Absorption and Excretion of the Soy Isoflavone Genistein in Rats[1]

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ABSTRACT Rodent models have been used to study the anticarcinogenic properties of the soy isoflavones, particularly genistein, but there is little information regarding the pharmacokinetics of the absorption and excretion of genistein. In this study, rats were given a single oral dose of genistein (20 mg/kg body weight) or an equivalent dose of its glycone forms, as an isoflavone-rich soy extract. Concentrations of genistein were measured in plasma, urine and feces at intervals up to 48 h after dosing. Plasma genistein concentration at 2 h after dosing was 11.0 ± 2.3 μmol/L in genistein-treated rats compared with 4.93 ± 0.22 μmol/L (P = 0.025) in soy extract–treated rats, but there were no significant differences at 8 h and later times. The mean urinary excretion rate during the first 2 h after dosing was more than 10 times higher in the genistein group compared with the soy extract group (0.27 ± 0.08 μmol/h and 0.020 ± 0.011 μmol/h, respectively, P = 0.017) but the percentage of dose recovered in urine over 48 h was not different between groups (19.9 ± 2.4% genistein treated; 17.5 ± 1.1% soy extract treated). There were no significant differences between groups in the recovery of genistein in feces (21.9 ± 2.8% and 21.1 ± 2.5% of dose, respectively). Only 6.1 ± 0.9% of the daidzein from the soy extract was recovered in the feces. The results suggest that the extent of absorption of genistein is similar for the glycone and aglycone forms. Although higher initial plasma concentrations may be achieved with the aglycone, similar long-term concentrations exist for both forms of isoflavone. J. Nutr. 126: 176–182, 1996.

INDEXING KEY WORDS:

• *genistein* • *daidzein* • *pharmacokinetics* • *rats* • *soy*

There is considerable interest in the potential role of flavonoids in human health, and evidence has accumulated for beneficial effects with regard to cardiovascular disease (Hertog et al. 1993), osteoporosis (Brandi 1992) and cancer (for review see Messina et al. 1994), with a role for isoflavonoids being suggested for the latter two. In the case of cancer there have been a number of studies of the effects of soy products or the soy isoflavones in rodent models (Pereira et al. 1994, Wei et al. 1995; for review see Messina et al. 1994). Although information is available regarding the pharmacokinetics of some flavonoids (Gugler et al. 1972, Hackett and Griffiths 1977) and the synthetic anti-osteoporotic isoflavone, ipriflavone, in animals (Yoshida et al. 1985b) and in humans (Saito 1985, Sato et al. 1986), there is limited information with regard to the two main soy isoflavones, genistein (4,7,4'-[trihydroxyisoflavone) and daidzein (7,4'-dihydroxyisoflavone). An assessment of the extent of absorption of daidzein has been made in rats (Yueh and Chu 1977), and Lundh (1995) has measured plasma concentrations of formononetin and daidzein in a number of farm animals following an isoflavone-rich clover meal. In humans, Xu et al. (1994) measured urinary and fecal excretion as well as plasma concentrations at two time points following a single soy meal, and Hutchins et al. (1995) compared urinary excretion following consumption of fermented and unfermented soyfoods. However, detailed information regarding the pharmacokinetics of the soy isoflavones in humans and in the rat is lacking.

Genistein is present in plants and foods such as soy and is consumed, therefore, almost entirely in glycosidic forms, the simplest of which is genistin, in which a glucose molecule is linked to the A ring of isoflavone at the 7 position (Barnes et al. 1994). It has been suggested that bacterial hydrolysis of the glycosidic link of the glycosidic forms must occur before absorption from the gastrointestinal system can take place. However, there is no information regarding the influence of glycosidic conjugation on the absorption processes. The aim of the present study, therefore, was to obtain

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information regarding the pharmacokinetics of the absorption and excretion of genistein in rats and to determine the influence of glycosidic conjugation on these processes.

MATERIALS AND METHODS

Reagents. Genistein, 4-ethyl phenol and β-glucuronidase were obtained from Sigma-Aldrich, Castle Hill, NSW, Australia; daidzein was purchased from Research Biochemicals International, Natick, MA, and equol from Plantech (UK), Reading, England. All other reagents were analytical grade. The concentrations of standard solutions of genistein, daidzein and equol were determined using the following molar extinction coefficient values: genistein, ϵ_{262} 37,260 (Walter 1941); daidzein, ϵ_{254} 27,542 (Budavari 1989); equol, ϵ_{284} 4,100 (value supplied by Dr. J. Ingham, Plantech UK, personal communication).

Preparation of isoflavone-rich extract from soy flour. Soy flour (Lowan Whole Foods, Nhill, Victoria, Australia) was refluxed for 20 min with 800 mL ethanol/L (5mL/g flour) to extract isoflavones. The extract was centrifuged for 20 min at 900 × g in a Beckman GPR centrifuge at 5°C, and the supernatant removed and concentrated in a rotary evaporator (Buchi, Flawil, Switzerland) at 50°C until the volume was reduced to approximately one twentieth that of the original extract. Free and total concentrations of daidzein and genistein were measured using HPLC as detailed below. Free concentrations were <1% of the respective total values, demonstrating that the isoflavones were present almost entirely in the glycone forms. The extract was diluted with water to give concentrations equivalent to 3.5 g genistein/L (and 3.4 g daidzein/L) in the aglycone forms and stored at -20°C.

Animals, diets and treatments. Male hooded Wistar rats were obtained at 10 wk of age from the CSIRO Division of Human Nutrition breeding facility (O'Halloran Hill, South Australia) and housed at 25°C with a 12-h light:dark cycle with free access to food and water. All experiments were approved by the Division's animal care and ethics committee and conformed to published guidelines (National Health and Medical Research Council, CSIRO and Australian Research Council, 1985).

Seventy-two rats were used, with equal numbers randomly assigned to genistein or soy extract treatment groups. For each treatment, six rats were killed by decapitation at 0 (untreated), 2, 8, 15, 24 and 48 h after dosing to obtain plasma for isoflavone measurement. For those rats killed at 48 h, urine and feces for isoflavone measurement were collected for 24 h before treatment and for the intervals 0–2, 2–8, 8–15, 15–24, 24–32 and 32–48 h after treatment.

Genistein for administration to rats was freshly prepared in 25 mmol/L Na_2CO_3 at a concentration of 3.3 g/L. Immediately before use, the soy extract was thawed and adjusted to 25 mmol/L Na_2CO_3 and 3.3 g genistein/L by addition of 60 μL 0.44 mol/L Na_2CO_3 per mL extract.

Rats were fed a gelatin-based flavonoid-free synthetic diet for 1 wk to allow elimination of circulating flavonoids likely to arise from the normal nonpurified diet. The composition of the diet is defined in **Table 1**. The rats were then transferred to metabolism cages and urine and feces collected for 24 h to provide pretreatment samples. Mean body weights on transfer were 377 ± 4 g for the genistein group and 377 ± 5 g for the soy extract group. Genistein or soy extract was then administered as a single oral dose by stomach tube (20 mg genistein/kg body weight) and urine and feces collection continued. At the appropriate termination times, rats were anesthetized (sodium pentobarbitone 50 mg/kg body weight) and blood for preparation of plasma taken from the abdominal aorta. Immediately after collection, plasma, urine and feces were frozen at -20C° for storage.

Extraction and hydrolysis of isoflavones. *Soy Extract.* For measurement of free isoflavones, an aliquot of the extract was diluted with water and applied to a C_{18} solid phase extraction cartridge (Lida Manufacturing, Kenosha, WI). The cartridge was washed with 200 mL methanol/L, and the isoflavones eluted

TABLE 1

Composition of the rat diet

Ingredient	g
Cornstarch	352
Sucrose	325
Water	310
Casein	125
Olive oil	50
Gelatin	50
Methyl cellulose	45
Mineral mix[1]	40
Vitamin mix[2]	7.9
Amino acid mix[3]	3.5
Choline chloride	2.0
Cholesterol	0.38
Butylated hydroxyanisole	0.10

[1] The mineral mixture contained: KH_2PO_4, 11.0 g; $CaCO_3$, 7.41 g; $MgSO_4 \cdot 7H_2O$, 6.37 g; $Ca_3(PO_4)_2$, 5.28 g; NaCl, 4.89 g; KCl, 4.23 g; $FePO_4 \cdot H_2O$, 0.58 g; $ZnSO_4 \cdot 7H_2O$, 0.24 g; NaF, 18.6 mg; $CuSO_4 \cdot 5H_2O$, 13.2 mg; $MnSO_4 \cdot H_2O$, 10.9 mg; $Al_2(SO_4)_3K_2SO_4 \cdot 24H_2O$, 3.9 mg; KI, 1.6 mg.

[2] The vitamin mix contained: Inositol 0.24 g; *p*-amino benzoic acid, 0.24 g; niacin, 0.21 g; calcium pantothenate, 0.14 g; menadione, 0.11 g; (±)α-tocopherol, 50 mg; thiamine, 48 mg; riboflavin, 48 mg; pyridoxine HCl, 48 mg; folic acid, 5 mg; retinyl acetate, 4.3 mg; biotin, 1 mg; vitamin B-12, 320 μg; cholecalciferol D-3, 150 μg.

[3] The amino acid mix contained: L-methionine, 2.8 g; L-phenylalanine, 0.62 g; L-tryptophan, 0.10 g.

with 800 mL methanol/L and measured by a HPLC procedure (see below). For measurement of total isoflavone concentration, an aliquot of the extract was mixed with 9 volumes absolute ethanol and 10 volumes 4 mol/L HCl and hydrolysed for 30 min in a boiling water bath (Pettersson and Kiessling 1984). Isoflavones were extracted using a C_{18} solid phase extraction cartridge as described for free isoflavones, eluted and assayed. Preliminary studies showed that the hydrolysis procedure yielded complete hydrolysis of glycosides and that there was full recovery of isoflavones from the C_{18} cartridge.

Plasma and urine. Duplicate 200 μL aliquots of plasma or urine were incubated overnight in 0.17 mol/L ammonium acetate pH 4.6 containing 1.67×10^6 units β-glucuronidase/L (final volume 600 μL) to hydrolyse conjugates as described previously (Record et al. 1995). Preliminary studies established that this protocol ensured complete hydrolysis. Samples were extracted twice with 500 μL diethyl ether and the pooled extracts dried at room temperature with a stream of nitrogen before reconstitution in 200 μL HPLC mobile phase and dilution as necessary for HPLC. Preliminary studies showed that 86.4 ± 2.6% of a genistein spike was recovered from plasma and 90.3 ± 2.5% from urine by this method. The results reported have not been corrected for these losses.

Feces. Feces samples were crushed to a fine powder under liquid nitrogen and duplicate samples (0.3–1.0 g) homogenized at room temperature in a total volume of 20 mL absolute ethanol using a model PT10 polytron (Kinematica G.m.b.H., Lucerne, Switzerland) for 30 s on setting 2. Samples were heated in sealed tubes for 30 min at 55°C to ensure complete extraction, cooled and centrifuged. Aliquots were taken for measurement of free and total genistein and daidzein as follows. For measurement of free isoflavones, extracts were diluted with HPLC mobile phase as appropriate and applied to the HPLC column without further treatment. For measurement of total isoflavones, an aliquot of each extract was mixed with an equal volume of 4 mol/L HCl in a screw-capped tube and heated in a boiling water bath for 30 min to hydrolyse any conjugates. Isoflavones were extracted with a C_{18} solid phase extraction cartridge and eluted with 800 mL methanol/L as described above for the soy extract. The eluates were diluted as required in mobile phase for HPLC. Preliminary studies showed that 99 ± 1% of a genistein spike was recovered from a feces extract processed by this method.

HPLC methods. Isoflavones were separated on a C_{18} column (5 μm, 4.6 mm × 250 mm, SGE Australia, Ringwood, Victoria, Australia) using a mobile phase consisting of 55:50:1 v/v/v methanol:0.1 mol/L ammonium acetate pH 4.6:25 mmol/L EDTA at a flow rate of 1 mL/min. Injection volume was 50 μL. Instrumentation consisted of a model 1550 pump, model 1600 autosampler and model 1260 electrochemical detector operating at a potential of 0.75 V (GBC Scientific Equipment, Dandenong, Victoria Australia). Peak area integration was by DP800 software (GBC Scientific Equipment).

This method allowed clear separation of genistein from daidzein, equol and 4-ethyl phenol, but only partial separation of the latter two compounds from each other. For urine and plasma, where β-glucuronidase was used to hydrolyse conjugates, daidzein could not be quantified due to an interfering peak.

Intra- and interassay coefficients of variation for the entire extraction methods and HPLC were assessed for each of the biological samples by including in each extraction/HPLC series duplicate aliquots of a pool of representative experimental samples. In all cases equal numbers of samples from each time point and each of the two treatments were included in each assay to minimize any influence of interassay variation. Intra- and interassay percentage coefficients of variation were evaluated according to Rodbard (1974) and were as follows for genistein: urine, 8.6, 13.5; plasma 6.8, 10.1; feces (free), 2.7, 5.1, feces (total), 2.7, 3.8, respectively. For daidzein measurements in feces, corresponding values were: 3.7, 7.9 (free) and 7.4, 5.6 (total), respectively.

Statistics. Comparisons at each time point were made using Student's unpaired *t* test with $P < 0.05$ considered significant.

RESULTS

Plasma concentrations of genistein following an oral dose of genistein or soy extract are shown in **Figure 1**. At 2 h after dosing, the mean concentration in genistein-treated rats was 11.0 ± 2.3 μmol/L compared with 4.93 ± 0.22 μmol/L ($P = 0.025$) in the soy extract–treated animals. There were no significant differences between treatment groups at any other time points examined, and at 48 h after dosing concentrations were below the limits of detection (<0.2 μmol/L).

Although caution is required when comparing urinary excretion rates between the various collection periods because the intervals vary in length, it is apparent that the pattern with time differed between the two treatment groups (**Fig. 2*a***). In the genistein-treated animals, the mean excretion rate was initially high and declined during the subsequent periods, whereas in the soy extract–treated animals, the mean rate was initially low, rose to a peak and then declined. The mean excretion rate during the first 2 h following treatment was more than 10 times higher ($P = 0.017$) for the genistein-treated group compared with the soy extract–treated group. The excretion rate also tended to be higher ($P = 0.083$) in the former group during the interval 2–8 h but was not significantly different. As expected based on data in Fig. 2*a*, cumulative ex-

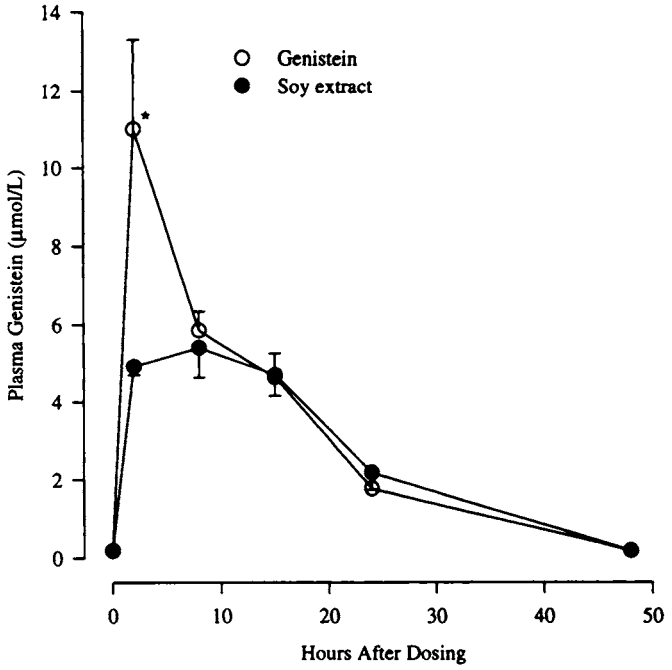

FIGURE 1 Concentrations of genistein in plasma of rats following a single oral dose (20 mg/kg body weight) of genistein as the aglycone or an equivalent dose as its glycones administered as an isoflavonoid-rich soy extract. Values are the mean ± SEM of six rats at each time point for each treatment group. *$P < 0.05$ compared with soy extract group at the same time.

cretion was significantly lower in the soy extract-treated group at the early time points ($P = 0.034$ after 2 h; $P = 0.032$ after 8 h) (Fig. 2*b*). After 48 h there was no significant difference between the groups with 19.9 ± 2.4% of the administered dose recovered for the genistein-treated group and 17.5 ± 1.1% for the soy extract-treated group.

Although equol could not be accurately quantified (see Methods), at the dilutions of extracts used for genistein measurement, equol (and 4-ethyl phenol) was detectable in urine from soy-treated rats only in samples collected after 24 h. This corresponded to an approximate concentration of equol of <20 μmol/L urine. Over the period 24–48 h, this represented excretion in urine of less than 2% of the mean daidzein dose as derived from the soy extract.

The fecal excretion of genistein is illustrated in **Figure 3**. This followed an expected pattern with the greater proportion excreted between 8 and 24 h (Fig. 3*a*). There were no significant differences between the two treatment groups at any time, with 21.9 ± 2.8% of the administered dose recovered for the genistein-treated group and 21.1 ± 2.5% recovered for the soy extract group over the 48-h post-dosing collection period (Fig. 3*b*). Interestingly, for animals dosed with soy extract, only 6.1 ± 0.9% of the administered daidzein was recovered in the feces. For feces, both free and total isoflavones were measured to determine if, for the soy extract–treated animals, any of the isoflavones remained in the glycosidic form after passage through the animal.

There were no significant differences between free and total values at any time point for the soy extract–treated group, with recovered free genistein after 48 h representing 20.1 ± 2.4% of the dose and total genistein 21.1 ± 2.5%, indicating that no significant fraction of the conjugate in the soy extract group escaped hydrolytic cleavage.

DISCUSSION

The present study is the first detailed description of the pharmacokinetics of genistein absorption and

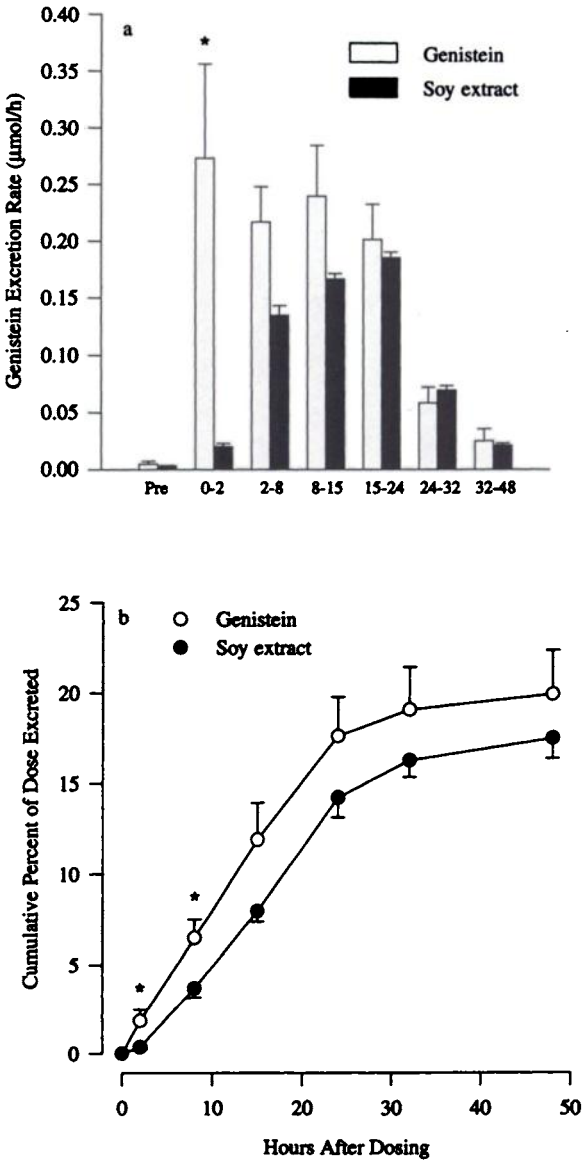

FIGURE 2 Urinary excretion rates (*a*) and cumulative excretion (*b*) in rats following a single oral dose (20 mg/kg body weight) of genistein as the aglycone or an equivalent dose as its glycones administered as an isoflavonoid-rich soy extract. Values are the mean ± SEM of six rats at each time point for each treatment group. Pre = pre-treatment. *$P < 0.05$ compared to soy extract group at the same time.

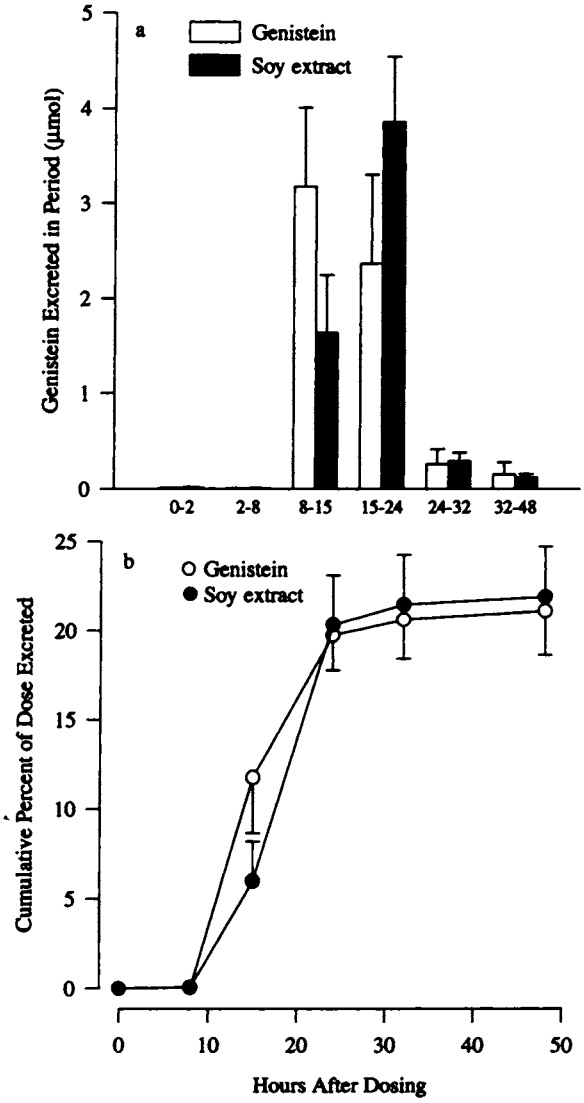

FIGURE 3 Fecal excretion for each collection period (*a*) and cumulative excretion (*b*) in rats following a single oral dose (20 mg/kg body weight) of genistein as the aglycone or an equivalent dose as its glycones administered as an isoflavonoid-rich soy extract. Values are the mean ± SEM of six rats at each time point for each treatment group.

excretion in rats. The urinary and fecal excretion of the related isoflavone, daidzein, has been measured over 24 h in rats (Yueh and Chu 1977), although a time course was not followed. In addition, doses of 50 and 500 mg/kg body weight were used, somewhat higher than the present study, and only in urine were concentrations of total, rather than free, isoflavone measured thus enabling only limited comparisons to be made. However, at an oral dose of 50 mg/kg body weight, ~15% of the dose was recovered in urine over the 24 h following dosing, an amount similar to that in the present study and suggesting significant absorption. Yoshida et al. (1985b) have examined in detail the pharmacokinetics of a related synthetic isoflavone following a single oral dose of radiolabeled compound in rats at a dose of 20 mg/kg body weight, the same as used for genistein in the present study. Ipriflavone (7-isopropoxy isoflavone), unlike the soy isoflavones, lacks hydroxyl groups and is not metabolized substantially by gut bacteria in rats (Yoshida et al. 1985a). It is, however, extensively metabolized by mammalian tissues (Yoshida et al. 1985a), again contrasting with the soy isoflavones which appear to undergo conjugation only with glucuronic acid and sulfate (Lundh 1995). Not withstanding these differences, the recovery of ~20% of administered genistein in feces in the present study is similar to that reported for ipriflavone (Yoshida et al. 1985a). Also in agreement with the results for rats dosed with genistein in the present study, plasma levels of radioactivity peaked at 1.5 h compared with 2 h in our study and declined with a half-life of 5.8 h compared with 8.8 h in our study. Similar results have been reported in humans (Saito 1985). In contrast with the present results, unmetabolized ipriflavone was present at a concentration of <1 μmol/L in the plasma of rats dosed with ipriflavone (Yoshida et al. 1985a) and represented only a very small fraction compared with the metabolites which, in total, reached a peak concentration of approximately 20 μmol/L, similar to the concentration we found for genistein. Consistent with this, whereas approximately 20% of the genistein dose was recovered in urine in the present study, less than 0.1% of administered ipriflavone was recovered as ipriflavone in urine, although more than half of the radiolabel, representing metabolites, was excreted by this route.

Thus the three studies, although differing in some respects, all suggest an appreciable absorption of orally administered isoflavones in rats. The marked difference between the fractions of genistein and of daidzein recovered in feces following dosing with soy extract in the present study is striking and may reflect a greater bioavailability of daidzein compared to genistein (Xu et al. 1994). However, the apparent strong resistance of ipriflavone to bacterial degradation (Yoshida et al. 1985a) compared with the soy isoflavones suggests an important role for hydroxyl substituents in determining this resistance, and the differences in recoveries of genistein and daidzein in feces may also reflect this.

The early differences in urinary excretion rates of genistein between rats dosed with the aglycone (pure substance) and those dosed with the glycoside (soy extract) are consistent with the need for bacterial hydrolysis of the glycosidic link of the latter form in the gut before absorption will occur. The differences in plasma concentrations of genistein between treatments were less dramatic and suggest some early absorption in soy extract-treated rats. A number of factors could be postulated to contribute to this—hydrolysis by the low pH of the stomach, deconjugation of glycosides by hydrolases possibly located in the upper gut wall or a limited ability of the gut to transport

conjugates. The relative contribution of these factors remains to be determined.

The production of isoflavonoid metabolites such as equol from daidzein and 4-ethyl phenol from genistein (Griffiths and Smith 1972) also appears to be totally dependent on the action of gut bacteria in rats (Axelson and Setchell 1981, Griffiths and Barrow 1972). In humans, the concentrations of excreted metabolites can vary over many orders of magnitude from individual to individual (Kelly et al. 1993), suggesting an important role also for human gut bacteria. However, in the present study, equol and 4-ethyl phenol were not detectable in urine or feces before 24 h following dosing with soy extract or genistein (data not shown), suggesting little bacterial action. Interestingly, Xu et al. (1994) were also unable to detect equol in any urine samples from the twelve subjects followed for up to 2 d in their study. It may be significant that in both humans (Setchell et al. 1984) and rats (Axelson et al. 1984), there seems to be a delay of a number of days following the introduction of dietary soy before high concentrations of equol appear in the urine. In both the present study and in that of Xu et al. (1994), only a single dose of soy was administered, and in the present study the rats had been consuming a synthetic flavonoid-free diet for 1 wk before administration of the isoflavone. The disappearance of the capacity of rats to produce equol following transfer to a synthetic diet has been previously noted (Axelson et al. 1984). It is possible that the synthetic diet may have altered gut conditions such as the redox state which have been suggested to influence bacterial populations (Setchell et al. 1984). Possibly a population of bacteria capable of metabolizing the isoflavones must be induced in the gut over a number of days before significant concentrations of metabolites are produced, absorbed and excreted in the urine. Alternatively, under some circumstances, particular bacterial populations may be capable of degradation of the isoflavones predominantly to products other than equol and 4-ethyl phenol, e.g., *O*-desmethyl angolensin (Kelly et al. 1993), and/or to products that have not yet been identified. Such possibilities warrant further study.

The results from the present study are also similar in some respects to those of a human study in which plasma, urinary and fecal concentrations of genistein were measured following a single soy-based liquid meal (Xu et al. 1994). In that study the highest dose consumed provided 2 mg isoflavones/kg body weight of which ~45% was genistein, substantially less than in the present study. Plasma concentrations of genistein were measured at 6.5 and 24 h after dosing, urine was collected at intervals up to 2 d and a total fecal sample was also obtained. At 6.5 h after dosing, plasma genistein concentration was 2.15 μmol/L, similar to the value of 5.4 μmol/L at 8 h in our study. However, in contrast to our study, Xu et al. recovered only 9% of the administered dose of genistein in urine and only 1–2% in feces, compared with ~20% for each of these in our study. In another human study (Hutchins et al. 1995), <2% of genistein consumed as either soybean pieces or as the fermented product tempeh was recovered in urine, substantially less than that reported by Xu et al. (1994). Hutchins et al. (1995) attributed their lower recoveries to physical factors related to the form of the soy-based meal that may have influenced its digestibility and hence the absorption of the isoflavones. If this is the case, it may explain the higher absorption in the present study in which a soluble soy extract was employed. However, as is evident from the comparisons between rats and dogs made by Yoshida et al. (1985b), and as might be expected, there are clearly both similarities and differences between species in their metabolism of isoflavones.

Based on the similarity in plasma genistein concentrations attainable in humans (Xu et al. 1994) and in rats in the present study, rats may provide a useful model for investigation of the biological effects of soy isoflavones. The current challenge is to relate dietary intake of isoflavones to the circulating concentrations required to attain maximum health benefit.

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