APPLIED MICROBIOLOGY, May 1968, p. 797-798

Vol. 16, No. 5
Printed in U.S.A.

Detection of a Fungal Estrogen (F-2) in Hay Associated with Infertility in Dairy Cattle[1]

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Received for publication 5 February 1968

Some of the problems of infertility in swine herds in the United States have been explained by the recent discovery of an estrogenic fungal metabolite (F-2) produced by *Fusarium graminearum* in stored maize (C. M. Christensen, G. H. Nelson, and C. J. Mirocha, Appl. Microbiol. **13**:653, 1965; C. J. Mirocha, C. M. Christensen, and G. H. Nelson, Appl. Microbiol. **15**:497, 1967; M. Stob et al., Nature **196**:1318, 1962). This metabolite causes an enlargement of vulva and uterus when consumed by rats, guinea pigs, or swine, and there is some evidence that consumption of F-2 by pregnant swine results in reduced size of litters. The fungal estrogen (F-2) and a chemically related material (F-3), also produced by *F. graminearum*, have been found in the feed of dairy cattle in Minnesota, and are suspected of playing some role in the infertility problems of these animals.

Recently, a farmer in the county of Cheshire, England, complained about an apparently bad lot of hay, which, when fed to dairy cattle, resulted in decreased fertility in these animals. This was the first report of an infertility problem in dairy cattle in England which appeared to be directly connected with the consumption of hay. The hay, known to be of poor quality, was chopped up, mixed as additional roughage with silage, and fed to the cattle. Good fertility records were kept on this particular herd of 150 cattle; before the incorporation of the hay into their diet, their artificial insemination index (AI) was about 1.2. The AI index refers to the number of inseminations necessary to obtain a successful pregnancy. During the period when the hay was fed, the index increased to a value as high as 4. After this observation, the animals were quickly taken off the hay and their AI index gradually returned to normal. When discussing this problem with farmers in the upper regions of Derbyshire, we found that cases of infertility during the indoor or winter feeding period were more frequent than suspected.

The hay described above was dried and stored for future experimentation. It was extracted with methylene chloride, concentrated under vacuum in a flash evaporator, and purified by use of the techniques of thin-layer, column, and gas-liquid chromatography as previously described (C. J. Mirocha, C. M. Christensen, and G. H. Nelson, Appl. Microbiol. **15**:497, 1967). The constituents of interest were eluted either off the thin-layer chromatograms or the silica-gel column and concentrated, and their trimethylsilyl ether derivative made by reacting with *N,O*-bis(trimethylsilyl acetamide). The ether derivatives were then identified by gas-liquid chromatography. Whenever possible, internal standards were used to facilitate final identification of the compounds. We tested for the following metabolites: F-2 [6-(10 hydroxy-6-oxo-trans-l-undecenyl) β-resorcylic acid lactone]; F-3 (partially characterized and structurally similar to F-2); ergosterol; radicicol (B. N. Mirrington et. al., Tetrahedron Letters **7**: 365, 1964); and coumestrol (E. M. Bickoff, A. L. Livingston, and A. N. Booth, Arch. Biochem. Biophys. **88**:262, 1960).

The methylene chloride extract and the acetonitrile fraction left after partitioning with petroleum ether (boiling point, 30 to 60 C) were analyzed for the above compounds. F-2 and ergosterol were found in the hay, but F-3, radicicol, and coumestrol were not. The presence of the trimethylsilyl ether of F-2, as well as other unidentified compounds, is illustrated in Fig. 1. The presence of F-2 was confirmed by adding a known amount of F-2 as an internal standard to the mixture and performing co-chromatography. Identical retention values were obtained, confirming the presence of F-2. No F-3 was found; its location, if it were present, is marked by an arrow in Fig. 1.

The compound labeled number 4 (Fig. 1) complicated resolution of the constituents of the

[1] Paper No. 6437, Scientific Journal Series, Minnesota Agricultural Experiment Station.

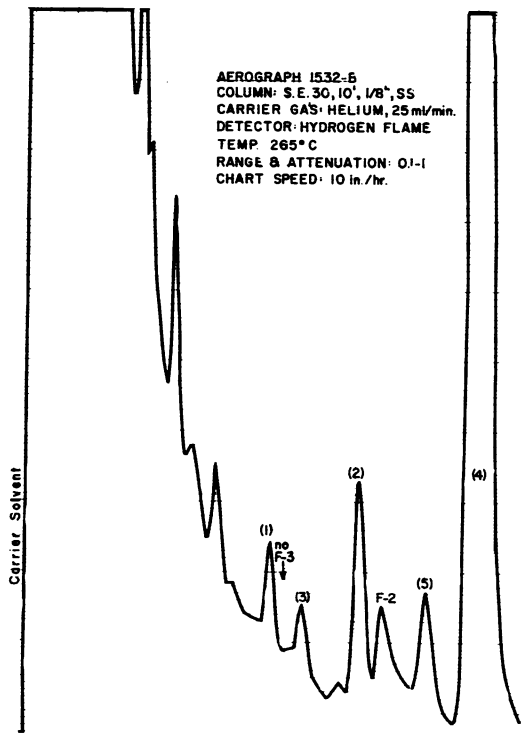

FIG. 1. *Gas-liquid chromatographic analysis of the constituents found in a sample of hay implicated in dairy cattle infertility problems. All of the constituents shown are the trimethylsilyl ether derivatives of the extract. F-3, if present in this sample, would have appeared in the position marked by the arrow. Constituents 1 through 5 are not identified. Constituent 4 had a strong absorption maximum at 240 mμ and, when separated on TLC, masked the presence of F-2.*

hay extract on thin-layer chromatograms because it did not resolve itself into a discrete band during development, but rather spread itself uniformly throughout the entire plate. This phenomenon, which was determined by charring the constituents on the thin-layer chromatograms with methanol-H_2SO_4 (50%), caused great difficulty in getting reliable spectra of other compounds. Compound number 4 eluted off the silica gel as a contaminant and masked the ultraviolet absorption of the desired compounds. Compound 4 has a very intense band of absorption around 240 mμ.

To circumvent the problem posed by compound 4, large amounts of F-2 were obtained by strip application onto silica-gel chromatoplates and subsequent elution after development. The F-2 in the concentrated eluate was taken up in a small volume of chloroform and was precipitated out with petroleum ether (boiling point, 30 to 60 C); crystals were obtained. Fortunately, compound 4 was left behind in the chloroform-petroleum ether mixture. The F-2 crystals in ethyl alcohol had an absorption spectrum identical to that of F-2, i.e., 236, 274, and 314 mμ.

In summary, a concentration of 14 ppm of F-2 was found in a hay sample which was implicated in an infertility problem in dairy cattle in England. The presence of the compound was demonstrated by gas-liquid and thin-layer chromatography and by ultraviolet absorption spectrophotometry. In addition, ergosterol, a common metabolite of *F. graminearum* was also found to be present in the hay sample. Samples of the hay, sent to the United States from England, were cultured on several agar media, and the predominant fungi found were *Aspergillus* spp. and *Penicillium* spp. At moisture contents that permit these fungi to thrive, *Fusarium*, if originally present, would die out relatively rapidly; thus, we do not know whether the hay previously had been invaded by *Fusarium*.

The presence of F-2 in this hay sample strongly supports the hypothesis that this fungal metabolite may be responsible for some infertility problems in dairy cattle. F-2 and a closely related compound (F-3) have been previously found, on three separate occasions, in feed (other than hay) thought to be involved in infertility and abortion problems in dairy cattle in Minnesota. Further investigations of this problem are being carried out, and the effect of crystalline F-2 on bairy cattle will be studied.

This investigation was supported by the Office of International Programs of the University of Minnesota (Ford Foundation) and by Public Health Service research grant UI-00307-02. We wish to thank Sonja Ullrich for making the chemical analyses and C. M. Christensen for the isolation of fungi reported in this paper.