Conjugation of the Plant Estrogens Formononetin and Daidzein and Their Metabolite Equol by Gastrointestinal Epithelium from Cattle and Sheep

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Conjugation of the plant estrogens formononetin and daidzein and their metabolite equol has been investigated in gastrointestinal epithelium of cattle and sheep to explain species differences in susceptibility to plant estrogens. Conjugative activity occurred in all tissues investigated except abomasum. In sheep, the activity was 3-20 times higher than in cattle in all epithelial tissues studied except small intestine, where bovine activity was twice as high. With 1-naphthol as a reference substrate, only minor differences in conjugation rate were found between sheep and cattle rumen epithelium. Furthermore, in sheep rumen wall, 1-naphthol was more efficiently conjugated than formononetin but was only half as efficient as equol. The results show that significant glucuronidation of plant estrogens, and probably other phenolic compounds, occurs in the gastrointestinal tract, which reduces the role of hepatic glucuronidation of ingested substances. The prevailing conception that sheep are more susceptible than cattle to plant estrogens was not explained by the results.

Some commonly grazed plants contain substances that induce estrus in immature animals or interfere with the normal reproductive processes (Price and Fenwick, 1985). Particular attention was paid to the effects that the isoflavonic plant estrogens (formononetin, daidzein, biochanin A, and genistein) had on reproduction when massive outbreaks of infertility were described in sheep that grazed on clover in Australia (Bennetts et al., 1946).

Formononetin is the major plant constituent responsible for reproductive dysfunction in sheep (Millington et al., 1964). This compound is metabolized by the microorganisms in rumen mainly to daidzein and further to the metabolite equol (Figure 1), which in turn produces the effect on estrus (Shutt and Braden, 1968).

Similar reproductive disturbances may also occur in cattle grazing on estrogenic clover (Kallela, 1968; Kallela et al., 1984; Thain, 1965) and on other estrogenic herbage (Adler and Trainin, 1960). However, the effect of plant estrogens in cattle is generally weaker than in sheep (Lightfoot, 1974; Austin et al., 1982).

That cattle are less susceptible to plant estrogens than are sheep has been suggested to be due to the ability of cattle to metabolize and, by conjugation, detoxify the plant estrogens and their metabolites more efficiently than sheep (Braden et al., 1971). However, in vitro studies with liver microsomes from sheep and cattle have shown that only minor differences exist between bovine and ovine metabolism of formononetin and daidzein and that such differences cannot account for differences in sensitivity between the two species (Lundh et al., 1988a). Some other tissue capable of metabolizing formononetin and daidzein could possibly be responsible for the differences in metabolism and sensitivity in cattle and sheep. One such tissue could be the gastrointestinal mucosa. Therefore, in the present study, the metabolism of formononetin and daidzein was investigated in preparations from the rumen wall and other parts of the gastrointestinal tract. For comparison, the conjugation rate of a standard substrate, 1-naphthol, was investigated.

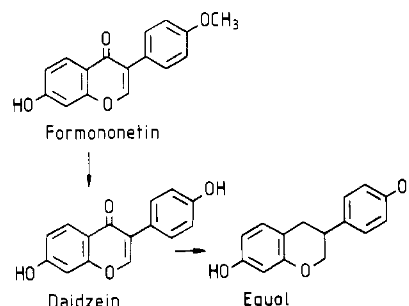

Figure 1. Major metabolic pathway of formononetin in cattle and sheep.

MATERIALS AND METHODS

Chemicals. Uridine 5′-diphosphoglucuronic acid (UDPGA), D-saccharic acid 1,4-lactone, Lubrol PX, β-glucuronidase, type IX (E.C. 3.2.1.31), 1-naphthol, and 1-naphthyl β-D-glucuronide were obtained from Sigma Chemical Co. Formononetin (7-hydroxy-4′-methoxyisoflavone) and daidzein (4′,7-dihydroxyisoflavone) were obtained from ICN Pharmaceutical Inc., Life Sciences Group, Plainview, NY. Equol (4,7-dihydroxyisoflavandiol) was donated by Prof. H. Adlercreutz, Helsinki, Finland. Organic solvents were purchased from Merck, Darmstadt, FRG, and the methanol used for HPLC analyses was obtained from May & Baker LTD, Dagenham, Essex, England.

Animals and Tissue Sampling. Tissue samples from mature female cattle and sheep were collected from randomly selected animals slaughtered commercially at the abattoir (Farmek, Uppsala). The samples were collected in the autumn at the end of the grazing period. Such animals generally come directly from natural pasture, which for sheep and cattle in Sweden consists of cutover pasture, meadowland, and, to a certain degree, the regrowth of the last ley. Farmers, however, generally try to minimize grazing on pasture with an abundance of red clover by sheep, as sheep show estrogenic symptoms more easily than cattle. A risk may therefore exist that cattle graze pasture containing plant estrogens with an ensuing induction of a conjugative activity to a greater extent than sheep. To mitigate the risk that all animals within the same group (cattle or sheep) had been fed on the same forage and to ensure that the groups were made up randomly, tissue samples from no more than one

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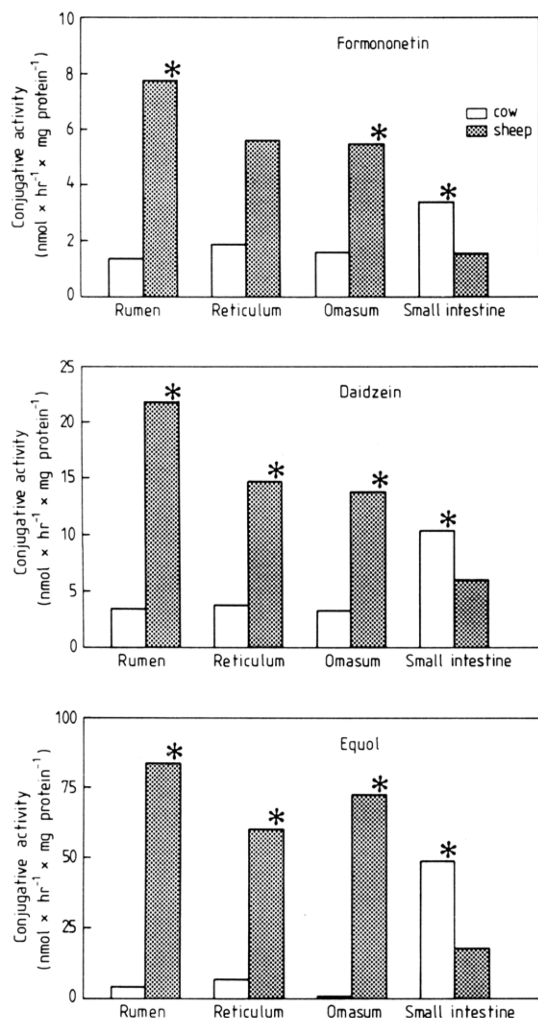

Figure 2. Conjugative activity toward formononetin, daidzein, and equol in various gastrointestinal tissue homogenates from cattle and sheep. The columns represent mean values of duplicates from three animals of each species. The columns marked with an asterisk are significantly greater (Mann–Whitney U test) than for the corresponding species ($P < 0.05$).

animal were collected on any given day. All animals were inspected preslaughter and judged to be healthy by a licensed veterinarian, and their carcasses passed examination by a licensed meat inspector.

Samples of the rumen (ca. 10 cm^2) were excised from the cranial sac (just below the reticulum), the whole reticulum and about 3–4 of the lamellae in the omasum were collected, and a section of the intestine (ca. 20 cm) was excised about 2 m down from the pylorus. The samples were rinsed with phosphate-buffered saline (PBS), pH 7.4, and kept in ice-cold PBS during transport to the laboratory.

Preparation of Tissue Homogenates. Papillae from the rumen were cut off with scissors; epithelium from the reticulum and omasum was scraped off with a surgical blade. The tissue samples were placed in an ice-cold buffer of sucrose (0.25 M), Tris-HCl (5 mM), and EDTA (1 mM), pH 7.4, and were first homogenized in an Ultra Turrax (Janke & Kunkel KG, IKA Werk, Staufen i. Breisgau) and then in a Potter-Elvehjem homogenizer. The intestinal mucosa was gently scraped off, almost in the same way as epithelium from the reticulum and the omasum, but was only homogenized with the Potter-Elvehjem homogenizer. The homogenate was adjusted to 20% (w/v) with sucrose–Tris buffer and was kept at −80 °C until analysis.

The conjugative activity against plant estrogens was measured by incubating duplicates of 0.5-mL aliquots of tissue homogenate (10% (w/v) for formononetin and daidzein and 2.5% (w/v) for equol analysis) together with 100 μL of substrate (200 μg/mL formononetin, daidzein, and equol), 100 μL of 20 mM UDPGA, and 100 μL of 40 mg/mL saccaric acid lactone (a β-glucuronidase inhibitor). In some incubations, 100 μL of β-glucuronidase (6000 U/mL) was incubated to confirm that the disappearance of the substrates really was the result of conjugation to UDPGA. The incubation volume was adjusted to 2 mL with 100 mM potassium phosphate buffer (pH 7.0) containing 8 mM magnesium chloride.

A zero sample, containing homogenate, 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 1 h. The tubes were sonicated in an ultrasound bath (Sonorex RK 100H, Bandelin Electronic, West Berlin) for 15 min, and the precipitates were spun down (1600g for 10 min). The ethanol fractions were transferred by Pasteur pipet to 100-mL round-bottom flasks. The pellets were washed with 5 mL of ethanol (95%), sonicated, and centrifuged as described above, and the ethanol was transferred to the same flasks as the first ethanol fraction. The two pooled fractions were evaporated with a rotavapor heated to a maximum of 60 °C. The nearly dry residue was taken up in 2 × 0.5 mL of ethanol and transferred to a centrifuge tube. The evaporating flask was rinsed with 2 × 2 mL of water, the latter was pooled with the ethanol phase, the sample was acidified by adding 100 μL of 1 M HCl, and the volume was adjusted to 5 mL with water.

The ethanol–water fractions were analyzed as described by Lundh et al. (1988a) and Pettersson and Kiessling (1984).

Liquid Chromatography. Following the incubation, the analysis of formononetin, daidzein, and equol was performed by high-performance liquid chromatography (HPLC) as described by Lundh et al. (1988a), except that UV detection at 280 nm (Waters LC spectrophotometer 481) was used in addition to fluorescence detection. The peak heights were calculated with a Spectra-Physics SP490 integrator.

Determination of 1-Naphthol Glucuronidation. The determination of uridine diphosphoglucuronosyltransferase (E.C. 2.1.1.17) using 1-naphthol as substrate was performed as previously described (Lundh et al., 1988a) (slightly modified from Mackenzie and Hänninen, 1980) except that 100 μL of the supernatant from a homogenate centrifugated at 400g for 2 min was used instead of microsomes.

Protein. Protein was analyzed according to Lowry (Lowry et al., 1951).

Statistical Analysis. Statistical differences between the two species were determined by using the Mann–Whitney U test (Siegel, 1956).

RESULTS

In a pilot study, we examined the conjugation activity toward the plant estrogens formononetin and daidzein and their metabolite equol in epithelial tissues from the four compartments of the complex stomach and the small intestine of cattle and sheep. Since no activity was found in the abomasum, this line was not investigated further. We also examined the conjugative activity in two different parts of the rumen, ventral and cranial, but found no differences in activity between the two parts (data not shown). The cranial part was chosen for further experiments because it was easier to locate a defined area for repeated sampling.

The conjugative activity in the epithelia from the rumen, reticulum, omasum, and small intestine of cattle and sheep is illustrated in Figure 2, expressed as the difference between tissue homogenates with and without UDPGA added. The results show that there is an evident conjugation capacity in epithelial tissue from rumen, reticulum, omasum, and small intestine. That in sheep was about 3–20-fold greater than in cattle in all parts of the gastrointestinal tract investigated, except in the intestinal mucosa, where the converse relationship was found, i.e., about twice as high in cattle as in sheep. The variation in activity (3–20) between the two species depended on which substrate was used, formononetin, daidzein, or equol (Figure 2).

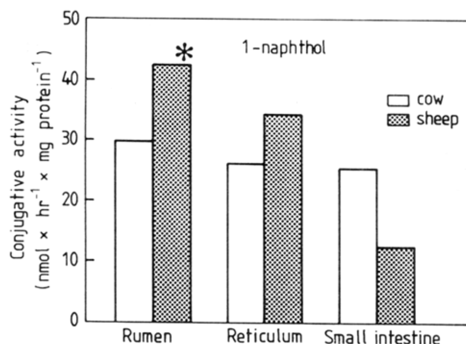

Figure 3. Conjugative activity toward 1-naphthol in some gastrointestinal tissue homogenates from cattle and sheep. The columns represent mean values from five experiments, except for sheep reticulum ($n = 4$) and cattle intestine ($n = 3$). The column marked with an asterisk is significantly greater (Mann–Whitney U test) than for the corresponding species ($P < 0.05$).

The conjugation rate also showed an appreciable variation within the different tissues, depending on which substrate was used. In the sheep homogenates, daidzein and equol were about 3 and 10 times more efficiently conjugated than formononetin.

That the plant estrogens were conjugated with UDPGA was confirmed by simultaneous incubations with β-glucuronidase. In these cases about 100% of the substrates was recovered (in free form). Recovery from the extraction procedure was also checked, the levels of formononetin, daidzein, and equol being 90.5 ± 4.2%, 99.0 ± 2.7%, and 95.5 ± 4.7%, respectively.

In order to compare the uridine diphosphoglucuronosyltransferase activity in sheep vs cattle, a standard substrate, 1-naphthol, was employed. With this substrate there were only minor differences in conjugation activity in rumen between cattle and sheep and no significant differences in reticulum and small intestine (Figure 3). Homogenates from five different animals were investigated. Some samples demonstrated no activity and are not included in Figure 3.

DISCUSSION

The results obtained when using plant estrogens as substrates show that sheep had a greater conjugative activity than cattle in almost all parts of the gastrointestinal tract studied. The exception was the small intestine, where activity in the cattle was about twice as high as in sheep. The conjugation rates varied remarkably, depending on which of the three estrogenic substrates was used, although the differences in chemical structure between parent compounds and the metabolites are small (Figure 1).

Equol was most efficiently conjugated, which is unexpected as it is the major metabolite responsible for reproductive dysfunctions in sheep (Shutt and Braden, 1968). The cause of the difference in conjugation rate between formononetin and daidzein is probably that daidzein has two hydroxyl groups that can bind to UDPGA, while formononetin has only one. Why equol is more efficiently conjugated than daidzein is more difficult to explain, since the only structural difference between these two substances is a keto group (Figure 1). It is well-known that uridine diphosphoglucuronosyltransferase can occur in multiple forms. Whether the higher conjugation rate of equol vis-à-vis daidzein is due to a different isoenzyme or merely to steric hindrance by the keto group is impossible to say without further investigation.

Even though conjugation rate is almost the same in rumen, reticulum, and omasum (Figure 2), the rumen is probably the most important tissue in the gastrointestinal tract as regards detoxication of plant estrogens. The ingested food stays in the rumen for a relatively long period (1–3 days, depending upon the nature of the diet and on the animal species), and the plant estrogens are metabolized mainly by the microorganisms in the rumen by demethylation and reduction. This leads to a change in the toxicity/activity of these substances (Nilsson et al., 1967; Nekby, 1985), including an increased disposition for conjugation. Furthermore, the absorption of the bulk of the plant estrogens probably occurs in the rumen (Cox and Braden, 1974). Therefore, the high bovine conjugative activity in the small intestine compared with the rumen and with other investigated parts of the gastrointestinal tract was unexpected. However, sheep chew their feed more thoroughly than cattle, which should result in a more rapid solubilization of the isoflavones. Furthermore, liquid retention time in the rumen is longer for sheep than cattle. Therefore, the rumen should be the major site of detoxification in sheep. In comparison to sheep, solubilization of isoflavones will be slower in cattle, but once solubilized they leave the rumen faster via outflow to the lower tract. This suggests that cattle, more than sheep, might depend on intestinal conjugation. On the other hand, we recently found in a feeding experiment that the maximum concentration of formononetin and daidzein in blood was reached within 1 h after feeding (Lundh, unpublished results). These findings probably reduce the value of the high bovine conjugation rate in the small intestine as a part of a defense mechanism against plant estrogens.

We have shown in a previous investigation (Lundh et al., 1988a) that the demethylation of formononetin is very slow in the liver and that further reduction to equol does not occur, which also has been suggested by Braden et al. (1967) and Batterham et al. (1965). Therefore, the major degradation of isoflavones is very likely to be a result of microbial action in the rumen and only to a very minor part of a corresponding degradation in the liver. Most of the isoflavones and their metabolites in blood from cattle and sheep are found as conjugates (Lundh et al., 1988b; Shutt et al., 1967; Braden et al., 1971) and the bulk of the conjugated fraction is found as glucuronides (Shutt et al., 1967). This indicates that conjugation with glucuronic acid is the major detoxification mechanism for these substances.

The liver is generally regarded as the major organ of glucuronidation of foreign substances, including plant estrogens and their metabolites (Shutt et al., 1967; Cox et al., 1984). In most investigations of enzymes involved in detoxification of foreign substances, the liver shows higher specific enzyme activities than other tissues. Therefore the detoxification by organs other than the liver has probably been underestimated. However, in recent years, interest in extrahepatic metabolism of foreign compounds has increased (for a review, see Burke and Orrenius, 1982; Vainio and Hietanen, 1980), and special attention has been directed to the contribution of the gut and the intestinal microorganisms in metabolism of dietary substances and xenobiotics (Rozman and Hänninen, 1986; Kiessling, 1989).

There are numerous reports of xenobiotic metabolism by laboratory animals but only a few studies on the xenobiotic metabolism in livestock (for example, Smith et al., 1984, 1985). Smith and Watkins (1984) and Watkins et al. (1987) have shown that a considerable amount of biotransformation, phase I and phase II, occurs not only in the liver but also in the kidney, gut, and rumen tissue. These workers also showed that there is an appre-

ciable variation in conjugation rate, depending on which substrates were used. Thus, the uridine diphosphoglucuronosyltransferase activity in bovine ileum varied between 2 and 110% of the hepatic rates, depending on aglycon. The substrate-dependent variation in conjugation rate also seems to vary, depending on the animal species investigated. Thus, in the present study, 1-naphthol was about 6-fold more efficiently conjugated than formononetin but was only half as active as equol in tissues from sheep rumen. In cattle, the corresponding figures were about 20- and 8-fold (Figures 2 and 3).

Our results, using plant estrogens and 1-naphthol as substrate, reveal a considerable conjugative activity in the gastrointestinal tract that cannot be ignored. Smith and Watkins (1984) and Watkins et al. (1987) have reported similar results on the conjugative activity against 1-naphthol in the rumen wall of sheep. In contrast to our results, they found a higher activity in ileum from sheep compared with cattle. In different parts of the small intestine, however, the metabolizing capacity can vary (Olsen et al., 1987). Our samples were taken from the upper part of the small intestine, in contrast to Smith and Watkins (1984), who used ileum, which may explain the differing results.

Our working hypothesis was that sheep should have a lower conjugative capacity against plant estrogens than cattle as they may develop estrogenic symptoms more easily. However, the present results demonstrate that the crucial sheep tissues have much higher conjugative capacity than the respective cattle tissues. There may arise an uncertainty as to the possible effect on conjugation capacity of different feeding patterns prior to sampling as discussed previously. Such differences should, however, not compromise the experimental results. Estrogen-rich food would, if anything, increase the enzymatic activity through induction more in cattle than sheep. Without this hypothetical induction the differences found could have been more pronounced.

In conclusion, the experiments reported demonstrate that plant estrogens—and probably also other phenolic substances—are conjugated with UDPGA already in the gastrointestinal tract, which in this way act as a first line of defense before the substances enter the blood circulation. However, the differences between cattle and sheep as regards conjugative activity in different parts of the gastrointestinal tract cannot explain why cattle have been reported to be less susceptible than sheep to plant estrogens. In vivo experiments with cattle and sheep fed estrogenic fodder may therefore reveal hitherto undiscovered metabolic differences. Furthermore, differences in receptor site susceptibility between cattle and sheep may be another explanation, which is now being investigated.

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Registry No. Formononetin, 485-72-3; daidzein, 486-66-8; equol, 531-95-3; 1-naphthol, 90-15-3.

Protein Digestibility of Alkali- and Fructose-Treated Protein by Rat True Digestibility Assay and by the Immobilized Digestive Enzyme Assay System[†]

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The effect of processing on the digestibility of various food proteins was examined by using the immobilized digestive enzyme assay (IDEA) system. The values obtained were compared to true digestibilities determined by rat bioassay. Sodium caseinate, egg white, soy protein, and whey were treated with either 0.2 N NaOH at 40 °C for 6 h or 0.5 M fructose (pH 7.0) at 90 °C for 4 h. Untreated proteins were also analyzed. Treatment of all samples ($n = 12$) with 4 M urea assured solubility. Regression analysis of data for all samples resulted in a correlation coefficient (r) of 0.83 ($p < 0.001$). The IDEA systems appears to be an accurate and reliable estimate of in vivo digestibilities. Furthermore, it offers a more rapid and less expensive alternative to animal bioassays.

The immobilized digestive enzyme assay (IDEA) system has been used as an in vitro measure of protein digestibility (Porter et al., 1984) and in detection of decreases in protein digestibility due to protein modifications (Chung et al., 1986). Porter et al. (1984) reported that the IDEA system gave digestibilities in agreement with FAO and literature values for a number of plant and animal proteins. In addition, it has been shown that loss of digestibility was correlated to degree of racemization, to lysinoalanine formation, and to loss of available lysine, indicating that the system is sensitive to protein modification resulting from alkali treatment or Malliard reactions (Chung et al., 1986).

In a cooperative study on assessment of protein nutritive value (Bodwell et al., 1989), 17 commonly consumed foods were analyzed for various parameters of protein quality with several in vivo and in vitro measurements. As part of that study, Thresher et al. (1989) using the IDEA system compared the protein digestibility of the foods to two independent in vivo estimates of digestibility (Eggum et al., 1989; Sarwar et al., 1989). Although good agreement was seen between digestibilities for some foods, true comparisons were hampered by the lack of protein solubility.

These observations suggested that the IDEA system might represent a rapid, facile and inexpensive predictor of protein digestibility compared to in vivo methods where complete protein solubility could be achieved. This report gives the results of those comparisons.

EXPERIMENTAL SECTION

Materials. Porcine pepsin, trypsin, chymotrypsin, and intestinal peptidases were obtained from Sigma Chemical Co. The intestinal peptidase was further purified by the method of Porter et al. (1984). Sodium caseinate, egg white, and soybean protein were obtained from U.S. Biochemicals. Whey protein (Vari-Dairy Plus) was obtained from Nutrisearch. Succinic anhydride, trimethylamine, 1-ethyl-3-[3-(dimethylamino)-propyl]carbodiimide (EDC), fructose, benzoyl-L-tyrosine ethyl ester, (BTEE), (p-toluenesulfonyl)-L-arginine methyl ester (TAME), L-leucylglycine, urea, and controlled-pore glass beads

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