DIGESTION AND INTESTINAL METABOLISM OF SOY ISOFLAVONOIDS AND ISOFLAVONOID METABOLITES.

DISSERTATION

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By

Kelly Robert Walsh

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Dissertation Committee:

Professor Mark L. Failla, Adviser

Professor Joshua A. Bomser

Professor Martha A. Belury

Professor Steven J. Schwartz

Approved by

_____

Adviser
Ohio State University Nutrition Program

ABSTRACT

Soy isoflavonoids are phytoestrogenic compounds that exist in three families defined by their parent aglycones daidzein, glycitein and genistein. β-, acetyl- and malonylglucosides of isoflavonoid aglycones predominate in non-fermented soy products, whereas fermented soy foods are rich in aglycones. Isoflavonoid glucosides must be converted to aglycones prior to absorption from the gut.

In this study, digestive stability and bioaccessibility of isoflavonoids and the microbial metabolite equol were examined using simulated digestion, contributions of the small and large intestines in absorption of soy isoflavonoids and isoflavonoid metabolite production were examined using swine with ileal canulae, and Caco-2 human intestinal cells were used to characterize transport and metabolism of equol.

Isoflavonoids and equol were stable during simulated digestion and partitioning of aglycones and acetylgenistin into the aqueous fraction of digesta was significantly affected by the concentration of bile present during the small intestinal phase of the procedure. Ileal effluent from swine contained only isoflavonoid aglycones, indicating that hydrolysis of isoflavone glucosides from the meal occurred. Similar amounts of isoflavonoid equivalents were present in pig urine during small intestinal digestion (canulae open) compared to complete digestion (canulae closed) 24 h after consumption

of the soy meal. The quantities of the microbial metabolites dihydrodaidzein, dihydrogenistein and equol in urines after complete digestion markedly exceeded those of parent aglycones. Accumulation of cellular equol equivalents was proportional to the initial medium concentration and reached a maximum within 1 h. Free equol in the basolateral compartment was greatest at 1 h and represented 20% of its initial concentration in the apical medium. By 4 h, 85% of equol was present as phase II conjugates with 50 and 35% of the starting amount located in the apical and basolateral compartments, respectively.

In summary, the small intestine has a critical role in generating the bioavailable form of dietary isoflavonoids. The bioavailability of isoflavonoids from foods containing fat and protein may exceed that of isoflavonoid supplements consumed without food due to enhanced bile secretion. Moreover, ingested equol has the potential to be absorbed and individuals classified as "non-producers" may actually efflux equol conjugates into the intestinal lumen with high efficiency.

Dedicated to my family

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VITA

June 1, 1972 .....Born – Auburn, NY

1995.....A.A.S. Electrical Engineering Technology
Cayuga Community College

2000.....B.S. Human Ecology, The Ohio State
University

2000 – present .....Graduate Research Associate, The Ohio State
University

PUBLICATIONS

Research Publication

1. Failla M.L., Chitchumronchokchai C. and Walsh K.R. (2004) Bioavailability of β-Carotene. Proc. International Symposium on Biotechnology for Food and Nutritional Security. 145-162.

2. Walsh K.R., Zhang Y.C., Vodovotz Y., Schwartz, S.J. and Failla M.L. (2003) Stability and bioaccessibility of isoflavones from soy bread during in vitro digestion. *J. Agric. Food Chem*. 51: 4603-4609.

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FIELDS OF STUDY

Major Field: Nutritional Biochemistry

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CHAPTER 1

INTRODUCTION

1.1 Overview

A number of epidemiological studies have revealed an apparent inverse relationship between fruit and vegetable consumption and the incidence and severity of certain chronic diseases and cancers (1-6). Elucidation of the mechanisms by which fruits and vegetables confer health-promoting effects on individuals is an active area of research. An expanding literature suggests that numerous non-nutritive components in plant foods likely participate in the prevention and treatment of disease. Such interest in the use of foods for treating and preventing disease led to the inception of the term "functional foods". A food is considered functional when it provides health-promoting properties beyond its basic nutriture (7). The functional properties of foods may be attributed to their macro and/or micronutrient profile, non-nutritive components, or a combination thereof. I am particularly interested in the bioactivities of a family of non-nutritive, plant food constituents known as isoflavonoids.

1.2 Isoflavonoids in Foods

Soy-based foods contain a variety of bioactive components including isoflavonoids. Isoflavonoids, along with coumestans and lignans, belong to a class of plant constituents collectively named phytoestrogens (8). Studies have shown that these

phytoestrogens may enhance cardiovascular, skeletal, and cognitive health (4,9-11). Other reports indicate that isoflavonoids also may act as chemopreventive agents (12,13). Isoflavonoids exist in three distinct families defined by their parent aglycones daidzein, glycitein and genistein (Figure 1.1) and are typically present in the range of 0.1 to 3.0 mg/g dry weight in soy foods (5,14,15). The aglycones also may exist as β-, acetyl- or malonylglucoside conjugates.

The cotyledon, hypocotyl (germ) and seed coat represent 91.5, 2.2 and 6.3% of the dry weight of the soybean, respectively (16). Approximately 88% of isoflavonoids from soybeans are present in the cotyledon and exist primarily as daidzin and genistin (16). The hypocotyl contributes approximately 12% of total isoflavonoids predominantly as glycitin with significant, albeit lower, quantities of daidzin and genistin (16). Isoflavonoid glucosides predominate in non-fermented soy products, whereas fermented soy foods are rich in aglycones (17). The organisms that facilitate the fermentation process secrete β-glucosidases that catalyze hydrolysis of isoflavonoid glucosides to aglycones (18). Common fermented soy foods include natto, miso and tempeh, whereas tofu, soymilk and textured vegetable protein are common unfermented soy foods (19,20).

1.3 Analytical Methods

Isoflavonoid analysis begins with extraction of these compounds from the matrix of interest. Following extraction, isoflavonoids are separated and identified based on their unique chemical properties. High-performance liquid chromatography (HPLC) is commonly used to separate, identify and quantify isoflavonoids. Chromatographic separation is achieved by maximizing interactions between the compounds of interest and the mobile and stationary phases. The stationary phase used during liquid

chromatography consists of a column containing densely packed solids with adsorbant ligands. The chemistry of the stationary phase ligands is selected based on the chemical nature of the compounds to be separated. Reversed-phase columns, which have an affinity for moderate to highly hydrophobic compounds, are commonly employed for isoflavonoid analysis. The mobile phase used during liquid chromatography consists of one solvent or a mixture of solvents whose composition may remain constant (isocratic) or change (gradient) during the course of the analysis. Separation is achieved as compounds that are adsorbed to the column partition into the mobile phase and elute from the column. The mobile phase employed for reversed-phase separation of isoflavonoids is generally a gradient that progresses from a highly hydrophilic to moderately hydrophobic nature during the analysis. Under these conditions, elution of compounds will progress from hydrophilic to hydrophobic as a function of time.

Following separation, isoflavonoids in the eluate can be identified by a variety of methods including electrochemical, ultraviolet/visible (UV/Vis) or fluorescent detection systems (21). UV/Vis detection is the most commonly used approach and offers a high degree of precision and reliability for identifying isoflavonoids (22,23). Absorption maxima for isoflavonoids are within the range of 240 to 280 nm (24). The precision of UV/Vis isoflavonoid identification is enhanced when a photodiode array (PDA) detection module is used. The PDA is far superior to single wavelength detection because absorption data from the eluate are collected over a defined spectrum. Isoflavonoids in the eluate can then be identified and quantified based on retention times and spectral patterns of pure reference standards.

A number of methods are available for extraction of isoflavonoids from foods. Extraction methods such as those described by Franke et al. (25) involve refluxing in ethanol. However, this process may convert the malonyl- and acetylglucosides to their respective β-glucosides (26). Murphy and colleagues (27,28) have developed isoflavonoid extraction methods from food that minimize interconversion of glucosides and offer better recoveries than previous methods. In general, isoflavonoids are extracted from homogenized food in acidified acetonitrile at room temperature. Extracts are subsequently filtered, dried, re-solubilized in methanol and analyzed by reversed-phase HPLC.

The appearance of isoflavonoids and their metabolites in plasma, tissues and urine following consumption is indicative of absorption of these compounds. Extraction of isoflavonoids from these complex biological matrices is more laborious than extraction from foods. The extraction process generally requires pre-separation with organic solvents and/or solid phase extraction (29,30). The samples are then incubated with β-glucuronidase/sulfatase to deconjugate phase II metabolites followed by extraction in organic solvent (31-33). However, it has been shown that boiling acid is more effective than β-glucuronidase/sulfatase in deconjugating phase II metabolites of isoflavonoids from tissues (34). Extracts are then filtered, dried, re-solubilized in solvent and analyzed by reversed-phase HPLC.

Although the concentrations of isoflavonoids in plasma and urine generally are within the limits of HPLC-UV/Vis detection, the quantities of their microbial metabolites are much lower and not readily detected by such analysis. Gas chromatography (GC) or liquid chromatography (LC) in tandem with mass spectrometry (MS) is a highly sensitive

approach for detecting microbial metabolites, as well as identifying specific phase II conjugates of isoflavonoids, in biological samples (35-37). LC-MS is favored because the compounds of interest do not require derivatization prior to analysis (38). LC-MS analysis consists of two basic steps in which compounds are first separated by LC followed by molecular fragmentation by the mass spectrometer (39). Compounds are identified based on fragmentation patterns and the mass to charge ratios (m/z) of reference standards. It is likely that advances in LC-MS technology will lead to discovery of additional microbial metabolites of isoflavonoids.

1.4 Digestion, Absorption and Transport of Isoflavonoids to Tissues

Data pertaining to the site(s) of dietary isoflavonoid absorption and metabolism during gastrointestinal transit are limited. It is well established that isoflavonoid glucosides must be deglycosylated to aglycones prior to absorption from the gut (40). Plasma concentrations of isoflavonoids rise sharply within 2 hours of consuming soy food, followed by another peak within 8-12 hours (41-44). The delayed elevation in plasma isoflavonoids supports the putative role of large intestinal microbes in deglycosylation of isoflavonoid glucosides and absorption of aglycones (45,46). It is also possible that the latter increase in plasma isoflavonoids is due to enterohepatic re-circulation (47). Concentrations of isoflavonoids in plasma are generally less than 10 μmol/L (48).

Recent reports have emphasized the possible role of the small intestine in the conversion of isoflavonoid glucosides to aglycones. Studies have shown that small intestinal tissue from rats is capable of deglycosylating isoflavonoid glucosides (49,50). This process appears to be independent of microbial colonization as demonstrated by

Bowey et al. (51) in germ-free rats. Microbes that inhabit the small intestine may be a source of β-glycosidase activity (43,52), although there is definitive evidence that the brush-border disaccharidase, lactase phlorizin hydrolase, deglycosylates isoflavonoid glucosides (53-55). These findings emphasize the role of host enzymes in the small intestinal mucosal epithelium in generating the bioavailable form of dietary isoflavonoid glucosides.

Bioaccessibility characterizes digestive processes that facilitate the uptake of dietary compounds and/or their metabolites by the absorptive intestinal epithelium (56). Bioavailability generally describes utilization of dietary compounds following transport across the brush-border and basolateral membranes of intestinal epithelial cells (57). A number of factors including the amount consumed, chemical speciation, soy food matrix and intestinal microflora influence absorption of dietary isoflavonoids from the gastrointestinal tract (58,59). Studies clearly indicate that isoflavonoid aglycones are preferentially transported across the apical surface of the absorptive small intestinal epithelium, whereas isoflavonoid glucosides remain in the intestinal lumen (40,60). Information pertaining to digestive stability and factors affecting the bioaccessibility, bioavailability and sites of uptake and absorption of dietary isoflavonoids are inconclusive.

1.5 Pre-absorptive Metabolism of Isoflavonoids

Isoflavonoid aglycones are also subject to metabolism by gut microflora (Figure 1.2). Some species of microbes reduce the aglycone daidzein to dihydrodaidzein, which can be converted to *O*-desmethylangolensin or equol. Likewise, the aglycone genistein can be metabolized to dihydrogenistein and 6'-hydroxy-*O*-desmethylangolensin

(61,62). Simons et al. (63) recently demonstrated that fecal microbes convert glycitein to dihydroglycitein which may be metabolized to 5'-methoxy-*O*-desmethylangolensin or dihydro-6,7,4'-trihydroxy-isoflavone.

Interest in the bioactivities of microbial metabolites of soy isoflavonoids is emerging. Recent reports suggest that the microbial metabolite equol has greater estrogenic activity and antioxidant capacity than its daidzein precursor (64). Human studies repeatedly demonstrate that only 30 - 50% of subjects are equol "producers", i.e., they have an intestinal microflora capable of converting daidzein to equol (65-68). We recently demonstrated that humans without a functional large intestine do not excrete equol into the urine (save one outlier) after consuming a daidzin-containing meal, thereby supporting the role of colonic microbes in the conversion of daidzein to equol (69). Plasma and urinary levels of equol are consistently much lower than daidzein in humans. One explanation for this is that microbial conversion of daidzein to equol is limited. However, it is also possible that the efficiency of absorption of equol from the intestinal lumen is low. If the latter possibility is so, equol supplementation may represent a useful strategy to provide all individuals with the health-promoting benefits of this metabolite. The potential efficacy of dietary equol will be dependent on its stability during gut transit and its transport across the absorptive intestinal epithelium.

1.6 Phase II Metabolism of Isoflavonoids

Isoflavonoids, like many xenobiotics and drugs, undergo extensive phase II metabolism within the intestinal mucosal epithelium (70-72). The family of phase II enzymes is an elaborate system that catalyzes conjugation of lipophilic xenobiotics with hydrophilic functional groups. This represents a detoxification process that increases the

hydrophilicity of the parent compound so that it may readily partition into aqueous excreta such as the urine, bile and sweat (73). Specifically, the phase II enzymes Glutathione S-transferase, UDP-glucuronosyltransferase and sulfotransferase catalyze the conjugation of compounds with glutathione, glucuronic acid and sulfate, respectively (74). Also, NAD(P)H: quinone oxidoreductase catalyzes quinone reduction.

Following conversion to phase II metabolites, conjugated isoflavonoids are transported across the apical and basolateral membranes (75). Conjugated isoflavonoids that are transported across the apical membrane into the small intestinal lumen ultimately may be excreted in the feces. The conjugated isoflavonoids also may be deconjugated by microbial β-glucuronidases with the possibility of the parent isoflavonoids being degraded by microflora, or, transported back into intestinal epithelial cells where they will be conjugated and recycled back to the lumen or absorbed (40,76). Conjugated isoflavonoids that are transported across the basolateral membrane partition in the plasma. Isoflavonoids in the plasma and urine are present predominantly as 7-*O*-glucuronides and 4'-*O*-glucuronides, and to a lesser extent, sulfated derivatives (77-80). There is some evidence that phase II conjugates of isoflavonoids are moderately bioactive *in vitro*, although *in vivo* bioactivities of conjugated isoflavonoids have yet to be confirmed (81,82). It has been demonstrated that chronic soy consumption results in significant increases in the activities of glutathione S-transferase, UDP-glucuronosyltransferase, and quinone reductase in female rats, suggesting that soy consumption may enhance intestinal detoxification of xenobiotics (83).

1.7 Determination of Bioaccessibility and Bioavailability of Isoflavonoids

In vivo and *in vitro* methodologies can be used to assess the bioaccessibility and bioavailability of isoflavonoids from food matrices or supplements. Controlled human studies are the most effective approach to accurately assess the relative bioavailability of isoflavonoids from soy foods or supplements. The standard markers for bioavailability following consumption of soy foods or isoflavonoid supplements include plasma (41,42,46,60,61,84-91) and urinary (42,46,61,84-87,90,92) isoflavonoid equivalent concentrations. Response curves from which the area under the curve is calculated is a more representative estimate of the relative bioavailability of isoflavonoids due to the biphasic peak appearance of these compounds in plasma (41,61,87,89-91). Administration of daidzein and genistein labeled with a stable isotope (^{13}C) also has been used to study the *in vivo* absorption of isoflavonoids in humans (90). The disadvantages to human studies are that they may be laborious, cost-prohibitive and allow limited collection of biological samples. Animal models are an alternative to human subjects and facilitate the use of radioisotopes, as well as availability of tissues of interest.

An alternative to human and animal bioavailability studies are *in vitro* methodologies that assess digestive stability, bioaccessibility and transport and metabolism of isoflavonoids. One such approach is simulated gastric and small intestinal digestion of foods or pure compounds of interest. These methodologies have been used to investigate digestion of proteins (93), starch (94), lipids (95), polyphenols (96) and carotenoids from various matrices (97-99). Simulated digestion procedures provide information about the stability of compounds during gastric and small intestinal phases of digestion.

Moreover, partitioning of compounds into the aqueous (bioaccessible) fraction of the digesta can be determined by exposing the digesta to high-speed centrifugation followed by appropriate analyses.

The strength of *in vitro* digestion models is enhanced when they are coupled with uptake and transport studies in Caco-2 cells. The Caco-2 cell line originated from human colonic adenocarcinoma and exhibits some morphological and functional characteristics that are similar to those of differentiated small intestinal epithelial cells (100). Confluent monolayers of these cells differentiate into polarized, absorptive, enterocyte-like cells with apical brush-border and basolateral membranes (101). Intact monolayers form within one week of seeding cultures and domes are formed when monolayers are grown on non-porous substrates due to Na^+/K^+-ATPase-mediated fluid and electrolyte efflux across the basolateral membrane (102,103). Differentiated cultures of Caco-2 cells also express functional levels of UDP-glucuronosyl transferases, sulfotransferases and multidrug resistance transporters (104,105). Caco-2 cells can be grown and maintained on semipermeable transwell inserts that are suspended in a cell culture well containing medium. This generates a three-compartment model in which localization of compounds into the cellular, apical (upper) and basolateral (lower) compartments can be investigated. Apical to basolateral flux of compounds is an *in vitro* indication of their bioavailability. Transepithelial vs. paracellular flux of compounds across the monolayer is determined by comparing transport rates of compounds of interest with known markers of paracellular flux (e.g., phenol red, Lucifer yellow, inulin, and dextrans).

1.8 Health Promoting Activities of Isoflavonoids

1.8.1 Soy and Cardiovascular Health

The role of soy in cardiovascular health has received a significant amount of attention in both the scientific and lay communities. This is evident by the approval of the FDA food claim in 1999 that permits labeling of soy foods with the statement that "Diets low in saturated fat and cholesterol that include 25 grams of soy protein a day may reduce the risk of heart disease (106)." The cardio-protective activities of soy may be due in part to its cholesterol-lowering potential. The mechanism(s) for the hypocholesterolemic effects of soy products is unclear and conflicting data exist as to whether the soy protein itself, other bioactive components of soy, or the combination of the two results in favorable serum lipid profiles (107-110). Some studies suggest that improved serum lipid profiles following chronic intake of soy may be solely due to the isoflavone content of soy products consumed, although the mechanism(s) of such effects are unclear (111-113). Other studies suggest that soy protein as well as other non-isoflavonoid components of soy may lower serum cholesterol (114-118). It has been suggested that protease-resistant soy peptide fragments decrease the bioavailability of dietary cholesterol by binding bile acids, thereby inhibiting cholesterol transfer to mixed micelles (119,120). We observed that soy protein isolate with or without isoflavonoids resulted in a 40% reduction in micellarization of cholesterol during simulated digestion (Walsh and Failla, 2004, unpublished observations). Moreover, a number of rodent studies have demonstrated the cholesterol-lowering and anti-atherosclerotic effects of partially digested soy protein (121-123).

1.8.2 Estrogenic Activity of Soy Isoflavonoids

A number of the proposed health-promoting benefits of soy isoflavonoids may be attributed to the estrogenic nature of these compounds. The estrogen receptor (ER) belongs to the family of nuclear receptors and two isoforms, ERα and ERβ, have been identified to date (124). Estrogen receptors are activated upon ligand binding in the cytosol, thereby initiating the sequence of homo- or heterodimerization of ERα and ERβ, migration to the nucleus and binding to estrogen response elements to drive transcription of target genes (125,126). Kuiper et al. (127) demonstrated that daidzein and genistein bind with higher affinities to ERβ than ERα, and effectively activate estrogen-dependent transcription *in vitro*. In addition to the role of isoflavonoids as ER agonists, isoflavonoids also may act as estrogen antagonists either by direct interaction with estrogen receptors or through inhibition of gene products involved with estrogen synthesis (128). In light of these findings, isoflavonoids may play a role in the progression of disease in estrogen sensitive tissues.

1.8.3 Isoflavonoids and Cancer

Soy isoflavonoids also have been implicated for their potential role in cancer prevention (129-135). Endocrine-responsive tissues such as mammary, ovary and prostate are likely candidates for the chemopreventative action of soy isoflavonoids due to the estrogenic nature of these compounds (136,137). A significant amount of attention has been directed towards the role of isoflavonoids in prostate cancer prevention since there appears to be an inverse relationship between soy consumption and the incidence of prostate cancer (138). Furthermore, it has been demonstrated that human prostatic fluid is enriched in daidzein and genistein following consumption of a soy beverage (139,140).

Studies have demonstrated that isoflavonoids inhibit proliferation of prostate cancer cells *in vitro* (141,142) and decrease prostate tumor growth *in vivo* (143,144). While the chemopreventive potential of dietary isoflavonoids is promising, further studies are required to confirm their efficacy for such effects.

1.8.4 Isoflavonoids and Skeletal Health

The role of isoflavonoids in skeletal health, particularly in post-menopausal women, is an active area of research (145-147). A number of epidemiological studies have demonstrated and inverse relationship between the incidence of osteoporosis and hip fractures with chronic consumption of soy (148,149). Recent reports indicate that daily supplementation of isoflavonoids by post-menopausal women for greater than 6 months attenuated bone loss (150,151). Furthermore, a number of studies in ovariectomized rats also have demonstrated that isoflavonoids offer protection against bone loss (152-154). However, not all studies confirm the "bone sparing" effects of isoflavonoids (155,156). Further research in humans is needed to qualify the role of isoflavonoids in skeletal health.

1.8.5 Isoflavonoids and Cognitive Function

Isoflavonoids also may have a role in enhancing cognitive function (157,158). File et al. (159) reported that individuals consuming a high soy diet (100 mg/d of isoflavonoids) for 10 weeks showed improvement in both short and long-term memory tasks. In a follow-up study, these researchers reported that post-menopausal women showed improvements in memory recall, attention span and task planning abilities after consuming daily soy supplements for 12 weeks (160). Moreover, Kritz-Silverstein and colleagues (161) reported that post-menopausal women showed improvements in verbal

memory recall after consuming isoflavonoid supplements twice daily for 6 months. These findings suggest that chronic soy consumption may enhance cognitive function; however, further research is needed to gain insight into the mechanisms for such effects.

1.8.6 Antioxidant Activities of Isoflavonoids

Isoflavonoids also are of interest in light of their proposed antioxidant capacity. Rufer and Kulling (162) reported that daidzein, genistein and glycitein are effective in attenuating *in vitro* oxidation of LDL. Yen and Lai (163) reported that daidzein and genistein inhibited the production of reactive nitrogen species following stress with lipopolysaccharide *in vitro* as well as in rats. While the literature is flooded with studies addressing the antioxidant capacity of isoflavonoids, a strong debate on their efficacy as antioxidants exists.

1.9 Adverse Effects of Isoflavonoids on Endocrine Functions

While the health-promoting benefits of isoflavonoids appear promising, there are concerns about the endocrine-modulating potential of isoflavonoids in pre-menopausal women (164). These concerns arise from early observations of reproductive abnormalities in sheep grazing on subterranean clover (165), which has been reported to contain 2% isoflavonoid aglycone equivalents per gram dry weight (166). However, it is interesting to note that the quantity of isoflavonoids consumed by the sheep was 1000 to 2000-fold higher than what humans typically consume (64). Nevertheless, a number of controlled human studies have addressed the effects of soy and/or isoflavonoid consumption on reproductive cycles in pre-menopausal women. Cassidy et al. (167,168) observed increased follicular phase length, delayed menstruation and decreases in lutenizing hormone and follicle stimulating hormone surges in pre-menopausal women

consuming soy products containing approximately 28 mg of isoflavonoid aglycone equivalents per day for 1 menstrual cycle. Prolonged menstrual cycle also has been observed in pre-menopausal women consuming up to 40 mg isoflavonoid equivalents per day for 1 menstrual cycle (169). Conversely, Lu et al. (170) did not observe changes in menstrual cycle length in pre-menopausal women drinking soymilk containing 130 mg isoflavonoid aglycone equivalents per day for one month. Other studies have shown that consumption of soy products containing up to 80 mg of isoflavonoid aglycone equivalents per day for 2 to 3 months did not result in changes in reproductive cycles in pre-menopausal women (171-173). Despite the conflicting data about isoflavonoids and reproductive function, pre-menopausal women concerned about fertility issues should consider consuming soy foods in moderation.

1.10 Isoflavonoids and Infant Health

Soy-based infant formulas are typically fed to infants that are not able to tolerate breast milk or bovine milk-based formulas. Such intolerances may be due to allergies or sensitivities to milk protein, lactose intolerance, or galactosemia (174). Infants fed soy-based infant formulas as their sole source of nutrition may be exposed to up to 45 mg of isoflavonoids per day, resulting in plasma concentrations of isoflavonoids that exceed those of adults by an order of magnitude per kilogram bodyweight (175,176). These findings sparked concern about potential adverse effects of soy isoflavonoids in infants. Soy-based infant formulas have been used for over 50 years, and to date, studies have failed to demonstrate adverse effects in infants and adults that were fed soy-based infant formulas (174,177-179). Moreover, it is possible that exposure to isoflavonoids early in life may offer protection against hormone-related diseases later in life (175). Despite the

controversy over soy-based infant formulas, the American Academy of Pediatrics maintains their position that soy-based infant formulas are safe and viable alternatives to milk-based infant formulas (180). Further long-term studies are required to confirm the safety of soy-based infant formulas.

1.11 Specific Aims

The general goal of my dissertation has been to assess the fate of dietary isoflavonoids and isoflavonoid metabolites during digestion and to characterize intestinal uptake of these bioactive compounds. My research goal was addressed through three specific aims. First, I examined the digestive stability and bioaccessibility of isoflavonoids. I hypothesized that isoflavonoids are stable during digestion and efficiently partition into the aqueous (bioaccessible) fraction of chyme. Simulated oral, gastric, and small intestinal phases of digestion were used to examine digestive stability of soy isoflavonoids and possible partitioning of hydrophobic isoflavonoid aglycones into mixed micelles. Samples of soy-rich bread were digested using simulated digestion methods previously described by Garrett et al. (99) and Muir and O'Dea (181) with slight modifications. Upon completion of the digestion procedure, the isoflavonoid composition of the soy "meal" was compared to that in the chyme and the aqueous (micellar) fraction of the chyme to determine digestive stability and bioaccessibility of these compounds, respectively.

Second, I characterized the gastrointestinal metabolism of isoflavonoids in swine. I hypothesized that the small intestine is the predominant site for deglycosylation of dietary isoflavonoid glucosides. Swine with ileal canulae were used as a model to examine intestinal absorption and metabolism of dietary isoflavonoids. All animals were

maintained in metabolic crates and fed soy-free diets for 5 d prior to administering soy test meals with ileal canulae open (small intestinal digestion only) or closed (fully-functional GI tract). Urine and ileal chyme were analyzed for isoflavonoids by HPLC. The presence of the isoflavonoid microbial metabolites dihydrodaidzein, dihydrogenistein, *O*-desmethylangolensin, equol and 6-OH-*O*-desmethylangolensin was determined by HPLC-MS/MS.

Finally, I characterized the transport and phase II metabolism of equol in cultured Caco-2 cells derived from human colon adenocarcinoma. I hypothesized that the daidzein metabolite equol passively diffuses into Caco-2 cells where it is extensively metabolized to phase II conjugates. The extent of phase II metabolism of equol by Caco-2 cells was determined by analyzing replicate samples by HPLC that were extracted with or without overnight incubation with β-glucuronidase. I then explored the potential use of equol as a dietary supplement by examining its digestive stability and bioaccessibility during simulated gastric and small intestinal digestion.

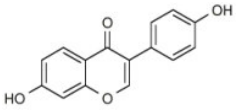
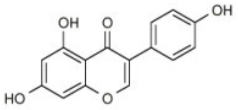
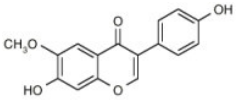
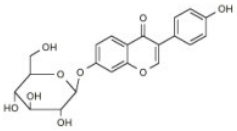
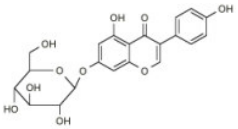
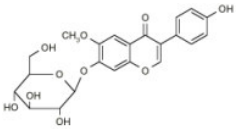
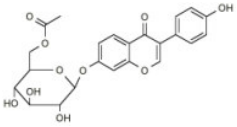
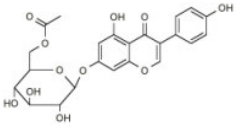
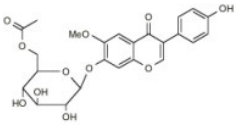
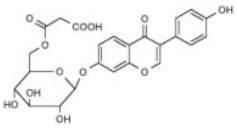
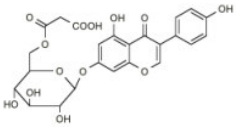
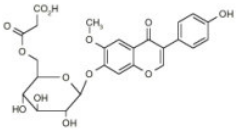
Daidzein Family	Genistein Family	Glycitein Family
		
Daidzein	**Genistein**	**Glycitein**
		
Daidzin	**Genistin**	**Glycitin**
		
Acetyldaidzin	**Acetylgenistin**	**Acetylglycitin**
		
Malonyldaidzin	**Malonylgenistin**	**Malonylglycitin**

Figure 1.1: Chemical Speciation of Isoflavonoids

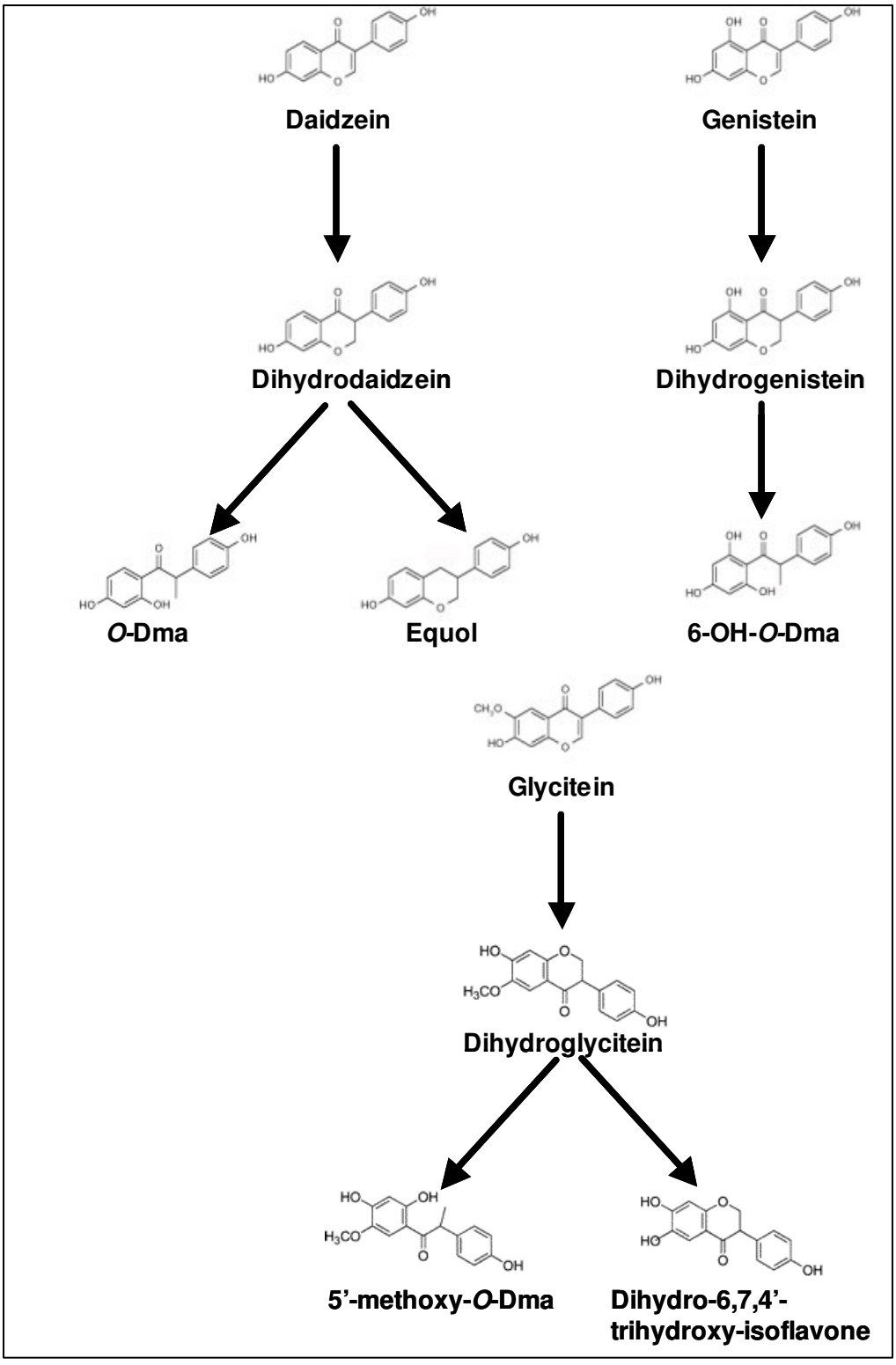


Figure 1.2: Chemical Speciation of Select Microbial Isoflavonoid Metabolites

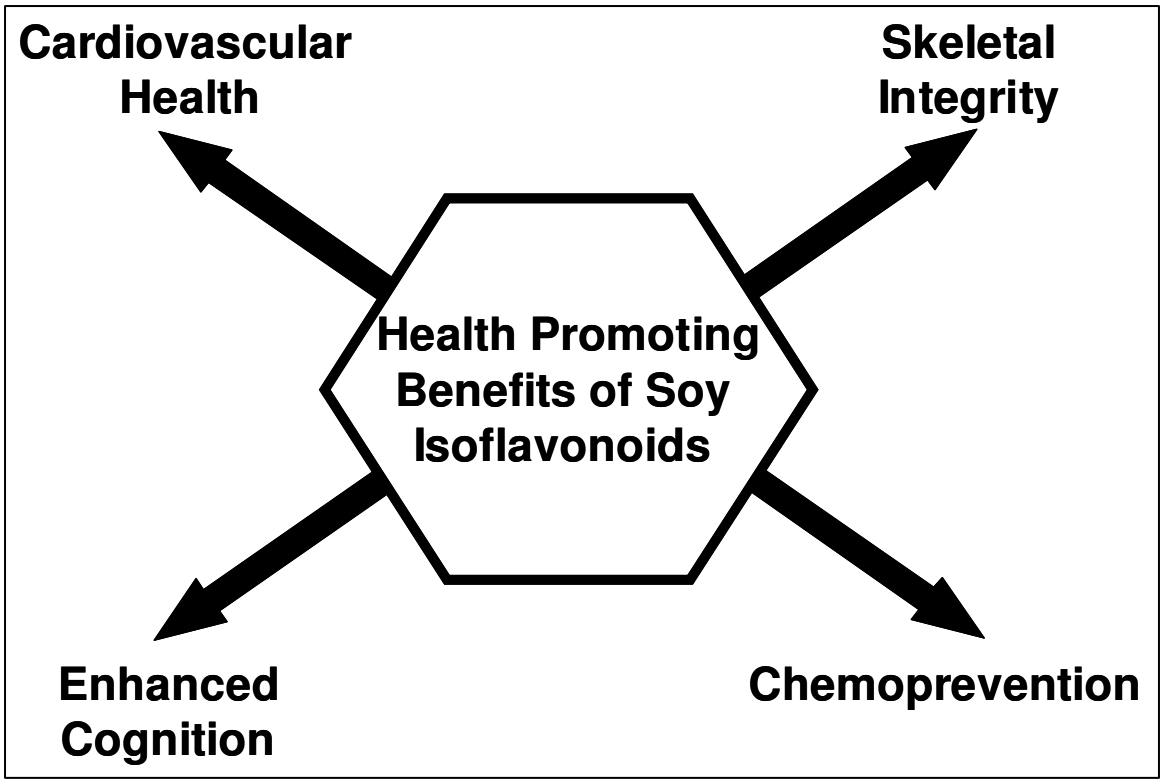


Figure 1.3: Health-promoting benefits of soy isoflavonoids.

CHAPTER 2

STABILITY AND BIOACCESSIBILITY OF ISOFLAVONES FROM SOY BREAD DURING IN VITRO DIGESTION

2.1 ABSTRACT

The impact of simulated digestion on the stability and bioaccessibility of isoflavonoids from soy bread was examined using simulated oral, gastric, and small intestinal digestion. The aqueous (bioaccessible) fraction was isolated from digesta by centrifugation and samples were analyzed by HPLC. Isoflavonoids were stable during simulated digestion. Partitioning of aglycones and acetylgenistin into the aqueous fraction was significantly ($P < 0.01$) affected by the concentration of bile present during small intestinal digestion. Omission of bile resulted in non-detectable genistein and < 40% of total daidzein, glycitein, and acetylgenistin in the aqueous fraction of digesta. Partitioning of these compounds into the aqueous fraction was increased by physiological concentrations of bile extract.

The results suggest that micellarization is required for optimal bioaccessibility of isoflavonoid aglycones. We propose that the bioavailability of isoflavones from foods containing fat and protein may exceed that of supplements due to enhanced bile secretion.

2.2 INTRODUCTION

Numerous studies suggest that estrogen-like isoflavonoids present in soy foods promote cardiovascular, skeletal, and postmenopausal health (4,9,12). Some data also suggest that these compounds may hinder the progression of certain forms of cancer, although further studies are needed to address the efficacy of isoflavonoids as chemopreventive agents (13). Unfortunately, consumption of soy foods by individuals eating a typical western diet remains low. The development of soy-enriched foods represents one strategy for increasing soy consumption. We have recently developed a soy-enriched bread that contains sufficient soy protein per serving to meet the FDA approved health claim (106). The potential health promoting activity of the bread is in part dependent upon the absorption of its isoflavonoids and their bioactive metabolites.

Isoflavonoids in soy products exist in either the free form (aglycone) or as acetyl, malonyl, or β-glucoside conjugates. The content of isoflavonoids varies from approximately 0.1 to 3.0 mg/g dry weight among soybean products (5,14,15). Conjugation patterns of the isoflavonoids are significantly affected by style and duration of cooking, as well as interactions with other ingredients (182). For example, the predominant isoflavonoids in soy flour are the malonyl glucosides. Baking at 190^{o}C does not alter the total isoflavone content in soy flour, but slight increases in β-glucoside conjugates with proportional declines in the malonyl glucosides of isoflavonoids have been reported (183). Malonyl glucoside isoflavonoids are also degraded more rapidly during cooking in the presence of sugar and fat (183).

The bioavailability and bioactivity of soy isoflavonoids depends to some extent on the quantity consumed, chemical speciation, and the physical properties of soy foods

(58,59). Previous studies have demonstrated that the aglycone, but not glycosylated isoflavonoids, are transported across the apical membrane of absorptive epithelial cells in the gastrointestinal tract (40,60). β-glucosidase activity along the brush border surface in the small intestine and resident microflora in the large intestine convert isoflavonoid glucosides to their aglycone derivatives (53,184). Within mucosal epithelial cells, isoflavonoids are extensively modified to glucuronide and sulfate conjugates (43,185). These conjugated compounds can be transferred to the plasma or exported to the intestinal lumen, which confounds determination of their bioavailability (41).

Bioaccessibility characterizes the potential of a dietary compound for apical uptake by absorptive epithelial cells, whereas bioavailability generally describes its utilization following transport across the brush-border and basolateral membranes (57). Information pertaining to factors affecting both the bioaccessibility and bioavailability of dietary isoflavonoids is limited. The specific aims of the present study were to assess the digestive stability and bioaccessibility of isoflavonoids from a soy-rich bread. Simulated oral, gastric, and small intestinal phases of digestion were used to determine digestive stability. Bioaccessibility was determined by examining the partitioning of isoflavonoids into the aqueous fraction of digesta. The potential role of micelles as delivery vehicles for aglycones was examined by varying the concentration of bile during the small intestinal phase of the *in vitro* digestion procedure.

2.3 MATERIALS AND METHODS

2.3.1 Supplies

Porcine pepsin, porcine lipase, porcine pancreatin, porcine bile extract, β-glucosidase (almond), daidzein (4',7-Dihydroxyisoflavone), genistein (4',5,7-Trihydroxyisoflavone), and glycitein (4',7-Dihydroxy-6-methoxyisoflavone) were purchased from Sigma Chemical Co. (St. Louis, MO). Daidzin (daidzein, 7-*O*-β-D-glucopyranoside), genistin (genistein, 7-*O*-β-D-glucopyranoside), acetyldaidzin (6"-*O*-acetyldaidzin), malonyldaidzin (6"-*O*-malonyldaidzin), acetylgenistin (6"-*O*-acetylgenistin), malonylgenistin (6"-*O*-malonylgenistin), acetylglycitin (6"-*O*-acetylglycitin), and malonylglycitin (6"-*O*-malonylglycitin) were purchased from LC Laboratories (Woburn, MA). Other reagents were purchased from Fisher Scientific Co. (Fairlawn, NJ); reagents used for high-performance liquid chromatography (HPLC) analysis were HPLC grade. Soy flour was purchased from Archer Daniel Midland Co. (Decatur, IL), soy milk powder was purchased from DevanSoy Farms (Carroll, Iowa), and wheat flour was purchased from General Mills (Minneapolis, MI).

2.3.2 Soy Bread (SB) Preparation

Soy-rich bread was developed and produced in our laboratory by incorporating soy milk powder into a standard bread formula and replacing 29% of the wheat flour with soy flour. The bread ingredients were combined, and the dough was kneaded in a 5-quart Kitchen Aid Mixer (KitchenAid Portable Appliance, St. Joseph, MI), proofed at 50^{o}C for 1 hr in an oven (Blue M Electric Company, Blue Island, IL), and baked at 165^{o}C for 50 min. The baked SB was allowed to cool at room temperature for 40 min. The macronutrient composition of the SB was 222 mg/g carbohydrate, 22 mg/g fat, 190 mg/g

protein, and 447 mg/g moisture. The crust was removed, and crumb samples were taken from the center of each loaf. Samples were stored in sealed polypropylene tubes at -20°C under nitrogen until analysis.

2.3.3 Preparation of Diluted SB Sample for Reference Starting Material

It was assumed that 1.0 g of SB was equivalent to a 1.0 ml volume for indicated manipulations. Homogenized SB in saline was prepared as a control for the investigation of the effects of *in vitro* digestion on isoflavonoid stability. Soy bread (10 g) and saline (100 mL) were combined in a 250 mL beaker. The bread was homogenized with a Tissumizer batch dispersing rotor-stator (SDT1800, Tekmar Company, Cincinnati, OH) and dispersing probe (S25KR18G, IKA-Werk, Wilmington, NC) for 30 sec at a setting of 85. After the sample was homogenized, the dispersing probe was submerged in clean saline and engaged for 3 sec to collect the residual food sample, which was added to the homogenized SB. The beaker used for collection of the residual food sample was rinsed with saline (30 mL), and the contents were pooled with the homogenized SB. The final concentration of the homogenized sample was 50 mg SB/mL. Aliquots (50 mL) were stored at -20ºC under nitrogen until analysis.

2.3.4 *In Vitro* Digestion.

Oral Phase

Bread samples were subjected to a modified version of the *in vitro* digestion method previously described by Garrett et al. (99). The primary modification involved the addition of an oral phase to the simulated gastric and small intestinal phases of digestion. The oral phase of digestion was performed in a manner similar to that described by Muir and O'Dea (181). Briefly, a single investigator conducted the oral

phase for all *in vitro* digestions to control for potential interindividual variation in saliva composition. After the oral cavity was rinsed with deionized water, 12.5 g of SB (22°C) combined with 10 mL saline (37ºC) was chewed 10 times and subsequently expelled into a tared beaker. The oral cavity was rinsed twice with 10 mL of saline (37ºC) and the contents were expelled after each rinse into the beaker containing chewed SB. Saline (45 mL; 37ºC) was added and the sample was stirred for 5 min at room temperature. The sample was homogenized as described in the reference SB sample preparation and diluted to a final volume of 175 mL with saline. An aliquot (35 mL) was transferred to a centrifuge tube, diluted to 50 mL with saline (50 mg SB/mL), and stored at -20ºC under nitrogen until analysis for determination of the possible impact of the oral phase of digestion on the chemical profile of isoflavonoids in SB.

Gastric and Small Intestinal Phases

The remaining sample from the oral phase of digestion was acidified with 1 mol/L HCl (pH=2.0 ± 0.1). Porcine pepsin (10 mg/mL final concentration) in 100 mmol/L HCl was added, and the sample was diluted to a final volume of 160 mL with saline. Aliquots (40 mL) were transferred to separate 50 mL tubes, blanketed with nitrogen, sealed, and incubated in a shaking (85 rpm) water bath (Versa-Bath S model 224, Allied Fisher Scientific, Indiana) at 37ºC. After 1 h, the reaction tubes were placed on ice and the pH was immediately increased to 6.0 ± 0.2 with 1 mol/L $NaHCO_3$. Porcine bile extract (2.4 mg/mL final concentration), porcine pancreatin (0.4 mg/mL final concentration), and porcine pancreatic lipase (0.2 mg/mL final concentration) in 100 mmol/L $NaHCO_3$ were added to the reaction tubes.

The pH of each tube was increased to 6.9 ± 0.1 with 1 mol/L NaOH, and the final volume was diluted with saline to 50 mL (equivalent to 50 mg SB/mL). All tubes were blanketed with nitrogen, sealed, and incubated in a shaking (85 rpm) water bath for 2 h at 37ºC.

Preparation of the Aqueous Fraction of Digesta.

Following the small intestinal phase of digestion, aliquots (10 mL) of digesta were transferred to 15 mL conical tubes and stored at -20ºC under nitrogen until analysis for digestive stability of SB isoflavonoids. Aliquots (12 mL) of digesta were also transferred to Quick-Seal™ ultracentrifuge tubes (Beckman Instruments, Inc., Palo Alto, CA), heat sealed (Beckman Instruments, Inc., Palo Alto, CA), and centrifuged at 167,000g for 35 min at 4ºC (Beckman L7 ultracentrifuge; 50Ti rotor). After centrifugation was completed, tubes were placed on ice and the aqueous fraction of the digesta was recovered using a syringe with an 18 gauge needle (Becton Dickinson and Company, Franklin Lakes, NJ). A minimum of 5 mL of each aqueous sample was filtered (0.2 µm Acrodisc® syringe filter, Gelman Laboratory) and stored in sealed 15 mL conical tubes at -20ºC under nitrogen until analysis.

Effect of Bile Extract on Isoflavonoid Bioaccessibility.

The effect of concentration of bile extract on isoflavonoid bioaccessibility was addressed by varying the amount of bile extract added to initiate the small intestinal phase of digestion. The final concentrations of bile extract examined were 0, 1.2, 2.4 (standard amount (99)), and 4.8 mg/mL.

2.3.5 Extraction of Isoflavonoids From Samples

SB Sample

SB (0.5 g) was ground to a fine paste and mixed with 100 mmol/L HCl (2 mL), acetonitrile (10 mL) and water (3 mL) in a 50 mL centrifuge tube (186,187). The mixture was shaken with a multiwrist shaker (Lab-line Instrument Inc, Melrose Park, IL) on setting 9 for 2 h before centrifuging (IEC HN-SII, Damon/IEC Division, Needhamhts, MA) at 430 x g for 30 min. An aliquot (1 mL) of the supernatant was then transferred to a glass vial, dried under nitrogen at room temperature, and resolubilized in 100% methanol (1 mL) (188). The mixture was vortexed (Model no. 231, Fisher Scientific, Fair Lawn, NJ) and filtered (0.2 µm syringe filter**,** Alltech Associates Inc, Deerfield, IL) prior to HPLC injection. The reliability of the extraction method was assessed by extracting after spiking digestion samples with known concentrations of pure isoflavonoids and determining their recovery. Recoveries for all test isoflavonoids exceeded 97 %.

Digested SB fractions.

Post-oral, small intestinal digesta, and aqueous fractions of digesta from simulated digestion of SB were thawed at room temperature and vortexed for 1 min. Aliquots (5 mL) were transferred to 50 mL centrifuge tubes and mixed with 100 mmol/L HCl (2 mL) and acetonitrile (5 mL). Samples were extracted as described above. Extracts were immediately filtered (0.2 µm syringe filter, Alltech Associates Inc, Deerfield, IL) and analyzed using HPLC.

2.3.6 HPLC Analysis

A Waters 2695 separation module (Milford, MA) and a Waters 2996 photodiode Array Detector (PDA) were used to quantify isoflavone content. Separation of isoflavonoids was achieved using a Waters Nova-Pak C_{18} reversed-phase column (150 x 3.9 mm; i.d. 4 µm, 60 Å pore size) with a Nova-Pak C_{18} guard column. The mobile phase consisted of 1.0% acetic acid in water (v/v) (solvent A) and 100% acetonitrile (solvent B) at a flow rate of 0.6 mL/min. The injection volume was 10 µL and components were eluted using the following solvent gradient: from 0 to5 min 15% B; from 5 to 36 min 15 to 29% B; from 36-44 min 29 to 35% B; from 44 to 45 min 35 to 15% B; and re-equilibration at 15% B for 5 min prior to the next injection (26). The PDA monitored a spectral range of 210 to 400 nm, and compounds in the eluate were detected from their absorbance at 260 nm.

2.3.7 Isoflavonoid Identification

Retention times and UV absorption patterns of pure isoflavonoid standards were used to identify isoflavonoids in samples following extraction and HPLC analysis (186,187). Identification of aglycones was confirmed by monitoring changes in β-glucoside and aglycone peak areas following enzymatic hydrolysis with almond β-glucosidase (189). To do so, prior to drying the SB extract under nitrogen, an aliquot (1 mL) of SB extract was suspended in 1 mL of 100 mmol/L acetate buffer (pH=5.0) containing 1 mg of β-glucosidase. The mixture was incubated at 37^{o}C in a water bath shaker overnight (186). Concentrations of aglycones and β-glucosides were calculated from the standard curves of these compounds. Concentrations of acetyl and malonyl glucosides were calculated from the curves for the corresponding glucoside.

2.3.8 Standard Solutions and Calibration Curves.

Stock solutions for the different isoflavonoids were prepared by dissolving 1 mg of the crystalline pure compounds in 80% methanol (100 mL). Each solution was then serial diluted with 80% methanol to prepare a standard curve. The concentration of working solutions was determined using the Beer-Lambert Law with UV absorbance readings in the range of 240-360 nm and their molar extinction coefficients in 80% methanol: daidzein λ_{max} = 254nm, ε=26000; genistein, λ_{max} = 262nm, ε=37300; glycitein, λ_{max} = 256nm, ε=22387 (14,25). We applied the λ_{max} and ε values of the aglycones to their respective conjugates for mass calculations with the assumption that the absorption spectra of the conjugates are not significantly different from the representative aglycone (190,191). Aliquots of working solutions were then analyzed by HPLC, and the relationship between HPLC peak area and concentration for each standard solution was calculated. These factors were used to calculate the concentrations of isoflavonoids present in the SB, oral phase, digesta, and aqueous fractions of digesta following HPLC analysis.

2.3.9 Statistical Analysis of Data

Four independent *in vitro* SB digestions were performed, and from each digestion, multiple oral (N = 3), digesta (N = 4), and aqueous fraction (N = 4) samples were analyzed for experiments addressing isoflavonoid stability. Independent digestions with variable concentrations of bile extract were performed, and aqueous fractions ($N \geq 5$) from each digestion were analyzed to characterize the effect on isoflavonoid bioaccessibility. All data are expressed as means ± SEM unless otherwise indicated. Means were compared using one way analysis of variance (ANOVA) followed by

Bonferroni posthoc comparison of statistical significance. Linear regression was performed when the bile extract concentration was varied for assessment of bioaccessibility. An α level of 0.01 was established *a priori* and results were considered statistically significant when $P < 0.01$. Statistical analyses were performed using SPSS Release 11.0 for Windows (SPSS Inc., Chicago, IL).

2.4 RESULTS AND DISCUSSION

2.4.1 Comparison of Isoflavone Profile of Ingredients and SB

The mass balance study demonstrated that 90% of total isoflavonoids from soy flour and soy milk powder were present in the SB product, indicating a general stability of isoflavonoids during processing (Table 2.1). This result is consistent with previous findings in which normal cooking conditions did not alter the total isoflavone content of soy foods (183).

The primary source of isoflavonoids in the SB is soy flour. Malonyl glucosides (49.0%) and β-glucosides (38.5%) were the prevalent isoflavonoids in soy flour with the aglycones representing less than 4% of the total isoflavone content. The isoflavone composition of soy milk powder differed in that β-glucosides (49.3%) and aglycones (30.2%) were most abundant with the malonyl and acetyl derivatives of daidzin and genistin accounting for only 22 and 17%, respectively, of the total. The differences in the isoflavonoid profile of the soy milk and soy flour likely result form altered processing of the beans (192). Soy flour is produced by grinding soybeans into powder, while preparation of soy milk powder involves cooking ground soybeans (95^{o}C, 7 min) followed by drying liquid soy milk (193).

The significant water-holding capacity of soy proteins in soy flour may regulate the internal temperature of the dough during baking and protect some of the malonyl glucosides from heat-induced decarboxylation (183).

Analysis based on derivative families of isoflavonoids revealed that daidzin was the most stable glucoside with a recovery of 101%. However, the amounts of the three malonyl glucosides in SB were 20 – 25% lower than those in the starting ingredients. The amounts of acetyldaidzin and acetylglycitin decreased by 55 and 89%, respectively, during preparation of SB, whereas the amount of acetylgenistin was similar in the ingredients and SB. The decreases in malonyl and acetyl glucosides were associated with a nearly 2-fold increase in the amounts of daidzein, genistein, and glycitein in SB as compared to starting ingredients. The high recovery of the daidzein (96%) and glycitein (105%) families in soy bread from the starting ingredients suggests that the lost malonyl, acetyl, and β-glucosides were converted to their respective aglycone during preparation of SB. However, the 14% decline in total content of compounds in the genistein family suggests that some genistin, malonylgenistin, acetylgenistin, and, perhaps, genistein were degraded during SB preparation. Similar results have been observed in the recovery of genistein and its derivatives following heating (194,195).

2.4.2 Stability and Bioaccessibility of Isoflavonoids From SB During Simulated Digestion

Generally, the bioavailability of a dietary compound is dependent upon its digestive stability, accessibility for uptake across the apical surface of intestinal epithelial cells, and the efficiency of its transepithelial passage for delivery to peripheral tissues. To our knowledge, this is the first study addressing the stability of individual

isoflavonoids during simulated digestion. In a recent report by Andlauer et al. (49) on absorption and metabolism of isoflavones in isolated rat small intestine, it was stated that isoflavonoids from tofu were stable during *in vitro* digestion, although no data were provided. The results reported below address the digestive stability of isoflavonoids from SB, as well as their potential accessibility for cellular uptake.

Daidzein, genistein, their corresponding β-glucosides, and glycitein in SB remained stable during simulated oral, gastric, and small intestinal phases of digestion with greater than 95% recovery of initial amounts (Figures 2.1 and 2.2). Stabilities of genistein and genistin during simulated gastric digestion are in line with previous findings in which these particular isoflavonoids remained stable following acid incubation for 4 h (196). Decreases in malonyldaidzin (Figure 2.2a) and malonylglycitin (data not shown) were associated with slight increases in the amounts of daidzein (Figure 2.2a) and glycitein, respectively, in the digesta. This suggests that some of the glucoside derivatives are converted to their corresponding aglycones during digestion. Neither glycitin nor acetylglycitin were detected after any phase of simulated digestion (data not shown).

Bioaccessibility refers to the release of a dietary compound from its parent matrix for diffusion through the aqueous milieu bathing the apical surface of absorptive epithelial cells. The aqueous fraction of digesta contains both water-soluble components and micellarized lipophiles (99). Isoflavonoid β-glucosides are more water-soluble than their respective aglycones (197). The majority of daidzin (93.7 ± 2.3 %), acetyldaidzin (71.4 ± 1.7%), malonyldaidzin (99.0 ± 2.1%), genistin (81.6 ± 1.3 %), and

malonylgenistin (86.4 ± 2.8%) was present in the aqueous fraction of the digesta following simulated digestion (Figure 2.2). In contrast, only 59.1 ± 2.3% and 33.0 ± 2.3% of daidzein and genistein, respectively, partitioned into the aqueous fraction following simulated digestion. The amount of glycitein (74.5 ± 2.3%) in the aqueous fraction of digesta exceeded that of malonylglycitin (58.8 ± 1.6%). The relatively high recovery of glycitein in the aqueous fraction of digesta may be explained by the possibility that more malonylglycitin actually partitioned into the aqueous fraction before hydrolysis to its parent aglycone. We next examined the possibility that some of the daidzein, genistein, and glycitein was micellarized in the aqueous fraction of digesta due to the poor solubility of isoflavonoid aglycones in an aqueous matrix.

Representative chromatograms of isoflavonoids present in the aqueous fraction following simulated digestion with 0 - 4.8 mg/mL bile extract are shown in Figure 2.3. Omission of bile extract during small intestinal digestion resulted in the complete absence of genistein and only 26.1 ± 0.3 % of daidzein and 38.9 ± 0.5 % of glycitein present in the aqueous fraction of digesta (Figure 2.4). Increasing the concentration of bile extract to 1.2 mg/mL, i.e., 50% of the amount used in the standard digestion procedure, did not significantly ($P > 0.01$) affect the amounts of the aglycones present in the aqueous fraction of digesta. However, the quantity of each aglycone in the aqueous fraction increased significantly ($P < 0.01$) when the concentration of bile extract was elevated to 2.4 (concentration used in the standard procedure) and 4.8 mg/mL. These higher concentrations of bile extract are within the range present in the duodenal lumen post-prandially (198). The amounts of the aglycones in the aqueous fraction of digesta were positively correlated ($R \geq 0.89$) with the concentration of bile extract during small

intestinal digestion. The amounts of both acetylgenistin and malonylgenistin in the aqueous fraction of the digesta also were positively correlated ($R \geq 0.85$) with the concentration of bile extract during small intestinal digestion (Table 2.2). These data suggest that at least some portion of isoflavonoid aglycones, as well as the acetyl and malonyl conjugates of genistin, were incorporated into mixed micelles during small intestinal digestion. Murota et al. (199) demonstrated that daidzein and genistein had approximately a 2- and 5-fold greater affinity, respectively, for incorporation into liposomal membranes in aqueous solution than their parent β-glucosides. Furthermore, the affinity of genistein for these large unilamellar vesicles was nearly twice that of daidzein. These findings support our hypothesis that micellarization enhances the bioaccessibility of aglycones during small intestinal transit.

Partitioning of daidzin, its malonyl and acetyl derivatives, and malonylglycitin into the aqueous fraction of digesta was not affected ($P > 0.01$) by changes in the concentration of bile extract during the small intestinal phase of simulated digestion (Table 2.2). Approximately 66% of genistin was present in the aqueous fraction of digesta when bile extract was omitted during small intestinal digestion. Addition of bile extract during the process significantly increased ($P < 0.01$) partitioning of genistin into the aqueous fraction to approximately 80%. The data also suggest that the acetylated derivatives of daidzin and genistin are less bioaccessible than their β-glucoside or malonyl conjugates at all concentrations of bile extract examined (Table 2.2). Recent observations suggest that a portion of isoflavonoid glucosides liberated from the food matrix may diffuse across the unstirred-water layer and undergo brush border

β-glucosidase activity (53,184). It is assumed that the generated aglycone then diffuses across the apical surface of the intestinal epithelium (40,60). Moreover, native flora in the distal small intestine and the large intestine convert isoflavonoid glucosides to aglycones and other bioactive metabolites with the potential for absorption (5,45,196,200,201). Setchell and colleagues (41) propose that the glucose moiety of isoflavonoid β-glucosides serves to protect the parent aglycone from microbial degradation during intestinal transit.

Information regarding the bioavailability of isoflavonoids from supplements compared with that of soy foods is limited (41). We found that daidzein was more soluble in the aqueous fraction of digesta and, therefore more accessible than genistein for potential uptake by absorptive epithelial cells. However, this only represents one of the factors that can affect bioavailability. Previous studies generally have demonstrated greater bioavailability of genistein than daidzein from soy foods or isoflavone supplements administered with a meal (41,42,60,86). This may be in part due to the greater lipophilic nature of genistein rendering it more effective in passive diffusion (202,203). In contrast, Xu et al. (84) reported that daidzein was more bioavailable than genistein in women and King (59) described similar findings in rats. Daidzein may be more accessible for uptake by enterocytes than genistein when the luminal concentration of bile is low (i.e., in the fasted state) in light of its greater solubility in the aqueous fraction of digesta.

In conclusion, daidzein, genistein, their corresponding β-glucosides, and glycitein in soy bread were stable during simulated oral, gastric, and small intestinal phases of digestion. The present study also suggests that micellarization during the small intestinal

phase of digestion enhances the bioaccessibility of isoflavonoid aglycones and especially genistein. Ingestion of foods containing protein and lipids induces release of bile into the duodenum (204). Thus, it is likely that the bioavailability of isoflavonoids from foods containing fat and protein exceeds that of isoflavonoid supplements consumed without food. The findings and implications resulting from the described *in vitro* experiments must now be validated in human studies.

Isoflavone	Soy Flour	Soy Milk Powder	Ingredients		Recoveries[b]	
	(nmol/g dry weight)		Total (nmol/g)	Soy bread (nmol/g SB)	Individual Compound	Family
daidzin	538.5 ± 43.6	874.9 ± 50.9	295.3	298.3 ± 35.5	101%	96%
M-daidzin	815.4 ± 10.6	321.0 ± 38.2	328.1	252.6 ± 0.75	77%	
A-daidzin	236.0 ± 3.3	85.1 ± 1.2	94.0	42.1 ± 1.2	45%	
daidzein	51.5 ± 3.5	534.9 ± 18.5	81.7	175.8 ± 2.3	215%	
genistin	1211.0 ± 44.8	1929.6 ± 40.2	659.6	482.2 ± 5.3	73%	86%
M-genistin	1280.0 ± 25.6	589.3 ± 6.0	525.2	403.9 ± 4.0	77%	
A-genistin	96.3 ± 0.6	64.9 ± 6.4	41.9	39.2 ± 1.4	94%	
genistein	92.9 ± 1.9	1140.5 ± 75.8	168.3	271.6 ± 7.9	161%	
glycitin	nd[c]	nd	nd	nd	nd	105%
M-glycitin	133.2 ± 6.0	31.7 ± 0.8	51.1	41.1 ± 0.29	80%	
A-glycitin	86.3 ± 3.8	69.8 ± 1.3	38.9	4.09 ± 0.32	11%	
glycitein	8.4 ± 0.6	48.2 ± 4.0	8.7	58.4 ± 0.64	671%	
total	4549.5	5689.9	2292.8	2069.3	90%	

[a]Soy bread was prepared using 249 g of soy flour and 83 g of soy milk powder (dry weight). The final weight (dry) of the loaf was 700 g ± 42 g.
[b]Recovery refers to the percentage of indicated compounds contributed by the ingredients that are present in the final soy bread product.
[c]nd, not detected.
An "M" or an "A" preceding the chemical species of isoflavonoid indicates a malonyl- and acetylglucoside form, respectively.

Table 2.1: Isoflavone mass balance in soy ingredients and soy bread (SB)[a]

Isoflavonoid	Bile extract concentration (mg/mL) during digestion			
	0	1.2	2.4	4.8
	% total present in the aqueous fraction of digesta			
daidzin	90.2 ± 2.1 [b]	99.2 ± 2.5 [b]	93.7 ± 2.3 [b]	97.4 ± 1.9 [b]
malonyldaidzin	95.0 ± 3.1 [b]	90.1 ± 3.6 [b]	99.0 ± 2.1 [b]	101.6 ± 8.6 [b]
acetyldaidzin	67.7 ± 2.3 [b]	70.9 ± 2.1 [b]	71.4 ± 1.7 [b]	75.7 ± 1.7 [b]
genistin	66.4 ± 1.7 [b]	78.1 ± 2.5 [c]	81.6 ± 1.3 [c]	81.5 ± 0.7 [c]
malonylgenistin	63.9 ± 1.9 [b]	72.8 ± 2.2 [b]	86.4 ± 2.8 [c]	90.1 ± 2.1 [c]
acetylgenistin	28.3 ± 0.5 [b]	33.8 ± 2.3 [b]	52.6 ± 1.3 [c]	77.2 ± 1.2 [d]
glycitin	nd[e]	nd	nd	nd
malonylglycitin	59.1 ± 0.9 [b]	58.6 ± 1.7 [b]	58.8 ± 1.6 [b]	60.6 ± 7.2 [b]
acetylglycitin	Nd	nd	nd	nd

[a]The standard concentration of bile extract during the small intestinal phase of *in vitro* digestion was 2.4 mg/mL (N = 13). Simulated digestions were also performed with bile extract concentrations at 0.0 (N = 5), 1.2 (N = 5), and 4.8 mg/mL (N = 9). Means within a glucoside group that do not share a common letter as superscript differ significantly ($P < 0.01$) from one another as determined by one-way analysis of variance (ANOVA) followed by Bonferroni correction. Data are mean percentages ± % SEM.
[e] nd, not detected.

Table 2.2: Effect of bile extract content on recovery of isoflavonoid glucosides in the aqueous fraction of digested soy bread[a]

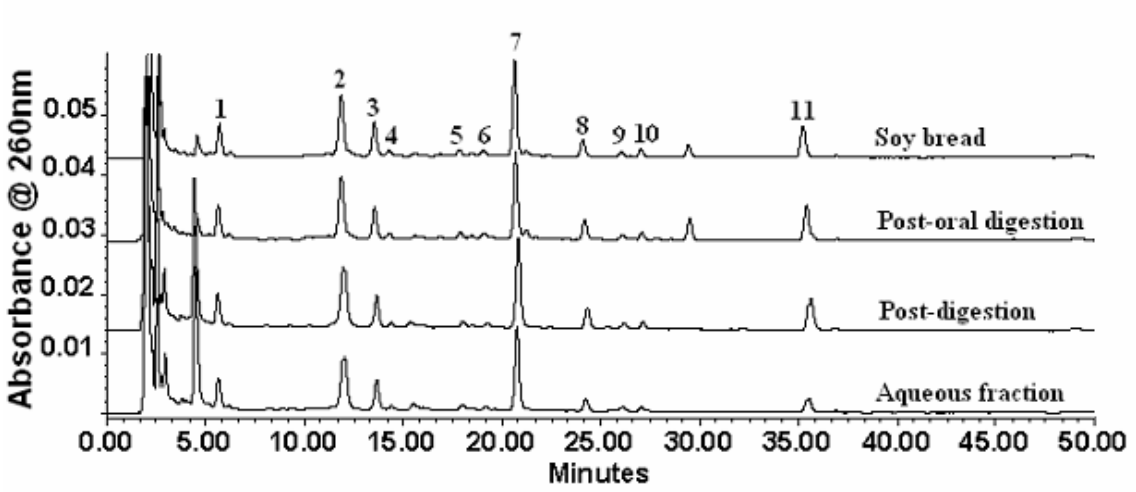


Figure 2.1: Reversed-phase HPLC analysis of isoflavonoids in starting and digested soy bread. Soy bread was exposed to simulated oral, gastric and small intestinal phases of digestion, and the aqueous fraction of digesta was prepared by high-speed centrifugation and filtration as described in the Materials and Methods. Representative chromatograms depict isoflavone profiles of extracts from soy bread and soy bread subjected to either oral or complete digestion, and the aqueous fraction of the digesta. Peak identification for representative chromatograms are as follows: (1) daidzin, (2) genistin, (3) malonyldaidzin, (4) malonylglycitin, (5) acetyldaidzin, (6) acetylglycitin, (7) malonylgenistin, (8) daidzein, (9) glycitein, (10) acetylgenistin, (11) genistein.

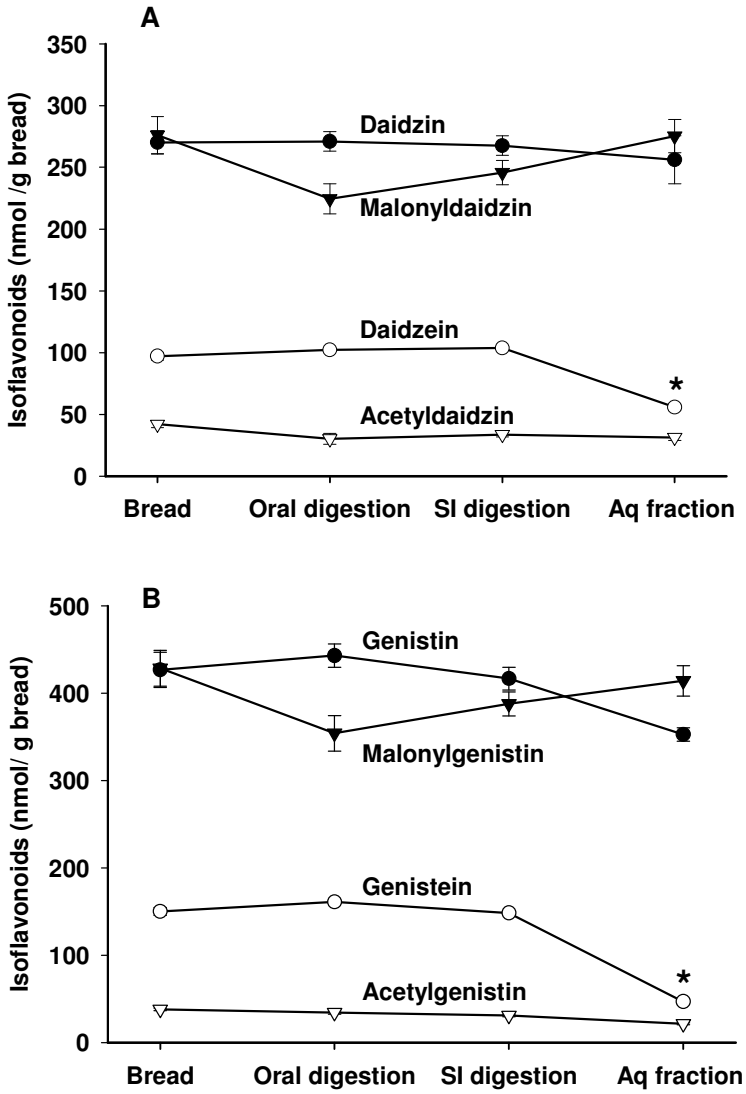


Figure 2.2: Stability of isoflavonoids in soy bread during *in vitro* digestion. Independent digestions of soy bread were performed, and samples were removed following completion of the oral and small intestinal phases of the procedure. The aqueous (Aq) fraction of small intestinal digesta was prepared by high-speed centrifugation and filtration as described in the Materials and Methods. Daidzein and its glucoside conjugates (panel A) and genistein and its glucoside conjugates (panel B) in soy bread

and the indicated samples were quantified by HPLC. Daidzein, genistein and their derivatives were stable during simulated oral, gastric, and small intestinal digestion. Data are means ± SE for starting soy bread meal (N = 12), post-oral digestion (N = 12), post-small intestinal digestion (N = 16) and aqueous fraction of digesta (N = 16). The presence of an asterisk (*) above error bars indicates that means differ significantly ($P < 0.01$) from the starting soy bread meal.

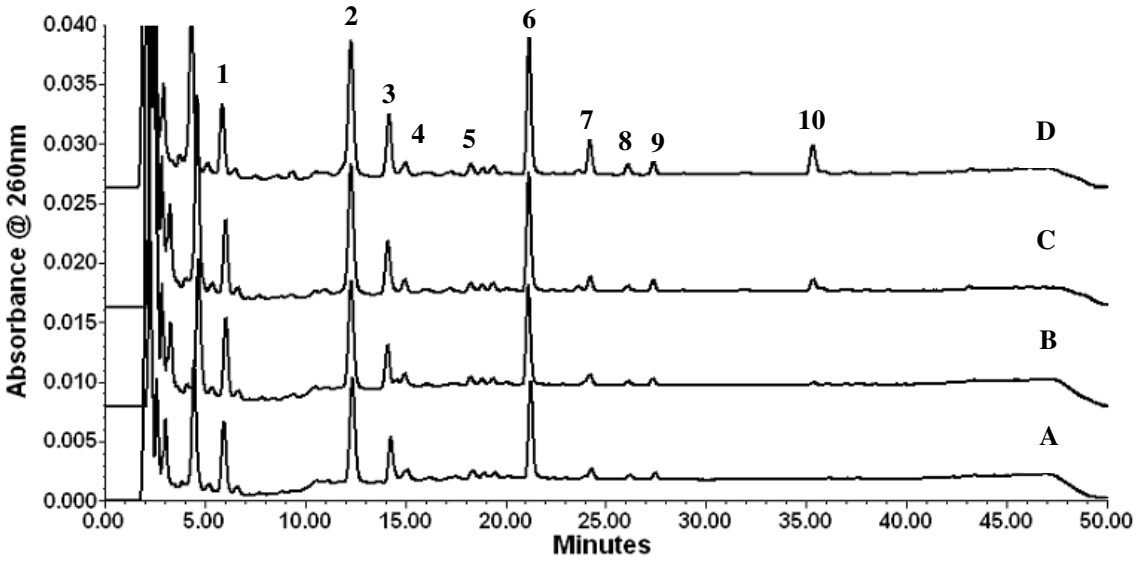


Figure 2.3: Effect of bile extract content during simulated digestion on isoflavone profile in the aqueous fraction of small intestinal digesta. Independent digestions were performed using 0 (A), 1.2 (B), 2.4 (C), and 4.8 mg/mL (D) of bile extract during the simulated small intestinal phase to investigate the effect on isoflavonoid bioaccessibility. Peak Identification for representative chromatograms are as follows: (1) daidzin, (2) genistin, (3) malonyldaidzin, (4) malonylglycitin, (5) acetyldaidzin, (6) malonylgenistin, (7) daidzein, (8) glycitein, (9) acetylgenistin, (10) genistein. Note that the area under the curve for daidzein, glycitein, acetylgenistin and genistein in the aqueous fraction increases as the concentration of bile extract present during the small intestinal phase of digestion is increased.

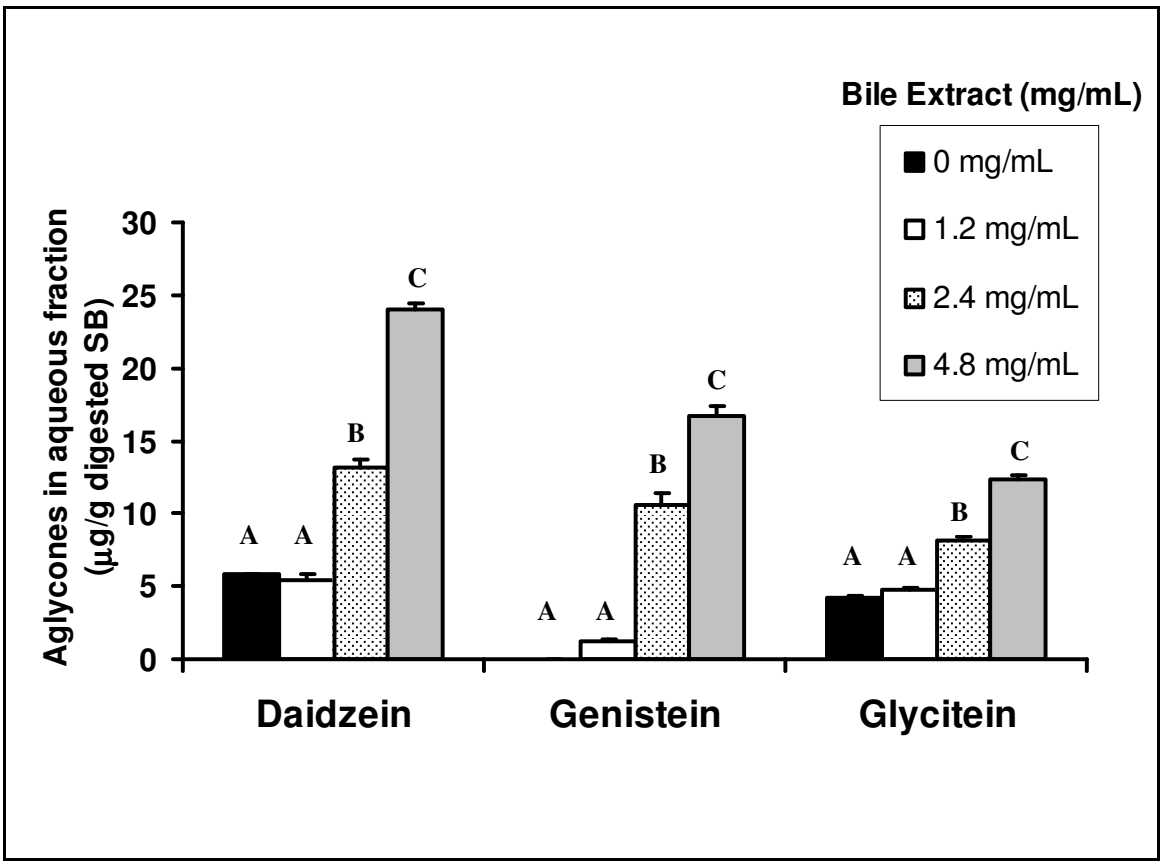


Figure 2.4: Bile extract increases partitioning of isoflavonoid aglycones from digested soy bread into the aqueous fraction. The concentration of bile extract regularly used during the small intestinal phase of *in vitro* digestion was 2.4 mg/mL (N = 13). Simulated digestions were also performed with bile extract concentrations at 0.0 (N = 5), 1.2 (N = 5), and 4.8 mg/mL (N = 9). The aqueous fraction of digesta was prepared by high-speed centrifugation and filtration as described in the Materials and Methods. Means within an aglycone group that do not share a common letter as superscript differ significantly ($P < 0.01$) from one another as determined by one-way analysis of variance (ANOVA) followed by Bonferroni correction. Data are means ± SEM.

CHAPTER 3

GASTROINTESTINAL METABOLISM AND ABSORPTION OF SOY ISOFLAVONOIDS IN SWINE

3.1 ABSTRACT

Dietary isoflavonoids (IFN) are metabolized by gut microbes and mammalian enzymes. We investigated contributions of small (SI) and large intestines (LI) in absorption of soy IFN and IFN metabolite production in swine (N = 4) with ileal canulae. All pigs were fed corn-casein diets for 5 d prior to feeding test meals of corn and 36% roasted soybeans. Urine and ileal effluent samples were extracted and analyzed for IFN by HPLC, and for the microbial IFN metabolites dihydrodaidzein (DHD), dihydrogenistein (DHG), *O*-desmethylangolensin (*O*-Dma), 6-OH-*O*-desmethylangolensin (6-OH-*O*-Dma) and equol by HPLC-MS/MS. IFN in the stomal effluent were present entirely as aglycones, indicating that significant hydrolysis of IFN glucosides occurred. Similar amounts of IFN aglycones were present in pig urine when digestion was limited to SI (ileal canulae open) compared to the entire GI tract (ileal canulae closed) for 0-24 h after consumption of the soy test meal. There was marked variation in amounts of DHD, DHG, *O*-Dma and equol in urines from different pigs after complete GI digestion, however the quantities of these microbial metabolites exceeded

those of the parent aglycones. These findings suggest that the SI participates in the absorption of dietary IFN and that extensive pre-absorptive metabolism of IFN occurs in weaned swine.

3.2 INTRODUCTION

Isoflavonoids are phytoestrogenic compounds present primarily as glucoside conjugates in non-fermented soy foods (192,201). Although numerous studies have addressed the health-promoting properties of isoflavonoids, details about their site(s) of absorption are limited. It is well established that isoflavonoid glucosides must be deglycosylated to aglycones prior to absorption (45). Plasma concentrations of isoflavonoids rise sharply in humans within 2 hours of consuming soy foods generally followed by a second peak at 8-12 hours (41-44). The latter increase in plasma isoflavonoids supports a role for the large intestinal microflora in the deglycosylation of isoflavonoid glucosides and the absorption of aglycones (45,46). Alternatively, it may represent enterohepatic re-circulation of absorbed isoflavonoids (47).

We previously reported that isoflavonoids are stable during simulated oral, gastric and small intestinal digestion of soy food, suggesting that enzymes located at the mucosal surface or secreted by intestinal microflora hydrolyze isoflavonoid glucosides (205). The role of the small intestine in isoflavonoid glucoside hydrolysis has been the subject of several reports. Andlauer et al. (49,50) demonstrated that the small intestine deglycosylated isoflavonoid glucosides during luminal perfusion in rats. Moreover, daidzein and genistein were excreted in the urine of germ-free rats following consumption of a meal containing isoflavonoid glucosides, suggesting that microbial activity is not essential for intestinal deglycosylation of glucosides (51). Lactase phlorizin hydrolase, a small intestinal brush-border membrane enzyme, has been shown to catalyze the deglycosylation of isoflavonoids (54,55,184).

Isoflavonoid aglycones are also metabolized by gut microflora. Some microbial species reduce the aglycone daidzein to dihydrodaidzein (DHD), which can be converted to *O*-desmethylangolensin (*O*-Dma) or equol. Likewise, the aglycone genistein can be metabolized to dihydrogenistein (DHG) and 6'-hydroxy-*O*-desmethylangolensin (61,62). Simons et al. (63) recently demonstrated that fecal microbes convert glycitein to dihydroglycitein which may be metabolized to 5'-methoxy-*O*-desmethylangolensin and dihydro-6,7,4'-trihydroxy-isoflavone.

In the present study, we investigated the gastrointestinal metabolism and absorption of isoflavonoids in swine with canulae surgically implanted at the terminal ileum. Swine were selected as a model because of the anatomical and physiological similarities of their digestive system and vasculature with those in humans (206). The intestinal mucosa from humans and swine also exhibit similar permeabilities to each other (207). Metabolism of isoflavonoids in the upper gut was determined by HPLC and HPLC-MS/MS analysis of ileal chyme collected after feeding a meal containing ground roasted soybeans. Isoflavonoid absorption in swine with open ileal canulae (i.e., chyme collected) was compared with closed ileal canulae (i.e., chyme allowed to enter the large intestine) by quantifying deconjugated isoflavonoid aglycones and metabolites in urine (208).

3.3 MATERIALS AND METHODS

3.3.1 Supplies

Daidzein, genistein, glycitein and their β-, acetyl- and malonyl-conjugates were purchased from LC Laboratories (Woburn, MA). The internal standard 2',4'-dihydroxy-2-phenylacetophenone was purchased from Indofine Chemical Company, Inc (Hillsborough, NJ). β-glucuronidase (G-1512) and the internal standard flavone (F-2003) were purchased from Sigma Chemical Co. (St. Louis, MO). C-18 solid phase extraction columns (#205462) and nylon syringe filters (0.2 and 0.45 μm) were purchased from Alltech Associates, Inc., Deerfield, IL. Advantasoy™ Clear isoflavonoid extract was provided by Cargill Health Food and Technologies (Wayzata, MN). Desitin zinc oxide ointment (Pfizer, Morris Plains, NJ) was purchased from a local pharmacy. Reagents for high-performance liquid chromatographic (HPLC) and mass spectrometric (MS) analysis were purchased from Fisher Scientific Co. (Fairlawn, NJ) and were HPLC grade.

3.3.2 Animals

Crossbred barrows (N = 6) weighing approximately 40 kg were surgically implanted with T-canulae at the distal ileum as previously described (209,210). Pigs were bathed each morning and zinc-oxide ointment was applied liberally to canula sites to minimize the potential for infection. The animals were maintained in individual stainless steel metabolic crates to control food and water intake, facilitate collection of urine and ileal effluent, and to prevent the animals from tampering with their canulae. Animals were adapted to their cages for 7 d before initiating feeding studies. Access to water throughout the study was *ad libitum*.

3.3.3 Diets and Experimental Design

The general experimental design is outlined in Table 3.1. All diets were prepared at the Ohio State University feed mill (Wooster, OH). The animals were maintained on a standard corn-soy diet prior to initiating the feeding studies. A washout period was initiated (day -5 to day -1) prior to feeding the soy test meal with pigs fed a soy-free diet containing 90.75% ground corn, 7.2% casein, 1.8% mineral mix and 0.25% vitamin premix at 0600 and 1800 h daily (Table 3.2). The soy test meal (550 g) contained 61.35% ground corn, 35.8% ground roasted soybeans, 2.6% mineral mix and 0.25% vitamin premix (Table 3.2). Chromium oxide (0.4%) was added to the soy test meal as a non-digestible indicator of gastrointestinal clearance of the soy test meal. The soy meal was fed at 0600 h on day +1 and ileal canulae remained open until 2200 h to collect chyme, thereby preventing its transfer to the large intestine. The pigs were fed the corn-casein meal at 1800 h and for both meals on day 2. The soy meal was fed again at 0600 h on day 3 with ileal canulae in the closed position to allow chyme to enter the large intestine. The corn-casein meal was fed for the remainder of the study.

The 16 h period for collection of ileal effluent was chosen after conducting a pilot study with ileal-canulated, but larger (approximately 50 kg), swine to determine the kinetics of small intestinal clearance of the test meal. Animals were fed the soy test meal and ileal effluent was collected and pooled every 2 h until 12 h post-ingestion. Approximately 85% of dietary chromium oxide in the soy meal was recovered in ileal effluent by 12 h in the pilot study.

Collection bags were changed as required (approximately 10 min intervals) and their contents were immediately pooled and refrigerated for each 2 h period for individual pigs. Pooled chyme samples were stored at -20°C until analysis.

Prior to initiating feeding studies, one of the animals removed its canula and was therefore euthanized. One of the animals developed a stomal blockage 8 h after the test meal was administered and was eliminated from the study. No problems were encountered with the remaining four animals for the duration of the study.

Urine collection was initiated 24 h prior to feeding the test meal (day –1) and continued for the duration of the study. Urine was filtered through glass wool to prevent particulate matter from entering collection vessels containing L-ascorbic acid (1.14 mmol) and boric acid (4.85 mmol) as preservatives (211). The collection vessels were replaced every 12 h and urine volumes were measured for each 12 h collection period. Aliquots of the two samples of 12 h urine were pooled proportionately for 24 h samples and stored at -20°C until analysis.

3.3.4 Incubation of Isoflavonoid Glucosides With Ileal Effluent

To examine the possibility of *ex vivo* microbial hydrolysis of isoflavonoid glucosides from the test meal in the collection bags, stomal effluent was collected from pigs (N = 4) fed a soy free (corn-casein) diet and mixed (5:1; v/v) with an aqueous solution of isoflavonoid glucosides (100 mg/mL, Advantasoy™ Clear in water). Aliquots (5 mL) of the effluent/isoflavonoid mixture were transferred to petri dishes, covered and incubated in an anaerobic chamber (Thermo Forma Scientific, Marietta, OH) at either 37°C for 0, 20, or 40 min, or at 0°C for 20 min. Samples were removed and stored at -20°C until analysis.

3.3.5 Extraction of Isoflavonoids From Diets and Ileal Effluent

Samples of feed were ground to a fine powder with a mortar and pestle prior to isoflavonoid extraction. Ileal effluent samples were thawed, weighed and homogenized with a blender (Osterizer Galaxie, Oster Corp.). Aliquots were freeze-dried for 2-3 days to complete dryness, finely ground, passed through a 0.5 mm mesh screen and stored at -20°C until analysis. Isoflavonoids from swine diets and ileal effluent samples were extracted into acidified acetonitrile as described previously (186,205). Extracted samples were centrifuged (Fisher Centrific Centrifuge, Fisher Scientific) at 450 x g for 30 min at room temperature. The solvent was evaporated from aliquots (1 mL) of the supernatants, residues were re-solubilized in methanol (1 mL) and filtered (0.2 μm pore size) prior to HPLC analysis.

3.3.6 Extraction of Isoflavonoids and Their Derivatives From Swine Urine

Isoflavonoids and their metabolites were extracted from swine urine as previously described with slight modifications (69,212). Urine samples collected from 0-24 h and 24-48 h were separately pooled to analyze isoflavonoid equivalents excreted daily for each pig. Microbial metabolites of isoflavonoids were extracted from pooled urine collected from 0-48 h. Aliquots of pooled urine (8 mL) were centrifuged (Avanti J-25, Beckman Coulter Inc., Palo Alto, CA) at 10,000 x g for 10 min at 4°C. Sodium acetate buffer (1.5 mL, 0.2 mol/L, pH 4.0) and internal standard (25 μmol/L 2',4'-dihydroxy-2-phenylacetophenone) were added to filtered (0.45 μm) aliquots (5 mL) of supernatants. Following sequential conditioning with methanol (6 mL) and sodium acetate buffer (6 mL, 0.2 mol/L, pH 4.0), urine samples were drawn through C-18 solid phase extraction columns using a vacuum manifold (Alltech Associates #210351). Columns

were rinsed with sodium acetate buffer (4 mL, 0.2 mol/L, pH 4.0), water (1 mL) and methanol (1 mL), respectively. Methanol (7 mL) was drawn through the columns to elute isoflavonoids and their derivatives into collection vials. Samples were heated to 37°C in a water bath and the solvent was evaporated under a stream of nitrogen. Residues were dissolved in 1 mL sodium acetate buffer (0.2 mol/L, pH 5.0) containing 200 units β-glucuronidase. Enzyme-treated samples were incubated with shaking (Versa-Bath S Model 224, Fisher Scientific) at 50 rpm for 18 to 22 h at 37°C. Isoflavonoids and their derivatives were extracted twice into diethyl ether (3.5 mL) and the organic phases were dried under a stream of nitrogen at room temperature. Residues were dissolved in 0.6 mL methanol:water (80:20; v/v) and filtered (0.2 μm pore size) before injection into the HPLC system.

3.3.7 HPLC Analysis of Isoflavonoids

Samples were analyzed for isoflavonoid content with a Waters 2695 HPLC (Milford, MA) and a Waters 2996 photodiode array detector (PDA) set to collect data from 210 to 400 nm. The stationary phase consisted of a Waters Nova-Pak C-18 reversed-phase column (150 mm x 3.9 mm i.d., 4 μm, 60 Å pore size) and a Waters Nova-Pak C-18 guard column (20 mm x 3.9 mm i.d., 4 μm). A mobile phase of 1.0% acetic acid in water (v/v) (solvent A) and acetonitrile (solvent B) at a flow rate of 0.6 mL/min (25°C ± 5°C) was used to separate isoflavonoids in extracted diet and ileal effluent samples. The injection volume was 10 μL and the following linear solvent gradient was used: 0 to 5 min, 85% A; 5 to 36 min, 85 to 71% A; 36 to 44 min, 71 to 65% A; 44 to 45 min, 65 to 15 % A; and, 45 to 50 min, 15 to 85% A. A mobile phase of 1.0% acetic acid in water (v/v) (solvent A), acetonitrile (solvent B), and methanol (solvent C)

at a flow rate of 0.55 mL/min (25°C ± 5°C) was used to separate isoflavonoids in extracted urine samples. The injection volume was 10 µL and the following linear solvent gradient was used: 0 to 1 min, 75% A, 12% B, 13% C; 1 to 14 min, 75 to 49% A, 12 to 25% B, 13 to 26% C; 14 to 15 min, 49 to 10% A, 25 to 45% B, 26 to 45% C; 15 to 19 min, no change; 19 to 20 min, 10 to 75% A, 45 to 12% B, 45 to 13% C; and, 20 to 25 min, no change. Elution profiles and UV absorption spectra of pure isoflavonoid standards were used to identify each isoflavonoid species.

3.3.8 HPLC-MS/MS Analysis of Microbial Metabolites of Isoflavonoids

HPLC-MS/MS was used for identification and quantification of equol, DHD, DHG, *O*-Dma and 6-OH-*O*-Dma in ileal effluent and urine. The same extraction procedures and operating parameters used for urine analyses by HPLC were implemented for HPLC-MS/MS analyses. A triple quadrupole mass spectrometer (Micromass Co. Ltd, UK) using positive ion electrospray ionization and selected reaction monitoring was used to detect DHD (m/z 257 > 123), DHG (m/z 273 > 123), *O*-Dma (m/z 259 > 121), and equol (m/z 243 > 133). These transitions were determined using the protonated molecule and the most abundant fragment ion established during analysis of their respective standards. The following system conditions were maintained for analyses: cone voltage of 35 V; capillary voltage, 3.0 kV; source temperature, 120°C; radio frequency lens 1, 50 V; desolvation gas temperature, 500°C at a flow of 16.3 L/min; and, collision energy, 25 eV. Due to limited availability of frozen aliquots of urine, isoflavonoid metabolites were not quantified in urine from pigs when ileal canulae were open.

3.3.9 Quantitation of Chromium Oxide

Chromium oxide content of the test meal and ileal chyme samples was determined by neutron activation analysis at the Experiment Station Chemical Laboratories at the University of Missouri (Columbia, MO).

3.3.10 Statistical Analysis

Data (N = 4) are expressed as means ± SEM when appropriate. A general linear univariate model was developed to examine the effect of canula status (open or closed) on urinary excretion of isoflavonoids. This model incorporated the type of isoflavonoid (daidzein, glycitein, genistein, total), pooled urine collection time (0 to 24 hours and 24 to 48 hours), canula status (open or closed) and subject number as fixed factors with urinary isoflavonoids as the dependent variable. Normal distribution of data was checked using normality plots and Kolmogorov Smirnoff tests. Equality of distribution was verified using box-plots and Levene's test. Urinary isoflavonoid metabolites were studied using a paired linear mixed model design with the amount of urinary isoflavonoid metabolite as the dependent variable, and the type of isoflavonoid metabolite and animal number as fixed factors. Following this test, LSD and Bonferroni post hoc comparisons of statistical significance were performed for individual pairwise comparisons of different isoflavonoid metabolites. Differences were considered statistically significant when $P < 0.05$.

3.4 RESULTS

The soy test meal included 35.8% roasted soybeans by weight (Table 3.2) containing 783.4 ± 30.6 μmol of isoflavonoids. The majority of the isoflavonoids (94.0 ± 3.1%) were present as glucosides (Table 3.3). The distribution of isoflavonoids was 375.2 ± 19.4, 90.9 ± 7.2 and 317.3 ± 4.0 μmol from the daidzein, glycitein and genistein families, respectively. β-, acetyl- and malonyl-glucosides accounted for 27.0 ± 1.1, 24.9 ± 0.5 and 42.1 ± 1.5% of the total isoflavonoids, respectively. Similar, but low, amounts of daidzein and genistein were present, whereas glycitein was not detected.

Ingested isoflavonoid glucosides were hydrolyzed to aglycones prior to reaching the ileal stoma (Figure 3.1). The total isoflavonoid content of the chyme collected during the 16 h collection period was 163.1 ± 51.5 μmol (range: 71.1 to 272.8 μmol) which represents 20.8 ± 6.6% of the test meal. Daidzein was more abundant in the effluent (91.1 ± 27.9 μmol) than genistein (30.8 ± 10.8 μmol) and glycitein (23.1 ± 6.6 μmol). A study addressing the kinetics of small intestinal clearance of the soy meal with a separate set of canulated swine revealed that isoflavonoid aglycones were detected in ileal effluent at all post-ingestion intervals between 2 and 12 h, with the majority reaching the terminal ileum between 8 and 12 h (Figure 3.2). To test the possibility that microflora hydrolyzed isoflavonoid glucosides, a glucoside-rich extract was incubated anaerobically with ileal effluent *ex vivo*. Following incubation for 20 min at 0°C and 40 min at 37°C, 106 and 94% of glucosides were recovered, respectively.

Since the collection bags were changed and placed on ice at approximately 10 min intervals, it is unlikely that deglycosylation of isoflavonoid glucosides occurred in the collection bag.

The major microbial metabolites of ingested isoflavonoids, DHD, DHG and equol, also were detected in ileal effluent from all swine (Table 3.4), but at much lower quantities than the isoflavonoid aglycones. 6-OH-*O*-Dma was detected in ileal effluent from a single animal, whereas *O*-Dma was not detected in ileal effluent from any swine.

Urine samples were incubated with β-glucuronidase to convert phase II conjugates of isoflavonoids to free isoflavonoid aglycones. Results are expressed as quantities of isoflavonoid aglycone equivalents. Daidzein, glycitein, genistein and their derivatives were not detected in urine prior to feeding the soy test meal (data not shown). Total mean urinary isoflavonoid equivalents were slightly, but not significantly, lower during the 24 h collection period after feeding the test meal with ileal canulae open (20.4 ± 2.8 μmol) and closed (24.4 ± 9.5 μmol) (Figure 3.3a). Urinary excretion of daidzein equivalents > genistein equivalents > glycitein equivalents during 24 h period after ingestion of the meal, and the quantities of the individual aglycone equivalents excreted were comparable ($P = 0.38$) for all animals with ileal canulae opened and closed after feeding the test meal. Urinary isoflavonoids (0–24 h) accounted for only 2.6 ± 0.4% and 3.1 ± 1.2% of the total isoflavonoids ingested from the test meal when canulae were open and closed, respectively. With respect to their aglycone equivalents in the meal, urinary recoveries of daidzein (2.6 ± 0.3% and 3.1 ± 1.3%), glycitein (3.6 ± 0.9% and 3.9 ± 1.5%) and genistein (2.4 ± 0.3% and 2.9 ± 1.1%) were not significantly different

when ileal canulae were opened and closed, respectively. There was no correlation between the amount of daidzein ($r^2 = 0.33$), glycitein ($r^2 = 0.02$) or genistein ($r^2 = 0.17$) in the ileal effluent and their respective quantities in urine.

When canulae remained closed throughout the 48 h collection period, urinary excretion of total isoflavonoid equivalents and each of the three aglycones were not significantly different on days 1 (0-24 h post ingestion) and 2 (24-48 h post ingestion) (Figure 3.3). In contrast, when ileal canulae were opened from 0-16 h after ingesting the test meal, urinary losses of total isoflavonoids (1.3 ± 0.1 μmol) on day 2 were significantly ($P = 0.003$) less than urinary excretion on day 1 (Figure 3.3). Similarly, urinary excretion of daidzein ($P < 0.001$), glycitein ($P < 0.001$) and genistein ($P < 0.001$) equivalents was significantly lower on day 2 than on day 1 when canulae had been open from 0-16 h after feeding the test meal.

DHD, DHG and equol were present in urine from all animals (Table 3.5). Mean amounts for urinary equol and DHG were similar and greater than DHD, although the difference was not statistically significant due to the relatively large variation in production of these metabolites between animals. *O*-Dma was detected in urine from three of the four animals in markedly lower quantities than equol ($P = 0.015$, LSD test) and DHG ($P = 0.016$, LSD test). Metabolites from the daidzein and genistein families accounted for approximately 12 and 9%, respectively, of their respective aglycone equivalents in the soy meal. 6-OH-*O*-Dma was not detected in any of the swine urine samples. The amounts of daidzein and genistein in ileal effluent were not correlated

($r^2 \leq 0.18$) with their respective metabolites in urine. The metabolite profile in urine from animals with canulae open after feeding was not determined due to failure to save sufficient volumes of samples.

3.5 DISCUSSION

Although it is recognized that dietary isoflavonoid glucosides must be converted to aglycones prior to absorption, the site for this conversion remains somewhat controversial. The present study offers insight into the relative participation of the small and large intestines in the absorption and metabolism of soy isoflavonoids in swine. To our knowledge, this is the first study to use ileal-canulated swine to address intestinal metabolism and absorption of soy isoflavonoids. One advantage of this model is that feeding studies were conducted using a crossover design. Additionally, there was no aversion to the soy test diet since the maintenance diet, like that used widely in the swine industry, contained a substantial proportion of roasted soy. The corn-casein diet used in this study was tolerated very well by all of the animals and appropriate for wash-out as indicated by the absence of isoflavonoids in urine following feeding of this diet for 5 d. The microbes and enzymes involved in metabolism of isoflavonoids appeared to remain stable during washout with the corn-casein diet as discussed below.

The marked deglycosylation of isoflavonoid glucosides from the test meal prior to reaching the ileal stoma suggests that the small intestine is the predominant site for hydrolysis of isoflavonoid glucosides in swine. We also have observed extensive small intestinal hydrolysis of isoflavonoid glucosides in human subjects with ileostomies (69). Similarly, Walle et al. (213) found that quercetin glucosides were completely hydrolyzed to aglycones prior to absorption in human subjects with ileostomies.

Conversion of isoflavonoid glucosides to aglycones may be mediated by the action of the brush-border enzyme lactase-phlorizin hydrolase or microbial β-glucosidases (54,55,184). Bacterial species with significant β-glucosidase activity include *Enterococci*, *Lactobacillus*, *Bacteroides* and *Bifidobacteria* (43). Of these species, significant numbers of *Enterococci*, *Lactobacilli* and *Bifidobacteria* reside in the small intestine of swine (214-217). The presence of small quantities of the isoflavonoid microbial metabolites DHD, DHG, 6-OH-*O*-Dma and equol in ileal effluent further supports the likelihood of microbial metabolism of dietary isoflavonoids within the small intestine. Differences in the amount of isoflavonoids excreted in urine collected 24-48 h after feeding the test meal when ileal canulae had been open (0-16 h) versus canulae closed confirmed that isoflavonoid aglycones reaching the terminal ileum are available for absorption or further metabolism in the large intestine.

The relative amount of total isoflavonoids recovered from the meal in urine 24 h post-ingestion when ileal canulae were open (2.6 ± 0.4%) versus closed (3.1 ± 1.2%) was lower than typically reported for humans (e.g., 20-50%) (218). However, the profile of individual isoflavonoid equivalents excreted in swine urine, i.e., daidzein > genistein > glycitein, was similar to that typically observed in human studies (42,84,86,208,219,220). Because urine was only collected for 48 h after ingestion of the soy meal, total recovery of isoflavonoids in the urine may be underestimated. It is noteworthy that the quantities of isoflavonoid metabolites in swine urine were several-fold greater than their respective aglycones. The typical corn-soy diet fed to swine provides high levels of isoflavonoids with respect to the amounts typically consumed by humans (221). It is possible that such chronic intake of high amounts of isoflavonoids is associated with increased metabolism

of isoflavonoid aglycones in swine. However, consumption of 100 mg/d of isoflavonoids per day from soy foods or supplements for ≥ 1 month did not result in changes in equol production or the bioavailability of isoflavonoids in humans, suggesting limited impact of chronic soy and/or isoflavonoid consumption on intestinal microbial populations that metabolize isoflavonoids (222,223).

Swine may represent a useful animal model to further test the pre-absorptive metabolism of isoflavonoids. We observed similar recoveries of daidzein (6.2 ± 3.2%) and equol (7.9 ± 4.3%) in 48 h urine pools in the current study. Lundh also reported that daidzein and equol in swine urine accounted for 6 and 22%, respectively, 8 h after feeding a meal containing 88 mg of daidzein equivalents from red-clover (224). These findings differ from those in humans where urinary excretion of isoflavonoid aglycone equivalents is higher than their respective metabolites (44,211,225). Also, it was recently reported that the urinary profile of daidzein and equol excreted by female piglets fed human infant formula containing soy protein isolate was similar to that of humans, with daidzein levels being 15 times greater than equol (226). However, the newborn pigs were weaned to the soy protein isolate formula at 2 d of age, whereas the swine in our study were 3 months old and maintained on a corn-soy diet from weaning to initiation of the experimental study. It also is interesting that Gu et al. (226) found that urinary *O*-Dma was 5 times higher than equol in the infant pigs, suggesting that microbial populations differ from those of the pigs in our study. Factors such as gender, age, gut microflora, maintenance diet, and diet matrix likely have contributed to the observed differences in recoveries of daidzein and equol in the studies with swine. Age, gender and food matrix also have been reported to moderately affect urinary excretion of isoflavonoids in humans

(227). Other factors such as fermentable carbohydrate in the meal also may influence the production of isoflavonoid metabolites (228). Our findings support extensive pre-absorptive metabolism of isoflavonoids in weaned swine.

In summary, the results from this study demonstrate that the small intestine is the predominant site for deglycosylation of dietary isoflavonoid glucosides in swine with ileal canulae. Although both the small and large intestine participate in the absorption of dietary isoflavonoids, the small intestine appears to have a key role in generating the bioavailable aglycones. Because the profile of urinary isoflavonoids and their metabolites observed in this study differs from that reported previously in humans, swine may not represent an ideal model for investigating the bioavailability of dietary isoflavonoids. However, the extensive metabolism of isoflavonoids observed in this study suggests that swine may be useful for studies addressing microbial metabolism of dietary isoflavonoids. Moreover, the availability of tissues makes swine an invaluable model for investigating isoflavonoid distribution and metabolism in peripheral tissues. Further studies addressing the pharmacokinetics of isoflavonoid transport and metabolism in swine are warranted.

	Meal		Canula Position		
Day	**0600**	**1800**	**Open (h)**	**Closed (h)**	**Urine Collected**
- 1	CC[a]	CC	------	24	+
+1	RSB[b]	CC	16	8	+
+2	CC	CC	------	24	+
+3	RSB	CC	------	24	+
+4	CC	CC	------	24	+

[a]Corn casein diet
[b]Roasted soy diet

Table 3.1: Experimental design.

Component	Corn casein diet (%)	Soy test diet (%)
Corn	90.75	61.35
Roasted soybeans	----------	35.80
Casein	7.20	----------
Chromic Oxide	----------	0.40
Salt	0.20	0.30
Dicalcium Phosphate	0.70	1.10
Limestone	0.65	0.55
Trace Mineral mix	0.10	0.10
Vitamin premix	0.25	0.25
Se premix	0.15	0.15

Table 3.2: Composition of swine diets.

Species	Daidzein (μmol/meal)	Glycitein (μmol/meal)	Genistein (μmol/meal)
Aglycone	23.8 ± 5.9	nd[b]	23.4 ± 0.4
β-glucoside	121.0 ± 4.7	42.6 ± 3.1	48.1 ± 0.7
Acetylglucoside	87.2 ± 1.3	18.9 ± 0.7	88.6 ± 1.8
Malonylglucoside	143.3 ± 7.5	29.4 ± 3.4	157.2 ± 1.1
Total glucosides	736.3 ± 24.3 (94.0 ± 3.1%)		
Total aglycones	47.1 ± 6.3 (6.0 ± 0.8%)		

[a]Data (N = 3) are expressed as means ± SEM.
[b]nd: not detected.

Table 3.3: Isoflavonoid content of the soy meal.[a]

Metabolites[a]	μmol ± SEM	Range	Producers[b]
DHD	0.30 ± 0.2	0.06 – 0.94	4/4
DHG	0.26 ± 0.2	0.02 – 0.88	4/4
***O*-Dma**	0.0	nd[c]	0/4
6-OH-*O*-Dma	0.003 ± 0.003	0.00 – 0.003	1/4
Equol	0.36 ± 0.2	0.17 – 0.81	4/4

[a]Data for ileal effluent (N = 4) are means ± SEM and represent total quantity for the 16 h collection period.
[b]Number of animals producing the indicated metabolites.
[c]nd: not detected.

Table 3.4: Isoflavonoid metabolites in ileal effluent of swine following consumption of a roasted soybean meal. Differences between means were not statistically significant as determined by one-way analysis of variance (ANOVA) followed by Bonferroni post-hoc comparison of statistical significance.

Metabolites[a]	μmol ± SEM	Range	Producers[b]
DHD	13.7 ± 10.1^{AB}	0.03 - 31.9	4/4
DHG	28.9 ± 21.7^{A}	0.03 - 65.9	4/4
***O*-Dma**	0.45 ± 0.2^{B}	nd[c] - 0.7	3/4
Equol	29.6 ± 16.3^{A}	0.05 - 48.1	4/4

[a]Data for urine (N = 4) are means ± SEM during 48 h collection with ileal canulae closed (fully-functional GI tract).
[b]Number of animals producing the indicated metabolites.
[c]nd: not detected.

Table 3.5: Isoflavonoid metabolites in urine of swine after ingesting a roasted soybean meal. Means that do not share a common letter as superscript differ significantly ($P < 0.05$) as indicated by Fisher F-test. While the differences between the individual isoflavonoid metabolites failed to achieve statistical significance under the Bonferroni post hoc comparison of statistical significance, the Fisher-protected LSD test indicated significantly higher amounts of urinary DHG ($P = 0.016$) and equol ($P = 0.015$) than *O*-Dma.

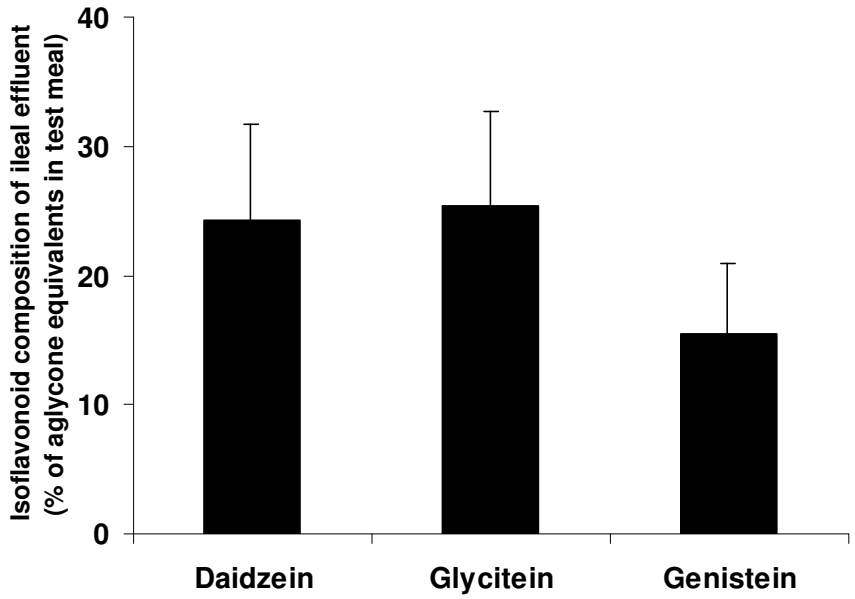


Figure 3.1: Dietary isoflavonoid glucosides are converted to aglycones prior to reaching the terminal ileum in swine with ileal canulae. Swine were fed the roasted soybean meal and ileostomal effluent was collected for 16 h. The test meal contained primarily isoflavonoid glucosides (94.0 ± 3.1%), whereas only isoflavonoid aglycones were present in chyme. Data are means ± SEM (N = 4) and expressed as percentage of each aglycone in chyme with respect to daidzein, glycitein or genistein equivalents in the soy test meal.

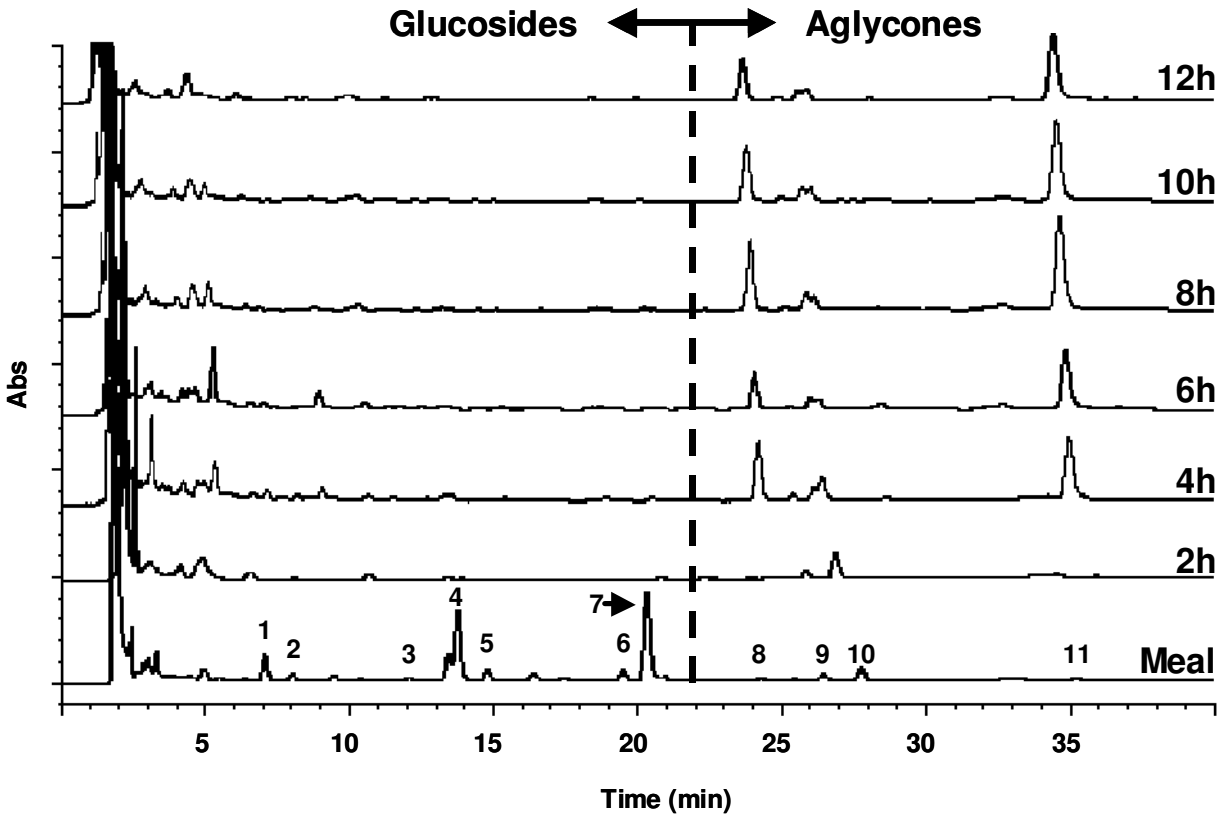


Figure 3.2: Representative reversed-phase HPLC chromatograms of the soy test meal and ileostomal effluent. Swine (N = 4) with ileal canulae were fed a soy test meal in a pilot study addressing the kinetics of small intestinal clearance of the meal. Ileal effluent was collected and pooled for each 2 h interval from 0-12 h. Peak identification: (1) daidzin, (2) glycitin, (3) genistin, (4) malonyldaidzin, (5) malonylglycitin, (6) acetyldaidzin, (7) malonylgenistin, (8) daidzein, (9) glycitein, (10) acetylgenistin and (11) genistein. Note that the soy meal contained predominantly isoflavonoid glucosides, whereas ileal effluent only contained isoflavonoid aglycones at all times.

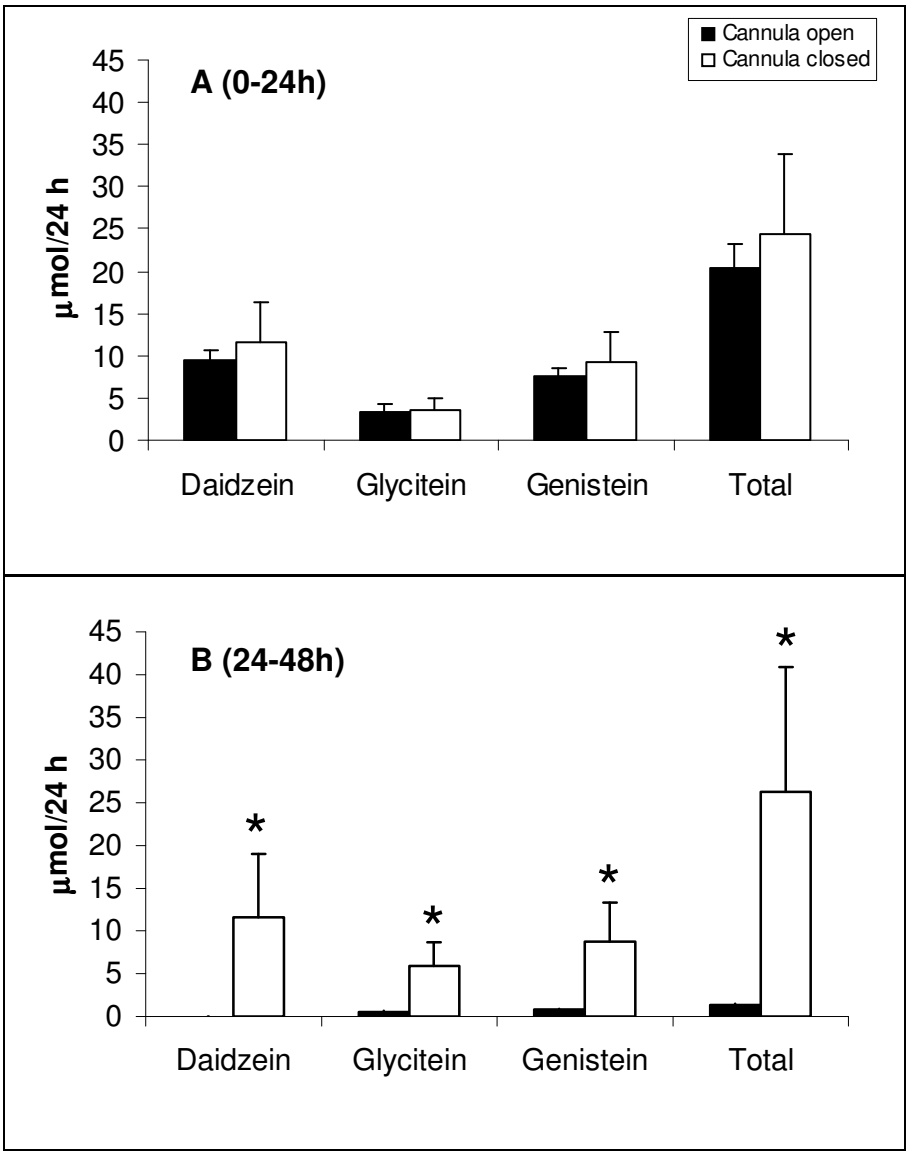


Figure 3.3: Urinary excretion of isoflavonoids in swine after feeding a soy meal. Swine were fed a soy meal containing 783 µmol of isoflavonoids and urine was collected for 48 h. Urinary excretion of isoflavonoids during the initial 24 h period after consuming a soy meal (Panel A) was similar when ileal canulae were open or closed. From 24 – 48 h after feeding the test meal (Panel B), total isoflavonoids excreted in urine with ileal

canulae closed markedly exceeded that when ileal canulae were opened from 0 – 16 h. Data are expressed as means ± SEM. The presence of an asterisk above the open bars indicates that the quantity of isoflavonoids in urine when canulae were closed was significantly ($P < 0.05$) greater than when canulae were open.

CHAPTER 4

DIGESTIVE STABILITY AND TRANSPORT AND METABOLISM OF EQUOL BY CACO-2 CELLS

4.1 ABSTRACT

Equol is a microbial metabolite of the isoflavonoid daidzein that has greater estrogenic activity and antioxidant capacity than its dietary precursor. Studies suggest that only 30 - 50% of humans possess an intestinal microflora capable of producing equol. We questioned if dietary supplementation of equol may represent a strategy to provide non-producers with the proposed health-promoting benefits of equol. Stability and partitioning of equol into the bioaccessible fraction during simulated digestion, as well as its uptake, transepithelial transport and efflux by differentiated Caco-2 cells, were examined. Recovery of equol following digestion was 106% ± 6% with ≥ 90% partitioned into the aqueous fraction of chyme regardless of the concentration of bile extract present during small intestinal digestion. Caco-2 cells were grown on culture dishes and transwell inserts to characterize uptake and transepithelial transport of equol, respectively. After 4 h, cellular equol equivalents were proportional to the initial concentration ($r^2 = 0.99$) of equol in the medium. Accumulation of equol was maximum at 1 h and declined proportionally as the time of incubation increased. The decline in cellular equol was associated with excretion of phase II conjugates across the apical and

basolateral membranes. By 4 h, 85% of equol in cultures was present as phase II conjugates with 50 and 35% of the starting amount located in the apical and basolateral compartments, respectively. The amount of conjugated equol present in the apical compartment was directly proportional ($r^2 = 0.99$) to incubation time. At 1 h, free equol in the basolateral compartment represented 20% of its initial concentration in the apical medium, but steadily declined thereafter suggesting basolateral uptake and conversion to conjugates. Pre-treatment with the MRP inhibitor MK571 decreased apical efflux of conjugated equol and increased the quantity of free equol in the apical, cellular and basolateral compartments. These data suggest that ingested equol has the potential to be absorbed and that some individuals classified as "non-producers" may actually efflux equol conjugates into the intestinal lumen with high efficiency.

4.2 INTRODUCTION

Equol is a microbial metabolite of the isoflavonoid daidzein that has received increasing attention from a number of investigators. Equol is optically active, and therefore can exist as either an R- or S- enantiomer (229). Setchell and colleagues (68) found that while there is no difference in the bioavailabilities of R- and S-equol, the S- enantiomer is the exclusive form generated by intestinal microbes.

The estrogenic and antioxidant activities of equol are of particular interest. Equol exhibits greater estrogenic activity than genistein in rats as assessed by uterine weights of immature animals (64). Muthyala et al. (230) demonstrated that the diastereoisomers of equol have selective affinity for binding to estrogen receptors *in vitro*. R-equol has a high affinity for ERα, whereas S-equol binds with higher affinity to ERβ. It also has been reported that equol induces ER-dependent transcription more effectively than other isoflavonoids (231). Lund et al. (232) also reported that the estrogen-like activity of equol may be related to its ability to complex with 5-α-dihydrotestosterone, but not with the androgen receptor. Indeed, subcutaneous administration of equol for 7 d decreased ventral prostate weight in rats suggesting that equol may be efficacious in prostate cancer prevention (232).

Equol is a reduced form of daidzein and as such has greater antioxidant capacity than its parent isoflavonoid (64,233,234). Equol has been shown to suppress superoxide formation, LDL oxidation and attenuate AAPH or Fe(II)-induced peroxidation *in vitro* (162,235-237).

Reeve et al. (238) found that topical application of a lotion containing 10 μM equol to albino mice conferred protection against UVA-induced lipid peroxidation. However, dietary administration of equol failed to reduce plasma malonyldialdehyde in rats (239).

In order to mediate its proposed activities, equol must first be produced from daidzein in the gut and subsequently absorbed and delivered to target tissues. While reports indicate that strict anaerobes residing in the large intestine are responsible for conversion of daidzein to equol, a specific microbial species that produces equol remains unknown (240,241). It is possible that conversion of daidzein to equol is mediated through the action of multiple species of bacteria. Decroos et al. (242) recently isolated a mixed equol-producing bacterial culture consisting of *L. mucosae*, *E. faecium*, *F. magna* and one unidentified species; however, these microbes were not able to produce equol from daidzein individually.

The standard markers for equol synthesis in the gastrointestinal lumen are plasma and urinary levels of this metabolite. Because the concentration of equol in human plasma and urine is much lower than daidzein in these fluids, microbial synthesis of equol is assumed to be limited. Only 30 - 50% of humans appear to possess microflora capable of converting daidzein to equol (64-68). However, it is possible that equol production may not be directly coupled with its absorption. We are not aware that poor efficiency of absorption of equol from the intestinal lumen has been considered as an alternative explanation for its low concentration in body fluids.

Because a limited number of individuals are classified as equol producers, we questioned if equol supplementation may represent a useful strategy for providing individuals with the health-promoting benefits of this metabolite. Transport of orally

administered equol to peripheral tissues requires stability during gut transit, as well as uptake by and transport across the absorptive intestinal epithelium. Bioaccessibility refers to the potential of an ingested compound to be taken up from the gastrointestinal lumen by absorptive epithelial cells (57). In the present study, simulated gastric and small intestinal digestion was used to examine the digestive stability and bioaccessibility of equol. Confluent monolayers of differentiated Caco-2 cells were used as a model for the small intestinal mucosal epithelium to characterize uptake, metabolism and transepithelial transport of equol.

4.3 MATERIALS AND METHODS

4.3.1 Supplies

Porcine pepsin, porcine lipase, porcine pancreatin, porcine bile extract, β-D-Glucuronide glucuronosohydrolase (β-glucuronidase) type H-5 from *Helix* pomatia (G-1512), Dulbecco's Modified Eagle's Medium and phenolsulfonphthalein (phenol red) were purchased from Sigma Chemical Co. (St. Louis, MO). Daidzin (Daidzein, 7-*O*-β-D-glucopyranoside), daidzein (4',7-Dihydroxyisoflavone) and equol (7-hydroxy-3[4'hydroxyphenyl]-chroman) were purchased from LC Laboratories (Woburn, MA). 2',4'-dihydroxy-2-phenylacetophenone was purchased from Indofine Chemical Company, Inc (Hillsborough, NJ). The multidrug resistance protein (MRP) family inhibitor MK571 was purchased from Calbiochem (La Jolla, CA). BODIPY® FL verapamil-HCL was purchased from Invitrogen Corp. (Carlsbad, CA). Nylon syringe filters (0.2 μm) were purchased from Alltech Associates, Inc (Deerfield, IL). Caco-2 human adenocarcinoma cells were purchased from American Type Culture Collection (Rockville, MD). Bicinchoninic acid protein assay reagents were purchased from Pierce

Biotechnology, Inc. (Rockford, IL). Glutamine, non-essential amino acid solution, penicillin G sodium and streptomycin sulfate, and fungizone amphotericin B were purchased from Gibco (Invitrogen Corp., Carlsbad, CA). Fetal bovine serum was purchased from Atlanta Biologicals (Lawrenceville, GA). All other reagents were purchased from Fisher Scientific Co. (Fairlawn, NJ). Reagents used for HPLC analysis were HPLC grade.

4.3.2 Simulated Digestion

Meal Preparation

Aliquots of saline (1 mL; 120 mmol/L) and a stock solution (2 mL) containing equol (2 mmol/L), daidzein (2 mmol/L) and daidzin (2 mmol/L) in ethanol and were combined in an 11 mL glass vial and sonicated in a water bath sonicator (Bransonic 12, Branson Cleaning Equipment Co., Shelton, CT) for 1 min. The addition of saline (1 mL) and sonication as described above was repeated seven times so that the final volume of the solution was 10 mL. Saline (158 mL) and the diluted stock solution of equol, daidzein and daidzin were combined in beaker and bovine milk (3.3% milk fat, 12 mL) was added while the mixture was stirred with a magnetic stir bar. The "meal" was subjected to gastric and small intestinal digestion as described below. An aliquot (30 mL) of the "meal" also was diluted to 50 mL with saline, blanketed with nitrogen and stored at -20°C until analysis.

Gastric and small intestinal phases

Simulated digestion was performed as described previously with slight modifications (99,205). To initiate the gastric phase of digestion, the test meal was acidified to a pH of 2.0 ± 0.1 with HCl (1 mol/L) and porcine pepsin (10 mg/mL final

concentration) in 100 mmol/L HCl was added. The meal was diluted to 200 mL with saline and 40 mL aliquots were transferred to 50 mL tubes, blanketed with nitrogen, and incubated with shaking (Versa-Bath S model 224, Allied Fisher Scientific, Indiana) at 85 rpm, at 37ºC. After 1 h, tubes were placed on ice and the gastric phase was terminated by increasing the pH to 6.0 ± 0.2 with $NaHCO_3$ (1 mol/L). Pancreatic lipase (0.2 mg/mL final concentration), pancreatin (final concentration ranging from 0-4.8 mg/mL), and bile extract (2.4 mg/mL final concentration) in 100 mmol/L $NaHCO_3$ were added to each tube and the pH was increased to 6.9 ± 0.1 with NaOH (1 mol/L). All tubes were diluted to a final volume of 50 mL with saline, blanketed with nitrogen and incubated with shaking (85 rpm) at 37ºC for 2 h.

Isolation of the aqueous fraction of the digesta

Bioaccessibility of equol, daidzin and daidzein was determined by examining their partitioning into the aqueous fraction of the digesta. Aliquots of the digesta were centrifuged at 167,000 x g for 35 min at 4°C (Beckman L7 ultracentrifuge; 50 Ti rotor). The aqueous fraction of the digesta was recovered using a syringe with an 18-gauge needle, filtered (0.2 µm Acrodisc syringe filter, Gelman Laboratory), blanketed with nitrogen and stored at -20°C until analysis. The participation of micelles in the partitioning of equol, daidzin and daidzein within the aqueous fraction of the digesta was determined by examining the impact of varying the concentration of bile extract during the small intestinal phase of simulated digestion.

4.3.3 Cell Culture

Caco-2 cells derived from human colon adenocarcinoma were used as a model for the small intestinal mucosa epithelium to investigate the uptake, transport and metabolism of equol. Details for growth and maintenance of these cells have been previously described (99,243). Experiments with Caco-2 cells (passages 21-35) were conducted when monolayers were 12-15 d post-confluent. For initial experiments addressing uptake of equol, cells were seeded in six-well plates (Falcon #353046, Becton Dickinson and Company, Franklin Lakes, NJ) at a density of 0.25 x 10^6 cells/well. For transepithelial transport studies, Caco-2 cells were seeded at a density of 0.25 x 10^6 cells/well on transwell inserts (Falcon #353493, Becton Dickinson and Company, Franklin Lakes, NJ). Cellular protein concentrations were determined by the bicinchoninic acid (BCA) method (Pierce).

4.3.4 Preparation of Test Medium

Equol was complexed with Tween 20 to yield a water-soluble delivery vehicle for cell experiments. Preparation of the Tween 20 complex was as reported by O'Sullivan et al. (244) with slight modifications. A stock solution of equol in ethanol was prepared, filtered (0.2 μm) and its concentration determined spectraphotometrically. Aliquots of the stock solution containing the desired mass of equol were dried under a stream of nitrogen and re-solubilized in ethanol containing 1% (v/v) Tween 20. The final concentration of the vehicle in medium did not exceed 0.01%. The ethanol was evaporated under a stream of nitrogen and 100 μL of serum-free Dulbecco's Modified Eagle's Medium (DMEM) was added. The solution was vortexed for 30 sec followed by sonication in a water bath sonicator for 30 sec. The addition of 100 μL of serum-free

DMEM, vortexing and sonication was repeated 4 times before final dilution with 4.5 mL of serum-free DMEM and vortexing and sonication as above. The solution was filter sterilized (0.22 μm) and diluted in sterile serum-free DMEM to the desired final concentration of equol.

4.3.5 Equol Uptake

Monolayers were washed once with serum-free DMEM (37°C) prior to initiating uptake experiments. Monolayers were incubated (37°C, 5% CO_2) for 4 h in serum-free DMEM with equol (2.5 – 20 μmol/L) to characterize the effect of dose on cellular uptake. Separate cultures were treated with equol (10 μmol/L) for 0.5 to 8 h to characterize the impact of time on cellular uptake. Replicate cultures treated with the vehicle (0.01% Tween 20) alone served as controls. To examine if cellular uptake of equol occurred by passive diffusion or facilitated transport, replicate cultures were incubated in serum-free DMEM with equol (10 μmol/L) for 1 h at either 4°C or 37°C. Samples were collected and analyzed by HPLC.

4.3.6 Transport and Metabolism of Equol

Caco-2 cells were cultured on transwell inserts for experiments examining transport and metabolism. Monolayers were washed as above before adding 1.5 mL of serum-free DMEM with equol (10 μmol/L) to the apical compartment and 2.5 mL serum-free DMEM to the basolateral compartment. Cultures were incubated (37°C, 5% CO_2) for 0.5 to 8 h before collecting apical and basolateral media and cells to characterize the distribution and speciation (free vs. conjugated) of equol. To determine if multidrug resistance proteins (MRP) participate in efflux of phase II conjugates of equol,

monolayers were pre-treated apically with 1 mL of serum-free DMEM containing the MRP family inhibitor MK571 (100 μmol/L) in DMSO (final concentration: 0.5%) for 1 h (245). Serum-free DMEM containing equol (0.5 mL, 10 μmol/L) was then added to the apical compartment and cultures were incubated (37°C, 5% CO_2) for 2 h. Samples were collected and analyzed by HPLC.

Basolateral to apical flux of the ABCC2 fluorescent substrate BODIPY® FL verapamil-HCL was monitored in the presence or absence of MK571 to confirm inhibition of the ABCC2 (MRP2) effluxer in the apical membrane of Caco-2 cells (246-248). BODIPY® FL verapamil-HCL was solubilized in Tween 20 (final concentration: 0.01%; v/v) as described above for equol. Monolayers were washed and 1.5 mL of serum-free DMEM with or without MK571 (100 μmol/L) was added to the apical compartment, and 2.0 mL of serum-free DMEM was added to the basolateral compartment. Cultures were incubated (37°C, 5% CO_2) for 1 h before the addition of 0.5 mL of serum-free DMEM containing 6 μmol/L BODIPY® FL verapamil-HCL to the basolateral compartment. The fluorescence of aliquots (1 mL) of apical media was determined after 4 h with excitation and emission wavelengths of 485 and 528 nm, respectively.

4.3.7 Sample Collection

Aliquots of media were collected at indicated times, centrifuged at 210 x g at 4°C for 5 min and the supernatants were stored under nitrogen at –20°C until analysis by HPLC. Monolayers were washed twice with cold (4°C) phosphate-buffered saline to remove adsorbed material, gently scraped with a rubber policeman and cells were

collected in cold (4°C) phosphate-buffered saline. Cell suspensions were transferred to 15 mL conical tubes and culture plates were scraped again to collect residual cells in cold (4°C) phosphate-buffered saline. The cell suspensions were centrifuged at 210 x g at 4°C for 5 min. Following centrifugation, the supernatants were discarded and the tubes were dried with cotton swabs. Pelleted cells were stored at -20°C under nitrogen until HPLC analysis.

4.3.8 Barrier Integrity of Cell Monolayers

Barrier integrity of Caco-2 monolayers grown on semi-permeable inserts was determined by quantitating paracellular flux of the non-permeable dye phenolsulfonphthalein (phenol red). Medium from the basolateral compartment was removed and replaced with phenol red-free and serum-free DMEM (2.5 mL). Medium from the apical compartment was aspirated and replaced with serum-free DMEM containing phenol red (500 μmol/L). Paracellular flux was determined by first collecting aliquots (250 μL) of medium from the basolateral compartment and immediately adding an equivalent volume of fresh DMEM without phenol red. NaOH (20 μL, 1 mol/L) was added to basolateral medium samples (150 μl) before measuring the absorbance at 560 nm (Synergy HT™ Multi-Detection Microplate Reader, Bio-Tek® Instruments, Inc., Winooski, VT). The concentration of phenol red in basolateral medium was calculated using a standard curve generated by adding known concentrations of phenol red to phenol red-free DMEM. The rate of apical to basolateral flux of phenol red did not exceed 0.02%/cm^2/hr for all Caco-2 cell monolayers grown on inserts.

4.3.9 High-Performance Liquid Chromatography Analysis

Equol, daidzein and daidzin concentrations were determined using a Waters 2695 separations module (Milford, MA), a Waters 2996 photodiode array detector, and a Nova-Pak C-18 reversed-phase column (150 mm x 3.9 mm i.d., 4 μm, 60 Å pore size) with an inline Nova-Pak C-18 guard column (20 mm x 3.9 mm i.d., 4 μm). The sample injection volume was ≤ 20 μL and data were collected from 210 to 400 nm. Equol, daidzein and daidzin were identified by retention times and UV absorption spectra of pure (≥ 98%) standards. Samples were quantitated using standard curves with HPLC peak areas as a function of concentrations that were determined spectraphotometrically.

Samples from simulated digestion

Analysis for isoflavonoids and equol in test meal, digesta (chyme) and aqueous fraction samples has been described elsewhere (Chapter 2; ref.205).

Samples from cell experiments

2',4'-dihydroxy-2-phenylacetophenone (final concentration: 200 μmol/L) in chloroform was added as an internal standard to the samples, and cell pellets were sonicated in 0.5 mL sodium acetate buffer (0.2 mol/L, pH 5) on ice for 10 sec. β-glucuronidase (400 units/mL) in 0.5 mL sodium acetate buffer (0.2 mol/L, pH 5) was added to sonicates. Samples were incubated with shaking on the highest setting (Varimax M48725, Thermolyne, Dubuque, IA) for 18-20 h at 37°C. After incubation, diethyl ether (4 mL) was added and samples were vortexed (DVX-2500 Multi-tube Vortexer, VWR International, Buffalo Grove, IL) at 2500 rpm for 1 min. Samples were centrifuged at 450 x g for 5 min and the organic phase transferred to 11 mL glass vials. The extraction was repeated once with the organic phase pooled with the initial extract.

Extracts were dried under nitrogen at 37°C, re-dissolved in 80% methanol (0.4 mL) in water (v/v) and filtered (0.2 μm syringe filter, Alltech Associates, Inc.) prior to analysis. Replicate aliquots of cell sonicates were extracted without treatment with β-glucuronidase to determine the amount of free equol in cells. Cellular levels of conjugated equol were calculated by subtracting the amount of free equol (- β-glucuronidase) from the amount of total equol (+ β-glucuronidase). Similarly, aliquots (0.5 mL) of culture medium were combined with β-glucuronidase (400 units/mL) in 0.5 mL sodium acetate buffer (0.2 mol/L, pH 5) and incubated and extracted as above. Replicate aliquots also were extracted without β-glucuronidase treatment to determine the amount of free equol in order to calculate the amount of conjugated equol.

HPLC separation was achieved using a mobile phase consisting of 1.0% acetic acid in water (v/v; solvent A), acetonitrile (solvent B), and methanol (solvent C) with the following linear solvent gradient at 0.55 mL/min (25°C ± 5°C): 0 to 1 min, 75% A, 12% B, 13% C; 1 to 14 min, 75 to 49% A, 12 to 25% B, 13 to 26% C; 14 to 15 min, 49 to 10% A, 25 to 45% B, 26 to 45% C; 15 to 19 min, no change; 19 to 20 min, 10 to 75% A, 45 to 12% B, 45 to 13% C; and, 20 to 25 min, no change.

4.3.10 Statistics

SPSS® release 14.0 for Windows® (SPSS, Inc., Chicago, Il) was used for all statistical analyses. All experiments had a minimum of 2 independent observations for each test group. Each experiment was replicated at least once such that $N \geq 6$ unless

otherwise indicated. Data were expressed as means ± SEM when applicable. Comparison of means between two groups was performed using independent Student's t-tests. Comparison of means between more than two groups was performed using one-way analysis of variance (ANOVA) proceeded by a Bonferroni post-hoc comparison of statistical significance. Linear regression was performed when correlational analysis was warranted. Results were considered statistically significant when $P < 0.05$.

4.4 RESULTS

4.4.1 Stability and Bioaccessibility of Equol During Simulated Digestion

Equol was stable during simulated gastric and small intestinal digestion as indicated by 101-107% recovery from the starting meal in the chyme (Figure 4.1). The majority (> 90%) was present in the aqueous fraction of the digesta and independent of the amount of bile extract present during the small intestinal phase of digestion (Figure 4.1). Recovery and partitioning of daidzin and daidzein (data not shown) were similar to previous reports (Chapter 2; ref. 205).

4.4.2 Transport and Metabolism of Equol by Caco-2 Cells

Initial experiments were conducted with Caco-2 cells grown on plastic to characterize the apparent uptake of equol from the apical compartment. Since all samples were treated with β-glucuronidase before extraction (i.e., the extent of phase II metabolism of equol was not determined), results are presented as equol equivalents. Accumulation of equol equivalents during 4 h incubation was directly proportional ($r^2 = 0.99$) to the initial concentration of equol in the medium (Figure 4.2). Intracellular quantities of equol equivalents represented 4.6 - 6.4% of the initial amount of equol in the medium. No gross morphological signs of toxicity were observed after incubation in

medium containing as much as 20 μmol/L equol. When Caco-2 cells were incubated in medium containing 10 μmol/L equol for 0.5 to 8 h, apparent uptake was rapid with maximum intracellular amounts accumulated within 1 h (Figure 4.3). By 8 h, intracellular equol equivalents decreased ($P < 0.05$) to 60% of the amount that accumulated at 1 h. This decrease in cellular equol equivalents suggests efflux or conversion of equol to metabolites not monitored in this study. The rapid accumulation of equol equivalents by Caco-2 cells also suggested that equol passively diffused into the cells. This possibility was supported by the observation that cellular levels of equol equivalents were 30% greater ($P < 0.05$) in Caco-2 cells incubated in medium with equol at 4°C compared to 37°C (Figure 4.4).

In order to examine equol metabolism and transport, Caco-2 cells were cultured on transwell inserts. These cultures also accumulated a maximum quantity of equol equivalents within the first hour of incubation from medium containing 10 μmol/L free equol (Figure 4.5a). Glucuronidase-sensitive (phase II) conjugates of equol accounted for all equol that was detected in cells from 0.5 – 2 h. Cellular equol equivalents declined rapidly after 1 h with the concentration of conjugated equol steadily increasing in both the apical (Figure 4.5b) and basolateral (Figure 4.5c) compartments. By 4 h, 72.6% of equol originally added to the apical compartment was present as phase II conjugates with 46.7 and 25.9% distributed into the apical and basolateral compartments, respectively. By 8h, approximately 98% of the equol in cultures existed as phase II conjugates. Approximately 20% of total equol in the culture was present in the basolateral compartment at 1 h. This appears to reflect transepithelial transport of free equol since barrier function of the monolayers was intact as assessed by apical to basolateral flux of

phenol red. The concentration of free equol in this compartment steadily decreased thereafter, suggesting uptake across the basolateral membrane with subsequent conjugation and efflux into the apical and basolateral compartments.

Conjugation of equol increases its water solubility and therefore, requires an effluxer for transport from the cell. To examine the participation of ABCC2 (MRP2) in apical efflux of equol conjugates, Caco-2 cells were pre-treated with 100 μmol/L MK571. Basolateral to apical flux of BODIPY® FL verapamil-HCL decreased by 43% after exposure to MK571 (Figure 4.6) confirming inhibition of ABCC2 (MRP2). Free, but not conjugated, equol was detected in cells (0.32 ± 0.01 nmol) after 2 h. In contrast, neither conjugated nor free equol were detected in control cells at this time. Pre-treatment with MK571 also decreased and increased equol equivalents in the apical and basolateral compartments, respectively, compared to that in control cultures (Figure 4.7). The proportion of total equol present in the free form in the apical compartment was significantly greater ($P < 0.001$) in cultures pre-treated with MK571 (52.6%) than controls (30.7%). Equol equivalents were similar in the basolateral compartment of control (33.5%) and MK571-treated (39.9%) cultures. However, the concentration of free equol in the basolateral compartment was several-fold greater ($P < 0.001$) in MK571-treated cells (2.3 ± 0.07 nmol) compared to control cultures (0.6 ± 0.06 nmol). Also, the concentration of conjugated equol in the basolateral compartment was 51% less in MK571-treated cultures than in control cultures.

4.5 DISCUSSION

The availability of equol for uptake by intestinal epithelial cells is dependent on both its stability and ability to partition into the unstirred water layer during digestion. We found that equol was stable during simulated digestion and efficiently partitioned into the aqueous (bioaccessible) fraction of digesta. We previously demonstrated that isoflavonoids from soy-enriched bread were stable during simulated digestion; however, partitioning of the daidzein, glycitein and genistein was partially dependent on the concentration of bile extract added during the procedure (205). This suggests that micellarization of isoflavonoid aglycones is required for optimal bioaccessibility. The finding that partitioning of equol into the aqueous fraction of the digesta was ≥ 90% regardless of the amount of bile extract added is intriguing since it is a relatively lipophilic compound (249).

To our knowledge this is the first study to address transport and metabolism of equol in Caco-2 cells. Here, we demonstrated that equol rapidly reaches its maximum concentration in Caco-2 cells and is extensively metabolized to phase II conjugates that are excreted across the apical and basolateral membranes of Caco-2 cells. We found that 33% of the equol initially added to the apical compartment was transported to the basolateral compartment after 6 h and that the majority (77%) of the equol was conjugated. These observations are similar to previous reports by Steensma et al. (250) involving the transport and metabolism of genistein and daidzein in Caco-2 cells. Although they mentioned that phase II metabolites of daidzein and genistein were formed, specific information about the extent of conjugation and the distribution of conjugates in the apical, cellular and basolateral compartments was not presented.

Murota et al. (199) also reported that 35% of daidzein and genistein added to the apical compartment was transported by Caco-2 cells to the basolateral compartment within 2 h with nearly one-third present as phase II conjugates.

We observed significantly higher intracellular quantities of equol in Caco-2 cells incubated at 4°C compared with those incubated at 37°C. This is presumably because equol passively diffused across the apical membrane and the activity of phase II enzymes, as well as efflux of conjugated equol, was attenuated by the lower temperature. There are multiple reports that the rate of transcellular flux of genistein from the apical to basolateral and basolateral to apical compartments are similar, thereby suggesting passive diffusion of this compound in Caco-2 cells (251,252). In contrast, Oitate et al. (253) found that cellular uptake of genistein increased proportionally with the medium concentration, but that transepithelial transport of this compound was saturable and temperature dependent.

Transport of equol from the apical to the basolateral compartment in the Caco-2 transport model represents the bioavailable fraction of equol. We found that 17% of the starting amount of equol in the apical compartment was transported to the basolateral chamber within 1 h. *In vivo*, this fraction of free equol would be expected to be available for uptake by peripheral tissues and/or metabolized and excreted in the urine or bile. As the duration of incubation increased in the closed Caco-2 culture system, the concentration of free and conjugated equol in the basolateral compartment decreased and increased, respectively. Ju et al. (254) found that equol significantly increase proliferation of MCF-7 estrogen-dependent human breast cancer *in vitro*; however, dietary supplementation of equol failed to increase proliferation of MCF-7 cells

implanted in ovariectomized athymic mice. The authors speculate that this lack of estrogenic response *in vivo* is due to "deactivation" of equol by phase II metabolism. Similarly, Selvaraj et al. (255) found that mice administered subcutaneous injections of equol had significantly higher uterine masses than mice that were fed equol. Collectively, these results suggest that phase II metabolism of equol likely decreases its bioactivity.

Equol is present in human plasma predominantly as phase II conjugates likely generated during first pass metabolism (64). Equol glucuronides are the predominant (>90%) form in humans (80) and rats (226) and free and sulfated equol also have been identified. The relative amount of free equol present in human plasma is lower than that for daidzein and genistein (78,79). The relatively low level of free equol may underestimate the quantity of the free compound actually transported across the basolateral membrane of intestinal epithelial cells since isoflavonoids are also subject to phase II metabolism in liver (256). We found that nearly 20% of equol from the apical compartment was transported to the basolateral compartment within 1 h. We are not aware of any reports addressing speciation of newly absorbed equol. However, it is noteworthy that free and glucuronidated genistein in portal blood of rats accounted for 9.3% and 31.3%, respectively, of the dose of genistein perfused through the small intestine of rats for 1 h (50).

Efflux of cellular equol across the apical membrane of Caco-2 cells is representative of excretion into the intestinal lumen *in vivo*. Efflux of conjugated equol into the apical compartment was evident within 0.5 h and increased to 73% of the starting concentration of equol by 8 h. Similarly, Murota et al. (199) reported that phase II

conjugates of daidzein and genistein appeared within 1 h in the apical compartment of Caco-2 cells grown on inserts and increased with the length of incubation. We observed that phase II conjugates of equol were not taken up by Caco-2 cells incubated in conditioned medium containing only conjugated equol (data not shown). This suggests that conjugated equol that is excreted into the intestinal lumen will not be taken up by intestinal epithelial cells. However, it is possible that equol conjugates will be hydrolyzed by β-glucuronidases secreted by microbial populations such as *Enterobacteria*, *Clostridium* and *Bacteroides* (43). Free equol may be further degraded by microflora or transported back into intestinal epithelial cells where it will be conjugated and recycled back to the lumen or absorbed (257).

Phase II metabolism of xenobiotics eliminates the potential for these compounds to passively diffuse across cell membranes (258). It has been demonstrated that multidrug resistance proteins, specifically ABCC2 (MRP2), facilitate efflux of glucuronidated and sulfated compounds (259). We found that the ABCC2 (MRP2) inhibitor MK571 effectively blocked accumulation of conjugated equol in the apical and basolateral compartments, and it also appeared to significantly inhibit glucuronidation of equol. Inhibition of glucuronidation of apigenin by MK571 in Caco-2 cells has been reported (260). Similarly, treatment of HT-29 intestinal cells with MK571 decreased cellular levels of the glucuronidated derivatives of the topoisomerase I inhibitor, NU/ICRF 505 (261). Cummings et al. (261) speculated that phase II conjugation likely occurred, but MK571-mediated inhibition of conjugate efflux was associated with conjugate cleavage by cytosolic β-glucuronidases.

This offers an explanation for our observation of free equol since Caco-2 cells express cytosolic β-glucuronidases (262). It is also possible that MK571 inhibited effluxers other than ABCC2 (MRP2) resulting in the observed decreases in conjugated equol in the basolateral compartment (263).

In summary, our results suggest that free equol passively diffuses across the apical membrane of Caco-2 cells with nearly 20% of the total also transported across the basolateral membrane within 1 h. Intracellular equol not transported across the basolateral membrane was efficiently converted to phase II conjugates that were effluxed across both the apical and basolateral membranes. The rapid phase II metabolism and apical efflux of equol may contribute to classification of ≤ 50% of individuals as "non-producers." The possibility that polymorphisms in genes coding for phase II enzymes and multidrug resistance transporters affect the efficiency of conjugation and efflux of equol from the intestinal mucosal epithelium merits consideration. Transfer of nearly one-fifth of free equol to the basolateral compartment within 1 h, as well as the high degree of digestive stability and bioaccessibility, suggest that oral supplementation of equol may provide individuals with the proposed health-promoting benefits of this isoflavonoid metabolite. Further studies in humans and animals are needed to confirm the bioavailability of orally administered equol and possible use as a dietary supplement.

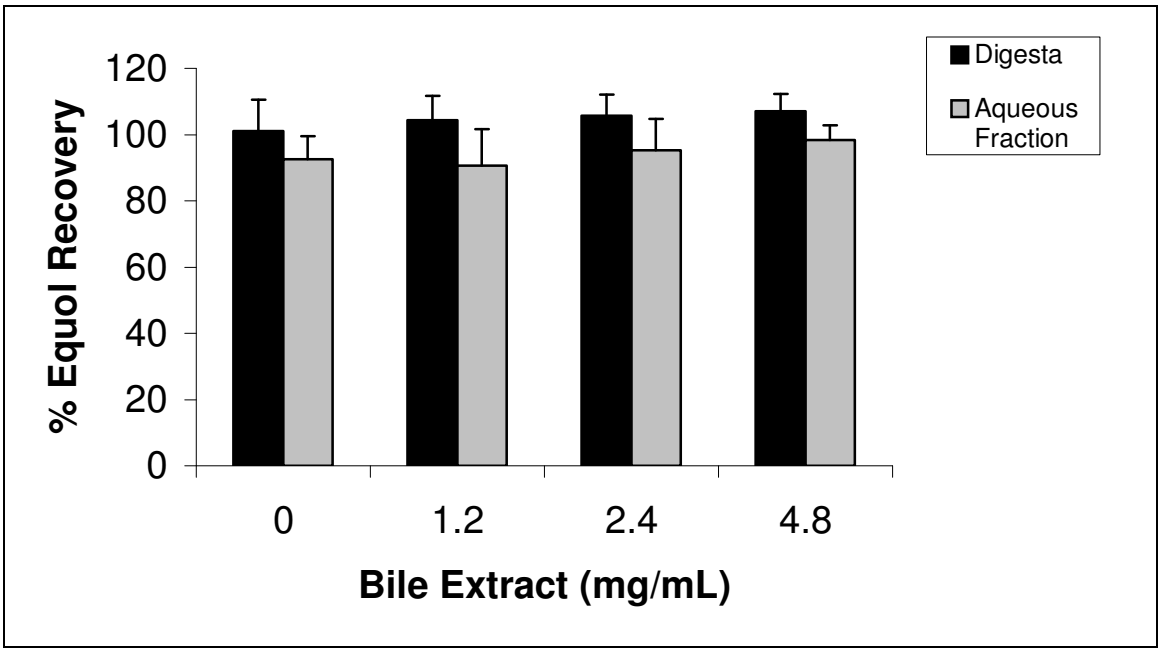


Figure 4.1: Stability of equol during *in vitro* digestion and efficient partitioning into the aqueous fraction of chyme is independent of the concentration of bile extract. The equol-containing meal was digested *in vitro* with the indicated quantities of bile extract. The aqueous fraction of the digesta was prepared by high-speed centrifugation and filtration as described in the Materials and Methods. Data (N = 6) are means ± SEM and expressed as % recovery of equol from the starting meal. Mean recoveries of equol in the digesta and the aqueous fraction were similar ($P > 0.05$) when the quantity of bile extract was varied as indicated by one-way analysis of variance followed by Bonferonni post-hoc test.

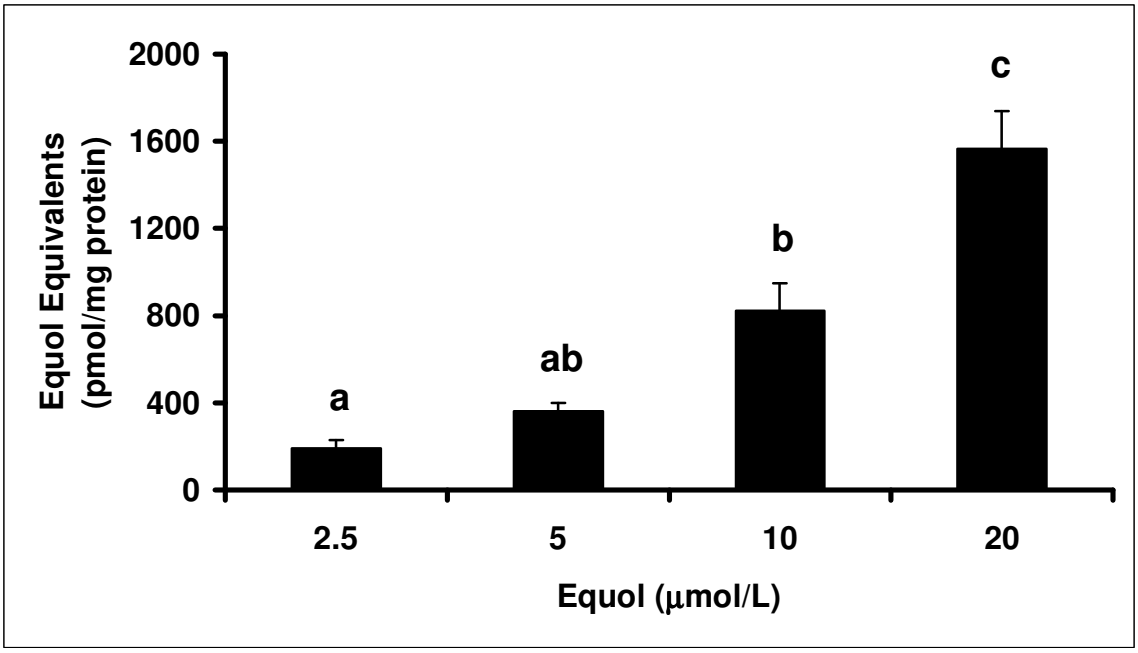


Figure 4.2: Apparent uptake of equol by Caco-2 cells is proportional to its concentration in media. Caco-2 cells were incubated in DMEM containing the indicated concentrations of equol for 4 h. Cells were collected, incubated with β-glucuronidase and analyzed by HPLC as described in Materials and Methods. Data are means ± SEM (N = 6 at each concentration). Means that do not share a common letter as superscript differ significantly ($P < 0.05$) from one another as indicated by one-way analysis of variance (ANOVA) followed by Bonferroni post-hoc comparison of statistical significance.

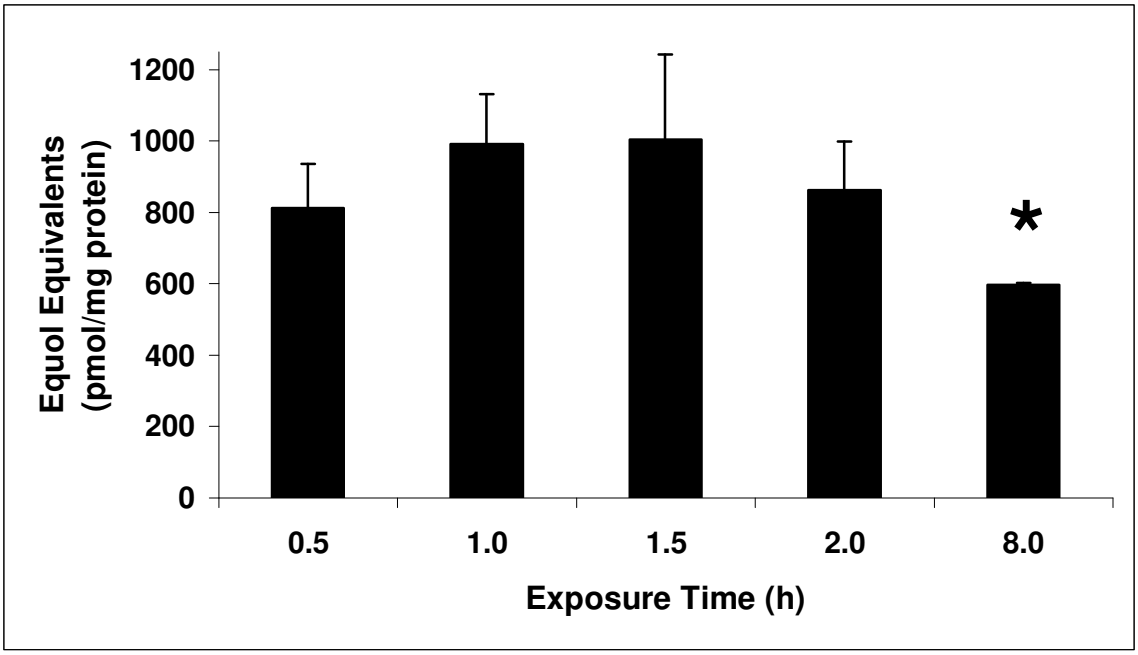


Figure 4.3: Accumulation of equol equivalents by Caco-2 cells is rapid but decreases with increased time in culture. Caco-2 cells were exposed to 10 μmol/L equol for the indicated times. Cells were collected, incubated with β-glucuronidase and analyzed by HPLC as described in Materials and Methods. Data are means ± SEM (N = 6 for each exposure time). The presence of an asterisk above the bar at 8 h indicates that the cellular content of equol equivalents was significantly less ($P < 0.05$) than at 1 h as determined by an independent T-test.

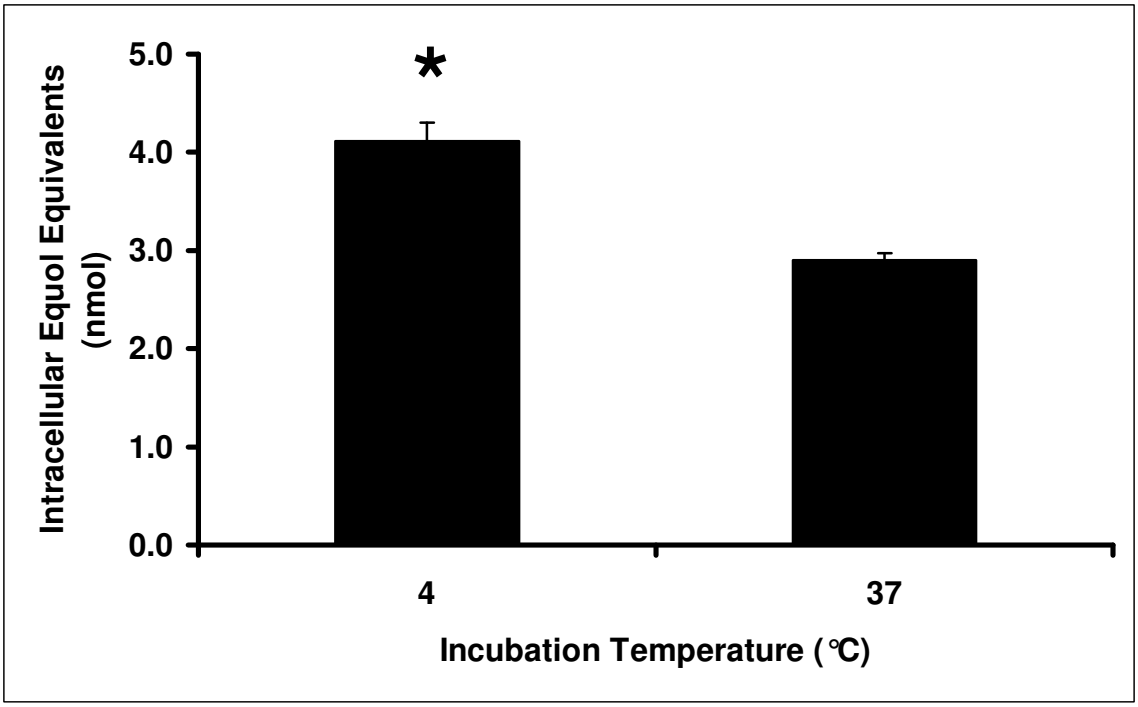


Figure 4.4: Cellular content of equol equivalents is greater after incubation at 4°C than at 37°C. Replicate cultures of Caco-2 cells were incubated in medium with 10 μmol/L equol at 4°C or 37°C for 1 h. Samples were collected and analyzed by HPLC as described in Materials and Methods. Data (N = 6) are means ± SEM and the presence of an asterisk indicates a significant difference ($P < 0.05$) as determined by an independent T-test.

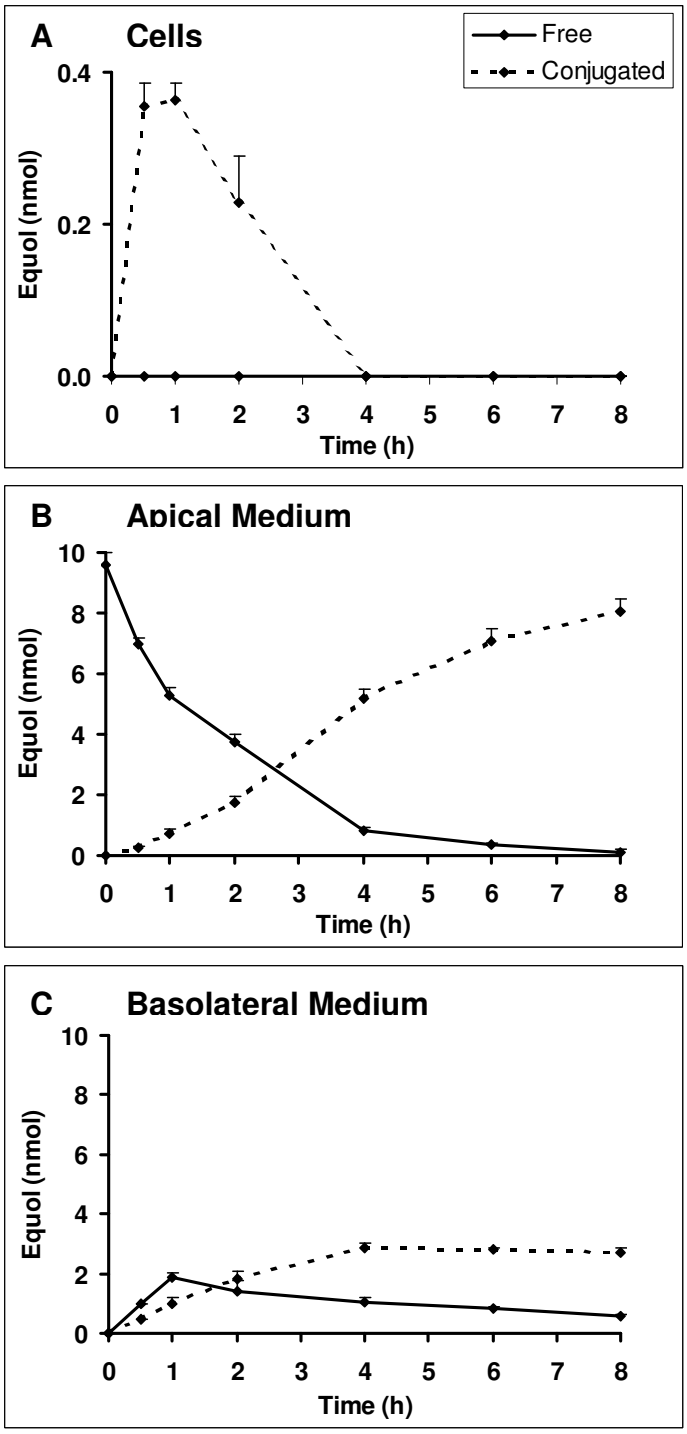


Figure 4.5: Uptake, metabolism and transport of equol by Caco-2 cells.

Equol (10 μmol/L) was added to the apical compartment of monolayers of Caco-2 cells on transwell inserts at 0 h. Cells and apical and basolateral media were collected from 0-8 h and analyzed by HPLC as described in Materials and Methods. Data are means ± SEM for 4 to 6 replicate cultures. Means within the cellular (panel A), apical (panel B) or basolateral (panel C) compartments that do not share a common letter as indicated below differ significantly ($P < 0.05$) from one another by one-way analysis of variance (ANOVA) followed by Bonferroni post-hoc comparison of statistical significance.

Cellular conjugated equol: 0h (A); 0.5h (B); 1h (B); 2h (B); 4h (A); 6h (A); 8h (A).

Apical free equol: 0h (A); 0.5h (B); 1h (C); 2h (D); 4h (E); 6h (E); 8h (E).

Apical conjugated equol: 0h (A); 0.5h (A); 1h (AB); 2h (B); 4h (C); 6h (D); 8h (D).

Basolateral free equol: 0h (A); 0.5h (B); 1h (C); 2h (BC); 4h (BD); 6h (BD); 8h (AD).

Basolateral conjugated equol: 0h (A); 0.5h (AB); 1h (B); 2h (C); 4h (D); 6h (D); 8h (D).

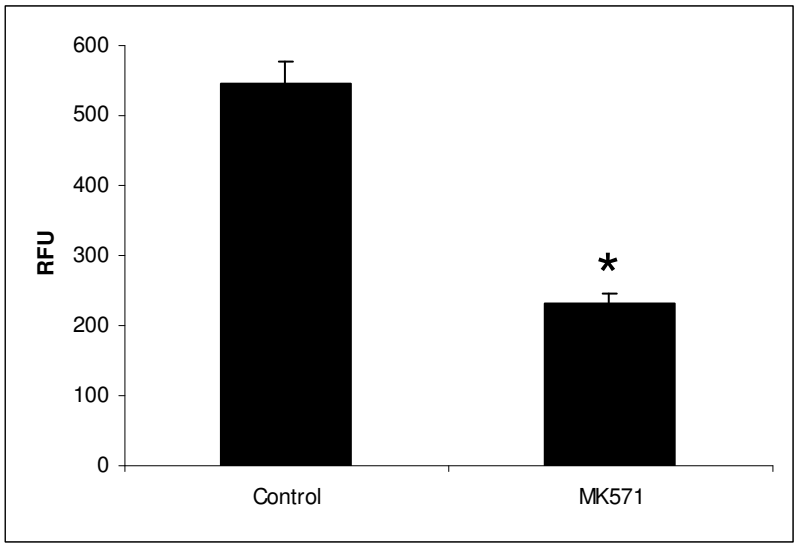


Figure 4.6: Basolateral to apical transport of BODIPY® FL verapamil-HCL is inhibited by MK571. Monolayers of Caco-2 cells on transwell inserts were treated with 100 µmol/L MK571 or vehicle only (control) and incubated (37°C, 5% CO_2). After 1 h, BODIPY® FL verapamil-HCL (final concentration: 1.2 µmol/L) was added to basolateral media and cultures were incubated (37°C, 5% CO_2) for 4 h. Basolateral to apical flux of BODIPY® FL verapamil-HCL was determined by monitoring the fluorescence of apical medium. Data are expressed as means ± SEM (N = 3) and differ significantly ($P < 0.05$) as determined by an independent T-test.

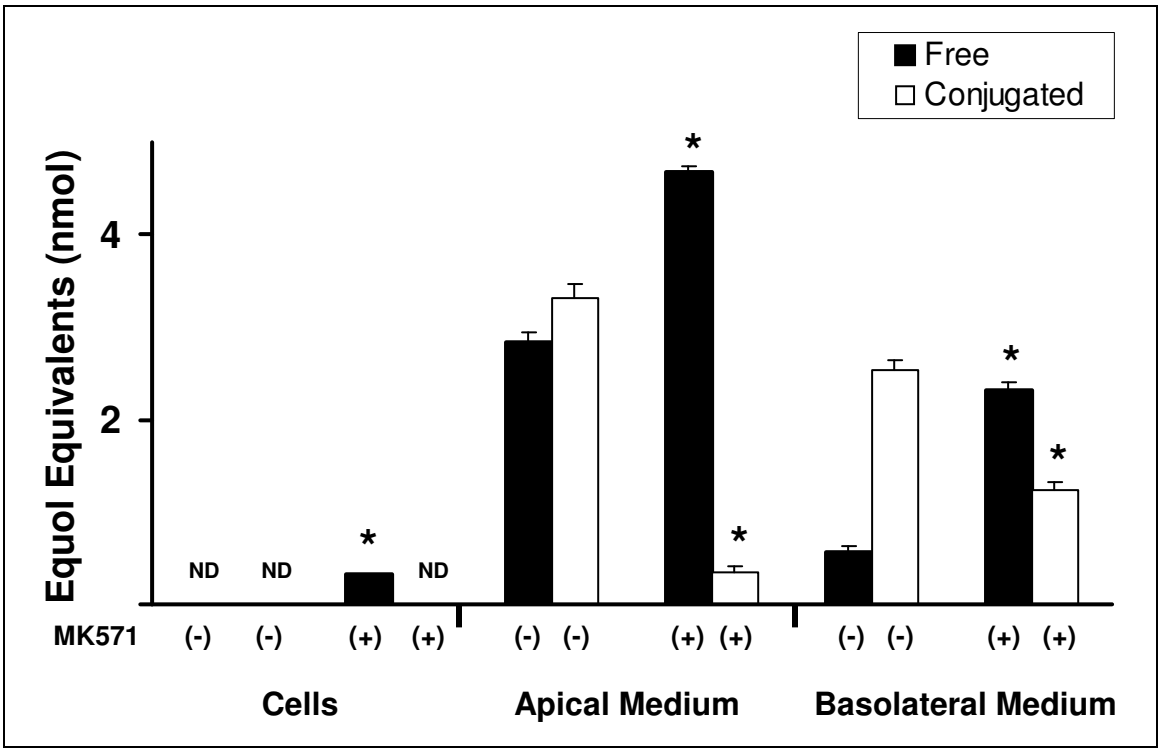


Figure 4.7: MK571 inhibits accumulation of conjugated equol in the cellular, apical and basolateral compartments of Caco-2 cells. MK571 (100 μmol/L) was added to the apical compartment of monolayers of Caco-2 cells on transwell inserts and cells were incubated for 1 h. Equol was added to the apical compartment and cells were incubated for 2 h. Cells and apical and basolateral media were collected analyzed by HPLC as described in Materials and Methods. Data (N = 4) are means ± SEM. The presence of an asterisk above bars indicates that inhibition of ABCC2 (MRP2) activity significantly altered the quantity of free or conjugated equol in the respective compartment. ND: not detected.

CHAPTER 5

EPILOGUE

I found that isoflavonoids are stable during simulated oral, gastric and small intestinal digestion; however, isoflavonoid glucosides are efficiently converted to aglycones in the small intestine during *in vivo* digestion. The small intestine therefore plays a critical role in generating the bioavailable form of dietary isoflavonoids. The small intestine also represents an important site for absorption of isoflavonoid aglycones. Moreover, the bioavailability of dietary isoflavonoids from foods containing fat and protein may exceed that of isoflavonoid supplements consumed without food due to enhanced bile secretion.

It is generally assumed that ≥ 50% of human subjects lack the required microflora to generate equol, i.e., they are classified as non-producers. I observed extensive phase II metabolism and efflux of equol across the apical membrane of Caco-2 cells. Thus, inter-individual variations in plasma and urinary equol are more likely due to a combination of different intestinal microflora profiles and phenotypic differences that modulate phase II metabolism and efflux of this compound. Despite the limited bioavailability of equol, supplementation has the potential to provide individuals with some degree of its health-promoting benefits.

The research presented here has generated a number of questions that merit future attention. Results from the experiments with soy bread clearly demonstrate that partitioning of isoflavonoid aglycones into the bioaccessible fraction of the digesta is dependent on the amount of bile extract present during the small intestinal phase of simulated digestion. A human study addressing the impact of foods that enhance bile secretion on the bioavailability of isoflavonoids is a logical extension of my *in vitro* work. It would be interesting to compare the bioavailability of isoflavonoids from supplements consumed with or without meals containing protein and fat. From a physiological point of view, chyme containing fat and protein will stimulate cholecystokinin release, resulting in contraction of the gall bladder and release of bile into the duodenum. Therefore, one would anticipate that the bioavailability of isoflavonoids would be enhanced when meals containing elevated protein and fat are consumed.

The swine study demonstrated that the small intestine has a predominant role for hydrolysis of isoflavonoid glucosides to aglycones. However, it remains unclear whether glucosidases of mammalian or microbial origin or both participate in such conversion. To clarify the origin of small intestinal β-glucosidase activity, the study could be repeated with and without an extended regimen of orally administered broad-spectrum antibiotics. If hydrolysis of isoflavonoid glucosides is due to microbial β-glucosidases, a higher ratio of glucosides to aglycones in ileal effluent should be the outcome when swine or human ileostomists are pretreated with antibiotics.

A crossover design could be implemented so long as the initial feeding study is done without antibiotic treatment. Numbers and taxonomic analysis of microorganisms in ileal effluent also would be valuable when addressing this question.

One of my novel findings is that swine had higher amounts of microbial metabolites in urine than the respective dietary isoflavonoid conjugates. Based on this observation, a study addressing intestinal microbial adaptation to chronic soy consumption in swine, as well as humans, is warranted. A crossover design will offer greater validity to interpretation of results since there is marked variation in isoflavonoid bioavailability and metabolism among individuals (4,67). Subjects, i.e., humans or swine, would first be maintained on soy-free diets for an extended period of time. If time, money and subject compliance were not an issue, a period of months would likely be ideal to allow intestinal microbial adaptation. Subjects would then consume one or more test diets containing soy isoflavonoids and isoflavonoid equivalents and microbial metabolites would be quantitated in plasma and urine. Next, subjects would consume soy-containing diets for the same amount of time to allow intestinal microbial adaptation before re-feeding soy test meals and examining plasma and urinary profiles of isoflavonoid equivalents and microbial metabolites. Also, analysis of fecal populations of microorganisms using denaturing gradient gel electrophoresis (DGGE) could be conducted to examine if the number of known species that convert dietary aglycones to metabolites was altered. Results from these experiments would provide considerable insight into the impact of chronic soy consumption on adaptations in intestinal flora responsible for metabolism of isoflavonoids.

I have provided the first characterization of transepithelial transport and metabolism of equol in Caco-2 cells. Currently, equol is readily available from commercial suppliers, and sources of other microbial metabolites of isoflavonoids are emerging. The model and experimental paradigm provides the opportunity to investigate the transport and metabolism of dihydrodaidzein, *O*-desmethylangolensin, dihydrogenistein, 6-OH- *O*-desmethylangolensin, dihydroglycitein, 5'-methoxy-*O*-desmethylangolensin, dihydro-6,7,4'-trihydroxy-isoflavone and other isoflavonoid metabolites as they become commercially available. It is perhaps more physiologically relevant to conduct these experiments in large intestinal cell lines such as HCT-116 or HT-29 due to the fact that these compounds are primarily produced in the large intestine. The uptake and extent of phase II metabolism and efflux of these compounds may offer insight to their *in vivo* pharmacokinetics.

Equol underwent extensive phase II metabolism following uptake by Caco-2 cells. Conjugated equol was excreted across both the apical and basolateral membranes while a fraction of apical equol was transported to the basolateral compartment. This fraction of free equol would likely be conjugated in the liver. Therefore, studies addressing the effects of free equol on cellular bioactivities may not be physiological relevant. Studies using conditioned medium rich in conjugated equol or co-culture systems could be used to examine the biological activities of conjugated equol. The co-culture approach involves the use of transwell inserts in which Caco-2 cells are cultured on the membrane and a different cell type is cultured in the basolateral compartment. Free equol is added to the apical compartment and incubated for various times to provide basolateral media with different ratios of free versus conjugated equol.

The intracellular activity (e.g., transcription of estrogen responsive genes) in the cells in the basolateral compartment would be investigated to assess the potential influences of equol speciation.

It would also be interesting to examine which multidrug resistance protein(s) is (are) responsible for efflux of conjugate equol. The use of inhibitors of these proteins offers some insight, although, their inhibitory action is not necessarily limited to a single transporter in this diverse family of gene products. A more targeted approach would involve the use of specific antibodies that bind exclusively to the transporter of interest and block its activity. Similarly, siRNA methodologies could be used such that the transcript of interest is hybridized to a small consensus sequence of RNA, thereby rendering it un-translatable. In either case, the specificity for knocking out multidrug resistance protein activity is a superior approach to the use of pharmacologic inhibitors.

Finally, soy foods are becoming more popular. Data pertaining to interactions between soy components and drugs are increasingly more important to the general population. Potential interactions of isoflavonoids and their metabolites with drugs merit investigation. For example, might these compounds affect the bioavailability of various drugs? The nuclear receptor PXR mediates expression of many genes involved in drug metabolism. In addition to estrogen receptors, isoflavonoids have been shown to activate PPAR and other nuclear receptors (264). This could be investigated by pre-treating Caco-2 cells with isoflavonoid aglycones and their metabolites, singly and in combination, and then characterizing uptake, metabolism and efflux of pharmacologic compounds of interest.

It is possible that isoflavonoids and their metabolites alter phase I and II metabolism of some drugs. It is also possible that they may affect efflux of some drugs through interactions with multidrug resistance transporters.

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