Dietary fiber and hormonal processes related to mammary carcinogenesis

The inverse association between dietary fiber and breast cancer risk revisited

Voedingsvezel en hormonale processen in verband met de ontwikkeling van borstkanker

(met een samenvatting in het Nederlands)

PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de Rijksuniversiteit Utrecht,
op gezag van de Rector Magnificus, Prof. Dr. J.A. van Ginkel,
ingevolge het besluit van het College van Decanen
in het openbaar te verdedigen op dinsdag 19 mei 1992
des namiddags te 2.30 uur

door

Cornelis Jacobus Maria Arts

geboren op 10 oktober 1951 te Boven-Leeuwen

Promotores	Prof. Dr. J.H.H. Thijssen Prof. Dr. W.H.P. Schreurs
Co-promotor	Dr. H. van den Berg

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Stellingen behorende bij het proefschrift
'Dietary fiber and hormonal processes related to mammary carcinogenesis: the inverse association between dietary fiber and breast cancer risk revisited'

1. De *in vitro* gemeten β-glucuronidase-activiteit in faecesmonsters is geen maat voor de verhouding tussen ongeconjugeerde en geconjugeerde oestrogenen.
(Dit proefschrift)

2. Bij de pathofysiologie van borstkanker zouden naast de oestrogeenblootstelling ook de progesteronniveaus belangrijk kunnen zijn.

3. Omdat meisjes op steeds jongere leeftijd hun menarche bereiken en vrouwen steeds later voor het eerst zwanger worden is het te verwachten dat de incidentie van borstkanker in de toekomst zal toenemen.

4. De fysiologische eigenschappen van voedingsvezel zijn sterk afhankelijk van de samenstelling en dus van de oorsprong van de vezel.

5. Dat 16α-hydroxyoestron een rol speelt in het ontstaan van borstkanker is twijfelachtig.
(Dit proefschrift)

6. Een hoge voedingsvezelinneming tijdens de ontwikkeling tot jonge vrouw zou in het latere leven borstkanker kunnen voorkomen.
(Dit proefschrift)

7. Een betere opleiding van artsen en verpleegkundigen op het gebied van voeding zou een zinvolle bijdrage leveren aan de beheersing van de kosten van de volksgezondheid.

8. De controle op het illegale gebruik van β-agonisten als anabolica bij de vleesproduktie dient ook bij andere dieren dan de vleeskalveren te worden geïntensiveerd in verband met de potentiële gezondheidsrisico's voor bepaalde bevolkingsgroepen.

9. Het verbod op het gebruik van natuurlijke anabole steroïden als groeibevorderaars heeft meer uit te staan met aspecten als gelijke mededinging dan met zorg voor de volksgezondheid.

10. De voorliefde van wetenschappers voor een overvloedig gebruik van hoofdletters in publikaties stoelt op typografische gebruiken uit de Romeinse tijd en is exemplarisch voor de vernieuwingsdrift van de wetenschap op het terrein van de formele communicatie.
(D.G. van der Heij).

Cor J.M. Arts
19 mei 1992

Woord vooraf

Dit proefschrift kwam tot stand dank zij de inzet en medewerking van velen.

TNO, met name de directie van het Instituut voor Toxicologie en Voeding (prof. dr. R.J.J. Hermus) en de afdeling Experimentele Biologie (hoofd: prof. dr. W.H.P. Schreurs), ben ik zeer erkentelijk voor het feit dat ik kon werken aan het onderzoek naar de relatie tussen voedingsvezel en borstkanker, dat tot deze promotie mocht leiden. Beste Wil, jij gaf en geeft me de vrijheid om dit naast de 'normale' werkzaamheden voor elkaar te krijgen.

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Contents

Part I: Introduction

Literature review of the parameters involved in this study

Introduction

It is commonly accepted that breast cancer is affected by lifestyle factors and that diet is one of the most important of these. Fat, protein, carbohydrate and particular minerals and vitamins are dietary components that might affect mammary carcinogenesis as reviewed recently (1). Other factors that have been associated with breast cancer risk are time of menarche and menopause, parity, age at first birth and familial history of breast cancer (2). This thesis focuses on the effects of dietary fiber on breast cancer risk.

Dietary fiber

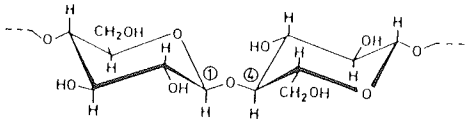
Definition

Trowell (3) defined dietary fiber as the plant cell wall remnants not digested by human alimentary enzymes. In addition to polysaccharides and lignin, this definition would also include indigestible protein, lipids and inorganic constituents of the cell wall. In 1976, Trowell et al. (4) substituted this definition for one which is chemically more precise, including only undigestible polysaccharides and lignin, but at the same time broader in that it also includes undigestible polysaccharides from sites other than the plant cell wall. These compounds cannot easily be differentiated analytically from the cell wall polysaccharides.

Chemical classification and characteristics (5-9)

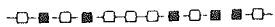
Dietary fiber can be divided into two major chemical classes: *polysaccharides* and *lignins*. Polysaccharides can be further subdivided into *cellulose* and non-cellulosic polysaccharides. The latter group can be further divided into *hemicellulose* and *pectic substances* and – a category of minor importance – mucilages and gums. The structural formulae of the various fiber components are given in Fig. 1, their chemical composition is given in Table 1 and their physicochemical properties are summarized in Table 2.

CELLULOSE

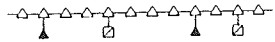


HEMI-CELLULOSE

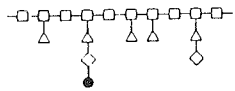
1.4, 1.3 - β - Glucan



Arabinoglucuronoxylan



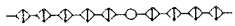
Xyloglucan



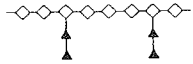
PECTIN

Galactomannan

Rhamnogalacturonan



Arabinogalactan



△ = D-Xylose
□ = 1,4-D-Glucose
◇ = D-Galactose
○ = L-Rhamnose
⊠ = D-Glucuronic acid
▲ = L-Arabinose
▩ = 1,3-D-Glucose
◆ = D-Mannose
● = L-Fucose
◇ = D-Galacturonic acid

LIGNIN

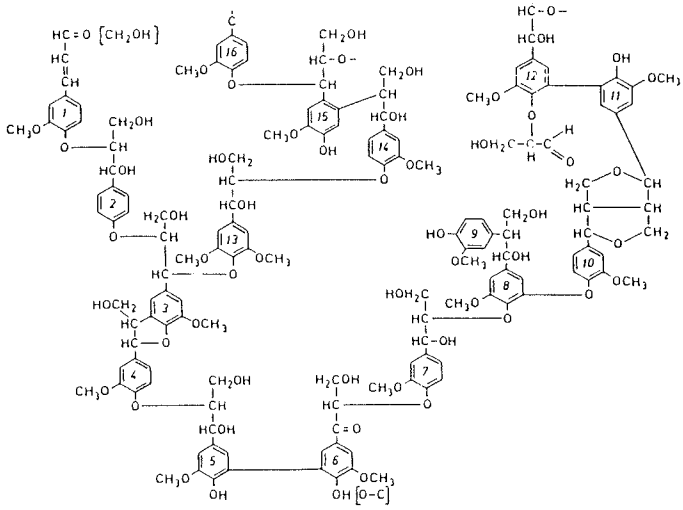


Fig. 1. Chemical structure of the main dietary fiber components.

Table 1. Chemical composition of fiber. After: D. Kritchevsky (5).

Component	Major constituents		Remarks
	primary chains	secondary chains	
Cellulose	glucose	none	linear polymer with β-(1,4) linkages
Hemicellulose	mannose, glucose, galactose, xylose, arabinose	arabinose, galactose glucuronic acid	mainly β-(1,4) pyranosides
Pectins	galacturonic acid	rhamnose, fucose, arabinose, xylose	mainly α-(1,4) galacturans, varying in methylation
Mucilages	galactose-mannose, glucose-mannose, arabinose-xylose, galacturonic acid	galactose	
Gums	galactose, glucuronic acid-mannose galacturonic acid, glucose	xylose, fucose, galactose	
Lignin	sinapyl alcohol, coniferyl alcohol, *p*-coumaryl alcohol	–	complex, cross-linked phenylpropane polymer

Table 2. Physico-chemical properties of dietary fiber. Modified from Bijlani (10).

Component	Water solubility	Water-holding capacity[1]	Breakdown by		Remarks
			EE[2]	BE	
Cellulose	insoluble	5.1	–	+	
Hemicellulose	slightly to moderately soluble	variable	–	+ +	
Lignins	insoluble	negligible	–	–	bind bile acids and estrogens, have antioxidant properties
Pectins	soluble in hot water	56.2	–	+ + +	solution forms gel on cooling, production of short-chain fatty acids when fermented
Gums	soluble		–	+ + +	gel formation
Mucilages	soluble	21.4 (guar gum)	–	+ + +	gel formation

[1] g water per g dry matter.
[2] EE, endogenous enzymes; BE, bacterial enzymes.

Cellulose, the most abundant molecule in nature, is the beta isomer of starch; it is a long (up to 10,000 sugar residues) linear polymer of 1,4β-linked glucose units. Cellulose can be hydrolyzed by cellulase-producing bacteria of the bacterial flora of the rumen of polygastric animals and even partly by those of the human colon. The presence of cellulase-producing bacteria in the rumen of polygastric animals explains why these animals can use grass, which mainly is composed of cellulose, as energy source. The cellulose molecules can orient together closely, attached through hydrogen bonds. This is why cellulose is insoluble in most solvents. In mixed linkage beta-glucans, containing both β1,4 and β1,3 links, this structure is disturbed so that these glucans are highly soluble in water. These β-glucans occur in endosperm cell walls of barley, where they constitute 70–80% of the cell wall dry matter. Starch is also a glucose polymer but it has α1,4 and α1,6 links (= amylose) which are readily hydrolyzed by salivary and pancreatic amylases, and by small intestinal glucoamylase and isomaltase, respectively.

Hemicelluloses are a heterogeneous group, defined originally as polysaccharides soluble in alkali but not in water. Pyranosidic β-1,4-linked sugars form their backbone, as in cellulose, but their monomeric composition as well as their molecular weight and branching varies widely. Typically, each molecule contains 50–200 monomeric residues. The hemicelluloses are subclassified on the basis of the principal monomeric sugar residue. Acidic or neutral forms differ in content of glucuronic and galacturonic acids. The hexose and uronic acid components of hemicelluloses are somewhat more accessible to bacterial enzymes than is cellulose.

Pectins or, more correctly, pectic substances are highly water-soluble and are almost completely metabolized by colonic bacteria. The backbone structure of pectin is an unbranched chain of axial-axial-1→4-linked D-galacturonic acid units. Long chains of galacturonan are interrupted by blocks of L-rhamnose-rich units which result in bends in the molecule. The galacturonic acid residues are either free (pectic acid) or present as methyl esters (pectin).

Mucilages and gums are difficult to define. Some are exudates, while others are components of the plant tissue that act as reserve polysaccharides. They are soluble in hot water.

Lignin is not a carbohydrate. It is a highly cross-linked, complex three-dimensional structure based on phenylpropane units including coniferyl, sinapyl, and *p*-coumaryl alcohols that have undergone a complex dehydrogenative polymerization process. Due to strong intramolecular bonding which includes carbon-to-carbon linkages, lignin is very inert and demonstrates greater resistance to digestion than any other naturally occurring polymer. There are several reasons for including lignin in the dietary fiber concept: its intimate structural relationship to dietary fiber polysaccharides (lignin is covalently linked to hemicellulose), its importance for digestibility of animal feeds and probably also for the physiological properties of dietary fiber in man, and its possible importance as a binder of bile salts in the human gastrointestinal tract.

The physiological effects of dietary fiber are largely attributable to its physicochemical properties. The different constituents of dietary fiber are physiologically diverse. The effects depend not only on the chemical nature of its constituents but also on such factors as physical structure (11, 12), particle size (13, 14), molecular weight and degree of esterification (13). This indicates that dietary fiber is a collective term and should not be seen as a uniform entity. The origin or chemistry of the fiber must be known for an insight into the physiological properties of that fiber (16).

Partly for convenience sake and partly because of a lack of more specific information, the physiological effects of dietary fiber will be considered under one heading. The most striking effects of a high fiber intake are an increased fecal weight, a shortened transit time and an increased water-holding capacity (17–22). The increased water content (dilution) and the accelerated transit of the intestinal contents make such (harmful) compounds as estrogens or carcinogens less available for (re)absorption by the intestinal mucosa, resulting in an increased fecal excretion of these compounds.

Dietary fiber affects the composition of the intestinal flora (23). A change from a grain to a beef diet results in profound alterations in types and amounts of bacteria in the colonic flora of rats within two weeks (24). The composition of the intestinal flora is important because of the changes in activity of different enzymes. The intestinal bacterial β-glucuronidase activity, an enzyme responsible for hydrolysis of glucuronides in the intestinal lumen (25), is higher in stool of humans on a high-meat diet than on a vegetarian, high-fiber diet (18, 26, 27). The activities of nitroreductase and azoreductase, responsible for reduction of nitro- and azo-compounds to aromatic amines (the final products and the highly reactive intermediates are mutagens and carcinogens (28)) have been found to be lower in cecal contents of rats fed a fiber-rich diet than in those of rats fed a fiber-free diet.

Dietary fiber, especially lignin, is able to bind steroids and lipophilic carcinogens (29–31), thus preventing these compounds to be (re)absorbed by intestinal mucosa so that they are excreted with feces.

When dietary fiber passes into the large intestine, it can be fermented by anaerobic microflora resulting in the production of short-chain fatty acids (SCFA) and a lower pH (32). Besides SCFA such as propionic acid, butyric acid and acetic acid, also gases such as hydrogen (H_2), methane (CH_4) and carbon dioxide (CO_2) are produced resulting in an increased flatulence.

In summary, the physiological effects of an enhanced fiber intake are: a shortened transit time; an increased fecal weight; an increased water uptake; alterations of the intestinal flora and changed intestinal bacterial enzyme activities; the binding of steroids, bile acids and carcinogenic compounds; a change in fermentation resulting in a lower fecal pH and the production of SCFA and gases.

Besides dietary fiber, plant food – in particular rye, buckwheat, linseed, wheat, soy, oat and barley – also contains other constituents which are introduced into the body (33). In this context the *lignans* and the *isoflavonic phytoestrogens* are of relevance. Two well-known lignans are enterolactone and enterodiol, which are formed from precursors, such as the plant lignans matairesinol and secoisolariciresinol, by microfloral actions.

The isoflavonic phytoestrogens are heterocyclic phenols with a close similarity in structure to estrogens, and their diphenolic character makes them also similar to lignans. They occur in numerous plants and many studies have shown that they have hormonal effects in animals, the most important being the so-called 'clover disease' (34). Some well-known phytoestrogens are: formononetin, equol, daidzein, genistein. Intestinal bacterial action is involved in the formation of equol and O-desmethylangolensin from formononetin and daidzein present in foodstuffs such as soy products. The structural formulae of some well-known lignans and phytoestrogens are shown in Fig. 2.

Women with a relatively low risk of breast cancer and a relatively high intake of grain products had a higher urinary lignan excretion than women with a low intake of

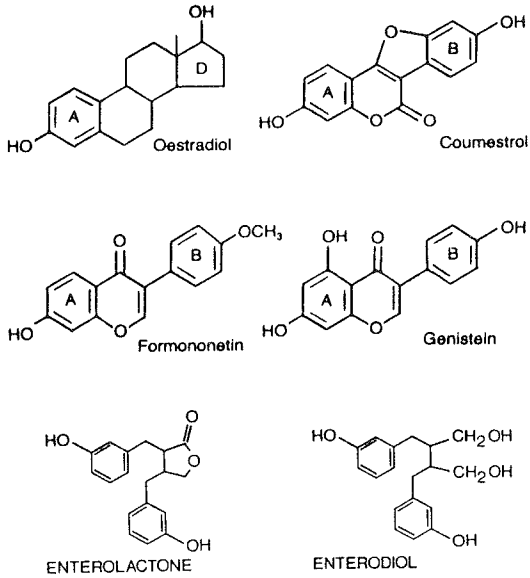


Fig. 2. Chemical structure of some well-known phytoestrogens (coumestrol, formononetin, genistein) and lignans (enterodiol, enterolactone) compared with the endogenous oestradiol-17β.

grain products and a high breast cancer risk. Women consuming various soy products had a very high excretion of isoflavones and were found to have a low risk for breast cancer (35). The biological effects of lignans and phytoestrogens have been reviewed recently (36). Briefly, the lignans enterodiol, enterolactone and matairesinol and the isoflavonic phytoestrogens daidzein, equol, O-desmethylangolensin and genistein have both a weak estrogenic and antiestrogenic activity. Besides, many plant lignans have been shown to have anticarcinogenic, antiviral, bactericidic and fungicidic activities. The estrogenic or antiestrogenic activities of lignans and phytoestrogens depend on their concentration and the presence of endogenous estrogens (35). Antiestrogenic activity can occur when other endogenous or exogenous estrogens are present (37). The most important biological effect of lignans and phytoestrogens in man seems to be the stimulation of SHBG synthesis in liver and thus the reduction of the biological effects of sex hormones. An increase of SHBG results in lowering of the percentage of free estradiol-17β and hence in a reduction of the bioavailability of this steroid. The lowered estradiol-17β bioavailability is supposed to be an important factor in reducing breast cancer. Other biological effects of lignans and phytoestrogens are the inhibition of enzymes involved in cell proliferation such as tyrosine-specific protein kinases. Several plant and mammalian lignans and isoflavones compete with estradiol-17β for the rat nuclear estrogen type II binding site resulting in an inhibition of cell growth.

The estrous cycle of the rat

The unmated female rats exhibit a four- to six-day estrous cycle which continues throughout the year, interrupted only by pregnancy (polyestrous all the year round). The cycle can be divided into four phases (38):

1. *Proestrus* (lasting 18 hours) is essentially a period of preparation, during which the ripening follicles grow and the output of estrogen increases. Under the influence of estradiol the wall of the uterus becomes hydrated and its cavity distended by fluid, while the vaginal epithelial cells multiply to form a thick layer from which the outermost cells are delaminated. Vaginal smears stained according to Papanicolaou (39) show mainly basophilic nucleated epithelial cells and sporadically polymorphic nucleated leukocytes and some non-nucleated orange-yellow-stained unnucleated cells (Fig. 3A). In the later stage of their growth, the follicles are stimulated by FSH, probably potentiated by LH in small quantities.
2. *Estrus* (lasting about 28 hours) is the period of sexual receptivity or heat, which results from the fact that the secretion of estrogen reaches a climax during this phase. As the output of this hormone increases, it is thought to inhibit FSH output and to increase the output of LH; the latter then evokes ovulation, and the secretion of estrogen diminishes. As a result of these interactions, the ova enter the oviducts at a time best calculated to ensure a good prospect of fertilization. During estrus the uterine lumen remains distended, but there is some degeneration of its epithelium, while increasing cornification of the vaginal

smear consisting of orange-yellow cells which are cornified and non-nucleated (Fig. 3B).

3. *Metestrus* (lasting about 8 hours) is marked by a heavy invasion of the vaginal epithelium by polymorphous nucleated leukocytes; these therefore predominate in the vaginal smears, although they are accompanied with some cornified and some nucleated cells (Fig. 3C). The microscopical image shows a 'clear picture'. Meanwhile the uterus becomes reduced in size towards the resting stage characteristic of diestrus, and there is by now a marked reduction of estrogen secretion.
4. *Diestrus* (lasting about 53 hours) is a stage marked by the appearance of both nucleated epithelial cells and, mainly, polymorphous nucleated leukocytes in the vaginal smear and a mucus-rich microscopical image (Fig. 3D), as well as by the formation of corpora lutea. These, however, are virtually functionless in the rat and begin to degenerate at three days after ovulation, which accounts for the short duration of the estrous cycle in these animals.

In effect, the cycle in the unmated female of this species is a purely follicular one, determined by the time required to ripen a new set of follicles; the influence of a luteal phase is seen if the animal is mated, the extent of the influence then depending on whether or not pregnancy ensues. Without mating, diestrus is followed by the proestrus of a new cycle, with FSH secretion increasing again as a result of the reduction of estrogen output; this simple type of cycle thus depends upon the reciprocal interaction of pituitary and ovarian secretions.

Estrogens

Definition

Estrogens are the steroids primarily responsible for the female characteristics such as breast development, (pubic) hair growth and body fat distribution. Estradiol-17β is the biologically most active estrogen followed by its metabolite estrone and estriol.

Synthesis

In premenopausal women the gonads are the main source of estrogen production, whereas the production in the adrenals and metabolism in peropheral tissues are quantitatively of minor importance. In postmenopausal women estrogen production by the ovaria is minimal. In these women the metabolic conversion of androstenedione to estrone in peripheral tissue (adipose tissue, muscle, kidney, liver) by aromatization is the main source of estrogen production. Estrone can be reduced to estradiol-17β by estradiol-17β dehydrogenase. Fig. 4 briefly schematizes the biosynthesis of sex steroids.

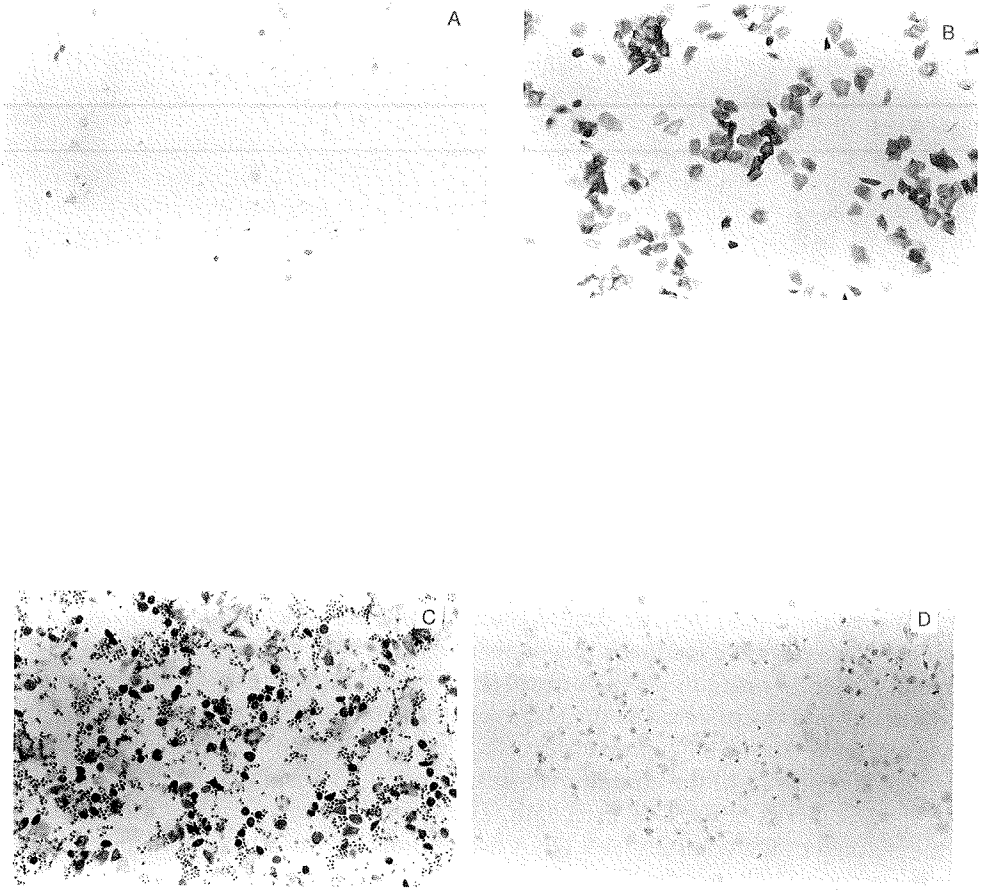


Fig. 3. The four different phases in the estrous cycle of the rat. The typical cells of each phase are indicated with an arrow. A. Proestrus with basophilic nucleated epithelial cells. B. Estrus with non-nucleated orange-yellow cells. C. Metestrus with many polymorphous nucleated leukocytes accompanied with endothelial cells. D. Diestrus with a mucus picture containing polymorphous nucleated leukocytes and nucleated epithelial cells.

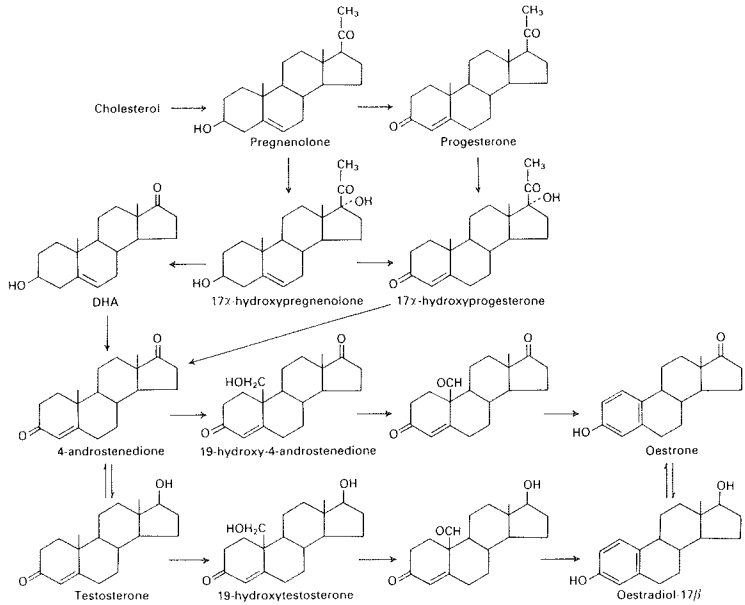


Fig. 4. Biosynthesis of estrogens.

Estrogen production by the gonads is under hypothalamic-pituitary control, regulated by a negative feedback mechanism. Estrogens are secreted in blood plasma, bound to proteins (in man mainly by sex hormone-binding globulin, in rats by albumin) and transported to the target organs. The free estrogens not bound to proteins can be absorbed by the target cells. In the cytoplasm estrogens are bound to estrogen receptors and the complex is transported to nucleoplasm where it can bind with specific places of the DNA strength. The various proteins synthesized as a result of the binding of the complex with DNA are responsible for estrogen properties such as stimulation of cell proliferation, an important item in this thesis.

Metabolism

The metabolism of estradiol-17β to its main metabolites is shown in Fig. 5. After oxidation to estrone, this metabolite can be converted to estriol via C16 hydroxylation. In this conversion 16α-hydroxyestrone seems to be a remarkable intermediate product as it is supposed to be a possible initiator of breast cancer (40,41). Another pathway for the conversion of estrone is by C2 hydroxylation to catecholestrogens. In this case 2-hydroxyestrone and 2-methoxyestrone are important products which seem to affect neurohypothalamic processes (42).

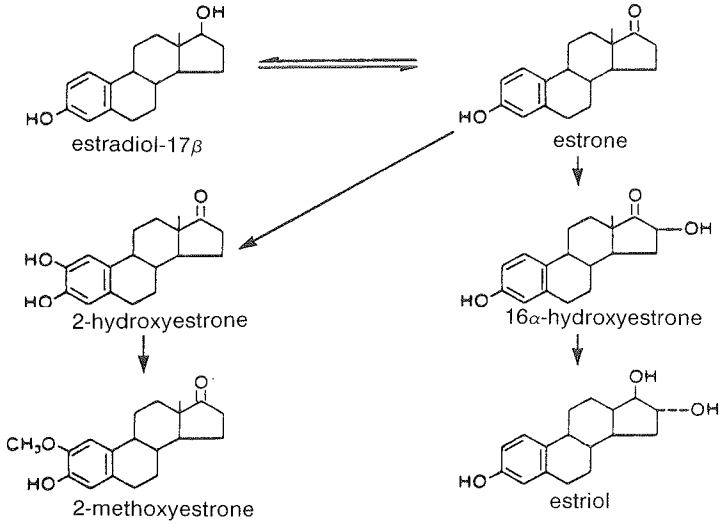


Fig. 5. Metabolism of estrogens.

Estrogens and cell proliferation

Hypotheses on the mode of action of estrogens in cell proliferation are manifold (43). The positive hypothesis proposes interaction of estradiol with the receptor that triggers the multiplication of its target cells directly (Fig. 6A) or indirectly by inducing the synthesis and/or release of growth factors (estromedins) or facilitating factors (plasminogen activator) which, in turn, cause proliferation of estrogen-sensitive cells in other organs (Fig. 6B). In the autocrine hypothesis estradiol induces the synthesis and secretion of a variety of growth factors by estrogen target cells themselves (Fig. 6C). The negative hypothesis proposes an estrogen effect on releasing the inhibition of the proliferation of their target cells by inhibiting the synthesis and/or release of a specific inhibitor of E2-sensitive cells secreted by an intermediary organ or by neutralizing the action of blood borne specific proliferation inhibitors (Fig. 6D).

It has been clearly shown that estrogens act proliferatively at an increased concentration in the physiological range. At supra-physiological levels cell proliferation will not be influenced or even decreased (44).

Peroxidase

Different peroxidases have been described (45). Next to lactoperoxidase, myeloperoxidase, eosinophyl peroxidase and thyroid peroxidase, also uterine peroxidase has been described. The latter peroxidase (donor: hydrogen-peroxide

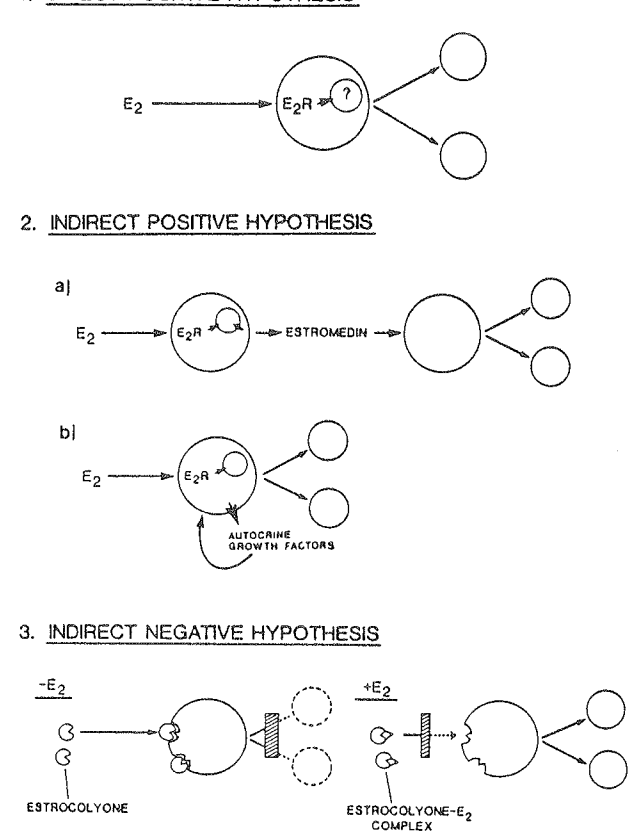


Fig. 6. Schematic representation of working hypotheses on the regulation of initiation of the proliferation of estrogen-sensitive cells. (For explanation see text; modified from 43.)

oxidoreductase, EC 1.11.1.7) has been proved to be a useful biomarker for estrogen exposure. Profound increase in the total content of this enzyme in the uterine tissue has been observed after treatment with estradiol, while peroxidase activity located in lung and spleen did not react to estradiol-17β treatment (46). Progesterone and testosterone injections did not stimulate peroxidase synthesis in immature or ovariectomized rats (47). It has been suggested that estrogen-induced peroxidase accumulation occurs only in tissues, such as uterus, vagina and carcinogen-induced mammary tumors, whose growth is affected by estrogen. This peroxidase can be induced by physiological doses of estrogen. It has been suggested that uterine peroxidase may function to terminate estrogen action in target tissues as peroxidase can metabolize estrogen to water-soluble products (48).

Peroxidase activity has been demonstrated in uteri of rats during proestrus, estrus and constant estrus and in mammary tumors. The method used was not sensitive

enough to demonstrate peroxidase activity in normal mammary tissue and in uterine tissue of rats during metestrus and diestrus (49). In this thesis, peroxidase activity was assessed by using a modified method of Lyttle et al. (49). Instead of guaiacol oxidation, tetramethyl-benzidine was used as the substrate and horseradish peroxidase as the standard. Samples and standards were incubated until the lowest standard points colored. With this sensitive peroxidase assay we were able to measure uterine peroxidase in uterine endothelial cells and in normal mammary tissue in all stages of the cycle.

Enterohepatic circulation

The enterohepatic circulation of estrogens is shown schematically in Fig. 7. Estradiol-17β, synthesized mainly in the ovaria, is transported via the blood to the liver. In the liver estradiol-17β is metabolized, mainly to estrone and to a lesser extend to estriol. The estrogens are conjugated, i.e. coupled to glucuronic acid and/or a sulfate group making them water-soluble. It is excreted in part via the kidney in urine. About 50% is excreted by the bile in the intestine. After deconjugation about 80% of these estrogens excreted via the bile are reabsorbed via the intestinal wall and the vena

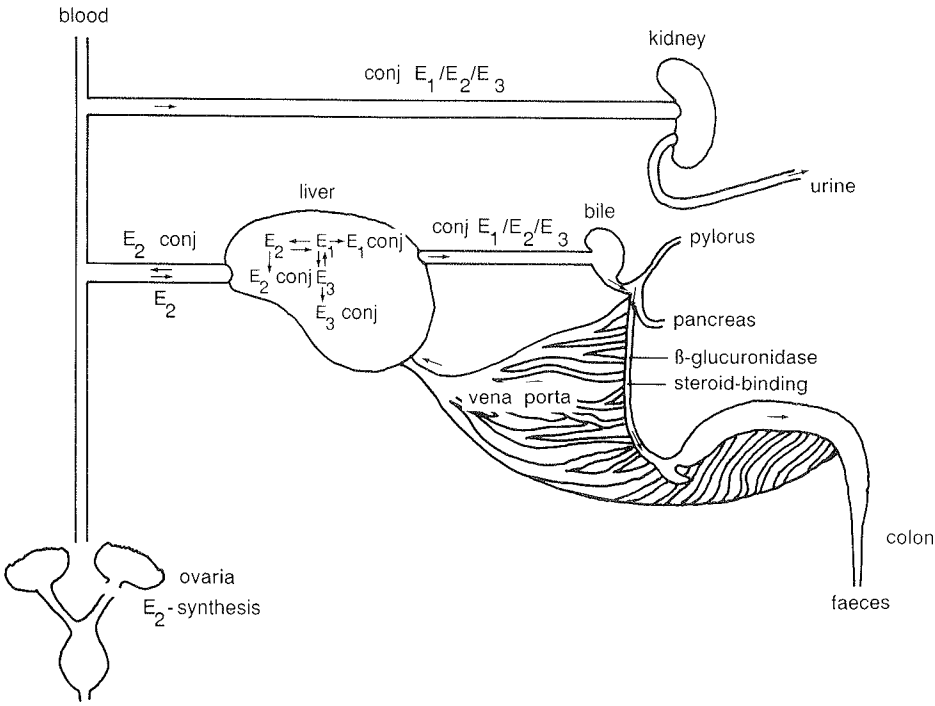


Fig. 7. Enterohepatic circulation of estrogens.

porta to the liver (50). Dietary fiber can interrupt the enterohepatic circulation in at least two ways. First, a high fiber intake results in an altered intestinal microflora through which bacterial enzyme activities are changed as well. The activity of intestinal β-glucuronidase is lower on high-fiber diets than on diets rich in meat and low in fiber. A decreased β-glucuronidase activity can result in a lowered deconjugation of conjugated estrogens and – as only unconjugated (free) estrogens can be reabsorbed – a decreased resorption and consequently in an increased fecal estrogen excretion. Second, estrogens can bind to poorly fermentable or unfermentable dietary fiber components such as lignin, hemicellulose and cellulose (29). As a consequence, estrogens bounded cannot be reabsorbed and are excreted in feces. As a result of the interrupted estrogenic reabsorption, less estrogens will be excreted in the urine and hence lower urinary estrogen levels can be expected. In vegetarians also lower plasma estrogen levels have been found (19).

Mammary development

Female rats have 6 pairs of mammary glands. At birth and during the first week of postnatal life, the mammary gland is composed of one or two main lactiferous ducts arising from the nipple and branching into 3–5 secondary ducts. The ducts are narrow and straight and end in small, club-shaped 'terminal end buds' (TEB). During the second week, further sprouting of ducts (up to the sixth generation) occurs. The density of TEB (number of TEB/mm^2), which increases steadily after birth, reaches its maximum value when animals are 21 days of age, with a concomitant increase in total area of mammary gland. Then further sprouting of lateral buds occurs and numerous TEB begin to cleave into 3–5 smaller buds, i.e. the 'alveolar buds' (AB). The differentiation of TEB into AB causes a progressive decrease in number of TEB and a concomitant increase in the number of ABs. AB increase in number with each successive estrous cycle until the animal reaches the age of about 60 days. Thereafter, the number of AB remains almost constant as long as the animal does not become pregnant.

After the beginning of the estrous cycle (between 35–42 days of age), the formation of 'lobules' (L) also starts. L are formed by further differentiation or cleavage of some AB into smaller alveoli, which remain grouped around a main duct. After rats have reached the age of about 60 days these structures are no longer recognizable as TEB but can be identified as 'terminal ducts' (TD). They have a smaller diameter than TEB, due to a progressive hypoplasia of the epithelium, which becomes two layers thick. TD do not undergo further morphologic changes as long as the animal remains virgin (51, 52).

In women, during childhood, the glands consist of a few branching rudimentary ducts lined by flattened epithelium, surrounded by collagenous connective tissue. With the onset of puberty, follicular ripening in the ovaries is accompanied with an increased output of estrogenic hormone. The rudimentary mamma begins to show growth activity both in the glandular tissue and in the surrounding stroma. The distal

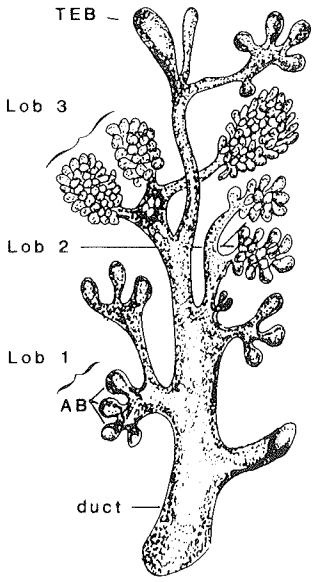


Fig. 8. The various topographic compartments of the human mammary gland: terminal end buds (TEB); alveolar buds (AB); lobuli 1, 2, and 3 (Lob 1, Lob 2 and Lob 3); and duct. Modified from 53.

or free end of each duct ends in a bulbous formation equivalent to the TEB in the rat. Main ducts give origin to new branches, and terminal ducts or lateral buds give origin to alveolar buds. Lobule formation occurs within 1 or 2 years after the onset of the first menstrual period (Fig. 8). Full differentiation of the mammary gland is a gradual process taking many years, and if pregnancy does not supervene, is never attained. Terminal ducts and TEB are mainly present in the virginal breast (53).

Some basic differences in developmental pattern between the human and the rat mammary glands have been observed: [1] in the human glands the ductal structures grow along connective tissue septa and rarely the lobular structures grow into adipose tissue. In the rat, instead, there is a constant growth of ductal and lobular structures into the adjacent adipose tissue; [2] in contrast to women, in the rat there is in general a good correlation between gland development and age. Different degrees of glandular development are seen in women of the same age which may depend on endocrinologic, genetic or socioeconomic differences, among other factors (53).

Mammary cancer

Susceptibility to malignant transformation has been correlated with DNA synthesis (54), cell proliferation (55) and the developmental stage of the gland at the time of carcinogen administration (51). Carcinogenic initiation occurs primarily in the epithelium of the TEB while they are developing into AB and TD; these structures

are considered to be equivalent to the terminal ductal lobular unit (TDLU) described in the human breast by Wellings et al. (56).

While mammary carcinomas mainly arise from undifferentiated structures of the gland (TEBs), benign lesions such as adenomas, cysts and fibroadenomas arise from structures that are more differentiated at the time of carcinogen administration. This indicates that the carcinogen requires an adequate structural target and the type of lesion induced is dependent on the area of the mammary gland attacked by the carcinogen. Thus, the more differentiated the structure at the time of carcinogen administration, the more benign and organized is the developing lesion.

The comparison between rats and man for the pathogenic pathways of mammary carcinogenesis is only tentative, because the role of the human terminal end bud is not known. Most of the data collected for the study of mammary carcinogenesis are derived from observations of postpubertal breasts. Then, most of the structures of the gland are mainly composed of TDs and ductules forming lobulus type I, which are the basic TDLU. The terminal ductal structures of the TDLU give rise to preneoplastic lesions which evolve to ductal carcinomas in situ, progress to invasive carcinomas and finally metastasize. Chemically induced mammary carcinogenesis requires active cell replication which is maximal in the TEB. In the human breast, the highest peak of cell replication occurs in the TD during early adulthood, decreasing considerably with age. This observation indicates that during early adulthood, when cell turnover is more rapid, more change exist for a neoplastic process to be initiated (53).

References

1. Adlercreutz, H. Diet and sex hormone metabolism. In: Rowland IR, ed. Nutrition, toxicity and cancer, CRC Press, London, pp. 137-195 (1991).
2. Kelsey JL, Gammon MD. Epidemiology of breast cancer. Epidemiol Rev 12: 228-240 (1990).
3. Trowell H. Ischemic heart disease and dietary fiber. Am J Clin Nutr 25: 926-932 (1972).
4. Trowell H, Southgate DAT, Wolever TMS, Leeds AR, Gassull MA, Jenkins DJA. Dietary fiber redefined. Lancet i: 967 (1976).
5. Kritchevsky D. Dietary fiber. Annu Rev Nutr 8: 301-328 (1988).
6. Kay RM. Dietary fiber. J Lipid Res 23: 221-242 (1982).
7. Asp NG. Dietary fiber- Definition, chemistry and analytical determination. Mol Aspects Med 9: 17-29 (1987).
8. Theander O. Chemistry of dietary fiber components. Scand J Gastroenterol 22 (Suppl): 21-28 (1987).
9. Jacqmain D. The composition of dietary fibers. In: Williams DL, ed. The status of dietary fiber: Physiology and current practice. Reckitt & Colman Foundation / Elsevier Librico, Brussels, pp. 15-42 (1984).
10. Bijlani RL. Dietary fiber: consensus and controversy. Progr Food Nutr Sci 9: 343-393 (1985).
11. Eastwood MA, Kay RM. An hypothesis for the action of dietary fiber along the gastrointestinal tract. Am J Clin Nutr 32: 364-367 (1979).
12. Robertson JA, Eastwood MA. An examination of factors which may affect the water holding capacity of dietary fibre. Br J Nutr 45: 83 (1981).
13. Heller SN, Hackler LR, Rivers JM, Van Soest PJ, Roe DA, Lewis BA, Robertson J. Dietary fiber; the effect of particle size of wheat bran on colonic function in young adult men. Am J Clin Nutr 33: 1734-1744 (1980).

14. van Dokkum W, Pikaar NA, Thissen JTNM. Physiological effects of fibre-rich types of bread. 2. Dietary fibre from bread: digestibility by the intestinal microflora and water-holding capacity in the colon of human subjects. Br J Nutr 50: 61-74 (1983).
15. Atallah MT, Melnik TA. Effect of pectin structure on protein utilization by growing rats. J Nutr 112: 2027-2032 (1982).
16. Eastwood MA. What does the measurement of dietary fiber mean? Lancet i: 1487 (1986).
17. Kay RM, Strasberg SM. Origin, chemistry, physiological effects and clinical importance of dietary fiber. Clin Invest Med 1: 9-24 (1978).
18. Reddy BS, Hedges AR, Laakse K, et al. Metabolic epidemiology of large bowel cancer. Fecal bulk and constituents of high-risk North American and low-risk Finnish population. Cancer 42: 2832-2838 (1978).
19. Goldin BR, Adlercreutz H, Sherwood L, et al. Estrogen excretion patterns and plasma levels in vegetarian and omnivorous women. New Engl J Med 307: 1542-1547 (1982).
20. Spiller GA, Story JA, Wong LG, Nunes JD, Alton M, Petro MS, Furumoto EJ, Whittam JH, Scala J. Effect of increasing levels of hard wheat fiber on fecal weight, minerals and steroids and gastro-intestinal transit time in healthy young women. J Nutr 116: 778-785 (1986).
21. Shetty PS, Kurpad AV. Increasing starch intake in the human diet increases fecal bulking. Am J Clin Nutr 43: 210-212 (1986).
22. Eastwood MA, Elton RA, Smith JH. Long-term effect of wholemeal bread on stool weight, transit time, fecal bile acids, fats, and neutral sterols. Am J Clin Nutr 43: 343-349 (1986).
23. Hill MJ, Goddard P, Williams REO. Gut bacteria and aetiology of cancer of the breast. Lancet ii: 472-473 (1971).
24. Weinstein WM, Onderdonk AB, Bartlett JG, et al. Experimental intra abdominal abscesses in rats. Development of an experimental model. Infect Immun 10: 1250-1255 (1974).
25. Goldin BR, Gorbach SL. The relationship between diet and rat fecal bacterial enzymes implicated in colon cancer. J Natl Cancer Inst 57: 371-375 (1976).
26. Reddy BS, Weisburger JH, Wynder EL. Fecal bacterial beta-glucuronidase: control by diet. Science 183: 416-417 (1974).
27. Reddy BS, Wynder EL. Large-bowel carcinogenesis: fecal constituents of populations with diverse incidence rates of colon cancer. J Natl Cancer Inst 50: 1437-1442 (1973).
28. McCalla DR, Reuvers A, Kaiser C. Mode of action of nitrofurazone. J Bact 104: 1126-1134 (1970).
29 Shultz TD, Howie BJ. In vitro binding of steroid hormones by natural and purified fibers. Nutr Cancer 8: 141-147 (1986).
30. Gulliver WP, Kutty KP, Laher JM, Barrowman JA. In vitro interaction of 7,12-dimethylbenz(a)anthracene and its biliary metabolites with dietary fibers. J Natl Cancer Inst 71: 207-210 (1983).
31. Smith-Barbaro P, Hanson D, Reddy BS. Carcinogen binding to various types of dietary fiber. J Natl Cancer Inst 67: 495-497 (1981).
32. Jacobs LR. Relationship between dietary fiber and cancer: Metabolic, physiologic, and cellular mechanisms. Proc Soc Exp Biol Med 183: 299-310 (1986).
33. Axelson M, Sjövall J, Gustafsson BE. Origin of lignans in mammals and identification of a precursor from plants. Nature 298: 659-660 (1982).
34. Braden AWH, Hart NK, Lamberton JA. The oestrogenic activity and metabolism of certain isoflavones in sheep. Aust J Agric Res 18: 335-348 (1967).
35. Adlercreutz H. Diet and sex hormone metabolism. In: Rowland IR, ed. Nutrition, toxicity and cancer. CRC Press, London, pp. 137-195 (1991).
36. Adlercreutz H, Mousavi Y, Loukovaara M, Hämäläinen E. Lignans, isoflavones, sex hormone metabolism and breast cancer. In: Hochberg RB, Naftolin F, eds. The new biology of steroid hormones. Serono Symposia Publications 74: pp. 145-154. Raven Press, New York (1991).
37. Mäkolä S, Pylkkänen L, Santti R, Adlercreutz H. Role of plant estrogens in normal and estrogen-related altered growth of the mouse prostate. In: Proceedings Interdisciplinary Conference on Effects of food on the immune and hormonal systems. Euro Food Tox III, May 23-24, pp. 135-139 (1991).
38. Barrington EJW. An introduction to general and comparative endocrinology. Clarendon Press, Oxford, pp. 129-130 (1975).

39. Papanicolaou GN. A new procedure for staining vaginal smears. Science 95: 438-443 (1942).
40. Bradlow HL, Hershcopf RJ, Martucci CP, Fishman J. Estradiol 16α-hydroxylation in the mouse correlates with mammary tumor incidence and presence of murine mammary tumor virus: A possible model for the etiology of breast cancer in humans. Proc Natl Acad Sci USA 82: 6295-6299 (1985).
41. Fishman J, Martucci C. Biological properties of 16α-hydroxyestrone: implications in estrogen physiology and pathophysiology. J Clin Endocr Metab 51: 611-615 (1980).
42. Fotherby K. Endocrinology of the menstrual cycle and pregnancy. In: Makin HLJ, ed. Biochemistry of steroid hormones. Blackwell Scientific Publications, London, pp. 409-440 (1984).
43. Soto AM, Sonnenschein C. Cell proliferation of estrogen sensitive cells: the case for negative control. Endocrine Rev 8: 44-52 (1987).
44. Schatz R, Soto AM, Sonnenschein C. Estrogen-induced cell multiplication: direct or indirect effect on rat uterine cells? Endocrinology 115: 501-506 (1984).
45. Bhattacharyya J, Datta AG. Uterine peroxidase: properties and possible functions. J Sci Industr Res 46: 535-540 (1987).
46. Lyttle CR, DeSombre ER. Generality of oestrogen stimulation of peroxidase activity in growth responsive tissues. Nature 268: 337-339 (1977).
47. Anderson WA, Kang YH, DeSombre ER. Endogenous peroxidase: specific marker enzyme for tissues displaying growth dependency on estrogen. J Cell Biol 64: 668-681 (1975).
48. Lyttle CR, Jellinck PH. Estrogen-induced metabolism of estradiol-17β in the rat uterus: a possible mechanism for the termination of estrogen action. Steroids 20: 89-92 (1972).
49. Lyttle CR, DeSombre ER. Uterine peroxidase as a marker for estrogen action. Proc Natl Acad Sci USA 74: 3162-3166 (1977).
50. Adlercreutz H, Martin F. Biliary excretion and intestinal metabolism of progesterone and estrogens in man. J Steroid Biochem 13: 231-244 (1980).
51. Russo IH, Russo J. Developmental stage of the rat mammary gland as determinant of its susceptibility to 7,12-dimethylbenz[a]anthracene. J Natl Cancer Inst 61: 1439-1449 (1978).
52. Russo J, Russo IH. DNA labeling index and structure of the rat mammary gland as determinants of its susceptibility to carcinogenesis. J Natl Cancer Inst 61: 1451-1459 (1978).
53. Russo J, Gusterson BA, Rogers AE, Russo IH, Wellings SR, van Zwieten MJ. Biology of disease: comparitive study of human and rat mammary tumorigenesis. Lab Invest 62: 244-278 (1990).
54. Nagasawa H, Yanai R, Taniguchi H. Importance of mammary gland DNA synthesis on carcinogen induced mammary tumorigenesis in rats. Cancer Res 36: 2223-2226 (1976).
55. Nagasawa H, Yanai R. Frequency of mammary cell division in relation to age: its significance in the induction of mammary tumors by carcinogen in rats. J Natl Cancer Inst 52: 609-610 (1974).
56. Wellings SR, Jensen MM, Martcum RG. An atlas of subgross pathology of the human breast with special reference to possible precancerous lesions. J Natl Cancer Inst 55: 231-273 (1975).

CHAPTER 2

Aim of the study

Rationale

Epidemiological and experimental studies have demonstrated that breast cancer risk is associated with diet (1–4). In countries with a Western-type diet (high fat and low fiber intake) the incidence of breast cancer is high, whereas in countries with a low fat intake (South America, Africa, Asia) incidence is low. Two life-span studies for spontaneous tumors in rats showed a negative correlation between dietary fiber intake and mammary cancer incidence.

Many studies have demonstrated that steroid hormones, especially estrogens, are involved in breast cancer development. Gorbach (5) hypothesized that dietary fiber affects intestinal microflora resulting in a lowered intestinal β-glucuronidase activity. This would result in an interrupted enterohepatic circulation of estrogens as only deconjugated (free) estrogens can be reabsorbed by the intestinal wall. An enhanced fecal estrogen excretion would result in a lowered exposure of mammary tissue to estrogens resulting in a decreased cancer risk.

A high fiber content in the diet implies that also other constituents of vegetable origin are available. The work of Adlercreutz et al. (6–8) has demonstrated that precursors of lignans and phytoestrogens can be present, especially in cereals. The precursors of lignans can be converted by intestinal microflora to lignans. The two major lignans demonstrated are enterodiol and enterolactone. These lignans and some phytoestrogens were found to have anti-estrogenic and anti-carcinogenic properties.

Fishman and Bradlow (9, 10) hypothesize that 16α-hydroxyestrone, a major metabolite in estrogen metabolism, is a risk factor in breast cancer development. It was found that 16α-hydroxyestrone can bind covalently to amino groups of DNA molecules (11). Furthermore, an increased 16α-hydroxylation was found in strains of mice with a high risk for mammary cancer development.

The experimental work of Russo and Russo (12–14) demonstrated that mammary tissue of rats is mainly susceptible to chemical induction of tumors during the time mammary tissue contains undifferentiated cells. Especially, the terminal end bud cells, which are rapidly dividing endothelial cells, are susceptible to tumor initiation.

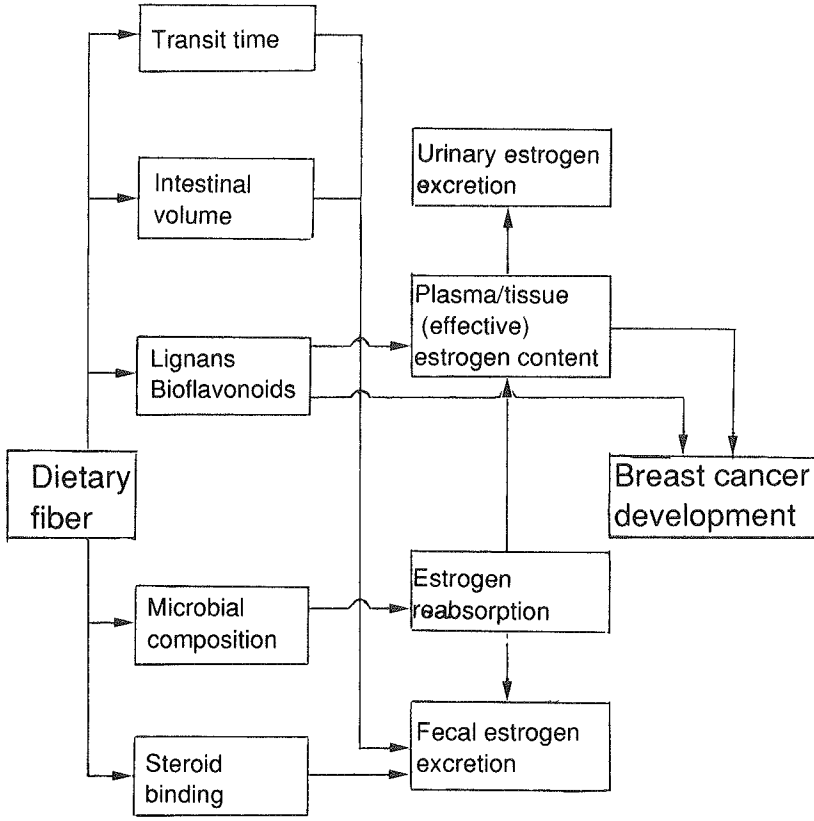


Fig. 1. Schematic presentation of the hypothetical role of dietary fiber in hormone metabolism and mammary cancer incidence.

Working hypothesis

The above findings have led to a working hypothesis on dietary fiber, steroid hormones and breast cancer. The protective effect of dietary fiber on mammary carcinogenesis may proceed via the following mechanisms.

1. A high fiber consumption is responsible for an increased fecal mass and increased bowel motility. It also decreases the activity of some important bacterial intestinal enzymes such as β-glucuronidase. This may result in an interrupted enterohepatic circulation of estrogens and a reduced enterohepatic uptake of any carcinogens present in the diet.
2. Another important aspect of a high fiber content in the diet is the fact that lignans, especially enterolactone and enterodiol, are formed by bacterial enzymes in the intestine. These compounds have been suggested to have anti-carcinogenic and anti-estrogenic activity.

3. A high fiber intake might result in decreased estrogen levels in blood and tissue. Less estrogen will be available as a substrate for 16α-hydroxyestrone synthesis. This compound may be a potential carcinogen, forming covalents bonds with DNA.

The working hypothesis with regard to the influence of a high fiber consumption on estrogen metabolism and its effects on breast cancer incidence can be presented schematically as shown in Fig. 1.

This working hypothesis lead us to the following questions.

1. Does dietary fiber (on the basis of wheat bran) alleviate the promotion of chemically induced mammary tumors?
2. Does dietary fiber influence estrogen balance (fecal and urinary estrogen excretion, and plasma estrogen levels) and estrogen production?
3. Is 16α-hydroxyestrone a mutagenic compound capable of initiating mammary tumors?
4. Does dietary fiber influence estrogen exposure and/or cell proliferation of estrogen-sensitive tissue?
5. Can the inverse association between dietary fiber consumption and breast cancer incidence be related to estrogen exposure or can other factors be involved?

These questions have prompted us to undertake a number of experiments which will be described and discussed in the second part of this thesis.

References

1. Goldin BR, Adlercreutz H, Gorbach SL, et al. The relationship between estrogen levels and diets of Caucasian American and Oriental immigrant women. Am J Clin Nutr 44: 945-953 (1986).
2. Goldin BR, Adlercreutz H, Gorbach SL, et al. Estrogen excretion patterns and plasma levels in vegetarian and omnivorous women. New Engl J Med 307: 1542-1547 (1982).
3. Fisher N, Berry CS, Fearn T, Gregory JA, Hardy J. Cereal dietary fiber consumption and divertical disease: a lifespan study in rats. Am J Clin Nutr 42: 788-804 (1985).
4. Sinkeldam EJ. Dietary fiber and cancer of the large bowel and breast. In: Leegwater DC, Feron VJ, Hermus RJJ, eds. Low digestibility carbohydrates. Proceedings of a workshop held at the TNO-CIVO Institutes, Zeist, Netherlands, 27–28 November 1986. Pudoc, Wageningen, Netherlands, pp. 96-102 (1987).
5. Gorbach SL. Estrogens, breast cancer, and intestinal flora. Rev Infect Diseases 6: S85-S89 (1984).
6. Adlercreutz H, Fotsis T, Heikkinen R, Dwyer JT, Woods M, Goldin BR, Gorbach SL. Excretion of the lignans enterolactone and enterodiol and of equol in omnivorous and vegetarian postmenopausal women and in women with breast cancer. Lancet ii: 1295-1299 (1982).
7. Adlercreutz H. Does fiber-rich food containing animal lignan precursors protect against both colon and breast cancer? An extension of the 'fiber hypothesis'. Gastroenterology 86: 761-766 (1986).
8. Adlercreutz H, Höckerstedt K, Bannwart C, Hämäläinen E, Fotsis T, Bloigu S. Association between dietary fiber, urinary excretion of lignans and isoflavonic phytoestrogens, and plasma non-protein bound sex hormones in relation to breast cancer. Progr Cancer Res Ther 35: 409-412 (1988).

9. Fishman J, Martucci C. Biological properties of 16α-hydroxyestrone: implications in estrogen physiology and pathophysiology. J Clin Endocr Metab 51: 611-615 (1980).
10. Bradlow HL, Hershcopf RJ, Martucci CP, Fishman J. Estradiol 16α-hydroxylation in the mouse correlates with mammary tumor incidence and presence of murine mammary tumor virus: A possible model for the etiology of breast cancer in humans. Proc Natl Acad Sci USA 82: 6295-6299 (1985).
11. Yu SC, Fishman J. Interaction of histones with estrogens. Covalent adduct formation with 16α-hydroxyestrone. Biochemistry 24: 8017-8021 (1985).
12. Russo IH, Russo J. Develoment stage of the rat mammary gland as determinant of its susceptibility to 7,12,-dimethylbenz(a)anthracene. J Natl Cancer Inst 61: 1439-1449 (1978).
13. Russo J, Russo IH. Influence of differentiation and cell kinetics on the susceptibility of the rat mammary gland to carcinogenesis. Cancer Res 40: 2677-2687 (1980).
14. Russo J, Tay LK, Russo IH. Differentiation of the mammary gland and susceptibility to carcinogenesis. Breast Cancer Res Treat 2: 5-73 (1982).
15. Russo J, Calaf G, Roi L, Russo IH. Influence of age and gland topography on cell kinetics of normal human breast tissue. J Natl Cancer Inst 78: 413-418 (1987).

Part II: Experimental

In vitro synthesis of 16-alpha-hydroxy-estrone by female rat liver microsomes: its possible role in the etiology of breast cancer

Cor J.M. Arts, Jan W.G.M. Wilmer, Albert Th.H.J. de Bie and Henk van den Berg

Abstract

Liver homogenates from female rat strains (Sprague Dawley, Wistar and Fisher) were incubated in a NADPH-regenerating medium in the presence of labeled and unlabeled estrone. Liver microsomes isolated from male rats and female mice were used as positive controls. Using HPLC and paper chromatography and under the experimental conditions used, it was found that liver homogenates from female rats were able to convert estrone to various metabolites such as 16α-hydroxyestrone. In a mutagenicity assay (Ames test), with 16α-hydroxyestrone as test substance, two strains (TA98 and TA1538) of the five strains tested showed a 2–3-fold increase in the number of his^+ revertants relative to the control values. Estrone did not cause any mutagens in the test used.

It is concluded that in this *in vitro* model female rats are able to synthesize 16α-hydroxyestrone. Whether this compound is a risk factor for breast cancer remains unclear.

Introduction

Estrone (E_1) is quantitatively the most important metabolite of estradiol (E_2) throughout the menstrual cycle and has been found in high concentrations in breast cyst fluid as well as in mammary tumors (1). 16α-Hydroxy-estrone (16α-OH-E_1) is an intermediate product in the conversion of E_1 to estriol (E_3) (2). It shows uterotrophic activity, decreases LH secretion and has a very low binding affinity for the rat uterine cytosol estrogen receptor. Furthermore, it shows a very low binding affinity for sex hormone-binding globulin (3, 4). Its most striking property is the formation of covalent bonds with amino groups of biological macromolecules (5–7). A close correlation between estradiol 16α-hydroxylation and mammary tumor incidence has been reported both for man and for animals. Women with breast cancer showed 50%

more 16α-hydroxylation than a control group free of overt disease (3). Mice strains with a high incidence of mammary tumors (C_3H) demonstrated elevated 16α-hydroxylation *in vivo* compared to strains with a low tumor incidence ($C_{57}Bl$) (8).

A discrepancy was found for the presence of 16α-hydroxylase activity in female rats. In contrast to male rats, Bradlow et al. (9) did not find activity in female Sprague Dawley rats whereas other workers (10, 11) have reported low activities of 16α-hydroxylase in *in vivo* experiments with these female rats.

In this study we report the *in vitro* formation of 16α-OHE1 from E1 using liver microsomes from various strains of female rats. Liver microsomes from male Sprague Dawley and Wistar rats and of female mice ($C_{57}Bl$ and C_3H) were used as positive controls. The genotoxic potential of 16α-OH-E_1, which might be indicative of its carcinogenicity, was studied in the Ames test with various strains of *Salmonella typhimurium* and a liver homogenate fraction (S9) of both male and female Aroclor-induced Wistar rats as metabolic activation system.

Experimental

Animals

Adult female $C_{57}Bl$ and C_3H mice (weighing about 16 g) were obtained from the Netherlands Cancer Institute (Antonie van Leeuwenhoekhuis), Amsterdam, Netherlands. Adult male and female Sprague Dawley rats (weighing about 325 and 200 g, respectively) and female Fisher rats (weighing about 200 g) were obtained from Charles River, Kent, England. Adult male and female Wistar rats (Cpb:WU, Wistar random, weighing about 350 and 200 g, respectively) were obtained from TNO Central Institute for Breeding of Laboratory Animals, Zeist, Netherlands.

Chemicals

All chemicals used were HPLC or Analar grade. Estrone, 16α-hydroxy-estrone, estradiol and estriol were purchased from Sigma Chemicals Co. (St. Louis, MO). [2,4,6,7-^{3}H(N)]-estrone (spec. act. 3.9 TBq/mmol) was purchased from Amersham, Houten, Netherlands and purified by HPLC before use. 16-Keto-estradiol was kindly donated by Dr J.H.H. Thijssen, Academic Hospital, Utrecht, Netherlands.

Preparation of microsomes

Animals (3 from each strain, species or sex) were anesthesized with ether and then decapitated. The livers were removed immediately, minced and homogenized in ice-cold 0.01 M phosphate-buffered saline (pH 7.0) with a Potter-Elvehjem glass-Teflon homogenizer. An aliquot of the homogenates, corresponding with 1 g of liver, was centrifuged at 20 000 *g* for 20 min; the resultant supernatants were centrifuged in a Beckmann ultracentrifuge for 50 min at 105 000 *g* (30 000 rpm) to give microsomal pellets. The microsomes were resuspended in 1 ml 0.01 M phosphate-buffered saline and used immediately for the incubation experiments.

Incubation of liver microsomes with estrone

Incubation was performed as described previously (12) with some minor modifications. Briefly, liver microsomes (0.2 ml) were incubated in 2 ml phosphate-buffered saline solution containing 10 μM E_1 and 2 μCi [2,4,6,7-^{3}H(N)]-E_1 for 60 min at 37 °C in the presence of 100 μl of a NADPH-generating system (2.4 mM NADP, 10 mM glucose-6-phosphate, 2 U/ml glucose-6-phosphate dehydrogenase). Incubation mixtures without liver microsomes served as blanks. The reaction was terminated by chilling in ice-water.

Extraction and purification of estrogens

Immediately after incubation, 8 ml of a 1:1 mixture of ethanol and acetone was added to the microsome suspensions and thoroughly mixed. After centrifugation at 800 *g* for 10 min at 4 °C, supernatants were decanted and evaporated at 40 °C under a mild stream of nitrogen. The residues were dissolved in 20% methanol. Thereafter reversed-phase C18 cartridges, activated by subsequent washing with 2.5 ml 100% methanol and 2.5 ml distilled water, were used for purification of the extracts. After transferring to a C18 cartridge and subsequently washing with 2 ml distilled water and 2 ml 50% methanol, the hormones were eluted with 2 ml methanol. The methanol fractions were evaporated to dryness at 40 °C under a mild stream of nitrogen. The residue was dissolved in 25 μl isopropyl alcohol by vortexing and 225 μl n-hexane was added.

High-performance liquid chromatography (HPLC)

HPLC was performed as described recently (13) with slight modifications. A Lichrosorb Diol column (Merck, Darmstadt, FRG; 125 mm × 4.6 mm) was used with isopropyl alcohol in hexane (1:9 v/v) as eluent at a flow rate of 1.2 ml/min. Base line separations between E_1, E_2, 16α-OH-E_1 and E_3 were obtained (Fig. 1A). Fractions with the same retention time as 16α-OH-E_1 were collected. One-third of the liquid fraction was used for counting tritium activity. The remaining part was evaporated to dryness and used for further identification by paper chromatography.

Paper chromatography

Paper (Whatman No. 1) was impregnated with a formamide/acetone solution (1:4 v/v) and dried between filter paper. HPLC fractions possibly containing 16α-OH-E_1 were chromatographed together with authentic standards of 16-keto-estradiol (16K-E_2) and 16α-OH-E_1. Elution was performed with chloroform. Detection of standards was done by spraying with sodium carbonate (20% w/v) and Folin Ciocalteu reagent diluted 1:3 in water. The paper was cut into 1 cm pieces; radioactivity was successively counted after addition of scintillation liquid (Atomlight). Peaks of radioactivity with the same retention time as authentic 16α-OHE_1 were supposed to contain both labeled and unlabeled 16α-OH-E_1.

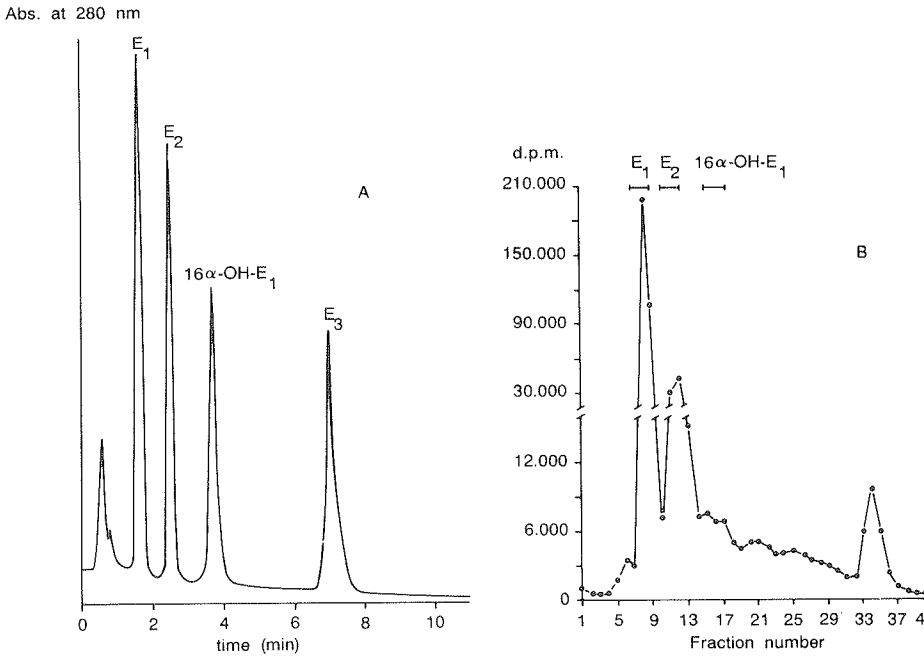


Fig. 1. Elution profiles after HPLC of: (A) standards of estrone (E1), estradiol (E2), 16α-hydroxyestrone (16α-OH-E1) and estriol (E3), and (B) a sample extract obtained after incubation of liver microsomes from female Fisher rats with labeled estrone.

Mutagenicity assay

Salmonella typhimurium strains TA 1535, TA 1537, TA 1538, TA 98 and TA 100 were obtained from Dr B.N. Ames, University of California. The mutagenicity assay was carried out as described by Ames et al. (14) using the plate incorporation procedure with and without a liver metabolic activation system (S-9 mix) of female and male, Aroclor-induced Wistar rats. Briefly, to 2 ml top agar (0.6% agar containing 0.5 mM histidin/biotin) were added in this sequence: 0.1 ml of the appropriate bacterial suspension, 0.1 ml of the appropriate concentration of estrone or 16α-OH-E_1 (dissolved in DMSO) and 0.5 ml of the S-9 mix, if any. The mixtures were then poured out onto minimal glucose agar plates. After a 3-day incubation period at 37 °C revertant colonies were counted. In some experiments test solutions, bacteria and S-9 mix were preincubated for 20 min at 37 °C. Thereafter, the mixtures were poured out onto minimal glucose agar plates, the plates incubated for 3 days at 37 °C and the revertant colonies counted.

Results

Addition of liver microsomes to a NADPH-regenerating medium containing an excess of E_1 results in the formation of polar and non-polar components. During

purification of the extracts with reversed-phase C18 cartridges, the relatively most polar components were removed by washing with 50% methanol in water. These polar components equal 15–20% of the original quantity of labeled compound. After reversed-phase C18 chromatography 60–80% of the original radioactivity added is recovered. During HPLC radioactivity is distributed among the different fractions. In Fig. 1B a typical HPLC profile is shown, obtained after incubation of liver microsomes from female Fisher rats with E_1. Of the total activity added, 14% was recovered in the E_1 fraction, 4% in the E_2 fraction and 1% in the 16α-OH-E_1 fraction.

The HPLC fractions containing the 16α-OH-E_1 were collected and re-chromatographed on the paper chromatography (PC) system. With this sytem a nearly quantitative separation between 16α-OH-E_1 and 16K-E_2 can be obtained, as both compounds elute in the same HPLC fraction. The radioactivity profiles obtained after PC for female Wistar and Fisher rats are shown in Fig. 2. For both strains radioactivity was found on the 16K-E_2 as well as on the 16α-OH-E_1 spot, equalling 0.01 to 0.001 % of the total radioactivity originally added. For female Wistar rats, the amount of radioactivity in the 16K-E_2 and the 16α-OH-E_1 position was about the same. For Fisher rats, most of the radioactivity applied was recovered on the spot with the Rf value of 16α-OH-E_1. In Table 1 the counts measured in the 16α-OH-E_1 fraction after HPLC and PC are presented for the various species/strains/sexes. These values are corrected for the loss during the clean-up procedure (50%) and for HPLC and PC aliquot factors (1.25 and 1.5, respectively).

The results of the Ames test showed a slight, but reproducible increase in the number of his$^+$ revertants with strains TA 1538 and TA 98 only in the presence of S-9 mix and with 16α-OH-E_1 as test substance (Table 2). Maximum values (2–3 times the control values) were obtained at dose levels of 500 to 2500 μg/plate. No significant increase in the number of his$^+$ revertants was observed in the absence of S-9 mix.

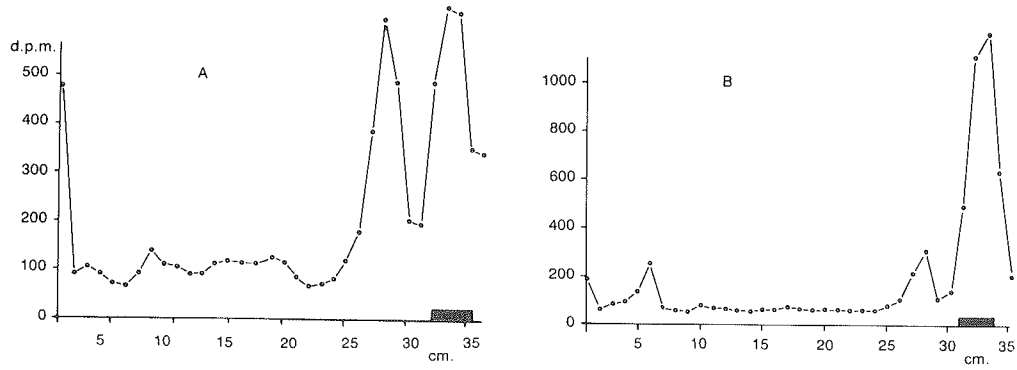


Fig. 2. Elution profiles after paper chromatography of the HPLC 16α-OH-E_1 fraction of samples obtained after incubation of liver microsomes from female Wistar rats (A) and female Fisher rats (B) with labeled estrone. The black bar indicates the spot of authentic 16α-hydroxyestrone.

Table 1. Radioactive counts (dpm), corrected for procedural loss, on the spot of 16α-OH-E_1 after paper chromatography ($n = 3$).

	Female	Male
Rats		
Wistar	6800 ± 1400	13000 ± 2500
Sprague Dawley	680 ± 75	5500 ± 1400
Fisher	12750 ± 940	–
Mice		
C57Bl	5100 ± 420	–
C3H	1570 ± 480	–

– not analyzed.

Table 2. Number[1] of his^+ revertants per plate of the *Salmonella*/microsome mutagenicity test (Ames test) with 16α-hydroxyestrone in the presence of S-9 mix from male Wistar rats (Aroclor-induced).

Dose (μg/plate)	*Salmonella* strain				
	TA1535	TA1537	TA1538	TA98	TA100
0	22 ± 3	15 ± 2	33 ± 3	48 ± 10	144 ± 13
62	19 ± 1	16 ± 6	–	48 ± 3	148 ± 24
185	23 ± 5	14 ± 2	–	59 ± 6	140 ± 6
500	–[2]	–	68 ± 5	68 ± 12	–
555	24 ± 1	23 ± 6	–	88 ± 9	162 ± 11
1000	–	–	87 ± 12	80 ± 5	–
1500	14 ± 3	13 ± 3	95 ± 12	110 ± 10	–
2000	–	–	69 ± 5	106 ± 17	–
2500	–	–	47 ± 4	95 ± 2	–

[1] Mean of triplicate values ± standard deviation.
[2] Test not performed at that dose/plate.

Table 3. Number[1] of his^+ revertants per plate of the *Salmonella*/microsome mutagenicity test (Ames test) with estrone.

Dose, μg/plate	TA1535		TA1537		TA1538		TA98		TA100	
	–S9[2]	+ S9	–S9	+ S9	–S9	+ S9	–S9	+ S9	–S9	+ S9
0	39 ± 2	21 ± 3	14 ± 2	20 ± 3	15 ± 3	48 ± 1	26 ± 7	66 ± 10	143 ± 4	149 ± 3
37	43 ± 4	17 ± 5	12 ± 5	14 ± 5	14 ± 3	46 ± 7	25 ± 3	65 ± 10	158 ± 24	159 ± 10
111	45 ± 5	20 ± 5	8 ± 5	12 ± 1	15 ± 2	44 ± 4	32 ± 6	54 ± 4	169 ± 15	159 ± 29
333	36 ± 8	26 ± 4	11 ± 3	12 ± 2	16 ± 4	57 ± 9	31 ± 5	62 ± 8	131 ± 4	170 ± 8
1000	36 ± 3	15 ± 4	13 ± 5	9 ± 1	16 ± 3	45 ± 2	31 ± 3	61 ± 5	155 ± 1	142 ± 7
3000	34 ± 6	18 ± 1	14 ± 6	14 ± 5	21 ± 3	45 ± 7	25 ± 6	62 ± 5	151 ± 11	140 ± 20

[1] Mean of triplicate values ± standard deviation.
[2] With (+) or without (–) the presence of S-9 mix derived from male Wistar rats (Aroclor-induced).

There was no effect of the type of liver homogenate used (either male or female; results not shown). With estrone as test substance, the Ames test did not show any mutagenicity with or without the presence of a S-9 mix (Table 3).

Discussion

The production of 16α-OH-E_1 by the hydroxylation of estrone was studied *in vitro* in a NADPH-regenerating system containing liver microsomes from various species. In the system described, a small proportion of 16α-OH-E_1 (<0.01%) is synthesized from estrone. A great part is not metabolized, reduced to estradiol (Fig. 1B) or metabolized to other compounds (15). 16K-E_2 is one of the compounds synthesized from estrone. In the HPLC system used, this metabolite elutes in the same fraction as 16α-OH-E_1. With PC and chloroform as the eluent a clear separation between 16K-E_2 and 16α-OH-E_1 can be obtained.

The disadvantage of an *in vitro* model is the absence of a normal physiological environment with the presence of compounds influencing the 16α-hydroxylase activity (16). However, with an *in vivo* experiment a direct production of 16α-hydroxyestrone is difficult to demonstrate due to the low concentrations synthesized.

A 2-fold (Wistar) to 8-fold (Sprague Dawley) higher amount of 16α-OH-E_1 is produced by liver microsomes from the male control rats than by those from the female strains (Table 1). This relatively higher 16α-hydroxylase activity was also found in male Sprague Dawley rats during metabolism studies of 4-(^{14}C)androstene-3,17-dione by others (10, 11). The elevated 16α-hydroxylase activity in male rats can be explained by circulating androgen and by the male-type pattern of blood GH levels (16). In this experiment, an extremely low production of 16α-hydroxyestrone was found in the Sprague Dawley female strain compared with the female Wistar and Fisher strains (Table 1), suggesting strain dependence of 16α-hydroxyestrone production.

In the mutagenicity assay (Ames test) only two strains (TA 98 and TA 1538) of the five strains tested showed a slight increase in the number of his$^+$ revertants at a non-physiological high concentration of 16α-OH-E_1. It is doubtful whether this is of any significance to support the postulated role of 16α-OH-E_1 as risk marker for breast cancer (3, 8). However, administration of estrone to rats by s.c. implantation (17), resulting in an estimated absorption of 6–7 μg estrone per day, caused mammary tumors in various female strains tested. As estrone itself seems not to be mutagenic ((18), Table 3), this may be explained by an increased concentration of 16α-hydroxyestrone resulting from the conversion of estrone.

Contrary to preliminary data (19), we now conclude that liver microsomes from female rats are able to synthesize 16α-hydroxy-estrone *in vitro*. The results of the Ames test do not support a strong direct role of 16α-OH-E_1 as initiator in the carcinogenesis process. Whether or not 16α-OH-E_1 is risk factor for breast cancer remains unclear.

References

1. Nunez M, Adeo AR, Landgren BM, Cekan SZ, Diszfalusy E. Studies of the pattern of circulating steroids in the normal menstrual cycle. Acta Endocrinol 86: 621-633 (1977).
2. Zumoff B, Fishman J, Bradlow H, et al. Hormone profiles in hormone dependent cancers. Cancer Res 35: 3365-3373 (1975).
3. Fishman J, Schneider J, Hershcopf RJ, et al. Increased estrogen 16-alpha-hydroxylase activity in women with breast and endometrial cancer. J Steroid Biochem 20: 1077-1081 (1984).
4. Fishman J, Martucci C. Biological properties of 16α-hydroxyestrone: implications in estrogen physiology and pathophysiology. J Clin Endocrinol Metab 51: 611-615 (1980).
5. Yu SC, Fishman J. Interaction of histones with estrogens. Covalent adduct formation with 16α-hydroxyestrone. Biochemistry 24: 8017-8021 (1985).
6. Bucala R, Fishman J, Cerami A. Formation of covalent adducts between cortisol and 16α-hydroxyestrone and protein: Possible role in the pathogenesis of cortisol toxicity and systemic lupus erythematosus. Proc Natl Acad Sci USA 79: 3320-3324 (1982).
7. Swaneck GE, Fishman J. Covalent binding of the endogenous estrogen 16α-hydroxyestrone to estradiol receptor in human breast cancer cells: characterization and intranuclear localization. Proc Natl Acad Sci USA 85: 7831-7835 (1988).
8. Bradlow HL, Hershcopf RJ, Martucci CP, Fishman J. Estradiol 16α-hydroxylation in the mouse correlates with mammary tumor incidence and presence of murine mammary tumor virus: A possible model for the etiology of breast cancer in humans. Proc Natl Acad Sci USA 82: 6295-6299 (1985).
9. Bradlow HL, Hershcopf R, Martucci C, Fishman J. 16α-Hydroxylation of estradiol: A possible risk marker for breast cancer. In: Angeli A, Bradlow HL, Dogliotti L (eds). Endocrinology of the breast: Basic and clinical aspects. Ann NY Acad Sci 464: 138-151 (1986).
10. Norstedt G, Mode A, Hoekfelt T, et al. Possible role of somatostatin in the regulation of the sexually differentiated steroid metabolism and prolactin receptor in rat liver. Endocrinology 112: 1076-1090 (1983).
11. Jansson JO, Ekberg S, Isaksson O, et al. Imprinting of growth hormone secretion, body growth and hepatic steroid metabolism by neonatal testosterone. Endocrinology 117: 1881-1889 (1985).
12. Milewich L, MacDonald PC, Guerami A, et al. Human fetal liver estrogen 16α-hydroxylase: Precursor specificity, kinetic parameters, and in vitro regulation. J Clin Endocrinol Metab 63: 180-191 (1986).
13. Arts CJM, Kemperman PTW, van den Berg H. Oestrogen radioreceptor assay for multi-residue screening of bovine urine for oestrogenic anabolic compounds. Food Add Contam 6: 103-116 (1989).
14. Ames BN, McCann J, Yamasaki E. Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian microsome mutagenicity test. Mutation Res 31: 347-364 (1975).
15. Adlercreutz H. Oestrogen metabolism in liver disease. J Endocrinol 46: 129-163 (1970).
16. Morgan ET, MacGeoch C, Gustafsson JA. Hormonal and developmental regulation of expression of the hepatic microsomal steroid 16α-hydroxylase cytochrome P-450 apoprotein in the rat. J Biol Chem. 260: 11895-11898 (1985).
17. Cutts JH. Estrogen induced breast cancer in the rat. Can Cancer Conf 6: 50-68 (1966).
18. Drevon C, Piccoli C, Montesano R. Mutagenicity assays of estrogenic hormones in mammalian cells. Mutation Res 89: 83-90 (1981).
19. Arts CJM, de Bie AThHJ, Donker GH, et al. Female rats are unable to synthesize 16α-hydroxyestrone in vitro. First European Congress of Endocrinology, Copenhagen, 21–25 June, Meeting Abstract (1987).

Influence of wheat bran on NMU-induced mammary tumor development, plasma estrogen levels and estrogen excretion in female rats

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Abstract

In our animal experiments the hypothesis was tested that a high-fiber diet reduces tumor promotion through interruption of the enterohepatic circulation resulting in lowered estrogen exposure of the estrogen-sensitive tissue. In the first experiment the development of N-nitrosomethylurea (NMU)-induced mammary tumors was investigated. One group of rats (HF) was fed a high-fiber diet (11% fiber, based on wheat bran), the other group (LF) fed a low-fiber diet (0.5% fiber, based on white wheat flour). Tumor incidence (90 and 80%, respectively) and latency (121 and 128 days, respectively) were similar in the HF and LF groups. Compared to the LF group, HF rats had lower tumor weights (0.16 g vs. 0.55 g; $P < 0.01$) and a slightly lower tumor multiplicity (1.8 vs. 2.8 tumors per tumor-bearing rat). These differences were smaller after adjustment for body weight.

In a second experiment, rats not treated with the carcinogen were kept on the same HF and LF diets. From these rats 24-hour urine and feces and orbital blood samples were collected for analysis of (un)conjugated estrogens. The excretion of both free and conjugated estrogens in fecal samples was about 3-fold higher in HF rats than in LF rats. During the basal period of the cycle, mean urinary excretion of estrone was significantly lower in HF rats (9.7 ng/day) than in LF rats (13.0 ng/day).

It is concluded that wheat bran interrupts the enterohepatic circulation of estrogens, but plasma levels are not affected. Whether the development of mammary tumors is reduced by the introduction of specific components of wheat bran, or by a reduced body weight due to a lower (effective) energy intake remains to be determined.

Introduction

There seems to be consensus that estrogens play a crucial role in the development of mammary tumors in animals or breast cancer in women (1). *In vivo* and *in vitro* experiments have shown a positive association between estradiol-17β (E_2) exposition and cell proliferation of estrogen-sensitive breast cancer cells (2–4). Moreover, epidemiological studies have demonstrated lowered plasma estrogen concentrations in women from populations at low risk of breast cancer, such as vegetarians versus omnivorous women (5), and Oriental immigrant women versus Caucasian Americans (6).

A negative association between dietary fiber and breast cancer has been reported (7, 8). A life-span study in rats showed that rats on high-fiber (wheat bran) diets developed fewer mammary tumors than those fed low-fiber diets, irrespective of the fat content of the diet (9, 10). In women who consumed a fiber-rich vegetarian diet, fecal estrogen excretion was higher, and urinary estrogen excretion and plasma E_2 concentration were lower than in omnivorous women (5, 11). These observations were attributed to decreased plasma estrogen concentrations resulting from altered enterohepatic circulation of estrogens (12). An alternative explanation for the negative association between intake of cereal products and (estrogen-sensitive) tumors may be the presence of anti-estrogenic or anti-oxidative compounds, such as lignans in vegetable products (13, 14). Furthermore, dietary fiber can bind lipophilic carcinogens presented to the gastrointestinal tract and enhance fecal excretion of these compounds (15).

To substantiate that the relation between dietary fiber and mammary tumor development is indeed mediated by plasma estrogens, we conducted two experiments. In the first experiment mammary tumor development was compared between female rats receiving a low-fiber diet versus a high-fiber diet using the direct-acting N-nitrosomethylurea (NMU) as tumor-initiating agent; in the second experiment, the effects of fiber on estrogen balance (urine, feces and plasma levels) were investigated.

Materials and methods

Animals

Newly weaned female F344 rats, 25 days of age, were obtained from Charles River Ltd. (Margate, Kent, UK). During the first two days after arrival the rats were fed the enclosed Charles River food and tap water (ad libitum). At 27 days of age and after computerized randomization to equalize for initial body weight, 62 animals were assigned to one of two groups of 31 animals each, i.e. the low-fiber (LF) and high-fiber (HF) group. Each rat was identified by earmark code. Individual body weights were recorded weekly.

Housing

The animals were housed in groups of 5 (one cage 6 animals) in suspended hanging-type stainless steel wire-mesh-bottom cages, in an air-controlled room (23 ± 1 °C) with a relative humidity of 50 ± 10% and a light/dark cycle of 12 h.

Composition and administration of the diets (study I and study II)

Except for fat, the composition of the LF and HF diets was similar to the low-fiber/high-fat and high-fiber/high-fat diets described recently (16), summarized in Table 1. To provide equal amounts of energy to both groups and taking into account the known higher average food consumption of the HF group compared to the LF group, rats in the HF group were fed a diet containing 15% of fat and rats in the LF group 18% of fat (containing equal amounts of lard and sunflower oil). The resulting diets were calculated to be iso-energetic according to the conversion factors for protein (4 cal/g), fat (9 cal/g) and carbohydrate (1 cal/g) used by Atwater & Bryant (17).

Table 1. Composition of the diets (% w/w unless indicated otherwise).

Ingredients added			Nutrient composition analyzed		
	LF diet	HF diet	ingredient	LF diet	HF diet
Casein	22.53	15.69	Protein	26.5	23.6
White wheat flour	54.72	42.00	Fat	18.4	15.0
Wheat bran	–	23.75	Carbohydrate	38.5	34.2
Mineral mixture[a]	4.24	3.63	Dietary fiber[d]	0.5	10.9
Vitamin ADEK mixture[b]	0.36	0.31	Moisture	10.6	10.7
Vitamin B mixture[c]	0.24	0.20	Ash	3.9	4.8
Lard	8.96	7.21	Vit A (IU/kg)[e]	7000	5000
Sunflower oil	8.96	7.21	Vit C (mg/kg)[e]	150	125
Energy (MJ/kg)	17.71	15.26	Vit E (mg/kg)[e]	12.5	10.0
			Palmitic acid[f]	16.2	16.0
			Stearic acid[f]	10.0	9.6
			Oleic acid[f]	30.3	30.1
			Linoleic acid[f]	38.2	39.2

[a] In mg per g mixture: KH_2PO_4 399, $CaCO_3$ 389, NaCl 142, $MgSO_4$ 58, $FeSO_4 \cdot 7H_2O$ 5.7, $ZnCl_2$ 0.9, $CuSO_4 \cdot 5H_2O$ 0.8, $MnSO_4 \cdot 2H_2O$ 4.6, $CoCl_2 \cdot 6H_2O$ 0.02, KI 0.007, $KCr(SO_4)_2 \cdot 12H_2O$ 0.08.
[b] Per g mixture: vitamin A 2112 IU, vitamin D-3 704 IU, vitamin E (50%) 30.0 mg, menadione sodium bisulfite (vitamin K-3) 1.0 mg, wheat starch 30.0 mg.
[c] In mg per g mixture: thiamin·HCl 3.00, riboflavin 2.25, pyridoxine·HCl 4.50, niacin 15.0, calcium pantothenate 6.0, biotin 0.075, folic acid 0.75, vitamin B-12 (0.1%) 37.50, choline chloride (50%) 931.
[d] Insoluble dietary fiber analyzed according to Hellendoorn et al. (51).
[e] Analyzed according to Speek et al. (49, 50).
[f] Fatty acid composition expressed as % w/w of the fatty acid methyl esters present.

All dietary ingredients were obtained from commercial sources, the diets were prepared in house and stored at +4 °C in the dark until use. At regular times batches were analyzed for protein, fat, and carbohydrate contents according to standard procedures (Table 1).

All animals had free access to tap water and were fed the experimental diets in powdered form ad libitum. Food consumption was recorded weekly to calculate food and energy intake.

Tumor initiation and development (study I)

At 49 days of age, after three weeks on the experimental diets, 20 (of 31) animals of both the LF and HF groups received a single intravenous injection into the tail vein with NMU (Sigma, St. Louis, MO; 50 mg/kg body weight, individually adjusted) under light ether anesthesia. From 39 days after NMU injection until the end of the study (180 days after NMU injection) all animals were examined twice a week for palpable mammary tumors.

At the end of the study or in case of a moribund condition ($n = 2$) animals were anesthetized with ether and blood was obtained by aorta puncture. Mammary tissues were examined macroscopically and both palpable and non-palpable mammary tumors were excised, weighed and histologically classified (18). Organs were examined for gross pathological aberrations.

Estrogen balance (study II)

At 118 days of age, 11 (of 31) young adult animals of both the LF and HF groups (the animals not used for study I) were individually housed in stainless steel cages. To record the phase of the cycle, vaginal smears were taken daily between 10.00 and 11.00 on 15 successive days and subsequently stained (19). From each animal, 24-h urine (collected on dry ice) and feces were collected. Orbital blood was sampled in heparinized tubes on days 2, 9 and 15 of the experiment. Blood plasma, urine and fecal samples were stored at –20 °C until analysis.

In blood plasma total (conjugated and unconjugated) estrone (E_1) and unconjugated (free) E_2 were estimated. In all urine samples collected total E_1 was analyzed. Total E_2 and total estriol (E_3) were analyzed in urine samples collected on days 1 through 8 of the experiment only. Fecal samples collected on days 2, 9 and 15 were analyzed for both conjugated and unconjugated E_1, E_2 and E_3.

The pH and β-glucuronidase activity were measured in fecal samples collected at random on 6 different days from cages containing NMU-treated animals fed the HF or LF diet.

Chemicals (study II)

E_1, E_2 and E_3 standards were obtained from Sigma Chemical Company, St. Louis, MO. The tritiated compounds [2,4,6,7-^{3}H]estradiol-17β, [2,4,6,7-^{3}H]estrone and

[2,4,6,7,-^{3}H]estriol, with a specific activity of 105 Ci/mmol each, were purchased from Dupont (New England Nuclear), 's-Hertogenbosch, Netherlands. Antisera were raised in rabbits against estradiol-17β-6-CMO-BSA, estrone-6-CMO-BSA and estriol-6-CMO-BSA. Cross-reactions, recorded according to Abraham (20), were $< 0.5\%$ for all related compounds tested. All other chemicals used were HPLC grade or Analar quality and purchased from E. Merck (Amsterdam, Netherlands), unless stated otherwise.

Sample treatment (study II)

Blood plasma. E_2 content was estimated after extraction of an aliquot of 500 µl of plasma using Extrelut columns (Merck, Darmstadt, Germany). Total E_1 was analyzed after hydrolysis of 200 µl of plasma sample in 200 µl of 0.15 M sodium acetate buffer pH 4.5 containing hydrolytic enzymes of Suc d'Helix Pomatia (Boehringer Mannheim, Germany) followed by extraction with Extrelut columns and determination by radioimmunoassay (RIA) as described (21).

Urine. An aliquot of 250 µl urine was hydrolyzed in 250 µl acetate buffer containing Suc d'Helix Pomatia. Extraction of estrogens and purification of the extract were done by transferring the hydrolysis solution onto activated reversed-phase C18 cartridges (J.T. Baker Chemicals, Deventer, Netherlands). The cartridges were successively washed with water and 50% methanol. Estrogens were eluted with 100% methanol and estimated by RIA.

Feces. The 24-h fecal samples were dismembered using a Mikro-Dismembrator (B. Braun, Melsungen, Germany). Fecal powder was weighed and 0.2 g of it was transferred to vials containing 2 ml acetate buffer. Unconjugated estrogens were extracted twice with 5 ml distilled diethyl ether. The conjugated estrogens were extracted with diethyl ether after completing deconjugation with Suc d'Helix Pomatia. The separated ether fractions were evaporated to dryness, residues were dissolved in 2 ml 20% methanol and transferred onto activated reversed-phase C18 cartridges. RIA was performed as described (21).

Fecal parameters. The freshly collected samples were dismembered, 1.5 g of the fecal powder was suspended in 4 ml 0.01 M KCl and pH was measured. For β-glucuronidase activity 0.5 to 1.5 g of the fecal powder was suspended in 15 ml cold 0.1 M phosphate buffer pH 6.5 by vortexing and ultrasonic vibrations for three 1-min bursts at 4 °C. The enzyme reaction was performed as described by Goldin and Gorbach (22).

The pH optimum of β-glucuronidase activity was recorded in feces of the rats on the HF and the LF diet using 0.01 M citrate buffer pH 4.0–5.5 and 0.01 M phosphate buffer pH 6.0–9.0.

Statistical analysis

Growth of the animals, expressed as body weight gain, was compared by analysis of variance (ANOVA) techniques.

Time to first tumor appearance (latency) was analyzed as follows. For animals without tumors 180 days after NMU injection a latency > 180 days was estimated with the method of Taylor (23). Subsequently the data were analyzed with ANOVA.

Tumor weights were analyzed by hierarchical analysis of variance using the method of Gower (24).

Tumor multiplicity was analyzed with a χ^2 test of homogeneity. The relevant two-way table was classified by fiber group and number of tumors, respectively.

Estrogen contents of plasma and urine were analyzed by ANOVA with the factors fiber (high vs. low) and cycle (peak vs. basal period) and their mutual interactions. Variables in fecal samples were analyzed with fiber as factor and rat and repeated measurements within rat as blocks.

Results

Study I: Tumor development

Animal growth and food intake

Food (energy) intake and weight gain decreased immediately after NMU administration. After recovery from this treatment (i.e., from day 68 until the end of

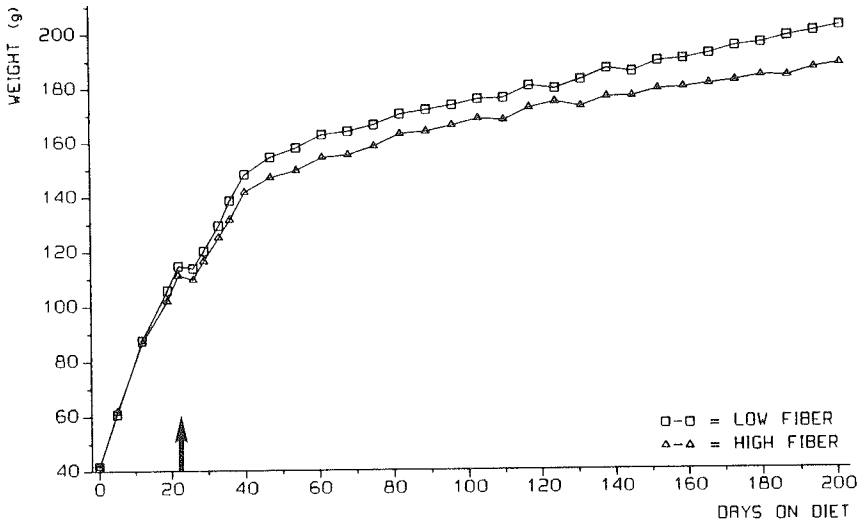

Fig. 1. Mean body weight of two groups of rats fed the LF diet or the HF diet. The arrow indicates the time of NMU injection.

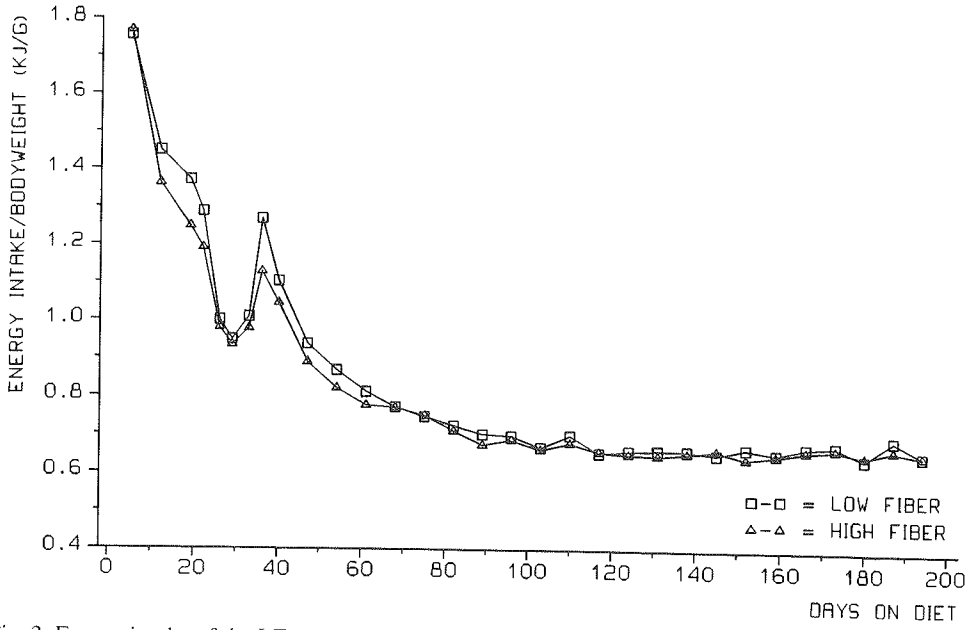

Fig. 2. Energy intake of the LF group and the HF group relative to body weight.

the study), as expected, mean daily food intake was higher for the HF diet than for the LF diet (7.6 ± 0.3 and 7.0 ± 0.3 g/day, respectively). However, in spite of the lower energy content of the HF diet, during this period mean energy intake of the LF group (124 kJ/day) was 6.5% higher than that of the HF group (116 kJ/day). This resulted in a slightly higher mean body weight of the animals of the LF group (Fig. 1). Energy intake relative to body weight of both groups was similar (Fig. 2). In the period between day 0 and day 22 of the experiment mean weight gain was 70 g (HF group) and 73 g (LF group; $P = 0.09$). In the period between day 22 and day 68, when NMU was administered, mean weight gain of the HF and the LF groups was 44 and 49 g, respectively ($P = 0.05$). In the period between day 68 and day 201 mean weight gain was 33 and 38 g for the HF and LF group, respectively ($P = 0.07$).

Pathology

The tumors are classified in Table 2. The types of mammary tumors in the LF and HF groups were similar, with adenocarcinoma of the tubulo-papillary type as the main type (87.5% in the HF group; 90.7% in the LF group). At 124 days after NMU injection, two animals of the LF group were sacrificed due to a moribund condition (large, bloody mammary tumors). These animals also showed a pale liver and enlarged adrenals. Post-mortem examination did not reveal gross changes in the major organs or organ systems in the other animals.

Table 2. Mammary tumors induced by NMU.

Type of tumor	Number of tumors (%)	
	HF group	LF group
Adenocarcinoma		
tubulo-papillary adenocarcinoma	28 (87.5%)	39 (90.7%)
cribriform-comedo carcinoma	1 (3.1%)	3 (7.0%)
compact-tubular adenocarcinoma	2 (6.3%)	1 (2.3%)
Fibroadenoma	1 (3.1%)	–

Tumor incidence, multiplicity and latency

Results concerning these parameters are summarized in Table 3. Incidences of the histologically verified tumors did not differ significantly 180 days after NMU administration (90% in the HF group vs. 80% in the LF group).

Mean tumor multiplicity was 2.8 in the LF group and 1.8 in the HF group at the end of the study. The numbers of animals bearing 1, 2, 3, and more than 3 tumors did not differ significantly between the experimental groups.

The mean time to first histologically verified palpable tumor appearance hardly differed between the LF group (128 days) and the HF group (121 days).

Weight of tumors

In the HF group only 7 out of 32 tumors (22%) had a weight exceeding 0.25 g, whereas 20 out of 42 tumors (48%) in the LF group exceeded that weight (Fig. 3). Mean tumor weight in the HF group (0.161 g) was lower ($P < 0.01$) than in the LF group (0.552 g) even when two tumors weighing 35 g each from two rats of the LF group were excluded.

Table 3. Effects of the HF and LF diets on tumor incidence, multiplicity and latency of NMU-induced mammary tumors.

Parameter	HF diet	LF diet	*P*
Tumor incidence (%)	90	80	n.s.[1]
Latency (days)	121	128	n.s.
Tumor multiplicity (*n*)	1.8	2.8	n.s.
Number of rats with:			
0 tumor	2	4	
1 tumors	8	7	
2 tumors	7	2	
3 tumors	2	3	
4 tumors	1	0	
5 tumors	0	2	
6 tumors	0	1	
7 tumors	0	1	

[1] n.s. = not significant.

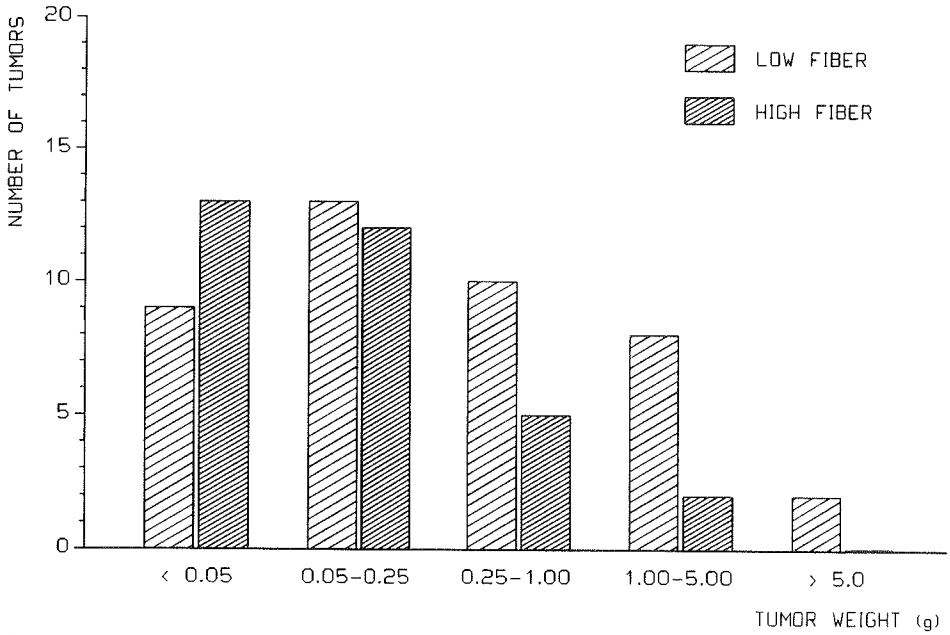

Fig. 3. Weight of tumors (classified into weight categories) in rats on the LF and HF diets.

Mean tumor burden (total weight of tumors per tumor-bearing animal) was 0.48 g and 1.61 g for the HF group and the LF group, respectively ($P = 0.02$, the two heavy tumors of the LF group (weighing 35 g each) being excluded). When tumor burden was adjusted for the difference in body weight between both groups at the time of section, significances disappeared.

Fecal parameters

In feces from the LF group β-glucuronidase activity was measured to be 538 ± 38 U/kg, while the activity in the feces from the HF group was found to be 83 ± 14 U/kg ($P < 0.001$). For β-glucuronidase activity in the feces from both the LF group and the HF group a pH optimum of 6–7 was found.

The pH of fecal samples of the HF group (7.9 ± 0.4) was lower ($P = 0.02$) than in samples of the LF group (8.4 ± 0.2).

Study II: Estrogen balance

Plasma estrogen levels

A relatively low estrogen production occurs during estrus, metestrus and the first day of diestrus, whereas on the second day of diestrus and in proestrus estrogen production peaks (25). Blood plasma samples collected during estrus, metestrus and the first day of diestrus were considered to reflect the basal period, whereas samples

Table 4. Mean concentrations of total (unconjugated and conjugated) estrone (E_1) and unconjugated estradiol-17β (E_2) in blood plasma (ng/ml) from untreated rats fed a HF diet or a LF diet.

	Cycle	HF group	LF group	SED[2]	*P*
Total E_1	OD[1]	0.26	0.26	0.04	n.s.[2]
	D_2P[3]	0.29	0.29	0.03	n.s.
Unconjugated E_2	OD_1	0.010	0.012	0.008	n.s.
	D_2P	0.042	0.029	0.006	0.02

[1] Estrus, metestrus and first day of diestrus (OD_1) were considered the basal period of the cycle ($n = 16$).
[2] SED, standard error of difference; n.s., not significant.
[3] The second day of diestrus and proestrus (D_2P) were considered the peak period of the cycle ($n = 8$).

collected on the second day of diestrus and during proestrus were considered to reflect the peak period. Only animals contributing two values from the basal period and one from the peak period were considered for analysis (8 HF and 8 LF animals).

As presented in Table 4, no differences in E_1 levels were found between the animals on the HF diet and those on the LF diet. A cycle effect was obtained for plasma E_2 levels ($P < 0.001$). In samples from the HF group collected during the peak period a higher ($P = 0,02$) higher mean E_2 concentration was found than in samples from the LF group, whereas during the basal period no difference between the two groups was found.

Urinary estrogen excretion

During the 15-day experiment the amount of urine excreted per rat was similar for both groups (HF 4.3 ± 0.7 ml/day, LF 4.4 ± 0.8 ml/day).

A 24-h sample does not necessarily represent a single phase of the cycle. For urine we considered, for each cycle, the sample with the lowest estrogen content as representative of basal excretion and the sample with the highest content as representative of the peak period. Analysis of variance indicated that both E_1, E_2 and E_3 excretions were dependent on cycle phase ($P < 0.001$). During the basal period only mean E_1 excretion was significantly lower in HF rats than in LF rats. No significant interaction could be demonstrated between the factors cycle phase and diet indicating that differences between the LF and HF groups are similar irrespective of the phase of the cycle. E_2 and E_3 excretions were similar for both groups (Table 5).

Fecal estrogen excretion

In the HF group mean wet weight of the feces per rat was 1.63 ± 0.2 g/day, and in the LF group 0.45 ± 0.09 g/day.

As the estrogen content in a 24-h fecal collection can represent a few days of the cycle and the enterohepatic circulation can influence fecal estrogen content, it was decided to ignore the estrous cycle in the statistical analysis.

Table 5. Mean 24-hour urinary excretion of total estrone, estradiol-17β and estriol in untreated rats fed a HF diet or a LF diet (ng/day per rat). [1]

	HF group	LF group	SED		P	
			within	between	within	between
Estrone						
Basal[2]	9.7	13.0	1.641	1.444	< 0.001	0.035
Peak	28.2	30.7				
Estradiol-17β						
Basal[3]	2.01	1.94	0.206	0.193	< 0.001	0.89
Peak	4.02	4.05				
Estriol						
Basal[3]	16.1	18.3	1.936	1.577	< 0.001	0.26
Peak	33.3	34.7				

[1] No significant interaction was found between diet (high vs. low) and cycle (basal vs. peak period). For SED, 'within' refers to the difference as a result of the cycle within the rat, whereas 'between' refers to the difference as a result of diet between the two groups of rats.
[2] $n = 3 \times 11$ values for each period.
[3] $n = 2 \times 11$ values for each period.

As shown in Table 6, fecal estrogen excretion of all unconjugated and conjugated estrogens, except conjugated E_2, was higher in rats on the HF diet than in those on the LF diet ($P < 0.001$). In contrast, concentrations of conjugated estrone and of both unconjugated and conjugated estradiol-17β and estriol were significantly higher

Table 6. Mean fecal estrogen excretion (ng/g and ng/day) of untreated rats on a HF diet or a LF diet.

	HF group[1]		LF group[2]		SED		P	
	ng/g	ng/24 h	ng/g	ng/24 h	ng/g	ng/24 h	ng/g	ng/24 h
Estrone								
Unconjugated	9.6	16.1	10.9	5.2	0.903	1.281	0.149	< 0.001
Conjugated	2.6	4.2	4.9	2.4	0.668	0.336	0.002	< 0.001
% Free	79	79	70	71	1.175	0.850	< 0.001	< 0.001
Estradiol-17β								
Unconjugated	7.3	12.3	6.2	2.3	0.433	1.281	0.023	< 0.001
Conjugated	1.1	1.72	2.2	1.73	0.314	0.195	0.002	0.96
% Free	87	87	73	57	1.68	1.806	< 0.001	< 0.001
Estriol								
Unconjugated	3.4	5.8	4.4	2.6	0.221	0.472	< 0.001	< 0.001
Conjugated	2.3	3.9	3.5	1.0	0.273	0.253	< 0.001	< 0.001
% Free	60	60	56	74	1.061	1.63	< 0.001	< 0.001

[1] 3 samples each from 11 rats, $n = 33$.
[2] 3 samples each from 10 rats and 2 samples from 1 rat, $n = 32$.

in fecal samples from the LF group. Percentages of unconjugated (free) estrogen (E_1, E_2 and E_3) were significantly higher in feces from HF rats.

Discussion

Although diet composition was such to provide iso-energetic diets to both groups, the actual energy intake was calculated to be 6.5% lower in the HF group than in the LF group. Mean body weight was 203 ± 13 g (LF group) and 189 ± 9 g (HF group) at the time of section. Tumor incidence and latency were similar in both groups. Compared to the LF group, HF rats had lower tumor weights and a slightly lower tumor multiplicity. These differences were reduced after adjustment for body weight at the end of the study, which makes the effects of wheat bran on tumor development rather inconclusive. A significantly lower urinary estrone excretion during the basal period and an increased fecal estrogen excretion was found in animals fed the HF diet. Whether the lower mean tumor weight is a result of a lower (effective) energy intake, lowered estrogen exposure or other wheat bran-induced factors will be discussed below.

In animal experiments on nutrition and mammary cancer, tumors are usually induced by administering either the direct-acting *n*-nitrosomethylurea (NMU) or the indirect-acting 7,12-dimethylbenz(a)anthracene (7,12-DMBA) to female rats. DMBA is administered intra-gastrically, absorbed by the intestinal mucosa and metabolized in liver to its ultimate carcinogenic form. Wheat bran and, to a lesser extent, white wheat flour contain DMBA-binding fiber components (15), and may therefore reduce effective carcinogen exposure of breast target cells. As we were especially interested in the effect of dietary fiber on tumor promotion, we used NMU as the carcinogen. NMU has been reported to give a high incidence (26) of estrogen-sensitive tumors (27).

The decreased tumor promotion can be explained by at least three factors: lower energy intake (28), decreased estrogen exposure (5), and the presence of anti-estrogenic (13) or tumor growth-inhibiting compounds (29) in the diet.

Energy intake

Energy intake affects cell proliferation. A dietary restriction of 25% resulted in a 3–5-fold reduced mammary alveolar cell proliferation in mice 2 days after estrus (30). Energy restriction further affects tumor incidence, tumor multiplicity, mean tumor weight and latency (28). However, the effects of energy restriction on tumor incidence were not observed until after 10% restriction, while a restriction up to 20% did not alter tumor multiplicity and tumor weight (31). In our study a difference in energy intake of 6.5% between the two groups was calculated (using Atwater's general factors excluding fiber content, the best approach found to estimate metabolizable energy for rats fed diets containing up to 12% (w/w) NDF from wheat bran (32)). Although it is not in agreement with previous findings (31), a reduction of energy intake of 6.5% might influence tumor development because differences in

sensitivity have been suggested between Fisher and Sprague Dawley rats (33). Furthermore, the reduced difference in tumor weight after adjustment for body weight suggests that the lower tumor weights in animals of the HF group is not a specific effect of fiber intake but rather an effect of lower energy intake. Future experiments on fiber and carcinogenesis may provide iso-energetic diets more exactly using the 'pair feeding' technique.

Estrogen exposure

A decreased exposure of target cells to endogenous estrogens may also explain the decreased tumor development in rats fed a HF diet. Although a nearly 3-fold higher fecal estrogen excretion (Table 6) and a lowered urinary estrone excretion were found in the rats of the HF group, plasma estrogen levels were similar in both groups during the basal period of the cycle. The enhanced fecal estrogen excretion and the decreased urinary estrone excretion of animals fed the HF diet are in line with the human studies (5, 6, 11). In these studies, however, plasma estrogen levels were decreased as well. The similar plasma estradiol-17β levels in the HF and LF animals during the basal period of the cycle suggests that estrogen production rate in rats fed the HF diet was increased by the negative feedback mechanism on the pituitary, leading to enhanced gonadotrophin secretion. This fits in with the observation that the mean plasma E_2 level during late diestrus and proestrus (peak period of the cycle) was higher in the HF than in the LF animals ($P = 0.02$), indicating that the pituitaries of rats on the HF diet are under a constant stimulation to produce gonadotrophins. If a HF diet indeed increases estrogen production, then in post-menopausal women a HF diet may contribute to the inhibition of breast tumor development by decreasing plasma estrogen levels, as their ovarian estrogen production is negligible and these women lack the negative feedback mechanism. In these women aromatization of C19 steroids in peripheral tissues is the main source of estrogen production, which is independent of the feedback mechanism.

It still remains unclear whether the high fiber intake influences estrogen metabolism and alters estrogen exposure in the mammary tumor tissues. The effect of dietary fiber on cell proliferation in estrogen-sensitive tissues, i.e. mamma and uterus, is presently being investigated.

A clear difference in β-glucuronidase activity between animals on the HF diet and those on the LF diet was reflected in fecal samples, as could be expected (34, 35). However, differences are not always so pronounced (36). Although in fecal samples from animals on the HF diet a relatively low β-glucuronidase activity was measured as compared with feces from the LF group, significantly higher percentages of free (unconjugated) E_1 and E_2 were measured in fecal samples from the HF group. This may be explained by the higher pH of the LF fecal samples (37, 38), or β-glucuronidase may not be the rate-limiting factor for estrogen deconjugation. Probably, fecal bulk, transit time (39) and binding capacity of the non-fermentable dietary fiber components (40) are crucial factors affecting the enterohepatic circulation of estrogens.

Anti-estrogenic and tumor growth-inhibiting factors

We cannot exclude the possibility that our results are also affected by differences in lignan (41, 42) and/or phyto-estrogen (43, 44) exposures or by other fiber-related compounds such as bioflavonoids (29, 45–47) introduced in the rat by the diet.

Lignans, such as enterolactone and enterodiol, are synthesized from precursors by bacterial enzymes in the gut (41). These precursors are present as dietary constituents of plant origin and were found in wheat bran and, to a lesser degree, in wheat flour (42). Lignans have anti-estrogenic and anti-oxidative properties (13). Phytoestrogens, such as coumestrol, genistein, daidzein and equol, are other important components of plant material (43, 44), possibly leading to hormonal imbalance. In addition, they may act as anti-estrogens by competing for receptor proteins in estrogen-sensitive tissues with the biologically more active endogenous estrogens which occur in much lower concentrations in blood plasma (48). The availability of the compounds mentioned may influence (general) cell proliferation and therefore be responsible for the observed differences in tumor development.

In conclusion, it seems that a high intake of dietary fiber (as wheat bran) reduces tumor promotion in estrogen-sensitive tissues. Our results suggest that this is not likely to be attributable to an interrupted enterohepatic circulation of the estrogens resulting in lowered estrogen exposure. Whether this can be explained by anti-estrogenic, other tumor-growth inhibiting compounds, or by a reduced body weight due to a lower (effective) energy intake, remains to be established.

References

1. Anonymous. Hormones and breast cancer. In: Pike MC, Siiteri PK, Welsch CW (eds.). Banbury Report 8. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1981).
2. Morozova TM, Mitina RL, Nagibneva IN, Ozhogina ZB, Salganik RI. The biochemical mechanisms of the stimulating action of estradiol on the growth of mammary tumours. Exp Clin Endocrinol 88: 293-302 (1986).
3. Podhajcer OL, Bravo AI, Dain L, Guman N, Bover L, Mordoh J. In vitro analysis of the cellular proliferative response to 17β-estradiol of human breast cancer. Cancer 61: 1807-1812 (1988).
4. Soto AM, Sonnenschein C. Cell proliferation of estrogen-sensitive cells: the case for negative control. Endocrine Rev 8: 44-52 (1987).
5. Goldin BR, Gorbach SL. Effect of diet on the plasma levels, metabolism and excretion of estrogens. Am J Clin Nutr 48: 787-790 (1988).
6. Goldin BR, Adlercreutz H, Gorbach SL, Woods MN, Dwyer JT, Conlon T, Bohn E, Gershoff SN. The relationship between estrogen levels and diets of Caucasian American and Oriental immigrant women. Am J Clin Nutr 44: 945-953 (1986).
7. van 't Veer P, Kolb CM, Verhoef P, Kok FJ, Schouten EG, Hermus RJJ, Sturmans F. Dietary fiber, beta-carotene and breast cancer: results from a case-control study. Int J Cancer 45: 825-828 (1990).
8. Rose DP. Dietary fiber and breast cancer. Nutr Cancer 13: 1-8 (1990).
9. Fisher N, Berry CS, Fearn T, Gregory JA, Hardy J. Cereal dietary fiber consumption and divertical disease: a lifespan study in rats. Am J Clin Nutr 42: 788-804 (1985).
10. Sinkeldam EJ. Dietary fiber and cancer of the large bowel and breast. In: Leegwater DC, Feron VJ, Hermus RJJ (eds). Low digestibility carbohydrates. Proceedings of a workshop held at the TNO-CIVO Institutes, Zeist, Netherlands, 27–28 November 1986, pp. 96-102. Pudoc, Wageningen, Netherlands (1987).

11. Goldin BR, Adlercreutz H, Gorbach SL, Warram JH, Dwyer JT, Swenson L, Woods MN. Estrogen excretion patterns and plasma levels in vegetarian and omnivorous women. N Eng J Med 307: 1542-1547 (1982).
12. Heneghan JB. Alimentary tract physiology: Interactions between host and its microbial flora. In: Rowland IR (ed.). Role of the gut flora in toxicity and cancer, pp. 39-77. Academic Press, London (1988).
13. Adlercreutz H. Does fiber-rich food containing animal lignan precursors protect against both colon and breast cancer? An extension of the 'fiber hypothesis'. Gastroenterology 86: 761-766 (1984).
14. Adlercreutz H, Höckerstedt K, Bannwart C, Hämäläinen E, Fotsis T, Bloigu S. Association between dietary fiber, urinary excretion of lignans and isoflavonic phytoestrogens, and plasma non-protein bound sex hormones in relation to breast cancer. Progr Cancer Res Ther 35: 409-412 (1988).
15. Gulliver WP, Kutty KP, Laher JM, Barrowman JA In vitro interaction of 7,12-dimethylbenz(a)anthracene and its biliary metabolites with dietary fibers. J Natl Cancer Inst 71: 207-210 (1983).
16. Sinkeldam EJ, Kuper CF, Bosland MC, Hollanders VHM, Vedder DM. Interactive effects of dietary wheat bran and lard on N-methyl-N'-nitro-N-nitrosoguanidine-induced colon carcinogenesis in rats. Cancer Res 50: 1092-1096 (1990).
17. Atwater WO, Bryant AP. The availability and fuel value of food materials. Rep Storrs Agric Exp Stat 1900: 73-110 (1899).
18. van Zwieten JM. The rat as animal model in breast cancer research. A histopathological study of radiation- and hormone induced rat mammary tumors. Thesis, Utrecht University. Martinus Nijhoff, Dordrecht, Netherlands (1984).
19. Papanicolaou GN. A new procedure for staining vaginal smears. Science 95: 438-439 (1942).
20. Walker WHC, Keane PM. Theoretical aspects of radioimmunoassay. In: Abraham GE (ed). Handbook of radioimmunoassay: Clinical and biochemical analysis, pp. 87-130. Marcel Dekker, New York (1977).
21. Arts CJM, van Baak MJ, van den Berg H, Schilt R, Berende PLM, den Hartog JMP. Concentrations of the endogenous steroid hormones oestradiol-17β, testosterone and progesterone in veal calves in connection with the control for illegal administration. Arch für Lebensmittelhyg 41: 58-62 (1990).
22. Goldin BR, Gorbach SL. The relationship between diet and rat fecal bacterial enzymes implicated in colon cancer. J Natl Cancer Inst 57: 371-375 (1976).
23. Taylor J. The analysis of designed experiments with censored observations. Biometrics 29: 35-43 (1973).
24. Gower JC. Variance component estimation for unbalanced hierarchical classifications. Biometrics 18: 537-542 (1962).
25. Yoshinaga K, Hawkins RA, Stocker JF. Estrogen secretion by the rat ovary in vivo during the estrous cycle and pregnancy. Endocrinology 85: 103-112 (1969).
26. Bunnik GSJ. Diet, hormones and NMU-induced rat mammary cancer. Thesis, Utrecht University, Netherlands (1988).
27. Rose DP, Noonan JJ. Hormone dependence of rat mammary tumors induced by n-nitrosomethylurea. Eur J Cancer Clin Oncol 17: 1357-1358 (1981).
28. Kritchevsky D, Welch CB, Klurfeld DJ. Response of mammary tumors to caloric restriction for different time periods during the promotion phase. Nutr Cancer 12: 259-269 (1989).
29. Markaverich BM, Roberts RR, Alejandro MA, Johnson GA, Middleditch BS, Clark JA. Bioflavonoid interaction with rat uterine type II binding sites and cell growth inhibition. J Steroid Biochem 30: 71-78 (1988).
30. Lok E, Nera EA, Iverson F, Scott F, So Y, Clayson DB. Dietary restriction, cell proliferation and carcinogenesis: a preliminary study. Cancer Lett 38: 249-255 (1988).
31. Klurfeld DM, Welch CB, Davis MJ, Kritchevsky D. Determination of degree of energy restriction, necessary to reduce DMBA induced mammary tumorigenesis in rats during the promotion phase. J Nutr 119: 286-291 (1989).
32. Zuleta A, Sambucetti ME. Calculation of the metabolizable energy of diets with wheat bran for rats. Nutr Reports Int 39: 1127-1138 (1989).

33. Cohen LA, Choi K, Wang C. Influence of dietary fat, calorie restriction, and voluntary exercise on N-nitrosomethylurea-induced mammary tumorigenesis in rats. Cancer Res 48: 4276-4283 (1988).
34. Reddy BS, Weisburger JH, Wynder EL. Fecal bacterial β-glucuronidase: Control by diet. Science 183: 416-417 (1974).
35. Reddy BS, Wynder EL. Large-bowel carcinogenesis: Fecal constituents of populations with diverse incidence rates of colon cancer. J Natl Cancer Inst 50: 1437-1442 (1973).
36. Mallett AK, Rowland IR, Bearne CA. Influence of wheat bran on some reductive and hydrolytic activities of the rat cecal flora. Nutr Cancer 8: 125-131 (1986).
37. Walker ARP, Walker BF, Walker AJ. Fecal pH, dietary fibre intake, and proneness to colon cancer in four South African populations. Br J Cancer 53: 489-495 (1986).
38. van Dokkum W, de Boer BJC, van Faassen A, Pikaar NA, Hermus RJJ. Diet, fecal pH and colorectal cancer. Br J Cancer 48: 109-110 (1983).
39. Wyman JB, Heaton KW, Manning AP, Wicks ACB. The effect on intestinal transit and the feces of raw and cooked bran in different doses. Am J Clin Nutr 29: 1474-1479 (1976).
40. Arts CJM, Govers CARL, van den Berg H, Wolters MGE, van Leeuwen P, Thijssen JHH. In vitro binding of estrogens by dietary fiber and the in vivo apparent digestibility tested in pigs. J Steroid Biochem Mol Biol 38: 621-628 (1991).
41. Axelson M, Setchell KDR. The excretion of lignans in rats. Evidence for an intestinal bacterial source for this new group of compounds. FEBS Lett 123: 337-342 (1981).
42. Axelson M, Sjövall J, Gustafsson BE, Setchell KDR. Origin of lignans in mammals and identification of a precursor from plants. Nature 298: 659-660 (1982).
43. Adlercreutz H, Fotsis T, Heikkinen R, Dwyer JT, Woods M, Goldin BR, Gorbach SL. Excretion of the lignans enterolactone and enterodiol and of equol in omnivorous and vegetarian postmenopausal women and in women with breast cancer. Lancet ii: 1295-1299 (1982).
44. Adlercreutz H, Musey PI, Fotsis T, Bannwart C, Wähälä K, Mäkelä T, Brunow G, Hase T. Identification of lignans and phytoestrogens in urine of chimpansees. Clin Chim Acta 158: 147-154 (1986).
45. Moses DF, Fridman O, De Nicola AF. Relationship between type I and type II estrogen binding sites in the rat uterus. Acta Physiol Pharmacol Latinoam 35: 451-457 (1985).
46. Suolinna EM, Buchsbaum RN, Racker E. The effects of flavonoids on aerobic glycolysis and growth of tumor cells. Cancer Res 35: 1865-1872 (1975).
47. Francis AR, Shetty TK, Bhattacharya RK. Modulating effect of plant flavonoids on the mutagenicity of N-methyl-N'-nitro-N-nitrosoguanidine. Carcinogenesis 10: 1953-1955 (1989).
48. Shutt DA. The effects of plant estrogens on animal reproduction. Endeavour 35: 110-113 (1976).
49. Speek AJ, Schrijver J, Schreurs WHP. Vitamin E composition of some seed oils as determined by high-performance liquid chromatography with fluorimetric detection. J Food Sci 50: 121-124 (1985).
50. Speek AJ, Schrijver J, Schreurs WHP. Fluorimetric determination of total vitamin C and total isovitamin C in foodstuffs and beverages by high-performance liquid chromatography with pre-column derivatization. J Agric Food Chem 32: 352-355 (1984).
51. Hellendoorn EW, Noordhoff MG, Slagman J. Enzymatic determination of the indigestible residue (dietary fibre) content of human food. J Sci Fd Agric 26: 1461-1468 (1975).

In vitro binding of estrogens by dietary fiber and *in vivo* apparent digestibility tested in pigs

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Abstract

Within the framework of experiments related to the association between dietary fiber and breast cancer, an *in vitro* test system was used to study the binding of estrogens to various fibers (e.g. cholestyramin, lignin, cellulose) and fiber sources (e.g. wheat bran, cereals, seeds and legumes). Furthermore, the *in vivo* apparent digestibility of the different fiber sources was tested using a mobile nylon bag technique in intestine-cannulated pigs. Estradiol-17β (E_2) bound more strongly to the various fibers than did estrone (E_1), estriol (E_3) or estrone-3-glucuronide (E_1-gluc). At increasing pH (>7) binding of both E_1 and E_2 to wheat bran decreased significantly. Cholestyramine and lignin bound almost all estrogens present in the medium. Linseed (91%), oats (83%), barley chaff (88%) and wheat bran (82%) are other excellent binders of E_2. Corn, rye and white wheat flour showed lower binding capacity with a relatively low affinity. Cereals with the highest percentage of lignin in the fiber ($>3\%$) were also the fiber sources with the lowest apparent digestibility. Estrogens bound with the highest affinity (relative to bovine serum albumin) to these fiber sources. Together with wheat bran and lignin, oats, linseed and soybean seem to be products with good perspectives for *in vivo* evaluation of the lowering effect of dietary fiber on estrogen exposure of estrogen-sensitive tissues.

Introduction

It has been well documented that estrogens are involved in breast cancer development [1]. Estrogens play an important role in cell proliferation [2–6] and hence in tumor initiation and/or promotion [7]. It has been hypothesized that fiber affects breast cancer risk [8, 9]. One of the mechanisms in which fiber might be involved is its influence on the enterohepatic circulation (EHC) of estrogens [10].

The estrogens, synthesized by the gonads and to a very small extent by the adrenals, are metabolized mainly in the liver, but also in the peripheral tissues [11].

The estrogen(s) (metabolites) are readily conjugated with glucuronic acid and/or sulfuric acid. About one third to half of the circulating estrogens are secreted in the bile, and 80% of this fraction is reabsorbed after hydrolysis in the intestinal canal [12]. When the hydrolysis reaction is blocked [13] or the reabsorption of estrogens by the intestine is lowered as a result of binding to fiber, fecal estrogen excretion increases, urinary estrogen excretion decreases and plasma estrogen levels might be decreased as well.

Vegetarians on a high-fiber diet excrete significantly more estrogens in their feces, resulting in lower plasma estrogen levels, than omnivores on a low-fiber diet [14, 15]. Comparable results have been described for rats receiving a high-fiber diet on the basis of wheat bran relative to those on a low-fiber diet on the basis of wheat flour [16]. It seems that reabsorbtion of estrogens, which is usually around 80%, can be diminished by dietary fiber. In this context, the binding of estrogens by dietary fiber plays an important role [17, 18], which might result in an interrupted EHC of estrogens.

An *in vitro* method for assessment of estrogen binding can be useful in selecting appropriate fiber sources for further *in vivo* studies on fiber-hormone interactions, i.e. the effect of fiber on hormone-sensitive tumors. Shultz and Howie [17] reported an *in vitro* study of estrogen binding to fibers. In this paper we extend these data to various cereals, seeds and legumes and fiber compounds. Relative binding affinity was measured by adding bovine serum albumin (BSA) to the incubation medium. As *in vivo* binding of estrogens to dietary fiber might be affected by the degree of fermentation we also measured the *in vivo* apparent digestibility of the various fiber types and/or their components in intestine-cannulated pigs. The fermentation grade of various wheat varieties were tested *in vivo* as well.

Materials and methods

Chemicals

All chemicals used were Analar grade and obtained from Merck, Darmstadt, FRG, unless stated otherwise. The tritium-labeled hormones [2,4,6,7-^{3}H(N)]-estradiol (spec. act. 115 Ci/mmol), [6,9-^{3}H(N)]-estrone-3β-D-glucuronide (spec. act. 12.6 Ci/mmol), [2,4,6,7-^{3}H(N)]-estrone (spec. act. 105 Ci/mmol), [2,4,6,7-^{3}H(N)]-estriol (spec. act. 105 Ci/mmol) and [1,2,6,7-^{3}H(N)]-progesterone (spec. act. 90.1 Ci/mmol) were purchased from New England Nuclear/Dupont, 's-Hertogenbosch, Netherlands. The labeled compounds were purified before use by HPLC as described previously [19]. The steroids 17β-estradiol (art. E 8875), estrone (art. E 9750), estriol (art. E 1253), progesterone (art. P 0130) and estrone-glucuronide (art. E 1252), as well as α-cellulose (art. C 8002), cholestyramine (art. C 4650), pectin (from apple; art. P 2157), cellulose (Sigmacell, Type 100; art. S 3755) and bovine serum albumin (BSA; art. A 7030) were obtained from Sigma Chemical Company, St. Louis, MO. Pepsin (porcine gastric mucosa) was obtained from Boehringer

Mannheim, FRG. Wheat, wheat bran, white wheat flour, soybean, rye, oat, barley, linseed, buckwheat and corn were obtained from a local miller (Van Rooy, Wageningen, Netherlands). Lignin was kindly donated by Westvaco, Charleston Heights, SC. Lignin sulfonate and lignin extracted from rye chaff were kindly donated by Dr M. van Oort, IGMB-TNO, Wageningen, Netherlands and pea bran by Dr J. Huisman of that Institute. The wheat cultivars Soft Red Winter (SRW), Soft White Winter (SWW), Durum, Hard Red Winter (HRW) and Hard Red Spring (HRS) were kindly donated by Dr J. de Waart, TNO-CIVO Institutes, Zeist, Netherlands.

Methods

Before performing the *in vitro* test the brans, cereals, seeds and legumes were ground using an ultracentrifugal grinder with ring sieves of 0.5 or 1 mm (Retsch B.V., Ochten, Netherlands). The *in vitro* binding method was a modification of Shultz and Howie's method [17]. In brief, of each grinded fiber source or fiber component 50 mg was weighed in duplicate into tubes (16 mm × 100 mm) and suspended in 400 µl of phosphate-buffered saline (PBS, pH 7.0). After incubation (30 min, 37 °C) pH was adjusted to 2.0 and to each tube 0.2 U of pepsin was added in 200 µl of PBS pH 2.0. After incubation (60 min, 37 °C) pH was adjusted to 7.0 and ^{3}H-labeled steroids were added. After another incubation (60 min, 37 °C) tubes were centrifuged and an aliquot of 400 µl of the supernatant and 4 ml of scintillation liquid (Safefluor; Lumac B.V., Olen, Belgium) were added in counting vials. Tubes

Table 1. Schematic presentation of the method.

	Total	− BSA	+ BSA
Binder (mg)	−	50	50
PBS[1] pH 7.0 (µl)	400	400	400
	Incubation, 30 min, 37 °C, shaking		
PBS[1] pH 2.0 + pepsin (µl)	200	200	200
	Incubation, 60 min, 37 °C, shaking		
NaOH 0.67 M (µl)	100	100	100
Tracer[2] (µl)	50	50	50
BSA[3]	−	−	250
PBS pH 7.0 (µl)	250	250	−
	Incubation, 60 min, 37 °C, shaking		
	Centrifugation, 20 min, 3200 × *g*,		
	4 °C in small tubes (12 mm × 75 mm)		

[1] 0.1 M sodium phosphate in saline pH 7.0 or by adding HCl pH 2.0.
[2] Each tracer was diluted to 25,000 dpm per 2.5 µl ethanol. One of the following solvents was added to the various binders: (1) 2.5 µl of ^{3}H-E_2, evaporated and solved in 5 µl ethanol and 45 µl PBS; (2) 2.5 µl of ^{3}H-E_2, 0.625 µl (= 500 ng) to 2.5 µl (= 2000 ng) of unlabeled E_2, evaporated and solved in 5 µl ethanol and 45 µl PBS: (3) 2.5 µl of ^{3}H-E_1, ^{3}H-E_3, or ^{3}H-E_1-glucuronide, evaporated and dissolved in 5 µl ethanol and 45 µl PBS.
[3] 2% bovine serum albumin (BSA) in 250 µl PBS.

for total counts (0% binding; containing no test binding substance) were treated similarly. The quantity of steroid bound was calculated as the difference between the amount of labeled steroid added (total counts) and that recovered in the supernatant. For testing relative binding affinity, incubation was performed with and without BSA for each fiber type. A scheme of the assay is presented in Table 1.

Intra-assay variance, which was calculated from the duplicate values of the percentages of estrogen binding to the various fiber types, was found to be lower than 5% for binding of E_1 ($n = 67$) and E_2 ($n = 106$), 6.5% for E_3 ($n = 78$) and 11% for E_1-gluc ($n = 66$). Inter-assay variance, which was calculated from the binding of E_2 to wheat bran (particle size 0.5 mm), white wheat flour and cellulose, was found to be 2.9% (wheat bran; $n = 27$), 3.6% (white wheat flour; $n = 12$) and 7.0% (cellulose; $n = 8$).

The effect of pH on binding was statistically analyzed with ANOVA techniques using pH as factor and white wheat flour and bran as variates for both E_1 and E_2.

In vivo apparent digestibility tests were performed using the mobile nylon bag technique (MNBT) described by Sauer et al. [20] as modified recently [21]. In brief, pigs fed a standard-type pig grower diet were fitted with a simple T-cannula in the duodenum. Of each fiber type tested (particle size 1 mm) 1 g aliquots were put into nylon bags (25 mm × 40 mm; pore size 48 μm) in quadruplicate. The nylon bags were pre-incubated in 1 liter 0.01 M HCl, containing 4000 U pepsine (Merck, art. 7189) for 5 h at 37 °C under shaking. Each of the nylon bags was inserted into the duodenal cannula of four pigs. The bags passed with the feces. Next, the contents of the nylon bags were lyophilized and weighed. The difference in weight of the contents of the nylon bags before and after *in vivo* MNBT is a measure for the apparent digestibility of the fibers.

The dietary fiber composition of cereals was analyzed using the AOAC method [22] for total dietary fiber, whereas neutral detergent fiber (NDF) and acid detergent fiber (ADF), analyzed according to a modification of the method of Van Soest [37], were used to analyze the amounts of hemicellulose, cellulose and lignin.

Results

Binding of estrogens

The data obtained for the relative binding of steroids to the various fiber sources are summarized in Table 2. Of all steroids tested, E_2 showed the highest binding to the various fiber sources, followed by E_1 and E_3. Binding of E_1-gluc to the fibers was lowest. Cholestyramine and lignin (from rice) bound almost all estrogens present in the medium. Linseed, oats, barley chaff and wheat bran are other good binders of E_2 and E_1.

When BSA was added to the incubation mixture binding was lowered for almost all fiber types tested. The extent of decrease in binding capacity in the presence of BSA was interpreted as indicative of binding affinity. As the affinity constants of E_1,

Table 2. Binding of estrogens to various fibers and fiber sources[1] without and in the presence of 2% (w/v) bovine serum albumin ($n = 2$).

Binder	E_1			E_2			E_3			E_1-gluc		
	−BSA	+ BSA	diff.[2]	−BSA	+ BSA	diff.	−BSA	+ BSA	diff.	−BSA	+ BSA	diff.
1. Wheat bran	68.4	66.8	1.6	82.0	68.1	13.9	46.5	46.0	0.5	30.7	29.1	1.6
2. White wheat flour	50.9	36.4	14.5	71.4	40.0	31.4	40.5	31.5	9.0	28.2	22.2	6.0
3. Wheat	65.2	42.4	22.8	78.6	42.7	35.9	49.8	36.9	12.9	27.7	18.5	9.2
4. Soybean	59.3	56.5	2.8	78.2	64.8	13.4	43.3	37.1	6.2	21.0	12.4	8.6
5. Buckwheat meal	65.2	38.8	26.4	79.4	41.3	38.1	46.3	28.7	17.6	21.5	11.9	9.6
6. Barley	65.9	46.2	19.7	80.8	45.9	34.9	55.6	39.8	15.8	32.2	21.5	10.7
7. Barley chaff	n.d.	n.d.	n.d.	88.5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
8. Oats	66.3	55.8	10.5	83.2	60.8	22.4	54.8	44.6	10.2	18.6	17.6	1.0
9. Corn	59.7	22.5	37.2	73.4	22.9	50.5	45.6	28.2	17.4	15.6	6.2	9.4
10. Rye	62.4	34.6	27.8	70.0	36.8	33.2	48.2	28.6	19.6	22.7	6.6	16.1
11. Linseed	82.7	72.0	10.7	90.6	70.6	20.0	48.0	38.1	9.9	24.9	25.7	- 0.8
12. Pea bran	59.5	n.d.	n.d.	73.5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
13. Rice (unpolished)	58.0	n.d.	n.d.	72.0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
14. Lignin from rice	95.1	92.9	2.2	98.9	97.8	1.1	97.8	95.3	2.5	85.7	85.1	0.6
15. Lignin sulfonate	83.0	81.8	1.2	82.9	80.4	2.5	82.9	83.6	- 0.7	85.2	83.2	2.0
16. Lignin (Westvaco)	n.d.	n.d.	n.d.	96.0	90.2	5.8	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
17. Cholestyramine	95.8	94.9	0.9	99.1	98.6	0.5	98.9	98.3	0.6	97.8	97.0	0.8
18. α-Cellulose	26.9	2.9	24.0	45.9	7.5	38.4	23.6	9.5	14.1	0	0	n.d.
19. Sigmacell	49.8	n.d.	n.d.	63.4	20.8	42.6	28.4	n.d.	n.d.	6.5	n.d.	n.d.

n.d.: not determined, analysis not performed.

[1] The binding of steroids to grains with a particle size of 0.5 mm are presented.

[2] Diff. = difference in binding of estrogens to the various sources with and without the presence of BSA.

E_2 and E_3 with regard to albumin are not equal (5×10^4, 1.8×10^5 and 1×10^5 mol·l., respectively [11]), the relative affinity can only be compared for each of the estrogens separately. A relatively small difference between the percentage of binding without or in the presence of BSA means a high affinity for binding of the estrogen to the fiber, whereas a larger difference suggests a lower affinity. As shown in Table 2, cholestyramine, lignin sulfonate, lignin (from rice), wheat bran, soybean, linseed and oats have the highest relative affinity for estrogens.

Apparent digestibility of fibers sources

The results of the MNBT test are presented in Table 3. Wheat and pea bran, soybean, oats, linseed and lignin showed the lowest apparent digestibility in the MNBT test suggesting a relatively low fermentability. In contrast, the apparent digestibility was 97% for white wheat flour and 80, 82 and 86% for corn, rye and wheat, respectively. After lyophilization, the residues in the nylon bags were also used to assess the *in vitro* binding of E_2. Residual binding of E_2 was clearly lower for digested white wheat flour than for undigested flour, for this *in vitro* binding test was performed with only a small amount of binder (16 mg in 320 µl of volume) as most of the white wheat flour was digested. Binding of E_2 to the residues of soybean, corn and rye were higher than without the *in vivo* MNBT test. Binding of E_2 to the other residues of the fiber sources was similar to binding to the undigested sources.

The apparent digestibility of various wheat varieties (SRW, SWW, Durum, HRW, HRS) was 86%, 86%, 85%, 85% and 83%, respectively. Before the *in vivo* fermentation tritiated E_2 was bound to the flours of these varieties for 60 to 68% without BSA in the incubation medium and for 31 to 35% in the presence of BSA.

Table 3. Apparent digestibility of various dietary fiber sources (particle size 1 mm) tested with the MNBT in cannulated pigs and residual *in vitro* binding with E_2

Binder	Apparent digestibility %; $n = 4$	Binding[1] %; $n = 8$
1. Wheat bran 0.5 mm	44 ± 1.0	78 ± 2.6
2. Wheat bran 1.0 mm	42 ± 2.3	79 ± 2.4
3. White wheat flour	97 ± 0.4	50 ± 4.9[2]
4. Soybean	57 ± 4.8	87 ± 2.6
5. Barley	73 ± 0.8	81 ± 1.5
6. Oats	59 ± 3.7	82 ± 0.9
7. Corn	80 ± 2.9	81 ± 2.2
8. Rye	82 ± 1.5	79 ± 1.9
9. Wheat	86 ± 2.0	80 ± 2.1
10. Linseed	54 ± 2.5	86 ± 2.9
11. Pea bran	44 ± 4.3	74 ± 2.2
12. Lignin (Westvaco)	54 ± 5.4	96 ± 0.5

[1] *In vitro* binding of tritiated E_2 with the residues of the fermented products.
[2] Binding performed with 16 mg, $n = 4$.

Table 4. Dietary fiber composition (% w/w) of the various binders analyzed with the AOAC method (total fiber) and a modified method of Van Soest [37].

Binder[1]	Total fiber	NDF	ADF	Hemi-cellulose	Cellulose	Lignin
1. Wheat bran	40.4	39.5	11.8	27.8	8.3	3.5
2. White wheat flower	3.7	2.2	0.2	2.0	0.2	< 1
3. Soybean	16.8	11.9	7.8	4.1	5.6	2.3
4. Buckwheat meal	2.6	2.1	0.6	1.5	0.5	< 1
5. Barley	17.2	16.4	5.9	10.6	4.7	1.2
6. Oats	26.6	26.5	12.4	14.1	9.4	3.1
7. Corn	9.3	9.8	2.5	7.3	2.1	< 1
8. Rye	14.7	12.5	2.6	9.9	1.6	1.0
9. Wheat	9.7	9.9	2.7	6.2	1.9	< 1
10. Linseed	22.3	19.9	13.0	6.9	9.9	3.1
11. Wheat, SRW	9.4	9.2	2.4	6.9	1.8	< 1
12. Wheat, SWW	10.8	9.3	2.0	7.3	1.8	< 1
13. Wheat, Durum	10.4	9.2	2.4	6.7	2.0	< 1
14. Wheat, HRW	11.2	10.0	2.3	7.7	2.0	< 1
15. Wheat, HRS	12.0	10.4	2.7	7.7	2.2	< 1
16. Wheat, European	10.7	8.8	2.0	6.8	1.7	< 1

[1] SRW, Soft Red Winter; SWW, Soft White Winter; HRW, Hard Red Winter; HRS, Hard Red Spring.

Tritiated E_2 was bound with the residues of the fermented products for about 88% without BSA and for about 80% in the presence of BSA indicating an increased capacity and affinity after fermentation.

Dietary fiber composition of the binders

The highest percentage of dietary fiber was measured in wheat bran (40%) followed by oats (26.6%) and linseed (22.3%). In these products the highest percentages of lignin are found (> 3%). In soybean the percentage of lignin is 2.3, and in the other cereals tested it is lower than 1.2 (Table 4).

The dietary fiber composition of the various wheat varieties is similar. The amount of fiber and hemicellulose of the HRW ans HRS varieties is 0.5 to 1% higher than in the other ones.

Binding properties

Fig. 1 shows the percentage of binding of tritiated E_2 to 50 mg of wheat bran suspended in various incubation volumes. The percentage of E_2 bound to wheat bran decreases when the concentration of the binder is lower, i.e. the incubation volume is increased. The solubility of tritiated E_2 was found to be 100% in the various incubation volumes.

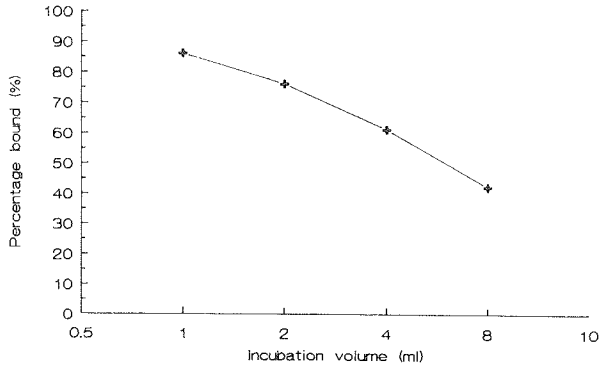

Fig. 1. Relative binding of estradiol-17β with wheat bran as function of incubation volume.

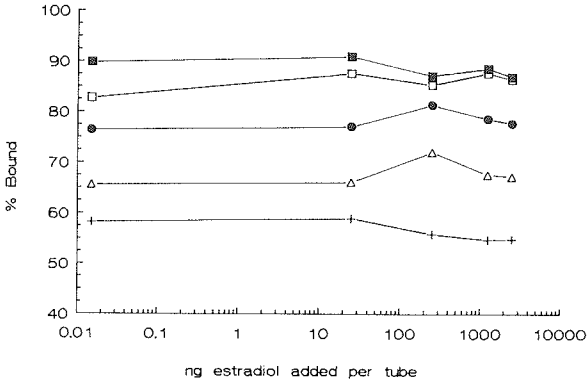

Fig. 2. Relative binding of estradiol-17β with wheat bran estimated at various levels of both unlabeled estradiol and wheat bran. Plus signs, 25 mg bran/tube; triangles, 50 mg; dots, 100 mg; open squares, 250 mg; filled squares, 500 mg.

Table 5. Percentage of binding of tritiated E_1 and E_2 to wheat bran and white wheat flour ($n = 2$) in relation to pH.

Estrogen	Binder	Percentage of binding at pH					SED	P
		4.6	5.0	6.0	7.0	8.0		
E_1	bran	70.9	73.2	71.6	66.9	61.2	0.953	<0.001
E_1	flour	55.9	57.7	55.9	57.5	56.1	1.298	0.52
E_2	bran	84.8	83.7	84.2	81.6	79.8	1.151	<0.05
E_2	flour	72.6	72.7	73.4	71.4	73.3	1.035	0.407

As shown in Fig. 2, a distribution of E_2 between the binder (wheat bran) and the liquid phase is found which seems to be independent of the amount of estradiol present, but dependent on the amount of binder in the medium.

It was found that the pH of the incubation mixture affects binding of both E_1 and E_2 to wheat bran while binding to white wheat flour was not influenced by pH. At higher pH binding of E_1 and E_2 to wheat bran was significantly lower (Table 5).

Discussion

The breast cancer hypothesis of Gorbach [10] is based on interruption of the EHC of estrogens by a change in intestinal microflora composition resulting in a lowered β-glucuronidase activity [23]. The conjugated estrogens excreted by the bile into the intestine cannot be reabsorbed since only free estrogens can pass the intestinal mucosa [12]. Other possibilities for interrupting the EHC of estrogens are an accelerated intestinal transit and a bulking effect of dietary fiber resulting in lower intestinal estrogen concentrations making them less available for reabsorption.

A more effective way of interrupting the EHC of estrogens and other compounds such as bile acids [24] seems to be binding to dietary fiber [17]. As binding capacity depends on fiber composition, i.e. the origin of dietary fiber, various cereals and fiber components were tested in an *in vitro* system.

Calculation of the affinity constant using Scatchard analysis [25] was not possible as, independent of the amount of estrogens, a constant percentage of the estrogens was bound to a particular amount of binder (Fig. 2). As the affinity constants for binding of estrogens to albumin are known [11], the affinity with which the estrogens are bound to the various fiber types was expressed relative to BSA. This protein does not bind to fiber: addition of fiber compounds to a 2% BSA solution did not affect the BSA concentration as measured with Lowry's method [26]. Although we realize that our *in vitro* experimental conditions represent a complex physicochemical system, we assume that measuring the binding relative to BSA gives an impression of the affinity of the individual steroids bound to the various binders.

Contrary to Shultz and Howie [17], we used 50 mg of binder substance in a total volume of 1.0 ml instead of the 5 ml they used. This may explain the lower percentage of binding of the steroids to bran and cellulose in the previous study [17] which is supported by our findings that an increased incubation volume decreases estrogen binding (Fig. 1).

In our *in vitro* binding assay E_2 was the steroid bound with the highest percentage to the various fiber types tested. In contrast, Shultz and Howie [17] found that the percentage of binding of E_2 to oat bran and corn bran was similar as that of E_1; for wheat bran and oat hulls percentage of binding of E_2 was lower and for cellulose higher than binding of E_1. We cannot explain these different findings.

Cholestyramine bound more of each of the estrogens tested than any of the natural types of fiber. This ion exchange resin is specifically designed to bind bile salts. The lignin used in our experiments exhibited substantial estrogen-binding

properties, as was found previously [17], making it an excellent candidate for further tests concerning its dietary effects on estrogen balance *in vivo*.

The various wheat cultivars are used for different purposes. Hard Red Winter and Hard Red Spring cultivars are used for bread and hard rolls. Durum wheat is used for pasta. Soft Red Winter and Soft White Winter are used for flat loaves, cakes, pastry and crackers (information from U.S. Wheat Associates). The varieties used for bread and hard rolls were found to have the highest content of fiber and hemicellulose. In our *in vitro* and *in vivo* tests no differences were found between the various wheat types.

A relatively high binding of an estrogen or a carcinogenic compound to a fiber source is only meaningful if the fiber source is not or only poorly fermented *in vivo*. Fiber sources showing the highest relative binding of estrogens also showed the lowest apparent digestibility in the MNBT test as well as a high binding affinity relative to BSA. A relatively low apparent digestibility was measured for wheat bran (42%), linseed (54%), oats (59%) and soybean (57%). These products also showed the highest amount of the unfermentable lignin and of the poorly fermentable hemicellulose (Table 4). The chemically inert lignin binds almost all estrogens with a relatively high affinity (Table 2). The relatively large amount of hemicellulose in oats has a high content of the polysaccharide (1→3)(1→4)-β-D-glucan. Experiments performed previously in our Institute have shown that oats diets significantly lowered rat serum cholesterol levels, in contrast to barley, wheat and rice [27]. The hypocholesterolemic effect of oats has been confirmed recently [28]. This effect may be ascribed to β-D-glucan [29].

Concerning the influence of pH on binding properties, our findings suggest that pH affects binding depending on the type of fiber involved. Binding of E_1 and E_2 to wheat bran was lower at higher pH, while binding of these estrogens to white wheat flour was not influenced by pH (Table 5). Opposite effects of pH on binding of (lipophilic) carcinogenic compounds such as dimethylbenz(a)-anthracene (DMBA; [30]) and 1,2-dimethylhydrazine (DMH; [31]) to various dietary fibers have been reported previously. As fiber consists of a matrix of polysaccharides and lignin and as fiber has cation exchange properties, adsorption may depend upon charges [32]. It might be concluded that pH affects binding depending on both the type of fiber and the compound to be bound.

When particular fiber components or fiber sources are evaluated *in vivo* it has to be considered that not only the binding of estrogens to these fibers can exert an effect on estrogen exposure of estrogen-sensitive tissues, but also other effects might be introduced. When a pure dietary fiber component like lignin is administered to a diet, it might be expected that the EHC of estrogens is interrupted by binding of estrogens to lignin followed by fecal excretion. When a dietary fiber source like wheat bran, oats or linseed is administered to a diet, various effects might be introduced: the dietary fiber will interrupt the EHC by binding the estrogens [17], the intestinal microflora will change [33], which results in changed activities of enzymes in the intestine, such as lower β-glucuronidase, azoreductase and nitroreductase activities [34]. The lower β-glucuronidase activity may diminish estrogen reabsorption. The

different fiber components in the products mentioned each have their own characteristics: pectin, cellulose and hemicellulose have a water-absorbing capacity, while pectin and cellulose are fermented (partly) to short-chain fatty acids resulting in lowered fecal pH [35]. A lower pH may increase the binding of estrogens to the products remaining after fermentation as shown. A very important effect of administering wheat bran, oats or linseed is the introduction of lignans, such as precursors of enterolacton and enterodiol, and phytoestrogens present in these cereals. These compounds have anti-estrogenic properties and might counteract estrogen exposure of estrogen sensitive tissues [36].

This *in vitro* test is much cheaper and less time-consuming than *in vivo* experiments. However, the *in vitro* binding procedure described has certain drawbacks: only insoluble dietary fibers can be tested; binding of estrogens to other products of the cereals, such as proteins and fat, might influence estrogen binding; the applicability to the *in vivo* situation is hard to interpret). It still can be contended that this *in vitro* binding study, in combination with the *in vivo* apparent digestibility tests, will contribute to an improved selection of specific dietary fibers for *in vivo* evaluation on hormonal balance. In conclusion, wheat bran, soybean, oats and linseed seem to be promising compounds for further *in vivo* experiments in which estrogen exposure should be lowered.

References

1. Anonymous. Hormones and breast cancer. In: Pike MC, Siiteri PK, Welsch CW, eds. Banbury Report 8. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1981).
2. Quarmby VE, Korach KS. The influence of 17β-estradiol on patterns of cell division in the uterus. Endocrinology 114: 694-702 (1984).
3. Schatz R, Soto AM, Sonnenschein C. Estrogen-induced cell multiplication: direct or indirect effect on rat uterine cells? Endocrinology 115: 501-506 (1984).
4. Morozova TM, Mitina RL, Nagibneva IN, Ozhogina ZB, Salganik RI. The biochemical mechanism of the stimulating action of estradiol on the growth of mammary tumours. Exp Clin Endocrinol 88: 293-302 (1986).
5. Soto AM, Sonnenschein C. Cell proliferation of estrogen sensitive cells: the case for negative control. Endocrine Rev 8: 44-52 (1987).
6. Brünner N, Bronzert D, Vindelov LL, Rygaard K, Spang-Thomsen M, Lippman ME. Effect on growth and cell cycle kinetics of estradiol and tamoxifen on MCF-7 human breast cancer cells grown in vitro and in nude mice. Cancer Res 49: 1515-1520(1989).
7. Albanes D, Winick M. Are cell number and cell proliferation risk factors for cancer. J Natl Cancer Inst 80: 772-775 (1988).
8. Rose DP. Dietary fiber and breast cancer. Nutr Cancer 13: 1-8 (1990).
9. Adlercreutz H. Western diet and Western diseases: some hormonal and biochemical mechanisms and associations. Scand J Clin Lab Invest. 50 (Suppl 201): 3-23 (1990).
10. Gorbach SL. Estrogens, breast cancer and intestinal flora. Rev Infect Dis 6 (Suppl 1): S85-S90 (1984).
11. Fotherby K. Biosynthesis of the oestrogens. In: Makin HLJ, ed. Biochemistry of steroid hormones, pp. 207-229. Blackwell Scientific Publications, Oxford/London (1984).
12. Adlercreutz H. Hepatic metabolism of estrogens in health and disease. New Engl J Med 9: 1081-1083 (1974).
13. Adlercreutz H, Martin F, Tikkanen MJ, Pulkkinen M. Effect of ampicillin administration on the excretion of twelve oestrogens in pregnancy urine. Acta Endocrinol 80: 551-557 (1975).

14. Goldin BR, Adlercreutz H, Gorbach SL, Warram JH, Dwyer JT, Swenson L, Woods MN. Estrogen excretion patterns and plasma levels in vegetarian and omnivorous women. New Eng J Med 307: 1542-1547 (1982).
15. Goldin BR, Adlercreutz H, Gorbach SL, et al. The relationship between estrogen levels and diets of Caucasian American and Oriental immigrant women. Am J Clin Nutr 44: 945-953 (1986).
16. Arts CJM, de Bie AThHJ, van den Berg H, van 't Veer P, Bunnik GSJ. Influence of wheat bran on NMU-induced mammary tumor development, plasma estrogen levels and estrogen excretion in female rats. J Steroid Biochem Mol Biol 39: 193-202 (1991).
17. Shultz TD, Howie BJ. In vitro binding of steroid hormones by natural and purified fibers. Nutr Cancer 8: 141-147 (1986).
18. Whitten CG, Shultz TD. Binding of steroid hormones in vitro by water-insoluble dietary fiber. Nutr Res 8: 1223-1235 (1988).
19. Arts CJM, Kemperman PTW, van den Berg H. Oestrogen radio receptor assay for multi-residue screening of bovine urine for oestrogenic anabolics. Food Additives Contaminants 6: 103-116 (1989).
20. Sauer WC, Jorgensen H, Berzins R. A modified nylon bag technique for determining apparent digestibilities of protein in feedstuffs for pigs. Can J Anim Sci 63: 233 (1983).
21. Sauer WC, den Hartog LA, Huisman J, van Leeuwen P, de Lange CFM. The evaluation of the mobile nylon bag technique for determining the apparent protein digestibility in a wide variety of feedstuffs for pigs. J Anim Sci 67: 432-440 (1989).
22. Anonymous. Official methods of analysis. AOAC, Washington, DC, p. 136 (1975).
23. Reddy BS, Weisburger JH, Wynder EL. Fecal bacterial β-glucuronidase: Control by diet. Science 183: 416-417 (1974).
24. Story JA, Kritchevsky D. Comparison of the binding of various bile acids and bile salts in vitro by several types of fiber. J Nutr 106: 1292-1294 (1976).
25. Scatchard G. The attractions of proteins for small molecules and ions. Ann NY Acad Sci 51: 660 (1949).
26. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin Phenol reagent. J Biol Chem 193: 265-275 (1951).
27. de Groot AP, Luyken R, Pikaar NS. Cholesterol lowering effect of rolled oats. Lancet ii: 303-304 (1963).
28. Shinnick FL, Ink SL, Marlett JA. Dose response to a dietary oat bran fraction in cholesterol-fed rats. J Nutr 120: 561-568 (1990).
29. Klopfenstein CF, Hoseney RC. Cholesterol-lowering effect of beta-glucan-enriched bread. Nutr Rep Int 36: 1091-1098 (1987).
30. Gulliver WP, Kutty KP, Laher JM, Laher JM, Barrowman JA. In vitro interaction of 7,12-dimethylbenz(a)anthracene and its biliary metabolites with dietary fibers. J Natl Cancer Inst 71: 207-210 (1983).
31. Smith-Barbaro P, Hanson D, Reddy BS. Carcinogen binding to various types of dietary fiber. J Natl Cancer Inst 67: 495-497 (1981).
32. Kritchevsky D, Story JA. Binding of bile salts in vitro by nonnutritive fiber. J Nutr 104: 458-462 (1974).
33. McCarthy RE, Salyers AA. The effects of dietary fibre utilization on the colonic microflora. In: Rowland IR, ed. Role of the gut flora in toxicity and cancer, pp. 295-314. Academic Press, London (1988).
34. Goldin BR, Gorbach SL. The relationship between diet and rat fecal bacterial enzymes implicated in colon cancer. J Natl Cancer Inst 57: 371-375 (1976).
35. Jacobs LR, Lupton JR. Relationship between colonic luminal pH, cell proliferation, and colon carcinogenesis in 1,2-dimethylhydrazine treated rats fed high fiber diets. Cancer Res 46: 1727-1734 (1986).
36. Setchell KDR, Adlercreutz H. Mammalian lignans and phyto-oestrogens. Recent studies on their formation, metabolism and biological role in health and disease. In: Rowland IR, ed. Role of the gut flora in toxicity and cancer, pp. 315-345. Academic Press, London (1988).
37. Van Soest PJ. Use of detergents in the analysis of fibrous feeds. J Ass Off Agric Chem 50: 50-55 (1967).

Effect of wheat bran on excretion of radioactively labeled estradiol-17-beta and estrone-glucuronide injected intravenously in male rats

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Abstract

Urinary and fecal estrogen excretion was studied in male rats fed a non-fiber wheat starch diet (dietary fiber < 1%; NF group; $n = 4$), a low-fiber wheat flour diet (dietary fiber 2%; LF group; $n = 4$) or a high-fiber wheat bran diet (dietary fiber 11.6%; HF group; $n = 3$). Short-term effects of the experimental diet on estrogen excretion were studied after intravenous (i.v.) injection of 5 μCi (0.185 MBq) of ^{14}C-E_2 into the tail vein of the rats fed the diets for 2 days. After 3 weeks on the experimental diets, the long-term effects were studied after injection of 5 μCi of ^{14}C-E_2 and 10 μCi of ^{3}H-E_1-gluc. The diet was found to affect estrogen excretion. The short-term effect indicated that rats fed the HF diet excreted twice as much labeled compounds in the feces during the first day after injection as the rats fed the NF or the LF diet. On the other hand, urinary excretion of labeled compounds was significantly higher in the NF and LF rats. The long-term effect resulted in steeper slopes ($P < 0.05$) of the fecal excretion profiles of rats fed the HF diet than for rats fed the NF or the LF diet, indicating an accelerated fecal excretion of labeled compounds in the HF rats. The kinetic profiles of ^{14}C and ^{3}H radioactivity in blood plasma indicated a fast decrease ($t_{1/2} < 2$ min) for both ^{14}C-E_2 and ^{3}H-E_1-gluc. It was concluded that, owing to the short-term effect of wheat bran intake, during the first 24 h after i.v. administration relatively large amounts of radioactively labeled compounds are excreted in feces of rats fed the HF diet. In contrast, excretion is lower in urine of these rats. When the microflora is adapted to the experimental diet the wheat bran diet still results in an accelerated fecal excretion of labeled compounds, which might be attributed to an interruption of the enterohepatic circulation of estrogens. This might result in lowered plasma and/or tissue estrogen levels and hence a decreased exposure of estrogen-sensitive tissue to estrogens, which might decrease risk on mammary (breast) cancer development.

Introduction

Estrogens have been reported to play a role in mammary carcinogenesis. An increased exposure of the estrogen-sensitive breast tissue would result in a higher risk of mammary cancer at the initiation and/or promotion phase (1). Estrogens synthesized in the ovaries, and partly in the peripheral tissues, are mainly metabolized in the liver, and about 50% is excreted via the bile into the intestine. Subsequently, from the estrogens in the gastro-intestinal tract about 80% is reabsorbed via enterohepatic circulation (2).

Dietary fiber can partly interrupt the reabsorption of estrogens, resulting in an increased fecal and a decreased urinary estrogen excretion, and lowered plasma estrogen levels (3). In rats fed a high-fiber (HF) diet based on wheat bran a 3-fold higher fecal estrogen excretion was measured than in rats fed a low-fiber (LF) diet based on white wheat flour (4). The composition of the gut flora is influenced by dietary factors, in particular by indigestible plant cell wall materials (5). This results in changed bacterial enzyme activities (lower β-glucuronidase, azo- and nitroreductase activity (6, 7)) after about 3 weeks on a HF diet (8). Whether the increased fecal estrogen excretion is a result of binding to dietary fiber (9, 10), of a changed (intestinal) estrogen metabolism, or both, is not known.

In this paper an experiment is described in which radioactively labeled estrogen excretion was studied in groups of male rats fed a non-fiber (NF) diet based on wheat starch, a LF diet based on white wheat flour, or a HF diet based on wheat bran. The study was performed to confirm previous findings (4), i.e. an increased fecal estrogen excretion and a lowered urinary estrogen excretion by rats fed a HF diet, and to exclude a changed (increased) estrogen synthesis by intestinal microflora.

Short-term effects of feeding the different diets were studied by quantifying the disposal of ^{14}C-estradiol-17β (^{14}C-E_2) injected i.v. into the rats fed the LF and HF diets for two days. Long-term effects of intestinal estrogen metabolism were studied by injection of ^{14}C-E_2 and ^{3}H-estrone-glucuronide (^{3}H-E_1-gluc) to rats fed the experimental diets for 3 weeks, assuming intestinal flora is adapted by then (8, 11). Estradiol-17β was used as the radioactively labeled hormone because it is the biologically most active estrogen. E_1-gluc is a quantitatively important metabolite in urine. From the results it is concluded that a HF diet increases and accelerates fecal estrogen excretion and lowers urinary estrogen excretion.

Materials and methods

Animals and diets

Until the start of the experiment, male Wistar rats (Charles River Wiga, Germany; 13 weeks of age) were housed in groups of 4 animals each in an air-controlled room (23 ± 1 °C) with a relative humidity of 50 ± 10% and a light/dark cycle of 12 h. Tap water and the NF diet based on wheat starch were administered ad libitum for 20 d.

Table 1. Composition of the three diets (% w/w).

Ingredients	NF diet[1]	LF diet	HF diet
Composition calculated			
Casein	22.53	22.53	15.69
Wheat starch	54.72	-	-
White wheat flour	-	54.72	42.00
Wheat bran	-	-	23.75
Mineral mixture	4.24	4.24	3.63
Vitamin ADEK prep.	0.36	0.36	0.31
Vitamin B mix	0.24	0.24	0.20
Lard	8.95	8.95	7.21
Sunflower oil	8.95	8.95	7.21
Composition analyzed			
Protein	21.3	27.1	23.2
Fat	17.7	18.4	16.0
Carbohydrate	48.4	41.3	38.5
Dietary fiber[2]	< 1	2.0	11.6
Energy (MJ/kg)	18.31	18.36	16.34

[1] NF, non-fiber; LF, low fiber; HF, high fiber.
[2] AOAC method (22).

At the start of the experiment the rats were divided into three groups on the basis of body weight and housed individually in cages arranged such that feces and urine could be collected separately. Group NF ($n = 4$) received the non-fiber diet based on wheat starch, group LF ($n = 6$) received the low-fiber diet based on white wheat flour and group HF (n = 6) the high-fiber diet based on wheat bran. The composition of the diets is given in Table 1. Water and the iso-energetically composed diets were administered ad libitum. Food intake was recorded weekly.

Injection of labeled estrogens and collection of samples

After 2 days on the experimental diets 4 rats of each group were mildly anesthesized with ether and injected i.v. into the tail vein with 100 µl of a solution containing 5 µCi (0.185 MBq) of [4-^{14}C]-estradiol-17β (Amersham, 's-Hertogenbosch, Netherlands; spec. act. 56 mCi/mmol), 20% ethanol and 0.1% bovine serum albumin (BSA) in saline. The labeled steroids were checked on purity before use by HPLC (^{14}C-E_2) or by solid-phase chromatography (^{3}H-E_1-gluc; reversed-phase C18 cartridges; Baker Chemicals, Deventer, Netherlands). One animal of the HF group died during anesthesia. During the following 7 days 24-hour urine and feces were collected. Urine was collected on dry ice, thawed and the volume measured; feces

were weighed both wet and dry after lyophilization. All samples were stored at –20 °C until further use.

After 20 days on the experimental diets all animals received a second injection with 200 µl of a solution containing 5 µCi of ^{14}C-estradiol-17β and 10 µCi of [6,9-^{3}H]estrone-3-β-D-glucuronide (Amersham, 's-Hertogenbosch, Netherlands; spec. act. 12.6 Ci/mmol). During the following 7 days, 24-hour urine and feces were collected.

Two animals of the LF group and two animals of the HF group were equipped with a permanent indwelling catheter in the right external jugular vein. Via the catheter these animals were administered 200 µl of injection solution containing both 5 µCi ^{14}C-estradiol-17β and 10 µCi ^{3}H-estrone-glucuronide. The cannulae of the catheter were washed 3 times with saline containing 0.1% BSA, then 200 µl of blood was sampled 5, 10, 15, 30, 60, 120, 180 and 240 min after administration of the labeled estrogens. Blood samples were transferred to Eppendorf cups containing 100 µl of 1.5% sodium citrate solution. After centrifugation the diluted plasma was stored at –20 °C until further use.

Quantitation of radioactivity and separation of metabolites by HPLC

In each urine sample and in plasma samples total radioactivity (^{14}C or ^{3}H and ^{14}C) was measured over a 10-min period (counting error < 1%) in a liquid scintillation counter (Wallac 1410, Pharmacia/LKB, Woerden, Netherlands).

Lyophilized fecal samples were dismembered (liquid nitrogen, 1 min, amplitude 12 mm) using a microdismembrator (B. Braun, Melsungen, Germany) and solubilized in 3 ml of a sodium acetate buffer pH 4.5. To 100 µl of the solution 2 ml of soluene (Packard Instrument, Groningen, Netherlands) was added and samples were incubated for 48 h. Hydrogen peroxide was added and after incubation for 24 h, 10 ml of scintillation liquid (Hionic fluor, Packard Instrument) was added. Total radioactivity (^{14}C or ^{14}C and ^{3}H) was measured. labeled compounds from the dismembered fecal solution were extracted with diethyl ether before (free fraction) and after (conjugated fraction) hydrolysis with a solution containing 4500 Fishman U β-glucuronidase and 45000 Roy U sulfatase activities (Suc d'Helix Pomatia; Boehringer Mannheim, Almere, Netherlands; 2 h, 50 °C, pH 4.5). These extracts were used for counting radioactivity as well as separation of the labeled estrogen metabolites by HPLC.

HPLC was performed as described previously (12). Briefly, a column (125 mm × 4.5 mm) was filled with Lichrosorb diol, the eluent being hexane/isopropanol (90:10, v/v), at a flow rate of 1.2 ml/min. Fractions of 0.5 min each were collected during a run of 20 min per sample. To each fraction scintillation liquid (Safefluor) was added and radioactivity was counted. A tentative identification of peaks of radioactivity of the samples was performed by comparing the elution times with elution times of authentic E_1, E_2, E_3 and 16α-hydroxyestrone (16α-OH-E_1), eluted under the same conditions as the samples.

β-Glucuronidase activity and pH of cecal and intestinal contents

At 7 days after the second injection the rats were killed by an excess of ether anesthesia. The cecal and intestinal contents were used for measuring β-glucuronidase activity and pH as described (4).

Statistics

Differences in fecal and urinary radioactivity excretion as well as differences in β-glucuronidase activity and pH were analyzed using Student's t-test. As slopes of excretion profiles were only calculated from straight lines after log transformation, these parameters were calculated from day 2 of the collection period to the time 0.1% of the radioactivity relative to the amount injected had been excreted. Slopes were calculated using BMDP program 3R (13), while clearance was calculated as 'ln 2/slope'. Slopes and clearance were compared among the groups by analysis of variance (ANOVA) techniques. A P value of < 0.05 was considered to reflect statistical significance.

Results

Body weight and food intake

At the start of the experiment body weight (mean ± SD) of the rats ($n = 4$ per group) was 344 ± 36, 347 ± 29 and 349 ± 29 g for the NF, LF and HF groups, respectively. Food and energy intake was similar for the NF and LF groups. A higher mass of food was consumed by the HF group, and during the first week energy intake was higher ($P = 0.05$) for the HF group than for the LF and NF groups. Food intake of all three groups was lower during the first week, when animals had to acclimatize from group accommodation to individual housing, than during the second week of the experiment (Table 2).

Table 2. Food and energy intake by male rats (weighing about 350 g) fed a non-fiber (NF), low-fiber (LF) or high-fiber (HF) diet. Values are mean ± SD.

	NF	LF	HF
Week 1			
Food (g/day)	10.0 ± 1.4	9.9 ± 0.7	13.4 ± 1.2
Energy (kJ/day)	182 ± 25.5	182 ± 12.6	218 ± 19.7*
Week 2			
Food (g/day)	13.9 ± 1.9	14.0 ± 0.8	16.8 ± 1.6
Energy (kJ/day)	254 ± 35.7	257 ± 14.3	275 ± 26.4

* Different ($P = 0.05$) from the NF and LF groups.

Table 3. Excretion of ^{14}C- and ^{3}H-labeled compounds (% of total radioactivity injected; mean ± SD) i urinary and fecal samples collected for 7 days after injection. Injection of ^{14}C-E_2 was performed when rats had been fed the non-fiber (NF; $n = 4$), low-fiber (LF; $n = 4$) and high-fiber (HF; $n = 3$) diets for 2 days. ^{14}C-E_2 and ^{3}H-E_1-gluc were injected when rats had been fed the experimental diets for 3 weeks. Values are mean ± SD.

	Injection of ^{14}C-E_2			Injection of ^{14}C-E_2 and ^{3}H-E_1-gluc					
	% ^{14}C recovered			% ^{14}C recovered			% ^{3}H recovered		
	NF	LF	HF	NF	LF	HF	NF	LF	HF
Urine	18 ± 7.2	14 ± 3.4	8.8 ± 2.1	19 ± 4.0	22 ± 3.6	17 ± 4.1	18 ± 3.9	22 ± 4.0	17 ± 3.1
Feces	90 ± 25	55 ± 20	89 ± 16	76 ± 8.2	71 ± 5.3	71 ± 2.2	76 ± 6.4	72 ± 5.8	73 ± 2.9
Total	107	69	97	96	94	88	94	94	89

Excretion of labeled compounds

After the first injection total urinary radioactivity excretion over the 7-day collection period was lower ($P < 0.05$) in the HF group than in the LF and NF groups (Table 3). After the second injection, when the animals had been fed the experimental diets for 3 weeks, no difference in total urinary ^{14}C and ^{3}H radioactivity excretion among the groups was observed. Excretion of ^{14}C and ^{3}H radioactivity, expressed as percentage of total radioactivity injected, was similar. Total fecal ^{14}C excretion was similar for the NF and HF groups, but lower for the LF group, after the first injection and similar for all 3 groups after the second injection. Recovery of ^{14}C and ^{3}H radioactivity in urinary and fecal samples ranged between 88 and 108% fo all groups, but total recovery of the LF group was only 69% after the first injection (Table 3).

The daily cumulative radioactivity excretion and the excretion profiles for ^{14}C after the first and after the second injection are presented in Figs. 1 and 2, respectively. Because the figures for ^{3}H radioactivity excretion were very similar to those of the ^{14}C excretion, the results are not shown separately. The relatively small amount of ^{14}C excreted in urine of the HF group after the first injection (Table 3) was excreted faster than in the other groups. The slope of the excretion profile of the HF group was steeper than for the other groups ($P < 0.05$; Fig. 2A).

Fecal ^{14}C excretion after the first injection varied largely among the groups (Figs. 1 and 2). In the HF group, 73% of total ^{14}C fecal radioactivity excreted over the 5-day period was excreted during the first 24 h and 20% during the second 24-h period. In the other two groups about 30% of total fecal radioactivity was excreted during the first day and 42% (NF group) or 32% (LF group) during the second day after injection (Fig. 1). The excretion rate of the HF group tended to be higher ($t_{1/2}$ = 0.54 days) than for the other groups ($t_{1/2}$ about 1.15 days). After 3 weeks the fecal excretion profiles of the NF and LF groups were similar and comparable with the

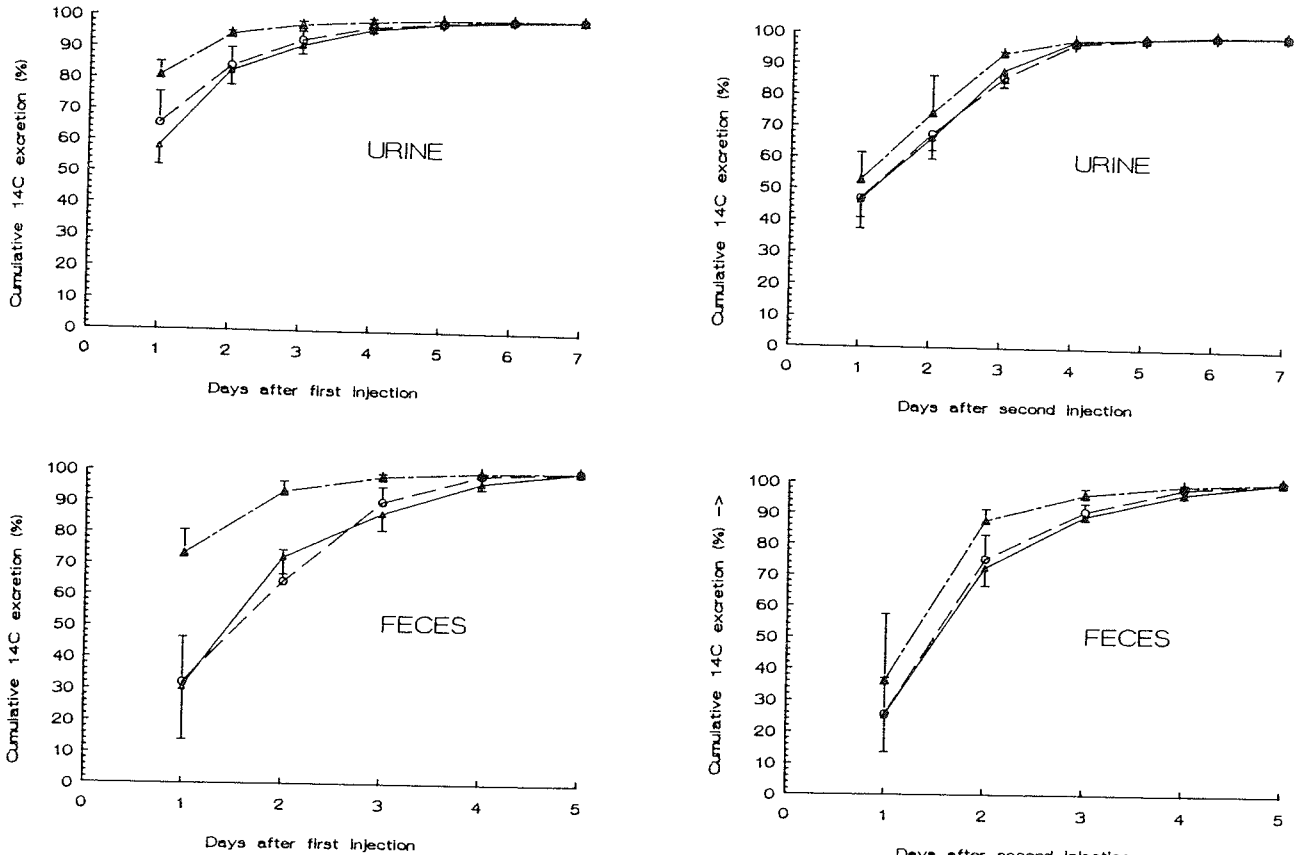
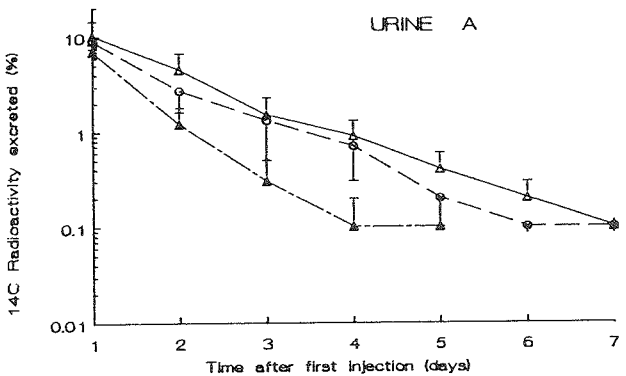
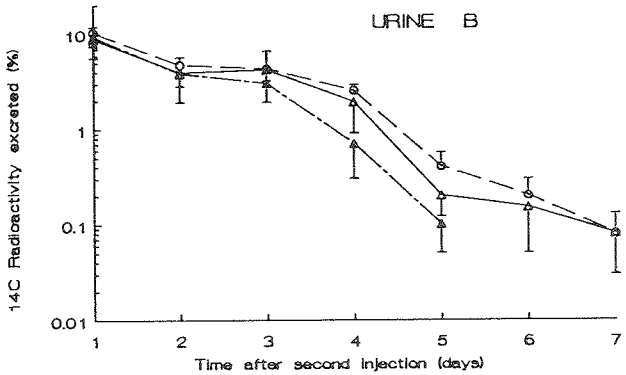
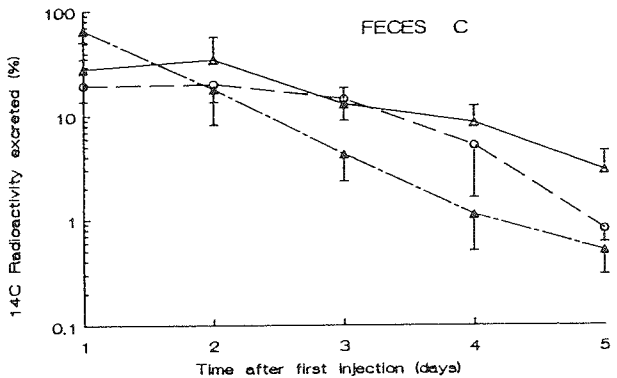


Fig. 1. Cumulative excretion pattern of ^{14}C radioactivity in urinary and fecal samples from rats fed 2 days or 3 weeks on a non-fiber (NF; wheat starch; open circles), low-fiber (LF; wheat flour; open triangles) or high-fiber diet (HF; wheat bran; solid triangles). Rats were injected with 5 μCi ^{14}C-E_2. The marks indicate means and SD. 100% of radioactivity equals total mean excretion per rat and per group. (Cf. Table 3.)







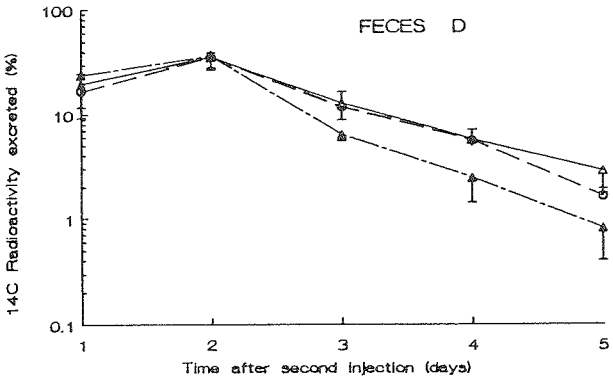


Fig. 2. Urinary (A, B) and fecal (C, D) excretion profiles of ^{14}C radioactivity after injection of 5 μCi ^{14}C-E_2 in rats fed the experimental diet for 2 days (A, C) or 3 weeks (B, C). NF, open circles; LF, open triangles; HF, closed triangles. The marks indicate means and SD. 100% of radioactivity equals total mean excretion per rat and per group. Cf. Table 3.)

excretion profile of the NF group after the first injection. The HF group, however, showed a remarkably different fecal excretion profile after 3 weeks on the HF diet as compared with the profile obtained after the HF diet was administered during 2 days. A straight profile was obtained after 2 days on the HF diet, while after 3 weeks the excretion profile showed a higher fecal radioactivity excretion on the second day than on the first day (Fig. 2). Slopes of the fecal radiactivity profiles of the HF group were steeper ($P < 0.05$) than those of the other two groups.

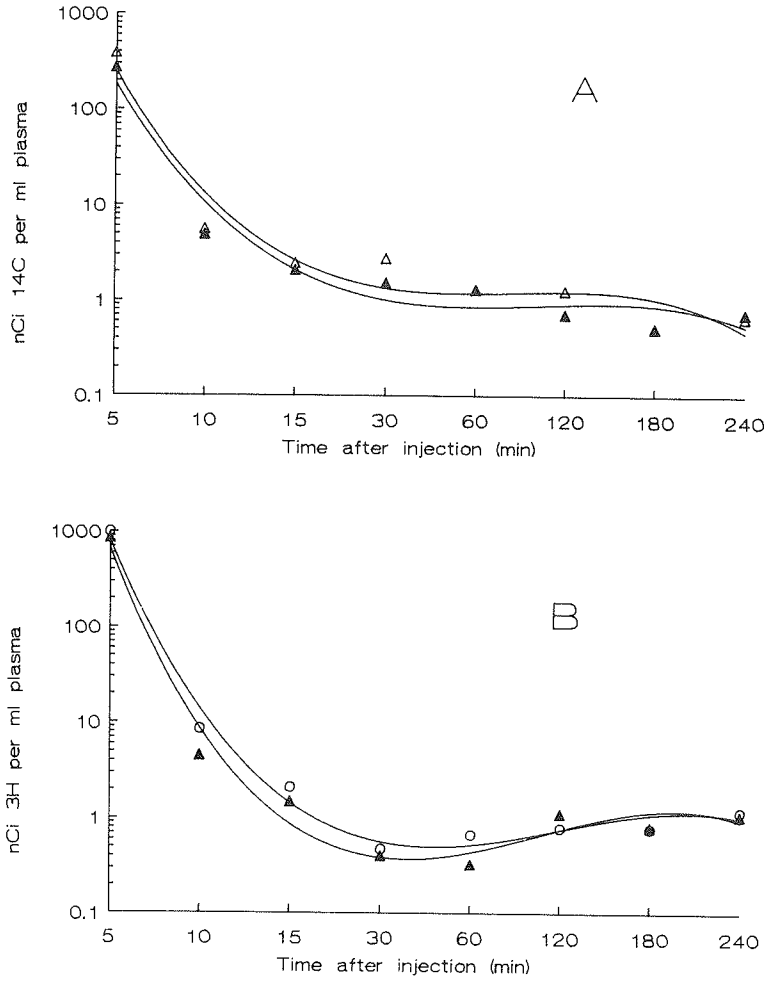


Fig. 3. Kinetic profile of ^{3}H and ^{14}C radioactivity in blood plasma of 2 animals of both the LF group (circles) and the HF group (triangles), as a result of injection of 5 μCi ^{14}C-E_2 (A) or injection of 10 μCi ^{3}H-E_1-gluc (B).

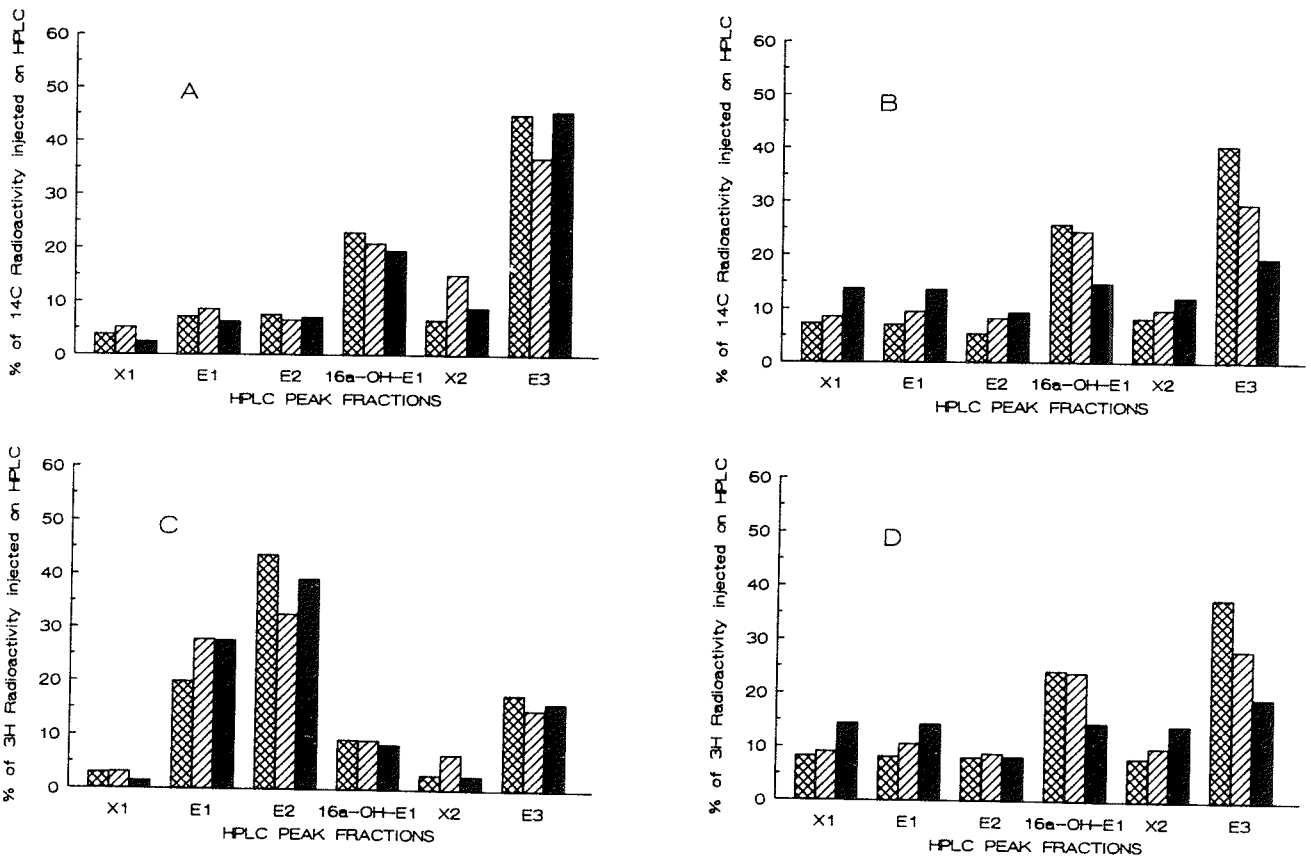


Fig. 4. Relative distribution of radioactivity among HPLC fractions: fecal samples collected 1 (A,C) and 3 days (B,D) after injection of 5 μCi of ^{14}C-E_2 (A, B) and 10 μCi of ^{3}H-E_1-gluc (C, D). HPLC fractions containing radioactive compounds with the same elution times as authentic E_1, E_2, E_3 and 16α-OH-E_1 were collected. Peaks indicated with X1 and X2 contain unknown compounds. Histograms: cross-hatched, NF group; hatched, LF group; solid, HF group.

Kinetics in blood plasma

Mean ^{3}H and ^{14}C radiactivity in blood plasma, sampled from 2 animals of both the LF and HF group, is shown in Fig. 3. A fast decrease of radioactivity was observed between 5 and 15 min after injection. During the subsequent 15 min, ^{3}H radioactivity decreased to about 0.4 nCi/ml and ^{14}C radioactivity to about 1 nCi/ml. ^{14}C radioactivity remained fairly constant between 15 and 180 min after injection (Fig. 3A). ^{3}H radioactivity increased slightly between 60 and 180 min after injection (Fig. 3B), which might be the result of enterohepatic circulation or of a delayed release of the labeled compounds by the tissues (e.g. adipose tissue).

Fecal estrogen metabolites

In the HPLC fractions obtained from feces collected during the first day after the second injection, ^{14}C (Fig. 4A) and ^{3}H radioactivity (Fig. 4C) gave different excretion profiles of estrogen metabolites. The metabolites 16α-OH-E_1 and E_3 were the major compounds in the HPLC profile after injection of ^{14}C-E_2, whereas E_1 and E_2 were the major compounds after injection of ^{3}H-E_1-gluc. Three days after the second injection no differences in HPLC profiles could be observed between excretion of ^{14}C (Fig. 4B) and ^{3}H (Fig. 4D) compounds. Peaks with the highest radioactivity primarily derived from compounds that elute during HPLC as authentic 16α-OH-E_1 and E_3. In feces of the NF and LF groups about 12% of total ^{14}C or ^{3}H radioactivity injected was excreted during the third day, whereas in the HF group about 6% was excreted during this period. Furthermore, in the HF group the relative amount of labeled compounds with elution time as authentic 16α-OH-E_1 and E_3 was lower and as X_1 and authentic E_1 higher than in the NF and LF groups.

Fecal weights

As shown in Table 4, total fecal excretion (both wet weight and dry weight) for the HF group was 3 to 4 times higher than for the NF and LF groups. The higher fecal wet weight of the HF group is only partly explained by the higher moisture content.

Table 4. Mean fecal excretion (g/day)[1] by groups of rats fed a wheat starch non-fiber (NF; $n = 4$), a wheat flour low-fiber (LF; $n = 4$) or a wheat bran high-fiber diet (HF; $n = 3$).[2]

Group	Wet weight	Dry weight	% moisture
NF	0.79 ± 0.35a	0.52 ± 0.16a	28 ± 7.5a
LF	0.80 ± 0.21a	0.63 ± 0.19a	22 ± 10 a
HF	3.05 ± 0.34b	1.97 ± 0.22b	35 ± 4.1a

[1] Mean fecal weights of samples collected 1, 6 and 7 days after the first injection.
[2] Mean ± SD values sharing the same letter within a column are not significantly different ($P > 0.05$).

Table 5. Intestinal and cecal β-glucuronidase (U/kg) and pH in rats 7 days after the second injection (see text for more details) as well as the amount of ether extractable (% free estrogen) as a percentage of radioactivity extracted before and after hydrolysis, from fecal samples of rats fed the experimental diets for 3 weeks. All values are mean ± SD.

β-Glucuronidase		pH		% of free estrogen					
				Day 1[1]		Day 2		Day 3	
int.[2]	cecum	int.	cecum	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
Non-fiber (NF) group									
41 ± 15a	675 ± 177a	6.5 ± 0.1a	7.8 ± 0.2a	60 ± 4.7a	60 ± 5.7a	66 ± 3.5a	63 ± 3.8a	43 ± 9.3a	41 ± 9.1a
Low-fiber (LF) group									
39 ± 14a	1074 ± 242b	6.7 ± 0.2a	7.4 ± 0.1b	54 ± 5.8a	51 ± 6.7a	55 ± 3.2b	51 ± 4.7a	43 ± 6.6a	40 ± 6.1a
High-fiber (HF) group									
85 ± 21b	608 ± 289ab	6.9 ± 0.5a	6.8 ± 0.2c	53 ± 4.5a	54 ± 2.3a	58 ± 7.6a	60 ± 5.0a	60 ± 3.4b	60 ± 2.8b

[1] Remarks: only the first 3-day samples collected were used for calculating % free estrogen, because in samples collected at later times after injection, radioactivity was too low for a reliable calculation. Mean ± SD values sharing the same letter within a row are not significantly different ($P > 0.05$).
[2] int. = intestine.

pH and β-glucuronidase activity

Mean cecal β-glucuronidase activity of the NF and HF groups was lower than for the LF group (Table 5). Cecal pH differed significantly among the groups, but pH of the intestinal contents did not.

In Table 5 the percentage of unconjugated (free) estrogen is also given, expressed as a percentage of ether-extractable radioactivity without hydrolysis. Percentages of free estrogen in feces collected during the first day were similar. During the second day, however, the percentage of free estrogen as a result of injection of ^{3}H-E_1-gluc was lower in the LF group than in the other two groups. During the third day the rats of the HF group showed a significantly higher percentage of free estrogen (as a result of injection of both ^{3}H-E_1-gluc and ^{14}C-E_2) than the animals of the NF and LF grou The higher percentage of free estrogen in the HF group does not agree with the previous finding, i.e. a lower β-glucuronidase activity.

Discussion

In our experiments differences in estrogen excretion as a result of ingestion of different diets were studied. To avoid possible effects of the estrous cycle on estrogen excretion, male rats were used instead of females, as the estrus cycle might influence intestinal motility (14) and hence reabsorption and fecal estrogen excretion. A difference in estrogen excretion between male and female rats can be expected as estrogen metabolism concerning 16α-OH-E_1 synthesis has been found to be strongly sex-dependent (15). Sex was not considered to influence any diet-induced differences.

Short-term effects

From the hypotheses stated previously (4, 16, 17) it could be expected that a high-fiber diet affects estrogen excretion resulting in an increased fecal estrogen excretion, a decreased urinary estrogen excretion and lower plasma estrogen levels. In the present study the short-term effect of a high fiber intake indeed resulted in a significantly lower urinary ^{14}C excretion, while fecal ^{14}C excretion was 2- to 4-fold higher in the HF group than in the NF and LF groups during the first day after injection. The accelerated fecal excretion can be explained by interruption of the enterohepatic circulation due to binding of estrogens to dietary fiber components as demonstrated *in vitro* (9, 10). It might be expected that during injection, as well as a few days after the first injection, the intestinal microflora of the animals fed the LF and HF diets was not changed relative to the microflora of the rats fed the NF diet which remained on the wheat starch diet (8). For this reason, other explanations for the interrupted enterohepatic circulation than binding of estrogens to fiber might be excluded.

Long-term effects

The long-term effect, measured after 3 weeks on the experimental diets, resulted on the first day in a higher fecal excretion of labeled compounds in rats fed the HF diet and a steeper slope ($P < 0.05$) of the ^{14}C and ^{3}H excretion profiles of this group (Fig. 2D). During the second 24-hour period a higher fecal excretion of labeled compounds was measured in all 3 groups than during the first day. This suggests reabsorption of labeled estrogens after previous intestinal bile excretion during the first 24 h, i.e. enterohepatic circulation (3). Thus fecal excretion of labeled compounds tended to be higher in the HF group, which confirms previous findings (4). It also fits in with the hypothesis that dietary fiber enhances fecal estrogen excretion resulting in a lowered exposition of estrogen to estrogen-sensitive tissue, and hence a reduced risk for mammary cancer development.

pH and β-glucuronidase

The intestinal flora may be expected to be adjusted to the experimental diets after 3 weeks (8, 11), resulting in a lower intestinal pH and lower β-glucuronidase activity in animals fed the HF diet. The results presented in Table 5 are in agreement with these expectations as well as with earlier findings (4) in which significantly lower β-glucuronidase activities were found in fecal samples from rats fed the HF diet than in rats fed the LF diet. The very low β-glucuronidase activity in the intestinal contents compared to that in cecal contents is in agreement with previous reports (18). In the cecum from animals fed the HF diet a significantly lower pH was measured, and also cecal β-glucuronidase activity tended to be lower than in the LF group (Table 5). A lower β-glucuronidase activity might result in decreased deconjugation of the estrogens excreted by the bile and hence a lowered reabsorption by mucosal cells (2).

However, in feces from animals of the HF group collected on the third day a higher percentage of free (unconjugated) labeled compounds was found than in non-hydrolyzed samples of the NF and LF groups. This confirms the validity of suggestions discussed previously (4). In our earlier study, significantly higher percentages of free (unconjugated) E_1 and E_2 were estimated in fecal samples from the HF group. This was explained by the higher pH of the LF fecal samples (less optimal pH for β-glucuronidase activity). It was suggested that other factors such as fecal bulk, transit time and binding capacity of the non-fermentable dietary fiber components, rather than β-glucuronidase activity as measured *in vitro* under standardized conditions determine fecal estrogen excretion.

Fecal estrogen metabolites

During the first day after injection of ^{3}H-E_1-gluc, E_1 and E_2 were the quantitatively most important metabolites excreted (Fig. 4C), whereas 16α-OH-E_1 and E_3 were dominant three days after injection (Fig. 4D). This difference in excretion of estrogen metabolites was not observed after injection of ^{14}C-E_2 (cf. Fig. 4A and 4B). It seems that the conversion of ^{3}H-E_1-gluc to 16α-OH-E_1 and E_3 takes more time than conversion to E_1 and E_2, in which process one or two enzymes are directly involved (19).

In a previous experiment (4) it was found that energy (i.e. fat) intake of a group of female rats fed a similar HF diet as used in this study, was 6–10% lower than for rats fed the LF diet. Although in this study a difference in fat intake between the groups was not measured during the experimental period (Table 2), it might be assumed that fat was utilized in a manner differently by rats fed the HF diet than by rats fed the LF diet. The relatively small amount of E_3 and 16α-OH-E_1 estimated in fecal samples from the HF group (cf. Fig. 4B and 4D) could thus be explained. Women consuming a low-fat diet and given radiolabeled E_2 (both orally and intravenously) excreted significantly lower levels of 16α-hydroxylated estrogens than women on a high-fat diet (20). The 16α-OH-E_1 and E_3 levels in fecal samples from rats fed the HF diet are of special importance regarding the discussion on its carcinogenic properties (15).

In the HPLC fraction in which 16α-OH-E_1 eluted (Fig. 4), 16-keto-estradiol eluted as well. This estrogen metabolite can quantitatively be synthetized to the same extent as 16α-OH-E_1 (15). In studies reviewed previously (2), 16-hydroxylated estrogens and E_3 were also found to be the main biliary metabolites of E_1 and E_2.

Blood plasma kinetics

The kinetic profiles of ^{14}C and ^{3}H radioactivity, shown in Fig. 3, indicate a fast decrease ($t_{1/2} < 2$ min) for both ^{14}C-E_2 and ^{3}H-E_1-gluc. Differences between the LF and HF groups were not observed. This clearance rate supports earlier findings in which a similar clearance was estimated after injecting ^{3}H-E_2 into female Sprague Dawley rats (21).

It can be concluded that, owing to the short-term effect of wheat bran intake, during the first 24-hour period after i.v. administration a relatively large amount of radioactively labeled compounds is excreted in feces of rats fed the HF diet. In contrast, in urine of these rats excretion is lower. Once the microflora has adapted to the test diets the wheat bran diet still results in an accelerated fecal excretion of labeled compounds.

This effect of dietary fiber might result in a lowered estrogen exposition of estrogen-sensitive tissue and hence a lowered risk of mammary cancer at the initiation or promotion phase.

References

1. Henderson BE, Ross R, Bernstein L. Estrogens as a cause of human cancer: The Richard and Hinda Rosenthal foundation award lecture. Cancer Res. 48: 246-253 (1988).
2. Adlercreutz H, Martin F. Biliary excretion and intestinal metabolism of progesterone and estrogens in man. J Steroid Biochem 13: 231-244 (1980).
3. Gorbach SL. Estrogens, breast cancer and intestinal flora. Rev Infect Dis 6 (Suppl 1): S85-S90 (1984).
4. Arts CJM, de Bie AThHJ, van den Berg H, van 't Veer P, Bunnik GSJ, Thijssen JHH. Influence of wheat bran on NMU-induced mammary tumor development, plasma estrogen levels and estrogen excretion in female rats. J Steroid Biochem Mol Biol 39 (1991) (in press).
5. Rowland IR, Mallett AK, Wise A. The effect of diet on the mammalian gut flora and its metabolic activities. CRC Crit Rev Toxicol 16: 31-103 (1985).
6. Reddy BS, Weisburger JH, Wynder EL. Fecal bacterial β-glucuronidase: Control by diet. Science 183: 416-417 (1974).
7. Reddy BS, Wynder EL. Large-bowel carcinogenesis: Fecal constituents of populations with diverse incidence rates of colon cancer. J Natl Cancer Inst 50: 1437-1442 (1973).
8. Goldin BR, Gorbach SL. The relationship between diet and rat fecal bacterial enzymes implicated in colon cancer. J Natl Cancer Inst 57: 371-375 (1976).
9. Arts CJM, Govers CARL, van den Berg H, Wolters MGE, van Leeuwen P, Thijssen JHH. In vitro binding of estrogens by dietary fiber and the vivo apparent digestibility tested in pigs. J Steroid Biochem Mol Biol 38: 621-628 (1991).
10. Shultz TD, Howie BJ. In vitro binding of steroid hormones by natural and purified fibers. Nutr Cancer 8: 141-147 (1986).
11. Mallett AK, Rowland IR. Factors affecting the gut microflora. In: Rowland IR (ed) Role of the gut flora in toxicity and cancer, pp. 347-382. Academic Press, London (1988).
12. Arts CJM, Kemperman PTW, van den Berg H. Oestrogen receptor assay for multi-residue screening of bovine urine for oestrogenic anabolic compounds. Food Add Contam 6: 103-116 (1989).
13. Dixon WJ, Brown MB, Engelman L, Hill MA, Jennrich RI. BMDP Statistical Software Manual. University of California Press, Berkeley, Los Angeles (1988).
14. Kumar D. In vitro inhibitory effect of progesterone on extrauterine human smooth muscle. Am J Obstet Gynecol 84: 1300-1304 (1962).
15. Arts CJM, Wilmer JWGM, de Bie AThHJ, van den Berg H. In vitro synthesis of 16α-hydroxyesterone by female rat liver microsomes: its possible role in the etiology of breast cancer. J Steroid Biochem 36: 527-531 (1990).
16. Goldin BR, Gorbach SL. Effect of diet on the plasma levels, metabolism and excretion of estrogens. Am J Clin Nutr 48: 787-790 (1988).
17. Goldin BR, Adlercreutz H, Gorbach SL, Warram JH, Dwyer JT, Swenson L, Woods MN. Estrogen excretion patterns and plasma levels in vegetarian and omnivorous women. N Engl J Med 307: 1542-1547 (1982).

18. Larsen GL. Deconjugation of biliary metabolites by microfloral β-glucuronidases, sulphatases and cysteine conjugate β-lyases and their subsequent enterohepatic circulation: In: Rowland IR (ed) Role of the gut flora in toxicity and cancer, pp. 347-382. Academic Press, London (1988).
19. Fotherby K. Biosynthesis of the oestrogens: In: Makin HLJ (ed) Biochemistry of steroid hormones, pp. 207-229. Blackwell Scientific Publications, Oxford (1984).
20. Longcope C, Gorbach S, Goldin B, Woods M, Dwyer J, Morrill A, Warram J. The effect of a low fat diet on estrogen metabolism. J Clin Endocrinol Metab. 64: 1246-1250 (1987).
21. Larner JM, Hochberg RB. The clearance and metabolism of estradiol and estradiol-17-esters in the rat. Endocrinology 117: 1209-1214 (1985).
22. Anonymous: Official Methods of Analysis. AOAC., Washington, D.C. (1975) p. 136.

Effects of wheat bran and energy restriction on onset of puberty, cell proliferation and development of mammary tissue in female rats

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Abstract

Delayed onset of puberty and mammary development is supposed to reduce the risk for mammary cancer. An animal experiment was performed to investigate the influence of dietary fiber, which is known to affect hormonal balance, on these characteristics. Fourty-five immature female rats were randomized into 3 groups which were fed ad libitum either a low-fiber diet (< 0.5% dietary fiber based on white wheat flour; $n = 15$), a high-fiber diet (9.2% dietary fiber based on wheat bran; $n = 15$), or an energy-restricted low-fiber diet providing 90% of the energy of the ad libitum low-fiber diet ($n = 15$). Energy intake in the second and third groups was similar. Wheat bran slightly delayed onset of puberty, whereas restricted energy intake delayed onset of puberty by about 6 days. At 48–58 days of age, 14 rats of the low-fiber group, 10 of the high-fiber group and 7 of the restricted group were in cycle. Development of mammary tissue was rudimentary in rats of the energy-restricted low-fiber group, stronger in the ad libitum high-fiber group and strongest in the ad libitum low-fiber group. Cell proliferation in mammary tissue was similar for both groups fed ad libitum, but significantly lower in the restricted group. Peroxidase activity, a biomarker for estrogenicity, was lower in the high-fiber group than in the two low-fiber groups.

It is concluded that wheat bran and, even more effectively, an imposed restricted energy intake delays onset of puberty and mammary development. This shortens the time for mammary cells to be initiated to tumor cells and hence reduces the risk for mammary cancer development. It seems that wheat bran acts via a reduced energy intake. However, a role of wheat bran in the delay of mammary development due to a reduced exposure to estrogen cannot be excluded.

Abbreviations

A-HF, ad libitum high fiber; A-LF, ad libitum low fiber; R-LF, restricted low fiber; TLI, thymidine labeling index; TEB, terminal end bud cells

Introduction

Epidemiological and experimental (animal) studies have indicated that various parameters affect the risk for mammary cancer development: a delayed menarche, early menopause [1], and dietary factors such as an increased fiber intake [2] and restricted energy intake [3].

In rats fed a high-fiber (HF) diet based on wheat bran, mean weight of NMU-induced mammary tumors was lower than in rats fed a low-fiber (LF) diet based on white wheat flour [4]. Estrogen acts on mammary cells as an inducer of cell proliferation at physiological levels [5]. It was postulated that cancer risk is proportional to the number of proliferating cells, which in turn depends on both the number of cells and the rate of cell division in the tissue [6]. To decrease cell proliferation of mammary tissue, exposure to estrogen has to be minimized. One way to decrease estrogen exposure might be adding estrogen-binding compounds, i.e. particular fibers, to the diet [7], which results in an enhanced fecal and a lowered urinary estrogen excretion [4]. Despite increased fecal estrogen excretion, no lowered plasma estrogen levels were observed in rats fed the HF diet [4]. A more sensitive test for the estrogen-mediated effect of fiber on estrogen-sensitive tissue could be found in studying the maturation of rats. Addition of wheat bran to a diet also results in a lowered energy intake and the introduction of lignan precursors. The precursors can be converted by intestinal microflora to lignans, absorbed by the intestinal wall and behave as anti-estrogenic compounds [8, 9]. All of these factors affect the parameters studied.

To investigate specifically the effects of both wheat bran and reduced energy intake, an experiment was designed in which three groups of immature rats were fed either a HF diet, a LF diet, or an energy-restricted LF diet. The parameters studied were onset of puberty, mammary development and cell proliferation of mammary tissue. Exposure of estrogen-sensitive tissue to estrogen was studied by measuring peroxidase activity as biomarker for estrogenicity [10, 11].

Materials and methods

Animals

Immature female Fisher rats (F-344), 24–25 days of age, were obtained from Charles River Wiga GmbH, Sulzfeld, Germany. The second day after arrival the rats were divided into three groups of 15 animals each after computerized randomization to match for initial body weight. The rats were housed individually in suspended hanging-type stainless steel wire-mesh-bottom cages, in an air-controlled room (23 ± 1 °C) with a relative humidity of $50 \pm 10\%$ and a light/dark cycle of 12 h. The animals were weighed weekly.

Diets

One group of rats was fed a high-fiber diet based on wheat bran (A-HF group) and another one a low-fiber diet based on white wheat flour (A-LF group), both given ad libitum. The rats of the third group (R-LF group) were fed a diet accounting for 90% of the amount of food consumed by the animals of the A-LF group on the previous day. The R-LF diet was composed such that the R-LF animals received approximately the same amount of energy as the animals of the A-HF group [12]. Water was supplied ad libitum. Food consumption of the A-LF and R-LF groups was recorded daily and food supply of the R-LF group was adjusted accordingly. Food consumption of the A-HF group was recorded weekly. The diets provided all animals with equal amounts of vitamins, minerals, protein and fat. The lower energy intake of the R-LF group was at the cost of a lower carbohydrate intake relative to the A-LF group. The composition of the diets is summarized in Table 1. Nutrient composition of the diets was analyzed as reported recently [4].

Onset of puberty

Vaginal membrane rupture, which indicates the onset of puberty, was controlled twice daily from 31 days of age. The estrous cycle was determined by vaginal smears, which were taken at 09.00 and 18.00 from 48 days of age. Smears were fixed in methanol for 5 minutes and subsequently stained according to Papanicolaou [13]. Phases of the cycle were recorded as proestrus, estrus, metestrus or diestrus [14].

Table 1. Nutrient composition of the diets (% w/w).

Ingredients	A-HF group	A-LF group	R-LF group
Casein	15.69	22.53	25.70
White wheat flour	42.00	54.72	49.03
Wheat bran	23.75	–	–
Mineral mixture	3.63	4.24	4.74
Vitamin ADEK prep.	0.31	0.36	0.40
Vitamin B mixture	0.20	0.24	0.27
Lard	7.21	8.96	9.95
Sunflower oil	7.21	8.96	9.95
Protein	22.3	25.6	28.1
Fat 16.1	18.5	20.5	
Carbohydrate	39.0	42.8	39.2
Dietary fiber	9.2	< 0.5	< 0.5
Moisture	9.9	9.7	9.2
Ash	4.5	3.9	4.2
Energy (MJ/kg)	164	184	190*

* With a food restriction of 10%, energy intake by animals of the R-LF group will be similar to that of the A-HF group.

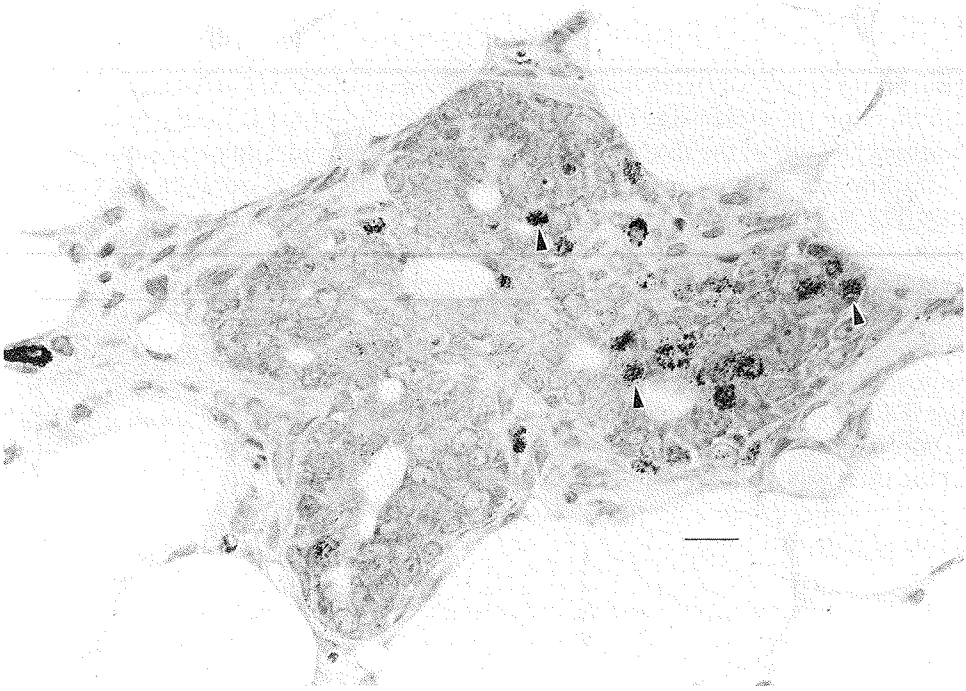

Fig. 1 (top of opposite page). A 5 μm section of a well developed mammary gland showing terminal end bud cells (TEB) of mammary tissue (staining by Toluidine Blue, bar represents 20 μm) of a rat injected with ^{3}H-methyl-thymidine (2 μCi/g body weight; killed 4 h after injection). The arrowheads indicate radioactively labeled nuclei.

Fig. 2 (bottom of opposite page). A 5 μm section of a mammary gland showing ducts (staining by Toluidine Blue, bar represents 50 μm) of a rat injected with ^{3}H-methyl-thymidine (2 μCi/g body weight; killed 4 h after injection). The arrowheads indicate radioactively labeled nuclei.

Cell proliferation

Nine animals of the A-HF group, nine of the A-LF group and seven of the R-LF group, while in metestrus, were injected i.p. with methyl-^{3}H-thymidine (2 μCi/g body weight, spec. act. 40–60 Ci/mmol, Amersham, 's-Hertogenbosch, Netherlands). The animals were killed 4 h after injection by ether anesthesia and aorta bleeding. Both right and left cervical, thoracic, abdominal and inguinal mammary glands, attached to the overlying skin, were dissected. The left part of mammary tissue was used for estimating peroxidase activity and the right part for measuring cell proliferation (thymidine labeling index, TLI).

The right mammary tissue was fixed in 4% aqueous phosphate-buffered formaldehyde solution (pH 7.0) and used for autoradiography. The tissues were dehydrated and embedded in Technovit 7100 plastic (Kulzer, Wehrheim, Germany). The blocks were sectioned semiserially at a thickness of 5 μm. For each block 3 preparations and per preparation 3 sections were obtained. Between sections 15 μm was omitted and of each preparation 50 μm was omitted. The sections were covered with liquid photographic emulsion (Kodak NTB-2, Eastman Kodak, New York) diluted 1:1 with a 2% aqueous glycerol solution. Autoradiograms were exposed in dry, light-tight boxes for 4 weeks at –28 °C, developed in Kodak D19, stained with 0.01% Toluidine Blue and embedded in DePeX mounting medium (BDH, Poole, England). TLI was determined by counting the number of labeled nuclei (indicated by an arrow in Figs. 1 and 2) and the total number of epithelial cells of the terminal end buds (TEB, Fig. 1) and terminal ducts (TD) or alveolar buds (AB, Fig. 2). Nine sections per mammary tissue and per animal were counted. TLI was expressed as the percentage of labeled nuclei in a total of 500 cells. The values obtained for each group were pooled and mean and standard deviation were calculated for each group.

Mammary development

The right part of the mammary tissue from rats of the A-HF group (n = 14), the A-LF group (n = 14), and the R-LF group (n = 12) was also used for establishing mammary development. The right glands from rats not used for measuring cell proliferation were dissected from the skin and fixed in acetone. The fixed tissues were further processed according to the AMEX method [13] and embedded in paraffin. Three 5 μm slides, obtained as described above, of each of the embedded

tissues were stained with hematoxylin and eosin. These slides as well as three slides of tissues of each of the rats used for measuring cell proliferation were used for classifying mammary tissues into two groups according to development of mammary tissue. Based on histological criteria the first group included poorly developed mammary tissue, i.e. individual transverse structures of collecting ducts and TEB. The second group showed moderately and well developed tissue, i.e. a rather large number of collecting ducts and TEB lying close together. The slides were screened blindly two times by two different investigators to obtain reliable scores.

Peroxidase activity in mammary and endometrial cells

The peroxidase (E.C. 1.11.1.7.) activity meant is called 'uterine peroxidase' which is soluble in 0.5 mol/l $CaCl_2$ and is a biomarker for estrogenicity [16]. For clarity sake this enzyme will be called peroxidase hereafter.

The left part of the mammary tissues, adipose tissue included, from the animals used for establishing mammary development was frozen in liquid nitrogen and stored at –80 °C. The left part of mammary tissues from animals injected with labeled thymidine was washed in saline (until the saline was free of radioactivity), and stored at –80 °C. Tissues were weighed, minced with scissors and suspended in 10 mmol/l Tris buffer (final volume 10 ml). The suspension was homogenized with a Potter homogenizer set at 1000 rpm in ice. Five ml of the homogenate was centrifuged for 45 minutes at 35 000 × *g* and 4 °C. The sediment was rehomogenized with 2.5 ml of the peroxidase extraction buffer (10 mmol/l Tris, 0.5 mol/l $CaCl_2$) and centrifuged again (35 000 × *g*, 20 min, 4 °C). The supernatant between pellet and fat layer was used for measuring peroxidase activity.

The entire uterus of the sacrificed rats was quickly dissected. Any adhering mesenteric and adipose tissues were removed and the uterus was chilled in 0.9% NaCl. Each uterine cornu from the oviduct to the cervix was opened longitudinally. Endometrial cells were collected by scraping with bent tweezers over the inner surface of the uterus [17]. The cells were washed by centrifugation and the pellet was suspended in 1 ml of 10 mmol/l Tris buffer pH 7.2 and used for the peroxidase assay (600 µl) and the DNA assay (see below). To 600 µl of the suspension 3 ml of peroxidase extraction buffer (final $CaCl_2$ concentration 0.5 mol/l) was added and peroxidase activity was measured according to Lyttle and Desombre [10] with some modifications. 3,3',5,5'-tetramethyl-benzidine (TMB, Sigma Chemicals, St.Louis, MO) was used as substrate and horseradish peroxidase (HRP, Serva, Heidelberg/New York) as standard. Tubes with samples and standards were incubated until the lowest standard points colored light blue. The reaction was stopped with 500 µl of 2 mol/l HCl. To remove denatured proteins tubes were centrifuged (15 min, 35000 × *g*, 4 °C). In the supernatants the intensity of the color was measured at 452 nm. Peroxidase activity was expressed per µg DNA as units of 'apparent HRP' equaling an absorption unit of color formation per minute.

DNA analysis

DNA was measured according to Labarca and Paigen [18] based on the enhancement of fluorescence seen when bisbenz-imidazole (Hoechst 33258) binds to DNA. Endometrial cells suspended in 10 mmol/l Tris buffer or an aliquot of the mammary tissue homogenate in Tris buffer were diluted in 0.05 mol/l sodium phosphate pH 7.4 containing 2.0 mol/l NaCl. Cells were homogenized in a Potter homogenizer. To 500 µl of the homogenate 1 ml buffer containing 1.5 µg Hoechst 33258 was added. After vortexing fluorescence was measured with a fluorometer (Perkin Elmer 204-A fluorescence spectrophotometer) with the excitation and emission wavelengths adjusted to 356 nm and 458 nm, respectively. The emission units were read on a standard curve for DNA calf thymus (type I, art. D1501; Sigma, St. Louis, MO) with a range of 20–2500 ng/tube.

Statistics

Differences in mean energy intake, body weight, onset of puberty, cell proliferation and peroxidase activity were tested using Student's t-test. Differences in development of mammary tissue and estrous cycle were estimated by Pearson's χ^2 test with the BMDP program 4F [19]. A difference at $P < 0.05$ was considered statistically significant.

Results

Energy intake and body weights

Initial mean weight of the rats in each of the groups was about 44 g. From the start of the experiment until the age of 48 days, energy intake in the A-HF and R-LF groups was similar, but significantly lower than in the A-LF group (Table 2). Although energy intake was similar, mean body weight of the R-LF group was slightly, but

Table 2. Mean food intake (g/day per rat) and energy intake (kJ/day per rat) by female rats ($n = 15$ per group) fed a HF or a LF diet ad libitum or an energy-restricted LF diet at 27 to 48 days of age.

Group	27–34 days (Week 1)		34–41 days (Week 2)		41–48 days (Week 3)	
	food	energy	food	energy	food	energy
A-LF	8.2 ± 0.9	151 ± 15.9[b]	9.6 ± 1.0	176 ± 18.8[b]	10.2 ± 1.2	187 ± 22.2[b]
A-HF	6.7 ± 1.1	109 ± 17.5[a]	9.6 ± 0.6	157 ± 9.4[a]	9.5 ± 0.7	155 ± 10.8[a]
R-LF	5.8 ± 0.2	110 ± 3.8[a]	8.4 ± 0.1	160 ± 1.3[a]	8.4 ± 0.3	159 ± 5.8[a]

Mean ± SD indicated with different superscripts in one column indicate a significant difference ($P < 0.05$).

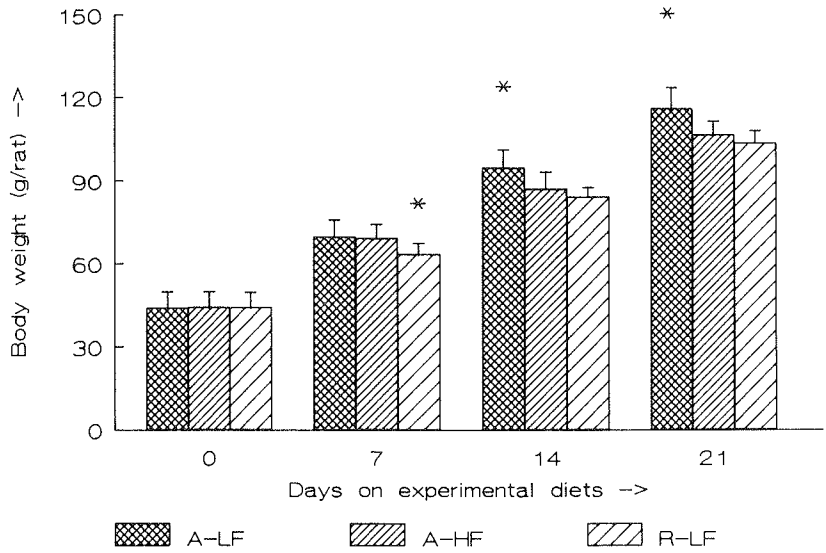

Fig. 3. Mean body weight with SD of rats fed ad libitum a low-fiber (A-LF) or a high-fiber diet (A-HF) or an energy-restricted low-fiber diet (R-LF). The asterisk indicates that mean body weight is significantly ($P < 0.05$) different from the other groups.

significantly, lower than in the A-HF group after the first week (Fig. 3). The higher energy intake by rats of the A-LF group resulted in a significantly higher mean body weight after the first week (compared with the R-LF group) and after the second week of the experiment (compared with both other groups).

Onset of puberty

The A-HF diet slightly delayed and the R-LF diet delayed vaginal opening significantly relative to the A-LF diet (Table 3).

Table 3. Age (mean ± SD in days) at onset of puberty and of being in estrous cycle of groups of rats (n = 15 per group) fed an A-HF, an A-LF or a R-LF diet.

Group	Mean age (days) at vaginal membrane rupture	Estrous cycle of animals at 48–58 days of age	
		in cycle	not in cycle
A-LF	34.2 ± 2.7	14	1
A-HF	37.0 ± 4.7 n.s.	10	5 n.s.
R-LF	40.7 ± 6.0**	7	8*

* $P < 0.05$; ** $P < 0.001$; n.s. = not significant, relative to the A-LF group.

Vaginal smears taken to assess the phase of the cycle indicated that, at 48–58 days of age, 14 rats out of 15 in the A-LF group had an estrous cycle, whereas just 10 animals of the A-HF group and 7 animals of the R-LF group were in cycle (Table 3).

Cell proliferation

With the procedure described, in only 2 out of 7 rats of the R-LF group mammary tissue had sufficiently developed to establish cell proliferation in TEB cells, while ducts could not be observed in 1 out of 7 slides prepared from the mammary tissues of these rats. In the A-HF group, 4 out of 9 tissues had sufficiently developed to show TEB and in 7 out of 9 to show ducts. In the A-LF group, in 8 out of 9 slides TEB and ducts could be observed. TLI (number of labeled cells in a total of 500 cells) in the TEB and ducts in mammary tissue did not differ between the A-HF and A-LF groups (Table 4). Cell proliferation in TEB from the R-LF group, but not in ducts, was significantly lower than in the two other groups.

Mammary development

Development of mammary tissue was significantly affected by the diet (Table 5). In rats fed the R-LF diet, mammary tissue showed a poor development in 9 out of 12 rats examined. Mammary tissue of the A-LF group was classified as moderately or well developed in 13 out of 14 rats. Mammary development in the A-HF group was intermediate: 6 animals showed a poor development and 8 animals showed a moderate or well development. A typical example of a well and a poorly developed mammary gland used for the quantitation is shown in Figs. 1 and 4, respectively.

Table 4. Cell proliferation (TLI = % of labeled cells in a total of 500 cells; mean ± SD) of TEB[1] and ducts[2] of mammary tissue from rats fed the A-HF wheat bran, the A-LF wheat flour diet or the R-LF diet.

	TEB	*n*[3]	Ducts	*n*
A-LF	13.4 ± 7.6^{a}	8/9	0.4 ± 0.2^{a}	8/9
A-HF	13.7 ± 5.4^{a}	4/9	0.4 ± 0.4^{a}	7/9
R-LF	4.2 ± 2.3^{b}	2/7	0.5 ± 0.4^{a}	6/7

[1] TEB = terminal end bud cells.
[2] Ducts = epithelial cells of the ducts.
[3] Number of animals in which mammary tissue was sufficiently present to calculate TLI (500 mammary tissue cells per animal) versus the number of rats injected with labeled thymidine when in metestrus or in the first day of diestrus.
Mean ± SD indicated with different superscripts in one column indicate a significant difference ($P < 0.05$).

Table 5. Development of mammary tissue in rats fed an A-HF, an A-LF or an R-LF diet.

Group	Poor[1]	Good[2]	Total	Significance of difference[3]
A-LF	1	13	14	–
A-HF	6	8	14	$P < 0.05$
R-LF	9	3	12	$P < 0.001$

[1] Poor: few or no collecting ducts and/or TEB cells present in the slides.
[2] Good: a sufficient number of islands of collecting tubes and TEB cells in the slides.
[3] Significance of difference from the A-LF group calculated according to Pearson's χ^2 test.

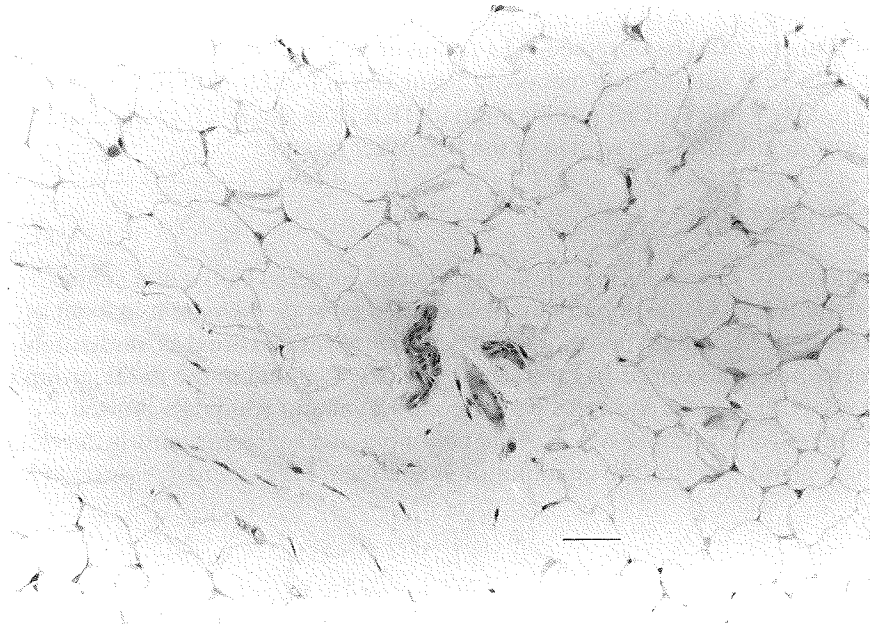

Fig. 4. A 5 μm section of a poorly developed mammary gland (staining by Toluidine Blue, bar represents 20 μm).

Peroxidase activity in mammary tissue and endometrial cells

Peroxidase activity in mammary tissue and endometrial cells in the three groups is shown in Table 6. A significantly lower peroxidase activity was found in mammary tissue from rats fed the A-HF diet than in mammary tissue from the A-LF and R-LF groups.

Peroxidase activity in endometrial cells was found to be 100-fold higher than in mammary tissue. Differences in activity in endometrial cells were not observed among the groups (rats in metestrus). In a pilot experiment, using female rats fed the

Table 6. Uterine peroxidase activity (mU/μg DNA) in mammary tissue and endometrial cells from rats fed the A-HF, the A-LF or the R-LF diets. Rats were in metestrus at the time of section.

Group	*n*	Mammary tissue	Endometrial cells
A-LF	10	4.7 ± 3.0^{b}	480 ± 391^{a}
A-HF	9	2.6 ± 0.6^{a}	535 ± 369^{a}
R-LF	7	4.0 ± 1.3^{b}	435 ± 366^{a}

Mean ± SD indicated with different superscripts in one column indicate a significant difference ($p < 0.05$).

LF diet ad libitum from 27 days to about 50 days of age, peroxidase activity was found to be 2–3-fold higher in endometrial cells from rats in proestrus (about 1400 mU/μg DNA) than in rats in metestrus (about 500 mU/μg DNA).

Discussion

Our experiments show clearly that both dietary fiber and energy restriction affect hormonal processes which finds expression in a delayed vaginal membrane rupture, i.e. a delayed puberty, a delayed onset of the estrous cycle and poor mammary development. An important consequence of ad libitum dietary fiber intake seems to be a reduced energy intake. An imposed lowered energy intake seems to have a still stronger effect, energy intake being similar for the A-HF and R-LF groups. A 'stress factor' evoked by the absence of sufficient food might play a crucial role in hormonal processes. However, it cannot be excluded that lignans introduced in the body by wheat bran [8] have a low estrogenic activity which compensates for the effects of lower energy intake [9, 20].

In the A-HF and R-LF groups energy intake was similar from the start of the experiment until the age of 48 days. However, a restricted diet has a direct effect on body weight; already after the first week mean body weight in the R-LF group was significantly lower than in the A-LF group. The A-HF diet has a delayed effect on body weight; mean body weight of the A-HF was similar to the A-LF group after the first week but lower after the second week (Fig. 3).

An A-HF diet and, even more effectively, a R-LF diet delays vaginal membrane rupture, i.e. onset of puberty. Eckstein et al. [21], using descendants of Wistar or Charles River rats, showed vaginal opening to occur at 35.6 days, which is in good agreement with our findings. We started measuring the estrous cycle when rats were 48 days old, at which time almost all animals of the A-LF group were in cycle. In the A-HF group, and in particular in the R-LF group, more animals were in an irregular or non-cyclic state indicating that wheat bran and, even more effectively, an imposed reduced energy intake affect those hormonal processes which regulate the estrous cycle. The amenorrheal state or delayed menarche has also been noticed in girls who sport intensively [22] or have a high intake of dietary fiber [23–25].

A clear effect of dietary fiber and restricted energy intake is the delay in mammary development (Table 5). This finding corroborates the result of human studies in which dietary intake based on cereal fiber, was related to breast development in girls aged 9 to 13 [24].

Cell proliferation of the epithelial cells in mammary tissue was not found to differ between the two groups fed ad libitum. This is in agreement with our previous finding that the incidence of NMU-induced mammary tumors was similar in two groups fed a HF or LF diet [4]. It has been suggested that mammary tumor induction by a carcinogen depends principally on the frequency of mammary cell division at the time a carcinogen acts on the gland [26]. In the energy-restricted rats cell proliferation was significantly lower. Lok et al. [27] also found a strong effect of energy restriction on proliferation of mammary cells of female mice, but this effect became only manifest when restriction was as large as 20%. Peroxidase activity has been suggested to be a measure of estrogen exposure [10]. In mammary tissue of the A-HF group a significantly lower peroxidase activity was measured than in tissue of the two low-fiber groups. This suggests that estrogen exposure in mammary tissue of the A-HF animals (when in metestrus) is lower, which is in agreement with earlier studies in which we found a 3–4-fold higher fecal estrogen excretion and a lower urinary estrogen excretion in rats fed the HF diet [4]. However, a lowered estrogen exposure should result in a lowered epithelial cell proliferation as well, which was not observed (Table 5). As mammary development is under specific control of oestradiol-17β [28], the poor mammary development in the A-HF and R-LF groups relative to the A-LF group can thus be explained by the lowered estrogen exposure. In the A-HF group this can be explained by the increased fecal estrogen excretion [4], which is still not compensated by a higher estrogen production or, as in the R-LF group, by a lower energy intake. A lower energy intake might inhibit gonadotropin (LH and FSH) secretion and thus decrease estrogen production [29].

The reduced risk for breast cancer in vegetarian women [30] or in women on a fiber-rich diet [2] might be explained as presented schematically in Fig. 5. According to the unifying hypothesis of de Waard and Trichopoulos [31], breast cancer is induced during puberty and adolescence. Only undifferentiated epithelial mammary cells (TEB cells) are believed to be susceptible to carcinogenic compounds [32]. Wheat bran, but also a restricted energy intake, shortens the time between onset of mammary development and fully differentiated mammary tissue, i.e. time after pregnancy. Thus the period during which mammary cells can be initiated to tumor cells is shortened. The presence of about 24% wheat bran (ca. 9.5% dietary fiber) in the diet results in a reduced energy intake of about 10% [4]. An energy restriction of 10% affects tumor promotion (reduction of tumor multiplicity and tumor burden) as reviewed previously [33]. Furthermore, it is known that wheat bran affects estrogen excretion [4, 34] and hence lowers exposure of mammary tissue to estrogen (Table 6). In the phase of mammary development at an age of about 50 days, the lowered estrogen exposure appears not to affect cell proliferation (Table 4). However, it cannot be excluded that mammary tumors are more sensitive to lower

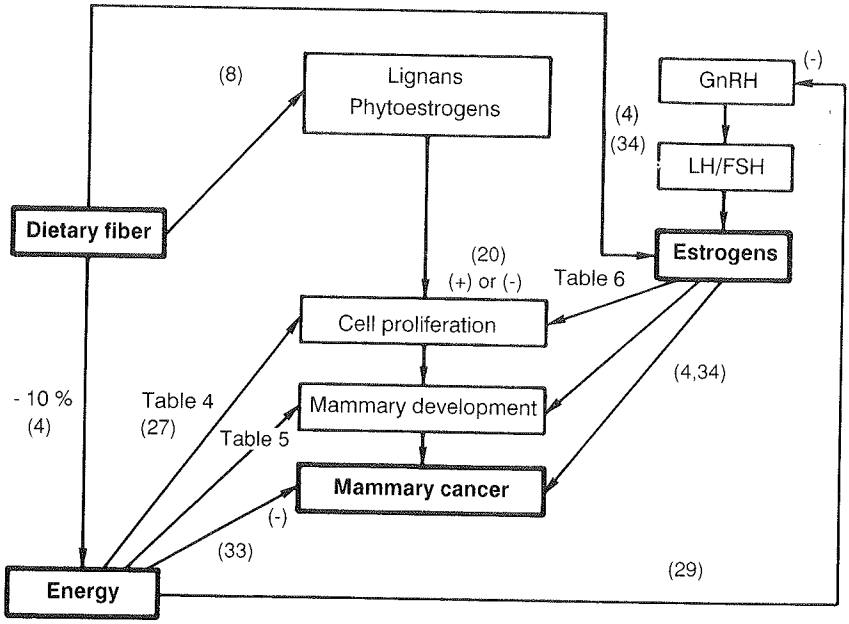

Fig. 5. Schematic presentation of the hypothesized pathway for the effects of dietary fiber and/or energy on mammary cancer risk. The numbers in parenthesis refer to the References. The (+) or (−) effects of lignans depend on the presence of endogenous estrogens. An explanation of this figure is given in the text. GnRH is gonadotropin-releasing hormone.

estrogen exposure resulting in a reduced tumor weight as found previously [4, 33]. A restricted energy intake, rather than an iso-energetic wheat bran diet, affects the parameters studied, i.e. onset of puberty, estrous cycle and mammary development. This might be explained by a stress factor, as mentioned before, or by the fact that wheat bran contains certain precursors, which can be converted into lignans [9]. In the absence of endogenous estrogens in the prepubertal period these lignans might exert estrogenic activity which compensates for the reduced energy intake [20]. However, when endogenous estrogens are present, as in the case of puberty and adolescence, the lignans will have anti-estrogenic activity [9], which might reduce the (effective) biologic activity of the estrogens, as discussed previously [4]. Another explanation is that a reduced energy intake inhibits gonadotropin secretion [29]. A lowered LH secretion might affect the estrous cycle and, due to a lowered estrogen production, delay mammary development.

References

1. Vihko R, Apter D. Endogenous steroids in the pathophysiology of breast cancer. Crit Rev Oncol/Hemat 9: 1-16 (1989).
2. van 't Veer P, Kolb CM, Verhoef P, Kok FJ, Schouten EG, Hermus RJJ, Sturmans F. Dietary fiber, beta-carotene and breast cancer: results from a case-control study. Int J Cancer 45: 825-828 (1990).
3. Sinha DK, Gebhard RL, Pazik JE. Inhibition of mammary carcinogenesis in rats by dietary restriction. Cancer Lett 40: 133-141 (1988).
4. Arts CJM, de Bie AThHJ, van den Berg H, van 't Veer P, Bunnik GSJ. Influence of wheat bran on NMU-induced mammary tumor development, plasma estrogen levels and estrogen excretion in female rats. J Steroid Biochem Mol Biol 39: 193-202 (1991).
5. Nicholson RI, Gotting KE, Gee J, Walker KJ. Actions of oestrogens and antioestrogens on rat mammary gland development: relevance to breast cancer prevention. J Steroid Biochem 30: 95-103 (1988).
6. Albanes D, Winick M. Are cell number and cell proliferation risk factors for cancer? J Natl Cancer Inst 80: 772-775 (1988).
7. Arts CJM, Govers CARL, van den Berg H, Wolters MGE, Van Leeuwen P, Thijssen JHH. In vitro binding of estrogens by dietary fiber and the in vivo apparent digestibility tested in pigs. J Steroid Biochem Mol Biol 38: 621-628 (1991).
8. Thompson LU, Robb P, Serraine M, Cheung F. Mammalian lignan production from various foods. Nutr Cancer 16: 43-52 (1991).
9. Adlercreutz H, Mousavi Y, Loukovaara M, Hämäläinen E. Lignans, isoflavones, sex hormone metabolism and breast cancer. In: Hochberg RB, Naftoli F (eds). The new biology of steroid hormones, pp. 145-154. Serono Symposia Publications 74. Raven Press, New York (1991).
10. Lyttle CR, Desombre ER. Uterine peroxidase as a marker for estrogen action. Proc Natl Acad Sci USA 74: 3162-3166 (1977).
11. Levy J, Liel Y, Glick SM. Peroxidase activity as a marker for estrogenicity: studies in uterus and mammary tumors. Isr J Med Sci 17: 970-975 (1981).
12. Atwater WO, Bryant AP. The availability and fuel value of food materials. Rep Storrs Agric Exp Stn 1899: 73-110 (1900).
13. Papanicolaou GN. A new procedure for staining vaginal smears. Science 95: 438-443 (1942).
14. Yoshinaga K, Hawkins RA, Stocker JF. Estrogen secretion by the rat ovary in vivo during the estrous cycle and pregnancy. Endocrinology 85: 103-112 (1969).
15. Sato Y, Mukai K, Watanabe S, Goto M, Shimosato Y. The AMEX method. Am J Path 125: 431-435 (1986).
16. Bhattacharyya J, Datta AG. Uterine peroxidase: properties and possible functions. J Sci Ind Res 46:535-540 (1987).
17. Bitton-Casimiri V, Rath NC, Psychoyos A. A simple method for separation and culture of rat uterine epithelial cells. J Endocr 73: 537-538 (1977).
18. Labarca C, Paigen K: A simple, rapid and sensitive DNA assay procedure. Anal Biochem 102: 344-352 (1980).
19. Dixon WJ, Brown MB, Engelman L, Hill MA, Jennrich RI. BMDP Statistical Software Manual. University of California Press, Berkeley (1988).
20. Mäkelä S, Pylkkänen L, Santti R, Adlercreutz H. Role of plant estrogens in normal and estrogen-related altered growth of the mouse prostate. In: Proceedings of the Interdisciplinary Conference on Effects of food on the immune and hormonal systems. Euro Food Tox III, Zurich, Switserland, May 23-24, pp. 135-139 (1991).
21. Eckstein B, Golan R, Shani J. Onset of puberty in the immature female rat induced by 5α-androstane-3β,17β-diol. Endocrinology 92: 941-946 (1973).
22. Frisch RE, Wyshak G, Vincent L. Delayed menarche and amenorrhea in ballet dancers. N Engl J Med 303: 17-19 (1980).
23. Pedersen AB, Bartholomew MJ, Dolence LA, Aljadir LP, Netteburg KL, Lloyd T. Menstrual differences due to vegetarian and nonvegetarian diets. Am J Clin Nutr 53: 879-885 (1991).

24. de Ridder CM, Thijssen JHH, van 't Veer P, van Duuren R, Bruning PF, Zonderland ML, Erich WBM. Dietary habits, sexual maturation, and plasma hormones in pubertal girls: a longitudinal study. Am J Clin Nutr 54: 805-814 (1991).
25. Hughes RE, Jones E. Intake of dietary fiber and the age of menarche. Ann Hum Biol 12: 325-332 (1985).
26. Nagasawa H, Yanai R. Frequency of mammary cell division in relation to age: its significance in the induction of mammary tumors by carcinogen in rats. J Natl Cancer Inst 52:609-610 (1974).
27. Lok E, Scott FW, Mongeau R, Nera EA, Malcolm S, Clayson DB. Calorie restriction ans cellular proliferation in various tissues of the female Swiss Webster mouse. Cancer Lett 51: 67-73 (1990).
28. Nicholson RI, Gotting KE, Gee J, Walker KJ. Actions of oestrogens and antioestrogens on rat mammary gland development: relevance to breast cancer prevention. J Steroid Biochem 30: 95-103 (1988).
29. Dubey AK, Cameron JL, Steiner RA, Plant TM. Inhibition of gonadotropin secretion in castrated male rhesus monkeys (*Macaca mulatta*) induced by dietary restriction: analogy with the prepubertal hiatus of gonadotropin release. Endocrinology 118: 518-525 (1986).
30. Barbosa JC, Shultz TD, Filley SJ, Nieman DC. The relationship among adiposity, diet, and hormone concentrations in vegetarian and non-vegetarian postmenopausal women. Am J Clin Nutr 51: 798-803 (1990).
31. de Waard F, Trichopoulos D. A unifying concept of the aetiology of breast cancer. Int J Cancer 41: 666-669 (1988).
32. Russo J, Wilgus G, Russo IH. Susceptibility of the mammary gland to carcinogenesis. Am J Path 96: 721-736 (1979).
33. Kritchevsky D. Fat, calories and cancer. Front Gastrointest Res 14: 188-198 (1988).
34. Adlercreutz H. Diet and sex hormone metabolism. In: Rowland IR (ed). Nutrition, toxicity and cancer, pp. 137-195. CRC Press, London (1991).

Effects of wheat bran on blood and tissue hormone levels in adult female rats

Cor J.M. Arts and Jos H.H. Thijssen

Abstract

The protective effect of dietary fiber on breast cancer development might be explained by the interaction between dietary fiber and hormonal processes. We studied the effects of dietary fiber and the effects of a reduced energy intake on exposure of mammary tissue to both estrogens and progesterone as well as blood plasma levels of these steroids. Adult female Fisher rats were fed ad libitum either a low-fiber diet (0.5% dietary fiber based on wheat flour) or a high-fiber diet (9.2% dietary fiber based on wheat bran). A third group was used to control for the reduced energy intake of the high-fiber group and was fed the low-fiber diet restricted. Energy intake was similar for the second and third group. Four out of 14 rats of the high-fiber group and 4 out of 15 rats of the restricted low-fiber groups were not in cycle after 7 weeks on the experimental diets, whereas rats of the ad libitum low-fiber group were all in cycle. This indicated that the estrous cycle was significantly affected by a reduced energy intake. Exposure of mammary tissue to estrogens did not differ among the groups as measured by estrone, estradiol-17β, estriol and peroxidase activity. During the peak period plasma LH levels tended to be higher in the high-fiber group than in the two low-fiber groups. FSH levels were not affected by the experimental diets. Progesterone plasma levels were clearly affected by dietary fiber during the basal period of the cycle. It is concluded that dietary fiber affects hormonal processes involved in breast cancer development. The lower plasma progesterone levels can be an important element in the discussion on the mechanism underlying the inverse relation between dietary fiber and breast cancer risk.

Abbreviations

A-HF, ad libitum high fiber; A-LF, ad libitum low fiber; R-LF, restricted low fiber; E_1, estrone; E_2, estradiol-17β; E_3, estriol; Pg, progesterone; DNA, deoxyribonucleic acid; LH, luteinizing hormone; FSH, follicle-stimulating hormone.

Introduction

In various studies an inverse relation has been observed between dietary fiber intake and breast or mammary cancer. A lower incidence of breast cancer was observed in vegetarian postmenopausal women [1], and a reduced risk in women with a relatively high intake of dietary fiber [2]. In two lifespan studies [3, 4], mammary cancer incidence was significantly lower in rats fed a high-fiber (HF) diet than in those on a low-fiber (LF) diet. Moreover, NMU-induced mammary tumors in rats fed a HF diet weighed significantly less than those in rats fed a LF diet [5]. Estrogens have been reported to play a crucial role in mammary carcinogenesis. An increased exposure of the estrogen-sensitive tissue to estrogens would result in a higher risk of mammary cancer at the initiation or promotion phase [6]. In a previous study [5], a clear interruption of the enterohepatic circulation of estrogens was observed resulting in a 3–4-fold higher fecal estrogen excretion in rats fed the HF diet than in those fed the LF diet, contrary to urinary estrone (E_1) excretion which was lower ($P < 0.05$) in the HF group. In spite of the increased fecal estrogen excretion, plasma estradiol-17β (E_2) levels were similar in both groups. This latter finding may be explained by a negative feedback mechanism, i.e. an increased estrogen production induced by a higher gonadotropin release by the pituitary in response to a fiber-induced negative estrogen balance.

Interpretation of data from studies on fiber and cancer is complicated by the fact that changing the fiber content of the diet affects both food intake and energy intake. Both reduced energy intake [7] and fiber (e.g. through interruption of the hormonal enterohepatic circulation) might affect mammary carcinogenesis. To differentiate between a fiber-induced and an energy-induced effect on estrogen balance we compared the estrus-related changes in blood plasma (LH, FSH, E_2 and Pg levels) between rats fed ad libitum a high-fiber (A-HF) or a low-fiber (A-LF) diet and introduced a third, 'restricted LF' (R-LF), group which was fed a restricted amount of food such that energy intake was similar for the R-LF and A-HF groups. Exposure of estrogen-sensitive tissues to estrogens and Pg was established by measuring the estrogen and Pg contents in mammary tissues. Peroxidase activity, a biomarker for exposure of tissue to estrogens [8, 9], was measured in mammary tissue and endometrial cells.

Materials and methods

Animals

Immature female Fisher rats (F-344), 26–27 days of age, were obtained from Charles River Wiga GmbH, Sulzfeld, Germany. The second day after arrival the rats were

housed in 9 groups of 5 animals each in suspended hanging-type stainless steel wire-mesh-bottom cages, in an air-controlled room (23 ± 1 °C) with a relative humidity of 50 ± 10 % and a light/dark cycle of 12 h.

At 98 days of age the population was divided into 3 groups of 15 animals each after computerized randomization to match for initial body weights. Then the rats were housed individually in smaller cages as previously and in the same room. The animals were weighed weekly.

Diets

Until 98 days of age all rats were fed ad libitum the low-fiber diet (A-LF). Thereafter one group of rats remained on the A-LF diet. A second group of rats was fed ad libitum a high-fiber diet based on wheat bran (A-HF group). The rats of the third group (R-LF group) received 90% of the amount of food consumed by the animals of the A-LF group on the previous day resulting in a similar energy intake for the A-HF and R-LF groups. This percentage of 90 was based upon findings of a previous study [5] in which a 10% lower energy intake was observed in the A-HF group relative to the A-LF group. Food consumption of the A-LF and R-LF groups was recorded daily. Food consumption of the A-HF group was recorded weekly. The diets, the energy value of which was calculated according to Atwater [10], were composed such that all animals were provided with equal amounts of vitamins, minerals, protein and fat. The lower energy intake of the R-LF group was at the cost of a lower carbohydrate intake relative to the diet of the A-LF group. The composition of the diets was similar to that reported previously [11]. Water was supplied ad libitum.

Experimental design

When animals had been fed the experimental diets for 5 weeks (age of the rats about 135 days), vaginal smears were taken daily on 3 successive days to assess the phase of the estrous cycle. Smears were fixed in methanol for 5 minutes and subsequently stained according to Papanicolaou [12]. The phase of the cycle was recorded as proestrus, estrus, metestrus or diestrus [13]. All rats were cannulated via the vena jugularis externa during the 7th week. One animal of the A-HF group died during cannulation. On 5 successive days in the 8th week of the experiment, 1.5 ml blood was collected in heparinized tubes. Just before blood collection, vaginal smears were taken to record the phase of the estrous cycle. During the following two weeks some animals were killed because of a moribund condition. The healthy rats, when in metestrus or on the first day of diestrus, were used for measuring estrogen and progesterone (Pg) contents in mammary tissue. Section of the rats and collection of the tissues was performed as described previously [11]. Uterine peroxidase activity (expressed as units per g DNA) was measured in mammary tissue and uterine epithelial cells as biomarker for exposure to estrogens.

Analysis of estradiol, progesterone and gonadotropins in blood plasma

E_2 was measured with a radioimmuno assay (RIA) [14] after previous extraction of 500 µl of plasma with Extrelut (Merck, Darmstadt, Germany) using a specific E_2-17β antiserum raised in rabbit against estradiol-17β-6-CMO-BSA. This antiserum showed 0.7% cross-reactivity with E_1 and 1.5% with E_3; cross-reactivity with other related compounds was $< 0.001\%$. Coefficients of variation (CV) of control samples were $< 10\%$ both within and between the tests at about 40 pg/ml. Recovery of spiked samples was between 90 and 110%.

Pg was measured with RIA [14] after previous extraction of 100 µl of plasma with ethylene glycol-coated Chromosorb (Chrompack, Middelburg, Netherlands). Pg antiserum used was raised in rabbit against progesterone-11α-hydroxyhemisuccinate-BSA. It showed cross-reactivity with 17-OH-progesterone (0.9%), cortisol (2.2%), corticosterone (4.5%), deoxycorticosterone (4%) and pregnenolone (4%). Validation tests showed a recovery of spiked plasma samples between 90 and 110%, and the CV within and between the tests of control samples was $< 10\%$.

The gonadotropins LH and FSH were analyzed directly using a RIA as reported recently [15].

Analysis of estrone, estradiol, estriol and progesterone in mammary tissue

The left part of the mammary gland (0.5–2.8 g) was used for steroid analysis. Extraction of the steroids from the tissue and further purification of the extract were performed as described previously [16] with some minor modifications. Extraction was performed with ethanol/acetone (1:1, v/v) for 10 min while vortexing at regular intervals. Fat was removed with 70% methanol at –20 °C for 1 h (2 times). Steroids were extracted twice with 5 ml of distilled diethyl ether, evaporated to dryness, dissolved in 2 ml of 20% methanol and transferred onto activated reversed-phase C18 cartridges (J.T. Baker Chemicals, Deventer, Netherlands). The cartridges were successively washed with water and 50% methanol, and steroids were eluted with 100% methanol. The methanol was evaporated and the steroids were dissolved in 1 ml of ethanol for measurement of the recovery and for estimation of Pg, E_1, E_2 and E_3. The RIAs were performed [14] using antisera against E_2 and Pg (see above). E_1 and E_3 antisera were raised in rabbit against estrone-6-CMO-BSA and estriol-6-CMO-BSA, respectively. Cross-reactivities for E_1 antiserum were 0.4% with E_2 and 0.1% with E_3. For E_3 antiserum cross-reactivities were $< 0.01\%$ with E_1 and 0.05% with E_2. Loss of steroids during extraction and clean-up procedure was assumed to be equal for all steroids estimated and was calculated after measuring the recovery of ^{3}H-estradiol for each sample separately. Recovery was $77 \pm 9.7\%$ (mean $\pm$ SD; $n = 37$). For each steroid, all analyses were conducted in a single run, with a CV of $< 5\%$ for

Table 1. The estrous cycle divided into a peak period (x) and a basal period (o) for each hormone analyzed. [1]

	M	D_1	D_2	P	O
E_2	o	o	x	x	o
LH	o	x	x	o	o
Pg	x	x	o	o	o
FSH	o	o	o	o	x

[1] M, metestrus; D_1, day 1 of diestrus; D_2, day 2 of diestrus; P, proestrus; O, estrus.

all steroids in the range of 20–80% B/Bo of the standard curves.

For each compound analyzed, the data obtained for each cycle were combined for the basal and peak period, as was done for E_2 in the previous report [5]. The peak and basal periods for each compound measured are given in Table 1.

DNA analysis

DNA was measured according to Labarca and Paigen [17] on the basis of the enhancement of fluorescence seen when bisbenz-imidazole (Hoechst 33258) binds to DNA. Analysis of DNA in mammary tissue and endometrial cells was performed as described previously [11].

Peroxidase activity in mammary tissue and endometrial cells

The right part of the mammary gland was used for estimating peroxidase activity, which was performed as described previously [11].

Statistics

Differences in energy intake, body weight and hormone concentrations in blood plasma (sampled during the estrous cycle) between the groups of rats were analyzed with Student's t-test. Differences in steroid concentrations in plasma and tissue as well as differences in peroxidase activity between the groups were estimated using one way analysis of variance with the BMDP program 7D [18]. Differences in estrous cycle were estimated using the Pearson's χ^2 test with the BMDP program 4F [18]. A difference at $P < 0.05$ was considered statistically significant.

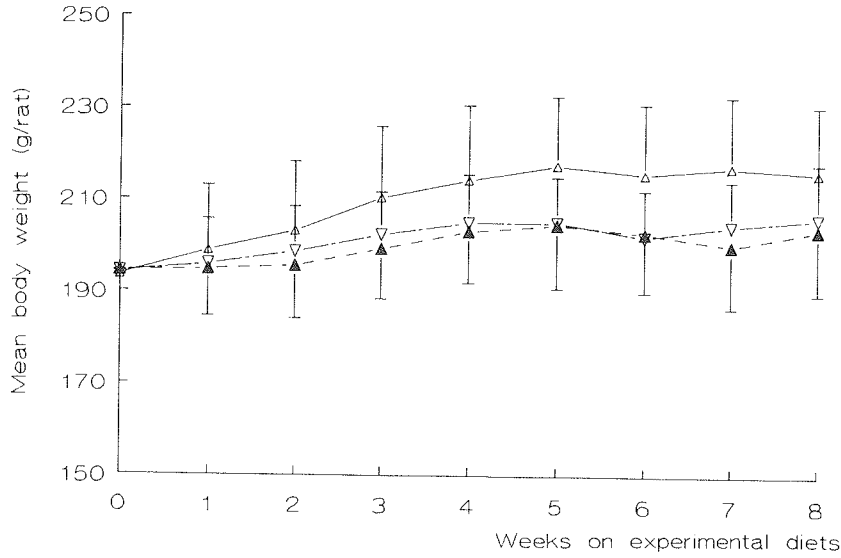

Fig. 1. Body weight (mean ± SD) of the rats fed ad libitum the HF diet (black triangles) or LF diet (small open triangles) or the LF diet restricted (large open triangles).

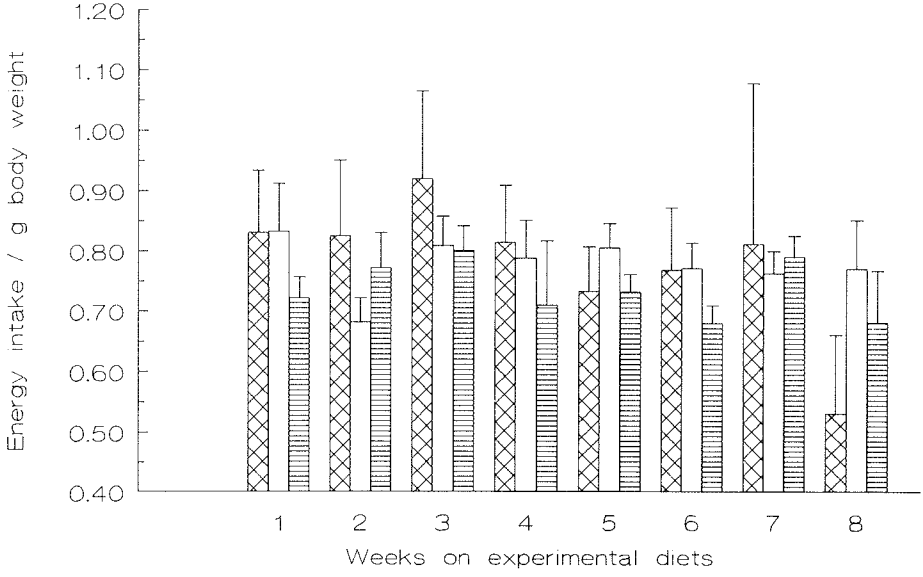

Fig. 2. Energy intake (kJ/day) relative to body weight (g/rat) of rats fed ad libitum the LF diet (cross-hatched columns) or HF diet (white columns) or the LF diet restricted (shaded columns). Each mark indicates mean ± SD.

Results

Body weight and energy intake

Until the start of the experiment, at 98 days of age, all rats received the LF diet ad libitum. Mean weight at the start of the experiment was 194 g for all 3 groups. During the experimental period weight gain was similar for the A-HF and R-LF groups, but significantly higher for the A-LF group (Fig. 1). At the beginning of the experiment, when the rats were switched from the A-LF to the A-HF and R-LF diets, energy intake of the latter two groups decreased. In the R-LF group a low energy intake was observed already in the first week, while in the A-HF group a lower energy intake was found in the second week of the experiment, suggesting that fiber acts more gradually than energy restriction. From the third week energy intake relative to body weight was similar among the groups. In week 8, when blood was sampled daily, energy intake of the A-LF group was lower than during the previous period (Fig. 2).

Estrous cycle

Rats of the A-LF group that received this diet from an immature age (27 days of age) until the end of the experiment (154 days of age) were all in cycle. According to the vaginal smears and plasma estradiol levels, 4 out of 14 rats of the A-HF group and 4 out of 15 rats of the R-LF group were not in cycle after 7 weeks on the experimental diets ($P < 0.05$ relative to the A-LF group).

E_2, Pg and gonadotropin contents in blood plasma

The mean concentrations of E_2 and Pg as well as of the gonadotropins (LH and FSH) in blood plasma sampled daily during one complete estrous cycle period are given in Table 2. For each hormone a particular period within the cycle was considered to reflect the basal period (i.e. relatively low concentrations in plasma for that analyte), whereas the other period within the cycle was considered to reflect the peak period, indicating relatively high levels for the compound analyzed. The results obtained from animals which were not in cycle were considered as basal values when no peak values were measured.

The basal period for E_2 was during estrus, metestrus and the day 1 of diestrus (OMD_1). The detection limit of the E_2 RIA was calculated to be 2 pg/ml (equal to 90% B/Bo, i.e. about 0.5 pg/tube). For statistical analyses, E_2 plasma levels below the detection limit were taken to be 1 pg/ml. Although the mean plasma E_2 in the A-LF group was significantly higher than in the A-HF and R-LF groups, the large variation of the assay at this concentration range (B/Bo 85-95%) must be taken into account. The peak period for plasma E_2 was on day 2 of diestrus and during proestrus (D_2P). Peak levels were found not to differ among the groups.

Table 2. Mean plasma E_2 (pg/ml), Pg, LH and FSH levels (ng/ml; mean ± SD) during the basal and peak periods of the cycle of rats fed the low-fiber and high-fiber diets ad libitum (A-LF group $n = 11$, and A-HF group $n = 13$, respectively) or the low-fiber diet restricted (R-LF, $n = 10$).

	Cyclic phase[1]	A-LF	A-HF	R-LF
E_2	OMD_1	2.6 ± 0.9*	1.3 ± 0.3	1.5 ± 0.5
	D_2P	19.5 ± 7.8	17.7 ± 7.4	17.0 ± 3.3
LH	OMP	0.44 ± 0.08	0.44 ± 0.11	0.40 ± 0.12
	D	0.73 ± 0.16	0.89 ± 0.19*	0.69 ± 0.25
Pg	D_2PO	2.5 ± 0.5	1.9 ± 0.7*	2.4 ± 0.5
	MD_1	12.6 ± 6.3	13.5 ± 5.6	12.2 ± 5.7
FSH	MD_2P	6.0 ± 0.5	6.1 ± 0.7	6.2 ± 0.9
	O	11.0 ± 1.6	11.7 ± 2.2	11.0 ± 1.2

[1] See 'Materials and methods' and Table 1 for explanation of the cycle periods.
* Different ($P < 0.05$) from the groups not marked with an asterisk.

LH, the pituitary hormone that stimulates estrogen secretion via a negative feedback action, increased during diestrus (D). During the peak period, plasma LH levels tended to be higher in the A-HF group than in the two LF groups. No differences were observed among the groups during metestrus, proestrus and estrus, i.e. during the basal period of the cycle (OMP).

Pg levels in blood plasma were lowest in rats of the A-HF group ($P < 0.05$) during the basal period, i.e. on day 2 of diestrus, proestrus and estrus (D_2PO). The peak period was during metestrus and on day 1 of diestrus (MD_1). In this period Pg levels were similar for all 3 groups.

FSH levels peaked during estrus (O) and were basal on the other 4 days of the cycle (MD_2P). FSH levels were not affected by the experimental diets.

Estrogen and Pg concentrations in tissues

Before dissection of the rats to collect mammary gland and endometrial cells, blood was sampled by aorta puncture. In plasma samples of these rats, which were in metestrus or on day 1 of diestrus, E_2 levels (basal period) did not differ among the groups. Exposure of mammary tissue to estrogens as measured by the concentrations of E_1, E_2, E_3 and peroxidase activity, did not differ among the groups. Exposure of endometrial cells to estrogens seemed to be affected significantly by dietary fiber as peroxidase activity in endometrial cells of the A-HF group was lower ($P < 0.05$) than in the A-LF and R-LF groups (Table 3).

Mean Pg concentrations (measured during the peak period) in mammary tissue and plasma were higher in the A-LF group ($n = 5$) than in the A-HF and R-LF groups ($n = 9$ each). As dissection of the rats was at metestrus (start of the Pg peak)

Table 3. E_2 and Pg levels in blood plasma and estrogen (E_1, E_2, E_3) and Pg contents in mammary tissue as well as peroxidase activity in mammary tissue and endometrial cells. Rats were fed the high-fiber and low-fiber diets ad libitum (A-HF and A-LF groups, respectively) or the energy-restricted low-fiber diet (R-LF group). Rats received the experimental diets for 8 weeks and were in metestrus or day 1 of diestrus at the time of dissection. Measured parameters are given as mean ± SEM.

Hormone	Units	A-LF (n = 5)	A-HF (n = 9)	R-LF (n = 9)
Estrogen exposure				
Plasma				
E_2	pg/ml	5.3 ± 1.6	4.2 ± 0.6	4.0 ± 0.8
Mammary tissue				
E_1	ng per g DNA	142 ± 22	172 ± 42	185 ± 35
E_2	ng per g DNA	57 ± 24	61 ± 20	45 ± 11
E_3	ng per g DNA	380 ± 42	468 ± 49	442 ± 51
Peroxidase act.	U per g DNA	22 ± 2.4	30 ± 3.9	31 ± 6.4
Endometrial cells				
Peroxidase act.	U per g DNA	2180 ± 660	648 ± 159*	2090 ± 566
Progesterone				
Plasma				
Pg	ng/ml	24 ± 5.8	8.8 ± 1.6	8.5 ± 1.9
Mammary tissue				
Pg	µg per g DNA	104 ± 19	47 ± 15	65 ± 21

* Different ($P < 0.05$) from the groups not marked with an asterisk.

or on day 1 of diestrus (top of the Pg peak) variation of the concentrations within the groups was large. For this reason no significant differences among the groups were observed (Table 3).

Discussion

Data collected in this study clearly indicate that the experimental diets used affect hormonal processes. Whether fiber or energy is the instrumental factor can be seen when Tables 2 and 3 are compared. Differences between the A-HF and the R-LF groups reflect the effects of fiber. When no differences are found between these groups, but both groups differ from the A-LF group, this difference can be ascribed to energy effects. Fiber effects are suggested by the increased plasma LH level (during the peak period of the cycle), the lowered plasma Pg concentration (during the basal period of the cycle; Table 2), and the decreased peroxidase activity in endometrial cells (Table 3) in the A-HF group relative to the R-LF group. The lower plasma E_2 level (basal period of the cycle) as well as the anestrous state of one-third

of the rats in the A-HF and R-LF groups seem clearly to be effected by the lower energy intake relative to the A-LF group.

The effects of diet on hormonal processes is corroborated by human studies reviewed recently [19]. It was found, for example, that vegetarians and Orientals consuming a low-fat, high-fiber diet have an increased fecal and a decreased urinary estrogen excretion relative to omnivorous women and to women in Western countries consuming high-fat, low-fiber diets. Contrary to recent findings [20], lower plasma estrogen concentrations have been established in women consuming a high-fiber diet [19]. When our data are considered (Table 2), the lower plasma estrogen levels might be due to a changed estrogen metabolism as a result of a lower energy intake [21]. However, a combined effect of an increased fecal estrogen excretion by interruption of the enterohepatic circulation, compensated by an increased ovarian estrogen production and a changed estrogen metabolism, seems to be at least as plausible. We measured a 3–4-fold higher fecal estrogen excretion in rats fed the A-HF diet than in the A-LF group, while plasma E_2 levels were similar [5]. It was postulated [5] that this increased excretion was compensated by a higher ovarian estrogen production due to the negative feedback action of E_2 on the pituitary resulting in elevated plasma gonadotropin (LH) levels. This hypothesis seems to be supported by our data showing a slightly higher plasma LH concentration in rats fed the HF diet during diestrus, i.e. the period just before the E_2 peak (Table 2). To test the increased production rate directly, we conducted additional experiments using ^3H labeled E_2 via direct infusion [22] and via Alzet mini-osmotic pumps (Alza Corporation, Palo Alto, CA). However, the low plasma activity (5–20 dpm/ml) and the great variation among the rats did not permit any conclusions. Introduction of a higher amount of ^3H-E_2 could disturb the equilibrium between the gonadal-pituitary axis and hence obscure the possible difference in estrogen production rate between the groups of rats fed the high-fiber and those on the low-fiber diet.

Peroxidase activity in mammary tissue of young rats (about 50 days of age) were lower in rats fed the A-HF diet [11], whereas in adult rats peroxidase activity in mammary tissue of rats was similar for all 3 experimental diets. In endometrial cells just the opposite was found. In young rats peroxidase activity was similar in endometrial cells of rats fed the A-LF, A-HF or R-LF diets, whereas in adult rats fed the A-HF diets a lower peroxidase activity was found. In mammary tissue E_2 concentrations were 2–3 times lower than E_1 concentrations, which supports the findings of Thijssen et al. [23] who measured a similar ratio between E_1 and E_2 in normal human breast tissue. In tumor tissue E_1 and E_2 levels were similar, whereas in endometrial tissue from premenopausal women the E_2 level was 3–7 times higher than E_1 [16]. With the findings of Thijssen et al. in mind, it might be supposed that the effects of dietary fiber on estrogen exposure, i.e. on peroxidase activity as measured in our study, are dependent on E_2 tissue concentration. The higher the concentration, the higher the effect. So, it might be expected that dietary fiber does not affect exposure of normal mammary tissue to estrogens, but that this effect does occur in tumor tissue due to the relatively high E_2 concentrations. Concerning the plasma estrogen levels a reduced energy intake seems to have a similar effect as a

high-fiber diet. However, a high-fiber diet has some extra. Fiber components can bind (lipophilic) carcinogenic compounds thus preventing them to be absorbed in the body through the intestinal wall [24, 25]. Dietary fiber, especially whole grains, provide the body with such products of vegetable origin as lignans and phytoestrogens, which are protective against breast cancer development [26].

Besides estrogens, exposure of mammary tissue to Pg is another hormonal parameter, which might be affected by dietary fiber. During the basal period of the cycle significantly lower Pg concentrations were measured in plasma of rats fed the A-HF diet. In future studies the Pg content in mammary tissue also has to be established when rats are in the basal period for Pg. Lower Pg concentrations, measured in plasma collected during the luteal phase, were not observed by Rose et al. [20] in women on bran diets. This can be explained by the fact that cycles of rats and women are hardly comparable. Danutra et al. [27] found differences in extent of Pg activity in saliva from adolescent girls living in Britain and Thailand. The age at which Pg secretion was established was earlier for the British girls than for the Thai girls. Whether this effect can be ascribed to differences in fiber intake is not clear yet. The lower Pg levels in our study might be explained by the interruption of the enterohepatic circulation of Pg or its metabolites resulting in an increased fecal Pg excretion which is not compensated by a negative feedback regulation as in the case of estrogens. This interruption can be caused by binding of Pg or its metabolites to dietary fiber components in a similar way as was reported for in vitro binding of estrogens to these fibers [28, 29; results of Pg binding not published]. Whether Pg alone or the E_2/Pg ratio is an important factor in breast cancer remains unclear to date. In the estrogen-window hypothesis of Korenman [30], Pg is supposed to play a protective role in carcinogenesis. This may be the case for endometrial cells, but for breast tissue this protective effect is doubtful. Key and Pike [31] proposed the 'oestrogen plus progestagen hypothesis' for breast cancer. They provided evidence that risk for breast cancer increased when Pg exposure was enhanced. In women, E_2 alone (in the follicular phase) induces some cell division, but E_2 and Pg together (in the luteal phase) much more so. The long-term use of combination-type oral contraceptives (estrogens and progestagens] was found to increase breast cancer risk [32], which supports the hypothesis of Key and Pike. More studies concerning the influence of Pg are necessary.

Mean plasma Pg levels measured during the peak period of the cycle (Table 2) are lower than those given in Table 3 (plasma collected before dissection when rats were in metestrus or on day 1 of diestrus, i.e. the peak period for Pg). This can be explained by the fact that plasma Pg levels were higher during day 1 of diestrus than at the start of the peak period, i.e. during metestrus. The mean value of the peak period (mean of metestrus and day 1 of diestrus) was always lower than the individual levels during day 1 of diestrus which mostly reflected the phase of the cycle when animals were dissected. In further studies exposure of tissue to Pg has to be studied in rats dissected on day 2 of diestrus, proestrus or estrus, i.e. during the basal period for Pg. As day 2 of diestrus and proestrus is the peak period for E_2, the estrous phase of the cycle seems to be ideal for studying both Pg and E_2.

Plasma E_2 levels measured during the basal period of the cycle were often much lower than the E_2 values given in Table 3 (4–5 pg/ml) and those found in a previous study [5] (about 10 pg/ml). This can be explained by the RIA used. Although this RIA is very sensitive, it is almost impossible to measure concentrations below 10 pg/ml accurately in 0.5 ml of blood plasma at most as all concentrations in the standard range of the RIA are higher than 80% B/Bo. Plasma E_2 concentrations in our studies were always much lower than those measured by Cohen et al. [33] who reported levels as high as 35 pg/ml in blood plasma. This also can be explained by the RIA techniques used, as Cohen et al. used a direct RIA with an E_2 antiserum that cross-reacted with E_1 for 25% (ICN 17β-estradiol antibody-coated ^{125}I-RIA kit; ICN Biomedicals, Costa Mesa, CA). As E_1 concentrations in rat plasma are 2–3 times higher than E_2 [5], Cohen et al. must have measured mainly E_1 instead of E_2.

It cannot be excluded that the results obtained are affected in part by stress. The anestrous state of some animals of the R-LF group might be a stress effect via the hypothalamic-pituitary axis resulting in decreased gonadotropin secretion as measured in rhesus monkeys on an energy-restricted diet [34]. The anestrous state found in rats fed the A-HF diets was also noticed in human studies. Women with a high intake of fiber more often have amenorrhea or a longer cycle than those with a low fiber intake [35]. The stress caused by the cannulation procedure or by sampling of a relatively large amount of blood (1.5 ml on 5 successive days) is not expected to influence directly estrogen or Pg levels in blood plasma or tissue.

It is concluded that dietary fiber affects hormonal processes related to breast cancer development. The fact that basal plasma Pg levels are decreased by dietary fiber intake sheds new light on studies in which fiber and breast cancer are involved.

References

1. Goldin BR, Gorbach SL. Effect of diet on the plasma levels, metabolism and excretion of estrogens. Am J Clin Nutr 48: 787-790 (1988).
2. van 't Veer P, Kolb CM, Verhoef P, Kok FJ, Schouten EG, Hermus RJJ, Sturmans F. Dietary fiber, beta-carotene and breast cancer: results from a case-control study. Int J Cancer 45: 825-828 (1990).
3. Fisher N, Berry CS, Fearn T, Gregory JA, Hardy J. Cereal dietary fiber consumption and divertical disease: a lifespan study in rats. Am J Clin Nutr 42: 788-804 (1985).
4. Sinkeldam EJ. Dietary fiber and cancer of the large bowel and breast. In: Leegwater DC, Feron VJ, Hermus RJJ, eds. Low digestibility carbohydrates. Proceedings of a workshop held at the TNO-CIVO Institutes, Zeist, Netherlands, 27–28 November 1986, pp. 96-102. Pudoc, Wageningen, Netherlands (1987).
5. Arts CJM, de Bie AThHJ, van den Berg H, van 't Veer P, Bunnik GSJ. Influence of wheat bran on NMU-induced mammary tumor development, plasma estrogen levels and estrogen excretion in female rats. J Steroid Biochem Mol Biol 39: 193-202 (1991).
6. Henderson BE, Ross R, Bernstein L. Estrogens as a cause of human cancer: The Richard and Hinda Rosenthal Foundation award lecture. Cancer Res 48: 246-253 (1988).
7. Kritchevsky D, Welch CB, Klurfeld DJ. Response of mammary tumors to caloric restriction for different time periods during the promotion phase. Nutr Cancer 12: 259-269 (1989).
8. Lyttle CR, Desombre ER. Uterine peroxidase as a marker for estrogen action. Proc Natl Acad Sci USA 74: 3162-3166 (1977).

9. Levy J, Liel Y, Glick SM. Peroxidase activity as a marker for estrogenicity: studies in uterus and mammary tumors. Isr J Med Sci 17: 970-975 (1981).
10. Atwater WO, Bryant AP. The availability and fuel value of food materials. Rep Storrs Agric Exp Stn 1899: 73-110 (1900).
11. Arts CJM, Govers CARL, van den Berg H, Thijssen JHH. Effects of wheat bran and energy restriction on onset of puberty, cell proliferation and the development of mammary tissue in female rats. Acta Endocrinol (in press).
12. Papanicolaou GN. A new procedure for staining vaginal smears. Science 95: 438-443 (1942).
13. Yoshinaga K, Hawkins RA, Stocker JF. Estrogen secretion by the rat ovary in vivo during the estrous cycle and pregnancy. Endocrinology 85: 103-112 (1969).
14. Arts CJM, van Baak MJ, van den Berg H, Schilt R, Berende PLM, den Hartog JMP. Concentrations of the endogenous steroid hormones oestradiol-17β, testosterone and progesterone in veal calves in connection with the control for illegal administration. Arch Lebensmittelhyg 41: 58-62 (1990).
15. van Nesselrooij JHJ, Bruijntjes JP, van Garderen-Hoetmer A, Tillapaugh-Fay GM, Feron VJ. Magnetic resonance imaging compared with hormonal effects and histopathology of estrogen-induced pituitary lesions in the rat. Carcinogenesis 12: 289-297 (1991).
16. van Landeghem AAJ, Poortman J, Helmond-Agema A, Thijssen JHH. Measurement of endogenous subcellular concentrations of steroids in tissue. J Steroid Biochem 20: 639-644 (1984).
17. Labarca C, Paigen K: A simple, rapid and sensitive DNA assay procedure. Anal Biochem 102: 344-352 (1980).
18. Dixon WJ, Brown MB, Engelman L, Hill MA, Jennrich RI. BMDP Statistical Software Manual. University of California Press, Berkeley, CA (1988).
19. Adlercreutz H. Diet and sex hormone metabolism. In: Rowland IR, ed. Nutrition, toxicity and cancer, pp. 137-195. CRC Press, London (1991).
20. Rose DP, Goldman M, Connolly JM, Strong LE. High-fiber diet reduces serum estrogen concnetrations in premenopausal women. Am J Clin Nutr 54: 520-525 (1991).
21. Longcope C, Gorbach S, Goldin B, Woods M, Dwyer J, Morrill A, Warram J. The effect of a low fat diet on estrogen metabolism. J Clin Endocrinol Metab 64: 1246-1250 (1987).
22. DeHertogh R, Ekka E, Vanderheyden I, Hoet JJ. Metabolic clearance rates and the interconversion factors of estrone and estradiol-17β in the immature and adult female rat. Endocrinology 87: 874-880 (1970).
23. Thijssen JHH, Blankenstein MA. Endogenous oestrogens and androgens in normal and malignant endometrial and mammary tissues. Eur J Cancer Clin Oncol 25: 1953-1959 (1989).
24. Gulliver WP, Kutty KP, Laher JM, Barrowman JA. In vitro interaction of 7,12-dimethylbenz(a)anthracene and its biliary metabolites with dietary fibers. J Natl Cancer Inst 71: 207-210 (1983).
25. Smith-Barbaro P, Hanson D, Reddy BS. Carcinogen binding to various types of dietary fiber. J Natl Cancer Inst 67: 495-497 (1981).
26. Adlercreutz H, Mousavi Y, Loukovaara M, Hämäläinen E. Lignans, isoflavones, sex hormone metabolism and breast cancer. In: Hochberg RB, Naftolin F, eds. The new biology of steroid hormones, pp. 145-154. Raven Press, New York (1991).
27. Danutra V, Turkes A, Read GF, Wilson DW, Griffiths V, Jones R, Griffiths K. Progesterone concentrations in samples of saliva from adolescent girls living in Britain and Thailand, two countries where women are at widely differing risk of breast cancer. J Endocrinol 121: 375-381 (1989).
28. Arts CJM, Govers CARL, van den Berg H, Wolters MGE, van Leeuwen P, Thijssen JHH. In vitro binding of estrogens by dietary fiber and the in vivo apparent digestibility tested in pigs. J Steroid Biochem Mol Biol 38: 621-628 (1991).
29. Shultz TD, Howie BJ. In vitro binding of steroid hormones by natural and purified fibers. Nutr Cancer 8: 141-147 (1986).
30. Korenman SG. Oestrogen window hypothesis of the aetiology of breast cancer. Lancet i: 700-701 (1980).
31. Key TJA, Pike MC. The role of oestrogens and progestagens in the epidemiology and prevention of breast cancer. Eur J Cancer Clin Oncol 24: 29-43 (1988).

32. Bergkvist L, Adami HO, Persson I, Hoover R, Schairer C. The risk of breast cancer after estrogen and estrogen-progestin replacement. N Engl J Med 321: 293-297 (1989).
33. Cohen LA, Kendall ME, Zang E, Meschter C, Rose DP. Modulation of N-nitrosomethylurea-induced mammary tumor promotion by dietary fiber and fat. J Natl Cancer Inst 83: 496-501 (1991).
34. Dubey AK, Cameron JL, Steiner RA, Plant TM. Inhibition of gonadotropin secretion in castrated male rhesus monkeys (*Macaca mulatta*) induced by dietary restriction: analogy with the prepubertal hiatus of gonadotropin release. Endocrinology 118: 518-525 (1986).
35. Pedersen AB, Bartholomew MJ, Dolence LA, Aljadir LP, Netteburg KL, Lloyd T. Menstrual differences due to vegetarian and nonvegetarian diets. Am J Clin Nutr 53: 879-885 (1991).

Part III: Discussion

CHAPTER 9

Dietary fiber and breast cancer pathogenesis: a hypothesis

Cor J.M. Arts and Jos H.H. Thijssen

Abstract

Epidemiological and experimental studies have demonstrated that dietary fiber reduces the risk for mammary cancer. It is hypothesized that the effects of dietary fiber on mammary carcinogenesis vary during a woman's life. The mechanism of protection by fiber (derived from cereals) is a complicated ensemble comprising at least the following factors: a reduced energy intake, a lowered estrogen and progesterone exposure, and anti-estrogenic and anti-carcinogenic properties of particular plant constituents. The fiber-based decreased risk for tumor initiation is related to the delay in menarche and breast development during puberty and adolescence. Tumor promotion can be inhibited by fiber during the pre- and postmenopausal periods. It is proposed that exposure of mammary tissue to estrogens is reduced during the postmenopausal period, but not during the premenopausal period. During the reproductive period the diminished exposure to progesterone may be relevant.

Introduction

There is general consensus as to the notion that hormones are involved in the development of breast cancer. Especially hormones of the gonadal-pituitary axis, i.e. estrogens, progestagens, prolactin and the gonadotropins LH and FSH, have received much attention. Evidence that diet can affect the production and excretion of hormones comes from several observational and experimental studies. With respect to estrogens, most data are based upon comparitive studies in Oriental and Caucasian women which differ in their dietary pattern, estrogen metabolism and breast cancer risk. Additional evidence comes from studies involving vegetarians and omnivores. Vegetarians generally have lower urinary estrogen excretion, lower plasma levels of estrone and estradiol and reduced risk for developing breast cancer (1–6). A high fat intake affects estrogen metabolism resulting in increased fecal excretion of 16α-hydroxylated estrogens (7). A high fiber intake enhances fecal estrogen excretion and declines urinary estrone excretion (3, 5).

Several recent hypotheses have proposed that breast cancer originates in utero (8), during puberty and adolescence (9) or during the premenopausal period (10). It has been supposed that breast cancer originates in utero through increased concentrations of estrogens in pregnancy, increasing the probability of daughters getting breast cancer by creating a 'fertile soil' for subsequent cancer initiation. The unifying hypothesis of de Waard and Trichopoulos supposing that breast cancer originates during puberty and adolescence was based on the findings that delayed menarche and early pregnancy decrease breast cancer risk. In support of this hypothesis is the finding that only undifferentiated endothelial cells can be initiated to tumor cells (11, 12). The hypothesis that breast cancer originates during the premenopausal period is based on clinical and epidemiological findings.

With these hypotheses in mind, in this report evidence will be furnished that dietary fiber, especially on the basis of grains, can decrease risk for mammary cancer in different phases of breast development.

Dietary fiber

As defined by Trowell et al. (13), dietary fibers are the plant cell remnants indigestible by human gastrointestinal tract enzymes. The two major classes of fiber are polysaccharides and lignin. The polysaccharides can be subdivided into cellulose, hemicellulose and pectin. The chemical and physiological properties of these compounds have been described extensively (14–18). Within the scope of this paper a few properties of fiber are relevant: (1) dietary fiber compounds have to be unfermentable by enzymes from the intestinal microflora; (2) the fiber compounds are able to bind steroid hormones and/or carcinogenic compounds. An additional property of dietary fiber is that also other plant constituents are introduced into the body, such as precursors of lignans and isoflavonic phytoestrogens, which are supposed to play also a role in reducing breast cancer risk (19).

Whether these properties find expression depends on the composition of dietary fiber, i.e. its origin. Dietary fiber from fruits and vegetables is mainly composed of cellulose and pectin, compounds which can be fermented partly or entirely and do not bind steroid hormones. Cereal fiber contains such compounds as lignin and hemicellulose bound to lignin, which make it unfermentable for intestinal microflora. These compounds are also capable of binding estrogenic steroids (20) and carcinogenic compounds (21, 22). Per gram (on a wet basis), cereals can introduce about 10 times more lignans (enterodiol and enterolactone) into the body than fruits and vegetables. Flaxseed (linseed) produces the highest concentration of lignans (almost 100 times greater than most foods) (23). Wheat bran, soybean, oats and linseed contain relatively much lignin in their fiber, can bind estrogens with a relatively high affinity and, tested in pigs, undergo the lowest apparent digestibility (20). From these findings it is concluded that these cereals are the most protective against breast cancer development.

Fiber and initiation of breast cancer

Breast cancer is thought to start with mutation of DNA in the nucleus of a normal epithelial cell. Sometimes the body is not able to repair the DNA damage, and after further cell division the source of a cancerous lesion has arisen from this initiated cell. The mutation can be caused by chemical, radioactive or other physico-chemical sources (24). Whether estrogens or their metabolites are directly involved in the initiation of breast cancer remains unclear. Bradlow, Fishman and co-workers (25–27) suggest that a major estrogen metabolite, 16α-hydroxyestrone, could play a role because (1) it can bind covalently to amino groups of DNA molecules (28–30); (2) its formation is positively related to a number of risk factors for breast cancer (27); (3) an increased 16α-hydroxylation was found in strains of mice with a high incidence of mammary tumors (26). In our study, using the Ames test (31), we did not find a clear indication that 16α-hydroxyestrone can induce mutation and thus is involved in breast cancer development.

All breast cells can be equally susceptible to mutation, but cells which a high proliferation rate are less sensitive to the natural DNA- repair process. The undifferentiated 'terminal end bud cells' are rapidly dividing cells which occur in rats at 40–70 days of age (11) and in women during puberty and adolescence (12). Late menarche and early pregnancy (< 20 years) are protective factors for breast cancer (32). The experimental work of Russo and co-workers (11, 33, 34) clearly indicated that in rats only the 'terminal end bud cells' are susceptible to chemical induction of mammary tumors. In women, time of menarche as well as breast development can be delayed by a high intake of dietary fiber, especially fiber derived from cereals (35). Similar results have been obtained in rat studies (36).

Fiber and promotion of breast tumors

After a latency of at least 20 years the initiated breast epithelial cells can undergo a reversible promotion stage (37). Whether the promotion phase reaches the irreversible progression phase depends on several factors including diet. The promotion of NMU-induced mammary tumors in rats can be delayed by a high fiber intake (5). Mammary tumors of rats fed a high-fiber wheat bran diet weighed significantly less than those of rats fed a low-fiber wheat flour diet. This was confirmed by a study of Cohen et al. (38). So, dietary fiber affects promotion of mammary tumors. It has been suggested (5) that promotion is decreased by the lower energy intake, by a decreased exposure of mammary tumors to estrogens or by the effects of lignans and phytoestrogens, which have anti-estrogenic or anti-carcinogenic properties.

In the animal model used in our studies (5, 36, 39) energy intake seems to be an important parameter which does not only affect body weight, but also hormonal processes involved in vaginal membrane rupture, i.e. start of puberty, mammary development and estrous cycle. Previous studies have shown an inhibitory effect of

reduced energy intake on development of mammary tumors in rats and mice (5, 40–42).

That a high fiber intake results in a decreased exposure of mammary tissue to estrogens in the mature period of the life of rats or during the premenopausal period of women seems to be very doubtful. In our studies an increased fecal estrogen excretion and a lowered urinary estrone excretion was established (5, 43). In contrast to the 3–4-fold enhanced fecal estrogen excretion and a slightly decreased urinary estrogen excretion in rats fed a high-fiber diet, almost identical plasma estrogen levels were measured in rats fed a high- or low-fiber diet. This indicates that estrogen production is higher in rats fed a high-fiber diet. Indeed, a higher level of luteinizing hormone (LH), a peptide hormone from the pituitary which regulates ovarian estrogen production, was measured in this group of rats (39). Rose et al. (6) found only slightly lower serum estrone and estradiol-17β levels in serum of women in the luteal phase who received a wheat bran diet for 2 months. Whether this lowered plasma estrogen level is relevant to accumulation of estrogens in breast tissue remains doubtful as estrogen concentrations in mammary tissue of rats fed a wheat bran diet were similar to those of rats fed a low-fiber diet (39). In this study it was also found that plasma progesterone levels were significantly lower in rats as a result of wheat bran diet. It can be expected that this parameter is an important factor in promotion of breast or mammary cancer. Dietary fiber components not only bind estrogens (20), but are also able to bind progesterone (unpublished data), indicating that the enterohepatic circulation of progesterone can be interrupted resulting in enhanced fecal excretion of progesterone metabolites, which is not compensated by an increased progesterone production as its production rate (in women in the luteal phase) is not under control of a negative feedback mechanism. This finding seems relevant in view of the 'estrogen plus progesterone' hypothesis of Key and Pike (44), implying that increased exposure to estrogen alone causes some increase in breast cancer risk but that this risk is much higher for exposure to estrogen plus progestagen together.

The effects of lignans and phytoestrogens on breast cancer have recently been reviewed extensively (19, 45). In concert, these compounds can act via their influence on particular enzymes involved in cell proliferation and via binding to the estrogen receptor, making endogenous estrogens less effective. In man, lignans and phytoestrogens can stimulate synthesis of sex hormone-binding globulin (SHBG) in the liver and thus reduce the biological effects of sex hormones. An increase of SHBG results in lowering the percentage of free estrogen resulting in a reduction of their biological activity. Linseed flour supplemented to a basal high-fat diet at a 5% or 10% level had significant inhibitory effects on mammary tumor initiation and promotion in a rat study (23).

The hypothesis

The effects of dietary fiber (on the basis of wheat bran) on mammary carcinogenesis vary during a woman's life as presented schematically in Fig. 1. The hypothesis presented elaborates on 'the unifying concept of the etiology of breast cancer' of de Waard and Trichopoulis (9) referred to above. We suppose that fibers from cereals have its greatest protective effects on mammary cancer when fiber is consumed in puberty and adolescence, as was corroborated in an animal study with dogs (46). That dietary fiber affects breast tumor initiation in utero as hypothezised by Trichopoulos (8) or during the premenopausal period (as hypothezised by Simpson et al. (10) can only be explained by binding of carcinogenic compounds to fiber components preventing them to absorb into the body or by the introduction of materials of vegetable origin with anti-estrogenic or anti-carcinogenic properties.

The risk for initiation of breast cancer during puberty and adolescence (9) can be decreased by late menarche and a delayed breast development (32, 47). Both can be reached by a high fiber intake in rats (36) and girls (35). In this period of life dietary fiber decreases exposure of mammary and uterine tissue to estrogens and probably also to progesterone. During a woman's early pubertal development, higher fecal

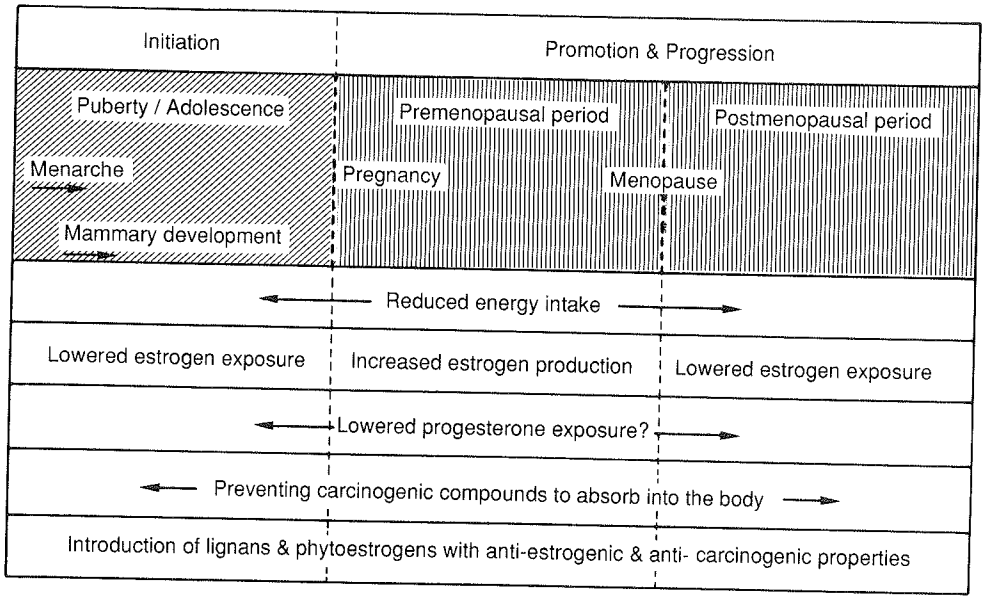

Fig. 1. A schematic presentation of the hypothesis of the effects of dietary fiber on mammary cancer development during a woman's life.

estrogen excretion is not yet compensated by an increased production resulting in a slower development to maturity and menarche. This delayed development can also be caused by a lower energy intake, accompanying a high fiber intake. In case of early pregnancy, a fast differentiation of mammary tissue reduces the time that mammary cells can be initiated to pre-cancerous lesions. This might be a reason why pregnancy protects against mammary cancer development (48).

Promotion of the initiated cells and progression can be delayed or prevented by dietary fiber via different mechanisms during the premenopausal and postmenopausal periods. During both periods energy intake is decreased by a high fiber intake, an important factor in reducing breast cancer risk as discussed before. Both during the premenopausal and postmenopausal periods fecal estrogen excretion, and probably also fecal progestagen excretion, increase and urinary estrone excretion and probably also urinary pregnanediol excretion, decline in women on a high-fiber diet. During the premenopausal period the increased fecal estrogen excretion is compensated by an increased estrogen production due to the negative feedback action of the gonadal pituitary axis. So, in this period it is very doubtful whether the increased fecal estrogen excretion results in a lowered exposure of breast tissue to estrogens. In our animal experiment (39) mammary tissue estrogen levels were similar in rats fed high-fiber and low-fiber diets. During the postmenopausal period the gonadal estrogen production is minimal (49), and the main source of estrogen production is peripheral aromatization of androstenedione to estrone and estradiol-17β, which is not under control of a negative feedback action. So, it can be expected that in the postmenopausal period exposure of breast tissue to estrogens can be decreased by dietary fiber. Plasma progesterone levels were lower in rats fed a high-fiber diet than in rats fed a low-fiber diet (39). When these results are extrapolated to the premenopausal period in women, exposure of breast tissue to progesterone is lower in woman on high-fiber diets as progesterone production seems not to be under the control of a negative feedback action. The lowered progesterone exposure fits well in with the 'estrogen plus progesterone hypothesis' of Key and Pike (44).

In addition to the hormonal effects, dietary fiber components can bind carcinogenic compounds, thus preventing intestinal absorption of carcinogens contained in the diet (21, 22), thus reducing the risk that these carcinogenic compounds reach particular cells in the body.

During the three stages of a woman's life (puberty/adolescence, premenopausal period, postmenopausal period) a high fiber intake also results in an increased intake of materials of vegetable origin such as phytoestrogens and lignans (19, 23, 45) which may also protect against breast cancer development (19) as discussed before.

Conclusions

It is proposed that dietary fiber, especially wheat bran, reduces breast cancer risk. The mechanism of protection is a complicated ensemble encompassing at least 4 parameters, i.e. energy, estrogens, progestagens and plant constituents. Which of these parameters is the most important is hard to say, but it is clear that the way of protection also depends on the stage of the woman's hormonal development. The risk for tumor initiation might be lowered when fiber intake is high during mammary development, i.e. during puberty and adolescence. The risk for tumor promotion and progression might be lowered during the pre- and postmenopausal periods. In the latter period the lowered exposure to estrogen might be an important parameter.

References

1. Goldin BR, Adlercreutz H, Dwyer JT, Swenson L, Warram JH, Gorbach SL. Effect of diet on excretion of estrogens in pre- and postmenopausal women. Cancer Res 41: 3771-3773 (1981).
2. Goldin BR, Adlercreutz H, Gorbach SL, Woods MN, Dwyer JT, Conlon T, Bohn E, Gershoff SN. The relationship between estrogen levels and diets of Caucasian American and Oriental immigrant women. Am J Clin Nutr 44: 945-953 (1986).
3. Goldin BR, Gorbach SL. Effect of diet on the plasma levels, metabolism and excretion of estrogens. Am J Clin Nutr 48: 787-790 (1988).
4. Barbosa JC, Shultz TD, Filley SJ, Nieman DC. The relationship among adiposity, diet, and hormone concentrations in vegetarian and nonvegetarian postmenopausal women. Am J Clin Nutr 51: 798-803 (1990).
5. Arts CJM, de Bie AThHJ, van den Berg H, van 't Veer P, Bunnik GSJ. Influence of wheat bran on NMU-induced mammary tumor development, plasma estrogen levels and estrogen excretion in female rats. J Steroid Biochem Mol Biol 39: 193-202 (1991).
6. Rose DP, Goldman M, Connolly JM, Strong LE. High-fiber diet reduces serum estrogen concentrations in premenopausal women. Am J Clin Nutr 54: 520-525 (1991).
7. Longcope C, Gorbach S, Goldin B, Woods M, Dwyer J, Morrill A, Warram J. The effect of a low fat diet on estrogen metabolism. J Clin Endocrinol Metab 64: 1246-1250 (1987).
8. Trichopoulos D. Hypothesis: does breast cancer originate in utero? Lancet 335: 939-940 (1990).
9. de Waard F, Trichopoulos D. A unifying concept of the aetiology of breast cancer. Int J Cancer 41: 666-669 (1988).
10. Simpson HW, Pauson AW, Griffiths K, Candlish W, Mcardle CS, Small RG. Genesis of breast cancer is in the premenopause. Lancet ii: 74-76 (1988).
11. Russo IH, Russo J. Development stage of the rat mammary gland as determinant of its susceptibility to 7,12,-dimethylbenz(a)anthracene. J Natl Cancer Inst 61: 1439-1449 (1978).
12. Russo J, Calaf G, Roi L, Russo IH. Influence of age and gland topography on cell kinetics of normal human breast tissue. J Natl Cancer Inst 78: 413-418 (1987).
13. Trowell H, Southgate DAT, Wolever TMS, Leeds AR, Gassull MA. Jenkins DJA. Dietary fiber redefined. Lancet i: 967 (1976).
14. Kay RM. Dietary fiber. J Lipid Res 23: 221-242 (1982).
15. Jacqmain D. The composition of dietary fibers. In: Williams DL (ed.). The status of dietary fiber: Physiology and current practice, pp. 15-42. Reckitt & Colman Foundation / Elsevier Librico, Brussels (1984).
16. Asp NG. Dietary fiber – Definition, chemistry and analytical determination. Mol Aspects Med 9: 17-29 (1987).
17. Theander O. Chemistry of dietary fiber components. Scand J Gastroenterol 22 (Suppl): 21-28 (1987).

18. Kritchevsky D. Dietary fiber. Annu Rev Nutr 8: 301-328 (1988).
19. Adlercreutz H. Diet and sex hormone metabolism. In: Rowland IR (ed.). Nutrition, toxicity and cancer, pp. 137-195. CRC Press, London (1991).
20. Arts CJM, Govers CARL, van den Berg H, Wolters MGE, van Leeuwen P, Thijssen JHH. In vitro binding of estrogens by dietary fiber and the in vivo apparent digestibility tested in pigs. J Steroid Biochem Mol Biol 38: 621-628 (1991).
21. Gulliver WP, Kutty KP, Laher JM, Barrowman JA. In vitro interaction of 7,12-dimethylbenz(a)anthracene and its biliary metabolites with dietary fibers. J Natl Cancer Inst 71: 207-210 (1983).
22. Smith-Barbaro P, Hanson D, Reddy BS. Carcinogen binding to various types of dietary fiber. J Natl Cancer Inst 67: 495-497 (1981).
23. Thompson LU, Robb P, Serraino M, Cheung F. Mammalian lignan production from various foods. Nutr Cancer 16: 43-52 (1991).
24. Pitot HC, Dragan YP. Facts and theories concerning the mechanisms of carcinogenesis. FASEB J 5: 2280-2286 (1991).
25. Fishman J, Martucci C. Biological properties of 16α-hydroxyestrone: implications in estrogen physiology and pathophysiology. J Clin Endocrin Metab 51: 611-615 (1980).
26. Bradlow HL, Hershcopf RJ, Martucci CP, Fishman J. Estradiol 16α-hydroxylation in the mouse correlates with mammary tumor incidence and presence of murine mammary tumor virus: A possible model for the etiology of breast cancer in humans. Proc Natl Acad Sci USA 82: 6295-6299 (1985).
27. Bradlow HL, Hershcopf RJ, Martucci CP, Fishman J. 16α-Hydroxylation of estradiol: A possible risk marker for breast cancer. In: Angeli A, Bradlow HL, Dogliotti L (eds.). Endocrinology of the breast: Basic and clinical aspects. Ann NY Acad Sci 464: 138-151 (1986).
28. Bucala R, Fishman J, Cerami A. Formation of covalent adducts between cortisol and 16α-hydroxyestrone and protein: Possible role in the pathogenesis of cortisol toxicity and systemic lupus erythematosus. Proc Natl Acad Sci USA 79: 3320-3324 (1982).
29. Yu SC, Fishman J. Interaction of histones with estrogens. Covalent adduct formation with 16α-hydroxyestrone. Biochemistry 24: 8017-8021 (1985).
30. Swaneck GE, Fishman J. Covalent binding of the endogenous estrogen 16α-hydroxyestrone to estradiol receptor in human breast cancer cells: characterization and intranuclear localization. Proc Natl Acad Sci USA 85: 7831-7835 (1988).
31. Arts CJM, Wilmer JWGM, de Bie AThHJ, van den Berg H. In vitro synthesis of 16α-hydroxyestrone by female rat liver microsomes: its possible role in the etiology of breast cancer. J Steroid Biochem 36: 527-531 (1990).
32. Staszewski J. Age at menarche and breast cancer. J Natl Cancer Inst 47: 935-940 (1971).
33. Russo J, Russo IH. Influence of differentiation and cell kinetics on the susceptibility of the rat mammary gland to carcinogenesis. Cancer Res 40: 2677-2687 (1980)
34. Russo J, Tay LK, Russo IH. Differentiation of the mammary gland and susceptibility to carcinogenesis. Breast Cancer Res Treat 2: 5-73 (1982).
35. de Ridder CM, Thijssen JHH, van 't Veer P, van Duuren R, Bruning PF, Zonderland ML, Erich WBM. Dietary habits, sexual maturation, and plasma hormones in pubertal girls: a longitudinal study. Am J Clin Nutr 54: 805-814 (1991).
36. Arts CJM, Govers CARL, van den Berg H, Thijssen JHH. Effects of wheat bran and energy restriction on onset of puberty, cell proliferation and the development of mammary tissue in female rats. Acta Endocrinol, in press (1992).
37. Bertram JS, Kolonel LN, Meyskens FL. Rationale and strategies for chemoprevention of cancer in humans. Cancer Res 47: 3012-3031 (1987).
38. Cohen LA, Kendall ME, Zang E, Meschter C, Rose DP. Modulation of N-nitrosomethylurea-induced mammary tumor promotion by dietary fiber and fat. J Natl Cancer Inst 83: 496-501 (1991).
39. Arts CJM, Thijssen JHH. Effects of wheat bran on blood and tissue hormone levels in adult female rats. Acta Endocrinol, submitted (1992).
40. Lok E, Nera EA, Iverson F, Scott F, So Y, Clayson DB. Dietary restriction, cell proliferation and carcinogenesis: a preliminary study. Cancer Lett 38: 249-255 (1988).

41. Kritchevsky D, Welch CB, Klurfeld DJ. Response of mammary tumors to caloric restriction for different time periods during the promotion phase. Nutr Cancer 12: 259-269 (1989).
42. Klurfeld DM, Welch CB, Davis MJ, Kritchevsky D. Determination of degree of energy restriction, necessary to reduce DMBA induced mammary tumorigenesis in rats during the promotion phase. J Nutr 119: 286-291 (1989).
43. Arts CJM, Govers CARL, van den Berg H, Blankenstein MA, Thijssen JHH. Effect of wheat bran on excretion of radioactively labeled estradiol-17β and estrone-glucuronide injected intravenously in male rats. J Steroid Biochem Mol Biol 42, in press (1992).
44. Key TJA, Pike MC. The role of oestrogens and progestagens in the epidemiology and prevention of breast cancer. Eur J Cancer Clin Oncol 24: 29-43 (1988).
45. Adlercreutz H, Mousavi Y, Loukovaara M, Hämäläinen E. Lignans, isoflavones, sex hormone metabolism and breast cancer. In: Hochberg RB, Naftolin F (eds.). The new biology of steroid hormones, pp. 145-154. Serono Symposia Publications 74. Raven Press, New York (1991).
46. Sonnenschein EG, Glickman LT, Goldschmidt MH, McKee LJ. Body confirmation, diet, and risk of breast cancer in pet dogs: a case-control study. Am J Epidemiol 133: 694-703 (1991).
47. Hughes RE, Jones E. Intake of dietary fiber and the age of menarche. Ann Hum Biol 12: 325-332 (1985).
48. Kvále G, Heuch I, Eide GE. A prospective study of reproductive factors and breast cancer. Am J Epidemiol 126: 831-841 (1987).
49. Fotherby K. Biosynthesis of the oestrogens. In: Makin HLJ (ed.). Biochemistry of steroid hormones, pp. 207-229. Blackwell Scientific Publications, London (1984).

Summary

Epidemiological and experimental studies have shown that breast cancer development can be affected by diet. In contrast with fat intake, which is positively associated with breast cancer incidence, it has been demonstrated that the association with dietary fiber is negative.

In Chapter 1 the parameters involved in this study are discussed. This chapter is not intended as a state-of-the-art report, but rather as background information supporting the understanding of the second and third parts of the thesis.

In Chapter 2 the aim of the study is explained and a working hypothesis is launched. The working hypothesis suggests that dietary fiber affects breast cancer carcinogenesis through its effect on the estrogen balance resulting in an increased fecal and a decreased urinary estrogen excretion and lowered plasma estrogen levels. Furthermore, it was hypothesized that, concomitantly with the lowered plasma estrogen levels, the exposure of mammary tissue to estrogens is decreased in rats fed a high-fiber diet as compared with those fed a low-fiber diet. The lowered estrogen exposure would result in decreased cell proliferation of estrogen-sensitive (tumor) tissue. The alleged carcinogenic potency of 16α-hydroxyestrone, a main metabolite in estrogen metabolism, was tested on its mutagenicity.

In an *in vitro* model (Chapter 3) using liver microsomes isolated from male rats and female rats and mice, it was found that the liver microsomes used were able to convert estrone to various metabolites such as 16α-hydroxyestrone. In a mutagenicity assay (Ames test), with 16α-hydroxyestrone as test substance, two strains (TA98 and TA1538) of the five strains tested showed a 2–3-fold increase in number of his^+ revertants relative to the control values. Estrone did not contribute to mutagenicity in the test used. It is concluded that, in this *in vitro* model, female rats are able to synthesize 16α-hydroxyestrone. Whether this compound is risk factor for breast cancer remains unclear.

In Chapter 4 the hypothesis was tested that a high-fiber diet reduces tumor promotion through interruption of the enterohepatic circulation resulting in lowered estrogen exposure of estrogen-sensitive tissue. In the first experiment the development of N-nitrosomethylurea (NMU)-induced mammary tumors was investigated. One group of rats was fed a high-fiber diet (11% fiber, based on wheat bran), the other group was fed a low-fiber diet (0.5% fiber, based on white wheat flour). Tumor incidence and latency were similar in both groups. Compared to the low-fiber group, rats fed the high-fiber diet had lower tumor weights (0.16 g vs. 0.55 g; $P < 0.01$) and a slightly lower tumor multiplicity (1.8 vs. 2.8 tumors per tumor-bearing rat). These differences were reduced after adjustment for body weight. In a second experiment, rats not treated with the carcinogen, were kept on the same high- and low-fiber diets. From these rats 24-hour urine and feces and orbital blood

samples were collected for analysis of (un)conjugated estrogens. The excretion of both free and conjugated estrogens in fecal samples was about 3-fold higher in rats fed a high-fiber diet than in rats fed the low-fiber diet. During the basal period of the estrous cycle urinary excretion of estrone was lower in the high-fiber group (mean 9.7 ng/day) than in the low-fiber group (mean 13.0 ng/day; $P < 0.05$). It is concluded that wheat bran interrupts the enterohepatic circulation of estrogens, but plasma levels are not affected, suggesting an increased estrogen production rate. Whether the development of mammary tumors is reduced by the introduction of specific components of wheat bran or by a reduced body weight due to a lower (effective) energy intake remains to be determined.

The effects of dietary fiber on estrogen balance, as described in Chapter 4, were studied further in male rats by injection of radioactively labeled estradiol and estrone-glucuronide (Chapter 5). The short-term effects measured in rats fed the high- and low-fiber diets for two days indicated that rats on the high-fiber diet excreted a twice as large an amount of labeled compounds in the feces during the first day after injection. On the other hand, urinary excretion of labeled compounds was significantly higher in the low-fiber groups. The long-term effect, measured after rats had been fed the experimental diets for 3 weeks, resulted in steeper slopes of the fecal excretion profiles of rats fed the high-fiber diet as compared with rats fed the low-fiber diets, indicating an accelerated fecal excretion of labeled compounds in the high-fiber group. It is concluded that, owing to the short-term effect of wheat bran intake, in the first 24 hours after estrogen administration relatively large amounts of radioactively labeled compounds are excreted in feces of rats fed the high-fiber diet. In contrast, excretion is lower in urine of these rats. When the microflora is adapted to the experimental diet the wheat bran diet still results in an accelerated fecal excretion of labeled compounds, which might be attributable to an interruption of the enterohepatic circulation of estrogens.

The interruption of the enterohepatic circulation of estrogens was thought to be caused by binding of estrogens to dietary fiber components. Therefore, an *in vitro* test system was used to study the binding of estrogens to various fibers (e.g. cholestyramin, lignin, cellulose) and fiber sources (e.g. wheat bran, cereals, seeds and legumes; Chapter 6). Furthermore, the *in vivo* apparent digestibility of the different fiber sources was tested using a mobile nylon bag technique in intestine-cannulated pigs. Estradiol-17β (E_2) bound more strongly to the various fibers than did estrone (E_1), estriol (E_3) or estrone-3-glucuronide (E_1-gluc). At increasing pH (> 7) binding of both E_1 and E_2 to wheat bran decreased significantly. Cholestyramine and lignin bound almost all estrogens present in the medium. Linseed (91%), oats (83%), barley chaff (88%) and wheat bran (82%) are also excellent binders of E_2. Corn, rye and white wheat flour showed a lower binding capacity with a relatively low affinity. Cereals with the highest percentage of lignin in the fiber ($> 3\%$) were also the fiber sources with the lowest apparent digestibility. Estrogens bound with the highest affinity (relative to bovine serum albumin) to these fiber sources. Together with wheat bran and lignin, oats, linseed and soybean seem to

be products with good perspectives for *in vivo* evaluation of the lowering effect of dietary fiber on estrogen exposure of estrogen-sensitive tissues.

The experiments described in Chapter 4 indicate that energy intake is an important factor in tumor promotion. For this reason, in subsequent experiments a third group of rats was fed restricted receiving the same amount of energy as the high-fiber group. An animal experiment was performed to investigate the influence of dietary fiber on maturation of young rats, as it is known that dietary fiber affects the balance of hormones involved in onset of puberty and mammary development (Chapter 7). Immature female rats were randomized into 3 groups which were fed ad libitum either a low-fiber diet ($< 0.5\%$ dietary fiber based on white wheat flour; $n = 15$), a high-fiber diet (9.2% dietary fiber based on wheat bran; $n = 15$) or an energy-restricted low-fiber diet providing 90% of the energy of the ad libitum low-fiber diet ($n = 15$). Energy intake in the second and third groups was similar. Wheat bran slightly delayed onset of puberty, whereas restricted energy intake delayed onset of puberty by about 6 days. At 48–58 days of age, 14 rats of the low-fiber group, 10 of the high-fiber group and 7 of the restricted group were in cycle. Development of mammary tissue was rudimentary in rats of the energy-restricted low-fiber group, stronger in the high-fiber group and strongest in the ad libitum low-fiber group. Cell proliferation in mammary tissue was similar for both groups fed ad libitum, but significantly lower in the restricted group. Peroxidase activity, a biomarker for estrogenicity, was lower in the high-fiber group than in the two low-fiber groups.

It is concluded that wheat bran and, even more effectively, an imposed restricted energy intake delays onset of puberty and mammary development. This shortens the time for mammary cells to be initiated to tumor cells and hence reduces the risk for mammary cancer development. It seems that wheat bran acts via a reduced energy intake. However, a role of wheat bran in the delay of mammary development due to a reduced exposure to estrogen cannot be excluded.

The effects of dietary fiber and of a reduced energy intake on exposure of mammary tissue to both estrogens and progesterone as well as on blood plasma levels of these steroids was studied in adult female rats fed the same diets as described in the previous chapter. Four out of 14 rats of the high-fiber group and 4 out of 15 rats of the restricted low-fiber group were not in cycle after 7 weeks on the experimental diets, while rats of the ad libitum low-fiber group were all in cycle. This indicated that estrous cycle was significantly affected by a reduced energy intake. Exposure of mammary tissue to estrogens did not differ among the groups as measured by estrone, estradiol-17β, estriol and peroxidase activity. During the peak period plasma LH levels tended to be higher in the high-fiber group than in the two low-fiber groups, which suggests that estrogen production is elevated in this group. FSH levels were not affected by the experimental diets. Plasma progesterone levels were clearly affected by dietary fiber during the basal period of the cycle. It is concluded that dietary fiber affects hormonal processes involved in breast cancer development. The lower plasma progesterone levels can be an important element in the discussion on the mechanism of the inverse relation between dietary fiber and breast cancer risk.

In the general discussion (Chapter 9) the working hypothesis is adapted. It is hypothesized that the effects of dietary fiber on mammary carcinogenesis vary during a woman's life. The mechanism of protection by fiber (on the basis of cereals) is a complicated ensemble comprising at least a reduced energy intake, a lowered estrogen and progesterone exposure and the anti-estrogenic and anti-carcinogenic properties of particular plant constituents. Risk for tumor initiation is decreased by delayed menarche and mammary development during puberty and adolescence. Tumor promotion can be reduced during the pre- and postmenopausal periods. It is supposed that exposure of mammary tissue to estrogens is reduced during the postmenopausal period and not during the premenopausal period of a woman's life.

It can be concluded that dietary fiber, especially wheat bran, reduces breast cancer risk. The mechanism of protection is a complicated one encompassing least four parameters, i.e. energy, estrogens, progestagens and plant constituents. Which of these parameters is the most important is hard to say, but it is clear that the degree of protection depends on the stage of hormonal development. Risk for tumor initiation might be lowered when a high fiber intake occurs during mammary development, i.e. during puberty and adolescence. Risk for tumor promotion and progression might be lowered during pre- and postmenopausal periods. In the latter period the lowered exposure to estrogen might be an important parameter.

Samenvatting

Epidemiologische en experimentele studies hebben aangetoond dat de ontwikkeling van borstkanker kan worden beïnvloed door voeding. Er is aangetoond dat voedingsvezel, in tegenstelling tot een hoge vetinneming, negatief geassocieerd is met borstkankerincidentie.

Voedingsvezel wordt gedefinieerd als voedingsstoffen die afkomstig zijn van plantecellen en in het maagdarmkanaal onverteerbaar zijn door enzymen van dierlijke (menselijke) oorsprong. Voedingsvezel kan chemisch worden onderverdeeld in polysachariden (cellulose, hemicellulose, pectine) en ligninen. In het maagdarmkanaal zijn bacteriën aanwezig die enzymen kunnen produceren die bepaalde voedingsvezelcomponenten wèl kunnen afbreken (ofwel fermenteren). Zo kan pectine, dat veel voorkomt in groenten en fruit, geheel gefermenteerd worden tot kortketenige vetzuren (azijnzuur, propionzuur, boterzuur) en gassen (waterstof, methaan). Cellulose kan geheel gefermenteerd worden door enzymen van bacteriën die voorkomen in het maagdarmkanaal van runderen en schapen. Daarom zijn grassen, waarin veel cellulose voorkomt, een belangrijke energiebron voor deze dieren. Lignine is chemisch inert en kan steroïden, galzuren en carcinogene stoffen binden.

De fysiologische eigenschappen van voedingsvezel – waterabsorptie; versnelde darmpassage van het opgenomen voedsel; vergroting van de faecesmassa; verlaging van de pH van de darminhoud; binding van steroïden, galzuren en carcinogene stoffen, zodat deze niet via de darmwand kunnen worden opgenomen – zijn sterk afhankelijk van de samenstelling en dus van de oorsprong van de voedingsvezel.

In hoofdstuk 1 worden een aantal parameters besproken die in dit onderzoek regelmatig terugkomen. Dit hoofdstuk is voornamelijk bedoeld als achtergrondinformatie voor het tweede en derde deel van dit proefschrift.

In hoofdstuk 2 wordt het doel van de studie verklaard en wordt een 'werkhypothese' gepresenteerd. De werkhypothese oppert dat voedingsvezel het borstkankerproces beïnvloedt via effecten van voedingsvezel op de oestrogeenbalans. Dit resulteert in een verhoogde faecale oestrogeenuitscheiding, een verminderde oestrogeenuitscheiding via de urine en een lagere plasma-oestrogeenspiegel. Verder wordt geopperd dat, samen met een lagere plasma-oestrogeenspiegel, de blootstelling aan oestrogenen van mammaweefsel van ratten die gevoerd worden met een vezelrijk dieet, lager is dan bij ratten op een vezelarm dieet. De verminderde oestrogeenblootstelling resulteert in een lagere delingssnelheid van voor oestrogenen gevoelig (tumor)weefsel. Bij het gezonde mammaweefsel zal dit resulteren in een verminderde kans op tumorinitiatie. Een eenmaal geïnitieerde cel zal minder gemakkelijk tot een tumor uitgroeien, dus de tumorpromotie wordt verminderd. Een

belangrijke metaboliet bij het oestrogeenmetabolisme met een veronderstelde carcinogene potentie, 16α-hydroxyoestron, werd getest op zijn mutageniteit.

In een *in vitro* model (hoofdstuk 3) waarbij levermicrosomen werden geïsoleerd uit mannelijke ratten en vrouwelijke ratten en muizen, werd aangetoond dat deze levermicrosomen in staat zijn oestron om te zetten in verschillende stofwisselingsprodukten, waaronder 16α-hydroxyoestron. In een mutageniteitsstudie (Ames-test), met 16α-hydroxyoestron als teststof, vertoonden twee van de vijf gebruikte bacteriestammen een 2- tot 3-voudige toename in het aantal histidine-terugmutanten ten opzichte van de controlewaarden. Oestron liet geen mutageen effect zien in de gebruikte test. In dit *in vitro* model werd aangetoond dat vrouwelijke ratten in staat zijn 16α-hydroxyoestron te synthetiseren. Of deze verbinding een risicofactor is voor de ontwikkeling van borstkanker blijft onzeker.

In hoofdstuk 4 wordt de hypothese getoetst dat een vezelrijk dieet tumorpromotie vermindert door onderbreking van de enterohepatische circulatie van oestrogenen, wat resulteert in een verminderde blootstelling aan oestrogenen van weefsel dat gevoelig is voor die stoffen. In het eerste experiment werd de ontwikkeling van door N-nitrosomethylureum (NMU) geïnduceerde mammatumoren onderzocht. Een groep ratten werd gevoerd met een vezelrijk dieet (11% vezel, gebaseerd op tarwezemelen), de andere groep met een vezelarm dieet (0.5% vezel, gebaseerd op witte tarwebloem). Tumorincidentie en -latentie waren in beide groepen gelijk. Het gemiddelde tumorgewicht van ratten op het vezelrijke voer was significant lager dan van ratten op het vezelarme voer (resp. 0.16 en 0.55 g). De tumormultipliciteit (het aantal tumoren per tumor dragend dier) was bij de vezelrijke groep wat lager. Het verschil in tumorlast (het totale tumorgewicht per tumor dragend dier) tussen de groepen verdween na correctie voor het verschil in lichaamsgewicht. In een tweede experiment werden ratten met eenzelfde vezelrijk en vezelarm dieet gevoerd. Van deze ratten, die niet werden behandeld met het carcinogeen, werd gedurende 15 dagen 24-uurs urine en faeces verzameld en werd tevens via een orbitale punctie drie maal, met steeds een tussenperiode van een week, bloed verzameld. De monsters werden gebruikt voor de analyse van ongeconjugeerde (vrije) en geconjugeerde oestrogenen. De excretie in faeces van zowel vrije als geconjugeerde oestrogenen bleek ongeveer 3 maal hoger te zijn bij ratten op het vezelrijke dieet dan bij ratten op het vezelarme dieet. Tijdens de basale periode van de cyclus was de oestronexcretie in urine van ratten op het vezelrijke dieet lager (gemiddeld 9.7 ng/dag) dan in urine van ratten op het vezelarme dieet (gemiddeld 13.0 ng/dag). Geconcludeerd werd dat tarwezemelen de enterohepatische cyclus van oestrogenen onderbreekt. Een verschil in oestrogeenspiegels in plasma werd echter niet gemeten. Dit suggereert een verhoogde oestrogeenproduktie bij ratten op een vezelrijk dieet en een vergelijkbare oestrogeenblootstelling bij beide groepen. De vraag blijft of de ontwikkeling van mammatumoren gereduceerd wordt door de introductie van specifieke componenten via de tarwezemelen of via een gereduceerd lichaamsgewicht als gevolg van een lagere (effectieve) energie-opname.

De effecten van voedingsvezel op de oestrogeenbalans, zoals beschreven in hoofdstuk 4, werden verder bestudeerd in mannelijke ratten door injectie van radioactief gemerkt oestradiol en oestron-glucuronide (hoofdstuk 5). De korte-termijneffecten, gemeten nadat ratten twee dagen met een vezelrijk of vezelarm dieet waren gevoerd, toonden aan dat ratten op het vezelrijke dieet tweemaal zoveel radioactief gemerkte verbindingen in de faeces hadden op de eerste dag na injectie. Aan de andere kant was de uitscheiding van gemerkte verbindingen via urine hoger bij ratten op het vezelarme dieet. Op langere termijn, nadat de ratten drie weken met de experimentele diëten waren gevoerd, resulteerde het vezelrijke dieet in een steilere helling van het profiel voor faecale excretie in vergelijking met ratten op het vezelarme voer. Dit wijst op een versnelde faecale uitscheiding van radioactief gemerkte verbindingen bij de groep op het vezelrijke voer. Geconcludeerd wordt dat de korte-termijneffecten van het dieet met tarwezemelen bestaan uit een verhoogde uitscheiding van radioactief gemerkte verbindingen gedurende de eerste 24 uur na toediening van de gemerkte oestrogenen. Daartegenover staat dat de excretie via de urine bij deze ratten lager is. Als de microflora aangepast is aan het experimentele dieet resulteert het tarwezemelendieet nog steeds in een verhoogde uitscheiding van gemerkte verbindingen via de faeces. Dit zou toegeschreven kunnen worden aan een onderbreking van de enterohepatische circulatie van oestrogenen.

Omdat werd verondersteld dat de onderbreking van de enterohepatische circulatie van oestrogenen werd veroorzaakt door binding van oestrogenen aan voedingsvezelcomponenten, werd een *in vitro* testsysteem gebruikt om de binding van oestrogenen aan verschillende vezelcomponenten (zoals lignine, cellulose) en vezelbronnen (zoals tarwezemelen, granen, zaden; hoofdstuk 6) te bestuderen. Bovendien werd *in vivo* de schijnbare verteerbaarheid van de verschillende vezelbronnen getest. Hiertoe werd een 'mobiele nylon-zaktechniek' gebruikt waarbij de proefdieren (biggen) een canule in de darm kregen. Oestradiol-17β bond sterker aan de verschillende vezels dan oestron, oestriol of oestron-3-glucuronide. Als het incubatiemedium basisch was (pH > 7) was de binding van zowel oestron als oestradiol aan tarwezemelen duidelijk lager. Cholestyramine en lignine bonden nagenoeg alle oestrogenen die in het medium aanwezig waren. Lijnzaad (91%), haver (83%), kaf van gerst (88%) en tarwezemelen zijn uitstekende binders van oestradiol-17β. Maïs, rogge en witte tarwebloem hebben een lagere bindings-capaciteit met een relatief lage affiniteit. Granen met het hoogste percentage lignine in de vezel (> 3%) waren ook de vezelbronnen met de laagste schijnbare verteerbaarheid. Oestrogenen binden met de hoogste affiniteit (gerelateerd aan runderserumalbumine) aan deze vezelbronnen. Samen met tarwezemelen en lignine schijnen haver, lijnzaad en sojabonen goede perspectieven te bieden voor een *in vivo* onderzoek naar het verlagende effect op de blootstelling aan oestrogenen van weefsel dat voor die hormonen gevoelig is.

De experimenten die worden beschreven in hoofdstuk 4 wijzen erop dat de energie-opname een belangrijke factor is bij tumorpromotie. Daarom werd in de vervolgstudies een derde groep ratten toegevoegd. Deze dieren werden onderworpen

aan energierestrictie: ze kregen zoveel van het vezelarme dieet dat hun energie-inneming gelijk was aan die van de groep op het vezelrijke dieet. Een experiment met ratten werd uitgevoerd om de invloed van voedingsvezel op de ontwikkeling van jonge ratten te bestuderen. Het is namelijk bekend dat voedingsvezel de balans beïnvloedt van die hormonen die betrokken zijn bij de aanvang van de puberteit en de mamma-ontwikkeling (hoofdstuk 7). Jonge onrijpe vrouwelijke ratten werden verdeeld in 3 groepen van elk 15 dieren. Ze werden ad libitum gevoerd met een vezelarm dieet ($< 0.5\%$ voedingsvezel gebaseerd op witte tarwebloem), een vezelrijk dieet (9.2% voedingsvezel gebaseerd op tarwezemelen) of een naar energie beperkt vezelarm dieet (90% van de hoeveelheid die door de groep op het ad libitum toegediende vezelarme dieet werd gegeten). De energie-inneming van de tweede en derde groep was gelijk. Tarwezemelen hadden een gering vertragend effect op de aanvang van de puberteit, terwijl een beperking van de energie-inneming een vertragend effect had van ongeveer 6 dagen. Op een leeftijd van 48–58 dagen waren 14 ratten van de vezelarme groep, 10 ratten van de vezelrijke groep en 7 van de energiebeperkte groep in oestrus-cyclus. De ontwikkeling van mamma-weefsel in de energiebeperkte groep bleef achter in vergelijking met die van de vezelrijke groep en in nog sterkere mate bij die van de ad libitum gevoerde vezelarme groep. De delingssnelheid van cellen van het mammaweefsel van de beide ad libitum gevoerde groepen was gelijk, maar duidelijk lager in de energiebeperkte groep. De peroxidase-activiteit, een biomarker voor de oestrogeenblootstelling, was lager in de vezelrijke groep dan in de twee vezelarme groepen. Geconcludeerd wordt dat tarwezemelen een gering en dat energiebeperking een duidelijk vertragend effect hebben op de aanvang van de puberteit en de ontwikkeling van mammaweefsel. Dit verkort de tijd dat mammacellen geïnitieerd kunnen worden tot tumorcellen. Eventueel geïnitieerde cellen kunnen gemakkelijker via een DNA-herstellingsproces ('DNA repair') genormaliseerd worden. Eén en ander reduceert het risico op ontwikkeling van borstkanker. De vertragende werking op de ontwikkeling schijnt het gevolg te zijn van een verminderde energie-inneming. Dat wil evenwel niet zeggen dat een verminderde blootstelling aan oestrogenen door de invloed van tarwezemelen kan worden uitgesloten.

Hoofdstuk 8 beschrijft een onderzoek naar de effecten van voedingsvezel en/of een beperkte energie-inneming op blootstelling van mammaweefsel aan zowel oestrogenen als progesteron en naar de niveaus van deze steroïden in bloedpasma. Volwassen vrouwelijke ratten werden gevoerd met dezelfde diëten als die beschreven in het voorgaande hoofdstuk. Nadat de ratten 7 weken lang het experimentele voer hadden ontvangen, waren 4 van de 14 ratten van de vezelrijke groep en 4 van de 15 ratten op een energiebeperkt vezelarm voer niet in cyclus, terwijl de ratten van de ad libitum gevoerde vezelarme groep alle in cyclus waren. De oestrus-cyclus wordt dus duidelijk beïnvloed door een verminderde energie-inneming. In het mammaweefsel van de 3 groepen waren de concentraties aan oestron, oestradiol-17β en oestriol en de peroxidase-activiteit gelijk, dus de oestrogeenblootstelling verschilde niet tussen de groepen. In het plasma van de ratten op het vezelrijke voer was de concentratie aan luteïniserend hormoon (LH) tijdens de piekperiode gemiddeld hoger dan in dat

duidelijk beïnvloed door een verminderde energie-inneming. In het mammaweefsel van de 3 groepen waren de concentraties aan oestron, oestradiol-17β en oestriol en de peroxidase-activiteit gelijk, dus de oestrogeenblootstelling verschilde niet tussen de groepen. In het plasma van de ratten op het vezelrijke voer was de concentratie aan luteïniserend hormoon (LH) tijdens de piekperiode gemiddeld hoger dan in dat van de twee vezelarme groepen. Dit suggereert dat de oestradiolproduktie hoger was in de vezelrijke groep, waarschijnlijk als gevolg van de negatieve-'feedback'-werking van oestradiol in de hypofyse-gonade-as. De niveaus van het follikelrijpingshormoon (FSH) werden niet beïnvloed door de experimentele diëten. Tijdens de voor progesteron basale periode van de cyclus was de progesteronconcentratie in plasma lager als gevolg van het vezelrijke dieet. Geconcludeerd kan worden dat voedingsvezel de processen beïnvloedt van die hormonen die betrokken zijn bij de ontwikkeling van borstkanker. De lagere progesteronniveaus in plasma kunnen een belangrijk element zijn in de discussie rond de beschermende effecten van voedingsvezel op de ontwikkeling van borstkanker.

In de algemene discussie wordt de werkhypothese aangepast. Verondersteld wordt dat de effecten van voedingsvezel verschillend zijn tijdens de verschillende fasen in het leven van de vrouw. Het beschermingsmechanisme van vezel (op basis van granen) is een gecompliceerd geheel, dat ten minste bestaat uit een verminderde energie-inneming, een lagere blootstelling aan oestrogenen en progesteron en anti-oestrogene en anti-carcinogene eigenschappen van bepaalde plantbestanddelen. Het risico op tumorinitiatie wordt verminderd door een vertraagde menarche en door een vertraagde mamma-ontwikkeling tijdens de puberteit en de adolescentie. Tumorpromotie kan zowel vóór als na de menopauze worden verminderd. Verondersteld wordt dat de blootstelling van mammaweefsel aan oestrogenen wordt verminderd na de menopauze, maar niet vóór de menopauze.

Geconcludeerd kan worden dat voedingsvezel, met name tarwezemelen, het risico op borstkanker vermindert. Het mechanisme achter deze beschermende werking is gecompliceerd en bestaat uit ten minste 4 factoren, te weten energie, oestrogenen, progestagenen en plantbestanddelen. Welke van deze het belangrijkst is, is moeilijk te zeggen. Het is echter duidelijk dat de mate van bescherming afhangt van de fase van hormonale ontwikkeling. Het risico op tumorinitiatie kan verlaagd zijn als tijdens de borstontwikkeling, dus tijdens de puberteit en de adolescentie, voldoende voedingsvezel wordt genuttigd. Het risico op tumorpromotie en -progressie kan worden verminderd vóór en na de menopauze. Na de menopauze kan de verlaagde oestrogeenblootstelling een belangrijke factor zijn.

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