Metabolism of Estrogenic Isoflavones in Domestic Animals (43828)

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Abstract. **The metabolism of estrogenic isoflavones in cattle and sheep is reviewed. Results from *in vitro* and *in vivo* studies are discussed, mainly regarding whether differences in sensitivity to phytoestrogens between cattle and sheep depend on differences in metabolism, particularly in conjugative capacity. Results from a feeding experiment with pigs fed red clover meal are presented. Levels of phytoestrogens in plasma from the pig are compared with those found in plasma from ruminants fed red clover silage. Some aspects relating to the possibility of pigs being exposed to risks when fed with feed containing estrogenic isoflavones are briefly discussed.**

[P.S.E.B.M. 1995, Vol 208]

A substantial work about the metabolism and estrogenic effects of isoflavones on sheep has been described since a massive outbreak of permanent and temporary infertility in sheep grazing on certain cultivars of subterranean clover (*Trifolium subterraneum*, L.) in Western Australia was reported in the 1940s (1). Since then, several different herbs have been shown to contain estrogenic substances that are capable of affecting the reproduction when consumed in large amounts (2). In Sweden, red clover (*Trifolium pratense*) is quantitatively the most important source of phytoestrogens, with a normal concentration of 0.5%–2.5% of dry matter (3). Dairy cows are allowed to graze on red clover pastures during the summer. During the indoor season, lasting about 7 months, the cattle are often fed silage with high contents of red clover. Thus, daily consumption of phytoestrogens may reach 50–100 g. Despite this, there have been no reports of permanent infertility in cattle, and, consequently, the estrogenic effects have been suggested to be generally weaker on cattle than on sheep (4, 5). Even if cattle are not affected to the same extent as sheep, the syndromes of infertility occurring in the former have been associated with consumption of estrogenic pasturage (6, 7, 8, 9, 10). The suggested differences in sensitivity to phytoestrogens between cattle and sheep have been proposed to depend on differences in metabolism, especially differences in detoxication capacity between the two species, cattle and sheep (11).

Ruminants

Rumen Metabolism. Metabolism in sheep has been reviewed a number of times (12, 13, 14), whereas very little has been described about metabolism in other species. However, rumen metabolism of isoflavones in other species has been suggested to be qualitatively similar to that in sheep (15).

The isoflavones occur in intact plants predominantly as glycosides (16) and are readily hydrolysed by plant enzymes or by microorganisms in the rumen. The major metabolic transformation of isoflavones is performed by microorganisms in the rumen (17). Biochanin A is demethylated to genistein and via ring cleavage to p-ethyl phenol and organic acids (Fig. 1). In contrast, formononetin, which is indirectly responsible for estrogenic disturbances in sheep (18), is mainly demethylated to daidzein and further via hydrogenation and ring fission predominantly to equol (Fig. 2). However, many metabolic interconversions are not completely defined. Intermediary compounds from ring cleavage of genistein to simpler phenol compounds have not yet been isolated. Furthermore, the metabolic pathway of formononetin may proceed, under some circumstances, via an alternative route involving reduction without prior demethylation, where

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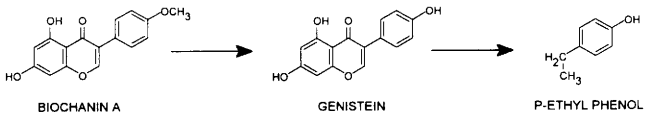

Figure 1. Rumen metabolism of biochanin A in sheep, suggested by Cox and Davies (13).

O-methyl-equol is the major excretory product of formononetin (12). The proportion of formononetin excreted in this form is very variable and factors that affect the extent to which formononetin is metabolized by this route have yet to be elucidated.

Hepatic Metabolism. The liver is generally regarded as the major organ for metabolism and detoxication of foreign substances. Therefore, the apparently lower susceptibility to phytoestrogens of cattle in comparison with sheep has been suggested to depend on differences in liver metabolism (11, 12). We have investigated the demethylating and conjugative rate of formononetin and daidzein in microsome preparations from sheep and cow livers (19).

Demethylating of formononetin to daidzein in liver microsomes was exceedingly small in both species. No further reduction of daidzein to equol appears to occur in the liver or is very small, since no equol was detected with the HPLC technique used (3). Furthermore, the results indicate that the liver contributes very little to the total degradation of phytoestrogens in ruminants, and rumen microorganisms probably account for most of the demethylation and reduction activity (17, 20).

The conjugative activity was much higher than the demethylating activity in both species. Both formononetin and daidzein was about 1.5 times more efficiently conjugated by the ovine liver than the bovine liver when uridine 5′-diphosphoglucuronic acid (UDPGA) was present (Table I).

In order to achieve a maximum glucuronidation rate, two different effectors, uridine 5′-diphospho-N-acetylglucosamine (UDPAG) and Lubrol PX were included in some incubations. When the effectors were added to the test tubes together with microsomes UDPGA and the β-glucuronidase inhibitor, *Saccharic lactone* (*S. lactone*), the conjugation pattern changed: total activity increased, but no significant difference between the two species was observed.

Metabolism of Isoflavones in the Gastrointestinal Epithelium. No explanation of the suggested differences in sensitivity to phytoestrogens between cattle and sheep could be established in the experiment with liver microsomes. Other tissues capable of detoxifying these substances must occur if the differences in sensitivity between cattle and sheep depend on their capacity to detoxify the estrogenic isoflavones. It is reasonable to believe that different dietary substances should be detoxicated before they reach the blood circulation. Therefore, the gastrointestinal epithelium

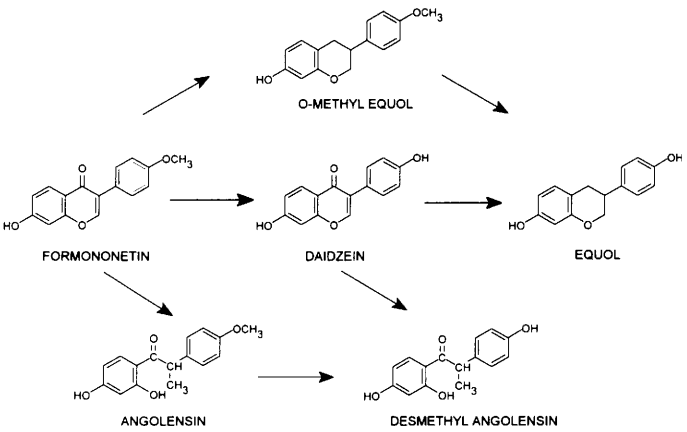

Figure 2. Rumen metabolism of formononetin in sheep, suggested by Cox and Davies (13). The major metabolic pathway of formononetin is via daidzein to equol, rather than via O-methyl equol and to desmethyl angolensin.

should probably be the most important site for detoxifying phytoestrogens and other harmful dietary substances before they enter the blood circulation.

In order to evaluate if differences in extrahepatic metabolism may explain the differences in susceptibility to phytoestrogens between cattle and sheep, the glucuronidation activity in the epithelium from the four compartments of the complex stomach and the small intestine have been investigated (21). This study was performed in a similar way to the liver study, but homogenates were used instead of microsomes. The results in Figure 3 show that a considerable conjugation activity takes place in the gastrointestinal epithelium, which cannot be ignored. The conjugation activity was 3 to 20 times greater in sheep than in cattle in all parts of the gastrointestinal tract, except in the intestinal mucosa, where the activity was about two times higher in cattle than in sheep.

Even though the conjugation rate is almost the same in rumen, reticulum, and omasum, the rumen is probably the most important tissue in the gastrointestinal tract as regards detoxification of these substances. The reason for this supposition is that ingested food stays in the rumen for a relatively long period (i.e., 1–3 days, depending upon the nature of the diet and animal species). The absorption of phytoestrogens takes place mainly in the rumen (22, 23), therefore, the higher bovine conjugation in the small intestine compared with the rumen and other organs was unexpected.

The conjugative activity differed depending on which substrate was used. The highest activity was observed when equol was used as substrate, about 3 to 10 times higher than when daidzein and formononetin were used. The large variations in conjugation rate between formononetin, daidzein, and equol are unexpected as the differences in chemical structure are very small. The differences in glucuronidation activity

Table I. Conjugation of Formononetin and Daidzein by Cow and Sheep Liver Microsomes[a]

Additions	Formononetin disappeared (μg/120 min)		Daidzein disappeared (μg/120 min)	
	Cow	Sheep	Cow	Sheep
UDPGA + β-Glu	0.6 ± 0.8	1.1 ± 1.0	0.4 ± 1.6	0.1 ± 0.8
UDPGA	3.7 ± 1.4	6.6 ± 0.8[b]	7.1 ± 0.9	10.0 ± 2.3[b]
UDPGA + (UDPAG + S. lactone)	7.4 ± 1.1	7.4 ± 2.6	13.0 ± 1.1	12.0 ± 2.0
UDPGA + (Lubrol + S. lactone)	7.9 ± 0.6	7.6 ± 1.8	12.7 ± 0.9	12.2 ± 1.3

[a] The figures are mean values ± SD of five to eight experiments.
[b] $P < 0.05$.
(From Lundh et al., J Agric Food Chem., **36**:22, 1988. With permission)

between formononetin and daidzein may be explained by differences in their chemical structure where formononetin has two methyl groups and daidzein only one, which can bind to glucuronic acid. The difference in chemical structure between daidzein and equol is small: equol has lost a keto group in Position 4 and has a single bound between C-2 and C-3 (Fig. 2). It is therefore more difficult to explain why these two substances differ so much in conjugation activity. However, it is well known that the glucuronosyltransferase exists in multiple forms. Whether the higher conjugation rate of equol in comparison with daidzein is due to different isoenzymes or merely to steric hindrance cannot be explained without further investigation. On the other hand, it seems rather logical that the conjugation rate of equol is higher than for formononetin and daidzein, because equol is the major substance absorbed from the rumen to the blood circulation.

Metabolism *in Vivo*. *In vivo* metabolism in ruminants of substances present in the feed is influenced by a complex pattern of variables. Even if the liver, gastrointestinal tissue, and to a certain extent, the kidney may be regarded as detoxification centers, other phenomena such as absorption, excretion, degree of conjugation, etc. may cooperate in an *in vivo* situation, which cannot be anticipated by means of only *in vitro* studies. Being able to determine the concentrations of phytoestrogens in blood samples taken from animals during feeding makes it possible to illuminate the complex interactions which occur *in vivo*.

In a comparative feeding study (23), five dairy cattle from a herd normally fed on red clover/grass silage (50%/50%) were used as experimental animals. The feed ratio for the cows was about 13–14 kg of silage, offered twice a day, corresponding to a daily mean intake of about 14–15 g of formononetin and 0.3 g of daidzein. The sheep were offered the same silage as the cows, and blood samples were collected after a 10-day period of prefeeding. To compare the plasma concentrations of the phytoestrogens in sheep with those in cattle, the sheep were offered the same amount of red clover silage, with consideration to the basal metabolic rate (which represents 0.247 kg of si-

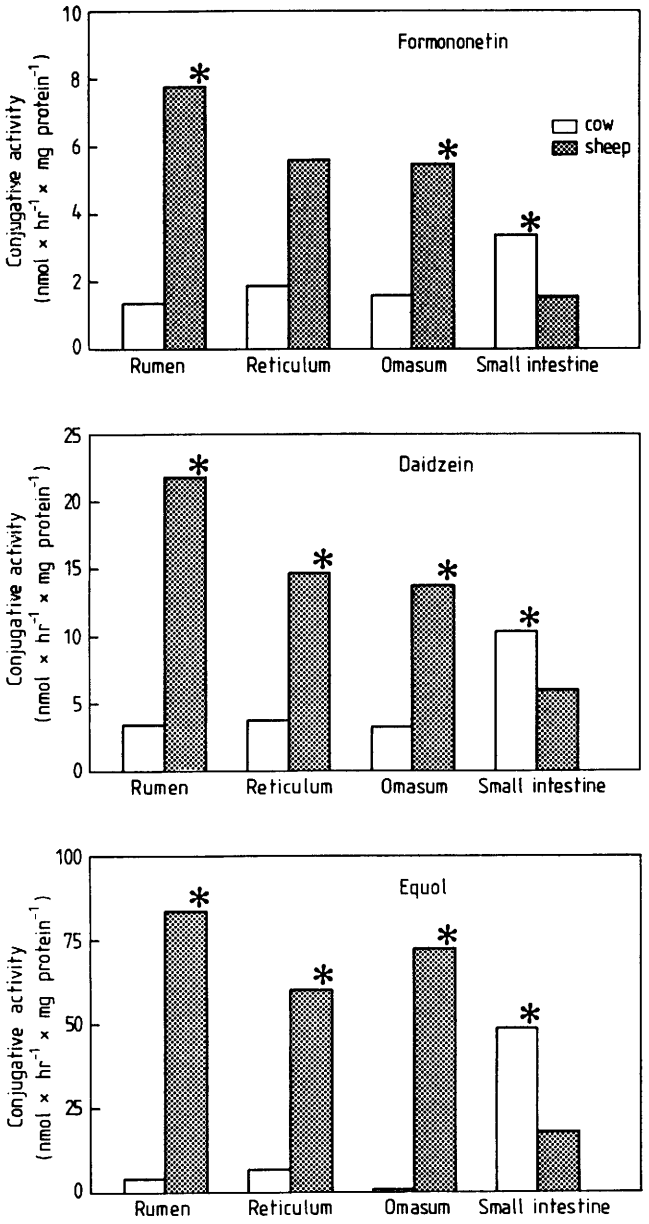

Figure 3. Conjugative activity toward formononetin, daidzein, and equol in various gastrointestinal tissue homogenates from cattle and sheep. The columns represent mean values of duplicates from three animals of each species. The asterisks mark a significantly greater activity than that in the other species. (From Lundh, J Agri Food Chem **38**:1012, 1990. With permission).

lage/liveweight$^{0.75}$) corresponding to a mean intake of about 3.2 g formononetin per day per sheep. Such high daily intake of formononetin is enough to cause apparent estrogenic symptoms in ovariectomized ewes (24) and adversely affect fertility in sheep (25). The analysis of total (conjugated and free) and free (unconjugated) phytoestrogens in blood plasma was performed by a HPLC-method previously described (26).

Formononetin and daidzein were absorbed very rapidly in cattle and reached a total (free and conjugated) maximum level within 1 hr after feeding. At this time, the plasma concentration was about three times higher in the cow than in the sheep, but declined to the same level as in sheep after about 2–3 hr (Fig. 4, A and B). The high value after 7 hr in bovine plasma, presented as a dotted curve in Figure 4 and 5, depends on the cows being accidentally fed with their other half of the daily silage ratio about 15–20 min before the last blood sample was taken. However, this result confirms that absorption of formononetin and daidzein is very rapid in the cow and take place already in the rumen.

The concentration of total equol in bovine plasma was almost constant during the whole sampling period. In the sheep, the initial equol concentration increased from about 80 μg/100 ml plasma, and did not reach the same level as in the cow until after 2–3 hr (Fig. 4C). These results show that the cattle are exposed to a higher concentration of equol for a longer time than the sheep since the plasma concentration of equol in the sheep blood decreases by about 50% during the night (14–16 hr after food intake), whereas the concentration remains fairly constant in cows. The differences in plasma concentration of total equol at the beginning of the sample time indicates that the clearence of equol is faster in the sheep. Concentrations of total equol in sheep plasma, similar to those presented in Figure 4C, are high enough to cause estrogenic symptoms in sheep (11, 27).

Glucuronidation is the major detoxication system for several potentially toxic endogenous and exogenous substances, including phytoestrogens. Formononetin, daidzein, and equol are mainly found as conjugates in blood. Not more than about 5% of the total amount was found as unconjugated substances in plasma at any time and the concentration of free formononetin was very low (Fig. 5A) (daidzein was detected in even lower concentrations, just around the detection limit). The free amount of equol constitutes about 5% of the total amount in cows and about 1% in sheep. The concentration of free equol was, however, about 10 times higher in plasma from cows than from sheep, whereas no differences in plasma levels of unconjugated formononetin and daidzein occurred (Fig. 5B).

In contrast to Braden *et al.* (11), who suggested that the weaker effects of phytoestrogens on cattle de-

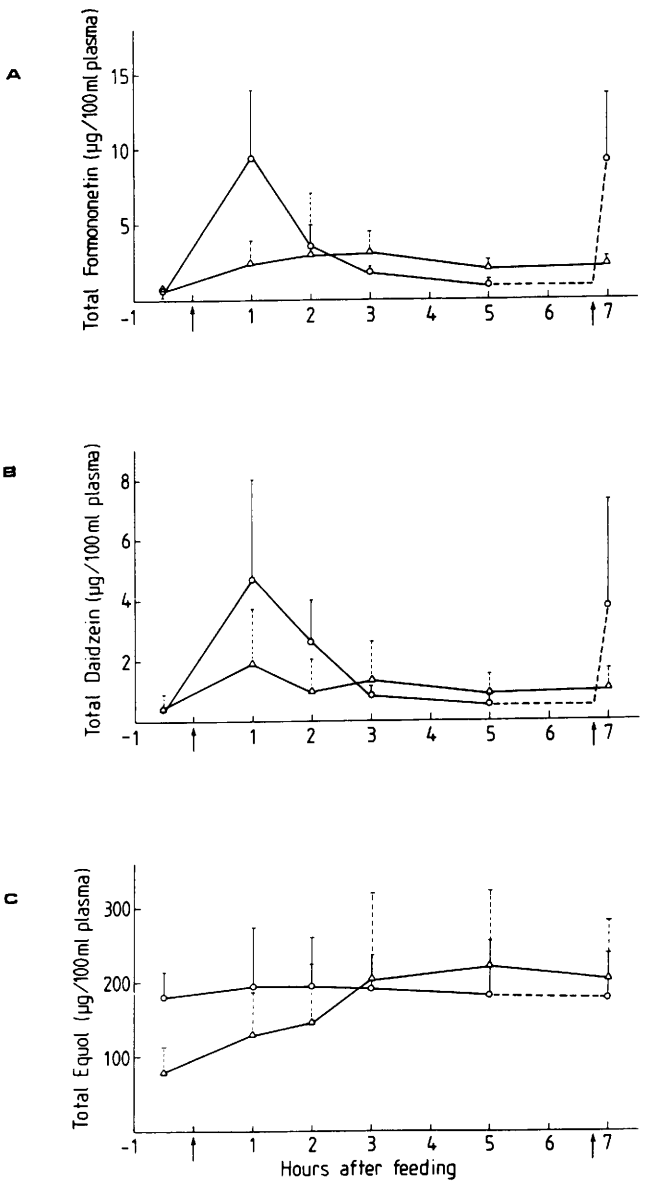

Figure 4. Concentrations of total (free and conjugated) phytoestrogens (A) formononetin, (B) daidzein, and their metabolite (C) equol in bovine (○—○) and (△—△) ovine blood plasma at different times after feeding on red clover/grass silage. Data represent mean ± SD from five animals. The first arrow indicates when the feed was offered to the cows and sheep. The line is dotted between 5 and 7 hr since the cows were offered the other half of their daily feed ration just before the last blood sample was taken which is indicated by the second arrow. (From Lundh et al., J Agr Food Chem **38**:1530, 1990. With permission).

pend on the circulating phytoestrogens and their metabolites being more efficiently conjugated in cattle than in sheep, our results suggest that sheep have a higher capacity to conjugate phytoestrogens. According to Shutt and Braden (27), equol is the main substance responsible for reproductive disturbances in sheep, and the prevailing opinion is that biologically active forms consist of free, unconjugated, and, to a certain degree, sulfonated substances. With this in mind and from the results presented here, the sheep

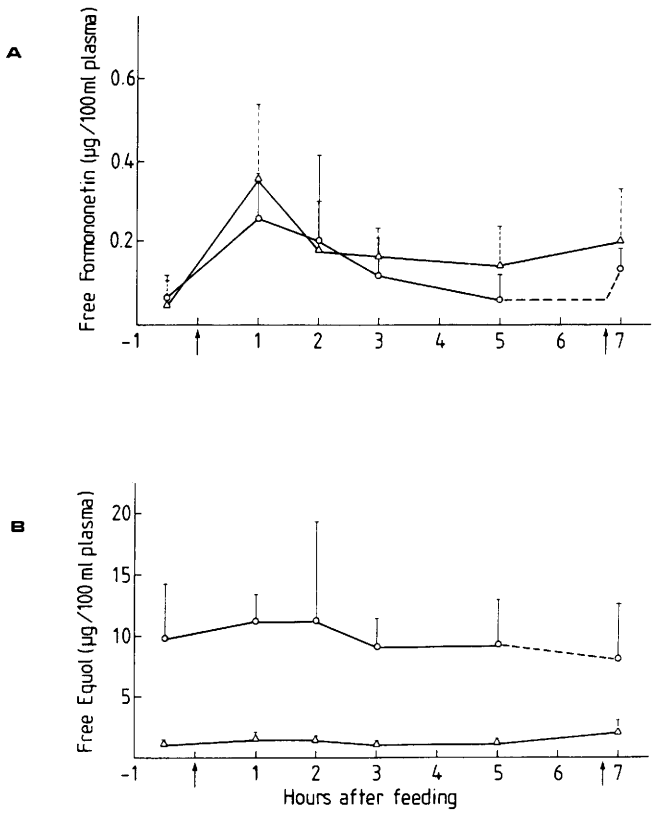

Figure 5. Concentrations of free (A) formononetin and (B) equol in bovine (○—○) and (△—△) ovine blood plasma at different times after feeding on red clover/grass silage. Explanations to the different signs is given in Figure 4. (From Lundh et al., J Ag Food Chem **38**:1530, 1990. With permission).

seems to be better equipped than the cow for detoxifying and eliminating phytoestrogens. Based on our *in vitro* studies with liver microsomes (Table I, cf.). gastrointestinal homogenates (Fig. 3, cf). and in *in vivo* experiments (Fig. 4 and 5, cf.), we assume that the sheep conjugates phytoestrogens more efficiently than the cow. Furthermore, the large amount of free equol in bovine plasma indicates that other factors than differences in conjugation rate between cattle and sheep explain the differences in sensitivity to phytoestrogens. Such factors might be on the receptor level. The concentration of estrogen receptors in uterus was about two to four times higher in sheep than in cattle (28, 29, 30).

Monogastric Animals

Metabolism in Pigs. The metabolism of phytoestrogens in pigs is not well documented and to my knowledge no studies on that subject have been described before. However, metabolic studies on such monogastric animals as rat (31), guinea pig (27), fowl (32), and humans (33) have been described in the literature. The major metabolic transformation of phytoestrogens in humans is performed by the intestinal microorganisms which probably is also the case for monogastric animals (33).

The pig may be exposed to high levels of phytoestrogens since their food might contain large amounts of legumes (such as red clover or soya products) containing estrogenic isoflavones. Furthermore, in Sweden, the usage of red clover products as food for pigs is predicted to increase in the future.

In a feeding experiment, two pigs were fed on a mixture containing 20% of red clover meal corresponding to a daily mean intake of 866, 88, 97, and 378 mg of formononetin, daidzein, genistein, and biochanin A respectively. The animals were prefed with the experimental diet 7 days before the blood samples were collected via permanent vein catheters, at different times after feeding. Formononetin was absorbed very rapidly and reached a total maximum level (free and conjugated) within 1 hr and remained at a rather stable concentration of 80–100 µg/100 ml plasma, which is about 10 times higher than the highest level found in bovine plasma. The high concentration of the formononetin in blood plasma immediately after feeding indicates that the absorption has already taken place in the stomach. Since the food stays in the stomach only for about 2 hr, absorption from the intestine is also most probable. Daidzein and equol show similar patterns to formononetin, but the concentrations were about 5–15 times lower (Fig. 6). Even total genistein was found in about similar concentrations as equol.

Very little formononetin occurred in unconjugated form, 0.2–0.6 µg/100 ml, which represents about 0.5% of the total amount (Fig. 7). Daidzein occurred in even lower concentrations (not shown). The free amount of equol in porcine plasma was about 13 times higher than that for formononononetin (Fig. 7). The free amount of equol constitutes about 30%–50% of the total amount in pigs. The corresponding values for sheep and cow were 1% and 5% which suggest fundamental differences in conjugation of equol between the pig and ruminants. The plasma concentration of total equol in the pig was about 10 to 15 times lower than in plasma from the sheep and the cow but the free amount of equol being at the same level as for the ruminants (about 2 and 10 µg/100 ml for sheep and cow, respectively).

Urine samples taken continuously for 8 hr via a ureter catheter show that the isoflavones are mainly excreted via the urine. About 55% of the ingested formononetin and daidzein, originated from the morning feed, was excreted within 8 hr feeding. The proportion of these excreted isoflavones were 72% of formononetin, 6% daidzein and the remaining 22% was identified as equol. Thus, the major part of the formononetin ingested by the pig was excreted unchanged without prior degradation. Furthermore, only trace amounts of the phytoestrogens were detected in blood plasma 3 days after the red clover feed was withdrawn.

Pigs are probably the most sensitive animals to the

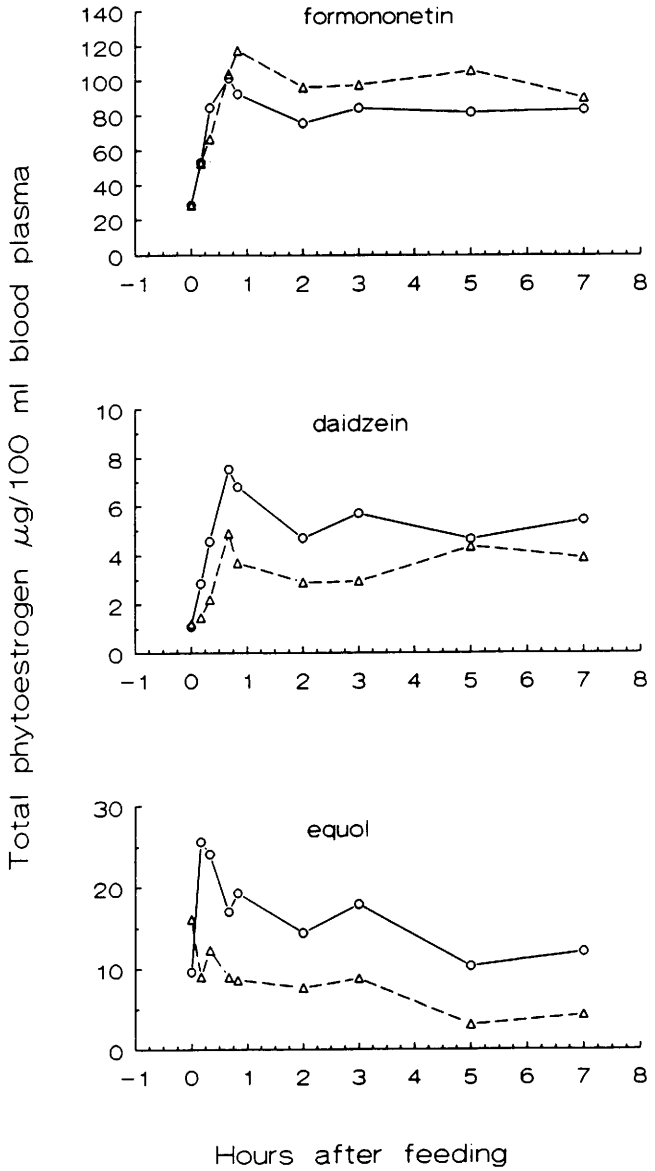

Figure 6. Concentrations of total (free and conjugated) phytoestrogens in blood plasma from two pigs (Pig 1, ○—○; Pig 2, △----△) at different times after feeding on 20% red clover meal.

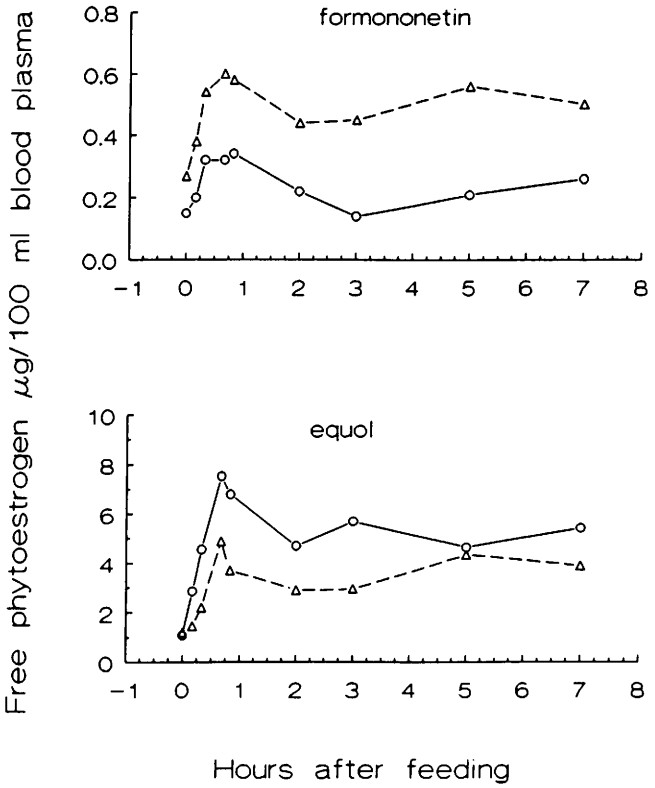

Figure 7. Concentrations of free formononetin and equol in blood plasma from two pigs (Pig 1, ○—○; and Pig 2, △----△) at different times after feeding on 20% red clover meal.

"mycoestrogen" zearalenone (34), which is produced by the mold fungus *Fusarium sp.* Hyperestrogenism is not uncommon in pigs fed on fusarium-infected feed as corn, barely or hay. Because of the high concentration of unconjugated equol shown in Figure 7, it would be interesting to conduct a more extensive study on the effects of phytoestrogens on reproduction in the pig.

Implication

As mentioned earlier, most of the phytoestrogens occur in the intact plant as glucosides, which are hydrolyzed in the rumen and further demethylated and reduced by the microorganisms (17). A very minor part of these hydrolyzed phytoestrogens is absorbed very quickly from the rumen, and reaches the blood circulation unconjugated. The bulk are, however, conjugated with glucuronic acid already in gastrointestinal epithelium. The substances remaining unconjugated when entering the blood circulation are mainly conjugated by the liver and perhaps also by other extrahepatic tissues as the kidney. The high conjugative activities of isoflavones in gastrointestinal epithelium from cattle and sheep implicate that the livers role as the predominantly detoxifying organ probably has been overestimated in ruminants. Detoxification by the means of conjugation in different tissues probably explains why no more than about 5% of the isoflavones are found in unconjugated form in blood plasma from sheep and cattle. The metabolite equol is excreted mainly via the urine, and 70% of the daily intake of formononetin was recovered as equol in urine from sheep fed on red clover (22).

According to Markiewicz *et al.* (35), the relative potency of equol is 0.061% of estradiol-17β. If we consider that during feeding with silage the concentrations of unconjugated equol in ovine plasma is about 20,000 pg/ml, the potency can be approximately 100 times higher than the activity of estradiol-17β during estrus (36).

The pig seems to differ markedly in comparison with ruminants regarding conjugation of equol. Only 50%–70% of total equol was found in conjugated form whereas the corresponding figure for conjugated equol in plasma from cow and sheep is 95%–99%. The sig-

nificance of the high plasma concentrations of unconjugated equol in pig reproduction is difficult to predict. Relatively high concentrations of total genistein were also found in plasma but whether the pig is sensitive to phytoestrogens or not is not known and further research is needed before any conclusions can be drawn about the effects of phytoestrogens and their metabolites on pig reproduction.

These investigations were supported by grants from the Swedish Council for Forestry and Agricultural Research, The Royal Swedish Academy of Sciences and the Futura foundation.

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