

Health aspects of residues of anabolics in meat

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INTRODUCTION

Anabolic agents with hormonal action are used worldwide to increase the efficiency and quantity of meat production. A Working Group on the Health Aspects of Residues of Anabolic Agents in Meat was held from 10 to 13 November 1981 at the National Institute of Public Health in Bilthoven, under the auspices of the WHO Regional Office for Europe.

Experts from ten European countries, together with a number of Dutch experts and representatives from the Commission of the European Communities, the European Food Law Association and the World Association of Veterinary Food Hygienists, discussed the benefits, risks and safety of anabolic agents in meat production, an issue of public health importance, highlighted by recent public concern over its illegal use in certain countries.

Dr H. Cohen, Director-General of the National Institute of Public Health, opened the meeting. He stressed the importance of placing the discussion of anabolic agents in its proper perspective, weighing the benefits to animal production against possible risks to the consumer. The recent findings of undesirably high levels of diethylstilbestrol (DES) in baby food has placed suspicion on some uses of hormones. Relevant scientific data on toxicology, mutagenicity and carcinogenicity need to be studied before decisions on the use of anabolics are made. Proper evaluation of new data is critical, and in this regard international cooperation is important.

Dr E.J. Ruitenberg was elected Chairman; Professor M.F. Nesterin acted as Vice-Chairman, Dr V. Silano as Secretary and Dr J.F. Roche as Rapporteur.

The meeting discussed the analytical problems and methods used to detect residues, the toxicological and public health aspects of residues, and the principles of legislative and administrative measures needed to ensure adequate control and safety of public health.

Recommendations were formulated pertaining to actions by regulatory agencies, research institutions and WHO which, when implemented, will contribute to the development of a more rational and better harmonized approach to the problem of public health risks from the use of anabolic agents.

PROBLEMS IN PERSPECTIVE

Two background papers by Professor B. Hoffmann (see Annex 1) and Professor M. Metzler (see Annex 2) and a review by Dr R.J. Heitzman were given, to put the benefits and problems associated with the use of anabolic agents in perspective.

Professor Hoffmann discussed the hazards to the consumer arising from the presence of residues in meat, the need for a strict regulatory approach to the problem, and the use of analytical methods for proper monitoring of residues.

Professor Metzler discussed the risks connected with the use of stilbene estrogens. He reviewed the existing toxicological data on DES and concluded that it should not be used in meat production. He also stressed the importance of assessing the safety of anabolic agents not only on the basis of their hormonal activity, but also on the basis of other biological activities which may cause toxic effects.

Dr Heitzman reviewed the efficacy and use of anabolic agents in meat production. By definition, anabolic agents are substances that increase nitrogen retention and protein deposition in animals. The overall anabolic response in farm animals is measured in terms of (a) increased average daily live weight; (b) improved feed conversion efficiency; and (c) better carcass quality. The efficacy of anabolic agents is best understood by considering the differences between animals of different sex and physiological status. Males are usually larger, have faster growth rates and use feed more efficiently than females, while castrated males occupy an intermediate position. The differences are caused in part by the sex steroids, the androgens and estrogens. After birth, growth is controlled by the presence and concentration of the circulating sex steroids. Androgen concentrations are highest in bulls and estrogen concentrations highest in pregnant cows. Both androgens and estrogens are necessary to achieve maximum growth rates in ruminants, and the administration of exogenous hormones should supplement the pre-existing endogenous hormones to give a suitable mixture of androgens and estrogens. Thus, the greatest benefits are seen in heifers and cows treated with androgens and in steers and veal calves treated with combined preparations of androgen and estrogen. The anabolic agents in common use are testosterone, estradiol, progesterone, zeranol and trenbolone acetate. Most countries prohibit the use of the synthetic estrogen compounds, DES, hexoestrol and dienestrol, which possess anabolic properties but present a public health hazard. Estrogens have been used as growth promoters in sheep and a few trials have also been carried out using combined implants in wethers. In trials with wether lambs implanted with a combination of trenbolone acetate and estradiol live weight gain, carcass weights and feed conversion efficiency were increased. Initial trials on the efficacy of anabolic agents

in bulls and pigs suggest that in these cases also some benefits may be achieved with combined preparations. The benefits of the use of anabolic agents in meat production are seen not only in increased growth and, sometimes, increased growth rates, but also in the much needed sparing of feedstuffs.

PRESENT USE IN EUROPE

European countries

Bulgaria. Anabolic agents are not permitted for use as growth promoters in animal production, except for experimental purposes.

France. Substances with estrogenic activity (i.e. a level of activity identical to or higher than that of estrone in biological assays) are prohibited. Samples from carcasses are checked by thin layer chromatography (TLC) and, if positive, the carcasses are discarded. Tolerance levels have been set for natural but not for artificial estrogens.

Federal Republic of Germany. There is a list of substances, including stilbenes, which may not be used as growth promoters. Other estrogens are regulated as follows: if an estrogen is five times less active than DES, as determined by the mouse uterus assay following oral application, its use in food animals is acceptable, providing it otherwise accords with existing drug laws; in all cases these compounds have to be applied under veterinary control. All estrogens exceeding that level of biological activity must be treated like the stilbene derivatives. Monitoring of stilbenes is by radioimmunoassay (RIA).

Ireland. Estrogens may not be used as growth promoters except under veterinary prescription. Natural estrogens and zeranol are used, as is trenbolone acetate. The monitoring of stilbenes by TLC and RIA is under consideration.

Italy. The use of anabolic agents as growth promoters in animal production is prohibited. Control is by bioassay and chemical assay.

Netherlands. The use of estrogen is illegal, but other compounds are permitted. Control programmes are based on histology and TLC, and RIA is currently being introduced. A control programme at farm level also exists.

Poland. The use of anabolic agents as growth promoters in animal production is not permitted, except for experimental purposes.

USSR. The use of anabolic agents as growth promoters in animal production is not permitted, except for experimental purposes.

United Kingdom. Anabolic agents are licensed under the Medicines Act. They must have proven efficacy and safety. There are regulations governing dose, formulation, route of administration and withdrawal times in designated species. Testosterone, progesterone, estradiol, zeranol and trenbolone acetate are licensed for use in beef cattle and zeranol is also used in sheep. Anabolic agents are used widely in steer beef production. Control is necessary but implementation is in its infancy. RIA is used for initial screening of stilbenes, trenbolone and zeranol in edible tissues and biological fluids, and confirmation of positive samples is obtained by equally sensitive methods, e.g. gas chromatography – mass spectrometry (GCMS). The use of anabolic agents is under continuing surveillance as part of the Meat Monitoring Programme organized by the Ministry of Agriculture and Fisheries.

Yugoslavia. The use of anabolic agents as growth promoters in animal production is not permitted. Experimental testing is well advanced.

European Economic Community (EEC)

On 31 July 1981, the EEC issued a directive banning the stilbene estrogens and thyreostatics from use in animal production. Progesterone, estradiol-17 β , testosterone, zeranol and trenbolone acetate are being reviewed at present to determine what position should be taken with regard to their use. In the meantime, national legislation in member countries operates. To implement the decision to ban certain compounds, proper control measures have to be created. Progress on standardizing methods, procedures and reagents has been made and will be implemented in the near future, in order to provide effective monitoring of residues in meat.

WHO

The Joint FAO/WHO Expert Committee on Food Additives (JECFA) reviewed the problem of hormones in animal production in March 1981. The JECFA agreed that:

(a) anabolic agents increase growth rate and feed efficiency in cattle following implantation;

(b) when properly used, the residue levels in meat and other edible tissues do not exceed 1 ng/g tissue;

(c) natural hormones (estradiol, testosterone and progesterone) give no cause for concern because the contribution of natural hormones from

meat of implanted animals is so small (<8% of intake for estrogenic substances) in relation to levels in the normal diet (estradiol in meat and dairy products, and plant estrogens);

(d) chemically modified hormones and synthetic anabolic agents give rise to specific problems including high potency and the consequent need for minimal residues, potential tumorigenic activity, and the presence of metabolites in meat that might have endocrinological or toxicological consequences;

(e) natural hormones used as anabolic agents cannot be distinguished from endogenously produced hormones;

(f) each synthetic compound should be evaluated by normal toxicological criteria as well as for its hormonal activity.

RESIDUES OF ANABOLIC AGENTS IN MEAT

The residues of anabolic agents found in meat fall into three groups:

(a) residues of estrogens, progestogens and androgens derived from the animals' own endocrine glands (endogenous natural steroids);

(b) residues derived from natural steroidal anabolic agents administered to animals during life (exogenous natural steroids); and

(c) residues derived from xenobiotic anabolic agents administered during life (exogenous xenobiotic agents).

Natural steroids are readily metabolized by the liver and for this reason have little or no activity when given by the oral route.

Three different xenobiotic agents have to be considered.

1. *Trenbolone acetate (TBA)*. This is a trienic steroid with androgenic activity which is readily metabolized by the liver and therefore has only weak activity after oral administration.

2. *Zeranol*. This is a non-steroidal chemical derivative of zearalenone which is derived from a variety of fungi. Zeranol has weak estrogenic activity, and is readily metabolized by the liver.

3. *Synthetic stilbenes, including DES, hexestrol and dienestrol*. These non-steroidal compounds have strong estrogenic activity and high oral potency

in ruminants. Features of these compounds not shared by TBA or zeranol are their high bioavailability and slow biodegradability and the fact that DES has been classified as a carcinogen for humans and laboratory animals (1).

HEALTH SIGNIFICANCE OF RESIDUES OF ANABOLIC AGENTS

Natural steroids

It is widely known that natural steroid hormones in excessive doses may increase the risks of particular types of cancer in humans and laboratory animals, although the mechanisms involved are not clear. Hormones may interact with other factors (chemical, physical or viral) to stimulate tumorigenesis or promote growth and metastasis of tumours, or they may act in other ways (2). When natural hormones influence the risk of cancer, they appear to do so under conditions in which the dose and duration of exposure are such as also to result in changes in hormonal status.

Under the conditions recommended for the use of anabolic agents, the treatment of animals with natural steroids results in residues in meat that are orders of magnitude lower than those that occur naturally in bulls and pregnant animals (Table 1). Residues of natural steroidal hormones derived from treated animals are therefore of negligible concern to human health because (a) they are readily degraded by the liver, (b) the consumer is producing far higher daily quantities of these hormones, and (c) the consumer is exposed to higher and widely variable levels from meat and milk of untreated animals.

The only cause for concern associated with the treatment of animals with natural steroids is that a depot of the administered agent might be consumed by humans. This could happen following incorrect administration by injection or implantation of the agent into edible tissues. For this reason, permitted procedures (e.g. implantation of a pellet into the base of the ear, to be discarded at slaughter) have to be clearly defined and controlled.

Xenobiotic anabolics

Residues of xenobiotic anabolic agents may pose questions of safety, first because of the potency of the hormonal activity and second because they themselves or their metabolites may exhibit toxic effects essentially unrelated to their hormonal activity. Their safety therefore needs to be assessed in both these respects by means of appropriate tests.

The hormonal activity of xenobiotic anabolic agents may mimic that of androgens, estrogens or progestogens, and activities of these kinds can be

Table 1. Human intake of anabolic steroids from meat compared with their endogenous production in humans of various ages

	Testosterone	Estrogen	Progesterone
<i>Production in humans (µg/day)</i>			
Adult male	6 480	136	416
Women — range during cycle	240	190–1 600	418–19 600
— late pregnant	320	64 300	294 000
— post menopausal	140	46	326
Pre-pubertal child	32	42	150
<i>Maximum amounts of hormone (µg) in 250 g meat</i>			
Untreated cattle	0.13 ^a	0.11 ^b	2.5 ^b
Treated steer	0.0006	0.005	0.15
Treated heifer	0.025	0.005	—

^a Mature bull.

^b Pregnant cow.

Sources: Henricks (3) and Reid (4).

measured by available biological techniques. By taking into account the quantity, bioavailability and biological activity of residues, their possible hormonal impact on the consumer can be assessed. Other types of hormonal activity, if any, would be detected in the course of the general safety assessment procedures outlined below. For a xenobiotic anabolic agent to be regarded as safe from a hormonal viewpoint, it must be clear that the levels of hormonal activity of the residues are well below the lowest level expected to cause hormonal disturbance in the consumer. In determining what safety factor should be applied, particular attention needs to be paid to young children, in whom the endogenous production of anabolic hormones is relatively low.

Provided that such residues are safe from the viewpoint of hormonal activity, the other aspects of their safety can be judged on the basis of precisely the same procedures as those employed for food additives, and food contaminants such as pesticide residues. This includes consideration of metabolic pathways in treated animals, the chemical nature and bioavailability of residues in meat, and data from appropriate laboratory tests including acute and chronic toxicity, mutagenicity, carcinogenicity and reproduction/teratogenicity. In the light of the results of these tests, it should be possible to determine whether an acceptable daily intake (ADI) for xenobiotic anabolic residues can be set.

It is common practice in the design of carcinogenicity tests to include a group of animals exposed at the maximum tolerated dose level. If this practice is followed with a xenobiotic anabolic agent, changes in tumour incidence in both directions are bound to occur because of its known hormonal activity. It is necessary to be able to distinguish between these effects and effects on tumour incidence unrelated to known hormonal activity. For this purpose it may sometimes be sensible to include, in the protocols for carcinogenicity studies on xenobiotic anabolic agents, control animals exposed to natural steroids with the same types of hormonal activity. The inclusion of controls treated with natural steroids would probably also be advisable in the case of mutagenicity and reproduction/teratogenicity tests involving exposure to high doses or levels of xenobiotic agents or their metabolites, because the effects of natural hormones in many of the available test procedures are not well documented.

The establishment of an ADI for residues of xenobiotic anabolic agents should be linked to clearly formulated proposals for excluding any possibility that depots of the treatment agent may be ingested by man or other animals.

In the case of DES and other stilbenes (e.g. hexestrol and dienestrol), moves to ban the use of these compounds have already been made or proposed in many countries on the grounds that, unlike natural steroids, they are not readily destroyed in the liver, so that residues in meat products are bioavailable to humans who ingest them. Another cause for concern is that these compounds are not readily biodegradable, so that their use may result in contamination of the food chain and other undesired

environmental effects. Thirdly, DES has been classified as a carcinogen in humans and in laboratory animals (1).

REGULATION OF THE USE OF ANABOLIC AGENTS

The need for regulation

The benefit of anabolic agents is clearly seen in increases in the efficiency and quantity of meat production, particularly with steers and veal calves (3-6). Farmers in many countries, therefore, are aware of the possibilities of profitable application of anabolic agents, and the effect of banning them may be to promote their illegal use. In these circumstances, consumer protection may best be assured by legal and controlled use of products proven to be safe. Illegal use is the only significant source of risk to which the consumer is likely to be exposed, and one which is exclusively related to harmful residues from illegal compounds and/or from improper administration.

Considering the importance of the international trade in meat in Europe, it is desirable to harmonize national regulations on the use of anabolic agents so that the same criteria pertain in all countries. These criteria could be examined by the Codex Alimentarius Commission. In the countries of the Council for Mutual Economic Assistance (CMEA) and in certain other countries anabolic agents are not used in animal production, although the subject is under active discussion. The EEC has issued a directive in this respect, banning the stilbene estrogens and thyreostatics. The use of estradiol, progesterone, testosterone, zeranol and trenbolone acetate as anabolic agents is under review.

In formulating regulations on anabolic agents, the health and protection of the consumer is of paramount importance. Such regulations should be based on thorough scientific evaluation of the different compounds, and should be reviewed periodically to take cognizance of new information. Proper facilities, manpower, and expertise should be provided to allow the regulations to be enforced. The Working Group considered it important that stilbene estrogens be banned in animal production. Natural hormones, under supervision and proper control, could be allowed. Trenbolone acetate and zeranol should be referred to JECFA for scientific evaluation as soon as possible.

Enforcement of regulations

Programmes to control and monitor the legal and illegal use of anabolic agents are required to enforce national regulations. An effective programme should include:

(a) Control of the manufacture and distribution of anabolic agents.

(b) Restriction of the use of anabolic agents to trained personnel.

(c) Control of the method of application to ensure that the site of application is discarded and does not enter the human food chain; in this respect intramuscular injections of crystalline suspensions are particularly dangerous. Implantation in the ear is the currently preferred method in cattle and sheep.

(d) Enforcement of withdrawal period. This can be done by proper certification of date of implantation, and random monitoring of carcasses for residue levels above the tolerance levels set for synthetic compounds. Tolerance levels need to be set for synthetic licensed products.

(e) Veterinary certification of exported meat, based on acceptable control measures.

The illegal use of banned products can be controlled by:

(a) Examination of excreta from animals on the farm.

(b) Random routine monitoring of excreta, bile and tissue at the slaughterhouse. Carcasses containing measurable amounts of banned products should not be permitted to enter the human food chain.

(c) Development and use of sufficiently sensitive assay methods to allow detection of banned compounds in meat destined for export.

MEASUREMENT OF ANABOLIC RESIDUES

Requirements

Analytical methods are required to measure residues of anabolic agents in edible tissues, biological fluids and faeces of farm animals. Without such methods effective regulation is not possible. The residues of substances which are not allowed should be measured by the most sensitive methods available. The residues of xenobiotic anabolics which are allowed should be determined using methods which are at least as sensitive as the established tolerance levels. The residues of natural steroids should be measured using methods which are sensitive enough to determine normal physiological levels

in untreated animals, although this is of low priority at present. The highest priority should be given to developing methods suitable for analysis of substances which are banned, especially DES and other stilbenes.

The evaluation of available assays should consider: (a) practicability (cost and speed of analysis); (b) reliability; and (c) sensitivity.

Radiometric studies have been reported for DES, trenbolone, zeranol, progesterone, estradiol and testosterone (see Annex 1, Tables 4 and 5). Data acquired by radiometric methods are important in establishing the sensitivity required of other methods which may be used for regulatory purposes.

Methods available

The analysis of residues of anabolic agents must meet the requirements set for residues of other chemical substances in food (1,2) and methods should be available in a standardized format, preferably approved by the International Organization for Standardization (ISO). Historically, bioassay and histological methods have proved useful in the investigation of veal calves treated with DES and other potent synthetic estrogens. These methods are limited, and for many substances or species they are not satisfactory. All other methods rely on an end-point analysis of a suitable extract of the sample. The quality of the extract and the time taken to obtain it both affect the overall suitability of the method.

The extraction and purification methods are:

- (a) partition, using solvents;
- (b) chromatographic procedures (thin-layer, high performance liquid, gas and paper chromatography).

The end-point detection methods are:

- (a) immunoassay – both RIA and EIA;
- (b) fluorescence analysis of fluorescent derivatives, especially combined with thin-layer chromatography;
- (c) electron capture in gas chromatography;
- (d) electrochemical and fluorometric analysis coupled with high performance liquid chromatography;
- (e) mass spectrometry coupled with gas chromatography.

For details of these methods see references 7-13.

At present, most countries rely on an immunoassay method for detection, in extracts which have been obtained by solvent/solvent partition extraction from very small amounts of sample (0.1-1 g). Some alternative chromatographic purification procedures are used prior to immunoassay to improve reliability. An EEC working document on radioimmunoassay methods for the stilbenes is available (No. 4694/VI/81-EN). Similar assay methods have been established for most anabolic agents except zeranol, for which methods are being developed.

All analytical methods need validation and for this purpose standards and reference materials must be available. It is recommended that a responsible body prepare and distribute appropriate samples. Reference materials should be obtained both from animals that have been treated with anabolic agents, and from animals that have not been treated. It is recommended that in the first instance a rapid method such as RIA be used to regulate meat and excreta of farm animals. A second method of adequate sensitivity is desirable for confirmation of positive results. To improve present techniques continuing research and development into analytical methodology is recommended.

FLOW OF INFORMATION

The flow of information on current knowledge and research needs to be improved. The meeting felt that WHO could play an important coordinating role by establishing a European collaborating centre on the safety of anabolic agents. This would allow WHO to act as a focal point for research, development, and the standardization of analytical methods and reagents. It would also help WHO to expand information on the effects of hormones on man (14).

Information presented to legislative personnel, if it is to lead to rational decision-making, needs to be precise, clear, accurate, and based on scientifically established facts. Information to the public, to the media and to special interest groups should be on a regular basis, factual and in a form that can be understood. Responses to requests from the public should be met rapidly and the setting up of public information offices should be considered. Information should also be published on a regular basis.

CONCLUSIONS AND RECOMMENDATIONS

1. Anabolic agents improve live-weight gain, carcass weight, feed efficiency and the percentage of meat in the carcass in some species under certain

husbandry conditions. They therefore have a useful role to play in animal production, provided they do not put the consumer at risk.

2. Producers are aware of the obvious benefits to be gained from the use of anabolic agents. To protect the consumer it is necessary to counteract dangerous and illegal practices which may be precipitated by a total ban on their use. It is recommended, therefore, that the legal and safe use of licensed anabolic agents be allowed under appropriate control.

3. The correct use and administration of exogenous natural steroid anabolic hormones poses no known public health problems to the consumer.

4. Stilbene estrogens should not be used in animal production as anabolic agents, because they are orally active, persistent in food, pose some environmental problems because of their low biodegradability, and because diethylstilbestrol (DES) is a known carcinogen.

5. Proper scientific evaluation of xenobiotic anabolic agents is required before their use can be permitted. The safety of trenbolone acetate and zeranol should be evaluated as soon as possible by appropriate international bodies, such as the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the Codex Alimentarius Commission.

6. The first priority for public health is to prevent the illegal use of banned products by establishing monitoring programmes at farms and/or slaughterhouses. Priority should be given to the development and use of analytical methods for the measurement of residues of banned substances. For this purpose the analysis should be performed using the method(s) with the highest sensitivity.

7. In the case of licensed products, it is recommended that the method and site of administration, and the withdrawal period, as laid down under the conditions of use for each product, be strictly observed and controlled to prevent excessive levels of residues entering the food chain.

8. Because of the importance of international trade in Europe, it is desirable to harmonize regulations on the use of anabolic agents, taking into consideration current and emerging legislative developments in the Council for Mutual Economic Assistance (CMEA), the European Economic Community (EEC) and in individual countries.

9. The primary aim of legislation on anabolic agents should be to protect the consumer. Adequate enforcement is essential and requires appropriate manpower, expertise and facilities.

10. For the purposes of evaluating anabolic agents and to assist in the enforcement of legislation, practical, reliable and sensitive analytical methods have to be developed, tested, standardized and rigorously applied. The analysis of residues of anabolic agents must meet the requirements generally set for residues of other chemical substances in food. Methods should be available in a standardized, preferably ISO, format.

11. At present the most suitable methods are either physicochemical or immunochemical; biological methods such as bioassays and histological methods have serious limitations.

12. Radioimmunoassay (RIA) is particularly useful for initial control; where appropriate, physicochemical methods can be used to confirm the presence of residues.

13. The required sensitivity and specificity of the method of detection will depend on the choice of sample, because the level of residue present is highest in excreta, lower in kidney and liver, and lowest in muscle. Excreta are most suitable for monitoring purposes. Large amounts of residues may be found at the site of administration but sampling at this site is not always possible, particularly after illegal administration.

14. To achieve comparability of results, it is recommended that international calibration of assay methods be carried out periodically.

15. It is recommended that, as soon as possible, a European collaborating centre on public health aspects of anabolic agents be established, to act as a focal point for further research on anabolics, including analytical methods, intercalibration, provision of reference materials and information exchange.

16. Information exchange among scientific institutions, decision-makers and the public should be improved. Rapid and full exchange of information on current research activities is essential. Information to decision-makers should delineate feasible alternatives for action and weigh the consequences of each one. Information to the public should be clear, honest, understandable and devoid of sensationalism.

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THE USE OF HORMONES IN FOOD-PRODUCING ANIMALS

B. Hoffmann^a

Introduction

Hormones, or substances with hormonal activity, are used in veterinary medicine for therapy, including preventive medicine, and in animal production for biotechnological purposes. As with all pharmacologically active substances, the use of hormones in food animals can only be considered acceptable if it conforms with public health interests. Particular attention has to be given to the type and quantity of residues formed after treatment.

Classification of anabolic compounds

On the basis of their chemical structure, two groups of compounds can be differentiated.

Group 1

Compounds in this group are proteins and peptides. When present as residues in food, either naturally or after treatment, these hormones are denatured and thus biologically inactivated during the digestive process. This was shown in experiments by Malven et al. (1) in rats and calves; no increase of prolactin, a polypeptide hormone, could be demonstrated in peripheral blood levels after oral application, in spite of the fact that highly sensitive radioimmunoassay techniques were used. Hence, these residues are of little, if any, consequence.

Group 2

This group consists of the corticoids, compounds such as prostaglandin- $F_2\alpha$ which exhibit luteolytic activity, and, of major concern in the present context, substances with sex-hormone-like activity (2). These are all compounds of lower molecular weight, and in contrast to compounds in Group 1, it has

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to be assumed that they can be absorbed from the intestinal tract without being subjected to molecular change; thus, their biological activity is maintained, at least as far as that stage.

For the purpose of classifying residues, the Working Group differentiated between residues derived from: (a) endogenous natural steroids; (b) exogenous natural steroids; and (c) exogenous xenobiotic agents. Xenobiotic substances which are rapidly hydrolysed into a naturally occurring hormone following administration, such as estradiol-monopalmitate, estradiol-benzoate and testosterone-propionate, may also be considered as exogenous natural steroids (cf. Table 2).

Use of hormones in food animals

The group of food animals can be divided into two subpopulations:

- animals raised for slaughter;
- animals kept mainly for breeding purposes, but that may be slaughtered because of reproductive behaviour, or for various managerial reasons.

Animals raised for slaughter

The prime reason for use of hormones is to stimulate growth and to gain extra protein during the feeding period, based on an improved food conversion rate (anabolic effect). These effects, which are in the order of 10–20%, can be achieved predominantly in *ruminants*. No changes in meat quality when compared with untreated animals could be demonstrated (for reviews, see Lu & Rendel (3) and Heitzman (4)).

In general the anabolic effect relates to the sex and age of the animal, the type or combination of compounds used, and the dose given (5,6). With respect to the latter it has been shown (7) that excessive amounts of estrogens may turn the anabolic into a catabolic effect. As is obvious from Table 1, the anabolic effects are strongest in immature and castrated animals.

Sex hormones have also been applied in *poultry* production. Use was restricted to compounds with estrogenic activity, and the effect was less to promote growth than to increase the deposition of fat in muscular tissues (for a review, see Hoffmann (8)).

In *pigs* various sex hormones have been examined for anabolic activity. As reported by Fowler (9) and van Weerden & Grandadam (10), an increase in the ratio of lean meat to fat rather than extra weight gain was observed.

Table 2 gives a list of those compounds which alone, or in combination, are commonly used in animal production to stimulate protein synthesis or to influence the carcass quality.

Table 1. Effectiveness of anabolic agents in cattle

Approximate slaughter weight	Animal	Hormonal activity			
		estrogen	androgen	androgen + estrogen	gestogen
500 kg	male	- +	-	- +	x
	castrated male	+ +	- +	+ +	x
	female	- +	+	+	+
180 kg	male and female	+	-	+ +	x

- not effective, - + marginal if any effects, + effective, + + highly effective, x no data available

Sources: Heitzman (4) and Hoffmann (6).

Breeding animals

Hormones are used in breeding animals to control reproductive function. The two regimes applied are (a) estrous synchronization; and (b) control of parturition. Table 3 lists the compounds which have been found effective.

Legal aspects of the use of hormones

It should be noted, first, that a distinction is frequently made between the application of a drug for therapeutic use by a veterinarian, and its use for promoting growth in food production.

The use of anabolic agents for growth promotion is generally accepted in the United States, and the compounds which can be applied legally under specified conditions are listed in Table 2.

According to the EEC regulation of 21 July 1981, the use of stilbene derivatives and of thyreostatic agents in food animals is prohibited for all purposes within the countries of the EEC. The use of estradiol-17 β , progesterone, testosterone, trenbolone and zeranol for growth promotion will continue under national legislation until a further decision has been made by the EEC Council in 1982. Except for the stilbene derivatives, the EEC regulation allows the use of hormonally active substances for other zootechnical purposes. National laws in EEC countries will have to be adjusted to meet the EEC regulation. Details of existing national regulations in a number of European countries have been given earlier in this report.

Table 2. Biological activity of various anabolic preparations

Classification according to the presence of:	Biological activity			Application in animal production	Examples of countries in which permitted as drug or growth promoter
	estrogenic	androgenic	gestogenic		
Exogenous natural steroids only	estradiol-17 β	testosterone		veal calf	Fed. Rep. of Germany, United Kingdom
	estradiol-17 β		progesterone	veal calf	Fed. Rep. of Germany, United Kingdom
Xenobiotic steroidal compounds (*)	estradiol-benzoate* ^a	testosterone-propionate** ^a		steer, heifer, lamb	United States
	estradiol-benzoate*		progesterone	steer, heifer, lamb	United States
	estradiol-monopalmitate* ^a			poultry	United States
	estradiol-17 β	trenbolone acetate (TBA)*		veal calf, steer	United Kingdom
Xenobiotic non-steroidal compounds (**)	diethylstilbestrol (DES)**			steer, heifer, lamb, veal calf, poultry	United Kingdom
	DES**	testosterone		steer, heifer	United Kingdom
	DES**	methyl-testosterone		pig	United Kingdom
	hexestrol**			steer, heifer, lamb, poultry	United Kingdom
	dienestrol diacetate**			poultry	
	zeranol**			steer, heifer, lamb, veal calf	Fed. Rep. of Germany, Ireland, United Kingdom, United States
	zeranol**	TBA		steer, heifer, lamb, veal calf	France, Ireland, United Kingdom

^a See text.

Analytical control methods

On a worldwide basis the distribution and availability of methods to control the use of anabolic agents must be considered inadequate. It can also be assumed with some confidence that the illegal use of anabolics in food-producing animals is common in many countries. This situation became evident in some western European countries in late 1980, when DES was detected not only in animals but also in baby food containing veal and poultry. In the United States, widespread illegal use of DES in cattle has been discovered following the total banning of DES in 1979 (see *FDA Veterinarian*, July 1980). On the other hand it can be seen from recent statistics issued by the Federal Republic of Germany that, with the introduction and application of practical analytical methods, adequate control is possible. There, due to the development of a routine control programme for DES in urine and faeces using

Table 3. Hormonally active compounds used in animal breeding

Control of:	Compounds		Mode of application	Animal species responding
	endogenous	xenobiotic		
Estrus (estrus-synchronization)	progesterone + estradiol-17 β		intravaginal, injection	cattle, sheep, goat
		various synthetic gestogens ^a	oral	cattle, sheep, goat, pig
	prostaglandin F ₂ α	prostaglandin F analogues	injection	cattle, sheep, goat, horse
Parturition	prostaglandin F ₂ α		injection	cattle, sheep, goat, pig, horse
		prostaglandin F analogues	injection	horse
		various synthetic corticoids ^b	injection	cattle, sheep, goat

^a E.g. chlormadinoneacetate.

^b E.g. flumethasone or dexamethasone.

thin-layer chromatography (TLC) and radioimmunoassay (RIA), the incidence of positive findings has been reduced significantly within a year.

Pharmacokinetic aspects

In order to establish adequate means for consumer protection, the following points have to be considered.

(a) Anabolic agents are metabolized when given to the animal, so the type of residue formation may vary between tissues. Any control method should therefore be based on the determination of a marker compound which, depending on the tissue examined, may be the anabolic agent itself or a main metabolite.

(b) Urine and faeces as well as tissues should be considered for residue analysis. As shown for DES in Table 4, concentrations in faeces are high,

Table 4. DES concentrations (ng/g) in tissues and faeces of two calves, 4 weeks and 3 months after intramuscular injection of 150 mg DES-propionate

Substrate examined	DES (ng/g) following slaughter after:	
	4 weeks	3 months
faeces	600	60
liver	2.3	0.24
kidney	1.5	0.17
muscle ^a	0.12-0.21	0.05

^a Not from the injection site.

Source: Karg (11).

making analysis relatively easy, while they are close to the limit of detection in other tissues, particularly muscle.

(c) In many cases anabolic agents form a depot at the site of application. With illegal treatments, the site of application will not necessarily be discarded at slaughter, constituting a major source of contamination. Analytical methods should therefore be sufficiently sensitive to detect the presence of such a depot.

Recommended sensitivities for analytic methods when applied to different tissues, have recently been drawn up by the EEC, with particular reference to DES (see also Hoffmann (12)):

<i>tissue</i>	<i>detection limit</i>
muscle	< 0.02 ng/g
liver, kidney	< 0.1 ng/g
urine	< 2.0 ng/g
faeces	< 5.0 ng/g

Methods available

Radioimmunoassay has been the methodological approach developed furthest and applied most widely for the detection of residues of anabolic agents

in tissues (13-23). The methodological aspects are covered in detail by Hoffmann (15). With increasing sensitivity the method of gas chromatography and mass spectrometry seems likely to become a useful and practical tool. At present, however, the only methods to have been routinely applied on a large scale are the determination of DES in urine and faeces by RIA and TLC.

Available data on residues

A summary of published results is given in Table 5. The concentration of residues depends on the dose given, or in case of endogenous hormones on the production rate; on the tissue examined; and on the time elapsed between treatment and slaughter. The DES data clearly demonstrate the presence of high concentrations at the injection site.

Finally, it may be concluded that providing an adequate waiting period is observed, it is not possible to distinguish reliably, on the basis of tissue hormone levels, between untreated animals and animals treated with naturally occurring hormones. (Note, however, that this statement does not include the site of application.)

Estrogens in children^a

The newly born infant usually exhibits the influence of the very high concentrations of foetal estrogens present during the terminal phase of intra-uterine life. Following parturition these estrogens are rapidly eliminated from the newborn. In consequence, the threshold levels of estrogens within the feedback mechanisms regulating the endocrine system, particularly the further development of the gonads, are lowered substantially. Children are about 20 times more sensitive to estrogens than adults. This situation lasts until puberty.

Only a few data on "dose-effects" are available. According to Bidlingmaier, 5 mg ethynylestradiol/m² body surface/day may suppress the anterior pituitary function in pre-pubertal children. The induction of estrogenic effects depends not so much on the amount of a *single* dose, but rather on the *time of exposure* (thus, the momentary, accidental consumption of a monthly supply of oral contraceptives by children can probably be regarded as harmless).

The clinical symptoms appearing after exposure to estrogen were defined by Knorr (25) as development of the mammary gland, pigmentation of the nipples and surrounding area, estrogenization of the vaginal epithelium

^a Based on Bidlingmaier (24) and Knorr (25).

Table 5. Concentrations (pg/g) of various anabolic agents (unconjugated form) in tissues of treated and untreated cattle

Compound determined	Animal	Tissue examined				Reference
		Muscle	Liver	Kidney	Fat	
Testosterone	bull	535	749	2 783	10 950	16
	heifer	92	193	595	250	
	veal calf	16	39	256	685	
	(treated) ^a	70	47	685	340	
Progesterone	pregnant cow				360 200	17
	heifer				16 700	
	veal calf				5 800	
	(treated) ^b				12 500	
Estradiol -17 β	pregnant cow	370 - 860				17
	veal calf (untreated and treated)	< 100	< 100	< 100	< 100	
	steer	—	19.7	30.7	—	
	heifer	12.0	38.3	39.8	—	
Estrone	pregnant cow	120 - 2 090				13
	veal calf (treated and untreated) ^c	100	100	100	100	
Trenbolone	steer ^d	50	230	50	80	18
	steer ^e	50	50	20	80	
	veal calf ^c	127	521	235	388	
	veal calf ^f	797	3 467	2 563	2 580	
	veal calf ^g	1 673	4 930	4 083	8 893	

Table 5 (contd)

Compound determined	Animal	Tissue examined				Reference
		Muscle	Liver	Kidney	Fat	
DES	veal calf ^h	90 (113 300) ⁱ	270	770	n.d. ^j	
	veal calf ^k	540	18900	8900	8 300	21,22
	veal calf ^l	120- 210	2 300	1 500	-	
	veal calf ^m	50	240	170	-	
Hexestrol	heifer ⁿ	30	70	140	52	23
	heifer ^o	35	77	50	110	

^a Slaughtered 77 days after implantation of 20 mg estradiol-17 β + 200 mg testosterone.

^b Slaughtered 70 days after implantation of 20 mg estradiol-17 β + 200 mg progesterone.

^c Slaughtered 70-77 days after implantation of 20 mg estradiol-17 β + 140 mg TBA.

^d Slaughtered 60 days after implantation of 40 mg estradiol-17 β + 200 mg TBA.

^e Implantation of 40 mg estradiol-17 β + 200 mg TBA, implant removal after 60 days and slaughtered after another 15 days.

^f Slaughtered 70-77 days after implantation of 200 mg estradiol-17 β + 1 400 mg TBA (10-fold of normal dose).

^g Slaughtered 70-77 days after implantation of 500 mg estradiol-17 β + 3 500 mg TBA (25-fold of normal dose).

^h Slaughtered 4 days after intramuscular injection of 100 mg DES.

ⁱ Muscle from the injection site.

^j n.d. = not detectable.

^k Slaughtered 7 days after intramuscular injection of 200 mg DES-dipropionate.

^l Slaughtered 28 days after intramuscular injection of 150 mg DES-propionate.

^m Slaughtered 90 days after intramuscular injection of 150 mg DES-propionate.

ⁿ Slaughtered 2 days after implantation of 60 mg hexestrol.

^o Slaughtered 7 days after implantation of 60 mg hexestrol.

and indications of menstrual-like bleedings. Some dose relationships were given for 10-year-old children for the extended application of ethynylestradiol:

- (a) 0.001 mg/day, no clinical abnormalities;
- (b) 0.005 mg/day, appearance of clinical symptoms;
- (c) 0.2–0.3 mg/day, inhibition of body growth.

The appearance of a slight gynecomastia can quite frequently be seen in pre-pubertal boys in the absence of any obvious evidence for an exogenous source of estrogens. Two of the few cases which could be considered as "estrogen accidents" were connected with the use of a DES-containing hair lotion and of a DES-containing tuberculostat.

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RESIDUES OF ANABOLICS IN MEAT:
RISKS FOR CONSUMERS

M. Metzler^a

Introduction

The safety of the food we eat is an important issue from both the public health and the ethical standpoints. The presence of harmful constituents in food potentially jeopardizes the health of millions of people, and may eventually lead to great damage, not only to the affected individuals but also to the economy of a country. This is amply demonstrated by the current instance of contaminated cooking oil in Spain. In most cases, however, it might be anticipated that the effect of poisoned food is not so obvious and therefore causes "silent damage", such as an increase in tumour frequency or some teratogenic effect. From an ethical standpoint, the consumer's trust in the safety of food, which he is unable to control, places a particular responsibility on the food producer and on the legislation intended to protect the consumer.

A particularly difficult situation with respect to food safety is created by the use of anabolic agents in meat production. These compounds have hormonal activity (estrogenic or androgenic), but are present as residues in meat only in minute amounts, provided they have been properly used. At first glance, no special risk appears to be involved for the consumer because we all have endogenous hormones (estrogens and androgens) in our bodies in amounts exceeding by orders of magnitude those ingested with meat. Moreover, numerous plants contain hormonally active compounds, frequently in amounts larger than those of the anabolic residues in meat. Therefore the additional body burden caused by anabolics from ingested meat should be negligible with respect to their hormonal effect.

However, a risk evaluation that considers only the hormonal activity of anabolics overlooks the fact that most of the compounds used, such as diethyl stilbestrol, hexestrol, zeranol, and trenbolone acetate, are not identical with the natural estrogens and androgens found in man. Therefore it is not justifiable to consider these compounds simply as "hormones";

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they rather represent individual chemical entities, which, in addition to their hormonal activity, may have all sorts of toxicological properties. It is this "non-hormonal" toxicology that should govern the risk assessment of anabolic agents.

This situation can best be illustrated by reference to diethyl stilbestrol (DES), which is certainly the most thoroughly investigated of all anabolics. Although its use as an anabolic is at present prohibited in the United States and in all EEC countries with the exception of the United Kingdom, it may be assumed to enjoy widespread illegal use. Moreover, the experience gained from DES can usefully be applied to a consideration of other anabolic compounds.

Toxicology of DES

DES has been quite extensively tested for carcinogenicity since the inception, around 1938, of its widespread use in human medication as an estrogen substitute. Today, there can be no doubt that DES is tumorigenic in several animal species (1-3). In humans, there appears to be an increased risk of endometrial cancer in women treated with DES for several years (1). Moreover, DES has been causally related to cancer of the lower genital tract of female offspring, both in humans and in several animal species (1-3). In addition, a high frequency of benign lesions has been observed in human and animal offspring after *in utero* exposure to DES.

To date, the mechanism of the direct as well as the transplacental carcinogenicity and teratogenicity of DES is an open question. Some investigators believe that the tumorigenic effect of DES is a consequence of its estrogenic activity, while others favour the hypothesis that DES, in addition to being hormonally active, also has the quality of a chemical carcinogen.

Although the mechanisms by which chemical carcinogens (such as benzo[a]pyrene and aflatoxin B₁) lead to tumour formation are not completely understood, it is generally believed that an early step in the chemical transformation of a cell involves damage to the genetic material, possibly leading to somatic mutations. Thus, there have been numerous efforts to clarify whether DES has genetic toxicity or not; most of the published results are listed in Table 1.

Of particular interest are the positive data on the chemical transformation of Syrian hamster embryo cells by DES (13,14). First, this effect is clearly not related to the estrogenicity of DES, because another potent estrogen, 17 α -ethynylestradiol, does not transform these cells even at concentrations exceeding that of a transforming concentration of DES by a factor of 5000 (13). Secondly, DES does not cause measurable mutations at two gene loci which are constantly mutated by other chemical carcinogens (14). This may indicate that DES can cause cells to become neoplastic, either without somatic mutation or by a mutational event at the chromosome level

Table 1. Tests on genetic toxicity of DES

Assay system for genetic damage	Result	Reference
<i>Salmonella</i> test (Ames test) under activating conditions	negative	4
Induction of unscheduled DNA synthesis in primary rat hepatocyte cultures	negative	5
Transformation of baby hamster kidney cells in culture	negative	6
Mutations in V79 Chinese hamster cells co-cultivated with primary hepatocytes	negative	7
Mutations in <i>Saccharomyces cerevisiae</i>	positive	8
Mutations in mouse lymphoma cells	positive	9
Induction of unscheduled DNA synthesis in HeLa cells with activating system	positive	10
Induction of sister-chromatid exchange in human fibroblasts <i>in vitro</i>	positive	11
Transformation of mouse fibroblast cells in culture	positive	12
Transformation of Syrian hamster embryo cells in culture	positive	13,14
Chromosome non-disjunction in HeLa cells	positive	15
Aneuploidy in mouse embryo cells <i>in vivo</i>	positive	16
Colchicine-like effects <i>in vitro</i>	positive	17
Mitotic aneuploidy in yeast	positive	18

rather than by a point mutation, frameshift mutation or small deletion. In any event, this demonstrates that a bacterial mutagenicity test like the Ames test certainly does not suffice to clarify the potential for genetic damage of a compound in a mammalian cell.

In addition to the results of assays for genetic damage, metabolic studies of DES implicate the potential of this compound to interact chemically with cellular macromolecules. As is the case with virtually all chemical carcinogens, electrophilic intermediates are formed in the metabolism of DES in several animal species and also in man (for a review, see 19). In accordance with the formation of reactive metabolites, covalent binding to nucleic acids and proteins has been observed in numerous metabolic studies using radioactively labelled DES (20). Some of the oxidative metabolites of DES have been found to be more genotoxic than DES itself, and metabolic activation of DES has been shown to be a prerequisite for genotoxicity in one assay (11).

Apart from the genetic toxicity of DES, other toxic effects of this substance have been reported. For example, DES inhibits DNA repair in human lymphocytes in culture (21), and alters the immunological response in mice (22,23). Moreover, there are reports that DES acts synergistically with X-rays (24) and chemical carcinogens (25) on mammary adenocarcinoma formation in female ACI rats. Prenatal exposure to DES and postnatal treatment with the chemical carcinogen 7,12-dimethylbenz[*a*]anthracene (DMBA) results in a significant increase in the number of mammary tumours as compared to rats treated with DMBA alone (26). In addition, the DMBA-induced tumours obtained after prenatal treatment with DES differ from those of control animals with respect to their hormone dependency for growth (27). Whether prenatal exposure to DES has similar effects in man can only be a matter of conjecture at present, because the exposed population has not yet reached the age where the risk of breast cancer is highest.

Are there threshold values for the effects of DES?

In view of the numerous adverse effects of DES, it is obvious that this compound should be avoided in food unless a "no effect" level can be established, below which it can be assumed with reasonable certainty that DES does not cause harm to the consumer.

For the estrogenic activity, it may be reasonably assumed and can be experimentally shown that, when the dose is lowered, a threshold will be reached at which the fraction of cellular receptors occupied by the estrogenic compound is too small to elicit a biochemical response. This threshold appears to be different between adult and immature organisms, as there is evidence that children are more sensitive than adults to estrogens (28). However, this is not a serious obstacle in setting a tolerance level for the *hormonal effect*, if care is taken that the tolerance level is sufficiently lower than the hormonally effective dose.

Unfortunately, the situation is completely different for the *genotoxic effect* of DES. For genotoxic compounds in general, both whole-animal carcinogenicity and mutagenicity studies, as well as biochemical studies on the covalent binding of the carcinogen to cellular macromolecules, have so far failed to provide evidence for a true threshold at low doses, i.e. a dose of carcinogen which causes no alteration at all of the genetic material of the cell (29,30). Of course, the genetic damage decreases with decreasing dose and, in the case of carcinogens, the latency period of the tumour lengthens and may eventually exceed the lifetime of the organism. At the molecular level, however, even minute doses of carcinogen appear to bind to the DNA in the same relative proportion as do larger doses (30). Applied to the situation of DES residues in meat, this means that even extremely small quantities will cause a certain amount of genetic damage. With continued exposure, as is the case with food constituents, the genetic damage must be expected to accumulate.

The situation is aggravated by the fact that chemical carcinogens have been shown to act synergistically, i.e. even doses which would not give rise to a tumour within the animal's lifetime may do so if other carcinogens, also in sub-tumorigenic amounts, are acquired by the organism. Examples of the synergistic action of DES with ionizing radiation and other chemical carcinogens have been cited above. Therefore, DES may contribute to the total body burden of carcinogens and thus enhance the probability of the consumer developing a tumour.

Because of the genotoxic effects of DES, it is not possible to set a tolerance level for this anabolic agent.

Implications for other anabolic agents

What should we learn from the experience with DES? First, as has already been mentioned, looking only at the hormonal activity of an anabolic is clearly too simplistic. A far greater risk for the consumer is the genotoxic potential, i.e. the carcinogenicity and mutagenicity of an anabolic agent. Unfortunately, this is more difficult to detect. Because of differences in susceptibility between species and strains, a carcinogenicity test with one or two species may well fail to reveal the tumorigenicity of a compound. *In vitro* assays for genetic toxicity are also not without problems, as they may give false negative results, either because the type of genetic damage assayed by the system is not produced by the particular compound, or because the metabolic activation of the tested compound does not operate properly in the test cells. Genotoxic potential is probably best revealed by metabolic studies with radioactively labelled compounds, as the structures of metabolites and the binding to cellular macromolecules often reveal the formation of reactive intermediates.

A second point worth noting from the DES experience concerns the extrapolation from animal carcinogenicity studies to man. Even when the tumorigenic effects of DES in certain animals had been found, these results were largely ignored and interpreted as peculiar hormonal disturbances which would not apply to man. The carcinogenic effect of DES on the human foetus was revealed only because of the medical use of DES during pregnancy and the rare type of tumour (vaginal and cervical clear-cell adenocarcinoma) evoked by DES. It is highly unlikely that such a favourable situation for the detection of carcinogenicity in man will arise for other anabolic agents. The finding of tumorigenicity in animals should be taken as serious evidence of a risk to man.

It may be appropriate in this context to remember that the natural steroid estrogens, estradiol-17 β and estrone, have also shown carcinogenic activity in several animal studies (1). The question of whether they are carcinogenic to man is at present a matter of debate. Interestingly enough, covalent binding to DNA (31) and transformation of mouse fibroblast cells

in culture (12) have recently been demonstrated, which implies the potential of natural estrogens for genetic damage. On the other hand, transplacental carcinogenicity studies in mice, which confirmed the teratogenic and carcinogenic effects of DES, have not revealed any carcinogenicity for estradiol-17 β even at the highest tolerated doses (32). Clearly more research is required to clarify further the carcinogenic risk of natural hormones to man.

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Annex 3

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