

# Sub-Group of the Veterinary Products Committee

Executive summary and critical evaluation of the scientific reasoning and methods of argument adopted in the opinion of the Scientific Committee on Veterinary Measures Relating to Public Health which assessed the potential risks to human health from hormone residues in bovine meat and meat products

October 1999

## EXECUTIVE SUMMARY

### Introduction

On the 30 April 1999, the European Commission published an Opinion of the Scientific Committee on Veterinary Measures relating to Public Health (SCVPH). The report discussed potential risks to human health from the residues in meat and meat products of hormonally active substances used for growth promotion purposes in cattle, in particular the six hormones, oestradiol-17 $\beta$ , testosterone, zeranol, progesterone, trenbolone acetate and melenogestrol acetate. The SCVPH concluded that risks associated with the consumption of meat from hormone-treated cattle may be greater than previously thought. At the request of the Minister of Agriculture, Fisheries and Food, a Sub-Group to the Veterinary Products Committee (VPC) was asked to review the SCVPH Opinion and the Committee's interpretation of the data cited in the report.

### Terms of Reference

To complete a balanced and critical evaluation of the scientific reasoning and methods of argument adopted in the SCVPH Report.

### Background

The use of hormonal growth promoters in food producing animals, including the six hormones covered in the SCVPH Report, has been banned in the European Community since 1988. Any third country which permits the use of growth promoting hormones is required to guarantee that no animals and no meat coming from animals to which they have been administered will be exported to the Community. In 1998 the World Trade Organisation (WTO) Appellate Body ruled that the EU had not undertaken a proper risk assessment prior to imposing the ban on imports of meat from animals treated with hormone growth promoters, and that the scientific reports referred to by the Commission did not provide acceptable support for the EU position. <sup>1</sup>As a result, the Commission is conducting a new risk assessment consisting of a number of related research projects. Most of these are unlikely to be finalised until late 1999, but some are being published as interim reports, including the SCVPH report which is the subject of this paper.

### Overview

It is the view of the Group that the SCVPH Report provides a wide-ranging though not fully comprehensive review of the many areas of work involved in the field, but arrives at selective conclusions. The Group identified areas underpinning the SCVPH's conclusions, which merited particular attention. These are covered in summary below and are supported by a paper containing more detailed specialised scientific reasoning and citing appropriate papers. The Group also identified specific areas where additional expert advice should be sought to add to the data on this subject and help prevent

selective conclusions being drawn in the future. These are also set out in the attached paper.

## Main Areas of Concern

### The Group

- concluded that the likely levels of **consumer exposure** to 17 $\beta$ -oestradiol, progesterone, testosterone, zeranol and trenbolone resulting from their use as growth promoters were very low **in comparison with the acceptable daily intakes** (ADIs) identified by the Joint WHO/FAO Expert Committee on Food additives (JECFA);
- concluded that the likely levels of **consumer exposure** to 17 $\beta$ -oestradiol, progesterone and testosterone resulting from their use as growth promoters were very low **in comparison with the amounts of these hormones produced naturally** by the bodies of some people;
- concluded that none of the publications reviewed in the Opinion provide any substantive evidence that Oestradiol is **mutagenic/genotoxic** at relevant levels of exposure from residues in meat. For the other five compounds there is no substantive evidence for mutagenic/genotoxic activity;
- had concerns about the validity and selective application of **a key analytical approach cited in the Opinion**, which was based on an assay for apparent oestrogenic activity done in genetically engineered yeast. The concerns were sufficient to throw doubt upon the values derived from this analytical technique and therefore also on the conclusions of the Opinion;
- found that much of the information cited on **'immunity'** and hormonally active substances comes from experiments and clinical observations in man and animals of uncertain significance; that it relied in part on unproven hypotheses, old studies or experiments, now superceded; and that questions of species specific effects and dose or exposure were largely ignored. References cited by the report as evidence of the role of sex hormones do not reflect modern physiological understanding of the immune system. Furthermore, the additional exposure to sex hormone activity represented by the residues of the 6 compounds in meat would be far below the level at which any relevant activity on the immune system and its functions has been demonstrated;
- found the SCVPH mentioned a possible link between certain common **cancers** and hormonally active residues in meat. The Group found the epidemiological evidence shows a link between overall meat and fat consumption and the occurrence of these neoplasms, but a link with small traces of hormones has not been directly examined. However, the tumours are found both in men and women, and they occur widely in countries where hormonal growth promoters are not permitted;
- found that the SCVPH suggests that many aspects of **human development and reproduction** could be affected by hormone residues in meat. The Group found that there is no evidence for such effects. The publications cited in the report to support this suggestion did not investigate low level residues, but instead studied the actions of high levels of exogenous hormones injected into animals. Contrary to current experience, the report suggests that there are no thresholds for developmental effects, but does not give any evidence. Epidemiological studies of human exposure that do not show an effect of exogenous hormones on the outcome of pregnancy are ignored in the report. The significance of endocrine disruption for human reproduction and development is still being intensely researched, but the available data do not support the particular suspicions

raised in the report, which are based on an incomplete review of the information.

### **Overall Conclusion**

Following a critical evaluation of the scientific reasoning and methods of argument adopted in the key papers cited in the SCVPH Report, the Group were unable to support the conclusion reached by the SCVPH that risks associated with the consumption of meat from hormone-treated cattle may be greater than previously thought. The Group found that they had sufficient concerns about the scientific reasoning in a number of key areas, as summarised above and more comprehensively described in the attached paper, to throw serious doubt on the conclusions of the SCVPH.

### **SUB-GROUP OF THE VETERINARY PRODUCTS COMMITTEE (VPC):**

ESTABLISHED TO COMPLETE A BALANCED AND CRITICAL EVALUATION OF THE SCIENTIFIC REASONING AND METHODS OF ARGUMENT ADOPTED IN THE SCVPH REPORT.

#### **CHAIRMAN:**

**Professor Antony Dayan**, member of the VPC, which provides expert advice to Ministers on the quality, efficacy and safety of veterinary medicinal products.

#### **MEMBERS:**

**Dr Edward Houghton** - Senior Assistant Director and Head of Research and Development at HFL (formerly the Horseracing Forensic Laboratory).

**Professor Mitch Dowsett** - Head of Biochemistry at the Royal Marsden Hospital.

**Professor James M Parry** - the School of Biological Sciences at the University of Wales at Swansea (Chairman of the Committee on Mutagenicity).

**Dr John McCaughey** - member of the VPC.

**Professor Nigel Brown** - Department of Anatomy, St George's Hospital Medical School.

### **CRITICAL EVALUATION OF THE SCIENTIFIC REASONING AND METHODS OF ARGUMENT ADOPTED IN THE SCVPH REPORT**

1 It is the view of the Group that the SCVPH Report provides a wide-ranging but not fully comprehensive review of the many areas of work involved in the field but arrives at selective conclusions. The Group identified the following areas as underpinning the SCVPH's conclusions about which they had sufficient concerns to throw serious doubt on the conclusions expressed in the Opinion.

### **HUMAN DIETARY EXPOSURE TO HORMONALLY-ACTIVE SUBSTANCES USED FOR GROWTH PROMOTION**

## Background

2. This paper compares the dietary exposure of consumers to growth promoters with the doses that might cause harmful effects. To do so it uses UK dietary data, the ADIs recommended by regulatory bodies, and information already available to the Sub-Group in the article by Hartmann et al [Hartmann S, Lacorn M & Steinhart H, 1998, "Natural occurrence of steroid hormones in food", Food Chemistry, **62**(1): 7-20].

## Acceptable Daily Intakes (ADIs)

3. Table I shows the acceptable daily intakes (ADIs) which have been set for the six growth promoting hormones permitted in the USA.

**Table I : Acceptable Daily Intakes for Hormone Growth Promoters**

Substance	ADI (mg/kg bw)	ADI ( $\mu\text{g}/60 \text{ kg person}$ )
17 $\beta$ -oestradiol	0.00005	3
Progesterone	0.03	1800
Testosterone	0.002	120
Zeranol	0.0005	30
Trenbolone acetate	0.00002	1.2
Melenogestrol acetate	No ADI set	No ADI set

## Oestradiol 17 $\beta$

4. The ADI for 17 $\beta$ -oestradiol was set by the Joint WHO/FAO Expert Committee on Food additives (JECFA) in February 1999. An uncertainty factor of 100 was applied to a no-observable-effect level (NOEL) of 0.3 mg/person/day (equivalent to 5  $\mu\text{g}/\text{kg bw}/\text{day}$ ) for increased serum corticosteroid-binding globulin in post-menopausal women. JECFA recommended maximum residue limits (MRLs) "not specified" for 17 $\beta$ -oestradiol residues in bovine muscle, fat, kidney & liver [summary & conclusions from the 52<sup>nd</sup> JECFA, to be published in WHO, Food Additive Series #43]. An MRL "not specified" means that no quantitative MRLs need be set for these tissues as the residues occurring subsequent to the recommended use of the drug are considered to be safe for consumers.

5. At the request of the European Commission (DG-XXIV), the Committee on Veterinary Medicinal Products (CVMP) is currently reassessing the safety of 17 $\beta$ -oestradiol and other sex hormones that have been approved for therapeutic use in food producing animals. At an earlier meeting (1994) CVMP were unable to set an ADI but considered that therapeutic and zootechnical uses presented no risk to consumers. 17 $\beta$ -oestradiol and its valerate and benzoate forms have subsequently been placed in Annex II of Regulation (EEC) 2377/90 for all food producing species [Commission Regulation (EC) 3059/94]. Annex II lists substances which will not leave harmful concentrations of residues in food if used as recommended and therefore do not need MRLs to be set. Use of 17 $\beta$ -oestradiol as a growth promoter is not permitted in the EU.

## Progesterone

6. The ADI for progesterone was set by JECFA in February 1999. An uncertainty factor of 100 was applied to the lowest observable effect level (LOEL) of 200 mg/person/day (equivalent to 3.3 mg/kg bw/day) for changes in the human uterus. JECFA recommended maximum residue limits (MRLs) "not specified" for progesterone residues in bovine muscle, fat, kidney & liver [summary & conclusions from the 52<sup>nd</sup> JECFA, to be published in WHO, Food Additive Series #43].

7. The CVMP are currently reassessing the safety of the therapeutic use of progesterone in food producing animals. At an earlier meeting, the CVMP considered that it was unnecessary to set an ADI or MRLs for progesterone, and recommended that progesterone should be placed in Annex II of Regulation (EEC) 2377/90 for all food producing species. The Standing Committee on Veterinary Products did not agree with this recommendation as they had concerns over the possible genotoxicity of progesterone. The future fate of therapeutic uses of progesterone awaits the outcome of the ongoing CVMP review of the sex hormones. Use of progesterone as a growth promoter is banned in the EU.

## Testosterone

8. The ADI for testosterone was set by JECFA, using an uncertainty factor of 1000 on a NOEL of 100 mg/person/day (equivalent to 2 µg/kg bw/day) for sexual function indices in a study of 5 eunuchs. JECFA recommended maximum residue limits (MRLs) "not specified" for testosterone residues in bovine muscle, fat, kidney & liver [summary & conclusions from the 52<sup>nd</sup> JECFA, to be published in WHO, Food Additive Series #43].

9. Therapeutic uses of testosterone within the EU are under consideration - France is the only EU country that uses testosterone in farm animals. The CVMP has deferred making a decision pending the provision of further residues data and oral bioavailability data. Use of testosterone as a growth promoter is not permitted in the EU.

## Zeranol

10. The ADI for zeranol was set by JECFA based on the no-hormonal-effect level in ovariectomised female cynomolgus monkeys [WHO, 1988, Food Additive Series #23]. MRLs have been set for bovine tissues: 2 µg/kg for muscle and 10 µg/kg for liver [Codex Alimentarius, 1995, ALINORM 95/21 Part 1; WHO, 1988, Technical Report Series, #763].

11. In the EU, zeranol is not permitted for any use in food producing animals although it may accidentally occur as a mycotoxin contaminant in some feeds.

12. In the UK, the Committee on Carcinogenicity (COC) considered the safety of zeranol in 1984 and the Committee on Toxicity (COT) also commented on this in 1987. The advice was that zeranol was non-genotoxic but there was insufficient information on carcinogenicity to allow the setting of an ADI.

13. In 1998, the Committee on Mutagenicity (COM) considered the possible mutagenicity of the mycotoxin zearalenone. (Note that zeranol [alpha-zearalenol] is metabolically interconvertible with zearalenone). The COM noted that it had been demonstrated that zearalenone could induce sister chromatid exchanges *in vitro* at high concentrations in the absence of metabolic activation and that it formed DNA adducts in the liver, kidneys and ovaries of mice exposed *in vivo*. In view of these results the COM

advised that it would be appropriate to regard zearalenone as being potentially genotoxic *in vivo*. It would be prudent to regard zeranol in a similar way, pending further investigation.

### **Trenbolone acetate**

14. In 1989, JECFA set an ADI for trenbolone acetate (TBA) by applying an uncertainty factor of 100 to the marginal effect level of 2 µg/kg bw/day for decreased testis weight and decreased serum concentrations of testosterone and oestradiol using TBA orally in growing male pigs in a 14 week study [WHO, 1990, Food Additive Series #25]. The ADI was in line with the no-hormonal-effect level of 2 µg/kg bw/day for β-trenbolone in monkeys [WHO, 1989, Technical Report Series #788]. TBA is an optically active compound. The 17alpha-epimer is the major metabolite in muscle from treated cattle [FAO Food and Nutrition paper 41/2].

15. In the EU, TBA is not permitted for any use in food producing animals.

### **Melengestrol acetate**

16. No ADI has been set for melenogestrol acetate (MGA), although the USA uses a tolerance value of 25 µg/kg for MGA in bovine fat [Code of Federal Regulations CFR21, Part 556]. JECFA plans to assess MGA at its meeting in February 2000.

17. In the EU, MGA is not permitted for any use in food producing animals.

### **Use of Food Intake Data to Estimate the Concentrations of Hormones that would cause ADIs to be Exceeded**

18. It is possible to calculate the maximum concentration of each hormone that may be in various foods without exceeding the ADI. There are different estimates available of the intakes of different foods. JECFA have proposed a series of standard portions for different foods that are used to set MRLs from ADIs. Using the JECFA standard portions to calculate daily intakes from residue concentrations tends to give an overestimate of the food intake of the average person. More accurate estimates can be obtained if food intake data from appropriate regional dietary surveys are used. UK surveys provide data on average intakes of food and on the intakes of extreme consumers (97.5 percentile) of certain foods.

19. The JECFA estimate assumes that an adult consumer has a bodyweight of 60 kg eats standard portions of various foods. The standard portions used are 300 g of muscle, 300 g of fish (with skin on), 100 g liver, 50 g kidney, 50 g fat (or skin + fat for pigs), 90 g poultry skin + fat, 1500 g of milk & dairy products, 100 g eggs & 20 g honey [36<sup>th</sup> JECFA, 1990]. Table II (below) gives an estimate of maximum concentration of each hormone that could be in each food. For each food under consideration, it is assumed that no other food in the diet contained residues of the hormone. The following formula has been used in the calculation:-

Max acceptable conc = (ADI in mg/60 kg person) ÷ estimated intake of food x 1000

### **Table II: Estimates of maximum acceptable concentrations (µg/kg) of**

### **hormones in JECFA standard portions of various foods eaten by**

### **adults**

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	<b>17<math>\beta</math>-oestradiol</b>	<b>Progesterone</b>	<b>Testosterone</b>	<b>Zeranol</b>	<b>Trenbolone</b>
Muscle	10	6000	400	100	4
Liver	30	18000	1200	300	12
Kidney	60	36000	2400	600	24
Fat	60	36000	2400	600	24
Fish	10	6000	400	100	4
Milk products	2	1200	80	20	0.8
Eggs	30	18000	1200	300	12
Honey	150	90000	6000	1500	60

20. Infants are assumed to eat roughly the same amounts of most animal-derived foods in proportion to their bodyweight as adults. The exception is milk. Infants drink about the same volume of milk in absolute terms as adults.

**Table III: Estimates of maximum acceptable concentrations ( $\mu\text{g}/\text{kg}$ ) of hormones in JECFA standard portions of various foods eaten by infants**

	<b>17<math>\beta</math>-oestradiol</b>	<b>Progesterone</b>	<b>Testosterone</b>	<b>Zeranol</b>	<b>Trenbolone</b>
Muscle	1.6	1000	66.6	16.6	0.6
Liver	5	3000	50	50	2
Kidney	10	6000	400	100	4
Fat	10	6000	400	100	4
Fish	1.6	1000	66.6	16.6	0.6
Milk Products	0.3	200	13.3	3.3	0.13
Eggs	5	3000	200	50	2
Honey	25	15000	1000	250	10

21. Recently the Advisory Group on Veterinary Residues (AGVR) compared the JECFA standard portions with the measured weekly dietary intakes of the adult UK population. (The intake by children has yet to be assessed by AGVR.) The intakes were measured over a week and so were divided by 7 to give a daily average over the week for comparison with the JECFA standard portions. AGVR noted that that for all the classes of foods, except honey, the 97.5<sup>th</sup> percentile chronic consumption of adults was less than the JECFA standard portion. Only small proportions of the population ate more than 300 g lean muscle (0.2%), 50 g animal fat (1.4%), 100 g eggs (0.5%) or 20 g honey (4.6%) on

an average day. No adults were found who consumed a daily average of more than 1.5 litres of milk or milk products, 100 g liver, or 50 g kidney. Table IV gives estimates of the concentrations of hormones in various foods that would be needed for the ADI to be exceeded if the daily intake for that food (averaged over a week) by extreme consumers (97.5<sup>th</sup> percentile) were to be consumed.

**Table IV: Estimates of maximum acceptable concentrations ( $\mu\text{g}/\text{kg}$ ) of hormones in average daily amounts of various foods eaten by UK adults who are extreme consumers**

Food	Extreme chronic intake of food (g/person/day)	17 $\beta$ -oestradiol	Progesterone	Testosterone	Zeranol	Trenbolone
Muscle	192.2	16	9370	624	156	6.2
Liver	35.4	85	50800	3390	847	34
Kidney	22.5	133	80000	5330	1333	53
Fat	44.4	68	40500	2700	676	27
Fish	?	-	-	-	-	-
Milk products	728.8	4.1	2470	165	41	1.7
Eggs	71.7	42	25100	1670	418	17
Honey	26.1	115	69000	4600	1150	46

22. Tables II, III & IV show that food residues from the use of 17 $\beta$ -oestradiol or trenbolone are more likely to cause toxicological problems than progesterone, testosterone or zeranol. Residue depletion data would be needed before the risk of the use of these hormones in food producing animals could be fully evaluated. However, even in the absence of residue data, it appears that the use of progesterone, testosterone or zeranol for growth promotion purposes would be unlikely to cause residue concentrations to be sufficiently high that the exposure of a consumer would be in excess of the ADI. On the other hand it is feasible that use of 17 $\beta$ -oestradiol or trenbolone in food animals could result in excessive levels of consumer exposure to residues. Thus it may be necessary to set MRLs for 17 $\beta$ -oestradiol & trenbolone.

23. If maximum residue limits (MRLs) were to be set for hormones they would need to be less than the concentrations shown in Table IV to ensure that the great majority of the UK population were not regularly exposed to more than the ADI for each hormone.

24. UK exposure data show that some people have occasional high intakes of foods that are greatly in excess of the 97.5<sup>th</sup> percentile average daily intakes of extreme consumers (shown in Table IV, above). For instance, the 97.5<sup>th</sup> percentile consumption of liver is 35.4 g/person/day, but the maximum intakes measured in any one day were 288 g for women and 418 g for men. It may be necessary to consider the toxicological significance of occasional high doses of the hormones.

### Comparison of Hormone Concentrations in Foods with ADIs

25. Table 10 in Hartmann et al, 1998 (sent out to members with the papers for the 1<sup>st</sup> meeting of the Hormones Working Group) gives estimates of the daily dietary intake and the endogenous production of the natural hormones progesterone, testosterone and

oestrogens (17 $\beta$ -oestradiol plus oestrone). None of the estimates of daily intakes of these hormones are close to being in excess of the ADIs, expressed in  $\mu\text{g}/60$  kg person (as shown in Table I, above). However, some of estimates of daily endogenous production are greatly in excess of the ADIs: progesterone in women; testosterone in men and to a lesser extent in women; and oestrogens for women and to a lesser extent for men and prepubertal children. This is not surprising as the hormones have a role to play in the body and must be present at hormonally active concentrations, whereas the ADIs are set at dietary levels which will have no observable additional hormonal effects.

26. Tables 1, 2, 3, 4, 5, 6, 7 & 8 in Hartmann et al, 1998, give information on the natural concentrations of hormones measured in various foods. Table 9 uses German food intake data to estimate the daily intakes of the hormones naturally present in meat/fish, milk products, eggs and vegetables for men, women, boys & girls.

27. Table V, below, shows the highest hormone concentrations reported. In most cases the information is taken from Hartmann et al. The figures for 17 $\beta$ -oestradiol in liver is taken from FAO Food & Nutrition Paper 41/12, which reports a higher concentration in pregnant cows than is reported by Hartmann et al. The kidney value is also taken from the FAO paper as Hartmann et al did not report kidney concentrations. The maximum amount of food containing the highest concentration of each hormone that a consumer could eat without exceeding the ADIs has been estimated in Table VI.

**Table V: Highest reported concentration ( $\mu\text{g}/\text{kg}$ ) of hormones naturally present in foods**

Food	17 $\beta$ -oestradiol	Progesterone	Testosterone
Muscle	2.45	27.4	2.8
Liver	1.027	1.85	1.16
Kidney	0.274	-	-
Fat	0.73	43.4	20.34
Fish	<0.03	0.51	0.07
Milk	0.06	12.5	0.15
Butter	<0.03	300	<0.05
Eggs	0.22	43.6	0.49
Potatoes	<0.03	5.07	<0.02

**Table VI: Maximum amount of food naturally containing the highest hormone concentration that may be eaten in a day with assurance that the ADI will not be exceeded**

Food	17 $\beta$ -oestradiol	Progesterone	Testosterone
Muscle	1.2 kg	66 kg	43 kg
Liver	2.9 kg	973 kg	103 kg

Kidney	10.9 kg	-	-
Fat	4.1 kg	41 kg	5.9 kg
Fish	>100 kg	3530 kg	1700 kg
Milk	50 kg	144 kg	800 kg
Butter	>100 kg	6.0 kg	>2400 kg
Eggs	14 kg	41 kg	245 kg
Potatoes	>100 kg	355 kg	>6000 kg

28. Table VI gives assurance of the safety to the consumer of the amounts of 17 $\beta$ -oestradiol, progesterone & testosterone naturally present in foods. The concentrations are sufficiently low that a consumer would need to eat an unrealistically large amount (more than 1000 g per day) of any of the foods to exceed the ADI of any of the individual hormones. The margin of safety is lowest in the case of 17 $\beta$ -oestradiol. The combined hormonal activity of 17 $\beta$ -oestradiol plus other dietary oestrogens (eg, oestrone and phytoestrogens) is not known. If MRLs were to be set for use of hormones in food producing animals, it would be advisable to take account of the dietary exposure as a result of the natural presence of some hormones in food.

#### **Food Residues Following the Use of Growth Promoters**

29. Residues data for some of the growth promoting hormonal agents are presented in a monograph prepared by the 32<sup>nd</sup> JECFA, 1987 [FAO Food & Nutrition Paper 41].

#### **Oestradiol**

30. Measurement of residues in steers following the use of a 17 $\beta$ -oestradiol implant, "Torelor", showed that much of the residue present in liver and kidney was in the form of conjugated 17 $\beta$ -oestradiol. Other residue was present as free 17 $\beta$ -oestradiol and oestrone. It is unclear whether the conjugated hormone and oestrone would be hormonally active when ingested by consumers, but it would be prudent to assume that all of these residues would be active. Table VII shows the extra residues resulting from implantation (peak mean levels minus the mean levels in untreated steers).

**Table VII: Additional oestrogen residues ( $\mu\text{g}/\text{kg}$ ) resulting from the use of Toreclor" (40 mg 17 $\beta$ -oestradiol + 200 mg progesterone) in steers**

	<b>Free 17<math>\beta</math>-oestradiol</b>	<b>Conjugated 17<math>\beta</math>-oestradiol</b>	<b>Oestrone</b>
Muscle	0.017	0.011	-
Liver	0.041	0.271	0.046
Kidney	0.037	0.076	-
Fat	0.112	0.014	0.071

31. The data on the endogenous amounts of total oestrogens, the amount in the diet and the residues resulting from use as growth promoters are brought together in Table VIII. Data from Hartmann et al have been combined with an estimate taken from Table VII using the JECFA adult portion sizes (300 g muscle, 100 g liver, 50 g kidney, 50 g fat) and assuming bodyweights of 60 kg for adults and 10 kg for children. For men, the intakes of oestrogens in the normal diet and the additional oestrogens due to use of 17 $\beta$ -oestradiol as a growth promoter take up only 3.3% and 1.7% of the ADI respectively.

**Table VIII: Exposure ( $\mu\text{g}/\text{person}/\text{day}$ ) to oestrogens (free & conjugated 17 $\beta$ -oestradiol + oestrone)**

	Endogenous	Normal diet	Due to use in cattle
Men	140	0.10	0.0517
Women	630	0.08	0.0517
Boys	100	0.08	0.0086
Girls	54	0.07	0.0086

### Progesterone

32. Results from residue studies of steers implanted with "Synovex-S" (20 mg oestradiol benzoate + 200 mg progesterone) showed peak additional mean progesterone levels (highest mean value minus the mean control level) of 0.31, 0.09, 0.15 & 1.19  $\mu\text{g}/\text{kg}$  in muscle, liver, kidney & fat, respectively. For men, the intakes of progesterone in the normal diet and the additional progesterone due to its use as a growth promoter take up only 0.59% and 0.009% of the ADI respectively.

**Table IX: Exposure ( $\mu\text{g}/\text{person}/\text{day}$ ) to progesterone**

	Endogenous	Normal diet	Due to use in cattle
Men	420	10.6	0.169
Women	19600	9.0	0.169
Boys	150	8.9	0.024
Girls	250	8.1	0.024

### Testosterone

33. Peak additional mean testosterone levels following use of "Synovex-H" (20 mg oestradiol benzoate + 200 mg testosterone propionate) in heifers were 0.0814, 0.0212, 0.262 & 0.3135  $\mu\text{g}/\text{kg}$  in muscle, liver, kidney & fat, respectively. For men, the intakes of testosterone in the normal diet and the additional testosterone due to its use as a growth promoter take up only 0.058% and 0.046% of the ADI respectively.

**Table X: Exposure ( $\mu\text{g}/\text{person}/\text{day}$ ) to testosterone**

	Endogenous	Normal diet	Due to use in cattle

Men	6480	0.07	0.0553
Women	240	0.05	0.0553
Boys	65	0.05	0.0092
Girls	32	0.04	0.0092

### Zeranol

34. Only small-scale residue studies were available for zeranol. The summaries given in the FAO document indicate that implantation with 36 mg zeranol would result in residues of approximately 0.2, 0.35, 0.075 & 0.2 µg/kg in muscle, liver, kidney & fat, respectively. Use of the JECFA portion sizes to estimate the dietary intake for adults gives a figure of 0.169 µg/person/day, which is about two-hundredth (0.56%) of the ADI of 30 µg/person/day.

### Trenbolone

35. Peak additional mean alpha-trenbolone levels following use of "Torelor" (40 mg 17β-oestradiol + 200 mg trenbolone acetate) in steers were 0.014, 1.871, 0.347 & 0.018 µg/kg in muscle, liver, kidney & fat, respectively. These concentrations are estimated to give a dietary intake for adults of 0.210 µg/person/day, which is approximately a sixth (17.5%) of the ADI of 1.2 µg/person/day.

### Melengestrol acetate

36. No residue data available.

37. Tables VIII, IX & X show that the contribution of residues resulting from the use of natural steroid hormones as growth promoters in cattle would be expected to contribute less than or levels of the same order of magnitude as natural dietary sources of the hormones. At worst the use of these natural hormones as growth promoters could double the dietary intakes of these substances. It should be noted however that the intakes from all sources are very low compared with the amounts naturally produced by the human body. The increases in consumer exposure to these hormones as a result of their use as growth promoters in cattle would be miniscule compared with the amounts of these hormones produced naturally in the body.

38. The use of the synthetic growth promoters, zeranol and trenbolone acetate, would give low concentrations of residues in food which when eaten would expose consumers to low doses which were easily within the range of the ADI for each substances. However, the residue data for zeranol was of poor quality and it should be noted that the COM had some outstanding concerns about the possible mutagenicity of zeranol.

39. Comparison of these estimates with the ADIs set by JECFA suggests that the use of 17β-oestradiol, progesterone, testosterone & trenbolone acetate as growth promoters in cattle would not cause consumers to be exposed to harmful levels of residues in food. However, before full assurance could be given of the safety of the use of any of these substances as a growth promoter, it would be necessary to thoroughly review both the published and the company-owned toxicological data. It would also be necessary to measure the residues left in food derived from treated animals kept under European conditions.

40. Further toxicological and residues information will be needed before assurance may be given about the safety of zeranol.

41. It was not possible to estimate the consumer risk from the use of melenogestrol acetate as a growth promoter.

### **Consumer Risk from Ingesting a Whole Implant**

42. Implants of growth promoters should be placed under the skin in a part of the animal such as the ear that can be discarded after slaughter. However, if an implant accidentally ends up in human food, it may expose the consumer to a large one-off dose.

43. Table XI gives estimates of the maximum dose that may be received by a consumer eating one fresh implant. The amounts of each substance in a variety of implants are listed in the FAO paper 41.

**Table XI: Dose from eating one fresh implant**

<b>Substance</b>	<b>Maximum amount in one implant (mg)</b>	<b>ADI (mg/person)</b>	<b>Consumer exposure as a proportion of the ADI</b>
17 $\beta$ -oestradiol	40	0.003	13333
Progesterone	200	1.8	111
Testosterone	200	0.12	1667
Zeranol	36	0.03	1200
Trenbolone acetate	300	0.0012	250000

44. No data were available for melenogestrol acetate.

45. In all cases, ingestion of an implant could give a one-off dose in excess of the ADI by several orders of magnitude, ranging from 111 times the ADI for progesterone to 250,000 times the ADI for trenbolone acetate.

46. It is recommended that, before any of these substances are approved for use as growth promoters, the risk from accidental ingestion of a whole implant should be considered. The risk of an implant ending up in human food should be estimated, and the toxicological significance of a one-off oral exposure to such a high dose should be addressed.

47. Conclusions:

- Endogenous production of 17 $\beta$ -oestradiol, testosterone and/or progesterone by some people is much higher than the likely consumer exposure resulting from the ingestion of residues of these hormones in food from treated animals.
- The amounts of 17 $\beta$ -oestradiol, testosterone and/or progesterone naturally present in the UK diet are unlikely to be harmful in themselves, but should be taken into account when considering the extra exposure from food residues due to the use of these hormones in

food producing animals.

- It is unlikely that the use of progesterone, testosterone or zeranol for growth promotion purposes could cause residues to be sufficiently high that the exposure of a consumer would be in excess of the ADIs set by JECFA. However, it is feasible that abuse of 17 $\beta$ -oestradiol or trenbolone could result in harmful levels of exposure to residues. Thus it is recommended that if the hormones ban is lifted, the setting of maximum residue limits (MRLs) for 17 $\beta$ -oestradiol & trenbolone should be considered.
- No conclusions can be made about the safety of residues resulting from the growth promotional use of melenogestrol due to absence of any useful data.
- There is a potential for adverse health effects in consumers if an intact growth promoting implant entered human food.

## **THE LIKELY INCREASE IN LEVELS OF CONSUMER EXPOSURE TO 17 $\beta$ -OESTRADIOL AS A RESULT OF ENDING THE MEASURES PUT IN PLACE TO COMBAT BSE**

### **Introduction**

48. The age and sex structure of the cattle population in the UK determines exposure to natural hormones in meat. Two schemes introduced in 1996 as part of the Bovine Spongiform Encephalopathy (BSE) control measures have had an influence on current intakes in food.

49. The Over Thirty Months Scheme (OTMS) prevents the sale of meat from cattle over 30 months of age to reduce any risk to humans from BSE and to raise public confidence in the safety of beef. In addition, a voluntary scheme was introduced, the Calf Processing Aid Scheme (CAPS), which pays aid on male calves, aged 20 days or less, withdrawn from production. This assists dairy and beef farmers by helping to balance the market, as these calves can no longer be exported. The calves do not go into the human food chain. The rules for the scheme are set out in two EC regulations: Council Regulation 805/68/EEC and Commission Regulation 3886/92/EEC as amended.

50. The purpose of this section is to provide a summary of endogenous production and to estimate the changes of exposure to 17 $\beta$ -oestradiol that might occur from the ending of the OTMS and CAPS. The underlying assumptions informing the following are in Annex 1 at the end of this paper.

### **Endogenous Production**

51. Natural hormones are not only part of the diet (exogenous) but are also produced by the consumer (endogenous production). The relative quantities from these sources vary with age, sex and intake.

52. The following tables of oestradiol-17 $\beta$  show (1) the estimated daily intakes of different age and gender consumer groups and (2) estimated daily production/daily intake. These are included to allow an assessment of the effects of changes in the market.

**Table (1) Estimated daily intakes of age and gender consumer groups in ug/d<sup>-1</sup>**

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	Meat/fish	Dairy products	Eggs	Vegetable*
Men	0.02	0.06	0.02	0.00
Women	0.01	0.05	0.02	0.00
Boys (prepubertal)	0.01	0.06	0.01	0.00
Girls (prepubertal)	0.01	0.05	0.01	0.00
Relative contribution	15-20%	60-70%	15-20%	<10%

From this table a prepubertal girl has a presumed intake of 0.07 ug/day (70ng/day).

53. The estimated endogenous production (Hartmann, Lacorn & Steinhart 1998) puts this into context.

**Table (2) Estimated (ug/d<sup>-1</sup>) daily production / daily intake of oestradiol-17B + oestrone**

	Daily production	Daily intake
	(ug/day)	(ug/day)
Men	140	0.10
Women	630	0.08
Girls (prepubertal)	54	0.07
Boys (prepubertal)	100	0.08

The lowest production of oestradiol-17B is that in the prepubertal girl. This is estimated at 54 ug/day (54,000 ng/day).

(Summary 0.07ug/day intake : 54ug/day produced or .13%).

## OTMS

54. The OTMS removes culled adult cows from the food chain. During pregnancy the concentration of oestrogens increases in these cows. Several surveys suggest that approximately 30% of these will be pregnant when killed ( e.g. Singleton and Dobson 1995 Vet. Record 136, 162-165). The effect of these animals entering the food chain, (as the OTMS is phased out and replaced by a date based scheme - effectively returning the market to pre-BSE status) will be to raise oestradiol-17B intake. To estimate the effects of these animals on overall intakes it is assumed that for a 450 kg cow (estimated 50% kill out) there would be 225 kg dressed carcass comprised of 18.5% bone, 66.8% red meat, 12.3% fat, 2.3% waste, (Source : Collaborative trial between University of Nottingham, Hoechst, IRAD, MLC (date unknown)) 5.5 kg liver and 0.6 kg kidney (Source : Meat Hygiene Ed J F Gracey published Bailliere Tindall 1986). The estimated

annual cull into the OTMS is 778,000. If 25% are in calf (and at stages of pregnancy evenly distributed over the three trimesters) then 194,500 cows would each add 7021.6 ng oestradiol-17B into the food chain. (A total of 1.366g. on an annual basis)

55. All slaughtered animals (approximately 3 million) contribute some oestradiol to the dietary intake from meat. Assuming that the non-pregnant heifer can be taken as an average then the base quantity is approximately 3.649g.

56. On this base the removal of these pregnant cull cows to the food chain has reduced the quantity of oestradiol in the food chain by 37% (1.366/3.649) which will be returned as the BSE controls are removed and the market returns to normal.

### **Implications of return to normal marketing**

57. From Table 1 above a prepubertal girl has a presumed intake of 0.07 ug/day (70 ng/day). In the extreme case the effect of altering her diet completely to pregnant cow beef would be to double this intake. However the bulk of the meat will still come from non pregnant animals therefore the actual increase will be 16.5 ng/day (approx 25% increase).

58. Table 2 shows that the lowest production of oestradiol-17B also occurs in the prepubertal girl. This is estimated at 54 ug/day (54,000 ng/day). When compared to the endogenous production the intake from meat and meat products is 0.13% (70 ng eaten; 54,000 produced endogenously). The additional intake arising from the slaughter of pregnant cows does not materially shift the balance (86.5 ng eaten; 54,000 produced (or 0.16%). This proportion become smaller when the contribution of other dietary components, milk, eggs and vegetables are also included.

### **CAPS**

59. The second change which will occur as the BSE controls are removed will be the return of dairy bull calves to the market as potential meat producing animals. These are not suitable for the production of quality meat and could benefit from the re-introduction of hormonal growth promoting implants. (Note these implants are banned within the EU therefore **this is a theoretical exercise**).

60. Assuming that there could be 100% use of Synovex implants (applied as recommended by the manufacturer) in these animals and that they were slaughtered 50 days after implantation then the addition would be an increase in the daily intake of 42.25ng per day for a consumer eating only this meat. However this figure needs to be adjusted to take account of other unimplanted cattle. Assuming that 700,000 were implanted out of a total of 3,500,000 then the figure would be reduced to 20% making a theoretical increase of 8.25 ng/day.

### **Implications**

61. The implications for the most vulnerable consumer need to be considered. The effect of the introduction of properly implanted animals would be a 12% increase (8.25ng/day increase over the normal dietary estimate 70ng/day (see above)) in intake. The lowest production of oestradiol-17B (see above) is that of the prepubertal girl estimated at 54 ug/day (54,000 ng/day). As above this would not materially shift the balance between dietary and endogenous oestradiol-17B.

### **GENOTOXIC EFFECTS OF OESTROGENS**

### Information necessary to define a genotoxic carcinogen

62. Genotoxic carcinogens are defined as agents which interact with the hereditary molecule DNA to produce modifications (adduct formation) of its components which following replication of DNA can lead to modification of the DNA sequence i.e. mutations. These mutations may involve discrete changes in the bases of the DNA (point mutations) or larger changes involving the transfer of whole regions between individual chromosomes (chromosome rearrangements). Not all the agent induced changes in DNA structure lead to mutations as all living cells possess highly effective mechanisms i.e. the repair systems which remove damage DNA and replace the damage with the original bases in an error-free manner. In general, it is only when the amount of damage to the DNA overwhelms the DNA repair systems that mutations are produced (for review see Friedberg et al. 1995).

Reference: Friedberg, E.C., G.C. Walker and W. Wiede, 1995, DNA repair and mutagenesis. **American. Soc. For Microbiology. Washington D.C., USA.** 698 pp.

63. When mutations are produced in key regulatory genes of the living cells they can lead to changes in cellular behaviour such as cellular immortality and uncontrolled proliferation, which are characteristic of tumours. Many methods are now available which allow the measurement of agent induced damage to DNA. Such measurements can be used to estimate exposure to the DNA and provide estimates of "potential" mutagenicity. However, as pointed out earlier not all DNA adducts lead to mutations and a critical requirement in the determination of genotoxic activity is that the assay systems used measure inherited changes, i.e. mutations.

64. At both the European and International Levels there have been extensive discussions and evaluations of test methods suitable for the detection and evaluation of genotoxic chemicals. The most recent International discussions were those of the International Conference on the Harmonisation of Genotoxicity Test Methods (ICH 1994, 1996).

References: 1) ICH (1994) Proceedings of the Second International Conference on Harmonisation of Technical Requirements for the Registration of Pharmaceuticals for Human Use (ICH), Orlando 1993, Queens University Belfast, UK, 221-257.

2) ICH (1996) Yokohama 1995, Queens University, Belfast, UK. 303-329.

65. International consensus amongst the scientific and regulatory bodies of all the major industrialised nations has led to a general acceptance that to define the potential genotoxicity of a chemical there is a need for information from both *in vitro* and *in vivo* test systems. The generally accepted test methods are outlined below:-

1) Bacterial point mutation assays using *Salmonella typhimurium*.

2) *In vitro* mammalian cell cytogenetics assay measuring the induction of chromosome damage.

3) *In vitro* mammalian cell point mutation assay - generally the mouse lymphoma tk<sup>+</sup>/tk<sup>-</sup> test system.

4) *In vivo* rodent bone marrow cytogenetics or micronucleus assay.

66. In the case of human pharmaceuticals ICH has recommended **either** an *in vitro* test for chromosome damage in cultured mammalian cells **or** an *in vitro* mouse lymphoma

assay tk<sup>+</sup>/tk<sup>-</sup> as an alternative method to detect chromosome damaging effects (clastogenic). The International cooperation of harmonisation of technical requirements for the registration of veterinary medicinal products (VICH) is currently discussing whether this approach can also be used for assessing veterinary medicines. It is important to note at this point that the internationally accepted methods detect and measure the ability of a chemical to induce **inherited** changes.

### **Evaluation of the potential genotoxicity of oestradiol**

67. Oestradiol has been evaluated for its potential genotoxicity in a series of widely used and well validated mutagenicity assays i.e.

- Ames Bacterial Mutation Assay
- Mouse lymphoma L5178Y TK<sup>+</sup>/TK27<sup>-</sup> point mutation assay.
- *In vitro* micronucleus assay for chromosome damage in Syrian hamster V79 cells.
- *In vivo* in rat bone marrow and male germ cell assay

All the above gave negative results.

Reference: Richold (1988). The genotoxicity of trenbolone, a synthetic steroid. **Arch. Toxicol.** 61, 249-258.

68. It should be noted that there are no reported results involving the evaluation of Oestradiol in a "standard" cytogenetics assay, such as one using cultured human lymphocytes. This is a major deficiency in the available data set. The SCVPH report uses a number of "non-standard" assay results in drawing its conclusions regarding the potential genotoxicity of Oestradiol. These individual methods and their results are reviewed below:-

Reference: 1. Thibodeau *et al.* (1998). Induction by estrogens of methotrexate resistance in MCF-7 breast cancer cells. **Carcinogenesis** 19, 1545-1552.

69. This paper demonstrated that Oestradiol and its metabolites induce methotrexate resistance in a cell line isolated from a breast carcinoma. The endpoint characterized i.e. increase in resistance to methotrexate is not generally recognised as being capable of defining the mutagenicity of a chemical. When methotrexate is withdrawn the resistance is lost i.e. it is not a stable endpoint. By definition, mutagenicity is the ability to induce **an inherited change** in the genes of an organism. The authors themselves conclude that the endpoint of methotrexate resistance could be the result of either genotoxic or non-genotoxic events.

Reference: 2. Rajah and Pento (1995). The mutagenic potential of antiestrogens at the HPRT locus in V79 cells. **Res. Comm. In Molecular Pathology and Pharmacology.** 89, 85-92.

70. This paper claims to demonstrate that Oestradiol at a concentration of 10<sup>-10</sup> M increases mutation frequency at the HPRT locus of Chinese hamster V79 cells. The increase reported is from 1.81 mutants/10<sup>6</sup> surviving cells to 6.11 mutants/10<sup>6</sup> surviving cells. The raw data obtained in the experiment is not reported and under the same treatment conditions no increases were reported for treatments with Oestradiol at 10<sup>-8</sup>M

and  $10^{-9}$  M (i.e. there was no dose related response).

71. There is a fundamental technical defect in this paper. i.e. relating to the actual number of cells evaluated for the mutagenic effects of Oestradiol. The experiment involved the treatment of 10 dishes each containing  $2 \times 10^5$  cells. i.e. a total number of  $2 \times 10^6$  cells. This means that over 10 dishes the authors only detected approximately 2 mutant colonies in the control plates and about 5 mutant colonies in the  $10^{-10}$  M treatment plates. The failure to demonstrate a dose-related increase in HPRT raises considerable doubts about the adequate performance of the test. The results presented are therefore not significant and do not unambiguously demonstrate the genotoxicity of Oestradiol.

72. There are a number of publications which demonstrate that Oestradiol induces both micro- and mini-satellite alterations.

References: 1. Hodgson *et al.* (1998). Estrogen-induced micro-satellite DNA alterations are associated with Syrian hamster kidney tumorigenesis. **Carcinogenesis**, **12**, 2169-2172. Treatment with 25 mg Oestradiol per Syrian hamster.

2. Paquette (1996). Enhancement of genomic instability by 17  $\alpha$ -oestradiol in mini-satellite sequences of X-ray-transformed mouse 10T $\frac{1}{2}$  cells. **Carcinogenesis**, **17**, 1221-1225.

73. Although micro and mini-satellite changes in DNA may have a role in cancer development, this is not an endpoint which is internationally recognised as a suitable indicator of genotoxic activity.

74. Oestradiol is reported in the SCVPH Opinion to induce chromosome aberrations at a concentration of  $2 \times 10^{-5}$  M in Chinese hamster V79 cells (Eckart and Stopper 1996).

Reference: Eckart and Stopper (1996). Genotoxic effects induced by  $\alpha$ -oestradiol *in vitro*. **Toxicology in vitro**, **10**, 637-642.

75. However, the paper actually demonstrated that the micronuclei are the result of whole chromosome loss resulting from a disturbance of mitotic cell division rather than chromosome structural damage.

76. Disturbances in chromosome segregation have been reported in a number of publications following Oestradiol exposure. However, this is generally assumed to be a process **not** dependent upon DNA reactivity and is probably thresholded and therefore not induced at low exposure concentrations.

77. A variety of publications demonstrate that Oestradiol induced oxidative damage and some DNA adducts.

References: 1) Cavalieri *et al.* (1997). Catechol estrogen - 3,4-quinones as endogenous tumour initiators. **PNAS**, **94**, 10937-10942.

2) Frenkel (1992). Carcinogen-mediated oxidant formation and oxidative DNA damage. **Pharmac. Ther.** **53**, 127-166.

78. However, oxidative damage to DNA is a natural phenomenon and living cells have extensive protective mechanisms against such damage. Therefore, the induction of oxidative damage is probably thresholded and at low concentrations the damage produced is within the range of that produced by endogenous metabolic reactions.

79. **It should be noted that Oestradiol does not induce mutagenic changes in any of the standard mutagenic assay which can be predicted to detect any increase in genetic damage produced by oxidative reactions. None of the publications reviewed above provide any substantive evidence that Oestradiol is mutagenic/genotoxic.**

#### **Evaluation of other hormones**

##### Testosterone

80. All the available genotoxicity data on testosterone is negative.

##### Progesterone

81. No data is available on the potential genotoxicity of progesterone.

##### Trenbolone

82.  $\alpha$  -TBOH all standard assay results are negative

$\beta$  -TBOH there are positive results reported in the base substitution mutation tester strain of the bacteria *Salmonella typhimurium*. However, positive results have not been reported in mammalian cells and in the somatic and germ cells of rodents.

##### Zeranol

83. There are no results for Zeranol available for any standard mutagenicity assay. There are positive results reported for a DNA damage indicator test in bacteria.

##### Melengestrol

84. No data is available on the potential genotoxicity of melengestrol.

85. In view of these deficiencies it is not possible to evaluate their genotoxic potential. However, we find it difficult not to believe that data on the genotoxicity of these hormones is not available from the manufacturers.

#### **General overview of Genotoxicity data**

86. When assayed in the internationally accepted test systems for the measurement of agent induced genotoxicity none of the hormones considered produced positive results (with the exception of  $\beta$  -TBOH which gave positive results in a strain of bacteria, but this result was not reproduced in either cultured mammalian cells or intact animals). **On the basis of these data, we conclude that there is currently no positive results from internationally accepted test systems which indicate that the hormones considered in the report are genotoxic.** The SCVMPH assessment of the genotoxicity of the hormones is based upon data generated by methodologies that:-

- 1) Are not internationally accepted as measures of genotoxic potential
- 2) Measure the induction of DNA adducts rather than mutations
- 3) Measure the induction of chromosome numerical changes (aneuploidy)

In the case of both 2) and 3) the endpoints measured are generally considered to be thresholded and thus do not represent genotoxic risks at low exposure doses.

**Recommendations as to future research needs and assessment method development**

87. Refer to Department of Health Advisory Committee on Mutagenicity for an expert opinion on the relevance of the endpoints of methotrexate resistance and microsatellite changes.

88. Obtain complete data package for all the hormones using the standard battery and assays.

89. Determine the aneuploidy activity of the hormones and evaluate the significances of aneuploidy in hormonally induced tumours

90. Ensure that EU Committees evaluating genotoxicity work with individuals with adequate expertise.

**ANALYTICAL METHODS FOR ASSESSING THE PLASMA LEVELS OF OESTRADIOL**

91. The Opinion places emphasis on plasma oestradiol data derived using a recently developed yeast cell assay (Klein et al, 1994). This information is important for the risk assessment because the mean plasma levels of oestradiol in the groups of humans considered at most risk from any additive hormonal exposure to exogenous oestradiol (i.e. prepubertal males and females) are much lower with the new assay than with conventionally used radioimmunoassays (RIAs): i.e. 0.6 and 0.08 pg/ml in the prepubertal males and females, respectively, compared with 8-23 and 5-14 pg/ml using RIAs (Klein et al, 1994). Thus it is argued that the amount of exogenous oestradiol to affect these subjects is much less than previously accepted. The new assay uses yeast genetically engineered to express human oestrogen receptor alpha together with a so called "reporter" system consisting of an oestrogen response element linked to  $\beta$  - galactosidase (as a convenient indicator). The quantification of oestradiol depends on its specific binding to, and activation of, the oestrogen receptor.

92. An advantage of the new assay is that is c.100-fold more sensitive than most radioimmunoassays (RIAs). There are, however, concerns about the reliability of this analytical approach, which has been very little used in peer-reviewed publications other than by the originators of the assay, despite its initial publication in 1994. These concerns throw doubt upon the values derived by Klein et al and therefore also on the conclusions of the opinion.

93. The new assay involves simple ether extraction of plasma, and the exposure of yeast to the crude extract reconstituted in buffer but no chromatographic purification. The ether extraction will select non-conjugated oestrogens but also any other lipophilic compounds, including, for example, phytoestrogens which are oestrogen-like compounds present in many vegetables. As explained above, the response of the yeast to the extracted compounds is due to their binding to transfected oestrogen receptor and consequential binding of the receptor-ligand complex to a transfected DNA sequence. Such activation can be achieved by a large variety of oestrogenic compounds other than oestradiol. Cross-reaction with some of these is considered and found to be small but many others are not assessed. Competitive binding by some of these "cross-reactants" may lead to positively biased results but others could lead to a reduced response by the yeast and to aberrantly low results. Evidence that these problems are not entirely theoretical is provided by the published description of the assay (Klein et al, 1994):

i) it is noted that "blood was collected in glass Vacutainers that were kept upright at all times to avoid contamination with substances in the rubber stoppers that otherwise cross-

reacted in the assay", revealing that unknown agents may react significantly.

ii) probably of more importance "standards were prepared in charcoal-stripped plasma to avoid a negative blank that occurred against an oestradiol standard curve that had not been exposed to plasma." Although the nature of the "blank" is not defined this phenomenon confirms that there are substances which can reduce the yeast response and give aberrantly low values if they are present at higher concentrations in samples than in the charcoal-stripped serum.

94. A set of data has been derived using this assay in plasma from postmenopausal women and reports values of c.2pg/ml. (Klein et al, J Clin Endocrinol Metab, 1995, 80, 2658-2660). In contrast the most sensitive RIAs report mean plasma levels of 6-10 pg/ml (e.g. Dowsett et al, Clinical Cancer Research 1995, 1, 1511-1515; Geisler et al, Brit. J. Cancer 1996, 74, 1286-1291; Klein et al, J Clin Endocrinol Metab, 1995, 80, 2658-2660). In this case there are data which strongly support the validity of the RIA results:

i) radioactive tracer studies have established transfer constants from the precursor androgens to oestrogens and also metabolic clearance rates (Lonning et al, J. Steroid Biochem. Md. Biol, 1997, 61, 255-260). In essence, these studies have established using definitive technology, the amount of oestradiol produced from all sources and the rate at which it is metabolized allowing a good approximation of its expected plasma concentration in post-menopausal women of c.6pg/ml.

ii) the application of a number of highly specific aromatase inhibitors to postmenopausal women has been shown to inhibit the enzyme in vivo by >97% (Dowsett et al, 1995; Geisler et al, Brit. J. Cancer 1996, 74, 1286-1291). The fact that RIA-measured oestradiol values are reduced to undetectable levels (<3pmol/L) indicates that the difference must be due to endogenously derived oestrogen. Given that in some cases these assays involve a chromatographic step the result is assured as due to oestradiol. (Data from these RIAs have been key components of the pharmacological development of aromatase inhibitors and the licensing of these as medicines by regulatory authorities in Europe and the US).

95. Unless further validity work is performed with the yeast assay (specifically chromatographic purification of extracts prior to analysis) most weight should be given to the most sensitive RIAs. (It is correct to say that there have been variable results from RIAs applied to low oestradiol concentrations, but the more recent use of the highly sensitive assays has provided much more consistency).

96. The Opinion recognises (section 3.2) that it is possible that the oestradiol levels in beef might also be lower if this assay was applied to meat but such data are not available. The opinion thereafter compares concentrations of oestradiol in beef with yeast-measured concentrations in plasma. This is inappropriate and may lead to a biased inappropriate perspective.

## **EFFECTS OF GROWTH PROMOTING HORMONES IN MEAT ON THE IMMUNE SYSTEM AND DISORDERS OF IMMUNITY AND THEIR ASSOCIATED DISEASES**

### **Immune System**

97. The SCVPH report attempts generally to associate the state of the immune system and disorders of immunity and their associated diseases with sex hormones. It assumes that all the growth promoting substances used in meat production will have the same

actions as the classical sex hormones, and suggests that ingested hormone residues in meat may alter the direction and level of activity of the immune system in harmful ways.

98. It notes experimental and clinical evidence that sex hormones may affect the immune system [Section 2.4]. It refers to the fact that autoimmune diseases, such as lupus erythematosus, occur much more commonly in women than men [Section 2.4.1] and assumes that sex hormones are the cause. None of the available and well-regarded alternative hypotheses are considered. In Section 2.4.2 there is a brief discussion of pregnancy, in which the temporary development of immune tolerance in the mother is required to prevent rejection of the fetus as a foreign "graft". In addition, the fairly recent, well-established increase in the incidence of allergic disorders, notably asthma and atopy, especially in children and adolescents, in countries with a Western life style is mentioned, and a possible link to meat in the diet is proposed, analogous to that demonstrated for colorectal cancer.

99. The discussion points out that there is no direct evidence of a causal relationship between any of the disorders and the growth promoting hormones, but it suggests that there might be associations between those substances ingested as residues in meat and the development of these diseases.

100. The classical sex hormones, oestrogens, testosterone and progesterone, and presumably substances with analogous actions, do affect the structure and functions of the immune system in laboratory and farm animals and in humans by a variety of means. In addition, some of the signalling substances of the immune system, certain cytokines, affect the functioning of the reproductive system, probably by altering the release and effects of a variety of hormones. In general terms, females show greater activity of the immune system than males, but both sexes have receptors for sex hormones on T- and B-cells and their precursors the principal cells of the immune system. Oestrogens tend to stimulate immune functions at low concentrations and to be inhibitory at high levels. Progesterone also stimulates immune responses in low doses, whereas androgens, such as testosterone, tend to reduce activity over a wide range of concentrations.

101. Amongst the clearest examples of the influence of sex hormones on immune defences are the alterations in the responses to infections to bacterial and other infections in man and animals produced by sex hormones; it is common to find that high doses of oestrogens, progestagens and androgens all tend to reduce resistance to micro-organisms and other parasites in farm and laboratory animals, and probably in humans, too, whereas low doses of oestrogens stimulate resistance.

102. The autoimmune diseases, such as lupus erythematosus and rheumatoid arthritis, are more common in women than men, and their experimental models show more marked disease effects in female than in male animals.

103. The major weaknesses in the SCVPH report include failure to take account of the relationship between dose and effect, ignoring differences between the actions of oestrogenic and androgenic substances, and the simplistic view that, because a substance has one action like a sex hormone, it will cause all of the same effects as the hormone. The most important point is that the maximum dose of a growth promoting substance ingested in the normal diet, even by an extreme meat eater, would be only a small fraction of the quantity of the hormones normally produced each day in the body, i.e. the increase in 'exposure' to hormonally active substances would be so small as to be negligible, whether one considers the hormones naturally present in cattle or the additional residues that might be present following use of growth promoting substances. It also ignores the fact that low levels of oestrogenic and androgenic substances tend to have opposite actions on the immune system, but, as both types of substance may be

used, it is quite unclear what effect mixed exposure might have, if there were sufficient present to exert any activity at all.

104. The rising incidence of asthma and other allergic diseases affects children and young adults of both sexes. It may be associated with changes in life style and in exposure to allergens, pollutants and infections in early life, as suggested by epidemiological studies showing that the diseases occur in wealthier countries. There is no evidence to associate it with eating meat from cattle treated with growth promoting substances, nor with any particular dietary factor. The fact that the increase has occurred in people of either sex also argues against a role for sex hormones.

105. The general role of sex hormones in affecting immune functions has been discussed in several recent reviews, which cover both qualitative and quantitative aspects in man and animals.

106. Actions of oestrogens, progesterone and androgens have been examined but not those of trenbolone, zearalenone and melenogestrol.

Ahmed SA, Talal N 1990. Sex hormones and the immune system-Part 2. *Animal Data. Balliere's Clin Rheumatol*, 4, 13-30.

Fox HS 1995. Sex steroids and the immune system. In *Non-Reproductive Actions of Sex Steroids*. Eds Bock GR and Goode JA. CIBA Foundation Symposium 191. Pp 203-217. Wiley, London.

Lahita RG 1990. Sex hormones and the immune system-Part1. Human data. *Balliere's Clin Rheumatol*, 4, 1-12.

## **2. Section 2.4 discusses 'sexual' hormones and the immune system**

107. It first points [Section 2.4.1] out that autoimmune diseases are much more common in women than men, and that administration of sex hormones can influence the onset and course of broadly similar conditions in animal models in NZB and NZW mice. Human pregnancy influences the evolution of these diseases.

108. The account does not consider the dose-response relationships of the experimental effects, which show that high doses are required to produce major effects, suggesting that exposure to hormonal residues in meat would be well below the level at which any detectable action would occur. It does not consider the conflicting nature of much of the evidence attempting to associate changes in the progression of the autoimmune disease lupus erythematosus in women with pregnancy and the associated controversy about the role of prolactin.

Khamashta MA, Ruiz-Irastorza G, Hughes GRV 1997. Systemic lupus flares during pregnancy. *Rheumat Dis Clin N America*, 23, 15-30.

109. In the case of pregnancy [Section 2.4.2], in which the mother becomes selectively tolerant of the fetus, the report correctly states that many factors are involved in producing that state, including sex hormones. Again, there is no consideration of relative exposures from residues in meat and the body's own production of such substances, which is considerably higher, nor is there consideration of the epidemiological evidence that associates successful pregnancy with a high quality diet, commonly including meat, e.g. several ecological and epidemiological surveys in the USA, where hormone treated beef is widely available.

110. The same section [2.4.2] describes the rising incidence of allergic diseases, especially in youngsters, and draws a parallel between it and the incidence of cancer of the breast and prostate, which it considers is related to meat consumption.

111. In the case of the allergic diseases, the report notes the lack of any direct evidence of a role of sex hormones in their causation or evolution, it does not comment on the evidence that they are as common in boys as in girls, and that many cases develop within the first year of life [Strachan, 1999]. As before, the question of relative exposure to endogenous and external sources of hormonal activities is not considered.

Strachan DP 1999. Lifestyle and atopy. *Lancet*, 353, 1457-8.

## **Cancer**

112. There appears to be a rising incidence of cancer of the breast, prostate and colon in industrialised countries in which meat consumption is also higher than elsewhere in the world. Some of the increase is probably due to our improved ability to make diagnoses [e.g. cancer of the prostate] and to more common screening [cancers of the breast and colon], but the causes of much of the increase remain unknown.

113. However, the detailed epidemiological results in most instances tend to support a weak link more with the fat associated with meat eating rather than with meat itself. There is no evidence to link these cancers to hormone residues in meat, rather than to the associated fat, and to the common negative relationship between the protection obtained by consuming more fruit and vegetables and less meat.

114. The recent DoH report [1998] on nutrition and the development of cancer presents a more rigorous analysis of the international epidemiological information than the SCVPH report. It notes the quite weak relationship between the consumption of red meat and the incidence of breast cancer [DoH Report, Section 5.2.2.5]. In the case of colorectal cancer [Section 5.4], there is at most a very weak link between red meat consumption and the incidence of cancer. Prostate cancer [Section 5.5] does show a weak link with meat consumption, all for unknown reasons. Surveying the incidence of these types of tumour in different countries, where dietary patterns are quite different, shows variations in the incidence of the cancers which might be due to many factors, that do not suggest any particular association with eating meat in those states where it may contain hormone residues.

115. The SCVPH evaluation has suggested links but without taking full account of all the evidence, especially the very weak nature of the association between the frequency of these tumours and meat eating, the roles of other associated factors, including fat consumption and changes in diet and diagnostic capacity, the relative exposure to hormonally active substances in the diet and from internal sources in the body.

DoH 1998. Nutritional Aspects of the Development of Cancer. Report on Health and Social Subjects 48. The Stationery Office, London.

## **DEVELOPMENT AND REPRODUCTION**

### **General**

116. Almost all aspects of mammalian reproduction and development involve some element of control by sex hormones. The critical hormones, their effects, and their normal concentrations vary widely across the genders, stages of development, and target tissues. There can also be complex interactions between hormones, and their precise

mechanisms are far from fully understood. It is prudent, then, to be concerned about human exposure to exogenous hormonally-active chemicals. In this area, however, the Report is far from comprehensive, and derives conclusions by inappropriate extrapolation from highly selected publications. There is a huge literature and intensely active research programmes addressing what has become known as "endocrine disruption" (see, for example, [www.epa.gov/endocrine](http://www.epa.gov/endocrine)). The current activities and uncertainties are not thoroughly addressed in the Report.

117. The Report suggests (1.3 para 2) that a variety of potential effects on development, including imprinting, perinatal development and puberty, are "non-traditional" areas of risk assessment. This is certainly not the case. These processes are monitored by orthodox developmental and reproductive toxicity testing, and are routinely assessed in regulatory risk assessment in both Europe and the USA. The Report further suggests (1.3 para 3) that for developmental and some other effects "...no threshold can be defined..". To the contrary, thresholds are almost always observed in dose-response studies of developmental disturbance by exogenous chemicals.

### Specific concerns

118. There are two recurrent limitations that run through this section of the Report: (a) the citation of a study demonstrating an adverse effect after high-dose exogenous hormone exposure, with no dose-response data, to support the assertion of a risk at very low doses; and (b) the ignoring of epidemiological data that do not demonstrate an effect of exogenous hormones on human development. Bearing in mind that the potential intake of exogenous hormones from meat is of the order of pg/kg, the following are examples of inappropriate extrapolation:

119. The study of Brawer et al (1978), cited for "...permanently affect the brain..." (2.2.1.2 para 2) administered 13mg/kg oestradiol valerate to young rats. [The study cited to support an effect on the vagina (Ma et al, 1998) did not actually look at this tissue].

120. In support of "hormonal imprinting" (2.2.2.4), the Report cites Mena et al (1992), who gave neonatal female rats 200mg/kg testosterone propionate, then assessed their response to an injection of 300ug/kg oestradiol.

121. In support of the assertion that adult function can be affected despite apparently normal puberty, the Report cites the study of Khan et al (1998), who gave 40mg/kg of either DES or oestradiol to hamsters at birth.

122. The Report cites the studies of von Saal on intrauterine position as evidence that "...no measurable threshold for these developmental effects is available" (2.2.1.3 para 5). There have been a number of studies, from several groups, on intrauterine position, none of which have measured the hormone levels in individual fetuses or their tissues, in order to correlate exposure with effect. Thus, no conclusions can be drawn about thresholds from these observations.

123. The Report asserts, without any supporting citation, (2.2.1.4 para 1) that "...even small additional concentrations of sex hormones (natural or synthetic) may deleteriously affect the development of these tissues (hypospadias or undescended testes)". Perhaps the most significant human information available to assess this possibility concerns the outcomes of pregnancy when the mother has continued to take oral contraceptives. A summary of eight birth defect monitoring programs, involving more than 2000 boys with hypospadias, showed no association with maternal use of oral contraceptives (*Kallen B, Mastroiacovo P, Lancaster PAL, Mutchinick O, Kringelbach M, Martinez-Frias ML, Robert E, Castilla E: Oral contraceptives in the etiology of isolated hypospadias.*

*Contraception* 1991;44:173-82). A meta-analysis of published reports specifically involving first-trimester sex hormone exposure also did not find any significant associations with fetal genital malformations (*Raman-Wilms L, Tseng AL, Wighardt S, Einarson TR, Koren G: Fetal genital effects of first-trimester sex hormone exposure: a meta-analysis. Obstet Gynecol* 85:141-9, 1995). These, and many other relevant epidemiological reports (e.g. *Kullander S, Kallen B: A prospective study of drugs and pregnancy. 3. Hormones. Acta Obstet Gynecol Scand* 55:221-4, 1976. *Michaelis J et al: Prospective study of suspected associations between certain drugs administered during early pregnancy and congenital malformations. Teratology* 27:57-64, 1983. *Heinonen OP et al: Birth Defects and Drugs in Pregnancy, Littleton, MA, Publishing Sciences Group, 1977.*) are not considered in the Report.

124. In contrast, in the Descriptive Epidemiology section of the Report (2.3.1), major prominence is given to precocious sexual development in Puerto Rico. There has been considerable controversy over the accuracy of these observations from the 1970's and 1980's. Regardless of that, and as the Report admits, there is no evidence of an environmental cause of any of the supposed effects, nor has any causal environmental oestrogenic contaminant been found in Puerto Rico.

125. The Report bases much of the concern about developmental effects of oestrogens on the actions of diethylstilbestrol (DES) in humans and other mammals. Aside from the issue of dose, it is not clear whether DES effects are appropriate models for steroidal hormones. DES is a nonsteroidal oestrogen, and is significantly more potent than oestradiol. This is due to a large part to the extensive binding of steroids to proteins, while DES remains largely free (*Henry EC, Miller RK: Comparison of the disposition of diethylstilbestrol and oestradiol in the fetal rat. Correlation with teratogenic potency. Biochem Pharmacol* 35:1993-2001, 1986).

126. There has been recent concern about an apparent decline to sperm counts in men from several developed countries, which the Report (2.2.3.2) suggests is due to "...oestrogenic or environmental chemicals during fetal and childhood development...", referring to Toppari et al (1996). This publication reviews the marked geographic differences in the prevalence of male reproductive disorders. The authors found that the reasons for these differences are currently unknown, and conclude that "An extensive research program is needed to understand the extent of the problem, its underlying etiology, and the development of a strategy for prevention and intervention", which is an appropriate conclusion that this Group would endorse.

127. In summary, hormones control reproduction and development, and there is no doubt that high levels of exogenous sex hormones can disrupt, in some cases permanently, many aspects of these processes. The scientific challenge is to understand the way in which humans, at all stages of development, handle the presence of low levels of complex mixtures of chemicals that can interact with hormonal pathways. There is no evidence that low-level environmental exposures to exogenous sex hormones have affected human reproduction or development.

## ANNEX 1

The following are from the Meat and Livestock Commission (MLC), Reading and Newcastle giving the figures for the UK.

**Table 1 Total Cows into Over Thirty Month Scheme (UK ) (MLC Reading)**

	August '97- Jul '98	Apr '98 - Mar '99

UK Total	720149	835688
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There is overlap between these two time periods but they indicate that when this scheme comes to an end some 778,000 cows may be slaughtered for human consumption. Most surveys suggest that approximately 30% of these will be pregnant (Singleton and Dobson 1995 Vet. Record 136, 162-165) when killed and hence will increase the total oestradiol-17B input to the food chain approximately as follows.

(Note source : The concentration of oestradiol 17B (ng/kg) in meat from pregnant cows/heifers is taken from Tables VII and VIII of FAO Paper 41 (page12). There is some variation in the concentrations and the following is an extract. (all figures are ng/kg).)

**Table 2 The concentration of oestradiol 17B (ng/kg) in pregnant cows/heifers**

	Control	120 days	180 days	240 days
Lean meat	5.54	13.3	27.3	32.7
Fat	13.4	48.1	71.5	67.5
Liver	1.54	82.5	380	1027
Kidney	2.89	118	230	274

The effect of these animals entering the food chain, (as the date based scheme is implemented) will be to raise oestradiol-17B intake. To estimate the effects of these animals on overall intakes it is assumed that for a 450 kg cow (estimated 50% kill out) there would be 225 kg dressed carcass comprised of 18.5% bone, 66.8% red meat, 12.3% fat, 2.3% waste, (Source : Collaborative trial between University of Nottingham, Hoechst, IRAD, MLC (date unknown)) 5.5 kg liver and 0.6 kg kidney (Source : Meat Hygiene Ed J F Gracey published Bailliere Tindall 1986) potentially adding the following E2-17B to the food chain.

**Table 3 Estimates of oestradiol-17B in meat from pregnant cull cows**

Tissue	Weight (kg)	E2-17B increase ng/kg (see above)	E2-17B increase net ng
bone	42	0	0
lean meat	150	18.89	2833.5
fat	28	48.96	1370.9
liver	5.5	489.96	2694.8
kidney	0.6	204.44	122.4
		Total	7021.6

(Note : the estimated increase has been calculated relative to the mean of the 120, 180 and 240 day concentrations.)

The quantity of oestradiol from control heifers as estimated from the same study is :-

**Table 4 Oestradiol -17B in meat from control heifers.**

Tissue	Weight	E2-17B ng/kg (see above)	E2-17B net ng
bone	42	0	0
lean meat	150	5.54	831
fat	28	13.4	375.2
liver	5.5	1.54	8.47
kidney	0.6	2.89	1.73
		Total	1216.4

The estimated annual cull into the OTMS is 778,000 (see above). If 25% are in calf (distributed evenly over the three trimesters then 194,500 cows would each add 7021.6 ng oestradiol-17B into the food chain. (A total of 1.365.7g. on an annual basis)

The annual UK kill taken from the residue programmes 1997 and 1998 is 2,260,814. Including the OTMS animals this figure rises to 3,167,276. There would therefore be 6% (approx.) adding extra oestradiol to the chain as a result of pregnancy i.e. 431ng per carcass overall.

Assuming that all slaughtered animals (approximately 3 million) each contribute this base quantity (total 3649.2 mg) then the pregnant cow slaughter (total 1365.7mg) therefore represents something around a 37% increase in oestradiol intake from meat and/or meat products. The significance of the increase for the intake of the individual consumer can be estimated using the EU projected intake figures.

Estimated intakes on EU theoretical consumer (Source: Intakes as per VMD residues document 1993)

The effect of these pregnant animals being re introduced to the food chain will be :-

**Table 5 Increase in oestradiol -17B consumer intake due to pregnant cows.**

(assuming only pregnant cow meat is eaten)

	Intake	E2-17B increase	Total
	(g/day or ml/day)	(pg/g)	(pg/d)
Lean meat	300	18.89	5667
Fat	50	48.96	2448

Liver	100	489.96	48996
Kidney	50	204.44	10222
		Total	67333
			(67.3 ng/day)

### Calf Processing Scheme

**Table 6 Actual Calf Processing Scheme Figures 1996 to date (ex MLC, Newcastle)**

	1996	1997	1998	1999 to date	Cumulative Total
England	285808	412935	457986	218449	1375178
Scotland	31171	55610	102751	38490	228022
Wales	71218	83120	53710	6474	215522
N. Ireland	16516	45567	52384	35684	150151
UK Annual	404713	597232	667831	299097	1968873

The returns show regional variations, for which MLC have no explanation but the trend is upwards therefore an annual estimate of 700,000 has been adopted for the calculation. These calves have the poorest conformation and are the most likely to benefit from the renewed use of implants therefore a 100% use is assumed. (Note : given EU resistance to this practice renewed use must be considered very unlikely.)

The contribution to the food chain from this source can be estimated on what appears to be a "worst case" basis using the results presented in Table X FAO, Food and Nutrition paper 41/2, paper 41, page 14).

	Control	Implanted	Quantity	Control	Implanted	Difference
	ng/kg	ng/kg	g/day	ng/day	ng/day	
Lean meat	7	60	300	2.1	18	
Fat	12	96	50	0.6	4.8	
Liver	32	193	100	3.2	19.3	
Kidney	13	134	50	0.65	6.7	
			Total	6.55	48.8	42.25

#### Footnote

1. Note that the hormones dispute, which hinges on the validity or otherwise of trade measures to protect health, has a different basis to the EU/US dispute over bananas, where the issue is whether measures which give preferential treatment to particular exporting countries are compatible with WTO rules.

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