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Possible health impact of animal oestrogens in food

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Oestrogens govern reproductive functions in vertebrates, and are present in all animal tissues. The theoretical maximum daily intake (TMDI) of oestradiol-17β by consumption of cattle meat is calculated to be 4.3 ng. Following the use of oestradiol-containing growth-promoting agents, TMDI is increased by a factor of 4.6 to 20 ng oestradiol-17β, assuming that single dosage and 'good animal husbandry' are observed. Pork and poultry probably contain similar amounts of oestrogens as untreated cattle. The mean concentration of oestradiol-17β in whole milk is estimated at 6.4 pg/ml. Scarce data available on eggs report up to 200 pg/g oestradiol-17β. The risk evaluation of oestrogenic growth-promoting agents is limited by analytical uncertainties. Residues of oestradiol-17α and the importance of oestrogen conjugates are widely unknown. The performance of mass spectrometry still needs to be improved for confirmation of oestrogen concentrations in most food. At present, the potential relevance of oestradiol acyl esters, the actual daily production rate of oestradiol in prepubertal children, and the role of oestradiol metabolites in cancer are obscure. The presence of different cytoplasmic oestrogen receptor subtypes and potential oestradiol effects in non-reproductive functions require further examination.

Key words: anabolics/food/oestradiol/oestradiol residues/public health

TABLE OF CONTENTS

Introduction

Oestrogens regulate most metabolic, behavioural and morphological requirements essential in reproduction of the female sex of vertebrates, including fish, amphibians, reptiles, birds and mammals (Meyer, 1999). Moreover, growing evidence arises on the important role of oestrogens in the male (Sharpe, 1998). In animals, oestradiol-17β is normally the most potent oestrogenic substance. In the first step of metabolic inactivation, the 17β hydroxyl group is either oxidized to its keto function (oestrone) or epimerized to a hydroxyl group in 17α-position (oestradiol-17α). Although oestrone (E1) is about two-fold and oestradiol-17α (E2-17α) about 10-fold less orally active than the parent compound oestradiol-17β (Herr *et al.*, 1970), they contribute to the total oestrogen activity in animals. In cattle fattening, additional application of oestrogens in anabolic preparations to increase live weight gain is licensed in several countries except the EU (e.g. USA, Canada), and leaves greater amounts of residues of oestrogens in edible tissues when compared with untreated animals. Thus, oestrogens are unavoidable constituents in non-vegetarian human nutrition. All foodstuff of animal origin contains oestradiol and its metabolites, but the concentrations vary with the kind of food, species, gender, age and physiological stage of the animal. In this review the oestrogen content of human foodstuff of animal origin is summarized and evaluated with respect to new scientific evidence on oestradiol toxicology. Special attention is given to the additional exposure to oestrogens that is caused by the use of oestrogenic anabolic preparations in meat production.

Table I. Analytical procedures and animal experiments performed in oestradiol quantification

Author(s)	Antigen or antiserum	Analytical method	Animals/animal experiments
Hoffmann *et al.* (1975)	E1- and E2-17β-6-CMO-BSA	Phenolate extraction Separation of E1/E2 by gel chromatography	a) Two pregnant heifers b) Six male veal calves, V-Implix-M^R (20 mg E2-17β), 72 days (five controls)
Henricks and Torrence (1978)	E1- and E2-17β-6-CMO-thyroglobulin	Phenolate extraction	18 heifers; 14 steers
Henricks *et al.* (1982)	E1- and E2-17β-6-CMO-thyroglobulin	Phenolate extraction	Eight heifers
Henricks *et al.* (1983a,b)	E1- and E2-17β-6-CMO-thyroglobulin	Phenolate extraction	a) 7 bulls; b) 39 cows (different stage of cycle or pregnancy); c) 24 heifers
Casal (1983)	Specific antisera for E2-17β and E1 (E. Lilly, IN, USA)	Organic solvent extraction, SPE (fluorosil), separation of E1/E2 by gel chromatography	15 steers, implanted with 24 mg E2, 197 days
Frank *et al.* (1983)	E1 or E2-17β bound to BSA at position 6 (E. Lilly, IN, USA)	Organic solvent extraction, SPE (fluorosil), separation of E1/E2 by gel chromatography	Steers implanted with Compudose (E2-17β) (number, implantation time and dose not specified)
Kushinsky (1983)	(Presented at the International Symposium on Safety Evaluation of Animal Drug Residues, Berlin 1983; cited in Hoffmann and Evers, 1986)		
Meyer *et al.* (1984)	E2-17β-17-hemisuccinate-BSA (detects all E2-17β, E2-17α and E1)	Phenolate extraction, SPE (RP-18), HPLC separation of E1/E2-17β/E2-17α	Eight female veal calves implanted with Synovex H (four treated with 10 mg E2-B + 100 mg T-P; 4 treated with 20 mg E2-B + 200 mg T-P; all 75 days); four controls
Meyer *et al.* (1985)	E2-17β-17-hemisuccinate-BSA (detects all E2-17β, E2-17α and E1)	Hydrolysis (glucuronidase/arylsulphatase) phenolate extraction, SPE(RP-18), HPLC separation of E1/E2-17β/E2-17α	Nine female calves, implanted with Compudose (20 mg E2-17β), 53 days
Fernández Suárez *et al.* (1988)	Antisera specific for E1 and E2-17β (no further specification)	Phenolate extraction, SPE (fluorosil), separation of E1/E2 by gel chromatography	19 pregnant cows
Tsujioka and Ito (1992)	Antisera specific for E1 and E2-17β (from Duildhay Antisera)	ether extraction, SPE(RP-18), separation of E1/E2 by gel chromatography	a) 24 pasture-fed cows and 45 steers; b) 19 steers implanted with Synovex S (20 mg E2-P + 200 mg progesterone); 30 and 60 days; 11 controls
Scippo *et al.* (1993)	Antisera specific for E1, E2-17β, and E2-17α (Laboratoire d' Hormonologie Marloie, Belgium and Biogenesis, Bournemouth, UK)	Hydrolysis (glucuronidase/arylsulphatase), ether extraction, SPE(RP-18)	Veal calves implanted with E2-containing implants (15–120 days); E2-17β: 80–103 treated; 21–34 controls; oestrone and E2-17α; 18 treated, three controls. 11 bulls injected with 20 mg E2-17β + 200 mg testosterone-esters
Fritsche *et al.* (1999)	E2-17β-17-hemisuccinate-BSA (detects all E2-17β, E2-17α and E1)	Hydrolysis (glucuronidase/arylsulphatase), phenolate extraction, SPE(RP-18), HPLC separation of E1/E2-17β/E2-17α	Nine steers implanted with Synovex S (20 mg E2-B + 200 mg progesterone); nine controls

BSA = bovine serum albumin; CMO = carboxymethyloxime; E1 = oestrone; E2 = oestradiol; HPLC = high-performance liquid chromatography; SPE = solid-phase extraction; T-P = testosterone propionate.
Implant suppliers: V-Implix-M®, Hydro-Chemie GmbH, Munich, Germany; Compudose, Vetlife Inc., Norcross, GA, USA; Synovex H and Synovex S, Fort Dodge Laboratories, Fort Dodge, IA, USA.

Analytical methods and problems of oestradiol quantification

This review compares data from 13 studies, which, to our knowledge, comprise the current knowledge on oestradiol concentrations in cattle tissues in the scientific literature (Table I). Ten studies provide detailed information on oestrogen concentrations in cattle tissues after application of anabolic preparations containing oestradiol-17β or oestradiol-17β-3-benzoate (E2-B). The other three studies relate to physiological oestrogen concentrations.

Immunological methods have been used widely in the determination of oestradiol-17β and its metabolites. The traditional radioimmunoassay (RIA), which has the basic problem of handling radioactive isotopes, is being replaced by enzyme immunoassay (EIA). Of the studies evaluated herein, only one (Fritsche *et al.*, 1999) applied an EIA method, whereas the other authors used RIA. Both procedures have a high sensitivity and do

not require expensive laboratory equipment. Their selectivity is based on the specificity of the antibodies used, and this can be improved via sample preparation by removal of possible interference. Immunization of host animals against protein (thyroglobulin or bovine serum albumin, BSA) adducts of oestrone or oestradiol-17β via the 6-position of the steroid [e.g. by a carboxymethyloxime (CMO) bridge] yields antisera of high selectivity between oestradiol-17β and -17α. However, the specificity of the elaborated assay systems has to be demonstrated separately. Antisera raised against oestradiol via the 17-hemisuccinate bridge allow a simultaneous quantification after complete separation of oestrone, oestradiol-17β and –17α prior to quantification. While gel chromatography will only be appropriate for the detachment of oestrone, high-performance methods (e.g. high-performance liquid chromatography; HPLC) are necessary for the separation of the oestradiol isomers. Phenolate extraction and clean-up by reversed-phase (RP) solid-phase extraction (SPE) will help to reduce matrix interference. Finally, some authors conducted hydrolysis of potential oestrogen conjugates (sulphates or glucuronides) to assure complete solvent extraction.

All analytical procedures of the cited studies have been validated; the accuracy (recovery rates) and precision have been specified. The antisera were available commercially, or were commonly used in scientific studies. There is no fundamental objection against the validity of the data. Nevertheless, there are some analytical problems which might contribute to variability of results:

(i) Reagent or matrix blank levels: with regard to oestrogens, there is no analyte-free matrix available by the help of which the blank signal of the quantification might be determined. Without knowledge of the matrix blank levels, the accuracy of the determination at low concentrations cannot be evaluated; therefore, low concentrations in particular might be measured inaccurately. The accuracy of the procedures was intended to be improved by sample preparation, but none of the techniques can assure complete disappearance of matrix interference.

(ii) Oestrogen conjugates: a complete hydrolysis of steroids from their glucuronides and sulphates is not guaranteed if not explicitly performed.

Despite the use of highly specific antisera, immunoassays cannot provide direct information on the analyte identity. For forensic purposes in residue control, mass spectrometric (MS) methods are preferred. In recent years, gas chromatography (GC)-MS methods have been developed (Daeseleire *et al.*, 1991, 1992) for the determination of oestradiol in bile and urine. As oestrogen concentrations in tissues are in the lower pg/g range, the determination limits of the GC-MS procedures often are too high. Most recently, MS techniques have been developed to measure oestradiol in muscle (Maume *et al.*, 2000). At the time when most of the cited residue studies were carried out, sophisticated GC-MS methods were not available.

Oestrogen concentrations in tissues of untreated and oestradiol-treated cattle

Oestrogen concentrations in cattle tissues as reported in those studies or study parts, where no comparison with treated animals is performed, are summarized chronologically in Table II. None of the cited studies presents data for oestradiol-17α.

Concentrations of oestrogens in treated animals compared with untreated control animals are presented in Table III. In addition to oestradiol-17β and oestrone, three of the 11 studies also consider residues of oestradiol-17α in all tissues. For each substance (oestradiol-17β, oestradiol-17α, oestrone or total oestrogens), and for each tissue, a 'treatment factor' is determined by division of the concentration measured in the treated group by the concentration in the control group. It was decided to present the treatment effect in this manner in order to eliminate potential relative differences in analytical procedures between the studies, and to focus on the relative treatment factor.

In steers, the lowest oestrogen concentrations in all tissues (<3 pg/g) were reported by some authors (Kushinsky, 1983, cited in Hoffmann and Evers, 1986; Fritsche *et al.*, 1999). The origin of oestrogens in steers (which are deprived of their testes, the major sex hormone-producing organs), was attributed to peripheral aromatase activity that might convert adrenal androgens into oestrogens. On the other hand, in other studies (Frank *et al.*, 1983; Henricks *et al.*, 1983a,b), oestradiol-17β and oestrone concentrations in steers were in a range typical for heifers and cycling cows (5–15 pg/g). This is in accordance with veal calves (male and female) which show a clear endogenous oestradiol production despite the absence of mature gonad function. Also, non-castrated males have a significant testicular oestrogen synthesis, but data on resulting tissue concentrations are rarely available.

While oestrogen concentrations in calves, cycling heifers and steers are in the same range, an accumulation of oestrone (and to a lesser extent of oestradiol-17α) takes place during pregnancy: beginning with the third month of pregnancy, increasing concentrations of oestrone sulphate and oestradiol-17α-conjugates are detectable, and reach 5000 pg/ml in plasma (Hoffmann, 1977) in the last days before parturition. The tremendous oestrogen production during pregnancy is ascribed to the growth of the placenta. Oestrogens also play an important role during mammo- and lactogenesis, and during priming of the genital tract before parturition (Döcke, 1994).

Data provided in Tables II and III are the results of experiments performed with quite low numbers of animals (except for Scippo *et al.*, 1993) and charted with the help of validated, albeit different, analytical procedures. Plausible mean concentrations (Table IV) were extrapolated for the oestrogen concentrations by combining data from Tables II and III.

At present, it is impossible to give a sound evaluation of the oestradiol treatment effect on oestradiol-17α concentration, because the three studies that focused on oestradiol-17α (or total oestrogens) yielded somewhat contradictory results. (Henricks *et al.*, 1983a,b; Meyer *et al.*, 1984, 1985; Scippo *et al.*, 1993). The analytical difference might result from different coverage of oestradiol conjugates. This observation is attributed to a possible lack of determination of oestradiol-glucuronides and -sulphates due to lacking conjugate hydrolysis by glucuronidase/arylsulphatase treatment or by sample preparation (Meyer *et al.*, 1985).

The relative contribution of oestradiol-17α to total oestrogen content was considered explicitly (Meyer *et al.*, 1984, 1985): in fat, the portion of single oestrogens was subject to great individual variations. After formation of the mean, oestrone (62%) was found predominantly, followed by oestradiol-17β (25%) and oestradiol-17α (13%). In muscle, no oestradiol-17α was traceable, and the other oestrogens appeared in equal

Table II. Concentrations of oestradiol and its metabolites in untreated cattle (pg/g)

Study/animal(s)	Residue	Muscle	Liver	Kidney	Fat
Hoffmann *et al.* (1975)					
Pregnant heifer #1, month 4	E2-17β	860	–	–	–
Pregnant heifer #1, month 9	E1	120	–	–	–
Pregnant heifer #2, month 9	E2-17β	370	–	–	–
	E1	2090	–	–	–
Henricks and Torrence (1978)					
Heifer	E2-17β	12	38	40	–
Steer	E2-17β	14	12	13	–
Henricks *et al.* (1982)					
Heifer	E2-17β	–	–	–	45–67
Henricks *et al.* (1983a,b)					
Heifer	E2-17β	12	38	40	–
Steer	E2-17β	14	12	13	–
Heifer	E2-17β+E1	13	71	71	–
Steer	E2-17β+E1	13	28	26	–
Bull, mature	E2-17β	–	–	–	21
	E1	15	13	3	36
Cow, follicular phase	E2-17β	–	–	–	24
	E1	31	23	14	28
Cow, luteal phase	E2-17β	–	–	–	10
	E1	19	14	15	26
Cow, pregnant, 1st trimester	E2-17β	–	–	–	20
	E1	13	25	10	18
Cow, pregnant, 2nd trimester	E2-17β	–	–	–	40
	E1	136	125	262	460
Cow, pregnant, 3rd trimester	E2-17β	–	–	–	68
	E1	208	252	550	3690
Frank *et al.* (1983)					
Cow, cycling	E2-17β	–	–	–	10
	E1	–	–	–	44
Cow, pregnant, 1st trimester	E2-17β	–	–	–	5
	E1	–	–	–	40
Cow, pregnant, 2nd trimester	E2-17β	–	–	–	22
	E1	–	–	–	964
Cow, pregnant, 3rd trimester	E2-17β	–	–	–	163
	E1	–	–	–	3870
Kushinsky (1983)[a]					
Cow, pregnant, 1st trimester	E2-17β	16	58	127	31
	E1	203	30	84	780
Cow, pregnant, 2nd trimester	E2-17β	27	380	230	72
	E1	482	115	166	2720
Cow, pregnant, 3rd trimester	E2-17β	33	1030	274	67
	E1	523	145	142	2770
Fernández Suárez *et al.* (1988)					
Cow, pregnant, 1st trimester	E2-17β	–	–	–	7
	E1	–	–	–	199
Cow, pregnant, 2nd trimester	E2-17β	–	–	–	30
	E1	–	–	–	934
Cow, pregnant, 3rd trimester	E2-17β	–	–	–	114
	E1	–	–	–	2430
Tsujioka and Ohga (1992)					
Cow, luteal stage	E2-17β	<det. lim.	9.1	<det. lim.	24
	E1	10	18	2.6	13
Cow, CL regressing stage	E2-17β	1.8	9.5	<det. lim.	17
	E1	13	20	1.9	15
Cow, follicular phase	E2-17β	<det. lim.	8.4	<det. lim.	16
	E1	11	16	3.2	20
Steer	E2-17β	1.3	12	11	9.3
	E1	4.5	6.7	7.8	11

[a]Cited from Hoffmann and Evers (1986).
CL = corpus luteum; det. lim = detection limit; E1 = oestrone; E2 = oestradiol.

Table III. Increases in tissue oestrogen concentrations (pg/g) caused by anabolic preparations

Study	Treatment	Muscle	Liver	Kidney	Fat
Veal calves implanted with E2-17β (20 mg; Hoffmann *et al.*, 1975)[a]					
E2-17β	treated	177	108	29	104
	control	113	73	11	129
	treatment factor	1.6	1.5	2.6	0.8
E1	treated	84	271	81	252
	control	75	204	47	275
	treatment factor	1.1	1.3	1.7	0.9
Steers implanted with E2-17β (dose not cited; Henricks *et al.*, 1983a,b)					
E2-17β	treated	17	79	–	54
	control	14	14	–	10
	treatment factor	1.2	5.6	–	5.4
E2-17α	treated	–	53	56	21
	control	–	<10	11	13
	treatment factor	–	> 5.3	5.1	1.6
E1	treated	8	57	–	55
	control	6	20	–	23
	treatment factor	1.3	2.9	–	2.4
Steers implanted with Compudose (E2-17β; Frank *et al.*, 1983)					
E2-17β	treated	3.4	10	21	15
	control	5.8	4.0	6.7	6.8
	treatment factor	0.6	2.5	3.1	2.2
E1	treated	10.4	9.0	19	35
	control	4.8	6.5	7.9	10.5
	treatment factor	2.1	1.4	2.4	3.3
Steers implanted with E2-17β (24 mg; Casal, 1983)					
E2-17β	treated	–	–	–	8.7
	control	–	–	–	3.2
	treatment factor	–	–	–	2.7
E1	treated	–	–	–	20
	control	–	–	–	7.9
	treatment factor	–	–	–	2.5
Steers implanted with E2-B (20 mg; Kushinsky, 1983)[a]					
E2-17β	treated	5.9	5.0	6.1	33
	control	0.84	0.91	1.6	1.8
	treatment factor	7.0	5.5	3.8	18
E1	treated	2.2	1.7	1.8	22
	control	1.6	0.66	1.2	8.5
	treatment factor	1.4	2.6	1.5	2.6
Heifers implanted with E2-B (20 mg; Kushinsky, 1983)[a]					
E2-17β	treated	10.4	3.3	15	56
	control	5.5	1.5	2.9	13.4
	treatment factor	1.9	2.2	5.2	4.2
E1	treated	5.2	1.5	3.9	32
	control	2.5	1.7	1.4	11
	treatment factor	2.1	0.9	2.8	2.9
Veal calves female; implanted with Synovex-H (Meyer *et al.*, 1984)					
Total (E2-17β+α+E1) (dose: 10 mg E2B)	treated	7	834	323	–
	control	2	600	270	–
	treatment factor	3.5	1.4	1.2	–
Total oestrogens (dose: 20 mg E2B)	treated	16	854	780	–
	control	2	600	270	–
	treatment factor	8	1.4	2.9	–
Veal calves male; implanted with Compudose (20 mg E2-17β; Meyer *et al.*, 1985)[b]					
Total (E2-17β+α+E1)	treated	36	1600	600	110
	control	35	2200	630	200
	treatment factor	1.0	0.8	0.95	0.55

(*continued*)

Table III continued

Study	Treatment	Muscle	Liver	Kidney	Fat
Steers; implanted with Synovex-S (20 mg E2-B; Tsujioka and Ito, 1992)					
E2-17β	treated	3.4	8.9	8.9	8.1
	control	1.5	9.7	1.6	4.7
	treatment factor	2.3	0.9	5.6	1.7
E1	treated	4.3	11.8	5.8	10.4
	control	3.8	12.1	5.4	10.4
	treatment factor	1.1	1.0	1.1	1.0
Veal calves implanted with E2-17β (dose not specified; Scippo *et al.*, 1993)[c]					
E2-17β	treated	11–280	5–1650	6.4–589	9.3–358
	control	3.5–33	5.3–53	7.8–69	5.6–50
	treatment factor	6	8	10	5
E2-17-α	treated	–	–	–	294–4160
	control	–	–	–	560–761
	treatment factor	–	–	–	2
E1	treated	3–72	73–284	34–144	29–149
	control	15–78	170–198	23–166	80–94
	treatment factor			(no change)	
Steers implanted with Synovex-S (20 mg E2-B; Fritsche *et al.*, 1999)					
E2-17β	treated	3.0	–	–	–
	control	2.8	–	–	–
	treatment factor	1.1	–	–	–
E2-17-α	treated	1.0	–	–	–
	control	2.6	–	–	–
	treatment factor	0.4	–	–	–
E1	treated	0.9	–	–	–
	control	2.2	–	–	–
	treatment factor	0.4	–	–	–

[a]Although the residues in the control group are markedly higher than in the other studies, the work seemed methodically correct.
[b]Oestrogen concentrations explicitly mentioned only in fat. Increase of E2-17β in fat from 9.5 to 31 pg/g (means), corresponding to a factor of 3.3.
[c]This is the only study which examined a number of samples (21–35 from control animals, 80–110 from oestradiol-implanted) and was high enough to see that the values appear almost normally distributed when plotted in logarithmic scale. The mean of the logarithms serves as the estimate of the treatment factors.

amounts. In liver and kidney, however, the situation was completely different: oestradiol-17α (76–100%) was primarily detectable, whereas oestradiol-17β and oestrone showed only low amounts (0–18% each).

Conclusion concerning natural concentrations of oestradiol-17β and oestrone

In non-pregnant cattle, the residues of oestradiol-17β and oestrone as detectable without explicit hydrolysis of conjugates are in the order of 10 pg/g. In liver and kidney, the total amount of residues will be higher due to the appearance of oestrogen conjugates. A specification of the total amounts (free + conjugated oestradiol-17β and oestrone) is not possible at present due to poor availability of data.

During pregnancy, oestradiol is accumulated in liver and kidney by a factor of 100–500, in fat by a factor of 5, and in muscle by a factor of 3. According to its more lipophilic character, oestrone concentrations in fat increase up to 300-fold in fat, and ~30-fold in other tissues when compared with the non-pregnant state. The marked increase is observed in the second trimester, and peaks at parturition. No accurate statement is possible with regard to oestradiol-17α during pregnancy.

The effect of oestradiol treatment on oestradiol-17β and oestrone concentrations in tissues varied between studies. Most often this can be explained by the small numbers of animals used, by the use of animals of different sex and age, and by differences in the anabolic preparations and time periods between implantation and slaughter. Thus, comparability of the studies is limited. On the other hand, the studies as a whole reflect the conditions of oestradiol for growth promotion (under defined experimental situations; for field conditions, see Chapter 4). Therefore, the treatment (enrichment) factors gained from each study for each tissue were combined by calculation of the arithmetic mean. Results expressed as mean treatment (enrichment) factor for oestradiol-17β and oestrone are listed in Table V.

After the application of oestradiol-17β or oestradiol-17β-3-benzoate, the residues of oestradiol-17β in tissues increase more if compared with oestrone. This corresponds to the concept that the slow-release implants result in a steady increase of oestradiol-17β plasma concentrations, of which a certain fraction is distributed and accumulated in the different tissues.

Table IV. Synopsis of oestradiol-17β and oestrone concentrations (pg/g) in cattle tissues (given as geometric mean and range)

Sex/age	Oestradiol-17β				Oestrone			
	Muscle	Liver	Kidney	Fat	Muscle	Liver	Kidney	Fat
Calf (Scippo *et al.*, 1993)	10.8 (3.5–33)	16.8 (5.3–53)	23.3 (7.8–69)	16.7 (5.6–50)	34.2 (15–78)	183 (170–198)	61.8 (23–166)	86.7 (80–94)
Heifer (Kushinsky, 1983[a]; Henricks *et al.*, 1983a,b)	8.1 (5.5/12)	7.5 (1.5/38)	10.8 (2.9/40)	29.5 (13/67)	2.5 –	1.7 –	1.4 n	11 –
Bull (Henricks *et al.*, 1983a,b)	–	–	–	21	15	13	3	36
Steer (see Table II)	5.0 (0.8–14)	7.2 (0.9–14)	3.3 (1.6–6.8)	5.3 (1.8–10)	3.8 (1.6–6.0)	9.8 (0.7–20)	4.8 (5.4–12)	12.1 (7.9–23)
Cow, cycling (see Table II)	1.8	9	–	17	17	18	7	24
Mean (non-pregnant cattle)	6.4	10.1	9.4	17.9	9.6	42.5	4.1	20.8
Pregnant heifer, 1st trimester	16	58	127	19	13/203	28	47	29
Pregnant heifer, 2nd trimester	27	380	230	45	309	120	213	1050
Pregnant heifer, 3rd trimester	33	1030	274	77	366	186	346	3440

[a]Cited in Hoffmann and Evers (1986).
Values in parentheses are ranges.

Table V. Mean treatment factors[a] for oestradiol-17β and oestrone in edible tissues after implantation with oestradiol-containing implants

	Oestradiol-17β				Oestrone			
Tissue	Muscle	Liver	Kidney	Fat	Muscle	Liver	Kidney	Fat
Treatment factor	3.7	4.2	6.2	6.4	1.2	1.4	1.6	1.9

[a]Treatment factor: mean factor by which the oestrogen concentrations increase after the use of oestradiol as an anabolic preparation.

The relative importance of growth promotion in cattle after oestrogen exposure

The increase of dietary oestradiol intake by consumption of products from treated compared with untreated cattle can be assessed as follows: an assumed overall mean tissue concentration of the respective substance (oestradiol-17β) is multiplied by daily intake figures of animal products. The difference between the results for tissues originating from treated or untreated animals poses the incremental increase of consumption (Table VI). The assumed overall mean tissue concentration is calculated as the mean of the tissue concentrations for calves, heifers, bulls and steers, as given in Table IV. For oestrone, the available data are contradictory: one study (Scippo *et al.*, 1993) reports concentrations that are 10- or 100-fold higher than in the other studies. Thus, a final conclusion on actual oestrone concentrations is not possible.

The quoted tissue concentrations were derived from the cited studies as most probable values. The maximum daily consumption figures (from JECFA, 1999) assume the consumption of 300 g meat, 100 g liver, 50 g kidney and 50 g fat. The resulting total ingestion would be 4.3 ng oestradiol-17β in the case of untreated animals, and 20 ng for treated animals; this corresponds to an overall increase factor of 4.6. For oestrone, the most probable values for tissue concentrations cannot be derived. Treatment factors (Table V) between 1 and 2 indicate a lower importance of the increase of its residues than of oestradiol-17β.

It was reported that the oestradiol-17α concentrations in liver and kidney are a factor of about 10- to 100-fold higher than oestradiol-17β and oestrone (Meyer *et al.*, 1984, 1985; Scippo *et al.*, 1993). Therefore, in the evaluation of the total oestrogen residues, oestradiol-17α cannot be ignored. In man, it is partly metabolized like oestradiol-17β (Breuer and Scott, 1966), and the affinity of oestradiol-17α to the human oestradiol receptor is identical for oestradiol-17α and oestrone (Hähnel *et al.*, 1973). By oral uptake in humans, oestrone (whether conjugated as sulphate or not) still showed almost half the biological activity of oestradiol-17β (Herr *et al.*, 1970). Hydrolysis of conjugates before absorption (Diczfaluzy and Levitz, 1970) and oxidation of oestradiol-17β to oestrone by the hepatic first pass (Rubens and Vermeulen, 1983) probably results in similar oral activity.

Major uncertainties in evaluating the effect of anabolic preparations

In liver and kidney, the oestrogen residues predominantly consist of oestradiol-17α. This is in complete accordance with the biological function of both tissues: in liver, the biologically most active oestrogen, oestradiol-17β, is transformed into the less active forms oestradiol-17α and oestrone, which are further metabolized to conjugates (glucuronides and sulphates) to become hydrophilic. Further, the conjugates are excreted via bile or urine, which explains the high concentrations found in kidney. The residue studies that ignore oestradiol-17α leave the most important residue in liver and kidney out of consideration.

The concentrations of oestrogen conjugates are of equal importance as the unconjugated steroids themselves due to hydrolysis of conjugates before resorption (see above). Especially in liver and kidney, glucuronides and sulphates may pose an important fraction of total residues. The studies

Table VI. Increases in oestradiol-17β ingestion caused by the use of oestradiol-containing implants

Parameter	Muscle	Liver	Kidney	Fat	Sum
Tissue concentration (pg/g)	6.4	10.1	9.4	17.9	-
Daily consumption (JECFA, 1999) (g)	300	100	50	50	500
Total amount ingested (ng) - untreated	1.92	1.01	0.47	0.90	4.30
Treatment factor[a]	3.7	4.2	6.2	6.4	-
Total amount ingested (ng) - treated	7.10	4.24	2.91	5.73	19.98

[a]Treatment factor: mean factor by which the oestrogen concentrations increase after the use of oestradiol as an anabolic preparation.
Overall increase in treatment factor = 4.6.

mentioned so far did not provide any precise statement on the fraction of conjugated oestrogens. In a radioactive tracer study (Kaltenbach *et al.*, 1976), at 3 h after i.m. injection of oestradiol-17β or oestradiol benzoate in a glycol/oil suspension into steers and heifers, 85–95% of the total radioactivity appeared as conjugated metabolites in the liver and kidney. However, most of the available studies on residues following oestradiol implantation do not ensure that the conjugates were hydrolysed.

The analytical procedures used to determine oestrogen concentrations are based on immunological techniques, and therefore do not provide any confirmatory statement on the identity of the residue. Further, low concentrations, in particular, have the risk of being overestimated due to unidentified matrix interference. Oestrogen concentrations in cattle tissues with and without application of oestradiol-containing implants must be confirmed by identifying analyses. The necessary sensitivity of GC-MS methods is currently under development.

Depending on the different agricultural practices in different parts of the world, a varying fraction of beef is derived from bulls. Intact male cattle are not devoid of the major sex hormone-producing tissue, the testis. Parallel to testosterone, bulls are suspected to produce relatively large amounts of oestrogens, but to our knowledge there are no data available in the literature on oestradiol concentrations in the edible tissues of bulls.

Data on residues in meat and meat products at the retail level: real-world studies versus controlled experimental studies

Reported data on oestrogen residues originate from animal treatments using preparations first licensed about 30 years ago. These were applied in single dosage, and samples were obtained after months of withdrawal within controlled animal trials. Nowadays, some preparations on the market show faster oestradiol release (Daxenberger *et al.*, 2000), and other recent studies investigated higher dosages and shorter withdrawal periods. There were at least six publications in *The Journal of Animal Science* alone during 1999, that recommend repeated or multiple treatments of different combined preparations to achieve optimal growth promotion and nutrient retention (Duckett *et al.*, 1999; Holzer *et al.*, 1999; Paisley *et al.*, 1999; Rumsey *et al.*, 1999a,b; Smith *et al.*, 1999). Such information is also available from the internet (NebGuide, 1997), but pharmacokinetic data have not been elaborated in any of these studies and published residue data from real-world treatments were not available.

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

Oestradiol-17β has been re-evaluated by the Joint Food and Agricultural Organisation/World Health Organization (FAO/WHO) Expert Committee on Food Additives (JECFA) during the 52nd meeting in Rome in February, 1999 (JECFA, 1999). During the meeting, the acceptable daily intake for oestradiol-17β was specified (0–0.05 μg/kg body weight). Maximum residue limits (MRL) were 'not specified'. MRL 'not specified' means that available data on the identity and concentration of residues of the veterinary drug in animal tissues indicate a wide margin of safety for consumption of residues in food when the drug is used according to good veterinary practice in the use of veterinary drugs (JECFA, 1999). The intake calculations ('Theoretical Maximum Daily Intakes'; TMDI) by JECFA were based on residue data provided by studies submitted to JECFA, but not published in peer-reviewed journals of scientific literature, and therefore cannot be evaluated herein. However, the incremental increase of oestrogen concentrations in edible tissues caused by application of oestradiol as growth promoter in cattle (Table 2, page 14f in JECFA, 1999) seems to be in a similar range as calculated in this review (Table VI).

Nevertheless, in our opinion the data supplied by JECFA reveal the same uncertainties as mentioned for the studies cited from the scientific literature:

(i) The contribution of oestradiol-17α is widely ignored.
(ii) The fraction of conjugated steroids is not precisely known, and the concentrations of the conjugates were often not measured
(iii) The residue data originate from laboratory-conditioned and not from real-world studies.
(iv) The data were not confirmed by GC-MS methods

Oestrogens in other foods of animal origin

Pork, poultry, fish and eggs

To our knowledge, there is only one systematic comparison available (Claus *et al.*, 1989) on oestradiol-17β and oestrone concentrations in the tissues of boars, gilts (female pigs) and barrows (castrated male pigs). Due to the removal of the major sex hormone-producing organs, barrows show only basal oestrogen concentrations that are similar to those measured in non-pregnant cattle. Concentrations in gilts were slightly higher. Boars behaved differently, revealing concentrations 10- to 100-fold

Table VII. Residues of oestradiol-17β and oestrone in pig tissues

Study/animals	Oestradiol-17β (pg/g)				Oestrone (pg/g)			
	Muscle	Liver	Kidney	Fat	Muscle	Liver	Kidney	Fat
Claus *et al.* (1989)[a]								
Boars	910	9700	11 600	430	150	3300	26 100	590
Gilts ($n=25$)	58	210	160	31	33	320	150	47
Barrows ($n=25$)	29	77	170	29	75	150	90	25
	Chops				Chops			
Hartmann *et al.* (1998)[b]								
Gilts ($n=2$)	<30				<20			
Barrows ($n=2$)	<30				<20			
	Ham[c]	Liver	Salami	Bacon	Ham[c]	Liver	Salami	Bacon
Undefined ($n=1$)	<30	<30	<30	<30	<20	<20	<20	<20

[a]Quantification by radioimmunoassay after hydrolysis of conjugates.
[b]Quantification by GC-MS after hydrolysis of conjugates.
[c]$n=2$.

higher than in gilts and barrows. Concentrations of oestradiol-17β and oestrone in the tissues of boars are unique for male animals, exceeding even those found in meat from pregnant cows (Table VII).

However, in another study using GC-MS after internal standardization with D3-oestradiol-17β, the measured oestrogen concentrations in gilts and barrows were not confirmed (Hartmann *et al.*, 1998). In all samples examined (two chops from gilts, two chops from barrows, two samples of ham, one sample of liver, and one of salami) oestradiol-17β and oestrone concentrations were below the detection limit of 30 pg/g and 20 pg/g respectively. This finding was attributed to overestimation of very low (pg/g) concentrations by RIA due to unspecified 'blind values' as observed in immunoassays after insufficient sample clean-up, e.g. by HPLC (Meyer *et al.*, 1985). Therefore, we conclude that in edible tissues of gilts and barrows (mature boars are not marketed due to unpleasant urine-like odour), the oestradiol-17β concentrations will be in the same range as in cattle.

Very limited data are available on oestrogens in tissues of poultry. Concentrations of oestradiol-17β measured by RIA in meat were between <4 pg and 20 pg/g in female chicken (Cantoni *et al.*, 1993), and reached 4 pg/g in female turkey (Cantoni *et al.*, 1994). Again, GC-MS could not confirm the values, since oestrogen concentrations in chickens, broilers and laying hens were below the detection limit of 30 pg/g (Hartmann *et al.*, 1998).

In general, it can be stated that the oestrogen residue concentrations in poultry meat may not exceed those in beef, but studies relating to gender, age or reproductive state are not available. The remarkable concentration of 730 pg/g oestradiol-17β was detected by GC in a sample of goose fat (Hartmann *et al.*, 1998).

Maternal transfer of nutrients, including steroid hormones, to embryos during gestation in viviparous amniotes is well known, but the underlying process is poorly understood (Janzen *et al.*, 1998). A considerable transfer of oestradiol-17β into the egg yolk is observed in oviparous animals (Lipar *et al.*, 1999). Unfortunately, a precise documentation of the oestrogen concentrations in eggs of the domestic fowl (*Gallus gallus*) does not yet exist. GC-MS data of oestradiol-17β concentrations in three eggs were reported to be below 30 pg/g, but concentrations reached 180 pg/g and 220 pg/g in another two eggs (Hartmann *et al.*, 1998). Thus, eggs may play a major role for the human dietary oestrogen consumption.

Oestrogens are also the most important regulators of metabolic requirements for reproduction in fish. Concentrations of steroid hormones in fish plasma vary widely, depending on the season and the reproductive stage (Meyer, 1999). To our best knowledge, no studies have been published dealing with oestrogen concentrations in the muscle tissue of fish. Oestradiol-17β and oestrone could not be identified by GC-MS in one herring and two carp samples (Hartmann *et al.*, 1998); however, these values must be seen as 'spot' observations and do not provide further information on season and reproductive stage.

Fish roe (caviar and caviar-like products) consists of complete oocytes of spawners, including the oestrogen-producing tissues, the follicles. Therefore, fish roe contains the highest amounts of oestrogen of all foodstuffs. Preliminary examinations performed in our laboratory by EIA after HPLC-separation of metabolites showed oestradiol-17β concentrations up to 1000 pg/g in roe from Russian sturgeon (unpublished data). Single fish species seem to regulate oestradiol at different levels, and oocytes have different size, yolk content and steroidogenic potential. As fish roe products do not play a major part of human nutrition, their overall contribution to oestrogen ingestion can be neglected, with the exception of certain unusual consumption habits. The section of the population that is most sensitive to oestrogens—prepubertal children—usually does not eat fish-roe products.

The phylogenetic origin of steroidal oestrogens remains unclear. Data on the physiological concentrations of oestradiol in cartilaginous fish, in acraniata and in invertebrates are very

Table VIII. Concentrations of oestrogens in unprocessed milk

Animal	Matrix	Concentration (pg/ml)	Analyte	Reference(s)
Cycling cow, follicular phase	Skim milk	1–3	Free E2-17β	MacDonald *et al.* (1982); Glencross and Abeywardene (1983);
Cycling cow, luteal phase	Skim milk	4–8	–	Abeywardene *et al.* (1984); Gyawu and Pope (1990)
Cow, castic ovarian follicle	Skim milk	2–5	Free E2-17β	Roy *et al.* (1985); Watson and MacDonald (1984)
Cow, whole cycle	Skim milk	2–5	Free E2-17β	Saumande and Batra (1984)
	8 – 25	Total E2-17β		
Cycling cow, before/at oestrus	Whole milk	20–30/80	Free E2-17β	Monk *et al.* (1975)
	30–40/50–60	Oestrone		
Early lactating cow (days 3–125)	Whole milk	13/28	Free E2-17β/E1	Erb *et al.* (1977)
	160	Free E2-17α		
Pregnant cow (whole pregnancy)	Whole milk	82–85	Free E2-17β	Monk *et al.* (1975)
	35–97	Oestrone		
Pregnant cow, days 40–60	Whole milk	150	Oestrone sulphate	Gyawu and Pope (1983)
day 240	Whole milk	Up to 1000		
Cycling goat, mid-luteal phase	Skim milk	1	Free E2-17β	Abeywardene and Pope (1990)
oestrus	Skim milk	8		
Buffalo, luteal phase	Whole milk	20–30	Free E2-17β	Batra *et al.* (1980)
oestrus and early pregnancy	Whole milk	50		

sparse, and the importance of oestrogens in molluscs (e.g. snails, octopus) is obscure, there being no data available on concentrations in edible tissues.

Milk and milk products

Lactogenesis and galactopoesis in the female mammal is dependent on the reproductive state of the animal, and thus is directly connected to the hormonal control of reproduction. Because of the permeability of the blood–milk barrier, lipophilic hormones that circulate in the blood plasma appear in milk. Consequently, the oestrogen content of milk reflects the ovarian function or pregnancy of the lactating animal.

During the past 25 years, RIA methods have been developed for the determination of oestrogen concentrations in milk and dairy products. Both systematic approaches for the description of oestrogens in commercial milk and specific studies on the dependence of oestrogen concentrations in milk on ovarian function are available in the literature (Table VIII).

In pregnant cattle, the oestrogens that are produced by the conceptus are known to pass into milk (predominantly oestrone sulphate). In common dairy herd management, milking of pregnant cows is terminated after the second trimester of pregnancy in order to guide the available nutrients into the developing embryo, and therefore cows in late pregnancy will not contribute to the total oestrogen content of bulk milk and the milk products manufactured from it.

In skim milk of cycling goats, oestradiol-17β concentrations were near 1 pg/ml in mid-luteal phase and peaked at oestrus to mean values of about 8 pg/ml (Abeyawardene and Pope, 1990). Full milk of buffaloes was found to contain about 20–30 pg/g oestradiol-17β during luteal phase and about 50 pg/g during oestrus. In early pregnancy the concentrations are similar to those in luteal phase (Batra *et al.*, 1980). The milk of both species seems to contain similar oestrogen concentrations as cow's milk. No information is available regarding milk from other animals that might play a role in human nutrition (e.g. sheep, mares, camels).

Commercial milk and milk products are produced from bulk milk, which is a mixture of milk originating from cows in all physiological stages. Oestradiol-17β overcomes processing and appears in commercial milk products (Table IX).

The total oestrogen content in milk and milk products can only be presumed. In skim milk, ~3.5 pg/g free oestradiol-17β will be present, which may correlate to a total (free + conjugated) concentration of 17.5 pg/g. In the cream fraction (butter, cheese) only unconjugated steroids appear. Oestradiol-17β concentrations in fresh cheese are 11 pg/g, in ripened cheese 25 pg/g, and in butter 82 pg/g. The concentration of oestradiol-17α is presumably 12-fold higher than the concentration of oestradiol-17β. Free oestrone concentrations are about two-fold that of free oestradiol-17β in skim milk, and seven-fold that of fat.

Confirmation of oestrogen concentrations in milk and its products by GC-MS was not possible due to lack of sensitivity (Hartmann *et al.*, 1998). Besides lacking MS-based confirmation, the oestrogen concentrations found in milk are subject to similar uncertainties, as observed for edible tissues (role of oestradiol-17α and oestrogen conjugates).

Estimation of the relevance of using anabolic agents

The relevance of the use of oestrogen-containing anabolic implants can only be evaluated from the limited data that have been presented here. This attempt is based on the following assumptions and limitations: An accurate assessment of the proportion of cows in slaughterhouses which are pregnant cannot be stated, as there will be huge regional and temporal variations due to different agricultural production habits. In good agricultural practice, it should not make sense to use pregnant cattle for

Table IX. Concentrations of oestradiol-17β in bulk milk and milk products

Product	Unit	Oestradiol-17β	Oestrone
Unprocessed whole milk[a]	pg/ml	12	60
Unprocessed skim milk[a]	pg/ml	7.0	15
Processed whole milk[a]	pg/ml	6.4	34
Processed skim milk[a]	pg/ml	3.5	10
Reconstituted non-fat milk[a]	pg/ml	1.2	9
Whey[a]	pg/ml	1.5	3.6
Cottage cheese[a]	pg/g	11	35
Butter[a]	pg/g	82	540
Ricotta, fresh cheese[b]	pg/g	8–12	–
(Half-)ripened cheese[b]	pg/g	14–27	–

[a]Data from Wolford and Argoudelis (1979).
[b]Data from Cantoni and d'Aubert (1995).

beef. As soon as pregnancy goes beyond the first months and no severe health problems occur, pregnant cows will be kept until parturition in order to gain the calf. The other meat that is outstanding for its high oestrogen concentrations originates from boars, but due to their potential off-flavour, boars are not regularly used for meat production in most countries.

As explained earlier (see Table VI), if cattle tissues are the only foods of animal origin that a consumer eats, she/he will increase their oestradiol-17β intake by a factor of 4.6 if the meat was produced with the help of oestrogenic anabolics. The higher the fraction of other animal foodstuff becomes, the less important becomes the contribution of oestradiol treatment of cattle. Assuming a daily consumption of 1 litre skim milk (17.5 pg/g oestradiol-17β), 100 g of ripened cheese (25 pg/g), 50 g butter (82 pg/g) and two eggs (100 g; 130 pg/g), the total oestradiol-17β intake by non-meat food is 37.1 ng. If 500 g of cattle tissues are ingested, an additional 4.3 ng of oestradiol-17β are consumed under 'regular' conditions, but 20 ng after the application of oestradiol as growth promoter. Under this scenario, the relative increase caused by growth promotion is 37.9% (corresponding to a total of 57.1 ng versus 41.4 ng). Depending on personal consumption habits, the use of oestrogenic growth promotants increases the total dietary oestrogen intake. This calculation assumes that anabolics are dosed only one-fold, and that good animal husbandry is observed. The real-world scenario is probably different.

Synthetic oestrogens used in animal production

Among synthetic oestrogens, only zeranol (α-zearalanol; Ralgro; Mallinckrodt Veterinary Inc., Mundelein, IL, USA) is licensed for cattle fattening in certain countries (but not in the European Union, where a general ban of hormonal growth promoters exists). For non-licensed, black-market drugs no sound evaluation on their use and their residues is possible. Therefore, in this treatise we focus on the semi-synthetic oestrogen zeranol, which is derived from the *Fusarium* toxin α-zearalenol by selective hydrogenation (reduction) of the non-aromatic carbon double bond. To our knowledge, a total of five studies on residue formation in cattle tissues after the use of zeranol are available in the scientific literature. In two studies (Janski, 1983; O'Keeffe, 1984) a polyclonal antiserum was applied, while in the others (Dixon and Russell, 1986; Dixon *et al.*, 1986) monoclonal antibodies were used for the quantification of zeranol in specific RIA methods. Zeranol residues were extracted with organic solvents and cleaned by separation of the phenolate anions under alkaline conditions. The extracts were purified by HPLC (RP-18; Janski, 1983) or by gel chromatography on Sephadex LH-20 material (O'Keeffe, 1984), or further cleaning was not used (Dixon and Russell, 1986; Dixon *et al.*, 1986). A more sophisticated method was presented by other workers (Chichila *et al.*, 1988) who measured zeranol residues with a GC-MS method (selected ion monitoring of trimethylsilyl derivatives) after solvent extraction and clean-up of phenolate anions on anion exchange solid-phase extraction cartridges. Table X gives the results of the studies. In addition to zeranol (α-zearalanol) itself, these authors measured the residues of its metabolites β-zearalanol (taleranol) and zearalanone (Chichila *et al.*, 1988).

Table X. Residues of zeranol in bovine tissues

	Concentration (ng/g)			
	Muscle	Liver	Kidney	Fat
Janski (1983)[a]				
Control	0.08	0.08	0.01	0.07
1- to 3-fold dose	0.04	0.28	0.10	0.03
6-fold dose	0.11	1.21	0.22	0.22
O'Keeffe (1984)[b]				
Control	0.001	0.14	0.028	0.064
Implanted	0.014	0.35	0.076	0.060
Dixon and Russel (1986)[c]				
Implanted	0.13	0.30	0.16	0.18
Dixon *et al.* (1986)[d]				
Control	0.28	0.10	0.10	0.075
After day 7	0.29	0.47	–	0.077
After day 14	0.57	0.73	–	0.19
After day 21	0.21	0.35	–	0.16
After day 30	0.28	0.81	–	0.11
After day 50	0.24	0.35		0.14
After day 70	0.73	0.20	0.13	0.073
After day 90	0.14	0.14	0.055	0.082
After day 120	0.28	0.10	0.084	0.066
Chichila *et al.* (1988)[e]				
24 mg	0.13	0.36	0.36	–
48 mg	0.21	0.66	<0.25	–
72 mg	0.16	0.68	<0.25	–
120 mg	0.16	3.56	0.67	–
68 mg	0.13	3.04	0.51	–

[a]Janski (1983): three male calves each implanted with 1, 2, 3, 4, 5, or 6 Ralgro implants (36 mg zeranol) at intervals of 65 days; four control animals; slaughter 65 days after last implantation.
[b]O'Keeffe (1984): 11 steers implanted with 35 mg zeranol; 11 control animals; slaughter 67 days after last implantation.
[c]Dixon and Russel (1986): four cows implanted with Ralgro 70 days before slaughter.
[d]Dixon *et al.* (1986): 27 steers implanted with Ralgro; tissue biopsy between day 7 and 120 after implantation; six control animals.
[e]Chichila *et al.* (1988): one steer each implanted with 24, 48, 72, 120 or 168 mg zeranol; implantation 5 days before slaughter.
Ralgro® (zeranol) supplied by Mallinckrodt Veterinary Inc., Mundelein, IL, USA.

Concentrations of taleranol in muscle were below the detection limit of 0.02 ng/g, in kidney were between the detection limit and 1.7 ng/g and were independent of dose, and in liver, in a dose-dependent manner, ranged from 1.1 to 4.0 ng/g. Concentrations of zearalanone ranged from 0.05 to 0.20 ng/g in muscle, between the detection limit and 0.31 in kidney, and between the detection limit and 0.47 ng/g in liver, where again a dose-dependent relationship was observed. If the relative uterotrophic activity in rat and mice after oral application of zeranol is set on 100%, zearalanone reaches 68% (rat) and 60% (mice), and relative oral activity of taleranol is 36% in rat and 30% in mice (Baldwin *et al.*, 1983). This indicates that zeranol metabolites contribute to the total hormonal activity of zeranol.

Three of these studies (O'Keeffe, 1984; Dixon and Russell, 1986; Dixon *et al.*, 1986) were included into the zeranol section of the JECFA evaluation 'Residues of some veterinary drugs in animals and foods' (JECFA, 1987). Regarding the specific residues of zeranol in tissues, this review is based on the same data material as the JECFA statement. Maximum residue limits (MRL) of 2 ppb in muscle and 10 ppb in liver have been adopted by the Codex Alimentarius Commission (ALINORM 95/21, 1995) on the basis of the JECFA evaluations. According to Table X, maximum concentrations observed after regular dosing of Ralgro (36 mg zeranol) reached 0.73 ng/g (ppb) in muscle and 0.81 ng/g in liver. Thus, the concentrations remained under MRL by a factor of at least 2.8 (muscle) and 12 (liver), indicating a wide safety margin. Even after 4.7-fold dose (168 mg), the zeranol residues in liver did not exceed 3.6 ng/g.

Moreover, in muscle no clear dose-dependent increase of residues was observed after overdose application. Hence the validity of the data is doubtful. The control concentrations range from 0.001 ng/g up to 0.28 ng/g, and cover a factor of about 300-fold.

Occurrence of zeranol in animals not treated with zeranol

When grown in rice culture, *Fusarium* spp. produce the three main toxins zearalenone, α-zearalenol and β-zearalenol. In the bile of Ralgro-negative cattle, the presence of α-zearalanol has been proved by GC-MS (Kennedy *et al.*, 1998). However, the concentrations did not exceed 4 ng/g, while values from 1 ng/g up to 14 ng/g were observed in Ralgro-treated animals. The hypothesis that zeranol is formed *in vivo* from α-zearalenol and zearalenone, but not from β-zearalenone (presumably by rumen microflora) was also confirmed (Kennedy *et al.*, 1998). As a consequence, zeranol is not compulsorily a xenobiotic substance, but might appear in animal tissues after *Fusarium* infection of animal feed. However, the concentrations in the control animals referred to by the studies cited in Table X are too high be explained by that origin.

Relevance of zeranol with respect to consumer safety

Following the use of zeranol, its concentrations in muscle and liver do not exceed 300 pg/g and 800 pg/g respectively (with single exceptions). Kidney and fat seem not to play an important role (Table X). The oral uterotrophic activity of zeranol in rats and mice is 0.25 and 0.5% that of diethylstilboestrol (DES) (Baldwin *et al.*, 1983). In women, DES is 4.5-fold more active in inducing withdrawal bleeding than oestradiol-17β after oral application (Herr *et al.*, 1970). Thus, the relative oral activity of zeranol when compared with oestradiol-17β (100%) should amount to 1–2%. After transformation of the assumed maximum zeranol residue concentrations (300 and 800 pg/g) by its relative activity, concentrations of oestradiol-17β equivalents after regular dose treatment with zeranol in muscle might be 3–6 pg/g and 8–16 pg/g in liver. Therefore, the maximum overall contribution of zeranol treatment to oestrogen residues in edible tissues is estimated to be of the same order of magnitude as implantation with oestradiol. Probably, the actual tissue concentrations of zeranol are smaller than given in Table X, which refers presumably to overestimated values.

The following gap of knowledge has to be highlighted: in a radioactive tracer study cited by JECFA (1987), total residues of [^{3}H]zeranol in liver reached peak concentrations of 8.2 and 7.3 ng/g at 5 and 15 days after implantation, and thus almost reaches the MRL of 10 ng/g. Unfortunately, the applied dose was not specified and therefore cannot be evaluated. In any case, there seems to be a major discrepancy in the detectable concentrations of zeranol (and its known metabolites) and the total concentrations of the tracer material. In muscle a similar difference of the values was not observed.

New scientific evidence on oestrogen pharmacology and toxicology

The EEC Scientific Working Group on Anabolic Agents presented a report to the EC Commission, in which it pointed out that 'assessment of toxicological risks is not static and may need to be examined and revised in due course, dependent on whether or not new information becomes available' (Lamming *et al.*, 1987). The JECFA evaluated oestrogen residues in meat from hormone-treated animals as safe for the consumer in 1987, and repeated its conclusion in 1999 (JECFA, 1987, 1999). However, in recent years new scientific evidence emerged that has not been regarded by JECFA.

Low daily production rate of oestradiol in prepubertal children

For the evaluation of toxicity of substances used in food-producing animals, the US Food and Drug Administration (FDA) ruled that '...no physiological effect will occur in individuals chronically ingesting animal tissues that contain an increase of endogenous steroid equal to 1% or less of the amount in microgram produced by daily synthesis in the segment of the population with the lowest daily production' (FDA, 1999). Regarding naturally occurring hormones such as oestradiol, daily production rate (PR) and exposure are the crucial elements of a risk assessment. PR of hormones are calculated from estimates of the metabolic clearance rate (MCR) and of the plasma concentration according to the following equation (Andersson and Skakkebæk, 1999):

$$\text{PR } (\mu g/day) = \text{plasma concentration } (\mu g/ml) \times \text{MCR } (ml/day)$$

We share the point of view that estimated MCRs for oestradiol in healthy prepubertal children have never been published, and most likely do not exist. MCRs applied by JECFA (1987) are suspected to be obtained from adults, but multiplied with the plasma concentrations of children (Andersson and Skakkebæk, 1999).

Simultaneously, it was emphasized that MCRs for sex steroids will be definitely higher in adults than in children. MCR is strongly affected by the body size/surface area relationship and will be overestimated 2- to 3-fold in a 3- to 4-year-old child when compared with an adult. Furthermore, children produce substantially more sex hormone-binding globulin, which binds oestradiol and protects it from being cleared. Finally, the enzymatic activity of sex steroid-metabolizing enzymes is markedly lower in prepubertal children. Altogether, the MCR of oestradiol in prepubertal children is presumably at least 2- to 4-fold lower than the MCR in adult women (Andersson and Skakkebæk, 1999), though the precise value is not known.

The second factor determining daily production rate is the endogenous plasma hormone concentration. Measurement of low peripheral concentrations of oestradiol-17β were traditionally performed with RIA, but the results were extremely variable (Potischman *et al.*, 1994). It was assumed that this was due to variable non-specific matrix interference, which disturbs especially the determination of low concentrations (Carlström, 1996). To date, no confirmatory MS-based techniques exist for precise determination of the extremely low prepubertal concentrations in the pg/g range or below, and uncertainty remains about the actual concentrations. While four RIA-based studies (Andersson and Skakkebæk, 1999) reported mean prepubertal children concentrations between 4.8 and 23 pg/ml, means of 0.6 pg/ml in girls and 0.08 pg/ml in boys were measured with the help of an ultrasensitive recombinant cell bioassay (Klein *et al.*, 1994). In prepubertal boys a PR between 0.04 and 0.1 μg/day was concluded. The PR of 0.04 μg/day correlates to a maximum tolerable intake level of 400 pg/day (1% of 0.04 μg). Assuming average concentrations of oestradiol-17β in meat from untreated cattle of 4.3 ng/500 g (see above), this threshold level is reached after consumption of 47 g meat. The threshold amount of meat from treated cattle (20 ng/500 g) would be exceeded after the ingestion of 10 g of meat.

The calculation is based on the oestradiol plasma concentrations as determined with the yeast reporter gene assay, which are subject to the principal uncertainty that the assay does not provide any information on the identity of the substance. Substances other than oestradiol extracted from plasma might induce or inhibit the assay signal by binding to the transfected oestrogen receptor.

Possible role of low doses of sex steroids

In recent years, nearly all body tissues have been identified as being susceptible to oestrogens (Oettel and Schillinger, 1999), but minimum effect oestrogen concentrations are widely uncertain. Oestrogen receptors and aromatase activity have also been identified outside the reproductive system in males, for example in brain, pituitary, thymus, heart, gut, bone, adipose tissue and muscle (Sharpe, 1998). The importance of oestrogens in bone growth and maturation during childhood has been emphasized, suggesting that low concentrations of oestradiol produced during childhood are responsible for epiphysial fusion, and the higher plasma oestrogen concentrations in girls are correlated to earlier termination of bone growth compared with boys (Cutler, 1997). The original understanding that low but rising concentrations of testosterone cause the pubertal growth spurt, and that high concentrations terminate it, has been displaced by the view that oestradiol is the active principle (Sharpe, 1998).

The section of the population that is presumably most sensitive to oestrogens are prepubertal girls with Turner syndrome, because they are lacking ovarian oestrogen production. In Turner syndrome girls, daily treatment with 25–100 ng ethinyl oestradiol/kg body weight increased leg growth rate (Levine Ross *et al.*, 1988) and growth hormone secretion, but reduced FSH secretion (Mauras *et al.*, 1989). The comparison of healthy prepubertal children with Turner syndrome girls highlights the important role of low oestrogen concentrations in childhood. In healthy, prepubertal girls transient signs of pseudo-precocious puberty were associated with accidental intake of oestrogens (Pasquino *et al.*, 1982). The concentrations of oestrogens that regulate growth and development during childhood are very low, but possible effects of dietary oestrogens on such low concentrations are unclear.

New insights into the role of oestrogens in cancer

Although it was widely assumed that oestradiol acts mainly as a growth factor in promoting cancers, new findings suggest that some of its metabolites may also initiate mutations (Service, 1998). Oestradiol and oestrone can be oxidized by cytochrome-P450 monooxygenases into the 2- or 4-catechol oestrogens. These can either be methylated by catechol-*O*-methyltransferase (COMT), or they are further oxidized by peroxidases or again by cytochrome enzymes. A third metabolic pathway of oestrogens is formation of 16α-hydroxyoestrone. The carcinogenicity of 4-hydroxyoestradiol in Syrian hamsters was demonstrated several years ago (Liehr *et al.*, 1986), but it was not possible to suggest a precise mode of action at that time. Later, it was shown that catechol oestrogen 3,4-chinones can act as endogenous tumour initiators by formation of depurinating DNA adducts in female Sprague-Dawley rats (Cavalieri *et al.*, 1997). Nowadays, it is suggested that oestradiol be viewed as a weak carcinogen and weak mutagen capable of inducing genetic lesions with low frequency (Liehr, 2000).

In conventional evaluation of carcinogenic incidents, it is not possible to specify any threshold levels because, in theory, one carcinogenic molecule may initiate cancer. In that context oestradiol should be discriminated a carcinogen, and consequently its exposure should be diminished as far as possible. On the other hand, oestradiol is a substance that is produced naturally in all human beings, and all exogenous exposure that does not markedly increase natural endogenous concentrations cannot be described as 'carcinogenic', because more cancers will not be observed. The crucial element of a risk assessment of dietary oestradiol is the endogenous concentration. The role of oestradiol in cancer development is not specified sufficiently. If findings that suggest a tumour-initiating potential of oestradiol are confirmed, the hormone has to be seen as substance that is undesirable in food.

Identification of new oestrogen receptor subtypes

Since the identification and characterization of a second human oestrogen receptor (ERβ) (Mosselman *et al.*, 1996), there has been speculation on its physiological function (Giguère *et al.*, 1998). Recently, it has become obvious that both ERα and ERβ are generally expressed in many tissues besides the reproductive systems (Gustafsson, 1999; Pfaffl *et al.*, 2000). The ability of both ER types to bind different ligands is not equal. It seems that ERβ is less specific, binding phyto-oestrogens (coumoestrol, genistein,

daidzein and others) with higher affinity than ERα (Kuiper *et al.*, 1998). With regard to residues of oestrogens in animal tissues after the use of oestradiol as growth promoter, the risk evaluation has been based mainly on effects on reproductive functions, while potential effects of low oestradiol concentrations on other parts of the body were widely ignored. The safety evaluation of oestrogens in cattle tissues did not regard the presence of different cytoplasmic ER subtypes, and ignored potential oestradiol effects in non-reproductive functions.

Formation of oestradiol fatty acid esters

Oestradiol-17β-fatty acid esters have been detected in bovine tissues (Paris *et al.*, 1994). Although they are presumed to be synthesized in hepatic and andrenocortical microsomes (Paris and Rao, 1989), their potential importance for residue formation of oestradiol is unclear. Although long-acting oestrogenic responses of oestradiol-fatty acid esters have been described (Vazquez-Alcantara *et al.*, 1989), there can only be speculation about the toxicological relevance of residues of these compounds. Oestradiol-17β-fatty acid esters have been identified in the plasma of women, but their concentrations are below that of oestradiol itself. However, adipose tissue seems to be a site of storage, containing nearly 1 pmol of oestradiol esters per gram of tissue. The concentrations decreased very slowly after menopause, still reaching 0.4 pmol/g more than 15 years later. The dramatically increased potency of oestradiol-fatty acid esters to stimulate mice uterine growth has been demonstrated (Hochberg, 1998). The basic possibility of storage of steroids as fatty acid esters was also confirmed for testosterone in rat by GC-MS (Borg *et al.*, 1995). The existence of enzymes that metabolize testosterone esters in the rat was demonstrated in the 1970s (Kishimoto, 1973), but very little knowledge exists relating to steroid esters in man. The formation of oestradiol residues as fatty acid esters after application of oestradiol to cattle is also unknown. The potential toxicological relevance of these residues is unclear.

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DISCUSSION

Discussion of this paper can be found on page S425.