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INFLUENCE OF WHEAT BRAN ON NMU-INDUCED MAMMARY TUMOR DEVELOPMENT, PLASMA ESTROGEN LEVELS AND ESTROGEN EXCRETION IN FEMALE RATS

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Summary—In our animal experiments the hypothesis was tested that a high-fiber (HF) diet reduces tumor promotion by 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 HF 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 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 HF and LF diets. From these rats 24-h 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 urinary excretion of estrone was lower in HF rats (mean 9.7 ng/day) than in LF rats (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. 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 vs omnivorous women [5], and Oriental immigrant women vs 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 (HF) (wheat bran) diets developed fewer mammary tumors than those fed low-fiber (LF) 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 concentrations were lower than in omnivorous women [5, 11]. These observations were ascribed 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

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Abbreviations: E_1, estrone; E_2, estradiol-17β; E_3, estriol; HF, high fiber; LF, low fiber; NMU, *N*-nitrosomethylurea; NDF, neutral detergent fiber; DMBA, dimethylbenz(*a*)anthracene.

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 LF diet vs a HF diet using the direct-acting *N*-nitrosomethylurea (NMU) as the 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, U.K.). 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 LF and HF groups. 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 LF/high-fat and HF/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% fat and rats in the LF group 18% 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 kcal/g), fat (9 kcal/g) and carbohydrate (4 kcal/g) from Atwater and Bryant [17].

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 and after 3 weeks on the experimental diets, 20 (of 31) animals of

Table 1. Composition of the diets [% w/w unless indicated otherwise]

			Nutrient composition analyzed		
Ingredients added	LF diet	HF diet	Ingredient	LF diet	HF diet
Casein	22.53	15.69	Protein[a]	26.5	23.6
White wheat flour	54.72	42.00	Fat[a]	18.4	15.0
Wheat bran	—	23.75	Carbohydrate[a]	38.5	34.2
Mineral mixture[b]	4.24	3.63	Dietary fiber[c]	0.5	10.9
Vitamin ADEK mixture[d]	0.36	0.31	Moisture	10.6	10.7
Vitamin B mixture[e]	0.24	0.20	Ash	3.9	4.8
Lard	8.96	7.21	Vit. A (IU/kg)[a]	7000	5000
Sunflower oil	8.96	7.21	Vit. C (mg/kg)[a]	150	125
Energy (MJ/kg)	17.71	15.26	Vit. E (mg/kg)[a]	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]Analyzed according to Speek *et al.* [49, 50].
[b]In mg per g mixture: KH_2PO_4 399; $CaCO_3$ 389; NaCl 142; $MgSO_4$ 58; $FeSO_4.7H_2O$ 5.7; $ZnCl_2$ 0.9; $CuSO_4.5H_2O$ 0.8; $MnSO_4.2H_2O$ 4.6; $CoCl_2.6H_2O$ 0.02; KI 0.007; and KCr $(SO_4)_2.12H_2O$ 0.08.
[c]Insoluble dietary fiber analyzed according to Hellendoorn *et al.* [51].
[d]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; and wheat starch 30.0 mg.
[e]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; and choline chloride (50%) 931.
[f]Fatty acid composition expressed as % w/w of the fatty acid methyl esters present.

both the LF and HF groups received a single i.v. injection into the tail vein with NMU (Sigma, St Louis, MO, U.S.A.; 50 mg/kg body wt, 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 a.m. 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 Co. The tritiated compounds [2,4,6,7-^{3}H]E_2, [2,4,6,7-^{3}H]E_1 and [2,4,6,7,-^{3}H]E_3, with a sp. act. 105 Ci/mmol each, were purchased from Dupont (New England Nuclear), 's-Hertogenbosch, The Netherlands. Antisera against the estrogens were raised in rabbits against E_2-6-CMO-BSA, E_1-6-CMO-BSA and E_3-6-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, The 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 previously [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, The Netherlands). The cartridges were successively washed with water and 50% methanol. Estrogens were eluted with 100% methanol and estimated using 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 previously [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 the pH was measured. For β-glucuronidase activity 0.5–1.5 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].

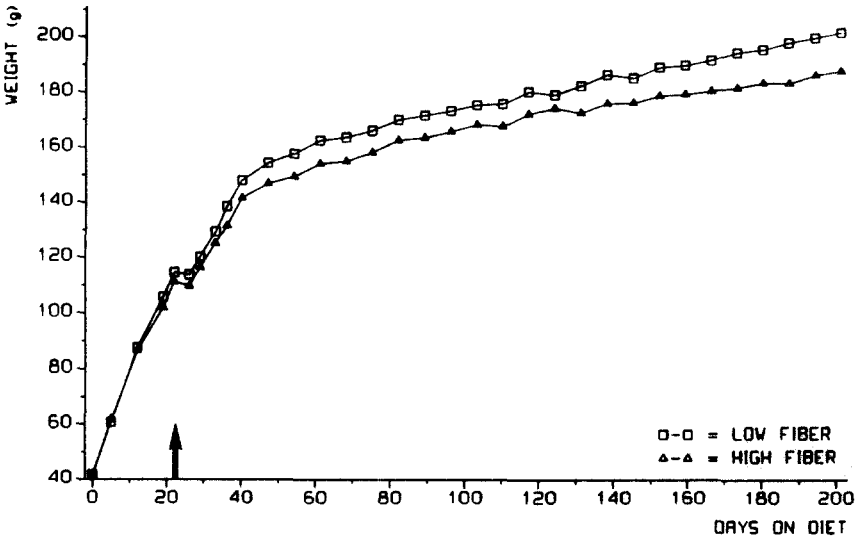

Fig. 1. Mean body weight of two groups of rats fed the LF or the HF diet. The arrow indicates the time of NMU injection.

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.

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

Tumor weights were analyzed with hierarchical analysis of variance using the method of Gower [24].

Tumor multiplicity was analyzed with a chi-square 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.

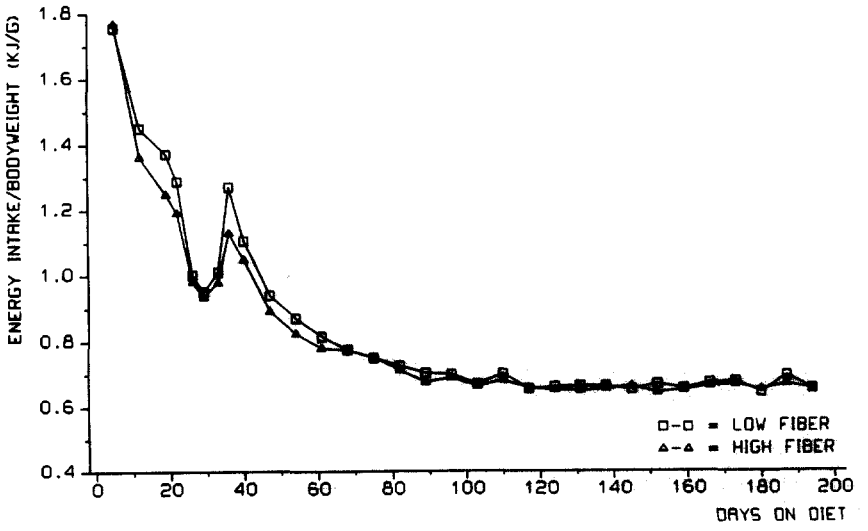

Fig. 2. Energy intake of the LF and the HF group relative to body weight.

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%)	—

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 the study) 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, due to 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), which can be explained by the slightly higher mean body weight of the animals of the LF group (Fig. 1). When body weight was taken into account, relative energy intake 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 did not reveal gross changes in the major organs or organ systems in the other animals.

Tumor incidence, multiplicity and latency

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

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	NS[a]
Latency (days)	121	128	NS
Tumor multiplicity (*n*)	1.8	2.8	NS
Number of rats with:			
0 tumour	2	4	
1 tumour	8	7	
2 tumours	7	2	
3 tumours	2	3	
4 tumours	1	0	
5 tumours	0	2	
6 tumours	0	1	
7 tumours	0	1	

[a]NS = non-significant

Mean tumor multiplicity was 2.8 in the LF group and 1.8 in the HF group at the end of the study. The ratio between the animals bearing 1, 2, 3, and >3 tumors did not differ between the experimental groups ($P > 0.10$).

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 significantly 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.

Mean tumor burden (total weight of tumors per tumor-bearing animal) was 0.48 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 weight and tumor burden were 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.

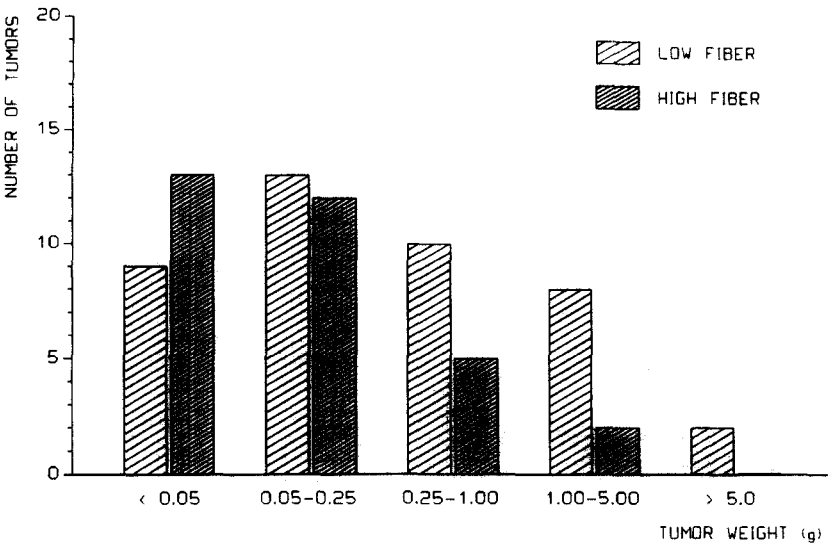

Fig. 3. Weight of tumors (classified into weight categories) in rats on the LF and HF diets.

The pH of fecal samples of the HF group (7.9 ± 0.4) was significantly ($P < 0.02$) lower 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 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 significant cycle effect was obtained for plasma E_2 levels ($P < 0.001$). In samples from the HF group collected during the peak period a significantly ($P = 0.02$) 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 levels

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).

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

	Cycle	HF group	LF group	SED	*P*
Total E_1	OD_1[a]	0.26	0.26	0.04	NS[b]
	D_2P[c]	0.29	0.29	0.03	NS
Unconjugated E_2	OD_1	0.010	0.012	0.008	NS
	D_2P	0.042	0.029	0.006	0.02

[a]Estrus, metestrus and 1st day of diestrus (OD_1) were considered the basal period of the cycle ($n = 16$).

[b]NS = non-significant.

[c]The 2nd day of diestrus and proestrus (D_2P) were considered the peak period of the cycle ($n = 8$).

Table 5. Mean 24-h urinary excretion of total E_1, E_2 and E_3 in untreated rats fed a HF or a LF diet (ng/day per rat)

				SED		P^a	
	Cycle	HF group	LF group	Within[b]	Between[c]	Within	Between
E_1	Basal[d]	9.7	13.0	1.641	1.444	< 0.001	0.035
	Peak	28.2			30.7		
E_2	Basal[e]	2.01	1.94	0.206	0.193	< 0.001	0.89
	Peak	4.02			4.05		
E_3	Basal[e]	16.1	18.3	1.936	1.577	< 0.001	0.26
	Peak	33.3			34.7		

[a]No significant interaction was found between diet (high vs low) and cycle (basal vs peak period).
[b]The difference as a result of the cycle within the rat.
[c]The difference as a result of diet between the two groups of rats.
[d]$n = 3 \times 11$ values for each period, and
[e]$n = 2 \times 11$ values for each period.

Fecal estrogen levels

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.

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 E_1 and of both unconjugated and conjugated E_2 and E_3 were significantly higher 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 E_1 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 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 contains 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]. Caloric 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 the tumor

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

	HF group[a]		LF group[b]		SED		P	
	ng/g	ng/24 h	ng/g	ng/24 h	ng/g	ng/24 h	ng/g	ng/24 h
E_1								
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
E_2								
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
E_3								
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

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

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 E_1 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 E_1 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 E_2 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 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 constant stimulation to produce gonadotrophins. If a HF diet indeed increases estrogen production, then this may contribute to the inhibition of breast tumor development by decreasing plasma estrogen levels, particularly in post-menopausal women, whose ovarian estrogen production is negligible and who lack the 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 HF 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 phytoestrogen [43, 44] exposures or by other fiber-related compounds such as bioflavonoids [29, 45–47] introduced in the rat by 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 study results suggest that this is not likely to be explained by 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.

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