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Received for review December 23, 1986. Accepted July 22, 1987. The present study was funded through a Natural Sciences and Engineering Research Council of Canada Strategic grant held by T.A.G.

Demethylation and Conjugation of Formononetin and Daidzein in Sheep and Cow Liver Microsomes

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Cattle are considered to be less susceptible than sheep to plant estrogens. Therefore, this study was designed to evaluate possible differences in microsomal metabolism of formononetin and daidzein, two isoflavones responsible for estrogenic symptoms in ruminants. The demethylation of formononetin to daidzein was very low in both species. Conjugation, the major mechanism, showed only a slight difference in activity between cow and sheep. About 20% formononetin and 40% daidzein were removed by glucuronidation in cow liver compared to 30 and 50% in sheep liver. When the conjugation enzyme UDP-glucuronosyltransferase was activated, no significant differences occurred. However, the conjugation was augmented, and the ultimate activity in cow and sheep liver rose to the same final level. In addition, NADPH-cytochrome *c* reductase, 7-ethoxycoumarin deethylase, and α-naphthol UDP-glucuronosyltransferase were about 2-6 times higher in sheep liver compared to cow liver. With the two isoflavones, however, the differences in capacity of the liver to metabolize these substances are not sufficient to explain the observed differences in estrogen susceptibility between cow and sheep.

Plant estrogens are substances found in certain plants. Their effect is similar to that of estrogen hormones. The most common types are the isoflavones formononetin, daidzein, genistein, and biochanin A. Another group possessing estrogenic activity is the coumestans, the best known compound in this series being coumestrol.

High levels of the estrogenic isoflavones formononetin and biochanin A have been found in certain cultivators of subterranean (*Trifolium subterraneum*) and red clover (*Trifolium pratense*) (Shutt et al., 1967, 1968, 1970; Francis et al., 1967). Many reports, especially from Australia, have described reproductive abnormalities in ewes associated with the content of plant estrogens in clover pasture [see for example Bennets et al. (1946), Lightfoot (1974), and Adams and Nairn (1983)]. Infertility can occur in animals grazing estrogenic clover or given clover silage during the mating season (Morley et al., 1966; Thomson, 1975). A return to normal fertility usually occurs after the ewes revert to nonestrogenic pasturage (Morley et al., 1966). A longer pasture period can even result in permanent infertility (Turnbull et al., 1966). Formononetin is the major plant constituent responsible for reproductive dysfunction in sheep (Millington et al., 1964).

In contrast to the many reports of the deleterious effects of plant estrogens on sheep, their effect on cattle has not been extensively studied. It has been reported, however, that reproductive disturbances can occur in cattle grazing on estrogenic clover (Kallela et al., 1968, 1984; Adler and Trainin, 1960) and other herbage (Rankin, 1963; Thain, 1965). The influence of these plant estrogens has been shown to cause a high frequency of ovarian cysts (Kallela et al., 1984; Adler and Trainin, 1960; Thain, 1965) and increased uterine weight in ovariectomized heifers grazing on red clover (Kallela, 1968). Even abortions in late gestation have been reported and suggested to be associated with estrogenic pasturage (Rankin, 1963). However, it has been found that the effect of plant estrogens on cattle is generally weaker than on sheep (Lightfoot, 1974).

The metabolism of formononetin and biochanin A is qualitatively similar in sheep and cattle (Figure 1), but the circulating isoflavones and their metabolites seem to be more efficiently conjugated in cattle (Braden et al., 1971). It has been suggested that the differences in degradation and conjugation rate may explain the apparently lower susceptibility to plant estrogens of cattle vis-à-vis sheep (Braden et al., 1971).

Relatively high concentrations of isoflavones have been detected in Swedish pasturage (Pettersson and Kiessling, 1984). Silage of red clover has also been more frequently

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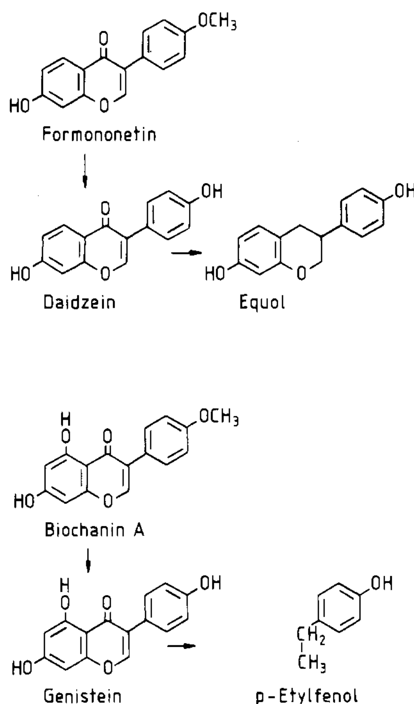

Figure 1. Major metabolic conversion route of formononetin and daidzein in cattle and sheep.

used as feed for dairy herds. It is therefore important to evaluate possible differences in order to explain why cattle are considered to be less susceptible than sheep to plant estrogens. Therefore, this study was designed to evaluate possible differences in demethylation and conjugation rate of formononetin and daidzein in ovine and bovine liver.

MATERIALS AND METHODS

Chemicals. Glucose 6-phosphate, glucose 6-phosphate dehydrogenase (EC 1.1.1.49), uridine 5′-diphospho-*N*-acetylglucosamine (UDPAG), uridine 5′-diphosphoglucuronic acid (UDPGA), nicotinamide adenine dinucleotide phosphate (NADPH), D-saccharic acid 1,4-lactone (S. lactone), Lubrol PX, β-glucuronidase (β-Glu) (EC 3.2.1.31.), α-naphthol, α-naphthyl-β-D-glucuronide, 7-ethoxycoumarin, and 7-hydroxycoumarin were obtained from Sigma Chemical Co. Daidzein (4,7-dihydroxyisoflavone) and formononetin (7-hydroxy-4-methoxyisoflavone) were obtained from ICN Pharmaceutical Inc., Life Science Group, Plainview, NY. All organic solvents were purchased from Merck.

Liver Samples. Liver samples from cows and sheep (ewes) were collected from randomly selected animals at the abattoir. No more than one liver was collected on any given day to ensure the groups were random and to reduce the influence of feeding background on the results. The livers were cut in small pieces and kept in an ice-cold buffer of sucrose (0.25 M), Tris-HCl (5 mM), and EDTA (1 mM), pH 7.4, during transport to the laboratory. No information was available about the age of the animals or about how long an interval elapsed between the time of slaughter and when the liver slices were chilled on ice. However, in all experiments, the livers were chilled within 30 min after the animal was killed. Such factors as mentioned above may explain differences in liver metabolism between individual animals. It is also well-known that diet affects the rate of metabolism in liver. Thus, differences in feeding during the days prior to slaughter may have contributed to the differences.

Microsome Preparation. About 10 g of liver was minced, washed with sucrose–Tris buffer, and homogenized in a Potter-Elvehjem homogenizer. The homogenate was adjusted to 10% (w/v) with buffer and centrifuged at 14000 *g* for 6 min. The pellet was discarded, and the supernatant fraction was centrifuged at 76000 *g* for 90 min. The resulting microsomal pellet was washed with sucrose–Tris buffer without EDTA (3 × 5 mL) and rehomogenized slightly. The microsome suspension, corresponding to 9 g of liver, was adjusted to 15 mL.

Incubations. The substrate concentration, 20.2 μg/incubation, was choosen to obtain about the same level as found in 100 mL of plasma from cattle fed with pasturage containing high levels of plant estrogens (Braden et al., 1971). The plant estrogens formononetin and daidzein were added to the tubes as an ethanol solution, and the solvent was allowed to evaporate. Then, 1.5 mL of 100 mM potassium phosphate buffer, pH 7.4, containing 8 mM magnesium chloride, was added, and the tubes were shaken for about 60 min at 37 °C in a water bath. Incubations with NADPH were carried out by adding a NADPH-generating system consisting of 25 mM NADPH, 1.5 mM glucose 6-phosphate, 0.6 mM nicotinamide, and 1 Kornberg unit of glucose 6-phosphate dehydrogenase. The other additions (listed in Table I) were 1 mM UDPGA, 600 units of β-glucuronidase, 0.25 mM UDPAG, 10 mM saccharic acid 1,4-lactone, and 0.01% Lubrol (w/v). The reaction was started by adding 0.2 mL of microsomal suspension, corresponding to 120 mg of liver, giving a total volume of 2 mL.

A zero sample, containing microsomes, buffer and substrate, was stopped at zero time by adding 7.5 mL of 95% ethanol. All other samples were stopped in the same way after 2 h.

Preparation of Extracts. The precipitate was spun down and extracted with 2 mL of 95% ethanol. The supernatants were pooled and evaporated to dryness in a rotavapor heated to maximum 60 °C. The pellet was dissolved in 3 mL of 0.4 M NaOH and extracted with 3 mL of benzene/petroleum ether (bp 40–60 °C) (1:1). The benzene/petroleum ether solution was then extracted with 3 mL of water. The water and sodium hydroxide fraction was pooled, extracted twice with 6 mL of ethyl acetate, and evaporated to dryness. The residue was taken up in 2 × 0.5 mL of methanol and transferred to a centrifuge tube. The evaporating flask was rinsed with 2 × 2 mL of water, the latter pooled with the methanol phase, and the volume was adjusted to 5 mL with water.

The methanol–water fractions were analyzed in principle as described by Pettersson and Kiessling (1984). Each sample fraction was injected through an activated Sep-Pak C 18 cartridge (Waters Associates). The tubes were washed with 2 × 0.5 mL of 20% methanol, which was added to the Sep-Pak. The isoflavones were eluted with 2 mL of 80% methanol in water.

Liquid Chromatography. Separation and quantification of formononetin and daidzein were performed by high-performance liquid chromatography as described by Pettersson and Kiessling (1984). The solvent system was 55% methanol in 10 mM sodium phosphate buffer, pH 6.5. The fluorescence was detected and measured with a Kratos FS-970 fluorimeter with excitation at 330 nm and an emission filter passing wavelength above 418 nm.

Enzyme Assays. The determination procedure for UDP-glucuronosyltransferase (EC 2.4.1.17) was that of Mackenzie and Hänninen (1980), but slightly modified. The reaction mixture in the cuvette contained (in 2 mL) 42.5 mM potassium phosphate buffer, pH 7.4; 3.4 mM $MgCl_2$; 50 μM α-naphthol (dissolved in 50% (v/v) dimethyl sulfoxide, final concentration in the cuvette being 0.001%); 0.012% w/v Lubrol; 0.5 mM UDPGA; and 50 μL of mi-

Table I. Metabolism 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
–	0.8 ± 1.0	1.3 ± 0.2	1.2 ± 1.6	1.0 ± 0.7
NADPH + β-Glu	1.1 ± 0.5	1.8 ± 1.2	0.0 ± 0.6	0.5 ± 1.0
UDPGA	3.7 ± 1.4	6.6 ± 0.8[b]	7.1 ± 0.9	10.0 ± 2.3[b]
UDPGA + β-Glu	0.6 ± 0.8	1.1 ± 1.0	0.4 ± 1.6	0.1 ± 0.8
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] $p < 0.05$.

crosomal suspension (approximately 4 mg of protein/mL). The formation of the glucuronide was continuously monitored at 30 °C in an Aminco Bowman spectrophotofluorimeter, with excitation at 293 nm and emission at 335 nm.

The O-dealkylation of 7-ethoxycoumarin was determined by the method of Prough et al. (1978). The formation of 7-hydroxycoumarin was measured at an excitation wavelength of 360 nm and emission wavelength of 460 nm.

NADPH-cytochrome *c* reductase (EC 1.6.2.4.) was determined according to Phillips and Langdon (1962).

Statistical Analysis. Statistical differences between sheep and cow were determined by using the Student's two-tailed *t*-test.

RESULTS

The metabolism of formononetin and daidzein has been studied by incubating microsomes from cow and sheep liver. The substrate disappearance was measured by the HPLC technique and is expressed as the difference between incubated and nonincubated samples (zero sample). The demethylating activity of formononetin was determined as formed daidzein. The recovery levels of formononetin and daidzein were 79.3 ± 6.9 and 79.2 ± 7.7%, respectively.

Metabolism of Formononetin. When NADPH was added to a microsomal suspension, only a very low demethylating activity of formononetin could be found. In incubations with cow liver, 0.45 ± 0.13 μg of daidzein was formed, and 0.76 ± 0.33 μg formed with sheep liver. The demethylating activity did not change even when higher concentrations of NADPH or glucose 6-phosphate were added.

According to Table I the major metabolic pathway of formononetin, in both cow and sheep liver, is conjugation. With UDPGA present, about 20% and 40%, respectively, more formononetin disappeared, compared with addition of NADPH. However, the highest conjugation activity was obtained when the effector UDPAG or the nonionic detergent Lubrol was added together with microsomes, UDPGA, and S. Lactone, an inhibitor of endogenous β-glucuronidase. In this case the conjugation rate was doubled when applied to cow liver, whereas only a slight increase was obtained with sheep liver. To confirm that formononetin was conjugated with UDP–glucuronic acid, a simultaneous incubation with β-glucuronidase was also done. In these cases almost all formononetin was recovered (Table I).

Metabolism of Daidzein. The metabolism of daidzein (Table I) shows the same pattern as with formononetin, but daidzein was more efficiently conjugated. However, the difference in conjugation between the two species was smaller with daidzein than with formononetin.

Table II. Activity of Three Microsomal Enzymes in Cow and Sheep Liver[a] **[Activity Expressed as nmol/(g of Liver) · min]**

enzyme	cow	sheep[c]
NADPH cytochrome *c* reductase	730.0 ± 306.2	1266.0 ± 247.3
7-ethoxycoumarin deethylase	11.8 ± 3.6	27.5 ± 8.6
UDP-glucuronosyl transferase	22.8 ± 22.8 (38.0 ± 13.1)[b]	221.2 ± 69.6
UDP-glucuronosyl transferase with Lubrol	86.0 ± 35.0	486.8 ± 106.4

[a] The figures are mean values ±SD of five experiments. [b] Two experiments gave zero activity. Figures within parentheses show mean values of the three remaining experiments. [c] $p < 0.05$.

Activity of Three Microsomal Enzymes Related to the Isoflavone Metabolism. In order to compare activities of enzymes involved in the drug-metabolizing system, NADPH-cytochrome *c* reductase, 7-ethoxycoumarin deethylase, and UDP-glucuronosyltransferase have been studied. The figures in Table II show that the activity of all three enzymes was greater in sheep liver than in cow. UDP-glucuronosyltransferase activity was particularly high, about 6–10-fold, depending on whether Lubrol was present or not. The activity of the other two enzymes was nearly twice as high in liver microsomes from sheep as from cow.

DISCUSSION

Degradation. Our results show that liver microsomes from both sheep and cow have very low demethylating activity when formononetin is used as substrate and the differences in activity between the two species are exceedingly small (Table I). Similar small differences in demethylating activity between the two species were observed by Nilsson (1963), though she obtained a higher total demethylating rate. The study with NADPH and liver microsomes shows that only 50% of the disappeared formononetin was converted to daidzein (0.45 and 0.76 μg). Moreover, it indicates that further metabolism of daidzein to equol does probably not occur, or is at most very slow in the liver (Table I).

The major metabolic degradation of isoflavones is more likely to be a result of microbial action in the rumen rather than in the liver. Conversion of formononetin and biochanin A has been demonstrated in vitro by Nilsson et al. (1967) and Nekby et al. (1985), using rumen fluid from sheep. Nekby et al. (1985) showed that the protozoa are more active than the bacteria. The bacterial fraction is mainly capable of demethylating formononetin and biochanin A and produces very little (if any) equol and *p*-ethylphenol, respectively. Equol, however, is formed both in the protozoal fraction and in intact rumen fluid.

Conjugation. Table I shows that both formononetin and daidzein were more efficiently conjugated by ovine liver than by bovine liver when only UDPGA was present. Furthermore, daidzein was more efficiently conjugated than formononetin, probably because daidzein has two free hydroxyl groups that can bind to UDPGA, while formononetin has only one.

When the effectors UDPAG and Lubrol were added in our experiments, the conjugation pattern changed; total activity increased, but no significant difference in final values between the two species was observed (Table I). Lubrol and UDPAG were about equally effective, which was not the case when steroids as estradiol, estrone, or testosterone were the substrates (Zakim and Vessey, 1976; Rao et al., 1977). This indicates that the isoflavones are

probably not glucuronidated by the same enzyme as estradiol.

The comparatively small species differences as regards conjugation rate were unexpected in view of the results presented in Table II, which shows that UDP-glucuronosyltransferase is at least 6 times more active in sheep than in cow. The only explanation so far may be that α-naphthol, used as substrate in Table II, has a different affinity to the enzymes in sheep and cow, whereas the isoflavones do not. Other enzymes in the drug-metabolizing system, NADPH cytochrome *c* reductase and 7-ethoxycoumarin deethylase (Table II), show smaller species differences in comparison with UDP-glucuronosyltransferase.

As was pointed out earlier (Materials and Methods), certain technical problems arise in experiments designed like this. Thus, dietary effects on the liver metabolism are very difficult to control. However, the number of randomly selected animals (5–8) in each group mitigates the feeding effect compared with individually fed animals. It is, however, obvious from our results that only minor differences exist between sheep and cow liver as regards metabolism of formononetin and that any such differences cannot account for the differences in sensitivity between the two species to the isoflavones studied. Therefore, further investigation must be carried out to elucidate why sheep are more sensitive to plant estrogens than are cattle. Above all, the conjugation rate in tissues other than liver, such as rumen wall and other regions of the digestive tract, will be further investigated.

Registry No. Formononetin, 485-72-3; daidzein, 486-66-8; NADPH-cytochrome *c* reductase, 9023-03-4; 7-ethoxycoumarin deethylase, 42613-26-3; UDP-glucuronosyltransferase, 9030-08-4.

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Received for review December 2, 1986. Revised manuscript received June 3, 1987. Accepted August 31, 1987. This investigation was supported by grants from the Swedish Council for Forestry and Agriculture Research, Dno. 580/83 D121:3.