Comparative Levels of Free and Conjugated Plant Estrogens in Blood Plasma of Sheep and Cattle Fed Estrogenic Silage†

Torbjörn J.-O. Lundh,*,‡ Hans I. Pettersson,‡ and Kjell A. Martinsson§

Department of Animal Nutrition and Management, Swedish University of Agricultural Sciences, Box 7024, S-750 07 Uppsala, Sweden, and Experimental Division of North Swedish Animal Husbandry, Northern District of Animal Husbandry Experiments, Swedish University of Agricultural Sciences, Rödbäcksdalen, Box 5097, S-900 05 Umeå, Sweden

Plasma concentrations of the plant estrogens formononetin, daidzein, and their metabolite equol were determined in blood samples from dairy cattle and sheep (wethers), fed on red clover/grass silage. Blood samples were taken before and at different times after feeding. The concentrations of total (free and conjugated) and free formononetin and daidzein were almost similar in the cattle and the sheep. The plasma level of total equol was lower in sheep than in cattle at the beginning of the feeding period, but reached the same level as in the cattle after about 2–3 h. The concentration of free equol was about 10 times higher in the bovine than in the ovine plasma during the whole experimental time. The results suggest that the differences in sensitivity, described in the literature, between cattle and sheep to plant estrogens are certainly not caused by differences in their capacity to detoxify the estrogenic compounds.

INTRODUCTION

The ingestion of clover pasture, containing high levels of plant estrogens, causes infertility in sheep (Bennetts et al., 1946). The main estrogenic compound in red and subterranean clovers is the isoflavone formononetin (7-hydroxy-4′-methoxyisoflavone) which is indirectly responsible for this reproductive dysfunction (Millington et al., 1964). Formononetin is metabolized by the rumen microorganisms mainly to daidzein (7-hydroxyisoflavone) and further to the isoflavan equol (7,4′-dihydroxyisoflavandiol), which causes the effect on estrus (Shutt and Braden, 1968). This has been extensively studied in sheep, but very little is known about corresponding effects in cattle. It has, however, been suggested by Braden et al. (1971) that the metabolism of the isoflavonic estrogens formononetin and biochanin A (5,7-dihydroxy-4′-methoxyisoflavone) proceeds along the same route in sheep and cattle (Figure 1). Even so, cattle seem to be less sensitive to plant estrogens than sheep, since cattle do not become permanently infertile as a result of clover diets (Lightfoot, 1974). However, Kallela (1968) has described changes in the genital tract of ovariectomized heifers grazing on red clover that are similar to those in sheep after the sheep have grazed on clover pasture. Also, other results indicate that plant estrogens in red clover and other herbage may cause an infertile syndrome in cattle (Kallela, 1984; Thain, 1965; Adler and Trainin, 1960; Rankin, 1963).

The differences in sensitivity to plant estrogens between cattle and sheep have been suggested to be due to a more efficient metabolism and detoxification of formononetin and the metabolites in cattle than in sheep (Braden et al., 1971). Furthermore, it has been suggested that the major detoxifying mechanism of the estrogenic substances should consist of conjugation to glucuronic acid by the liver (Shutt et al., 1967; Cox et al., 1984).

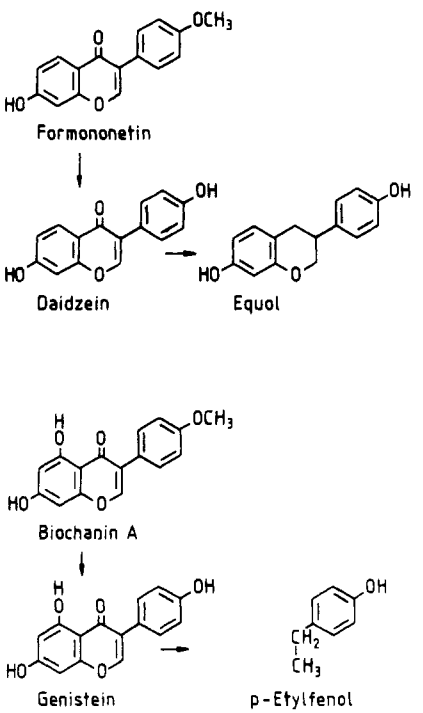

Figure 1. Major metabolic conversion route of formononetin and biochanin A in sheep and cattle.

Dairy cattle are very often fed on high ratios of silage. The admixture of large shares of red clover in the silage has become more frequent in Sweden and involves a very high ingestion of plant estrogens by cattle. The questions now arise if dairy cattle tolerate such high concentrations of plant estrogens without estrogenic effects and in which way these plant estrogens act differently in cattle and sheep. Previous studies in our laboratory have dealt with the metabolism of the estrogens in the liver (Lundh et al.,

† This investigation was supported by grants from the Royal Swedish Academy of Agriculture and Forestry and from the Swedish Council for Forestry and Agriculture Research.

* Address correspondence to this author.

‡ Swedish University of Agricultural Sciences, Uppsala.

§ Swedish University of Agricultural Sciences, Umeå.

0021-8561/90/1438-1530$02.50/0

1988a) and in the gastrointestinal epithelium from the two species (Lundh, 1990). In neither of these studies, however, could the results explain the differences in susceptibility.

We must, nevertheless, be aware that factors other than those of a purely metabolic nature, e.g., absorption and excretion, may contribute to dynamic conditions which cannot be predictable from the metabolism of the estrogens by isolated tissues. These conditions should, however, be reflected in the levels of the various estrogens and their metabolites in the blood system of the living animal. The present study was, therefore, designed so that the plasma levels of the estrogenic compounds in dairy cows and wethers fed the same estrogenic silage could be compared.

MATERIALS AND METHODS

Animals. Five 2–4-year-old dairy cows, Swedish red and white breed (mean liveweight 512–578 kg), were fed on silage, containing 50% grass and 50% red clover, for about 4 months (as a part of another study at the Röbäcksdalen experimental station, Swedish Unversity of Agricultural Sciences). During the first 3 months the cows were fed with an ad libitum supply of silage but during the last 4 weeks prior to blood sampling they were pen-fed twice a day with 12.5–14 kg of silage (daily intake 25–28 kg). The cows were also offered 1 kg of hay (timothy), 1 kg of dried molassed beet pulp, and 6.4–10.4 kg of concentrate mixture (83% grain, 11% soya meal, and 6% rape seed meal) per day.

Five 1–2-year-old wethers, predominantly fine wool (mean liveweight 58–80 kg), were pen-fed once a day with 5.2–6.6 kg of the same silage as offered to the cows during 10 days prior to blood sampling. To compare the plasma concentrations of the plant estrogens in sheep with those in cattle, the wethers were offered the same amount of silage as the cattle with consideration to the basal metabolic rates, which represents 0.247 kg of silage/liveweight$^{0.75}$.

The animals were fed in a similar way on the day for blood sampling as during the prefeeding period. The cattle were offered their first half of the daily feed ration at 7 a.m. and the other half at about 2 p.m. The last blood samples were taken about 20 min after the cows had been offered their second half of the feed rations.

The wethers were offered their daily silage ratio at about 8 a.m. and consumed about 50% of the silage within 3 h and finished their feed intake after 6–7 h.

Sample Collection. Jugular blood samples were collected in two 10-mL heparinized vacutainer tubes about 30 min before the feed was offered (which also represents about 14–16 h after the last feed intake during the previous day) and 1, 2, 3, 5, and 7 h after feeding. The red blood cells were centrifuged at 4 °C, and the plasma was drawn off and kept frozen at −20 °C until analyses.

Analytical Methods. The contents of plant estrogens in silage, hay, and concentrate mixture were determined by high-pressure liquid chromatography (HPLC) as described by Pettersson and Kiessling (1984). Finely chopped or ground samples were extracted with a mixture of ethanol and HCl which was heated to boiling. The mixture was filtered and cleaned up by injection through an activated Sep-Pak C18 cartridge (Waters Associates). The plant estrogens were separated on a μBondapak C18 column (Waters Associates) with a mobile phase consisting of 58% methanol in 10 mM sodium phosphate buffer, pH 6.5, and quantified by UV and fluorescence response.

The determination of free and conjugated plant estrogens and their metabolites (formononetin, daidzein, and equol) in blood plasma was carried out by the method of Lundh et al. (1988b), which is briefly described below. Blood plasma was diluted with acetate buffer, pH 5.5, and β-glucuronidase was added for determination of total (free and conjugated) compounds. β-Glucuronidase was omitted in samples where only the free plant estrogens were determined. The samples were incubated at 37 °C overnight and extracted with ethyl acetate and further with petroleum ether/benzene. After evaporation of the organic solvent, the residue was dissolved in 80% methanol and injected onto a μBondapak C18 column. The plant estrogens were eluated from the column by a linear gradient between 40% and 80% methanol for 30 min and quantified by UV and fluorescence response.

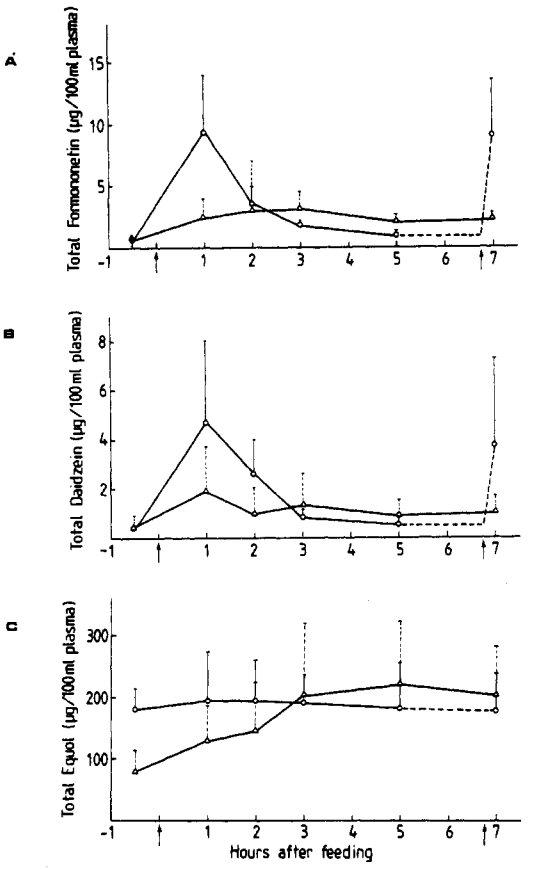

Figure 2. Total amount (free and conjugated) of the plant estrogens (A) formononetin, (B) daidzein, and their metabolite (C) equol in bovine (○) and ovine (△) blood plasma at different times after feeding on red clover/grass silage. Data represent mean ± SD from five animals. The dotted bars represent SD for the wethers, and the unbroken bars are SD for the cows. The first arrow (↑) indicates when the feed was offered to the cows and wethers. The curve that represents bovine plasma is dotted between 5 and 7 h since the cows were offered the other half of their daily feed ratio just before the last blood sample, which is indicated by the second arrow in each figure.

Statistical Analysis. Differences in plant estrogen plasma concentrations between the two species were analyzed by Student's *t*-test (Bailey, 1984).

RESULTS

The contents of isoflavones in the silage were determined by a HPLC technique to 530 mg of formononetin, 12.6 mg of daidzein, 364 mg of biochanin A, and 37.1 mg of genistein per kilogram wet weight, but no coumestrol was detected. That led to a daily intake of 13.8–14.8 g of formononetin and 0.32–0.35 g of daidzein for the cows and 2.7–3.5 and 0.06–0.08 g per day for the wethers. No detectable amounts of estrogens were found in the concentrate mixture given to the cows.

Total Amount of Plant Estrogens. Analyses of the isoflavones formononetin, daidzein, and their metabolite equol were performed in blood plasma since equol has been suggested to be the compound responsible for the reproductive dysfunctions in sheep grazing on clover-rich pasture. The total amounts (free and conjugated) of the plant estrogens formononetin, daidzein, and their metabolite equol found in bovine and ovine blood plasma at different times after feeding are shown in Figure 2. Formononetin and daidzein were absorbed very rapidly in

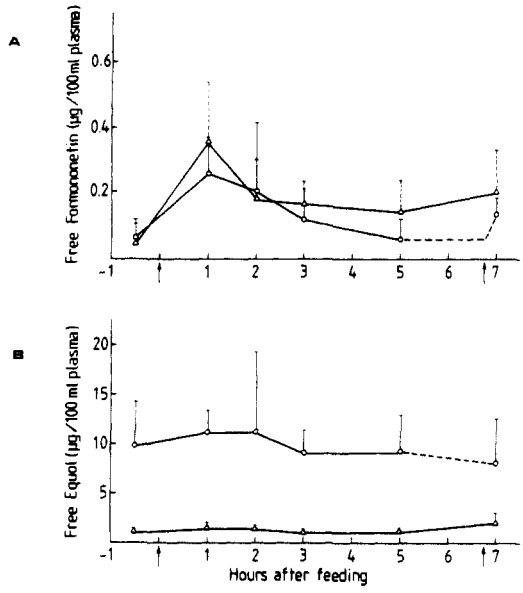

Figure 3. Free amount of (A) formononetin and (B) equol in bovine (O) and ovine (Δ) blood plasma at different times after feeding on red clover/grass silage. Explanations of the different signs are given in Figure 2.

cattle and reached the maximum level within 1 h after food intake. At this time, the plasma level was about 3 times higher in the cows than in the wethers and declined to about the same value as in the wethers after about 2 h. The high value after 7 h in bovine plasma depends on the cows being accidentally fed with their other half of the daily silage ration about 15–20 min before the last blood samples were taken. However, this result confirms that a very rapid demethylation and absorption of formononetin occur already in the rumen.

The concentration of equol in bovine plasma at different times after feeding revealed no absorption peak as for the parent compounds. The plasma level showed almost the same value at the beginning of the sampling time (which represents about 14–16 h after the latest food intake) as after 7 h and was about 2 times higher in the cows ($P < 0.05$) than in sheep at the beginning of the sampling time. In sheep the equol concentration increased successively and reached almost the same level as in the cows after about 3 h.

The results demonstrate very high individual variations in plasma levels. A part of these variations in plasma level can be derived from different feeding rates. All of the cows except one had eaten their feed rations within 1 h, which could be seen in individual plots of plasma estrogen levels (not shown). The wether's eating period was more lengthy compared with the cows and showed almost the same pattern of plasma estrogen levels versus feeding time.

Free Amount of Plant Estrogens. Very low amounts of unconjugated formononetin, only about 3–10% of the total amount, were found, and no significant differences were present between the two species (Figure 3A). The plasma level of free daidzein was found in even lower concentration than formononetin and varied between undetectable amounts and about 0.05 µg/100 mL plasma.

The plasma level of free equol showed almost the same pattern as for the total amount, and there were no pronounced peaks or depressions with time. The free amount constitutes about 5% of the total amounts in cows and about 1% in sheep. The cows demonstrate, however, about 10 times more ($P < 0.05$) free equol in plasma than the wethers. The results are shown in Figure 3B.

DISCUSSION

The plant estrogens formononetin and biochanin A, which can be found in high concentrations in red clover pasture, are demethylated and reduced by the microorganisms in sheep rumen (Nilsson et al., 1967; Nekby, 1985). This metabolic degradation of plant estrogens has been shown to occur in both sheep and cattle. The same metabolites were found in cattle and sheep fed red clover, and equol was the major metabolite in plasma. This suggests that degradation of clover isoflavones in cow is similar to that in sheep (Shutt, 1976).

In previous studies we have tried to find an explanation of the differences in sensitivity between the two species. These in vitro studies (Lundh et al., 1988a; Lundh, 1990), however, provided no satisfactory explanation. The purpose of the present study was therefore to get a clear view of the plasma concentrations of the free and conjugated plant estrogens that may affect the estrogen-sensitive tissues in sheep. To achieve a realistic feeding situation, we used dairy cows that were fed a moderately estrogenic silage for about 4 months. Since the cows used in this study represented a livestock of high milk production animals, they needed a supplement to the silage to cover their nutritional requirements. This was given as a concentrate mixture, beet pulp and hay (see Materials and Methods). The silage ratio (25 kg, 50% red clover and 50% grass) represented a feed ratio often used for dairy cows in Sweden. The wether's daily intake of silage was calculated with consideration to their body weight (silage/body weight$^{0.75}$) so as to be comparable with that for the cows. This implied a daily mean intake of 3.2 g of formononetin per day and wether, which is almost the daily intake reported to adversely affect fertility in sheep (Thomson, 1975).

Conjugation is probably one of the most important mechanisms in most animal species to detoxify different foreign substances ingested, including plant estrogens. The results in this investigation establish the importance of this mechanism and show that conjugates represent more than 90% of the circulating isoflavones and their metabolite equol, which is in accordance with other investigations [see, for example, Shutt et al. (1967, 1968) and Lundh et al. (1988b)]. Furthermore, our results indicate that sheep and cattle are almost equally effective in conjugating the isoflavones formononetin and daidzein (Figure 2A,B). The cows, however, have a more rapid initial conjugation capacity with a maximum of these conjugated plant estrogens in plasma within 1 h after the forage was offered (Figure 2A,B). In the wethers, there is a less pronounced increase of the substances in plasma compared with the cows. This may partly depend on a slower feed consumption rate. The reason for this conclusion is that plasma levels similar to those in sheep were seen in one cow which had eaten its food more slowly (50% after 3 h) compared with the other cows, which had finished their feed ration within 1 h. Another difference in the feeding between the cows and wethers was that the wethers were offered their daily silage ration only once a day (in the morning at 8 a.m.), just after the first blood sample was taken. The cows, on the other hand, were fed twice a day with half their daily silage ration in the morning (7 a.m., after milking time) and the other half in the afternoon, 7 h later (2 p.m.). Such differences in feeding may affect the absorption and excretion rate to some degree and explain why the levels of formononetin and daidzein fluctuate less in ovine plasma than in bovine plasma.

The total amount (free and conjugated) of equol in the cows' plasma (Figure 2C) was almost the same at each

sampling. In the sheep, on the other hand, the initial value was only half of that in cows but rose to about the same level after 2–3 h. This difference at the beginning of the sampling time, which occurred about 14–16 h after the latest ingested feed (see Materials and Methods) reveals basic differences between the two species with regard to the elimination rate of equol. Even if the concentration of equol is almost the same in cows and sheep some hours after feeding, the plasma level in the cows never drops below 180 μg/100 mL plasma. The sheep are exposed to the same high amounts of equol as the cows, but only for a much shorter time, which indicates a faster clearance of equol in the sheep. In spite of this, the equol level is probably high enough to induce estrogenic symptoms in the sheep (Braden et al., 1971; Shutt et al., 1968). Another unexpected result is that the amount of unconjugated equol was about 10 times higher in bovine than in ovine plasma (Figure 3B), whereas no significant differences in plasma levels of unconjugated formononetin (Figure 3A) and daidzein occurred.

According to Shutt et al. (1968), equol is the main substance responsible for reproductive dysfunctions in sheep, and the prevailing opinion is that the biologically active forms consist of unconjugated and, to a certain degree, sulfoconjugated substances. In light of this, and from the results pesented in this study, the sheep seem to be better equipped than cows, or at least equally equipped, to detoxify and eliminate the plant estrogens. The explanation of the generally weaker effects of plant estrogens in cattle than in sheep, mentioned by Shutt (1976) and Austin et al. (1982), is based upon the investigation of Braden et al. (1971), who suggested that the circulating plant estrogens and their metabolites are more efficiently conjugated in cattle than in sheep and also that formononetin is metabolized faster in cattle than in sheep. Our contradictory results, that sheep conjugate plant estrogens more efficiently than cattle, are, however, in accordance with our earlier in vitro studies, where we demonstrate that only minor differences in liver metabolism of formononetin and daidzein exist between the two species (Lundh et al., 1988a). Furthermore, the conjugation activity was higher in sheep than in cattle in almost all gastrointestinal tissues studied (Lundh, 1990).

In vivo metabolism in ruminants of substances present in the feed is influenced by a complex pattern of variables. Most of the plant estrogens occur in the intact plant in bound form, as glucosides, which are hydrolyzed in the rumen and broken down by the microorganisms (Nilsson et al., 1967). A very minor part of these hydrolyzed (unbound) plant estrogens are absorbed, probably already in the rumen, and reach the blood circulation unconjugated. The bulk are, however, conjugated with glucuronic acid already in the gastrointestinal epithelium (Lundh, 1990). The substances remaining unconjugated when entering the blood circulation are conjugated mainly with glucuronic acid by the liver (Shutt et al., 1967; Lundh et al., 1988a) and perhaps also by extrahepatic tissues (Watkins et al., 1987; Smith et al., 1984). Detoxification by means of conjugation in different tissues probably explains why no more than 10% of the plant estrogens are found in free form in blood plasma. Obviously the absorption and excretion rates also influence the plasma levels of plant estrogens at different times after feeding. All these factors, metabolic rate, absorption and excretion, feeding rate, and chewing behavior, differ from one animal to another and explain the large individual differences observed in this investigation.

The total amount of formononetin and daidzein in ovine blood plasma in this study is much lower than has been reported by Braden et al. (1971) and Shutt et al. (1968). Such differences, which perhaps are influenced by individual variations, may also depend on the composition of the forage. Thus, Braden et al. (1971) reported that higher levels of formononetin and their metabolites were found in plasma from animals fed Tallarook subterranean clover than in plasma from animals fed red clover, although the formononetin content was almost the same in the two plant species.

Our results suggest that cattle and sheep are almost equally effective in detoxifying the isoflavones formononetin and daidzein present in silage consisting of red clover and grass on a 50/50 basis. On the other hand, the high amount of free (unconjugated) equol in bovine plasma indicates that the low sensitivity to plant estrogens in cattle is probably not caused by a higher conjugation activity than in sheep. Cattle are not, however, totally insensitive to the plant estrogens as shown by Kallela (1968, 1984), but are probably only temporarily affected, since no reports about irreversible sterility in cattle have been published.

So far, no satisfactory explanation can be offered as to the differences between cattle and sheep in their sensitivity to plant estrogens. A possible explanation could be differences in estrogen receptors between the two species, a possibility that is now being investigated.

ACKNOWLEDGMENT

We thank Prof. H. Adlercreutz, Department of Clinical Chemistry, Unversity of Helsinki, Meilahti Hospital, Helsinki, Finland, for providing the equol standard; S. Brulin, Röbäcksdalen experimental station, for skillful assistance with blood sampling; T. Westerlund for technical assistance; and Prof. K.-H. Kiessling, Department of Animal Nutrition and Management, Swedish University of Agricultural Sciences, for constructive critical reading of the manuscript.

LITERATURE CITED

Adler, J. H.; Trainin, D. A. Hyperoestrogenic Syndrome in Cattle. *Refu. Vet.* **1960**, *17*, 108–115.

Austin, A. R.; Aston, K.; Drane, H. M.; Saba, N. The Fertility of Heifers Consuming Red Clover Silage. *Grass Forage Sci.* **1982**, *37*, 101–106.

Bailey, N. T. J. *Statistical Methods in Biology*, 2nd ed.; Hodder and Stoughton: London, 1984.

Bennetts, H. W.; Underwood, E. J.; Shier, F. L. A Breeding Problem of Sheep in the South-West Division of Western Australia. *J. Agric., West. Aust.* **1946**, *23*, 1–12.

Braden, A. W. H.; Thain, R. I.; Shutt, D A. Comparison of Plasma Phyto-oestrogen Levels in Sheep and Cattle After Feeding on Fresh Clover. *Aust. J. Agric. Res.* **1971**, *22*, 663–670.

Cox, R. I.; Collins, W. J.; Wilson, P. A.; Wong, M. S. F. Potential Biological Inactivation of Pasture Oestrogens in the Animal. Immunization or Metabolic Deflection. In *Reproduction in Sheep*; Lindsay, D. R., Pearce, D. T., Eds.; Cambridge University Press: Cambridge, 1984; pp 368–370.

Kallela, K. The Oestrogenic Effect of Red Clover Pasture on the Ovariectomized Heifer. *Nord. Veterinaermed.* **1968**, *20*, 185–192.

Kallela, K.; Heinonen, K.; Saloniemi, H. Plant Oestrogens; the Cause of Decreased Fertility in Cows. *Nord. Veterinaermed.* **1984**, *36*, 124–129.

Lightfoot, R. J. A Look at Recommendations for the Control of Infertility due to Clover Disease in Sheep. *Proc. Aust. Soc. Anim. Prod.* **1974**, *10*, 113–121.

Lundh, T. J.-O. Conjugation of the Plant Estrogens Formononetin, Daidzein and their Metabolite Equol by Gastrointestinal Epithelium from Cattle and Sheep. *J. Agric. Food Chem.* **1990**, *38*, 1012–1016.

Lundh, T. J.-O.; Pettersson, H.; Kiessling, K.-H. Demethylation and Conjugation of Formononetin and Daidzein in Sheep and Cow Liver Microsomes. *J. Agric. Food Chem.* **1988a**, *36*, 22–25.

Lundh, T. J.-O.; Pettersson, H.; Kiessling, K.-H. Liquid Chromatographic Determination of the Estrogens Daidzein, Formononetin, Coumestrol, and Equol in Bovine Blood Plasma and Urine. *J. Assoc. Off. Anal. Chem.* **1988b**, *71*, 938–941.

Millington, A. J.; Francis, C. M.; McKeown, N. R. Wether Bioassay of Annual Pasture Legumes. *Aust. J. Agric. Res.* **1964**, *15*, 527–536.

Nekby, C. Plant estrogens—Their Metabolism and Physiological Actions in Ruminants; Report 142; Department of Animal Nutrition and Management, Swedish University of Agricultural Sciences: Uppsala, 1985.

Nilsson, A.; Hill, J. L.; Lloyd Davies, H. An *In Vitro* study of Formononetin and Biochanin A Metabolism in Rumen Fluid from Sheep. *Biochim. Biophys. Acta* **1967**, *148*, 92–98.

Pettersson, H.; Kiessling, K.-H. Liquid Chromatographic Determination of the Plant Estrogens Coumestrol and Isoflavones in Animal Feed. *J. Assoc. Off. Anal. Chem.* **1984**, *67*, 503–506.

Rankin, J. E. F. Natural Oestrogens Associated with Abortion in Cattle in Northern Ireland. *Br. Vet. J.* **1963**, *119*, 30–34.

Shutt, D. A. The Effects of Plant Oestrogens on Animal Reproduction. *Endeavour* **1976**, *35*, 110–113.

Shutt, D. A.; Braden, A. W. H. The Significance of Equol in Relation to the Oestrogenic Responses in Sheep Ingesting Clover with a High Formononetin Content. *Aust. J. Agric. Res.* **1968**, *19*, 545–553.

Shutt, D. A.; Axelsen, A.; Lindner, H. R. Free and Conjugated Isoflavones in the Plasma of Sheep Following Ingestion of Oestrogenic Clover. *Aust. J. Agric. Res.* **1967**, *18*, 647–655.

Smith, G. S.; Watkins, J. B. Biotransformations of Xenobiotics in Tissues of Cattle and Sheep. *Can. J. Anim. Sci.* **1984**, *64*, (suppl). 278–280.

Thain, R. I. Bovine Infertility Possibly Caused by Subterranean Clover: A Preliminary Report. *Aust. Vet. J.* **1965**, *41*, 277–281.

Thomson, D. J. The Effect of Feeding Red Clover Conserved by Drying or Ensiling on Reproduction in Ewe. *J. Br. Grassl. Soc.* **1975**, *30*, 149–152.

Watkins, J. B.; Smith, G. S.; Hallford, D. M. Characterization of xenobiotic biotransformation in hepatic, renal and gut tissues of cattle and sheep. *J. Anim. Sci.* **1987**, *65*, 186–195.

Received for review December 19, 1989. Revised manuscript received March 2, 1990. Accepted March 25, 1990.

Registry No. Formononetin, 485-72-3; daidzein, 486-66-8; equol, 531-95-3; biochanin A, 491-80-5; genistein, 446-72-0.