the determination of a NOEL since changes in the seminal vesicles were observed at the lowest dose. PACC decided that provided adequately low doses are/were used, the male monkey study as provided or the female pig study as proposed, would be adequate for the determination of a NOEL.
2/85	In considering Wellcome Australia's application for an extension of the use of testosterone cypionate, PSC noted a further submission that provided details on the site of injection, the MRL proposed by PACC and an outline of the current field use. PSC decided the data was adequate to warrant the extension to the S6 entry to cover use of the compound as a growth promotant. The S6 testosterone entry was amended to include: (d) in oil preparations for growth promotion purposes labelled for injection at the base of the ear in sheep.
	PACSC considered additional zeranol toxicology data from Coopers Animal Health noting that a NOEL had not been demonstrated in any of the long term studies, and that zeranol was not mutagenic or teratogenic. A request for a nil withholding period was considered. The PACSC requested a detection method that would measure residues of zeranol down to 0.005mg/kg along with studies using this method, as well as a 2-year rat study demonstrating a NOEL based on oestrogenic effects. PACSC recommended that the MRL for cattle should remain the same but become provisional pending resolution of the above points (ie 0.02 mg/kg for cattle meat, with a 65-day withdrawal period).
5/86	PACSC noted that testosterone preparations were being directly or indirectly promoted for growth promotion purposes despite there being no NHMRC or TCVD recommendations for this use. Concern was expressed at the potential for such misuse to be detrimental to the public image of chemicals and in particular hormones. It was agreed that the matter be

[26] Appendix 2, part I covers substances which are exempted from the requirements of a maximum residue limit

	referred to TCVD for appropriate action to curtail such improper practices.
6/86	PACC agreed to defer further consideration of zeranol until November 1986 as the review of the data was not complete.
9/86	PACC considered additional data from Coopers Animal Health to support a nil withholding period for zeranol. PACSC noted that a NOEL for zeranol had not been demonstrated in an appropriate 2-year chronic study. On the basis of the data provided it was agreed to extend the provisional status for zeranol to November 1988 pending submission of the chronic rat study and clarification of the residue trials. The provisional MRL for sheep meat of 0.02 mg/kg, with a 40-day withdrawal period, was deleted as zeranol was not registered for use in sheep.
11/86	Hoescht Australia Ltd provided additional trenbolone acetate studies, including: 14-week pig study; a 2-generation rat study, a 1-generation rat study and overseas residue data. Discussion on the establishment of MRLs was deferred pending clarification of the apparent absence of a NOEL in a pig study.
2/87	PACC deferred further discussion on the establishment of MRLs for trenbolone acetate, pending clarification of the findings of a pig study. PACSC agreed that further consideration would require submission of an appropriate assay with an endpoint relevant to the hormonal effect of trenbolone acetate and demonstrating a true NOEL.
2/88	PACSC set the NOEL for zeranol at 0.015 mg/kg/d based on a 2-year rat study, and the ADI at 0.0002 mg/kg/d. PACSC amended and confirmed the MRL for zeranol in cattle meat at 0.005 mg/kg and in cattle offal at 0.02 mg/kg.
2/88	PACSC considered additional information on the hormonal effects of trenbolone acetate. It was noted that a true NOEL in a pig study using a hormonally mediated effect as a toxicological endpoint. In view of this, the Committee agreed that the following NELs and ADIs could be established: NOEL = 0.1 mg/kg/d for α-trenbolone and 0.001 mg/kg/d for β-trenbolone ADI = 0.0001 mg/kg/d for α-trenbolone and 0.00001 mg/kg/d for β-trenbolone PACSC requested that the company provide a 90-day study in an appropriate species with orally administered α-trenbolone as requested by JECFA and data on the effect of cooking on residues. From the available information PACSC determined that residues would not result in consumption of trenbolone acetate above the ADI. The following provisional MRLs were recommended, pending submission of the requested data. The residue is defined as α- + β-trenbolone. New entry: trenbolone acetate P0.001 cattle meat P0.005 edible offal of cattle
6/88	PACSC received a request from Upjohn Animal Health Division to establish an MRL for melengestrol acetate when used in cattle for indirect growth promotion. PACSC noted that there was a zero tolerance for melengestrol acetate in the US as it was a suspected carcinogen. PACSC rejected the submission because of toxicology and residue deficiencies.
8/88	SCA noted that a routine analytical method for zeranol and trenbolone to the MRLs as proposed (0.005 mg/kg) was not yet feasible. Given the EC position and the need for stringent monitoring, SCA had agreed that clearance be withheld until an appropriate analytical method had been developed. PACSC agreed that this matter would need to be discussed by the Public Health Committee.
	SCA informed PACSC that a routine analytical method for trenbolone to the MRL as proposed by PACSC (0.001 mg/kg) was not yet feasible. Clearance had been withheld by SCA and the matter referred to NHMRC for reconsideration.
11/88	PSC recommended the following: • the name osestradiol-17-beta in Schedule 6 be amended to oestradiol • amend paragraph (b) of the progesterone entry, and paragraph (c) of the testosterone entry to: in combination with oestradiol or trenbolone in ear implants for growth promotion in bovine cattle. • amend paragraph (b) of the trenbolone entry to read: in combination with oestradiol, progesterone or testosterone in ear implants for growth promotion in bovine cattle.
8/89	PACSC received additional MGA toxicology studies. PACSC reaffirmed its position that the product is unsuitable for the proposed use on the grounds that: the compound is readily absorbed; the compound is hormonally active; finite residues occur at levels that are likely to be hormonally active; and if modern contemporary studies were carried out it is likely that there would be hormonal effects at levels below the limit of detection. PACSC considered

	that it is not possible to correlate the finite residue levels with the toxicological and hormonal effects.
11/89	DPSC noted a company report of toxicological data in support of a provisional MRL and clearance of melengestrol acetate for use in cattle. DPSC noted that this would have a S4 classification.
	In February 1988 PACSC recommended MRLs for trenbolone and amendments to those for zeranol. PHC did not adopt these because the analytical method at the time was not capable of detecting residues at the level of the proposed MRLs and because of potential problems with overseas trade. PACSC requested DPIE to supply the methods of analysis used by AGAL.
2/90	PACSC considered the extension of the expiry date for the trenbolone acetate provisional MRL "P0.001 mg/kg cattle and P0.005mg/kg cattle, edible offal of". PACSC agreed to extend the provisional MRL to 31 December 1990.
6/91	PACSC endorsed the extension of the provisional MRLs for trenbolone acetate (P0.001 mg/kg cattle meat; P0.005 mg/kg cattle, edible offal of) to 31 December 1992.
8/91	PACSC considered the status of the PMRLs for trenbolone acetate in cattle meat and edible offal, but confirmed that it was not in a position to support clearance of any product without an analytical method that is capable of monitoring residue levels at the recommended provisional MRL.
11/91	PACSC considered Pitman-Moore's request for advice on the extent of residue data necessary to support a new 72 mg formulation of Ralgro® (zeranol) when used in feedlots for growth promotion in cattle. PACSC agreed that it was not possible to devote the time to providing replies on these matters.
	PACSC examined information on trenbolone acetate, including the radioimmunoassay for trenbolone residues in the muscle of cattle submitted by Hoechst in support of clearance of Finaplix® and Revalor®. Recognising the long standing difficulties that had been encountered in procuring valid regulatory analytical method, the Committee agreed that it would consider amending the proposed MRLs for trenbolone as follows: Trenbolone 0.002 Cattle meat (β-trenbolone) 0.01 Cattle, edible offal (α-trenbolone) PACSC considered that a valid analytical method to support such MRLs should be made available, taking into account the above methods of analysis. Due to the absence of a valid regulatory method of analysis, PACSC was unable to support the final clearance of trenbolone containing products.
2/92	PACSC considered the AGAL NDRC/analytical Method No. 168 "Determination of trenbolone in bovine liver by HPLC" provided to support clearances of Finaplix® and Revalor®. PACSC confirmed the existing MRLs for trenbolone acetate and agreed that it was not necessary to differentiate between α-trenbolone and β-trenbolone in the MRL Standard. MM 0812 0.001 Cattle meat MO 0812 0.005 Cattle, edible offal of PACSC also confirmed the withholding period of 9 weeks. Residue definition: sum of trenbolone acetate and 17 alpha- and 17 beta-trenbolone, both free and conjugated, expressed as trenbolone.
	PSC considered a request from the Veterinary Chemicals Advisory Committee to delete from the S6 entry for testosterone the paragraphs relating to "teaser rams" and "growth promotion purposes". The committee agreed to the proposal.
5/92	PSC noted that no comments were received in response to its pre-meeting gazettal notice to delete the paragraphs from the S6 entry for testosterone entry relating to "teaser rams" and "growth promotion purposes". PSC proposed the recommendation be forwarded to PHC for ratification.
8/92	PACSC noted that in February 1992 they confirmed the existing MRLs for trenbolone acetate on the basis of AGAL NRDC Method No 168 and that this is a proprietary method that is not generally available. Therefore PACSC decided that such a method would be unacceptable as a regulatory method for this reason and recommended to delete the previous entry and add: MO 1281 0.01 Cattle, Edible offal or MM 0812 0.002 Cattle meat
11/93	PACSC considered an application from Roussel Uclaf Australia Pty Ltd seeking clearance

	of Revalor-H® (trenbolone acetate) for improved growth promotion and finishing of heifers. PACSC noted: that the product contains 17β-oestradiol which is covered by the current Table 5 entry; considered that the proposed restraint statements were appropriate; and could not support the sponsor's argument for a nil withholding period as there is no data earlier than the first analysis point (15 days). PACSC agreed to the clearance of the product and confirmed the MRL for trenbolone recommended in August 1992.
2/96	NDPSC considered correspondence relating to the misuse of veterinary injectable testosterone by humans for body building purposes. They considered that as the growth promotant scheme was not associated with human abuse and was a trade issue, regulatory mechanisms employed in situations of human abuse of drugs were more appropriate and responsive to the public health program. In addition to this, members noted information received on the alternative pellet form, where the location of the implant in the head area would not impact on the quality of the carcase following slaughter. NDPSC recommended the S6 entry be amended to: TESTOSTERONE in implant preparations for use in animals.
5/96	Elanco Animal Health requested the NDPSC to consider rescheduling of its controlled release ear implant products, Compudose 100, Compudose 200 and Compudose 400, containing 12 mg, 24 mg and 45 mg respectively of oestradiol. NDPSC considered that on the toxicological information provided, the rescheduling of oestradiol when the only active ingredient in an ear implant preparation for growth promotion in bovine cattle from S6 to S5 was appropriate. NDPSC also noted that testosterone, progesterone and trenbolone, either in combination or alone, were also included in S6 in ear implants for growth promotion. The NRA member agreed to ascertain the impact on the monitoring of the products by the NRA if the proposed S5 classification were to proceed for ear implant preparations containing oestradiol, progesterone, trenbolone and testosterone.
	NDPSC agreed to amend the following testosterone entries: • S4 entry to read: #TESTOSTERONE **except** when included in Schedule 6. • S6 entry to read: TESTOSTERONE in implant preparations for use in animals. • Appendix D, paragraph 5 by adding to the list of poisons: TESTOSTERONE:
8/96	NDPSC considered it was appropriate to reschedule implant preparations containing oestradiol, trenbolone and progesterone from S6 to S5 because they did not present a serious poisoning hazard normally associated with a S6 product if accidentally ingested by humans. S4 amendments were made to the oestradiol, progesterone and trenbolone (trenbolone, trienolone) entries to read: **except** when included in Schedule 5. New S5 entries: OESTRADIOL in implant preparations for growth promotion in animals. PROGESTERONE (a) in implant preparations or controlled release pessaries for synchronisation of oestrus in cattle, sheep or goats; or (b) in implant preparations for growth promotion in cattle. TRENBOLONE in implant preparations for growth promotion in animals. S6 amendments: Oestradiol, progesterone and trenbolone were deleted
	NDPSC considered a response from the NRA expressing concern that the NDPSC had not rescheduled to S4 pelleted formulations of testosterone at the same time as the liquid injectable formulations. Due to the suggestion that pellet formulations were being abused, NDPSC agreed that further information should be sought from the NRA and the Ministerial Council on Drugs and Safety to ascertain whether further consideration needed to be given the rescheduling of formulations.
5/97	NDPSC considered resheduling testoterone pellets from S6 to S4 due to the potential for abuse of such products, but in the absence of any proven evidence of such abuse, this did not proceed.
11/99	NDPSC considered the evaluation of toxicological data associated with an application by Pharmacia & Upjohn Pty Ltd to register the product MGA Liquid Premix containing 1.14 g/L of melengestrol acetate. NDPSC noted that the product is a feed additive intended for use in heifers to increase weight gain, improve feed efficiency and suppress oestrus. Based on the toxicological profile of melengestrol acetate, NDPSC decided on a new S6 entry 'MELENGESTROL ACETATE when used as an animal feed additive', and a new S4 entry 'MELENGESTROL **except** when included in Schedule 6'.
5/01	ACPH concluded that there is no appreciable health risk to consumers from eating meat

	from animals treated with HGPs. A formal review of HGPs was considered unnecessary, however international scientific assessments and developments should be monitored.
10/01	ACPH reaffirmed its previous opinions relating to the safety of HGPs and amended its previous conclusion to '… registered HGPs according to good veterinary practice'.
5/02	ACPH supported the continuing review of HGPs and proposed that TGA evaluate 17 recent EC studies. ACPH indicated that Australian regulatory agenicies examine their collective position to ensure the continuing registration of HGPs is defensible.
10/02	ACPH was satisified that the human safety of HGPs was continuing to be addressed by the relevant government agencies and that the TGA was progressing the scoping study

VPHC - Veterinary Public Health Committee; PSC – Poisons Schedule Committee; TCVD - Technical Committee on Veterinary Drugs; NHMRC – National Health and Medical Research Council; PACC – Pesticides and Agricultural Chemicals Committee; PACSC – Pesticides and Agricultural Chemicals Sub Committee; DPSC – Drugs and Poisons Schedule Committee; NDPSC – National Drugs and Poisons Schedule Committee; SCA – Standing Committee on Agriculture; PHAC – Public Health Advisory Committee; ACPH – Advisory Committee on Pesticides and Health

ANNEX 4: MINUTES OF THE ADVISORY COMMITTEE ON PESTICIDES AND HEALTH RELATING TO THE HEALTH ASSESSMENT OF HGPS

ACPH 21 (2nd May 2001)

5.1 Hormone Growth Promotants – European Union (EU) Assessment

PURPOSE

The committee considered and discussed the outcomes and implications of the recent EU report on the potential risk to human health from hormone residues in bovine meat and meat products, in the light of how hormone growth promotants (HGPs) are regulated in Australia.

BACKGROUND

HGPs are used to increase the growth, feed efficiency and carcass leanness of cattle, and are typically administered via subcutaneous implants under the ear of the animal. They include naturally-occurring compounds such as 17-β-oestradiol, progesterone and testosterone, and synthetic compounds like zeranol, trenbolone acetate and melengestrol acetate.

The human safety of the above HGPs has been evaluated by the Codex Alimentarius Commission Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 1981, 1983, 1988 and 1999 and 2000. JECFA has set ADIs for all of these HGPs while the Australian TGA has set ADIs only for zeranol and melenestrol acetate (see Table 1). JECFA and the NRA have both set MRLs for only 2 HGPs, zeranol and trenbolone acetate (see Table 2).

Table 1: ADIs for hormone growth promotants (mg/kg bw/d)

Compound	TGA	JECFA
17β-oestradiol	-	0.00005
Progesterone	-	0.03
Testosterone	-	0.002
Zeranol	0.0002	0.0005
Trenbolone acetate	-	0.00002
Melenestrol acetate	0.00005	0.00003

Table 2: MRLs for hormone growth promotants (mg/kg)

Compound	NRA	JECFA
17β-oestradiol	-	#
Progesterone	-	#
Testosterone	-	#
Zeranol	0.005 cattle meat 0.02 edible cattle offal	0.002 meat 0.01 liver
Trenbolone acetate	0.002 cattle meat 0.01 edible cattle offal	0.002 meat 0.01 liver
Melenestrol acetate	-	-

residues generated according to good husbandry practices were considered unlikely to adversely effect human health

In 1989, the EU prohibited the use of HGPs on farm animals and banned the importation of beef and beef products from countries which allowed the use of growth promotants. Following a protest by the US and Canada, The World Trade Organisation (WTO) ruled against the EU ban in 1997 asserting that it was not based on scientific evidence or a proper risk assessment. The EU was allowed 15 months to conduct a risk assessment of hormone-treated meat. The subsequent EU risk assessment on human residues in bovine meat and meat products [*The opinion of the scientific committee on veterinary measures relating to public health (SCVPH): Assessment of potential risks to human health from hormone residues of bovine meat and eat products; 30th April 1999*] arrived at the following conclusions:

- There is a substantial body of evidence suggesting that 17β-oestradiol is a complete carcinogen (tumour initiator and promoter) but the available data does not allow a quantitative risk estimate;
- Data pertaining to the 5 other growth-promoting hormones (progesterone, testosterone, zeranol, trenbolone acetate & melengestrol acetate) does not allow a quantitative risk assessment;
- Potential developmental, immunobiological, neurobiological, immunotoxic, genotoxic and carcinogenic effects can be envisaged for all 6 hormones, with prepubertal children considered to be a high risk group, however the available data do not allow a quantitative risk assessment;
- In view of the intrinsic properties of the hormones, and taking into account epidemiological findings, no threshold levels or ADIs can be established for all 6 hormones.

Following the SCVPH report, the EU moved to impose a definite ban on the use of 17β-oestradiol and its ester-like derivatives in farm animals except for therapeutic purposes. A provisional ban was also placed on progesterone, testosterone, zeranol, trenbolone acetate and melengestrol acetate pending further scientific evidence.

DISCUSSION

From the onset, the Committee recognised the complexity and potentially contentious nature of the issue and consequently the discussion was varied and extensive, with several options put forward for how the Committee could proceed in addressing HGPs. The polarisation in scientific and regulatory opinion that exists regarding the safety of HGPs was appreciated, however there was little support for the EU position either on the basis of their scientific assessment or the resulting risk management procedures. The Committee was generally critical of the scientific basis of the report and also appreciated the inherent difficulty in making an assessment of any incremental risk to humans. The Committee noted that hormones such as 17β-oestradiol, progesterone and testosterone are naturally occurring constituents of the diet (eg meat, milk, soy-based products), are endogenously produced in quite significant amounts, and that the levels in treated and untreated animals overlap (ie there is a large variation in natural hormone levels).

The Committee appreciated that, by their very nature, hormones interact with the genetic apparatus and induce cell proliferation and growth. A hormonal substance could therefore promote carcinogenicity in hormone-sensitive tissues through such a proliferative mechanism. However, it was noted that the EU risk assessment was significantly focussed on the conclusion that 17β-oestradiol acts, and was so defined, as a genotoxic carcinogen. This EU assessment contrasts with the position of JECFA who have used the term genotoxic

potential, a term which is supported by the International Agency for Research on Cancer (IARC).

Among concerns raised in the debate over possible impacts of HGPs on human cancer incidence, is the increased incidence of hormone-related cancers, such as breast cancer, in certain countries. The EU assessment included consideration that increasing affluence may be contributing to this rising incidence through an increase in meat consumption, with reduced consumption of vegetables. However, the EU assessment did not consider alternative factors, such as the increased incidence of certain tumours being related to total calorific intake and the release of insulin and insulin-like growth factors that in turn interact with an individuals genetic constitution, hormone levels and hormone receptors.

The Committee reiterated a view that HGP residues in meat from treated animals would make a fractionally small contribution to overall hormone intake or exposure from natural sources. Furthermore, both natural hormone production and dietary intake are subject to wide fluctuations. Previous assessments had therefore concluded that incremental risk from HGPs would be extremely small.

A new issue to arise from the EU assessment from a scientific perspective is that while HGP exposure may be quite small, the excess or incremental risk associated with carcinogenicity cannot be addressed because of the new concept that such hormonal substances may initiate carcinogenesis via a genotoxic mechanism. Due to the insufficient information available to make an assessment of that incremental risk, the EU had adopted the 'precautionary principle' which is not accepted as an international standard. The EU also considered social, economic and political factors in their risk assessment.

The Committee was informed that the outcomes of the European human risk assessment were probably influenced by political pressure from lobby groups who seek to maintain high levels of subsidies which are afforded to the European farming community. Consumer organisations and other interested parties also applied emotive pressure to the debate and the EU may have contributed to the polarisation of the assessment by funding groups which were essentially supporters of the EU conclusions.

The Committee noted that the selective and incorrect citing of scientific literature was a major deficiency of the EU's evaluation. Various international experts that are aware of all the relevant scientific literature have indicated that some issues used by the EU to substantiate their conclusions have largely been discounted by the scientific community. The Committee appreciated the difficulty in interpreting the EU's position, and the scientific data generally, without enlisting the input of experts with an intimate knowledge of the mode of action of HGPs and possessing expertise in genotoxicity. This was the approach employed by JECFA, whose experts were able to critically analyse the data and give an appropriate weighting to the findings.

The Committee was mindful of a variety of other criticisms of the EU assessment. The human health aspects of the EU report, particularly the epidemiology, did not present a critical view of the data. For example, a Puerto Rico study cited in the assessment did not adequately investigate other possible causes for the effects which were attributed to hormones, and its findings have been largely discredited.

Another problem with the EU assessment was its failure to acknowledge the potential impact of the black market trade in growth promoting agents, including the illegal use of diethylstilbestrol (DES). The Committee was advised that many of the issues referred to in the EU documents were associated with the alleged illegal use of DES.

A Member reported that a residue chemist from the German Federal Institute for Health Protection of Consumers and Veterinary Medicine (BgVV) has shown that for some dietary intake in Germans, the consumption of meat from animals that have been castrated and treated with small amounts of hormones to induce growth, is lower than in those people who have consumed 'normal' animals that have matured under the influence of natural hormones originating from their reproductive tissues.

The conclusion of the EU that no ADI can be set for any of the six HGPs when they are administered to bovine animals for growth promotion purposes was criticised as it suggested that uses are considered when deriving an ADI. The Committee was unaware of this practice but noted that this would discredit the EU's process if uses were taken into consideration in their hazard assessment.

The Committee was advised that regardless of the scientific evidence, internationally there are emerging concerns regarding HGPs and these need to be appropriately addressed. Within Australia HGPs are largely a trade issue and there already exists mechanisms to control their distribution and use in meat production such as individually identifying each animal (eg details of ownership, origins and treatment). The EU, as a consequence of its policy on HGPs, audits suppliers such as Australia, Canada and New Zealand. Australia invests large sums of money into programs designed to accommodate this relatively small European market. The Committee was informed that representatives from the EU visited Australia in 2000 to audit the meat and milk industry. The European representatives also intended to audit the whole veterinary registration system which was not divulged prior to their arrival. They had problems in understanding the manner in which Australia regulates agricultural and veterinary chemicals given the involvement of 8 states and the Commonwealth in the process. Superimposed onto this is the fact that most veterinary chemicals in the EU are only obtained by prescription, whereas in Australia HGPs are open sellers that are strictly controlled at the point of the wholesaler. Due to regular national and international inquiries relating to Australia's position on 17β-oestradiol, it was suggested that that it be put under review or that Australian formulates a health position on it. The benefit of the first option would be that it could indicate to Europe that Australia is being pro-active in this area. The Committee also recognised that 17β-oestradiol is used therapeutically to rectify reproductive conditions in animals and that synthetic oestrogens are widely used in Australia in humans for therapeutic purposes.

The Committee heard that although the focus has been primarily on meat as a source of hormone intake, a paper by Setchell et al (1997)[27] published in *the Lancet*, illustrated soy-based infant formulas can be a significant source of oestrogenic substance intake in infants. The British Department of Health gave formal advice in 1996 regarding soy-based infant formula because some children that consumed high levels showed adverse effects. The EU risk assessment of meat-derived HGP intake did not take into account these other significant potential sources of hormonal exposure. The Committee considered that exposure of pre-

[27] Setchell KDR, Zimmer-Nechemias L, Cai J & Heubi JE (1997) Exposure of infants to phyto-oestrogens from soy-based infant formula. Lancet. **350**: 23-27.

pubertal children to phyto-oestrogens via a diet high in soy-based commodities and milk was likely to have a greater health impact than the consumption of meat from HGP-treated animals.

The Committee was advised that neither the NHMRC, The Commonwealth Department of Health or ANZFA had investigated the human health aspects of soy-based commodities. The Committee appreciated the political difficulties in addressing the issue of soy as the same consumer organisations that are anti-HGPs are pro-soy. The Committee was reminded of a preliminary conference report that was considered several meetings ago which reported that the penises of males that had been fed high levels of soy were shorter than normal children, a finding which was largely ignored by the press. The effect of phytoestrogens on the WA sheep industry was also raised as it almost wiped-out reproduction in sheep grazing on subterranean clover where it rendered the ewes sterile and caused the wethers to grow teats. The Committee was aware that extracts of red clover are sold as dietary supplements to treat menopausal symptoms in women, illustrating that these dietary hormones are biologically active.

The Committee discussed a variety of options for dealing with HGPs. A suggestion was made that all HGPs be put under review because although the focus is on 17β-oestradiol there are questions being raised about the other natural and synthetic HGPs. The Committee was aware that no new data relating to zeranol or trenbolone had arisen and therefore there was no reason for it to alters its opinions of these two HGPs from a registration perspective. A suggestion was made for the Committee to formally look at additional contemporary studies pertaining to trenbolone and zeranol but not melengesterol acetate, because of its potent oral activity and some potential problems with the toxicological data. The Committee was cautioned about this approach because the only mechanism to access those additional studies would be to call them in through the NRAs review program, irrespective of zeranol and trenbolone being off patent.

The Committee considered that as no new data from sponsors were available for any HGPs, a formal recommendation that they be reviewed would be of little consequence. A better option would be to adopt a watching brief, given the changing attitudes of the European press, developments occurring internationally and the amount of information arising from scientific assessments elsewhere. The Committee considered this to be suitably pro-active and that it should address any concerns that the issues are not being treated seriously in Australia. For this reason it was agreed that the ACPH leave HGPs as a continuing item on its agenda.

A representative from ANZFA suggested that the Committee undertake a brief critique from an Australian perspective of new major scientific opinions and work which could be used as a tool to support the watching brief that the Committee has set. Specific reference was made to the five EU conclusions and the importance of the Committee's views of each of these.

The Committee came to the conclusion that at this stage a formal review of HGPs would not be recommended but that it continue to monitor the scientific evaluations done at an international level and to monitor the international position, that it recognises that in the interim period or as an ongoing basis that there will need to be trade-related maintenance of the monitoring programs that are put in place because of the impact of the EU on trade. The Committee also recommended the development of a brief position paper based on the currently available information but appreciated that this option has resource implications which will need to be discussed and resolved between the TGA and NRA.

RESOLUTION NO 21/1

The Committee:

- AFFIRMED a view that that there is no appreciable health risk to consumers from eating meat from animals treated with HGPs;
- CONCLUDED that a formal review of HGPs is unnecessary at this stage, but that this issue be adopted as a continuing agenda item so as to monitor international scientific assessments and developments;

- RECOGNISED that in the interim period, or on an ongoing basis, that there will need to be trade-related maintenance of the current monitoring programs because of the impact of the EU on trade;

- SUPPORTED the generation of a brief position paper based on the minutes of this meeting but APPRECIATED that this option has resource implications which will need to be discussed and resolved between the TGA and NRA.

ACPH 22, 18th October 2001

5.1 Hormone Growth Promotants

PURPOSE

The Committee considered developments relating to the scientific assessment of hormone growth promotants (HGPs).

BACKGROUND

At the previous Meeting of the ACPH (2nd May 2001), the Committee:

- Affirmed a view that that there is no appreciable health risk to consumers from eating meat from animals treated with HGPs;

- Concluded that a formal review of HGPs is unnecessary at this stage, but that this issue be adopted as a continuing agenda item so as to monitor international scientific assessments and developments;

- Recognised that in the interim period, or on an ongoing basis, that there will need to be trade-related maintenance of the current monitoring programs because of the impact of the EU on trade;

- Supported the generation of a brief position paper based on the minutes of this meeting but appreciated that this option has resource implications which will need to be discussed and resolved between the TGA and NRA.

DISCUSSION

In compliance with the Committee's previous resolution to monitor international scientific assessments and developments, a variety of recent publications pertaining to HGPs were noted by Members. These included EC reports relating to inspections of Australia in 1999 and 2000 by European auditors, a number of scientific publications on residue levels associated with the use of HGPs in cattle, comparative data on oestrogenic potencies of some of the naturally-occurring HGPs, general reviews on the biochemistry and physiology of the anabolic hormones, and a review on the genotoxic potential of HGPs and their metabolites. A number of weaknesses were noted in the scientific publications and Members agreed on the importance of critically evaluating such data since it is likely to be cited irrespective of its quality. The main conclusion which could be drawn from these publications is that more work needs to be undertaken. The Committee thanked the secretariat for bringing these publications to their attention and requested that papers continue to be provided to Members.

The Committee was informed that following discussion of the resource implications of the previous proposal to draft a position paper on HGPs, formal approval has been given by the NRA to develop such a paper. The Committee re-affirmed its support for this position paper and noted that staff from the CRIH section of the TGA will table a draft paper at the 23rd meeting. This paper will be based on the minutes of the 21st meeting and will aim to encapsulate the various issues that drive the debate internationally. The paper would also distil many of the scientific developments that have and are occurring internationally and would help to focus the advice of ACPH and serve as resource if the TGA/DHAC were pressed from external sources to explain Australia's position, noting that ACPH advice does not currently support the position taken by the EU.

There was some discussion regarding the content of the position paper. Members were informed that a detailed critique of the European position would not be undertaken given the scope of such a task and that the majority of data has already been reviewed at the international level. It was suggested that the paper should address non-HGP uses of hormones such as 17β-oestradiol, as the EU audit of Australia focussed on the fact that 17β-oestradiol was not just being used as a HGP. The Committee anticipated that it may be asked in the future to defend the continuing use of 17β-oestradiol in growth promoting agents because IARC have concluded that it is a genotoxic carcinogen. The Committee was advised that Australia gives appropriate consideration to IARC reviews, but notes that IARC classifications are not intended to be used as recommendations for any specific regulatory action.

The Committee noted that Agriculture, Fisheries and Forestry Australia (AFFA) had expressed concern about the potential for the position paper to draw attention to the issue. It was suggested that this concern might be alleviated by structuring the paper as a summary of the issues and highlighting the main points in the debate without necessarily coming to a conclusion. The Committee was advised that AFFA had assured the EU auditors that the current control measures provide sufficient guarantee that beef exported to Europe is free from HGPs. Members considered that a position paper would strengthen AFFA's position and suggested that it be developed collaboratively. By keeping the issue constantly under review the ACPH could monitor emerging information and be in a position to advise government on any needed changes to policy on HGPs. If concerns were raised in relation to a particular member of the HGP, the TGA would be able to advise the NRA to include the substance in its review program.

A number of other points relating to HGPs were raised and/or discussed:

- The Committee was reminded of a British study indicating that children raised on large amounts of soy-based products can be at risk of adverse health effects. Although many of these plant-derived oestrogenic compounds have potent biological activities, humans are regularly exposed to fluctuating, sometimes high levels of naturally occurring hormones. The potential for these naturally-occurring substances to elicit hormonal effects in humans is likely to be greater than any potential effect of HGP residues received via the ingestion of meat.
- Humans have also been exposed to low levels of compounds like zeranol for thousands of years given its natural occurrence in a fungus which grows on a number of plant products. The Committee was informed that a substantial amount of work had been undertaken in Queensland where a survey of various grains had detected fungal-related compounds including zeranol. It was suggested that the intake of zeranol and zeranol-like compounds centuries ago would have been much greater than that occurring now given that society has improved its storage and handling capacity.
- Overseas, there appears to be a preoccupation with the misuse of both existing and registered products, combinations of registered products that were never intended to be combined, and also the use of unregistered agents or those that have been withdrawn from the market such as diethylstilboestrol (DES). It is sometimes the perception of consumers that unregistered products are used widely, or that unapproved doses and combinations are used. This perception has periodically caused regulatory bodies a degree of distress and at the same time inflamed the view of the consumers about the safety of agricultural products.
- The government has implemented a comprehensive monitoring and control program to ensure that trade issues are being addressed.
- Some of the data on relative potencies of HGPs (and EDCs) are based on *in vitro* assays using cell cultures. While these comparative data are useful, they are not necessarily reflective of comparative potencies *in vivo.*
- Data relating to the bioavailability of HGPs to sensitive tissues and the effect of tissue and serum protein binding is incomplete, and this can complicate any quantitative risk assessments or comparisons.

The Committee agreed that the issue could not be progressed any further at this stage and indicated that the Secretariat brief is to keep Members informed of any scientific developments. It was anticipated that the position paper on HGPs would be tabled at the next meeting and there will also be further information provided to the ACPH. Members agreed to provide any advice to the secretariat if they became aware of it via their contacts or readings. The Committee confirmed its previous position on HGPs and endorsed the suggestion that their previous conclusion stating that *there is no appreciable health risk to consumers from eating meat from animals treated with HGPs* be amended to read that that *there is no appreciable health risk to consumers from eating meat from animals treated with registered HGPs according to good veterinary practice*.

RESOLUTION 22/1

The Committee:

- NOTED a variety of recent scientific publications on HGPs;

- REAFFIRMED its support for the development of a position paper on HGPs;
- REAFFIRMED its previous conclusions relating to the safety of HGPs; and
- AMENDED its previous conclusion that *there is no appreciable health risk to consumers from eating meat from animals treated with HGPs* to, *there is no appreciable health risk to consumers from eating meat from animals treated with registered HGPs according to good veterinary practice*.

ACPH 23, 2nd May 2002

5.2 HORMONE GROWTH PROMOTANTS

PURPOSE

The Committee considered, and was given the opportunity to provide feedback on, the position paper on hormone growth promotants (HGPs) drafted by the TGA, and to discuss any developments relating to the risk assessment of HGPs.

BACKGROUND

The human safety of HGPs has now been considered at ACPH 21 and 22 (2nd May and 18th October 2001, respectively). One of the main outcomes of these meetings has been the conclusion that there is "*no appreciable health risk to consumers from eating meat from animals treated with registered HGPs according to good veterinary practice*". Additionally, the Committee supported the generation of a position paper on HGPs and agreed that this issue be kept on the agenda so as to monitor and respond to any scientific developments.

The TGA has drafted a scientific position paper on HGPs used in cattle which summarises the available scientific information relating to the risk assessment of HGPs and briefly reviews the status of these compounds internationally. In response to the Committees' decision to monitor and review relevant material on HGPs, a number of recent publications relating to the biochemistry, genotoxicity and tissue distribution of various HGPs, and the safety of hormones in food, were provided to Members.

DISCUSSION

Members noted the TGA's draft position paper on HGPs used in cattle and a number of recent scientific papers relating to hormones in food including hormones used as growth promotants, and exogenous oestrogens in plant products. Particular attention was paid to a paper by Stephany (2001)[28] regarding approaches taken to hormones in meat in Europe and the US. This paper examined HGP levels in treated *versus* untreated US and European meat. This paper revealed that very little analytical testing has been undertaken in Europe since 1994, and also suggested that illegal use of hormonal and other growth-promoting agents occurs in some member states. Other documents noted by the Committee included a 1999 JECFA residue monograph prepared by Dr Dieter Arnold on 17β-oestradiol, progesterone

[28] Stephany RW (2001) Hormones in meat: different approaches in the EU and in the USA. APMIS 109(Suppl 103): S357-64

and testosterone, and a recent EC document[29] on the potential risk to consumers from hormone residues in bovine meat.

There was some discussion on an EC document entitled *Hormones in bovine meat: background and history of WTO dispute.* This document indicated that the EC had requested data from Australia, Canada, New Zealand and the US supporting their risk assessments and marketing authorisations. This document also indicated that to date, no information had been received from Australia (or any of the other countries), implying that Australia does not have the necessary information to support its risk assessment decisions on HGPs. Members were informed that the EC's request was ostensibly for any additional data to that which is in the public domain, that is related to the assessment of chemicals in Australia, Canada, New Zealand and the US or was submitted as part of the WTO hearing. At the end of 1998, the EC funded a number of studies to fill what it perceived as some of the data gaps, and the majority of these are now complete and available in the public domain.

Members noted that European auditors had visited Australia in 1999 and 2000 to assess the regulation of HGPs (and veterinary drugs), and are due for a further visit at the end of 2002. Members were advised that Agriculture, Fisheries and Forestry Australia (AFFA) had argued that the auditors should be solely focussed on the regulation of HGPs in Australia, however the auditors contended that it is necessary to look more widely to how all veterinary products are regulated in Australia. The auditors also had some concerns regarding the methods of cattle mustering as part of the HGP monitoring process. It was suggested that previous worksharing arrangements between the TGA and the German BgVV could be used to illustrate to the auditors that, as far as possible, Australia had a very similar standard of regulation and used OECD guidance in its toxicological assessment procedures.

The Committee was informed that the current European position is set out in the recent EC document[2]. Notwithstanding the WTO dispute, any member state has the right to protect its consumers against chemical residues in food on non-scientific grounds (eg. political, social). Outside of Europe, this was considered to be a very difficult concept to work with. The EC commissioned a series of 22 studies to examine various aspects of HGPs such as the analytical methodology for detecting very low levels of residues, and issues of genotoxicity and carcinogenicity, particularly in relation to 17β-oestradiol. The EC was concerned by the determination of IARC that 17β-oestradiol is carcinogenic. In 1999, JECFA concluded that 17β-oestradiol had genotoxic potential, however the EC went one step further and labelled it a genotoxic carcinogen. The EC does not appear to consider that progesterone and testosterone are problems, while they have some questions regarding the toxicology of trenbolone and zeranol which they have grouped with 17β-oestradiol. They also have questions regarding the completeness of the data package for melengestrol acetate. The Committee was advised that the endpoint for trenbolone acetate a very sensitive endpoint viz. a reversible change noted by electron microscopy and set on the most sensitive species which was the pig.

It was proposed that the TGA independently evaluate the published studies cited in the recent EC document[2] and determine whether Australia would concur with the interpretation of the data collected. Some of the studies cited by the EC were judged by several Committee

[29] Opinion of the Scientific Committee on Veterinary Measures Relating to Public Health. Review of previous SCVPH opinions of 30 April 1999 and 3 May 2000 on the potential risks to human health from hormone residues in bovine meat and meat products. European Commission (adopted April 10, 2002).

Members to shed little further light on the matter and the point was re-iterated that these studies should not be accepted without appropriate assessment to judge their scientific merit.

It was suggested that Australian regulatory authorities should carefully examine their collective position and ensure that the continuing registration of HGPs (particularly 17β-oestradiol) in Australia can be defended. The Committee heard that a mitigating factor is that levels of 17β-oestradiol in treated beef are small when considered in relation to the total dietary load. If 17β-oestradiol were banned, it would still be difficult regulating any illegal use because it is not possible to differentiate treated animals from untreated animals. Members recognised the difficulty in regulating compounds that occur naturally in food.

Whether Australia can sustain registration of HGPs in the face of criticism depends on the outcome in Europe. A number of factors were raised which support Australia's current position and differentiate it from other countries, such as the US, that use HGPs: HGPs are not as widely used as in the US; HGPs are only registered for use in cattle and not in sheep, pigs or poultry; only 5 agents are registered (17-β-oestradiol, progesterone, testosterone, zeranol, trenbolone acetate); Australia has a reasonably rigorous residue monitoring program; the detection of violative levels of the naturally-occurring hormones is difficult because the levels in treated *versus* untreated meat overlap; no growth promoting agents are used in lactating cows (which is important in relation to dairy products). If agencies were concerned about regulating the intake of hormonal substances, particularly those that exert oestrogenic effects, then a whole series of other dietary sources would need to be examined (eg. eggs, soy-based products, dairy products, legumes).

The validity of the criticisms levelled at the JECFA assessments of HGPs by the EC were considered. Members were advised that JECFA had evaluated all of the epidemiological data that it could, all of the data that would allow it to set an endpoint in humans from clinical-type data, and had used this information to set an ADI for these hormonal substances. JECFA only evaluated what was available and concluded that 17β-oestradiol had genotoxic potential. There are more studies continually being done, especially when it appears that the EC may have a vested interest in being able to state unequivocally from scientific studies that the use of HGPs is resulting in food residues that interfere with the development of pre-pubertal children and are carcinogenic - therefore they could sustain the current ban based on the concerns of consumers and scientists. One of the EC criticisms of JECFA was the figures used for production rates of hormones in children was too high. The Committee was advised that the methodology for detecting very low levels of the naturally-occurring hormones relates to a published method which uses genetically modified yeast. This method has not been adequately validated and in some cases it has been shown to be invalid. Another criticism of JECFA was that its lowest limits of detection were not low enough.

There is often the misperception in Australia that hormones are widely used, both legally and illegally. The NRA representative suggested to the ACPH that there is a problem with people's perception that antibiotics used in poultry are synonymous with hormones. Members noted that porcine somatotropin is not registered for use in pigs and cattle. It was suggested that information relating to what and how hormones are used, the types and extent of residue surveys conducted, and the levels of use in Australia should be promulgated via ANZFA given the disquiet expressed by some part of the community.

The point was made that the TGA/ACPH are not the only group that should carry this issue. The Committee suggested that ANZFA, NRA and TGA need to collaborate on this issue and

be more proactive than they have been in the past. It was unclear to some Members whether ANZFA was aware that HGPs were under continuing review via the ACPH. It was suggested that given the complexity and enormity of this issue, 'someone' needs to be responsible for coordinating Australia's position so that the end result is available to reassure the community, consumers and government that this issue is being addressed.

Members supported the TGA's draft position paper on HGPs used in cattle and suggested a number of minor changes to improve its usefulness, consistency and readability. An additional section could be added aimed at the lay reader. Information from Stephany (2001) relating to the illegal use of HGPs in Europe could also be added. It was suggested that the paper be brought back to Members at a later meeting for their endorsement. A small section on the use of HGPs (in Australia) could also be added. The NRA anticipated that the use of HGPs in beef cattle in Australia would be high.

The Committee was advised that the NRA are concerned about the live cattle export to Indonesia. Indonesia is apparently requesting information on whether it is safe to consume ears and offal from HGP-treated cattle. Under no account would the Committee recommend the consumption of ears from treated cattle, given that HGPs are administered via subcutaneous implants in the ears. Members heard that the chemical industry is intending to generate residue studies on cattle ears, particularly in relation to the synthetic HGPs. A potential problem could be the duplication of studies which have been already conducted by the EC.

The Committee agreed that the TGA should liaise with ANZFA and the NRA on the TGA's draft position paper. If supported by the NRA, the TGA would review the studies which the EC had cited in their recent document. This review would then be brought back to the Committee at a future meeting. The other important aspect is that the Committee sees this as an emerging issue. If appropriate, the TGA needs to meet with the NRA and ANZFA and determine how the government should handle this issue. It is important to be able to justify the continued use of these compounds (particularly 17β-oestradiol) and to monitor the US approach to this issue in light of the recent EC document. It may be timely to revisit trenbolone and zeranol. The outcome of this issue could impact on how Australia handles the future registration of growth promoting agents such as melengestrol acetate and ractopamine hydrochloride.

RESOLUTION 23/2

The Committee:

- SUPPORTED the on-going ACPH review of HGPs and indicated that the TGA, NRA and ANZFA need to collaborate and be more pro-active in addressing this complex issue;

- PROPOSED that the TGA evaluate the published studies recently cited by the EC, and bring this review back to the Committee at a future meeting;

- SUPPORTED the TGA's draft position paper on HGPs used in cattle and suggested a number of minor changes to improve its usefulness, consistency and readability. The Committee also suggested that the TGA liaise with ANZFA and the NRA regarding the position paper, and bring it back to the ACPH for endorsement at a future meeting;

- SUGGESTED that ANZFA promulgate information regarding hormones in food; and

- SUGGESTED that Australian regulatory agencies carefully examine their collective position to ensure that the continuing registration of HGPs (particularly 17β-oestradiol) is defensible.

ACPH 24, 18th October 2002

5.1 Hormone Growth Promotants

PURPOSE

The Committee considered developments relating to the scientific assessment of hormone growth promotants (HGPs).

BACKGROUND

The human safety of residues of HGPs in meat has been an ongoing agenda item since ACPH 21 (1st May 2001). At the last meeting (ACPH 23, 2nd May 2002), the Committee expressed its support for the continuing ACPH review of HGPs and for the development and updating of the TGA's draft position paper on HGPs used in cattle. It had been suggested that the TGA liaise with ANZFA [now Food Standards Australia New Zealand (FSANZ)] and the NRA regarding the position paper, and present it to the ACPH for endorsement at a future meeting. Furthermore, it had been indicated that Australian regulatory agencies should carefully examine their collective position to ensure that the continuing registration of HGPs (particularly 17β-oestradiol) is defensible.

Prior to the release of their original 1999 risk assessment, the EC commissioned 17 new studies to fill data gaps that it had identified. Recently (10th April 2002), the EC released a second risk assessment[30] which considered data from these commissioned studies, in addition to other scientific literature that had become available since its 1999 review. The second risk assessment reaffirmed the conclusions established in 1999.

At ACPH 23, it was proposed that the TGA evaluate the published studies cited in the EC's second risk assessment, and present its findings to the Committee at a future meeting.

DISCUSSION

The Committee noted a number of recent scientific publications relating to the safety, biochemistry, genotoxicity and tissue distribution of various HGPs.

The Committee was informed that following ACPH 23 (2nd May 2002), the TGA had written to the NRA recommending that the EC's latest risk assessment and the 17 new studies supporting it, be reviewed. While the TGA's proposal was considered by the NRA to be well

[30] Opinion of the Scientific Committee on Veterinary Measures Relating to Public Health. "Review of previous SCVPH opinions of 30 April 1999 and 3 May 2000 on the potential risks to human health from hormone residues in bovine meat and meat products". European Commission (adopted April 10, 2002).

based, it was recognised that other government agencies are also involved in this issue. A meeting was held at AFFA on the 1st August 2002 with representatives from the NRA, TGA, AQIS, NRS, OCVO and Food Safety. After discussion of technical, trade and policy issues, it was agreed that the NRA would commission a "scoping study" of the EC's risk assessment, recent JECFA and IARC considerations of the HGPs, and any other recent substantial work. The executive from Safemeat[31] was subsequently advised of this scoping exercise.
The Committee noted that the TGA had commenced evaluating 11 of the 17 EC-commissioned studies that were available in the scientific literature. The majority of these had been provided to Members at previous meetings. The Committee was advised that it had been difficult identifying all 17 studies due to the lack of transparency in the EC's latest risk assessment. In fact, there was still a proportion of the studies that remained unidentified. The Committee agreed that it was important to confirm or obtain the identity of the 17 studies but recognised that it could be difficult to gain access to this European data.

The Committee was informed that it was likely that EC auditors would visit Australia in 2003 and that the scoping study would be useful in substantiating Australia's position on the human safety of HGPs. The TGA indicated that the draft report, incorporating both the scoping study and the TGA's previous position paper (tabled at ACPH 23), would be tabled at ACPH 25.

The Committee expressed satisfaction that the human safety of HGPs was continuing to be addressed by the relevant government agencies and that the TGA was progressing the scoping study.

RESOLUTION 24/1

The Committee:

- NOTED a number of recent scientific publications relating to the safety, biochemistry, genotoxicity and tissue distribution of various HGPs; and

- WAS SATISFIED that the human safety of HGPs was continuing to be addressed by the relevant government agencies and that the TGA was progressing the scoping study.

[31] Safemeat is a partnership between the Australian meat and livestock industry and State and Federal governments. http://www.safemeat.org/

ANNEX 5: HGP POSITION PAPER

Position Paper

HORMONE GROWTH PROMOTANTS USED IN CATTLE

Drafted in March 2002 by the Chemical Review and International Harmonisation Section of the Office of Chemical Safety.

Introduction

Hormone growth promotants (HGP) are used to increase the growth, feed conversion efficiency and carcass leanness of cattle, and are typically administered via subcutaneous implants in the ears. HGPs include naturally-occurring steroids such as 17β-oestradiol, progesterone and testosterone, and their synthetic counterparts zeranol, melengestrol acetate and trenbolone acetate (Fig 1). Zeranol is an oestrogenic derivative of the myco-oestrogen zearalenone which is produced by *Fusarium* moulds. Although hormonal implants are widely used in beef-exporting countries such as the USA, Canada, New Zealand and Australia, they have been officially banned in Europe on safety concerns since 1988. The consequence of the European ban has been an ongoing protest from the USA and Canada which has reinforced the polarisation in scientific and regulatory opinion regarding the safety of HGPs. This report summarises the scientific information relating to the risk assessment of HGPs and briefly reviews the status of these compounds internationally.

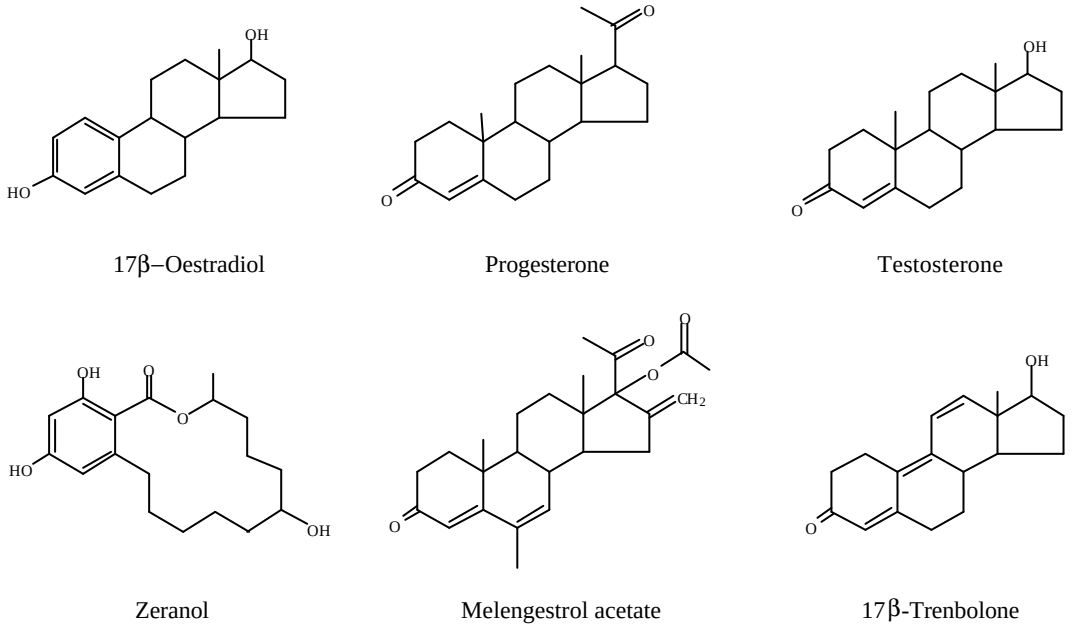


Fig 1: Structures of natural and synthetic HGPs

Assessment of the human safety of HGPs

Difficulties associated with assessing the safety of HGPs

The human risk assessment of HGPs is complicated by the difficulty of assessing the risk associated with low levels of HGP residues when humans are constantly exposed to relatively high and fluctuating levels of endogenously- and exogenously-derived hormones.

Compounds such as 17β-oestradiol, progesterone and testosterone are naturally occurring constituents of the diet (eg. meat, fish, milk, eggs) and are also endogenously produced in significant amounts. There are numerous plant-derived oestrogenic compounds that have potent biological activity (eg. isoflavones, coumestans, lignans) that are also consumed as part of the diet. Humans have been exposed to low levels of compounds like zeranol for thousands of years given its natural occurrence as a fungal contaminant (mainly *Fusarium spp*) on a number of plant products (eg. wheat, barley, maize). The potential for these naturally-occurring substances to elicit hormonal effects in humans is likely to be greater than any potential effect of HGP residues received via the ingestion of meat. Studies have shown that there is a large variation in natural hormone levels and therefore residues in meat from HGP-treated animals would make a fractionally small contribution to overall hormone intake or exposure from natural sources. Therefore, previous assessments had concluded that incremental risk from HGPs would be quite small.

Technically, it has been difficult to detect any significant fluctuations in oestradiol, progesterone and testosterone residues in treated *versus* untreated cattle, given their high natural background concentrations in animal tissue. A range of assays have been developed to measure the six HGPs (reviewed by Doyle 2000)[32] and these show varying degrees of sensitivity and reliability. Indeed, one of the ongoing problems, which is not particularly unique to these compounds, has been the variability in measurements performed by different laboratories.

Health concerns associated with HGPs

The main health concerns that have been raised in relation hormonal compounds used as growth promotants (and also as therapeutic agents) is their carcinogenic and endocrine-disrupting potential. Normally, hormones at very low concentrations (nM) interact with the genetic apparatus to induce cell proliferation and growth. Therefore, a hormonal substance could promote carcinogenicity in hormone-sensitive tissues through such a proliferative mechanism. It is clear that high levels of some of the endogenously-derived hormones (eg 17β-oestradiol) have been linked to certain types of cancer in genetically-susceptible individuals. However, the key factor is whether or not very low levels, the levels that would be found in HGP-treated cattle, can cause adverse health effects in humans.

The International Agency for Research on Cancer (IARC) has evaluated the carcinogenicity of 17β-oestradiol, progesterone and testosterone in 1974 (Vol 6) and 1979 (Vol 21). Zeranol was evaluated in 1993 (Vol 56) and hormonal contraceptives containing oestrogen and progesterone were assessed in 1999 (Vol 72). IARC has concluded that 17β-oestradiol, and testosterone have carcinogenic potential in experimental animals and therefore are likely to pose a carcinogenic risk to humans. There is limited evidence for the carcinogenicity of

[32] Doyle E (2000) Human safety of hormone implants used to promote growth in cattle. FRI Briefings.

zeranol and progesterone in experimental animals. Progesterone contraceptives have been classified as possibly carcinogenic in humans (Group 2B) while combined oral contraceptives (oestrogen/progesterone) have been classified as carcinogenic to humans (Group 1). Post-menopausal oestrogen therapy has been classified as carcinogenic to humans (Group 1). Although Australia gives appropriate consideration to IARC reviews, IARC classifications are not intended to be used as recommendations for any specific regulatory action.

Scientific assessments of HGPs

Various international agencies and national regulatory bodies have evaluated the safety of HGPs. There is also plethora of published scientific studies relating to the biochemistry and physiology of HGPs. The weight-of-evidence indicates that registered HGPs do not pose a threat to human safety when used according to good veterinary practice. However, this view is not supported by all countries (see European position below).

The human safety of 17-β-oestradiol, progesterone, testosterone, zeranol, melengestrol acetate and trenbolone acetate used as growth-promoting agents in cattle has been evaluated by the Codex Alimentarius Commission Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 1981, 1983, 1988 and 1999 and 2000. JECFA has set acceptable daily intakes ADIs[33] for all six HGPs, while the Australian Therapeutic Goods Administration (TGA) has set ADIs for zeranol, trenbolone (α and β)[34] and melengestrol acetate (Table 1). The absence of Australian ADIs [and maximum residue limits (MRLs)[35] – see Table 2] for oestradiol, progesterone and testosterone is due to their exemption from the MRL standard because any residues are identical to, or indistinguishable from, natural food components.

Table 1: ADIs for HGPs (mg/kg bw/d)

Compound	TGA	JECFA
17β-oestradiol	-	0-0.00005
Progesterone	-	0-0.03
Testosterone	-	0-0.002
Zeranol	0.0002	0-0.0005
Trenbolone	0.0001 (α-trenbolone) 0.00001 (β-trenbolone)	0-0.00002[#]
Melengestrol acetate	0.00005	0-0.00003

[#] = The JECFA ADI covers both the parent molecule (trenbolone acetate) and the two main metabolites (α and β trenbolone).

JECFA and the National Registration Authority for Agricultural and Veterinary Chemicals (NRA) have set MRLs for zeranol and trenbolone acetate (Table 2). Acceptable residue levels for oestradiol, testosterone and progesterone were not set by JECFA as residues from these compounds were considered unlikely to pose a hazard to human health when used according to good veterinary practice.

[33] The acceptable daily intake (ADI) for humans is considered to be a level of intake of a chemical that can be ingested daily over an entire lifetime without any appreciable risk to health.

[34] α and β trenbolone are the major metabolites of trenbolone acetate. The β epimer has approximately 10 times more hormonal activity than the α epimer.

[35] The maximum residue level (MRL) is the maximum concentration of a residue resulting from registered use of an agricultural or veterinary chemical. The MRL is set at a level which is not likely to be exceeded if the agricultural or veterinary chemical is used according to good agricultural or veterinary practice.

Table 2: MRLs for HGPs (mg/kg)

Compound	NRA	JECFA
17β-oestradiol	-	#
Progesterone	-	#
Testosterone	-	#
Zeranol	0.005 cattle meat 0.02 cattle, edible offal of	0.002 bovine meat 0.01 bovine liver
Trenbolone acetate	0.002 cattle meat 0.01 cattle, edible offal of	0.002 muscle (β-trenbolone) 0.01 liver (α-trenbolone)
Melengestrol acetate	*	-

= Residues generated according to good veterinary practices were considered unlikely to adversely effect human health; * = There are no registered products in Australia containing MGA, hence no need for any MRLs; the NRA did consider a registration application but it was withdrawn and the product never registered. The ADI was established during the evaluation of that application.

The Codex Committee on Residues of Veterinary Drugs in Foods (1987) also concluded that implants containing approved doses of HGPs and administered as recommended would not result in residues that would adversely affect human health.

The US FDA has set maximum safe tissue residue levels for trenbolone (α & β) and zeranol (Table 3) and allowable incremental increases in hormone levels above those normally present for oestradiol, progesterone and testosterone (Table 4). The US ADI for total trenbolone residues is 0.0004 mg/kg bw/d while a tolerance for total trenbolone residues in uncooked edible tissues of cattle was not considered necessary.

Table 3: FDA maximum safe tissue residue levels for trenbolone and zeranol (mg/kg)

Compound	Muscle	Liver	Kidney	Fat
Trenbolone (α & β)	0.05	0.1	0.15	0.2
Zeranol	0.15	0.3	0.45	0.6

Table 4: FDA allowable incremental increases above background levels for oestradiol, progesterone and testosterone (mg/kg)

Compound	Muscle	Liver	Kidney	Fat
Oestradiol	0.00012	0.00048	0.00036	0.00024
Progesterone	0.003	0.012	0.009	0.006
Testosterone	0.00064	0.0026	0.009	0.0013

The European position

The current polarisation in scientific and regulatory opinion surrounding the use of HGPs stems from the 1988 EC ban which prohibited the use of HGPs on farm animals, and this was extended in 1989 to include the importation of beef and beef products from countries which allowed the use of growth promotants. The catalyst for this ban was the synthetic hormone diethylstilbestrol (DES) which reached notoriety in the late 1970s following reports of abnormal sexual development in Italian babies after eating tinned baby food made from French veal contaminated with traces of DES. In 1981, the EC banned the use of DES and recommended that 17β-oestradiol, progesterone, testosterone, zeranol, and trenbolone acetate

be reviewed. The conclusion of the subsequent review[36] was that the three natural hormones (17-β-oestradiol, progesterone, testosterone) would not present any harmful effects to the health of the consumer when used under the appropriate conditions as growth promoters in farm animals, but zeranol, and trenbolone acetate required more data.

Following a protest by the US and Canada, The World Trade Organisation (WTO) ruled against the EC ban in 1997[37] asserting that it was not based on scientific evidence or an adequate risk assessment. The EC was allowed 15 months to conduct a risk assessment of hormone-treated meat.

The subsequent EC risk assessment on human residues in bovine meat and meat products[38] arrived at the following conclusions:

- There is a substantial body of evidence suggesting that 17β-oestradiol is a complete carcinogen (tumour initiator and promoter) but the available data does not allow a quantitative risk estimate;
- Data pertaining to the 5 other growth-promoting hormones (progesterone, testosterone, zeranol, trenbolone acetate & melengestrol acetate) does not allow a quantitative risk assessment;
- Potential developmental, immunobiological, neurobiological, immunotoxic, genotoxic and carcinogenic effects can be envisaged for all 6 hormones, with prepubertal children considered to be a high risk group, however the available data do not allow a quantitative risk assessment;
- In view of the intrinsic properties of the hormones, and taking into account epidemiological findings, no threshold levels or ADIs can be established for any of the 6 hormones.

Following their risk assessment, the EC moved to impose a definite ban on the use of 17β-oestradiol and its ester-like derivatives in farm animals except for therapeutic purposes. A provisional ban was also placed on progesterone, testosterone, zeranol, trenbolone acetate and melengestrol acetate, pending further scientific evidence. Internationally there appears to be very little support for the EC position either on the basis of their scientific assessment or the resulting risk management procedures.

In 1999, the Sub-Group of the Veterinary Products Committee from the UK Ministry of Agriculture, Fisheries and Food, critically evaluated the scientific reasoning and methods of argument adopted in the EC's risk assessment[39]. The group were unable to support the conclusion that risks associated with the consumption of meat from hormone treated cattle may be greater than previously thought. Additionally, they had concerns regarding the scientific reasoning in a number of key areas to throw serious doubt on the conclusions of the EC.

[36] EEC Scientific Working Group on Anabolic Agents, chaired by Dr GE Lamming. Scientific report on anabolic agents in animal production. Vet. Rec. 1987: 389-392.
[37] EC measures concerning meat and meat products (hormones). AB-1997-4. Report of the Appellate BodyWT/DS26/AB/R. WT/DS48/AB/R. 16 January 1998.
[38] The opinion of the Scientific Committee on Veterinary Measures Relating to Public Health (SCVPH): Assessment of potential risks to human health from hormone residues of bovine meat and eat products; 30th April 1999.
[39] Sub-Group of the Veterinary Products Committee, Ministry of Agriculture, Fisheries, and Food. Executive summary and critical evaluation of the 1999 opinion of the scientific committee on veterinary measures relating to public health. 1999. http://www.vmd.gov.uk/old_vmd_web_pages/finalrep.htm

A new issue to arise from the EC assessment from a scientific perspective is that while HGP exposure may be quite small, the excess or incremental risk associated with carcinogenicity cannot be addressed because of the new concept that such substances may initiate carcinogenesis via a genotoxic mechanism. Due to the insufficient information available to make an assessment of that incremental risk, the EC had adopted the 'precautionary principle' which is not accepted as an international standard. An additional argument put forward by EC-sponsored scientists is that although the parent molecules are safe, HGP metabolites could be a problem. Until such times as data is generated indicating that these metabolites are safe, then the precautionary approach should be maintained.

A variety of additional criticisms have been levelled at the EC's risk assessment. It has been claimed that the risk assessment was biased due to political pressure from lobby groups that want to maintain the high levels of subsidies which are afforded to the European farming community. Consumer organisations and other interested parties also applied emotive pressure to the debate and the EC may have contributed to the polarisation of the assessment by funding groups which were essentially supporters of the their position. Another criticism is the selective and incorrect citing of scientific literature.

There was a failure by the EC to acknowledge the potential impact of the black market trade in growth promoting agents, including the illegal use of DES. Many of the issues referred to in the EC documents were associated with the alleged illegal use of DES. An additional criticism was that the EC's risk assessment was significantly focussed on the conclusion that 17β-oestradiol acts as a genotoxic carcinogen as it was so defined. This EC assessment contrasts with the position of JECFA who have used the term genotoxic potential, a term which is supported by IARC.

Another factor which has at times exacerbated this debate is the issue of the misuse of both existing and registered products, combinations of registered products that were never intended to be combined, and also the use of unregistered agents or those that have been withdrawn from the market, such as DES. It is sometimes the perception of consumers that unregistered products are used widely, or that unapproved doses and combinations are used. This perception has periodically caused regulatory bodies a degree of concern and at the same time reinforced the view of some consumers regarding the safety of agricultural products.

The US position

Since the 1950s, the US Food and Drug Administration (FDA) has approved the use of oestradiol, testosterone, progesterone, trenbolone acetate and zeranol as growth-promoting agents[40] in cattle based on the conclusion that residues would not constitute a health risk to consumers. Tolerance levels for these compounds have been set (Table 3 & 4). The US has been highly critical of the EC's position and asserts that it is an unscientific and unjustified barrier to US exports.

The Australian position

HGPs have been approved for use in Australia since the mid 1970s. Implants containing 17β-oestradiol, progesterone, testosterone, trenbolone acetate or zeranol, or various combinations thereof, are registered for use in all states and territories. However, the Tasmanian

[40] Code of Federal Regulations (CFR) Part 522, 556 & 558.

government moved to prohibit the use of HGPs in 2000 purely as a marketing strategy to gain access to niche markets such as Japan and Europe. This move has been criticised by other states because Australia already has an accreditation program to enable access to these HGP-free markets.

As Australia does not ban the use of HGPs, it invests large sums of money into operating a hormone-free cattle program to accommodate the European market. This program controls and monitors the distribution and use of HGPs in meat production. The *HGP Free Accreditation Scheme*[41] is a national system that allows the full traceability of all animals within the scheme through the use of the National Livestock Identification Scheme (NLIS). Cattle within the HGP Free Accreditation Scheme must not have been treated with HGPs at any time in their lives. The seriousness of Australia's commitment to protecting EC trade is evidenced from a recent case where a Victorian Company was fined by the NRA for keeping inaccurate records regarding the supply of HGPs. The EC, as a consequence of its policy on HGPs, audits suppliers such as Australia, Canada and New Zealand. European auditors have inspected Australia in 1999[42] and 2000[43] and are due for another visit in 2002.

Toxicological assessments for trenbolone acetate and zeranol have been conducted by the TGA as part of the approval process for product registration. ADIs have been set for both compounds (Table 1). No ADIs have been set for the natural HGPs as they are exempt from the MRL standard because residues would be indistinguishable from those found naturally in food. The TGA's Advisory Committee on Pesticides and Health (ACPH) considered the human safety of HGP residues at their 21st and 22nd meetings (1st May and 18th October 2001, respectively). They concluded that there is no appreciable health risk to consumers from eating meat from animals treated with registered HGPs according to good veterinary practice. They recognised that this conclusion was incompatible with the EC's position and supported the continuation of the current HGP Free Monitoring Program, as an interim or ongoing measure, to accommodate the European market. The seriousness of this issue was reflected in their decision to keep it as a continuing agenda item so as to monitor international developments and scientific assessments. If there was any new evidence to suggest that HGP residues were a health concern, there would be a recommendation that this issue be considered within the NRA's Chemical Review Program.

Summary

(1) There is unlikely to be any appreciable health risk to consumers from eating meat from cattle that have been treated with HGPs according to good veterinary practice.
(2) Australia recognise that this position is incompatible with the EC's current risk assessment and risk management framework, therefore the current accreditation and monitoring program for HGP-free cattle will be maintained so that Australia can access the European market.
(3) Australian regulators will continue to closely monitor the scientific evaluations undertaken at an international level and to monitor the international position.

[41] HGP Free Accreditation Scheme Rules (July 2000) Australian Quarantine and Inspection Service (AQIS). Agriculture, Fisheries and Forestry Australia (AFFA).
[42] Final report of a mission carried out in Australia from 11 to 23 November 1999 relating to the production of fresh bovine meat for export to the European Union. DG/SANCO)/1170/1999 – MR final
[43] Final report of a mission carried out in Australia from 9 to 21 November 2000 in order to evaluate the operation of controls over residues in products of animal origin and over the production of fresh meat, game meat, and milk and milk products. DG(SANCO)/1217/2000 – MR final.

(4) If any new evidence emerged indicating that residues of HGPs were unsafe to the consumer, there would be a recommendation that this issue be considered within the NRA's Chemical Review Program.

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