

Isoflavone content of infant formulas and the metabolic fate of these phytoestrogens in early life¹⁻⁴

Kenneth DR Setchell, Linda Zimmer-Nechemias, Jinnan Cai, and James E Heubi

ABSTRACT Soy-based infant formulas have been in use for >30 y. These formulas are manufactured from soy protein isolates and contain significant amounts of phytoestrogens of the isoflavone class. As determined by HPLC, the isoflavone compositions of commercially available formulas are similar qualitatively and quantitatively and are consistent with the isoflavone composition of soy protein isolates. Genistein, found predominantly in the form of glycosidic conjugates, accounts for >65% of the isoflavones in soy-based formulas. Total isoflavone concentrations of soy-based formulas prepared for infant feeding range from 32 to 47 mg/L, whereas isoflavone concentrations in human breast milk are only $5.6 \pm 4.4 \mu\text{g/L}$ ($\bar{x} \pm \text{SD}$, $n = 9$). Infants fed soy-based formulas are therefore exposed to 22–45 mg isoflavones/d ($6\text{--}11 \text{ mg} \cdot \text{kg body wt}^{-1} \cdot \text{d}^{-1}$), whereas the intake of these phytoestrogens from human milk is negligible (<0.01 mg/d). The metabolic fate of isoflavones from soy-based infant formula is described. Plasma isoflavone concentrations reported previously for 4-mo-old infants fed soy-based formula were 654–1775 $\mu\text{g/L}$ (\bar{x} : 979.7 $\mu\text{g/L}$; Lancet 1997;350:23–7), significantly higher than plasma concentrations of infants fed either cow-milk formula ($\bar{x} \pm \text{SD}$: $9.4 \pm 1.2 \mu\text{g/L}$) or human breast milk ($4.7 \pm 1.3 \mu\text{g/L}$). The high steady state plasma concentration of isoflavones in infants fed soy-based formula is explained by reduced intestinal biotransformation, as evidenced by low or undetectable concentrations of equol and other metabolites, and is maintained by constant daily exposure from frequent feeding. Isoflavones circulate at concentrations that are 13 000–22 000-fold higher than plasma estradiol concentrations in early life. Exposure to these phytoestrogens early in life may have long-term health benefits for hormone-dependent diseases. *Am J Clin Nutr* 1998;68(suppl):1453S–61S.

KEY WORDS Phytoestrogens, isoflavones, soymilk, infants, estrogens, genistein, soy-based formula

INTRODUCTION

The increasing awareness that diet plays an important role in many of the common diseases that afflict Western populations (1) has led to the recognition that there are many classes of bioactive nonnutrients in foods that may play a beneficial role in disease prevention. Among these components are the dietary estrogens, or phytoestrogens, of the isoflavone class (2–5). These nonsteroidal estrogens have structural homology to

steroidal estrogens and are found in relatively high concentrations in soybeans and all soy-protein products (6–8). The list of biological properties associated with isoflavones is vast and includes both hormonal and nonhormonal actions.

In addition to behaving as an estrogen agonist and antagonist (3–5), one of the isoflavones, genistein, which is abundant as glycosidic conjugates in soybeans (9), is a potent inhibitor of tyrosine kinases (10) and can interfere with cell signal-transduction pathways (11). The myriad of biological properties associated with isoflavones provide plausible explanations for mechanisms whereby a diet containing these bioactive dietary estrogens may be of benefit in preventing many hormone-dependent diseases, including cancer, osteoporosis, and cardiovascular disease (2, 3, 12, 13). The hypocholesterolemic action of soy protein is well established (14), although the effect is not entirely attributable to the presence of isoflavones. Although there is convincing evidence from many *in vitro* studies (15, 16) and from studies of classic animal models of chemically induced breast cancer (17–19) that isoflavones have anticancer effects, conclusive data supporting a role for phytoestrogens in cancer prevention in humans are scant (20). However, recent studies in humans showed that a diet containing soy protein causes significant modifications to the menstrual cycle, including a prolongation in cycle length and a suppression of the usual midcycle surge in pituitary gonadotropins (21), effects that may be beneficial in reducing risk for breast cancer. These endocrine effects are not surprising given the high urinary and plasma concentrations of isoflavones relative to estradiol found in adults ingesting modest amounts of soy-protein foods (12, 22–25). Interestingly, all of the above effects are abolished when soy proteins are devoid of isoflavones (17, 26), and these observations have spurred considerable consumer interest in the health benefits and utilization of soyfoods in the Western diet.

¹From the Clinical Mass Spectrometry Center, Children's Hospital Medical Center, Cincinnati.

²These studies were published in part previously in Lancet 1997;350:23–7.

³Supported in part by funding from Wyeth Laboratories, Philadelphia; Protein Technologies International Inc, St Louis; and the National Institutes of Health (grant M01 RR-08084 for support of the General Clinical Research Center of Children's Hospital Medical Center, Cincinnati).

⁴Address reprint requests to KDR Setchell, Clinical Mass Spectrometry Center, Children's Hospital Medical Center, 3333 Burnet Avenue, Cincinnati, OH 45229. E-mail: setck0@chmcc.org.

Although most study is devoted to the beneficial effects of phytoestrogens, their potential for causing deleterious or toxic effects requires consideration. Several examples of negative effects of mature animals consuming relatively large quantities of dietary estrogens have been reported (27–29). The etiology of reproductive dysfunction in clover disease in sheep (27) and venoocclusive disease in captive cheetahs (29) was attributed to the ingestion of diets containing an abundance of isoflavones. However, species differences in metabolic handling or the huge doses and prolonged exposure account for these negative effects. These findings and concerns about the possible effects of environmental toxins, such as polychlorinated biphenyls and structurally related compounds (30, 31), not surprisingly have led to questions regarding the safety of dietary estrogens (32).

Although there is little evidence to suggest that ingestion of isoflavones in amounts consistent with those present in most soy-protein foods (0.1–4.0 mg/g) has any adverse effects in humans, the potential for these compounds to create steroid hormone imbalances or to compete for the normal steroid, drug, and xenobiotic metabolizing enzymes is presently unknown. Many issues still need to be addressed. It is unclear whether patients with menstrual cycle disorders, endometriosis, or estrogen-receptor-positive breast cancer may be disadvantaged by a diet rich in weakly estrogenic compounds. Furthermore, the recent trend in the commercialization of dietary supplements of soy isoflavones, with the potential for self administration of large doses, is cause for concern, especially given the paucity of data on dose-response effects and safety of phytoestrogens in humans.

The safety of soy-based infant formulas has been debated (33–35) because these infant foods, which are made from soy protein isolates, contain significant amounts of phytoestrogens (2, 36, 37), which are absorbed by infants and excreted in urine (38). There are limited data on the exact composition of isoflavones in soy-based infant formulas or on their metabolic fate in early life. This overview summarizes and extends previously published studies on phytoestrogen exposure in early life (33, 34, 36–40).

SUBJECTS AND METHODS

All studies were approved by the Investigational Review Board of the Children's Hospital Medical Center, Cincinnati.

Phytoestrogen composition of infant formulas

The isoflavone composition of a selection of major commercial brands of soy-based infant formulas was determined with reversed-phase HPLC; data from these analyses were summarized and reported previously (37). Specifics of the methods used, not mentioned previously, are now discussed and a more comprehensive presentation of the analytic findings is reported. Isoflavones were extracted into ice-cold 80% methanol from powdered and liquid formulas to minimize degradation of heat-labile malonylglycosides. This extraction was performed under sonication for 2 h in a sonic bath filled with ice water. After lipids were removed by partitioning into hexane, individual isoflavones and their conjugates were separated by gradient elution on a C₈ column, detected by their absorption at 260 nm (6), and quantified from the peak area response relative to the internal standard, with correction for differences in responses between the internal standard and the individual isoflavones. Pure standards of the malonyl- and acetylglycosides were unavailable; therefore, the response factor of the corresponding

β-glycoside was used. Concentrations are expressed as mg isoflavone/g for powdered formula and as g/L for liquid formulas. The actual concentration of the feed given to the infants was calculated from the manufacturers' instructions for preparation of the infant formula.

The isoflavone composition of Nursoy powder formula (Wyeth Laboratories, Philadelphia), Isomil powder formula and Ready to Feed liquid formula (Ross Products Division Abbott Laboratories, Columbus, OH), ProSobee liquid formula concentrate (Mead Johnson Nutritional Group, Evansville, IN), and All-soy liquid formula concentrate (Carnation Nutritional Products Division, Nestlé Food Company, Glendale, CA) was described. In addition, samples of cow-milk formula (Similac; Ross Products Division Abbott Laboratories) were analyzed by HPLC.

Plasma concentrations of isoflavones in infants fed soy-based formula, cow-milk formula, and human breast milk

Daidzein, genistein, equol, and desmethylangolensin were measured in plasma samples from healthy, full-term, 4-mo-old infants who had been exclusively fed (from the first week of life) a typical soy-based infant formula, a cow-milk formula, or human breast milk (37). Plasma isoflavone concentrations were quantified by gas chromatography–mass spectrometry (GC-MS) after liquid-solid extraction, enzymatic hydrolysis, liquid-gel chromatographic isolation of the unconjugated isoflavones, and conversion to the volatile *tert*-butyldimethylsilyl (*t*-BDMS) ether derivatives. Selected ion monitoring of specific ions at *m/z* (mass-to-charge ratio) 425 (daidzein and the internal standard, dihydroflavone), *m/z* 555 (genistein), *m/z* 470 (equol), *m/z* 472 (dihydrodaidzein), and *m/z* 543 (desmethylangolensin) afforded detection of the individual isoflavones and metabolites; these were quantified by comparing the ratio of the peak area response of the characteristic ion with the peak area response for the internal standard and interpolating this ratio against a calibration curve constructed from known amounts of the pure standards.

Phytoestrogen concentrations in human breast milk

Samples of aspirated human breast milk were analyzed by GC-MS by the same method used for plasma isoflavone analysis (37). To determine whether phytoestrogens can be transferred from human breast milk to infants breast-fed by women consuming soyfoods, pilot studies were carried out in lactating women consuming soy isoflavones. Breast milk was collected by aspiration from 9 healthy, lactating women and from 1 woman before and 3 d after she consumed 10 g toasted soy nuts (Express Snacks; Hershey Import Company, Inc, Rahway, NJ) containing 3.0 mg total isoflavones/g.

RESULTS

A typical HPLC separation of individual isoflavones in a sample of soy-based infant formula is shown in **Figure 1**. Concentrations of individual isoflavones measured by HPLC are summarized for the 5 different commercial infant formulas in **Table 1**. From these data, the average intake of total isoflavones was calculated and found to be related to the proportion of soy protein isolate incorporated in the various soymilk formulas. Typical volumes of milk consumed by infants over the first 4 mo of life are shown in **Table 2**. For comparison, the concentrations of isoflavones measured in 9 individual samples of human breast milk are shown in **Table 3**. Plasma concentrations of daidzein,



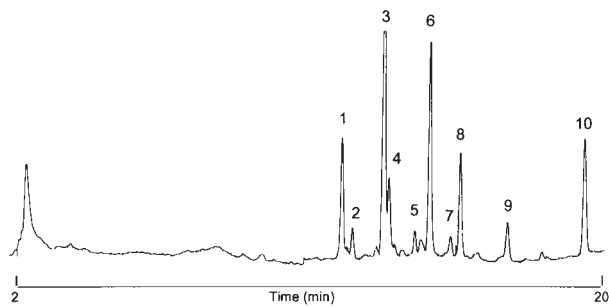


FIGURE 1. A typical reversed-phase HPLC analysis of the phenolic fraction of an extract of a powdered soy-based infant formula showing the composition of isoflavones. Isoflavones were separated by gradient elution on a C₈ column and detected by absorption at 260 nm. The following isoflavones are indicated: 1) daidzin, 2) glycitin, 3) genistin, 4) malonyldaidzin, 5) acetyldaidzin, 6) malonylgenistin, 7) daidzein, 8) acetylgenistin, 9) genistein, and 10) internal standard.

genistein, and equol in infants fed soymilk formula, cow-milk formula, or breast milk are not reported here because these are detailed in a previous publication (37).

DISCUSSION

Composition of isoflavones in soy-based infant formulas

More than 50 y ago, genistein and its β-glycoside, genistin, were isolated from soybeans (9, 41). Since then, many groups have shown that soy-protein products contain variable but significant amounts of isoflavones (6–8, 42, 43). It is therefore not surprising that soy-based infant formulas contain phytoestrogens (36, 37, 39, 40) because these infant foods are currently prepared from soy protein isolates; earlier forms of some milk products,

however, used soy flour as the protein source and probably contained higher concentrations of isoflavones.

The qualitative composition of isoflavones in all soy-based infant formulas is remarkably similar (37) and is characterized by the presence of a mixture of conjugated and unconjugated isoflavones (Figure 1). The β-glycosides, genistin and daidzin, and the 6''-O-malonylglycosides and 6''-O-acetylglycosides of genistein and daidzein were the principal isoflavones identified in all of the soy-based formulas (44–46). Glycitin is also present. The aglycones daidzein and genistein typically account for 3.2–5.8% of the total isoflavones in soy-based formulas (37). Although there are some differences in qualitative composition between formulas, conjugates of genistein predominate in all cases and account for >65% of the total isoflavones. The composition of all of the soy-based formulas examined is consistent with the reported findings for soy protein isolates (42, 46).

The minor differences observed among the different types of soy-based formulas relate mainly to differences in the relative proportions of the malonyl- and acetylglycosides of daidzein and genistein. The malonylglycosides are particularly heat labile and decompose to their corresponding acetylglycosides, whereas the β-glycosides, daidzin and genistin, are heat stable (46). Consequently, the variability in composition among the individual brands of formula is probably related to interbatch differences in the isoflavone content of soybeans or to effects of processing (47, 48), particularly with regard to the extent and duration of heat exposure and changes in pH. The liquid formulas contained slightly lower proportions of malonylglycosides, presumably because of the heat sterilization step used in the manufacture of these formulas. However, the temperatures typically used in the processing or cooking of soy proteins or soyfoods do not substantially change the total amount of isoflavone present but may affect the conjugation profile (25, 49).

Total mean values for isoflavone concentration expressed per gram of formula were, as expected, higher in the powdered soy-

TABLE 1
Isoflavone composition of 5 commercially available soy-based infant formulas¹

Isoflavone identified	Powdered formulas		Liquid formula		
	Nursoy	Isomil	ProSobee concentrate	Isomil Ready to Feed	Allsoy concentrate
Aglycones (μg/g or mg/L)					
Daidzein	7.4 ± 1.0 ²	10.9 ± 2.1	2.2 ± 0.6	1.0 ± 0.1	0.9 ± 0.3
Genistein	6.2 ± 2.1	8.7 ± 1.2	1.0 ± 1.0	1.0 ± 0.1	1.2 ± 0.3
Conjugates (μg/L or mg/L)					
Daidzin	61.2 ± 6.5	73.3 ± 8.2	27.4 ± 7.6	12.1 ± 0.2	15.7 ± 2.9
Daidzein 6''-O-malonylglycoside	—	—	—	—	—
Daidzein 6''-O-acetylglycoside	10.2 ± 1.0	7.0 ± 1.1	—	—	0.6 ± 0.4
Genistin	141.9 ± 18.7	149.6 ± 3.9	63.4 ± 15.6	25.6 ± 0.4	37.0 ± 5.4
Genistein 6''-O-malonylglycoside	44.7 ± 6.2	32.9 ± 2.0	—	—	1.7 ± 1.1
Genistein 6''-O-acetylglycoside	26.4 ± 0.9	19.0 ± 1.3	—	1.2 ± 0.3	0.5 ± 0.2
Glycitin	13.2 ± 1.1	15.7 ± 2.1	6.2 ± 0.6	2.6 ± 0.1	6.1 ± 1.2
Total isoflavones (mg/g or g/L)	307.3 ± 27.8	316.9 ± 13.1	91.0 ± 18.2	43.5 ± 0.7	63.7 ± 9.2
Composition (% of soy isolate)	15.9	14.6	4.0	1.9	2.8
Total isoflavones (mg/g soy protein)	1931 ± 175	2170 ± 90	2275 ± 455	2284 ± 37	2275 ± 328
Average isoflavone concentration of prepared infant food (mg/L) ³	46	47	45	44	32

¹Nursoy, Wyeth Laboratories, Philadelphia; Isomil, Ross Products Division, Abbott Laboratories, Columbus, OH; Prosobee, Mead Johnson Nutritional Group, Evansville, IN; Allsoy, Carnation Nutritional Products Division, Nestlé Food Company, Glendale, CA.

² $\bar{x} \pm SD$.

³ Infant formulas were prepared according to the manufacturers' directions.

TABLE 2
Soy-based infant formula intake and isoflavone exposure

Infant age	Volume mL/d	Isoflavone intake ¹ mg/d	Normal body weight kg	Dose ² mg · kg body wt ⁻¹ · d ⁻¹
1 wk	500–550	22.5–24.8	2.5–3.8	5.7–7.3
1 mo	700–800	31.5–36.0	2.9–5.0	6.0–11.9
2 mo	800–830	36.0–37.0	3.6–5.9	6.1–10.0
4 mo	800–1000	41.0–45.0	4.8–7.5	6.0–9.3

¹ Based on isoflavone concentration of 45 µg/L for soy-based infant formula.

² For comparison, the average daily exposure for adults consuming 57–85 g soyfoods containing 50–100 mg of isoflavones is 0.7–1.4 mg · kg body wt⁻¹ · d⁻¹.

based formulas than in the liquid formulas and these values were directly proportional to the amount of soy protein isolates used in the formulas (Table 1). The average isoflavone concentration of the formulas prepared according to the manufacturers' directions was 32–46 mg/L; 4 formulas had similar mean total isoflavone concentrations (43–46 mg/L). The lower mean total isoflavone concentration of the Allsoy liquid concentrate is explained by the smaller proportion of soy protein isolate incorporated in its manufacture. Overall, these values are similar to values first reported for total isoflavones in Prosobee (38.9 µg/g) and Isomil (41.6 µg/g) > 10 y ago, determined by methods that did not discriminate among isoflavone conjugation states (36). This finding indicates the relative consistency of the isoflavone content of soy-based formulas over time. The isoflavone concentrations of soy-based infant formulas reported here also are comparable with those given in a recent report (40). Variations are to be expected among different brands or within different batches of the same brand because the isoflavone content of soybeans can vary according to geographic location, climate, and growing conditions (48).

The average exposure of newborn infants and neonates to phytoestrogens can be calculated from the daily intake of milk (Table 2). In a 4-mo-old infant consuming 800–1000 mL formula, the total isoflavone intake will be 35–50 mg/d. When calculated on a body weight basis, using standard pediatric growth tables for full-term, normal-for-gestational-age infants, this corresponds to an exposure of 6–9 mg isoflavones · kg body wt⁻¹ · d⁻¹ at 4 mo of age. Body weight and milk intake are proportionally lower in younger infants, and consequently the exposure to phytoestrogens is relatively constant in the neonatal period in infants fed soy-based infant formulas (Table 2). The daily intake of isoflavones from soy-based infant formula (35–50 mg) is comparable with that of adults consuming modest amounts (56 g) of soy-protein foods (50 mg) and probably similar or higher than in Japanese adults consuming a traditional diet (50). Recent estimates from dietary questionnaires of soy-protein intake by Japanese men and women suggest the present daily intake to be ≈8–11 g protein, which suggests that the isoflavone intake by the Japanese population is likely ≈20–25 mg/d (51). When values are expressed relative to body weight, the infant exclusively fed soy-based formulas is exposed to a dose that is 5–10-fold higher than the 0.7-mg · kg body wt⁻¹ · d⁻¹ intake shown to exert significant physiologic and beneficial effects on the hormonal regulation of women's menstrual cycles (21, 26).

We have been unable to detect isoflavones in cow-milk formulas by using HPLC because of the insensitivity of this technique, but phytoestrogens were reported to be present in trace amounts in cow milk (52). The previous finding that infants fed cow-milk formulas excrete significant amounts of isoflavones in urine, including the bacterially derived metabolite equol, confirms the presence of isoflavones in cow milk (38).

It was suggested recently that human milk is a useful source of phytoestrogens (53), but our data and those of others (54, 55) do not support this contention. Table 3 gives the values for total and individual isoflavones in midstream breast milk obtained from 9 healthy, omnivorous, lactating women, and confirms that there are only traces of phytoestrogens in human milk. The concentrations were too low to be detected by the HPLC methods we used and measurement was possible only with GC-MS. The mean total isoflavone concentration was 5.6 ± 4.4 µg/L, which agrees with values reported elsewhere (54, 55). Equol, an intestinally derived isoflavone (56, 57) not present in soy-based formulas, was detected in 7 of 9 breast-milk samples (37); interestingly, we also found equol in amniotic fluid collected during early gestation (KDR Setchell, unpublished observations, 1997), confirming a preliminary report of the placental transfer of phytoestrogens (58).

The transfer of isoflavones into breast milk was also shown in feeding studies, in which lactating women were challenged with soyfoods (Figure 2). Isoflavone concentrations increased ≤10-fold when lactating women consumed the equivalent of 30 mg isoflavones, which confirms the findings of Franke and Custer (54) in similarly designed studies. Infants' daily intakes of phytoestrogens from human milk are calculated to be 0.005–0.01 mg/d, which is trivial when compared with the amounts provided by soy-based infant formulas. Furthermore, isoflavones are predominantly found as glucuronide conjugates in human milk, whereas they occur mainly as glycosidic conjugates in soymilk (37). It is not known how these compositional differences may influence bioavailability. Nevertheless, the available data provide little reason to be concerned about the maternal-infant

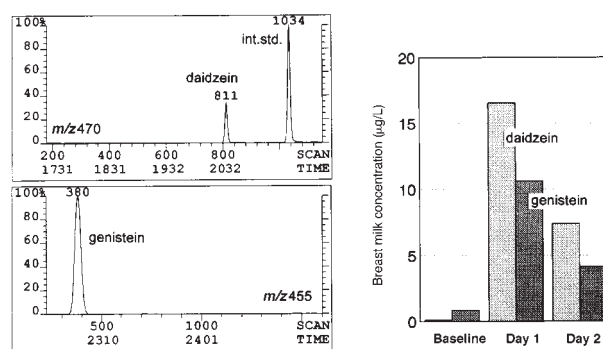


FIGURE 2. Isoflavones in human breast milk during soy intake. Shown are typical selected ion current recordings obtained by gas chromatography–mass spectrometry analysis of the *tert*-butyldimethylsilyl ether derivatives of isoflavones isolated from a sample of human breast milk, confirming the presence of detectable amounts of daidzein [mass-to-charge ratio (*m/z*) 470] and the recovered internal standard (int. std.) and genistein (*m/z* 455). The concentrations of these isoflavones measured in breast milk aspirated from a healthy, lactating woman before (baseline) and for 2 d after the ingestion of soy nuts containing 30 mg total isoflavones confirms the transfer of isoflavones to human breast milk.

TABLE 3
Concentrations of isoflavones in human breast milk from 9 healthy, lactating women¹

Isoflavone	Subject									$\bar{x} \pm SD$
	1	2	3	4	5	6	7	8	9	
	$\mu\text{g/L}$									
Daidzein	3.2	6.1	5.7	3.1	3.2	1.8	1	2.5	1.3	3.1 ± 1.8
Genistein	0.7	6.4	2.7	0.9	1	0.8	0.6	0.9	0.7	1.6 ± 1.9
Equol	0.8	1.4	3	0.7	0.8	0.8	ND	ND	0.6	1.1 ± 0.8
Total	4.7	13.6	11.4	4.7	5	3.4	1.6	3.3	2.6	5.6 ± 4.4

¹ Isoflavone concentrations were measured by gas chromatography–mass spectrometry with selected ion monitoring after hydrolysis of conjugates. ND, not detected.

transfer of phytoestrogens from human breast milk, even in women consuming phytoestrogen-rich diets while breast-feeding; on the basis of the weak estrogenic activity of isoflavones (3), it is doubtful that the dietary intake from human milk is sufficient to exert significant biological effects. Moreover, human breast milk contains >50 different steroid hormone metabolites, including estrogens and progesterone metabolites (59). Estrogen concentrations in breast milk in the first few days of lactation (3–120 nmol/L, or 1–33 $\mu\text{g/L}$) are similar to isoflavone concentrations, but decline thereafter (59).

Metabolic fate of isoflavones in infants

Earlier studies clearly indicated that the glycosidic conjugates of isoflavones in soy-based infant formulas are readily hydrolyzed by intestinal glucosidases (Figure 3), thereby giving rise to the aglycones, which are then absorbed and excreted in urine (38). Bacterial β -glucosidase activity shows an age-dependent increase in infants, being lower than in adults (60). By a few months of age there is significant activity to account for the hydrolysis of the glycosidic bonds of the conjugated isoflavones in soy-based infant formulas. The high variability in the previously reported concentrations of daidzein and genistein in infant urine (38) is accounted for in part by the fact that accurately timed daily collections were not obtained and the urine values represented spot samples. Urinary concentrations of daidzein and genistein in infants were slightly lower than urinary values of adults consuming a similar daily intake of isoflavones (8, 12, 22, 61–63), which could indicate poor renal clearance in early life. These studies showed that there was limited biotransformation beyond the initial hydrolysis of the glycosidic moiety because equol was not detected in the urine of infants fed soy-based formulas whereas it was present in the urine of infants fed either cow-milk formula or human breast milk (38).

Ethical and practical considerations make it difficult to determine accurately the bioavailability of isoflavones in infants, but

an indication of the extent of intestinal absorption can be gleaned from measurement of plasma concentrations. To our knowledge, there have been no previous reports of the plasma concentrations of isoflavones in infants fed soy-based formulas or other dietary regimens. This information is essential for assessing whether phytoestrogens circulate at concentrations sufficient to have physiologic effects.

Typical GC-MS ion recordings of ions specific to the M-57 (loss of C_4H_6) fragment of the *t*-BDMS ether derivatives of isoflavones are shown for plasma from one infant fed soy-based infant formula (Figure 4). These profiles reveal intense signals that correspond to daidzein, genistein, and the internal standard and a relatively weak signal for equol, consistent with a low concentration. The selected ion current recordings were obtained from only 2.5 μL plasma and the integrated peak areas for each of the isoflavones are shown. Although equol was detected by mass spectrometry (ion current recording at m/z 470), the intensity of the signal was 2 orders of magnitude lower than that for either daidzein or genistein, indicating that only traces are present in the plasma of infants fed soy-based formula.

Selected ion recordings for equol (m/z 470) obtained from comparable amounts of plasma from infants fed soy-based formula, cow-milk formula, and breast milk are shown in Figure 5. The peak area for equol in the plasma from infants fed cow-milk formula was 1–2 orders of magnitude higher than that for infants fed soy-based formula or breast milk. Equol was detected in the plasma of all infants fed cow-milk formula, in 4 of 7 infants fed soy-based formula, and in only 1 of 7 infants fed breast milk (37). The mean (\pm SD) plasma concentration of equol was 16.9 ± 2.0 nmol/L (4.11 ± 0.49 $\mu\text{g/L}$); interestingly, this was higher than either the mean plasma daidzein (8.1 ± 1.1 nmol/L, or 2.06 ± 0.29 $\mu\text{g/L}$) or genistein (11.6 ± 2.5 nmol/L, or 3.16 ± 0.68 $\mu\text{g/L}$) concentration in these infants. The lack of equol in the plasma of infants fed soy-based formula or breast milk is consistent with our previous findings from urinary analyses (38) and is explained by reduced intestinal biotransformation resulting from the lack of fully developed microflora in early life or inactivity of the enzymes essential for the further metabolism of isoflavones. This is exemplified by our inability to detect other intestinally derived bacterial metabolites such as desmethylangolensin and dihydrodaidzein in plasma (64–66).

Plasma concentrations of daidzein, genistein, and equol in 4-mo-old infants fed soy-based formula, cow-milk formula, and breast milk were also reported previously (37); concentrations ($\bar{x} \pm SD$) for genistein and daidzein during soy-based formula feeding were 683.9 ± 442.6 and 295.3 ± 59.9 $\mu\text{g/L}$, respectively. These values were significantly greater ($P < 0.05$) than the mean values for plasma genistein and daidzein in infants fed either cow-milk formula or breast milk. Plasma total isoflavone con-

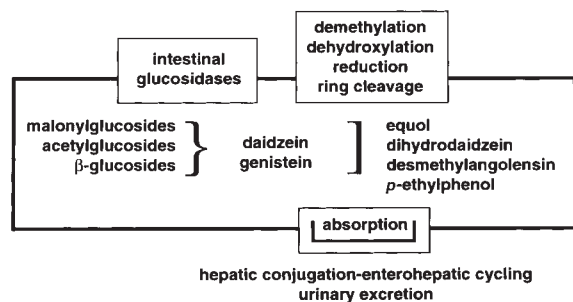


FIGURE 3. Metabolism of isoflavones in humans.

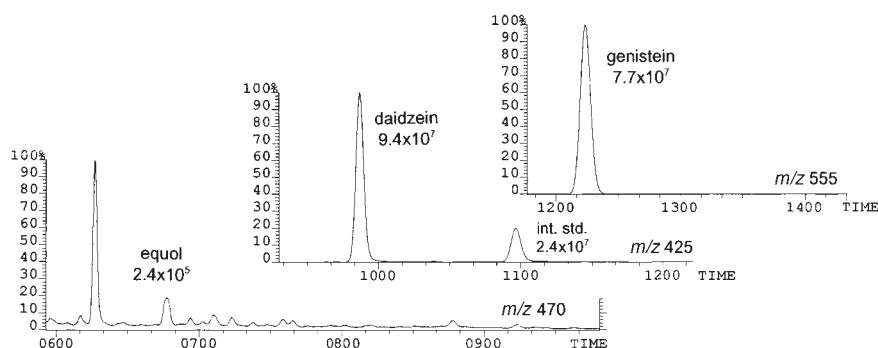


FIGURE 4. Typical selected ion current recordings obtained from gas chromatography–mass spectrometry analysis of the *tert*-butyldimethylsilyl ether derivatives of isoflavones isolated from the plasma of a 4-mo-old infant fed exclusively soy-based infant formula. A 2.5- μ L plasma sample was injected on the column and the ions of mass-to-charge ratio (m/z) 470, m/z 425, and m/z 555, respectively, were monitored for the specific detection of equol, daidzein and the internal standard (int. std.), and genistein. The integrated ion currents for each compound is indicated. The channel recording equol (m/z 470) is amplified 100-fold relative to the other channels. The internal standard was dihydroflavone.

centrations ranged from 552 to 1775 μ g/L (\bar{x} : 980 μ g/L) in infants fed soy-based formula, which is 2–5-fold higher than peak plasma concentrations in adults after single-bolus, oral administration of 50 mg of the pure compounds (25) and greater than values reported for adults (50–200 μ g/L) consuming similar intakes of isoflavones from diets of soy-based foods (22, 24). These values are also higher than plasma isoflavone concentrations of Japanese adults, which were found to be 40–240 μ g/L (67). The higher plasma concentrations can in part be attributed to the higher per-body-weight dose experienced by the infants fed soy-based formula compared with adults consuming comparable daily intakes of isoflavones. By contrast, the mean (\pm SD) total plasma isoflavone concentration of infants fed breast milk was 4.7 ± 1.3 μ g/L; for infants fed cow-milk formula, concentrations were approximately twice as high: 9.3 ± 1.2 μ g/L. Circulating concentrations of isoflavones in infants fed breast-milk and cow-milk formulas are $<1/200$ th and $1/100$ th, respectively, of the concentrations attained when infants are fed soy-based formula (37).

No attempt was made to determine the extent of conjugation of isoflavones in infant serum. However, in common with endogenous estrogens (68), and on the basis of previous studies of phytoestrogens in adults (23, 24, 69), it is assumed that isoflavones circulate predominantly as glucuronides and to a lesser extent as sulfate conjugates in infants. A recent report suggested that unconjugated isoflavones were not present in infant

plasma, but this would seem improbable and may reflect methodologic deficiencies in the measurement of this fraction (40). There is virtually no information on the biological activity of glucuronides and sulfate conjugates because of the lack of standards for testing. Although conjugation serves in part to facilitate elimination of steroids, it does not necessarily render these compounds inactive. The enterohepatic recycling of phytoestrogens (3, 25), in common with endogenous estrogens (70), retains these metabolites *in vivo*, where repeated deconjugation during recycling would release the unconjugated isoflavone.

The extent of protein binding of a steroid is also a key determinant of its availability to the cell and hence the steroid receptor. Estradiol binds efficiently to serum proteins and there is a dynamic equilibrium between unbound and bound hormone concentrations, with $<3\%$ of the total unbound and therefore available for cellular uptake and subsequent binding to estrogen receptors (71). In general, xenoestrogens show less binding to serum proteins and are therefore more available to the target cells for receptor occupancy (72, 73). Studies of the protein binding of several phytoestrogens, including genistein, daidzein, and equol, have shown lower affinity relative to estradiol (74), which would serve to increase their availability to the estrogen receptor and therefore could lead to an underestimation of their biological potency. In our studies, no attempts were made to determine the extent of protein binding of isoflavones in infant serum,

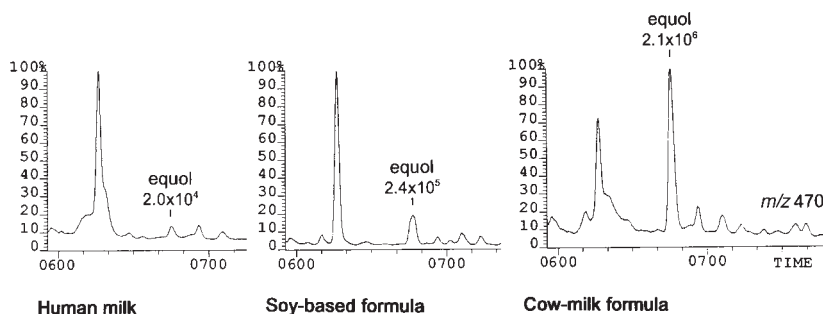


FIGURE 5. Selected ion current gas chromatography–mass spectrometry recordings for mass-to-charge ratio (m/z) 470 arising from the fragmentation of the *tert*-butyldimethylsilyl ether derivative of equol. A 2.5- μ L plasma sample was injected on the column. Compared are the integrated signal responses obtained from equivalent amounts of plasma from 4-mo-old infants fed exclusively human milk, soy-based infant formula, or cow-milk formula. These recordings indicate the greater amount of equol in the plasma of infants fed cow-milk formula than in those fed either soy-based formula or human milk.

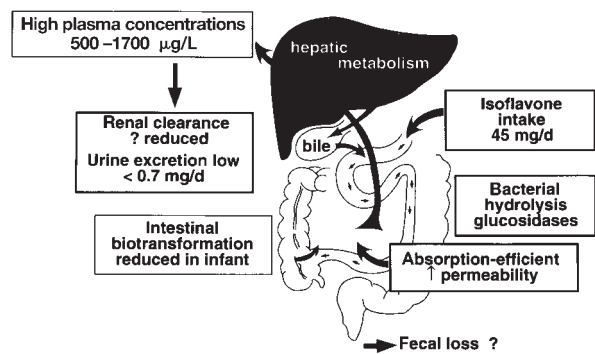


FIGURE 6. Summary of the physiologic behavior and fate of ingested isoflavones by infants fed soy-based formula.

but this needs to be considered when evaluating biological potency. Furthermore, the extent of binding and selectivity toward the newly described and cloned estrogen receptor ER β (75) remains to be clarified. It is possible that phytoestrogens may exert their effects selectively through pathways distinct from estrogen binding to the classic receptor, ER α .


The high plasma isoflavone concentrations observed in infants fed soy-based formulas indicate that the absorption of soy isoflavones from the intestinal tract is efficient and that these bioactive compounds have a high bioavailability. This may be because of reduced metabolic biotransformation and degradation beyond the initial cleavage of the glycosidic bond, thereby making more of the aglycones of daidzein and genistein available for absorption. In comparison, in adults there is extensive metabolism to many other isoflavonoid metabolites (56, 64–66). The relatively long plasma half-life of daidzein and genistein, 7–8 h in adults (25), combined with the fact that infants are continually exposed to phytoestrogens from soy-based infant formulas during regular and frequent daily feeding consequently leads to the high steady state plasma concentrations. The metabolic fate of isoflavones in infants is summarized in **Figure 6**. The average daily intake of isoflavones from soy-based formula is similar to that of an adult consuming a typical soyfood-containing meal. However, the circulating plasma concentrations of isoflavones in infants are an order of magnitude higher than those observed in adults with similar intakes.

On the basis of our findings for 4-mo-old infants, the plasma total isoflavone concentration in infants fed soy-based formula is 13000–22000 times higher than the plasma concentration of estradiol in early life, which is 147–294 pmol/L (40–80 pg/mL) (76). Even allowing for the weak estrogenic activity of isoflavones compared with estradiol, it is difficult to believe that isoflavones, circulating at these high concentrations, are biologically inert in infants, particularly given their weaker binding to serum proteins (74). Experimental data from *in vitro* and *in vivo* animal studies (5, 17, 25) together with human dietary intervention studies have shown significant biological effects with similar intakes of phytoestrogen (21, 26), all relating to potential health benefits.

Concerns about possible adverse effects of exposure of infants to phytoestrogens in soy-based formulas are founded on hypothetical possibilities and are related to knowledge of the role of estrogens at critical stages of development and in mediating reproductive or neuroendocrine disruption in various animal

species (77). Clover disease in sheep (27) and venoocclusive disease with infertility in cheetahs (29) were both found to be caused by dietary isoflavones. However, sheep grazed on amounts of isoflavones that would be difficult for humans to consume on a daily basis given the usual concentrations found in soyfoods; cheetahs, in common with most feline species, lack hepatic UDP-glucuronyltransferase, a key metabolizing enzyme for steroid hormones in most species, including humans (68). These 2 examples illustrate the need to consider dosage and species differences in metabolism when making extrapolations to humans.

Timing of exposure is also a critical factor in predicting potential steroid hormone effects (77). The devastating effects of diethylstilbestrol taken in early pregnancy only became apparent in offspring, who were predisposed to reproductive dysfunction and adenocarcinoma later in life (78, 79). This genetic imprinting and the effects of phytoestrogens on sexual differentiation in many mammalian and avian species (80–84) relate to prenatal rather than postnatal exposure. Unfortunately, there appears to be no ideal animal model for the human neonate; therefore, it is difficult to extrapolate these animal data to infants. Soy-based formulas are consumed postnatally, not prenatally. Any negative effects from phytoestrogens might be expected to be enhanced by exposure of the fetus to isoflavones from soy products consumed during pregnancy. Recent studies using animal models of chemically induced breast cancer point to beneficial rather than negative effects resulting from both neonatal and prepubertal exposure to genistein (18, 19). These animals were found to be more resistant to chemically induced breast cancer later in life.

In the absence of practical examples to support adverse effects of soy-based infant formulas, despite their use for > 30 y, it could be argued that long-term benefits may ensue from infant exposure to soy-based formulas containing isoflavones because this could confer protection later in life against hormone-dependent diseases. In this regard, we speculate that the low incidence of hormone-dependent diseases in China and Japan, where soy is a staple, may in part be a consequence of a lifetime exposure to phytoestrogens from the traditional diet. The concept of early-life diet influencing later disease outcome is gaining credence (85). Interestingly, the incidence of such diseases is increasing and this trend appears to be related to a move toward a more westernized diet in these countries (86). 

REFERENCES

1. National Academy of Sciences. Diet, nutrition, and cancer. Washington, DC: National Academy Press, 1982.
2. Setchell KDR. Naturally occurring non-steroidal estrogens of dietary origin. In: Estrogens in the environment: influence on development. New York: Elsevier, 1985:73–106.
3. Setchell KDR, Adlercreutz H. Mammalian lignans and phytoestrogens. Recent studies on their formation, metabolism and biological role in health and disease. In: Rowland IA, ed. The role of gut microflora in toxicity and cancer. New York: Academic Press, 1988:315–45.
4. Setchell KDR. Non-steroidal estrogens of dietary origin: possible roles in health and disease, metabolism and physiological effects. Proc Nutr Soc N Z 1995;20:1–21.
5. Adlercreutz H. Phytoestrogens: epidemiology and a possible role in cancer protection. Environ Health Perspect 1995;103:103–12.
6. Coward L, Barnes NC, Setchell KDR, Barnes S. Genistein and daidzein, and their β -glycoside conjugates: anti-tumor isoflavones in soybean foods from American and Asian diets. J Agric Food Chem 1993;41:1961–7.

7. Wang H-J, Murphy PA. Isoflavone content in commercial soybean foods. *J Agric Food Chem* 1994;42:1666-73.
8. Franke AA, Custer LJ, Cerna CM, Narala K. Rapid HPLC analysis of dietary phytoestrogens from legumes and from human urine. *Proc Soc Exp Biol Med* 1995;208:18-26.
9. Walter ED. Genistein (an isoflavone glucoside) and its aglucone, genistin, from soybeans. *J Am Oil Chem Soc* 1941;63:3273-6.
10. Akiyama T, Ishida J, Nakagawa S, et al. Genistein: a specific inhibitor of tyrosine-specific protein kinase. *J Biol Chem* 1987;262:5592-5.
11. Barnes S, Peterson TG. Biochemical targets of the isoflavone genistein in tumor cell lines. *Proc Soc Exp Biol Med* 1995;208:103-8.
12. Setchell KDR, Borriello SP, Hulme P, Kirk DN, Axelson M. Nosteroidal estrogens of dietary origin: possible roles in hormone-dependent disease. *Am J Clin Nutr* 1984;40:569-78.
13. Adlercreutz H. Western diet and Western diseases: some hormonal and biochemical mechanisms and associations. *Scand J Clin Lab Invest* 1990;201:3-21.
14. Anderson JW, Johnstone BM, Cook-Newell ME. Meta-analysis of the effects of soy protein intake on serum lipids. *N Engl J Med* 1995;333:272-86.
15. Peterson TG, Barnes S. Genistein inhibition of the growth of human breast cancer cells: independence from estrogen receptors and the multi-drug resistance gene. *Biochem Biophys Res Commun* 1991;179:661-7.
16. Peterson TG, Barnes S. Isoflavones inhibit growth of human prostate cancer cell lines without inhibiting epidermal growth factor receptor auto-phosphorylation. *Prostate* 1993;22:335-45.
17. Barnes S, Grubbs C, Carlson J, Setchell KDR. Soybeans inhibit mammary tumor growth in models of breast cancer. In: Pariza MW, ed. *Mutagens and carcinogens in the diet*. New York: Wiley-Liss, 1990:239-53.
18. Murrill WB, Brown N, Zhang J-X, Manzolillo PA, Barnes S, Lamartiniere CA. Prepubertal genistein exposure suppresses mammary cancer and enhances gland differentiation in rats. *Carcinogenesis* 1996;17:1451-7.
19. Lamartiniere CA, Moore JB, Holland M, Barnes S. Neonatal genistein chemoprevents mammary cancer. *Proc Soc Exp Biol Med* 1995;208:120-3.
20. Messina M, Messina V. Increasing use of soyfoods and their potential role in cancer prevention. *J Am Diet Assoc* 1991;91:836-40.
21. Cassidy A, Bingham S, Setchell KDR. Biological effects of a diet of soy protein rich in isoflavones on the menstrual cycle of premenopausal women. *Am J Clin Nutr* 1994;60:333-40.
22. Morton MS, Wilcox G, Wahlqvist ML, Griffiths K. Determination of lignans and isoflavonoids in human female plasma following dietary supplementation. *J Endocrinol* 1994;142:251-9.
23. Adlercreutz H, Fotsis T, Lampe J, et al. Quantitative determination of lignans and isoflavones in plasma of omnivorous and vegetarian women by isotope-dilution gas chromatography-mass spectrometry. *Scand J Clin Lab Invest* 1993;53:5-18.
24. Coward L, Kirk M, Albin N, Barnes S. Analysis of plasma isoflavones by reversed-phase HPLC-multiple reaction ion monitoring-mass spectrometry. *Clin Chim Acta* 1996;247:121-42.
25. Setchell KDR. Phytoestrogens: biochemistry, physiology, and implications for human health of soy isoflavones. *Am J Clin Nutr* 1998;68(suppl):1333S-46S.
26. Cassidy A, Bingham S, Setchell KDR. Biological effects of isoflavones in young women: importance of the chemical composition of soybean products. *Br J Nutr* 1995;74:587-601.
27. Bennetts HW, Underwood EJ, Shier FL. A specific breeding problem of sheep on subterranean clover pastures in western Australia. *Aust Vet J* 1946;22:2-12.
28. Leopold AS, Erwin M, Oh J, Browning B. Phytoestrogens: adverse effects on reproduction in California quail. *Science* 1976;191:98-9.
29. Setchell KDR, Gosselin SJ, Welsh MB, et al. Dietary estrogens—a probable cause of infertility and liver disease in captive cheetahs. *Gastroenterology* 1987;93:225-33.
30. Davis DL, Bradlow HL, Wolff M, Woodruff T, Hoel G, Anton-Culver H. Medical hypothesis: xenoestrogens as preventable causes of breast cancer. *Environ Health Perspect* 1993;101:372-7.
31. Soto AM, Chung KL, Sonnenschein C. The pesticides endosulfan, toxaphene and dieldrin have estrogenic effects on human estrogen-sensitive cells. *Environ Health Perspect* 1994;102:380-3.
32. Barrett J. Phytoestrogens, friends or foes? *Environ Health Perspect* 1996;104:478-82.
33. Irvine C, Fitzpatrick M, Robertson I, Woodhams D. The potential adverse effects of soybean phytoestrogens in infant feeding. *N Z Med J* 1995;108:208-9.
34. Robertson IGC. Phytoestrogens: toxicity and regulatory recommendations. *Proc Nutr Soc N Z* 1995;20:35-42.
35. Department of Health. Advice on soya-based infant formula. Department of Health, 1996. (Document 96/244.)
36. Setchell KDR, Welsh MB, Lim CK. HPLC analysis of phytoestrogens in soy protein preparations with ultraviolet, electrochemical and thermospray mass spectrometric detection. *J Chromatogr* 1987;368:315-23.
37. Setchell KDR, Zimmer-Nechemias L, Cai J, Heubi JE. Exposure of infants to phytoestrogens from soy-based infant formula. *Lancet* 1997;350:23-7.
38. Cruz MLA, Wong WW, Mimouni F, et al. Effects of infant nutrition on cholesterol synthesis rates. *Pediatr Res* 1994;35:135-40.
39. Knight DC, Eden J, Kelly G. The phytoestrogen content of infant formulas. *Med J Australia* 1996;164:575 (letter).
40. Huggett AC, Pridmore S, Malnoe A, Haschke F, Offord EA. Phytoestrogens in soy-based infant formula. *Lancet* 1997;350:815-6.
41. Walz E. Isoflavone—a saponin glucoside in soya. *Justus Liebigs Ann Chem* 1931;489:118-55 (in German).
42. Eldridge AC. Determination of isoflavones in soybean flours, protein concentrates and isolates. *J Agric Food Chem* 1982;30:353-5.
43. Murphy PA. Phytoestrogen content of processed soybean products. *Food Technol* 1982;34:60-4.
44. Farmakalidis E, Murphy PA. Isolation of 6'-O-acetyl daidzein and 6'-O-acetyl genistein from toasted defatted soy flakes. *J Agric Food Chem* 1985;33:385-9.
45. Kudou S, Flueury Y, Welti D, et al. Malonyl isoflavone glycosides in soybean seeds (*Glycine max* MERRILL). *Agric Biol Chem* 1991;55:2227-33.
46. Barnes S, Kirk M, Coward L. Isoflavones and their conjugates in soy foods: extraction conditions and analysis by HPLC-mass spectrometry. *J Agric Food Chem* 1994;42:2466-74.
47. Anderson RL, Wolf WJ. Compositional changes in trypsin inhibitors, phytic acid, saponins, and isoflavones related to soybean processing. *J Nutr* 1995;125:581S-8S.
48. Eldridge A, Kwolek WF. Soybean isoflavones: effect of environment and variety on composition. *J Agric Food Chem* 1983;31:394-6.
49. Coward L, Smith M, Kirk M, Barnes S. Chemical modification of isoflavones in soyfoods during cooking and processing. *Am J Clin Nutr* 1998;68(suppl):1486S-91S.
50. Messina M. Isoflavone intakes by Japanese were overestimated. *Am J Clin Nutr* 1995;62:645 (letter).
51. Nagata C, Takatsuka N, Kurisu Y, Shimizu H. Decreased serum total cholesterol concentration is associated with high intake of soy products in Japanese men and women. *J Nutr* 1998;128:209-13.
52. Bannwart C, Adlercreutz H, Wahala K, et al. Identification of the phyto-estrogen 3',7'-dihydroxyisoflavan, an isomer of equol in the human urine and cow's milk. *Biomed Environ Mass Spectrom* 1988;17:1-6.
53. Slavin JL. Phytoestrogens in breast milk—another advantage of breast-feeding? *Clin Chem* 1996;42:841-2.
54. Franke AA, Custer LJ. Daidzein and genistein concentrations in human milk after soy consumption. *Clin Chem* 1996;42:955-64.
55. Morton MS, Leung SSF, Davies DP, Griffiths K, Evans BAJ. Determination of isoflavonoids and lignans in human breast milk from

- British and Chinese women by gas chromatography–mass spectrometry. *Am J Clin Nutr* 1998;68(suppl):1537S (abstr).
56. Axelson M, Kirk DN, Cooley G, Farrant RD, Lawson AM, Setchell KDR. The identification of the weak estrogen equol (7-hydroxy-3-(4'-hydroxyphenyl) chroman) in human urine. *Biochem J* 1982;201:353–7.
 57. Axelson M, Sjøvall J, Gustafsson B, Setchell KDR. Soya—a dietary source of the non-steroidal oestrogen equol in humans and animals. *J Endocrinol* 1984;102:49–56.
 58. Adlercreutz H, Yamada T, Wahala K, Watanabe S. Maternal and neonatal phytoestrogens in Japanese women at birth. *Steroids* 1997;62:72 (abstr).
 59. Sahlberg B-L, Axelson M. Identification and quantification of free and conjugated steroids in milk from lactating women. *J Steroid Biochem* 1986;25:379–91.
 60. Mykkanen H, Tikka J, Pitkanen T, Hanninen O. Fecal bacterial enzyme activities in infants increase with age and adoption of adult-type diet. *J Pediatr Gastroenterol Nutr* 1997;25:312–6.
 61. Lu L-JW, Broemelin LD, Marshall MV, Sadagopa Ramanujam VM. A simplified method to quantify isoflavones in commercial soybean diets and human urine after legume consumption. *Cancer Epidemiol Biomarkers Prev* 1995;4:497–503.
 62. Hutchins AM, Slavin JE, Lampe JW. Urinary isoflavonoid phytoestrogen and lignan excretion after consumption of fermented and unfermented soy products. *J Am Diet Assoc* 1995;95:545–51.
 63. Xu X, Wang H-J, Murphy PA, Cook L, Hendrich S. Daidzein is a more bioavailable soy-milk isoflavone than is genistein in adult women. *J Nutr* 1994;124:825–32.
 64. Bannwart C, Adlercreutz H, Fotsis T, Wahala K, Hase T, Brunow G. Identification of *O*-desmethylangolensin, a metabolite of daidzein, and of matairesinol, one likely plant precursor of the animal lignan enterolactone, in human urine. *Finn Chem Lett* 1984;4–5:120–5.
 65. Joannou GE, Kelly GE, Reeder AY, Waring MA, Nelson C. A urinary profile study of dietary phytoestrogens. The identification and mode of metabolism of new isoflavonoids. *J Steroid Biochem Mol Biol* 1995;54:167–84.
 66. Kelly GE, Joannou GE, Nelson C, Reeder AY, Waring MA. Metabolites of dietary (soya) isoflavones in human urine. *Clin Chim Acta* 1993;223:9–22.
 67. Adlercreutz H, Markkanen H, Watanabe S. Plasma concentrations of phyto-oestrogens in Japanese men. *Lancet* 1993;342:1209–10.
 68. Mackenzie PI, Rodbourne L, Stranks S. Steroid UDP-glucuronyl transferases. *Steroid Biochem Mol Biol* 1992;43:1099–105.
 69. Axelson M, Setchell KDR. Conjugation of lignans in human urine. *FEBS Lett* 1980;122:49–53.
 70. Adlercreutz H. Studies of oestrogen excretion in human bile. *Acta Endocrinol Suppl (Copenh)* 1962;72:1–220.
 71. Sheehan D, Young M. Diethylstilbestrol and estradiol binding to serum albumin and pregnancy plasma of rat and human. *Endocrinology* 1979;104:1442–6.
 72. Ekins RP, Edwards P, Newman B. The role of binding proteins in hormone delivery. In: Albertini A, Ekins RP, eds. *Free hormones in blood*. New York: Elsevier Biomedical Press, 1982:3–44.
 73. Mendel C. The free hormone hypothesis: a physiologically based mathematical model. *Endocrinol Rev* 1989;10:232–74.
 74. Nagel SC, vom Saal F, Welshons WV. The effective free fraction of estradiol and xenoestrogens in human serum measured by whole cell uptake assays: physiology of delivery modifies estrogenic activity. *Proc Soc Exp Biol Med* 1998;217:300–9.
 75. Kuiper GGJM, Enmark E, Pelto-Huikko M, Nilsson S, Gustafsson J-A. Cloning of a novel estrogen receptor expressed in rat prostate and ovary. *Proc Natl Acad Sci U S A* 1996;93:5925–30.
 76. Winter JSD, Hughes IA, Reyes FI, Faiman C. Pituitary-gonadal relations in infancy: 2. Patterns of serum gonadal steroid concentrations in man from birth to two years of age. *J Clin Endocrinol Metab* 1976;42:679–86.
 77. Kavlock RJ, Daston GP, DeRosa C, et al. Research needs for the risk assessment of health and environmental effects of endocrine disruptors: a report of the US EPA-sponsored workshop. *Environ Health Perspect* 1996;104:715–39.
 78. Greenwald P, Nasca PC, Burnett WS, Polan A. Prenatal stilbestrol experience in mothers of young cancer patients. *Cancer* 1973;31:568–72.
 79. Herbst AL, Kurman RJ, Scully RE, Poskaner DC. Clear-cell adenocarcinoma of the genital tract in young females. Registry report. *N Engl J Med* 1972;287:1259–64.
 80. McLusky NJ, Naftolin F. Sexual differentiation of the central nervous system. *Science* 1981;211:1294–303.
 81. Mori T, Nagasawa H. *Toxicity of hormones in perinatal life*. Boca Raton, FL: CRC Press, 1988.
 82. Toran-Allerand CD. Gonadal hormones and brain development: implications for the genesis of sexual differentiation. *Ann NY Acad Sci* 1984;435:101–11.
 83. Whitten PL, Lewis C, Russell E, Naftolin F. Potential adverse effects of phytoestrogens. *J Nutr* 1995;125:771S–6S.
 84. Levy JR, Faber KA, Ayyash L, Hughes CL Jr. The effect of prenatal exposure to the phytoestrogen genistein on sexual differentiation in rats. *Proc Soc Exp Biol Med* 1995;208:60–6.
 85. Colditz GA, Frazier AL. Models of breast cancer show that risk is set by events of early life: prevention efforts must shift focus. *Cancer Epidemiol Biomarkers Prev* 1995;4:567–71.
 86. Hadfield P. Japanese swallow Western diseases. *New Scientist* 1995;Sept 2:5.

