IN VITRO METABOLISM OF FORMONONETIN AND BIOCHANIN A IN BOVINE RUMEN FLUID\textsuperscript{1,2,3}


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ABSTRACT

The phyto-estrogens formononetin (7-hydroxy-4'-methoxyisoflavone) and biochanin A (5,7-dihydroxy-4'-methoxyisoflavone) were independently incubated in vitro at 39°C in bovine rumen fluid from a fistulated steer receiving an alfalfa hay diet. Formononetin was incubated in studies 1 and 2, whereas biochanin A was incubated in study 3. The isoflavones were separated and quantified by high performance liquid chromatography. In study 1, formononetin concentration, 14.80 μg/ml at time 0, declined to 1.16 μg/ml by 12 h and to .76 μg/ml by 24 h. Daidzein (7,4'-dihydroxyisoflavone), .18 μg/ml at time 0, peaked at 12.92 μg/ml at 6 h and decreased to 1.30 μg/ml by 24 h. Equol (7,4'-dihydroxyisoflavonan), detected at 6 h, peaked at 16.94 μg/ml at 18 h and dropped to 12.64 μg/ml at 24 h. In incubation study 2, formononetin declined from 17.57 μg/ml at time 0 to 7.08 μg/ml by 6 h. Daidzein concentration was 1.75 μg/ml at time 0 and increased to 12.03 μg/ml by 6 h. Equol was detected at 3 h and increased to 2.32 μg/ml at 6 h. The half-lives were 4.3 for formononetin and 9.8 h for daidzein in this in vitro system. In study 3, biochanin A, 8.54 μg/ml at time 0, decreased to 0 μg/ml by 12 h in incubation 3, whereas genistein (5,7,4'-trihydroxyisoflavone), 3.17 μg/ml at 1 h, peaked at 7.35 μg/ml at 4 h and decreased to .32 μg/ml at 24 h. Equol was not detected in incubation study 3. The half-lives of biochanin A and genistein were 3.9 and 5.5 h, respectively. Bovine rumen fluid metabolism of formononetin yielded daidzein and equol, but the only identified product of biochanin A was genistein.

(Key Words: Rumen Fluid, Plant Estrogens, Isoflavones, Formononetin, Genistein.)

Introduction

Phyto-estrogens have estrogen-like activity in immature laboratory animals and may interfere with normal reproduction in ruminants. Infertility in sheep has been associated with consumption of specific varieties of subterranean clover (Bennetts et al., 1946). The infertility syndrome in ewes grazing estrogenic pastures for several years is characterized by cystic glandular hyperplasia in the cervix and uterus (Adams, 1976). The estrogenic activity in many clover species is the result of elevated concentrations of the isoflavones (Guggolz et al., 1961; Beck, 1964). Ruminal microorganisms can demethylate the isoflavones (Nilsson, 1961, 1962) to a compound of greater or lesser estrogenic activity. In vitro studies using ovine rumen fluid have shown that formononetin (7-hydroxy-4'-methoxyisoflavone) is converted to daidzein (7,4'-dihydroxyisoflavone; Lindner, 1967; Nilsson et al., 1967) and biochanin A (5,7-dihydroxy-4'-methoxyisoflavone) is converted to genistein (5,7,4'-trihydroxyisoflavone; Nilsson, 1961; Lindner, 1967; Nilsson et al., 1967; Batterham et al., 1971). The isoflavones that are consumed by ruminants have been shown to be absorbed from the gastrointestinal tract (Braden et al., 1967).

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\textsuperscript{3} Mention of a trademark of a proprietary product does not constitute a guarantee or warranty of the product by The Texas Agric. Exp. Sta. and does not imply its approval to the exclusion of other products that also may be suitable.

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Formononetin also has been shown to be demethylated and reduced by ovine rumen microflora to equol (7,4'-dihydroxyisoflavone; Nilsson et al., 1967; Shutt and Braden, 1968; Batterham et al., 1971). Equol is readily absorbed from the gastrointestinal tract, and high blood concentrations are responsible for infertility in sheep (Lindner, 1967). Equol has been found in the urine of sheep (Braden et al., 1967; Shutt and Braden, 1968) and the cow (Klyne and Wright, 1959). All of the known metabolites of the isoflavone phyto-estrogens have been identified in the blood plasma of sheep (Nilsson et al., 1967; Batterham et al., 1971; Braden et al., 1971). The estrogenic activity of the isoflavones is 10^-5 times that of diethylstilbestrol, and activity of equol is one-quarter that of the isoflavones (Shutt and Braden, 1968).

The agronomic value of subterranean clover as a potential forage for grazing cattle and the lack of information regarding degradation of formononetin and biochanin A in bovine rumen fluid indicate the need for increased information on bovine ruminal metabolism of isoflavones. The objective of this research was to determine the in vitro metabolism rate and to define the metabolic products of formononetin and biochanin A in bovine rumen fluid.

Materials and Methods

In vitro incubations were carried out in bovine rumen fluid collected from a ruminally fistulated steer maintained on a diet of alfalfa hay for at least 4 wk prior to collection. The rumen fluid was strained through eight layers of cheesecloth and maintained in a water bath at 39°C. Anaerobic conditions were maintained by bubbling CO₂ through the rumen liquor and capping each digestion tube with a vented stopper to allow pressure release.

In incubation study 1, 400 μg of formononetin (7-hydroxy-4'-methoxyisoflavone) were incubated in triplicate for 0, 6, 12, 18 and 24 h. In incubation study 2, 400 μg of formononetin were incubated in triplicate for 0, 1, 2, 3, 4, 5, 6, 12, 18 and 24 h. In incubation study 3, 400 μg of biochanin A (5,7-dihydroxy-4'-methoxyisoflavone) were incubated in triplicate for 0, 1, 2, 3, 4, 5, 6, 12, 18 and 24 h.

Stock solutions of formononetin and biochanin A were prepared at concentrations of 400 μg/ml in filtered, heat-sterilized and centrifuged rumen fluid. The stock solution was maintained at 4°C until used in the incubations. The volume of stock solution in each digestion tube was 1 ml, and 9 ml of rumen fluid were added to each tube. All reactions were stopped by addition of 10 ml methanol, and the tubes were centrifuged at 11,000 x g for 30 min. The eluate was centrifuged for an additional 30 min at 11,000 x g. A subsample of the eluate was vacuum-filtered through a metricel .2-μm membrane filter. The samples were stored at -20°C.

A Beckman 5 model 332 high performance liquid chromatograph (HPLC) system, equipped with a model 160 absorbance detector (254 nm), model 110A analytical pumps and using a 25-cm x 4.6-mm i.d. stainless steel column prepacked with Altex Ultrasphere ODS was used for isoflavone analysis (Smith et al., 1986). A mobile phase of methanol-water (2:1, adjusted to pH 3.5 with acetic acid) at 1.0 ml/min flow rate maximized isoflavone peak shape and separation. Compounds were quantified with a Hewlett-Packard model 3390A integrator. Sample injection volume was 20 μl. Retention times were 3.8, 4.8, 6.8 and 10.2 min for daidzein, genistein, formononetin and biochanin A, respectively. Equol eluted after 9.8 min and was detected at 214 nm using the same HPLC system and an acetonitrile-water (35:65) mobile phase. Standard curves of the pure isoflavones, genistein, daidzein, formononetin and biochanin A, obtained from K and K Labs, Plainview, NY, were prepared by dissolving the standard in methanol (1, 4, 10 and 16 μg/ml) and determining peak area. Pure equol was obtained through Paul E. Juniewicz, Johns Hopkins University, and a standard curve was prepared in the same manner.

The data were analyzed using regression analysis (Steel and Torrie, 1960).

Results

Within 6 h of incubation in live bovine rumen fluid, formononetin was almost completely demethylated to daidzein, and conversion of daidzein to equol had begun (Figure 1). Incubation study 2 was designed to observe changes during the first 6 h of exposure to live bovine...
Figure 1. Incubation (1) of formononetin (20 µg/ml initial concentration) in live bovine rumen fluid at 39°C.

Figure 2. Incubation (1 and 2) of formononetin (20 µg/ml initial concentration) in live bovine rumen fluid at 39°C.

Figure 3. Incubation (3) of biochanin A (20 µg/ml initial concentration) in live bovine rumen fluid at 39°C.
TABLE 1. REGRESSION EQUATIONS FOR RATE OF DECLINE AND HALF-LIFE OF ISOFAVONES IN LIVE BOVINE RUMEN FLUID

<table>
<thead>
<tr>
<th>Isoflavone</th>
<th>a</th>
<th>b</th>
<th>r</th>
<th>r²</th>
<th>Half-life, h&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formononetin</td>
<td>9.13</td>
<td>-0.55</td>
<td>.97</td>
<td>.94</td>
<td>4.3</td>
</tr>
<tr>
<td>Diadzein</td>
<td>25.28</td>
<td>-1.48</td>
<td>.99</td>
<td>.99</td>
<td>9.8</td>
</tr>
<tr>
<td>Biochanin A</td>
<td>8.46</td>
<td>-1.08</td>
<td>.89</td>
<td>.79</td>
<td>3.9</td>
</tr>
<tr>
<td>Genistein</td>
<td>16.34</td>
<td>-1.87</td>
<td>.95</td>
<td>.91</td>
<td>5.5</td>
</tr>
</tbody>
</table>

<sup>a</sup>Half-life = a + b (initial concentration/2).

ing more than 70% of the extracted phyto-estrogens, was detected in the blood plasma (50 μg/100 ml) of ewes grazing red clover (Shutt and Braden, 1968). Shutt and Braden (1968) concluded that in sheep the estrogenic activity of clover containing formononetin was due to equol. Formononetin, daidzein, biochanin A, genistein and equol all have been detected in the blood plasma of heifers grazing red clover (Braden et al., 1971).

Studies involving bovine rumen metabolism of isoflavones are limited. Nilsson (1961) reported in vitro demethylation of biochanin A to genistein in bovine rumen fluid, but gave no information with respect to demethylation rates. Braden and Shutt (1970) compared plasma levels of isoflavones and their metabolites in sheep and cattle 24 h after feeding red clover. The levels of equol were similar in both sheep and cattle. Both biochanin A and formononetin were present in the red clover; therefore, origin of the equol was unknown. In our study, in vitro metabolism of formononetin in bovine rumen fluid produced daidzein and equol, whereas the metabolism of biochanin A yielded only genistein. Batterham et al. (1971) reported a 10:90 formononetin:daidzein ratio after 6 h in ovine rumen fluid. This represents a faster rate of in vitro conversion in sheep than we noted for cattle. Batterham et al. (1971) reported at 50:50 ratio of 4 h and a 30:70 ratio at 6 h for biochanin A:genistein. This is a slower rate of biochanin A demethylation than we noted for bovine rumen fluid in vitro. Bovine rumen metabolism of formononetin and biochanin A had the same end products as were reported for sheep but conversion rates differed.

Our data using bovine rumen fluid indicate that formononetin, daidzein and equol are available for absorption from the gastrointestinal tract for up to 24 h after ingestion. Subterranean clovers containing high concentrations of formononetin potentially could affect the fertility of grazing cattle. Further studies are needed to clarify the effects of formononetin and its metabolites upon endocrine functions in cattle.

**Literature Cited**


