Metabolites of dietary (soya) isoflavones in human urine

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Abstract

This study was undertaken to better understand the metabolic fate of dietary isoflavones in humans. Twelve volunteers were challenged with soya flour and urinary diphenol levels were then determined by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS). The presence of previously described urinary diphenols was confirmed, i.e. the isoflavones, daidzein and genistein; the isoflavonoid metabolites, equol, dihydrodaidzein (Int-O-D), O-desmethyl-angolensin (O-Dma); the lignan, enterolactone. Diphenols detected for the first time were the isoflavone, glycitein and five novel isoflavonoid metabolites which are tentatively identified as 6’-hydroxy-O-desmethylangolensin (6’OH-O-Dma), dihydrogenistein (Int-O-G), dehydro-O-desmethylangolensin (dehydro-O-Dma) and two isomers of tetrahydroidaidzein. Urinary excretion rates of the three isoflavones (daidzein, genistein, glycitein) over a 3-day period following soya challenge showed moderate variation (4x, 6x and 12x, respectively) between the 12 individuals suggesting some individual variabilities in ability to deconjugate and to absorb dietary isoflavones. However, urinary excretion rates of each of three major isoflavonoid metabolites (equol, O-Dma, 6’OH-O-Dma) showed more marked variation (922x, 17x, 15x, respectively); while some of this variability may reflect varying individual ability to ferment dietary isoflavones per se, an inverse relationship was found between urinary levels of equol and both O-Dma and 6’OH-O-Dma suggesting individual variability in the preferred metabolic pathways of dietary isoflavones.

Key words: Isoflavonoids; Daidzein; Genistein; Equol

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1. Introduction

Isoflavones are plant diphenols, with at least 230 types described [1]. A range of biological functions in plants has been ascribed to isoflavones, with anti-microbial activities being important [1]. A small number of isoflavones additionally display oestrogenic activity in animals, an effect which has been attributed to the similar spatial arrangement of functional groups on both isoflavones and oestrogens, allowing those isoflavones to bind to oestrogen receptors [2,3].

Isoflavones occur principally, although not exclusively, in legumes (*Leguminosae* family). Moreover, they are at particularly high levels in certain legumes which are regularly consumed by man and animals [4]. Indeed, many traditional human diets such as those in India, Asia, Africa, South and Central America and the Mediterranean, which have relatively high legume consumption (soya, lentils, chick peas, beans, etc.) consequently have high isoflavone contents, particularly those with oestrogenic activity [4].

There is growing interest in the importance of dietary isoflavones to human health. Rather than being just passive dietary components, isoflavones and in particular the oestrogenic isoflavones, have been hypothesised to have a range of beneficial physiological effects, largely related to their influence on sex hormone metabolism [5–9].

Interest in isoflavones as dietary components with active physiological effects stems from the 1950s with the finding that infertility in sheep grazing on subterranean clover was a direct result of high isoflavone levels in the pasture [10,11]. The principal isoflavones found in subterranean clover (*Trifolium*) are genistein (4',5,7-trihydroxyisoflavone) and daidzein (4',7-dihydroxyisoflavone) which are present principally as glycoside conjugates (genistin, daidzin) or their respective 4'-methyl ethers, biochanin A and formononetin [11–14]. Upon ingestion the isoflavones are subjected to acidic hydrolysis and enzymatic hydrolysis and demethylation (from both plant and gut flora enzymes) to yield free aglycones and further metabolites [12–14]. Genistein is metabolised to the non-oestrogenic compound, para-ethylphenol, while daidzein is metabolised to the oestrogenic compounds, equol (4",7-dihydroxyisoflavan) and *O*-desmethylangolensin [1-(2',4'-dihydroxyphenyl)-2-(4'-hydroxy-phenyl)propan-1-one] (O-Dma) [13,14]. The aglycones and their metabolites are absorbed and appear in blood and urine as glucuronide conjugates [12,13]. Those aglycones (genistein, daidzein) and metabolites (equol, O-Dma) with affinity for oestrogen receptors, then result in a range of pathophysiological effects in both ewes and wethers (including permanent infertility) as a result of the unresponsiveness of tissues such as the hypothalamus to endogenous oestrogen [15].

The metabolism of dietary isoflavones in humans is not as well understood as it is in sheep although the available evidence points to similar pathways in both species. Following ingestion of isoflavone-rich foods such as soya the isoflavones daidzein and genistein and the daidzein metabolites equol, O-Dma, as well as two intermediate compounds between daidzein and O-Dma (Intermediate O) and daidzein and equol (Intermediate E), have been described in human urine [16–20].

In view of the known in vivo potency of these isoflavones [15], the relatively high levels of these compounds in many traditional human diets suggests that a better understanding of isoflavonoid metabolism in humans is needed.
The purpose of the study reported here was to investigate both the scope and uniformity of the metabolic response in humans to dietary isoflavones.

2. Materials and methods

2.1. Experimental procedures

Participants

The participants were 12 healthy Caucasian men and women aged between 25 and 51 years of age, of normal height and weight. None of the participants was taking any medication including oral contraceptives; none had received antibiotic therapy within 6 months prior to the study. All participants were volunteers and provided informed consent. The study was approved by The University of Sydney Human Ethics Review Committee.

Diet

All participants had a typical Western European omnivorous diet, which included little or no soya products and only moderate legume intake estimated at 5–10 g (garden peas, French beans) daily. During the study, participants maintained their normal diet and had no more than moderate alcohol intake. Each participant included 40 g of soya flour in the form of a prepared cake in their daily diet for 2 consecutive days. The soya was a commercial brand of full-fat soya flour and the one batch of flour was used for all participants. The soya was analysed for isoflavone content as detailed later.

Urine sampling

Urine samples (24 h) were collected from each participant on four occasions. If the 2 consecutive days of soya challenge are designated as days 1 and 2, then the urine collections were on days 0, 3, 4 and 5. Sample sizes varied between 600 and 1,850 ml. The collection was made into 5-l polypropylene containers with no preservative added. The containers were kept cool and delivered to the laboratory on a daily basis. The volume of urine was recorded immediately and a 200-ml aliquot stored at −20°C until ready for analysis. The analysis of samples commenced within 3 months of collection.

2.2. Analytical methods

Reference standards and compound identity

Equol, daidzein and genistein were kindly supplied by Dr Lamberton, Division of Organic Chemistry, CSIRO, Australia. Dihydrodaidzein was a gift from Professor T. Hase, Department of Chemistry and Professor H. Adlercreutz, Department of Clinical Chemistry, University of Helsinki, Finland. O-Desmethylandolensin and enterolactone were identified here by comparison of GC and GC-MS data from those in the literature [19,22–24]. Glycitein was extracted from soya and used as a reference standard after structural confirmation by NMR and GC-MS as follows. Isoflavones were extracted from soya
hypocotyl and hydrolysed by the method detailed later for the extraction and hydrolysis of daidzein and genistein from soya flour. GC and GC-MS showed that the extract consisted mainly of daidzein and glycitein and the glycitein was isolated by re-extraction with methanol followed by recrystallisation in aqueous (90%) methanol. The melting points and spectral analyses obtained were as follows: m.p. 290°C (dec.); UV \( \lambda_{\max} \) 256,319 (log \( \epsilon \) 4.35, 3.98); diacetate m.p. 208–210°C; \(^1\text{H}-\text{NMR} \delta \) p.p.m. 8.02 (1H, s, H-2), 7.78 (1H, s, H-5), 7.62 (2H, \( dJ = 8 \text{ Hz}, H-2',6' \)), 7.18 (2H, \( dJ = 8 \text{ Hz}, 3',5' \)), 3.95 (3H, s, OCH\(_3\)), 2.37 (3H, s, OAc), 2.32 (3H, s, OAc). GC and GC-MS data were as follows. As the TMS ether derivative MU 30.37: \( M^+ 428 \) (100%), \( m/z 192 \) (85%), \( m/z 184 \) (43%), \( m/z 398 \) (35%), \( m/z 413 \) (22%) and \( m/z 383 \) (11%). As the diacetate derivative MU 30.20: \( M^+ 368 \) (13%), \( m/z 284 \) (100%), \( m/z 326 \) (28%), \( m/z 166 \) (26%), \( m/z 123 \) (9%), \( m/z 255 \) (7%), \( m/z 151 \) (5%), \( m/z 241 \) (4%) and \( m/z 118 \) (4%). The NMR and mass spectral data of glycitein diacetate obtained here are in agreement with data reported previously [24].

**Isolation of diphenols from human urine**

**Enzyme hydrolysis and extraction.** Aliquots of urine (40 ml) were adjusted to pH 4.6 with 70% acetic acid and 30,000 counts/min \(^3\text{H}\)oestradiol glucuronide (New England Nuclear) and 60 \( \mu \)g of oestriol added as internal standards. Enzymatic hydrolysis of the glucuronides was achieved using \textit{Helix pomatia} juice (Calbiochem) in 0.1 M sodium acetate buffer, pH 5.0; 1000 Fishman units of beta-glucuronidase aryl sulphatase were added per ml of urine and incubated at 37°C for 24 h, after which time a fresh amount of enzyme was added and incubated for a further 24 h. Following extraction with diethyl ether (2 \( \times \) vol) and evaporation of the organic phase under nitrogen, the dry residue was dissolved in 1 ml of ethanol and stored at 5°C prior to partition chromatography.

**Liquid-gel chromatography on Sephadex LH-20.** The diphenol fraction, free of neutral steroids, was isolated by straight-phase partition chromatography on Sephadex LH-20. A siliconised Pasteur pipette (5 ml Corning) carrying a plug of glass wool at its narrow end served as a column, where the suspended slurry (1 g) was allowed to settle to 6.0 cm by gravity. A 250-\( \mu \)l aliquot of the 1 ml ethanol sample was evaporated, re-dissolved in 50 \( \mu \)l of chloroform/heptane/methanol (10:10:1, by vol.) and applied to the column. The majority of urinary steroids were eluted with 14 ml of chloroform/heptane/methanol as eluent; the oestriol/diphenol fraction then was eluted with 4 ml of methanol. The latter fraction was evaporated to dryness under nitrogen, dissolved in ethanol (600 \( \mu \)l) and a 200-\( \mu \)l aliquot taken for derivatisation for GC and GC-MS analysis. GC-MS analysis confirmed that no major steroid contaminants were present in the diphenol fraction and that further elution with methanol gave no other compounds of interest.

**Derivatisation**

Trimethylsilyl ether (TMS) derivatives were prepared by adding \( N,O\)-bis(trimethylsilyl)trifluoroacetamide (BSTFA)-pyridine (100 \( \mu \)l, 4:1, v/v) to the dry residues obtained by LH-20 chromatography and heating at 60°C for 1 h.

**GC and GC-MS**

Preliminary identification of major diphenols was based on predetermined reten-
tion indices expressed as methylene units (MU) [25]. They were obtained as the TMS ethers, on a Hewlett-Packard 5710A gas chromatograph equipped for flame ionization and connected to a Shimadzu CR4A integrator for quantitation of areas and for computation of the MU values. Capillary GC was performed on a 30 m SE-30 column using helium as carrier gas with a flow rate of 1 ml/min. Samples (1 μl TMS ethers) were applied to the column via an all-glass solid injector modified for a syringe-type plunger. Analysis was performed using temperature programming from 197°C to 270°C at 1°C/min. The flash heater was set at 250°C and the detector temperature at 300°C. Quantitation of the compounds was obtained by GC, expressed relative to the amount of cholesterol butyrate (50 ng) co-injected with each derivatised sample.

GC-MS was performed on a Finnigan TSQ-70 mass spectrometer using electron ionisation mode. This was carried out with repetitive scanning over the mass range of 80–800 Da and under the following conditions: temperature of transfer line, 280°C; ionization voltage, 70 eV; and ionization current, 1,200-1,800 μA. Urinary diphenols were considered identified when GC retention parameters and mass spectra were identical to those of the reference standards.

Control samples
A urinary sample with no detectable levels of equol, daidzein and genistein was spiked with 60 μg of each of these three standards and used as a control sample in every batch of urine samples assayed. The combined mean recovery of the standards in the spiked samples following enzyme hydrolysis, extraction and purification on LH-20 was 87% (range 82–93%; n = 11) as obtained by GC; the three spiked compounds were the only compounds identified by GC.

[^H]oestradiol glucuronide and oestriol were added to each sample at the beginning of the analysis. After enzyme hydrolysis and extraction, the mean percentage recoveries determined by scintillation counting and GC were 80% and 83%, respectively, which was in agreement with the recoveries obtained from the control samples above.

Quantitation of isoflavone levels in soya
Soya flour (5 g) was refluxed in 80% ethanol (100 ml) for 2 h and filtered and evaporated to 5 ml. The extract was diluted to 20 ml with 0.1 M acetate buffer (pH 5) and to this solution 1,400 Fishman units of β-glucosidase (Sigma G-0395) were added and incubated for 48 h at 37°C. The solution was extracted twice with 2× volume of diethyl ether and evaporated to dryness under nitrogen. Daidzein, genistein and glycitein levels in the soya flour were quantitated by GC and their identity confirmed by GC-MS.

3. Results

3.1. Isoflavone content of soya

Isoflavone levels in the soya flour were calculated to be 98 mg (genistein), 80 mg (daidzein) and 3 mg (glycitein) per 100 g.
Fig. 1. The urinary diphenol GC profile (TMS ethers) of baseline urine (day 0) of one subject and after (day 3) ingestion of soya flour in two subjects. Cholesterol butyrate (IS); $C_{22}H_{46}$ (C-22); $C_{34}H_{70}$ (C-34); O-desmethylangolensin (Dma); equol (Eq); enterolactone (Ent); daidzein (D); oestriol (O3); genistein (Gen); glycine (Gly); dehydro-O-desmethylangolensin (1); 6'-hydroxy-O-desmethylangolensin (2); unknown MU 26.12 (3); unknown MU 26.41 (4); dihydrodaidzein (keto form) (5); tetrahydrodaidzein (6); dihydrodaidzein (enol form) (7); tetrahydrodaidzein (8); dihydrogenistein (9).
3.2. Diphenols in human urine

Two examples of the urinary diphenol profiles obtained by GC following soya challenge are shown in Fig. 1. Levels of diphenols identified in the urine of all 12 volunteers obtained before and after ingestion of soya flour are summarised in Tables 1 and 2.

Daidzein (MU 28.60), genistein (MU 29.15), glycine (MU 30.35), equol (MU 25.45), O-desmethylangolensin (MU 24.85) and enterolactone (MU 27.74), were identified in human urine by GC and GC-MS of the basis of MU values and mass spectral data [18–24].

Five additional diphenols were observed with isoflavonoid-like mass spectra. The first of these novel compounds, (MU 26.03), on the basis of mass spectral data of the TMS ether was characterised as (1-(2',4',6'-trihydroxyphenyl)-2-(4'-hydroxyphenyl-prop-l-one)) (6'-hydroxy-O-desmethylangolensin; 6'-OH-O-Dma) (M+ 562, base peak (bp) m/z 369). Two other compounds were observed at MU 27.45 (M+ 488, bp m/z 298) and MU 25.92 (M+ 472, bp m/z 281) and were identified by GC-MS as (4',5,7-trihydroxyisoflavonone) (dihydrogenistein) and (1-(2',4',6'-trihydroxyphenyl)-2-(4'-hydroxyphenyl-prop-2-ene-1-one) (dehydro-O-desmethylangolensin), respectively. The remaining two novel compounds were

<table>
<thead>
<tr>
<th>Diphenol (MU value)</th>
<th>Days in relation to soya challenge</th>
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<td>0</td>
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<tr>
<td>Isoflavones</td>
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<tr>
<td>Daidzein (28.60)</td>
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<td>(1.8–6.6)</td>
<td>(7.3–25.6)</td>
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<td>Genistein (29.15)</td>
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<td>(0.8–1.96)</td>
<td>(2.7–19.6)</td>
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<td>Glycitein (30.35)</td>
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<tr>
<td>(0.02–2.4)</td>
<td>(0.6–6.4)</td>
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<td>O-Dma (24.85)</td>
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<td>(2.3–44.4)</td>
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<td>Equol (25.45)</td>
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<td>(ND-0.7)</td>
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<td>6’OH-O-Dma (26.03)</td>
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<td>Dihydroadaidzein</td>
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<td>(26.65 + 27.11)</td>
<td>(0.02–3.2)</td>
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<tr>
<td>Enterolactone (27.74)</td>
<td>11.03</td>
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<td>(0.9–35.3)</td>
<td>(7.5–26.9)</td>
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ND, not detected.
Table 2
Total urinary excretion of isoflavones, their metabolites and enterolactone in 12 subjects in a 3-day period immediately following a soya challenge (data expressed as $\mu$mol of compound per 72 h total urine volume)

<table>
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<tr>
<td>Daidzein</td>
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<td>28.52</td>
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<td>40.63</td>
<td>22.34</td>
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<td>11.60</td>
<td>10.35</td>
<td>12.98</td>
<td>24.86</td>
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<td>18.17</td>
<td>3.52</td>
<td>17.87</td>
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<td>12.62</td>
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<td>5.44</td>
<td>15.69</td>
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<td>7.00</td>
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<td>8.75</td>
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<td>73.08</td>
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</table>

ND, not detected.
observed at MU 26.77 and MU 27.15; (M⁺ 474, bp m/z 280) displaying similar fragmentation patterns as exhibited by the two isomers of cis/trans (4',4,7-trihydroxyisoflavan) (tetrahydrodaidzein; MU 26.05 and 26.39) which were obtained from daidzein. The complete mass spectral data pertaining to the characterisation of these novel compounds will be presented elsewhere.

GC and GC-MS also revealed the presence of two peaks at MU 26.65 and MU 27.11, which on derivatisation with BSTFA or BSTFA in pyridine and quantitation, appeared to be interconvertible and therefore related. Compound MU 26.65 was identified on the basis of mass spectral data (M⁺ 400, bp 192) and by comparison with a reference compound as the keto form of dihydrodaidzein (intermediate O). Compound MU 27.11 was characterised as the enol form of dihydrodaidzein with a molecular ion at m/z 472 (also the bp), a difference of 72 Da, which accounts for the extra TMS ether. The urinary levels of dihydrodaidzein quoted in this study are the combined levels of these two tautomers. Two further compounds with isoflavone-like mass spectra were characterised at MU 26.12 and 26.41, respectively; no attempt was made in this study to identify these two compounds.

A detailed account of data presented in Tables 1—3 follows:

(a) Isoflavones: Daidzein, genistein and glycitein were detected in the urine of all 12 subjects prior to the soya challenge; total urinary levels of these three isoflavones ranged between 3.3 and 8.2 µmol/day with daidzein being the predominant isoflavone in each case. Following the soya challenge, each subject showed elevated urinary levels of all three compounds, peaking on day 3 and returning in most cases to basal levels by day 4.

(b) Isoflavonoid metabolites: The metabolites equol, O-Dma and 6'-OH-O-Dma were detectable prior to soya challenge in the urines of 9, 10 and 10 of the 12 subjects, respectively. (The limit of detestability for equol by the GC procedure used in this study was established to be 0.02 µmol.) Following the soya challenge, all three metabolites were found in the urine of each subject at elevated levels; the levels peaked on day 3 and then fell progressively over days 4 and 5.

The minor metabolites identified here were detected only in urine collected on day 3 of the study. The tetrahydrodaidzein isomers and dihydrogenistein were identified

<p>| Table 3 |
|---|---|---|
| Comparison of the urinary excretion rates of isoflavones and isoflavonoid metabolites in individuals over the 72 h following 2 consecutive days of soya challenge. Individuals are grouped as either low-equol (less than 8 µmol in 72 h) or high-equol (over 25 µmol in 72 h) producers. |</p>
<table>
<thead>
<tr>
<th>Mean (S.D.) excretion (µmol)</th>
<th>Low-equol producer (n = 8)</th>
<th>High-equol producers (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Equol</td>
<td>1.53 (2.60)</td>
<td>64.89 (59.23)</td>
</tr>
<tr>
<td>O-Dma</td>
<td>21.72 (17.93)</td>
<td>6.97 (6.47)</td>
</tr>
<tr>
<td>6'-OH-O-Dma</td>
<td>14.29 (9.66)</td>
<td>5.25 (4.78)</td>
</tr>
<tr>
<td>Daidzein</td>
<td>23.05 (12.43)</td>
<td>14.95 (6.69)</td>
</tr>
<tr>
<td>Genistein</td>
<td>11.36 (7.81)</td>
<td>10.88 (6.59)</td>
</tr>
<tr>
<td>Male/female</td>
<td>4.4</td>
<td>2.2</td>
</tr>
</tbody>
</table>
in the urine of single subjects only (subjects nos. 12 and 3, respectively); dehydro-O-desmethylangolensin was observed in the urine of four subjects (subjects nos. 1, 3, 8 and 9).

(c) Enterolactone: Enterolactone (MU 27.70, M⁺ 442, bp m/z 180) was present in relatively high levels in the urine of all 12 volunteers both before and following the soya challenge; enterolactone levels were unaffected by the soya challenge.

(d) Interrelationship of urinary diphenols: Individuals were grouped according to their equol excretion rates over the 3 days immediately following the soya challenge, and the relationships between equol and the other diphenols compared and statistically analysed by coefficient of linear regression. These data are summarised in Table 3. A higher rate of excretion of equol appeared to be associated with relatively lower levels of excretion of O-Dma \((r = -0.3)\) and 6’OH-O-Dma \((r = -0.18)\).

4. Discussion

The data presented here confirm that soya is a rich source of the isoflavones genistein and daidzein and that these undergo varying degrees of intestinal metabolism in humans following ingestion, with both those isoflavones and their metabolites then being absorbed and excreted in the urine.

In soyabean, various processed soya products and most other legumes studied which are used as human foodstuffs, the principal isoflavones present are daidzein, genistein and glycitein, with low levels of pratensein and prunetin [4,23,26–30]. The isoflavones are present in the plant principally as the glycosides (daidzin, genistin, glycitin) with only approx. 1% present as free aglycones [27,28]. Formononetin and biochanin A which are common in some other legumes mainly used as animal foodstuffs [4], appear to be at low or negligible levels in soya [29]. The isoflavone content of soya varies greatly with soya plant variety as well as with the form of processing used to make the soya flour [27]. In this study, the batch of soya flour used had daidzein and genistein levels of 80 and 98 mg/100 g flour respectively which are at the higher end of the range of levels reported by others [30].

Current knowledge of isoflavone metabolism stems largely from sheep studies. Once ingested, free aglycone forms of genistein, daidzein, biochanin A and formononetin produced by acid or enzymatic hydrolysis of the conjugated isoflavones are available for absorption, appearing in blood and urine as glucuronide conjugates [12,13]; biochanin A and formononetin also may be demethylated by gut bacteria to genistein and daidzein, respectively [31]. Daidzein then is further reduced by gut flora to equol and various other metabolites including angolensin and O-Dma [13,14,31–33]; it has been calculated that about 70% of ingested formononetin in sheep is converted to equol [12]. Genistein is reported to be metabolised largely within the gut by ring cleavage to the non-oestrogenic compound, para-ethyl phenol [13,32,33]. Glycitein metabolism has not been described in sheep or other animals.

In humans, the isoflavones formononetin, daidzein and genistein, and the isoflavonoid metabolites equol, dihydrodaidzein, O-Dma and methylequol have been identified in urine [5,6,16,17,19,20,34]. Human faecal flora have been shown to be able to produce equol from soya-rich broth [5] and enzymes capable of carry-
ing out the reduction and deoxygenation reactions in the conversion of daidzein to equol have been identified in human gut flora [35], so it is reasonable to suppose that the metabolism of dietary isoflavones in humans has some common ground with that in sheep.

The relatively low levels of daidzein, genistein, equol and O-Dma found in this study to be present prior to soya challenge in the urine of all 12 subjects on a typically Western omnivorous diet is consistent with the relatively low legume content of that diet. Others [5,6,19,20] similarly report generally low urinary isoflavone levels in non-vegetarian European and North American individuals. However, it is interesting to note that the baseline urinary isoflavone levels found in this study are somewhat higher than in other studies of Western communities [5,6,19,20]. To what extent this apparent difference is due either to methodological factors or to real dietary differences is unclear. None of the 12 test subjects reported knowingly eating any legumes (soya, chick peas, lentils) or legume products (tofu, soymilk, etc.) with relatively high isoflavone content for the week prior to the study, so the urinary isoflavones more than likely derived from leguminous vegetables such as peas, broad beans and peanuts which generally were consumed three to five times weekly in modest amounts by all of the subjects. However, the baseline urinary isoflavone levels in this study were still below those of Japanese maintaining a traditional Japanese diet or of vegetarian subjects in Western communities with significant dietary soya intake on a regular basis [9,20].

The baseline urinary enterolactone levels reported here also were comparatively higher than those reported in other Western surveys of omnivorous individuals [6,20] and may reflect a relatively high consumption of cereal grain in the form of wholemeal bread and breakfast cereals which was reported by most of the 12 subjects. The levels are well below those found in individuals in Finland and the USA with a high cereal grain intake such as in a macrobiotic diet [20], but well above Japanese levels where that country's typical diet is low in cereal grains [9].

The responses of the subjects in this study to a soya challenge produced two noteworthy results. The first was the broad range of metabolites appearing in the urine. A proportion of dietary genistein and daidzein appeared to escape complete metabolic degradation within the intestine, being absorbed as the aglycones which were largely cleared in the urine within 24 h of absorption. However, a proportion of genistein and daidzein underwent more complete intestinal fermentation to yield various intermediate products and end-products which also were readily absorbed and then largely cleared in the urine within 24 h. We have confirmed the presence of equol, dihydrodaidzein and O-Dma as major metabolites of daidzein in humans, as well as describing for the first time four compounds which tentatively have been identified as minor daidzein metabolites, 6'-OH-O-Dma, dehydro-O-Dma and two isomers of tetrahydrodaidzein. The inter-relationships of the various daidzein metabolites cannot be commented on here, but the range of metabolites observed in this study suggests that there may be a number of alternative metabolic pathways; the results of this study also hint at the possibility of an inverse relationship between equol production and that of O-Dma which is in support of alternative metabolic pathways. The metabolic processes in humans associated with genistein are less clear than those with daidzein. para-Ethylphenol, the principal genistein metabolite in
sheep, has not been reported in human urine. We are, however, able to identify for
the first time the metabolite, dihydrogenistein. Glycitein metabolism has not been
described in humans and other animals; in this study, much of the glycitein content
of the soya challenge was recovered in the urine as the aglycone, suggesting little if
any further metabolism of this particular isoflavone.

The second noteworthy result is the high variability of the metabolic response to
isoflavones. In previous studies, others have noted the large variation in urinary
equol excretion in response to soya [5,9], an effect confirmed here. In this study, all
12 test subjects showed substantial rises in urinary levels of the three isoflavones
(daidzein, genistein, glycitein) following the soya challenge, indicating uniform com-
pliance with the test, as well as absorptive ability of all individuals for isoflavones.
Moreover, all 12 subjects showed at least moderate rises in urinary levels of the
isoflavonoid metabolites, O-Dma and 6'OH-O-Dma, indicating the ability in all 12
subjects to metabolise dietary isoflavones. However, the results of this study suggest
that while all 12 individuals had the capacity to metabolise dietary isoflavones, there
were differences in the particular metabolic pathways used. The reasons for this
variability are unclear; this study suggests that gender is an unrelated factor,
although genetic factors may be important [9]. In sheep, the overall efficiency of me-
tabolism of dietary isoflavones is influenced by the type of diet fed, although the rel-
ative proportions of the different metabolites appears to remain unaltered [31]. This
factor has been ascribed to different rates of passage of the different diets through
the rumen which is the principal site of detoxification in the sheep [12]. Setchell et
al. [5] have cited factors such as the composition of the intestinal microflora, the in-
testinal transit time and variability in the redox level of the large intestine as possible
causes of the variable rates of equol production in humans. Diet is known to influ-
ence the level of activity of intestinal bacterial enzymes in humans, including β-
glucosidase which is required for hydrolysis of the dietary isoflavones [35]. However,
a number of the subjects in this study have been tested repeatedly by the authors over
several years and have demonstrated consistent patterns of urinary excretion of the
various isoflavonoid metabolites (unpublished), suggesting that the factor responsi-
ble for the individual variability may be inherent and less likely to be due to in-
discriminant dietary factors.

A number of studies have inferred that dietary isoflavones play an important
physiological role in human health [5–9]. Isoflavones exhibit an affinity for
oestrogen receptor sites and may therefore be considered to function as anti-
oestrogens through competitive inhibition [2,36,37]. The complexing of the
isoflavone with the cytosol oestrogen receptor is thought to inhibit the biosynthetic
process associated with the receptor as well as inhibiting the replacement of the
receptor leading to a diminished concentration of the cytosol receptors. Genistein,
daidzein and equol have relatively strong affinities for oestrogen receptors, with O-
Dma showing much weaker binding affinity and glycitein appearing to be non-
oestrogenic [2,24,36,37]. Notwithstanding the weaker affinity of the isoflavones and
their metabolites for oestrogen receptors relative to oestradiol [2,36,37], the finding
that all individuals in this study challenged with soya showed urinary isoflavonoid
metabolite levels greatly in excess of the classical oestrogens [38], points to a poten-
tially significant in vivo effect in those individuals on endogenous oestrogen metabo-
lim and function.
5. References
