Nonsteroidal estrogens of dietary origin: possible roles in hormone-dependent disease[1,2]

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ABSTRACT Equol, a nonsteroidal estrogen of dietary origin, was recently identified in human urine, and is excreted in amounts comparable to the classical steroidal estrogens. We confirm here that phytoestrogens which are abundant in dietary soya protein are converted by human gastrointestinal flora to this weak estrogen. After the ingestion of meals containing cooked soya protein the urinary excretion of equol in four of six subjects studied increased by up to 1000-fold and this compound was the major phenolic compound found in the urine. These data also indicate that some subjects are unable to either produce or excrete equol despite the challenge of a diet containing soya. In view of the increasing use of commercial soya products in the diet and the capacity of human bacterial flora to synthesize this weak estrogen from the abundance of phytoestrogens in soya, the potential relevance of these observations to the diseases implicating steroid hormones is discussed. *Am J Clin Nutr* 1984;40:569–578

KEY WORDS Soyabean, dietary estrogens, phytoestrogens, gut bacterial metabolism, urinary equol, menstrual cycle disorders, breast cancer

Introduction

Our recognition of the presence of relatively large quantities of a variety of phenolic compounds in biological fluids (1–3) has aroused interest in the potential biological and physiological importance of this type of compound in man (4).

Several of these phenolic compounds, which are excreted in quantitites much greater than the classical estrogenic (phenolic) steroids, were recently identified as belonging to the chemical class called lignans (2, 3, 5). As a consequence of our studies on lignans it was apparent that in addition to estrogens, several other phenolic compounds were present in biological fluids, and structural elucidation studies revealed the identity of one of these to be equol, [7-hydroxy-(4′-hydroxyphenyl)chroman] (6).

Equol, a phytoestrogen that is structurally similar to estradiol-17β, (**Fig 1**) was first discovered in the urine of mares (7) and later found in urine from goats (8), cows (9), hens (10, 11), and sheep (12, 13) but had never previously been identified in man. Of particular significance, is the fact that this phytoestrogen possesses weak estrogenic activity (12–14), while also behaving as an antiestrogen in exhibiting a competitive binding with estradiol-17β for uterine cytosol receptors (14, 15). It was subsquently shown to be the "contraceptive" agent responsible for an infertility syndrome, referred to as "Clover disease," which was widespread in Australian agricultural animals (16–18).

Equol is not present in plants in significant quantities, but other phytoestrogens related in structure, such as formononetin, daidzein, and genistein (Fig 1) are found extensively throughout the plant world (19). In animals, equol is formed in the gastrointestinal tract as the result of the bacterial degradation of these phytoestrogens which are ingested in relatively large quantities in the feed (13, 20–24), and data presented here

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Received December 19, 1983.

Accepted for publication May 1, 1984.

confirm that equol may be formed similarly in man.

From previous observations, soya was found to be a foodstuff rich in precursors which could be readily converted into the weak estrogen equol (6, 24, 25). In view of the increasing use of soyabean as a protein food source in the Western world, and the general acceptance of diet as a major factor in disease, the role of biologically active phenolic compounds, such as phytoestrogens (26, 27) and lignans (2–5), which are present in our diet (25, 28), requires future consideration. The potential relevance of exposure to phytoestrogens in patients with menstrual cycle irregularities, infertility, and breast cancer, and in infants is discussed.

Experimental

Subjects

Six healthy laboratory personnel, four men and two women (age range 22 to 39 yr) were studied. Urine samples (24 h) were collected over a 14-day period, the volumes recorded, and aliquots taken and stored at −20°C. After the first 3 days of "normal" unrestricted diet, soya was substituted at one main meal for a period of 5 consecutive days. Textured soya (Natural Protoveg) was obtained from Direct Foods Ltd, Petersfield, Hants, UK and its chemical composition consisted of 52% protein, 3.5% carbohydrate, and 1% fat. Each subject received 40 g dry weight per day which was cooked according to the manufacturers instructions. On day 9 of the study the diet reverted to normal.

In vitro incubations with fecal flora

One g of freshly voided feces was added to each of a 9-ml volume of sterile distilled water, trypticase soy broth (BBL) with the addition of 1 g of soy protein, and brain-heart infusion broth (Difco). Uninoculated broths served as negative controls. All broths were incubated anaerobically for 3 days at 37°C. Postincubation the sample was treated as described below.

Determination of equol

The technique for the determination of equol in urine samples and fecal incubations is described in detail elsewhere (6, 24, 25) and is briefly outlined.

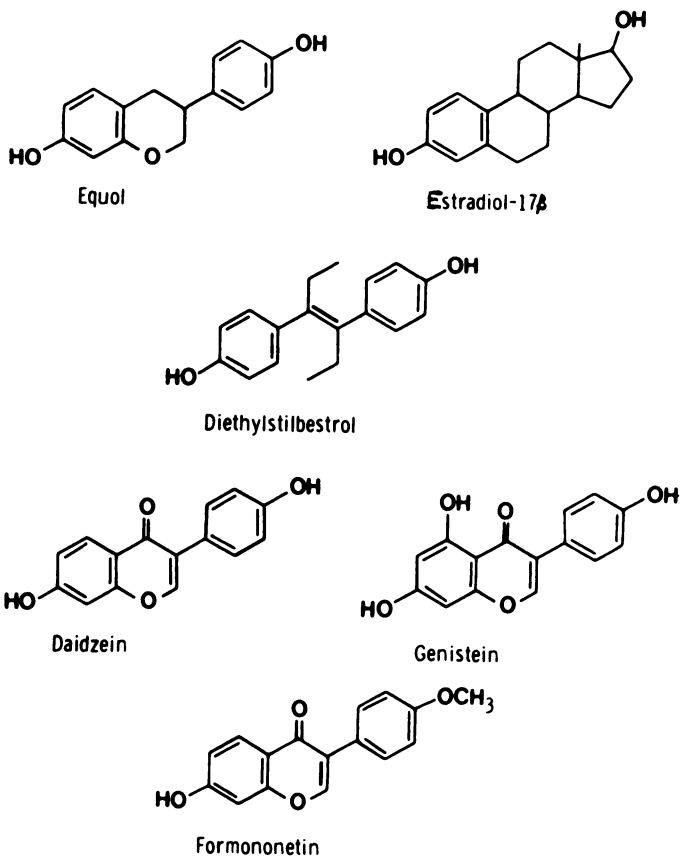

FIG 1. A comparison of the chemical structures of *1*) the phytoestrogen equol, formed in the gastrointestinal tract of man and animals, *2*) estradiol-17β, *3*) the potent synthetic oestrogen, diethylstilbestrol, and *4*) several phytoestrogens of plant origin.

Urine. Equol, which is present in urine as the glucuronide conjugate (6, 24, 25), was extracted together with steroids and lignans using reverse phase octadecylsilane bonded silica cartridges. After hydrolysis of the conjugate by a β-glucuronidase preparation (*Helix pomatia*) and reextraction using the same extraction procedure, equol was isolated and purified by gel chromatography using either a straight phase partition system on Sephadex LH-20 (29) or by ion-exchange on a lipophilic ion exchange gel, TEAP-Sephadex-LH-20 (24). 5α-Androstane-3β,17β-diol was added as an internal standard and the fraction containing equol was converted to the trimethylsilyl ether derivative. Quantification of equol was performed by gas chromatography (24) with flame ionization detection or by selected ion monitoring gas chromatography mass spectrometry using the ions m/z 386 and 346, which are characteristic ions in the mass spectra of the trimethylsilyl ether derivatives of equol (6) and 5α-androstane-3β,17β-diol, respectively. Authentic samples of equol were obtained from the MRC Steroid Reference Collection, Westfield College, London and from Prof H Adlercreutz (Department of Clinical Chemistry, Meilahti Hospital, University of Helsinki, Finland).

Fecal cultures. The incubation sample (2 ml) was diluted with an equal volume of distilled water and centrifuged at 3500 rpm for 5 min. The supernatant was passed through a cartridge of reverse phase octadecylsilane bonded silica to extract equol. Equol was recovered from the cartridge with methanol, isolated by gel chromatography, and analyzed by gas chromatography as the trimethylsilyl ether derivative after addition of the internal standard as described above.

Results

The gas chromatographic analysis of the urine from one of the subjects (JG) before and 3 days after the ingestion of soya protein, is compared in **Figure 2**. Before soya ingestion, equol was not detectable in urine (<5 μg/day), whereas after soya it became the major component of the phenolic fraction. Its excretion in the urine from this subject was 5.3 mg/day at this time, which is considerably greater than the two recently discovered phenolic lignans, enterolactone and enterodiol (1–5), and vastly in excess of any of the urinary estrogens, which could not be detected by gas chromatography.

The urinary excretion of equol in four of the six healthy adults studied, before, during, and after the ingestion of cooked soya protein (40 g/day) is shown in **Figure 3**. Basal levels of equol ranged from undetectable (<5 μg/day) to a maximum of 80 μg/day. After the consumption of soya, four of the six subjects (two men and two women) showed a marked increase in the urinary excretion of equol within 1 day, with the excretion increasing by 50- to 1000-fold. The maximum urinary excretion of equol ranged from 3.5 to 7.0 mg/day during the period of soya ingestion. After the diet reverted to normal and in the absence of any known source of soya, the urinary excretion of equol gradually returned to <100 μg/day within several days. In two of the subjects, both men, equol was not detected in the urine and a challenge of soya appeared to have no effect on equol excretion in one of these subjects and a negligible effect in the other (subject MM, Fig 3).

In vitro incubation of soya protein with the fecal flora obtained from one of these subjects (JG) confirmed their ability to form equol. Equol was only found in the soya broth after incubation with fecal bacteria. A comparison of the gas chromatographic analysis of the phenolic compounds isolated from a sample of (*a*) stool + diluent + soya and (*b*) stool + diluent is shown in **Figure 4**. For reference (*c*) authentic equol and the internal standard, 5α-androstane-3β,17β-diol are shown. Equol was not detectable in the sample of stool analyzed but was the major component after coincubation of the stool with a soya rich broth.

Discussion

Our earlier studies have shown that in addition to estrogens in biological fluids, there are many other phenolic compounds (1–4, 6) and that these are excreted in much higher concentrations than the classical steroidal estrogens (30, 31).

We recently reported the identity of two novel diphenolic compounds (2), enterolactone [2,3-bis(3-hydroxybenzyl)butyrolactone] and enterodiol [2,3-bis(3-hydroxybenzyl)butane-1,4-diol], which are chemically classified as lignans, and after these studies an isoflavan, equol, which is also diphenolic in structure was identified (6). Equol is classified as a phytoestrogen and possesses weak estrogenic activity, of the order of 10^{-3} of that of estradiol-17β (14, 15). Structurally these compounds are similar to estradiol-17β (Fig 1) and the potent estrogen, diethylstilbesterol (DES) in possessing a phenyl substituent which is considered as one of the

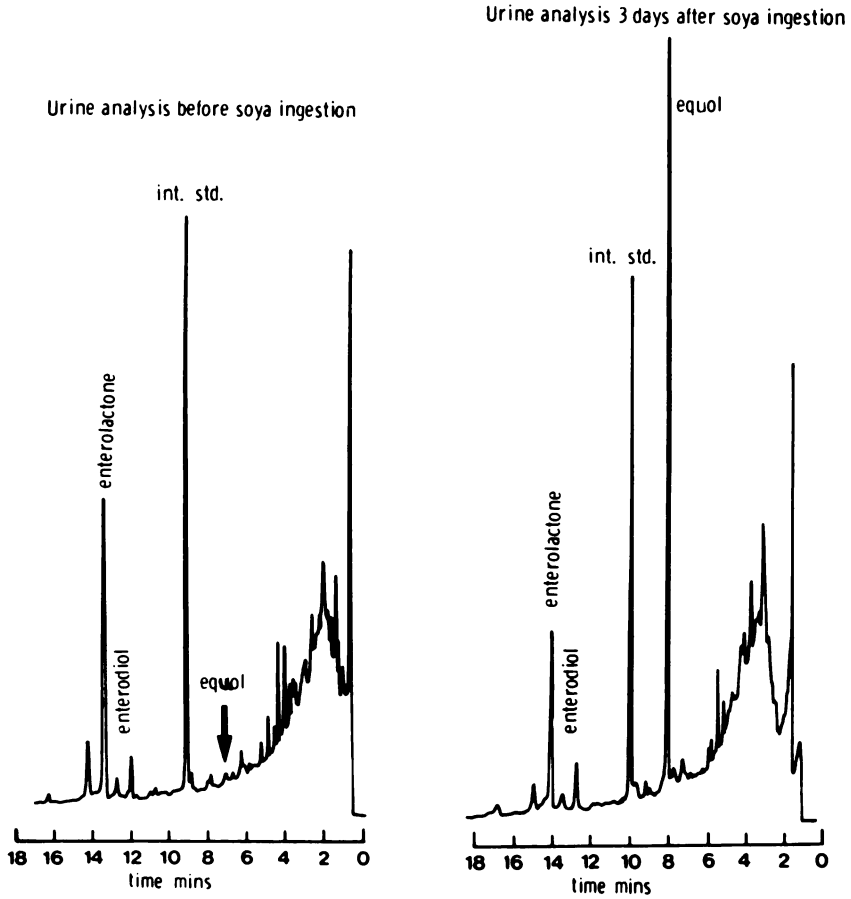

FIG 2. A comparison of the gas chromatograms of the trimethylsilyl ether derivatives of phenolic compounds isolated from the urine of an adult before and after the ingestion of 40 g of cooked soya protein. Gas chromatography was carried out on a 25 m chemically bonded silicone SE-30 column using isothermal operation at 250°C. 5α-Androstane-3β,17β-diol was the internal standard added and the identity of equol was confirmed by gas chromatography–mass spectrometry by comparison with the authentic compound.

prerequisites for estrogenic activity (32). In the last decade considerable attention has focussed on the potential deleterious effects in humans of the use of DES as a growth promoter in animal feed (33, 35). This follows the adverse effects reported after the therapeutic use of the drug for prostatic cancer (36–38) and of its carcinogenicity when administered in low doses in animals (39–41).

Although there are no reports of the effects in humans of DES in food, until its withdrawal from use in the Western world, animal tissue was strictly monitored at levels as low as 1 ppb. The level of naturally occurring nonsteroidal estrogens in many foods is substantially higher than the concentration of DES in animal tissues and the implications of this, as far as human disease is concerned, requires careful consideration, particularly when it is becoming increasingly accepted that many diseases common to the Western world are associated with dietary factors (42).

Our earlier studies using rats (6, 24) revealed that equol was excreted in urine and bile in amounts in excess of the lignans enterolactone and enterodiol. These studies also proved that, in rats, equol was formed by gut bacteria and that it undergoes an enterohepatic circulation, in common with many endogenous compounds, including estrogens (43, 44). In animal experiments designed to determine the source of precursors

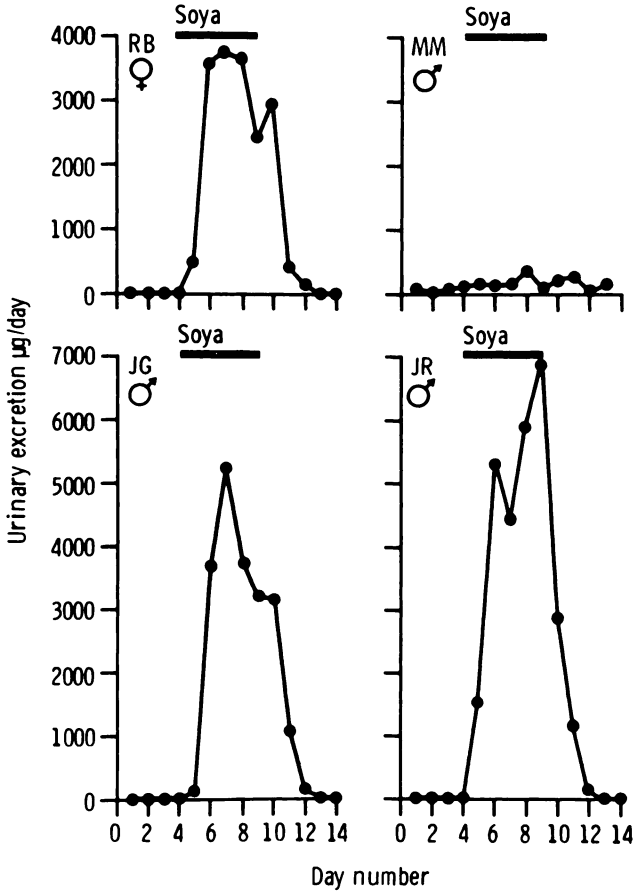

FIG 3. The urinary excretion (μg/day) or equol in four subjects over a 14-day period in which cooked soya protein (40 g) was ingested for 5 consecutive days.

to these compounds (25, 28) it was found that when soyabean was fed to rats a marked increase in the urinary excretion of equol occurred (6, 25). Data presented here confirm that soya is rich in precursors which are also used by human gut flora to give rise to substantial quantitites of equol. In vitro studies establish the ability of human fecal microflora to perform the necessary reactions to degrade phytoestrogen precursors to equol, however, in vivo it was found that only four of the six subjects studied excreted equol in the urine. The reason for this is unknown, and in view of the limited number of subjects studied, it is impossible to determine what proportion of the population are "nonresponders." Since both males and females are capable of excreting large quantities of equol after soya ingestion it would appear to be sex independent. The rate of formation of equol from daidzein, the phytoestrogen precursor in soya (25), is presumably influenced by the composition of intestinal microflora, the intestinal transit time, and alterations in the redox level in the large intestine, factors that may be strongly influenced by diet.

The urinary excretion of equol in humans consuming diets with no obvious major source of soya protein is less than 80 μg/day and this is in accord with levels reported recently (6, 45). After the consumption of a single meal consisting of 40 g of soya each day for 5 consecutive days the peak urinary excretion of equol exceeded 3.5 mg/day, representing in some cases an increase of up to 1000-fold in this weak estrogen. Compared with levels of the principal urinary estrogen, estrone-glucuronide, which in the follicular phase of women is excreted in

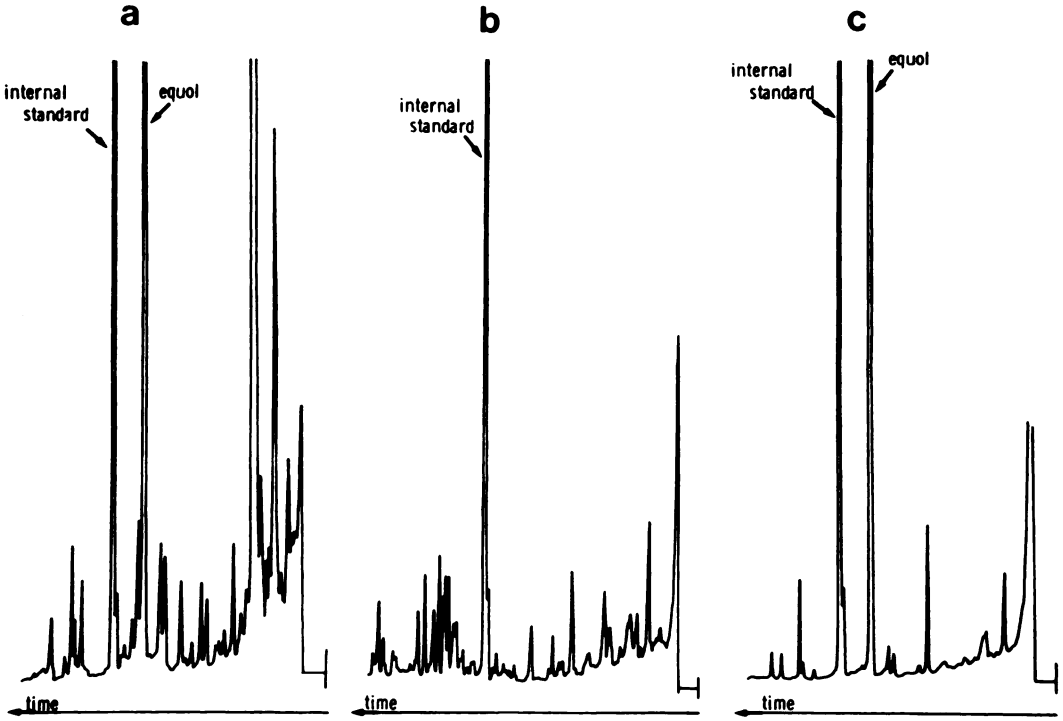

FIG 4. Gas chromatograms of the trimethylsilyl ether derivatives of phenolic compounds isolated from (*a*) human fecal flora, diluent, and soya protein, and (*b*) human fecal flora and diluent after a 3-day incubation period. For comparison, the trimethylsilyl ethers of an authentic sample of equol and the internal standard are shown in chromatogram (*c*). Gas chromatography was performed using a 25 m chemically bonded silicone SE-30 column and temperature programmed conditions from 185 to 275°C in increments of 2°C/min.

amounts ranging from 2 to 27 μg/day (29, 30), equol excretion after the ingestion of soya protein is significant and raises the question of the physiological relevance of a large amount of this weak estrogen.

The presence of phytoestrogens in soyabeans has been recognized for some time (46–48) and its estrogenicity has been reported (49) after observing that cake containing soya produced uterotrophic effects in rats (50). Daidzein and genistein (Fig 1) have both been reported to occur in soyabean in substantial amounts (46–48) [although we have been unable to confirm the presence of the latter phytoestrogen in the preparations of soya used in these studies (25)], and it was suggested that because of the estrogenicity of these compounds, soya cake might be as beneficial as diethylstilbestrol as a growth promoter in agricultural animals (48).

Our earlier finding of large amounts of equol in the urine of rats (24) and our recent identification of daidzein as the major phytoestrogen in soya flour (25) indicate that daidzein is converted by gut microflora into the more potent estrogen equol. The effects reported earlier in rats were therefore probably induced by equol rather than genistein and/or daidzein as was originally suggested (49).

In view of the various reproductive disorders in animals that have been associated with the ingestion of a variety of phytoestrogens (16, 18, 49–52), consideration should be given to the possible effects in man of the large quantities of equol that are derived from the ingestion of soyabean products. There may be some value therefore, in assessing the dietary status and determining the levels of phytoestrogens in biological fluids of patients with menstrual cycle disorders and in cases of infertility where there are no obvious physiological abnormalities. The potential value of plants as sources of antifertility agents (19, 53) gained impetus

after reports of the "contraceptive" action of equol in Australian sheep grazing on *Trifolium subterraneum*, a species of clover containing large quantities of the phytoestrogen, formononetin (16) (Fig 1). Furthermore a reduction in sperm count has been reported in sheep grazing for prolonged periods on this clover (54). In view of these effects in animals, it is conceivable that similar actions may occur in humans consuming diets rich in phytoestrogen precursors, provided that the intestinal flora responsible for their conversion to equol are both present and active.

Breast cancer, one of the most common causes of death from cancer in women, has long been known to be associated with hormone activity (55), but diet is also suspected to be a major factor in its etiology (42, 56, 57). Animal models, such as the 7,12-dimethyl-bene(α)anthracene-induced rat mammary carcinoma which is estrogen dependent (58), have helped in the understanding of the hormone dependence of mammary tumors, and estrogen receptors similar to those found in the uterus and vagina have been found in human breast cancer tissue (59, 60). Although the mechanism of action of estrogens on tumor growth is not fully understood, estrogen receptors are sensitive to both estrogens and antiestrogens (32). Estrogens bind to the appropriate cytoplasmic receptor, eliciting its translocation to the nucleus with subsequent retention of the estrogen-receptor complex. These events lead to an increase in RNA synthesis, which in turn results in protein synthesis and cell growth. Conversely, antiestrogens are considered to exert their effect by decreasing the cytoplasmic estrogen-receptor concentration (61), thereby producing an insensitivity of the target tissue to estrogen stimulation (62) and by forming complexes with the receptor preventing the initiation of biosynthetic events leading to tissue growth.

Several phytoestrogens have been demonstrated to inhibit the binding of estradiol-17β to uterine cytosol receptors (14, 15, 26) indicating that structurally a steroid nucleus is not essential for binding to the estrogen receptor. Structurally, the distance between the C-3 and C-17 hydroxyl groups of estradiol-17β is similar to that of the two hydroxyl groups of the aromatic rings of phytoestrogens (compare structures in Fig 1), a factor that is esential for strong binding to the estrogen receptor. The effect of equol in binding to the receptor in the nucleus but failing to stimulate DNA synthesis to the same degree as estradiol-17β, is supportive of an antiestrogenic role for this phytoestrogen (14).

As far as mammary tissue is concerned, several phytoestrogens have been shown to bind to estrogen receptors of human breast cancer cells (63). However, to our knowledge equol has not been tested but the plant phytoestrogens, coumesterol, commonly found in legumes and zearalanol, a mycotoxin that contaminates grains, have been shown to bind competitively to the estrogen cytosol receptor of both rat and human mammary tumour tissue (27, 63). Estrogens exert dose dependent dual effects upon tumour induction and growth. High doses inhibit tumour development and suppress growth (64, 65) while physiological doses stimulate growth of human tumour cells (66, 67). The significance therefore of naturally occurring phytoestrogens, which may also exhibit a similar dual role and which are consumed in our diet and synthesised in the gut, to the etiology of human breast cancer or its therapy is not known. In view of the affinity of these diphenolic compounds for estrogen receptors, the effects of exposure to high levels of a compound such as equol, which as we have demonstrated can occur after the ingestion of soyabean products, require examination. So while we hypothesize that repeated soya consumption in man may result in reproductive disorders due to the estrogenic effects of equol or other phytoestrogens, similar to its action in animals, conversely, its antiestrogenic effects may be beneficial with respect to breast cancer development or in its treatment.

Finally, with the recent introduction of soya milk products for infant feeding it would be of interest to determine how the newborn infant handles and metabolizes the phytoestrogens which are present in large quantities in the soya [25, 46–49]. Although the gut is sterile at birth, during the 1st wk of life it rapidly develops a bacterial flora (68). Whether in early life the bacterial enzymes which in adults are responsible for

the conversion of daidzein-glycoside in soya to equol are present, remains to be established. If so, the infant may be subjected to concentrations of this weak estrogen which are well in excess of endogenous estrogen levels, and if not, it is probable that the precursor, daidzein, itself a weak estrogen, may be absorbed and excreted in the urine as the glucuronide conjugate, as shown in adults (25, 69). Therefore, the potential effects of subjecting infants as well as adults to relatively large amounts of dietary phytoestrogens remains to be evaluated.

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