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Estrogenic Metabolite Produced by *Fusarium graminearum* in Stored Corn[1]

C. J. MIROCHA, C. M. CHRISTENSEN, AND G. H. NELSON

Institute of Agriculture and College of Veterinary Medicine, University of Minnesota, St. Paul, Minnesota 55101

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A derivative of resorcinylic acid, produced by the fungus *Fusarium graminearum*, has been found to be responsible for the estrogenic signs in swine and laboratory rats. An estrogenic response in rats can be incited by injecting intramuscularly as little as 20 μg of the estrogen (F-2). Stimulation in growth of rats was noted at the lower concentrations (20 to 40 μg) of a series. Up to 3,500 ppm of the estrogen was produced on a solid corn medium. The compound is relatively stable to heat and ultraviolet irradiation. Methods of analysis have been developed and include: extraction procedures, evaluations by ultraviolet absorption spectrophotometry, thin-layer chromatography, and gas-liquid chromatography.

The first report of an estrogenic condition in swine associated with consumption of fungus-infested feed was made by McNutt (4) in 1928. He observed that sows which had consumed moldy feed developed enlarged, tense, and elevated vulvae, enlarged mammary glands, and in more severe cases a prolapse of the vagina and rectum. In 1952, McErlean (3) reported a similar condition of swine in Ireland associated with the feeding of *Fusarium*-infested barley. Recently, Stob et al. (7) isolated an anabolic and uterotrophic compound from corn infected with *Fusarium* which appeared to be responsible for the estrogenic syndrome.

Christensen et al. (1) isolated an estrogenic metabolite produced by *Fusarium* which they called F-2. It was found in autoclaved corn inoculated with *Fusarium*, in samples of corn feed from farms in Minnesota reporting the estrogenic symptoms in their swine herds, as well as in commercially prepared pelleted feed. When this material was injected into virgin white weanling female rats, it caused greatly enlarged uteri. When fed ad libitum to swine, it caused swollen, edematous vulvae in females, shrunken testes in young males, enlarged mammary glands in the young of both sexes, and abortion in pregnant gilts or sows. The estrogen F-2 is produced by certain isolates of the fungus *F. graminearum* when grown on autoclaved moist corn.

To date, this estrogenic metabolite has been important in explaining diseases in the field of veterinary medicine heretofore called idiopathic, although it may also have potential unexplored significance in the area of public health and potential use in medicine.

The early work on the chemical isolation and structure of the estrogenic metabolite was reported by F. N. Andrews and M. Stob (Belgian Patent 611630, 1961; U.S. Patent 3,196,019, 1965) and its partial characterization was reported by Christensen et al. (1). Preliminary information on the chemical structure of the estrogen reveals it to be one of the enatiomorphs of 6-(10-hydroxy-6-oxo-trans-1-undecenyl)-β-resorcylic acid lactone, called by the trivial name of zearalenone (8).

Compounds similar in structure, but perhaps not in biological activity, to the estrogenic factor have been reported before, e.g., curvularin, produced by the fungi *Curvularia* sp., *Penicillium steckii* Zaleski, and *P. expansum* Link (6), and radicicol and monorden, produced, respectively, by *Nectria radicicola* (5) and *Monosporium* sp. (2), and which appear to be identical. These compounds are closely related to F-2 in structure, differing only in the presence of an epoxy group and one extra carbonyl and in the position of the conjugated double bonds.

MATERIALS AND METHODS

All solvents used in this study were of analytical reagent grade except for petroleum ether (bp 30 to 60 C) which was of a practical grade.

Ultraviolet absorption spectra were recorded with a Beckman DB recording spectrophotometer.

Reagents (chlorotrimethylsilane and 1,1,1,3,3,3-Hexamethyldisilazine) for the synthesis of the tri-

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methylsilylether of the estrogen (F-2) were purchased from Eastman Organic Chemicals, Rochester, N.Y.; each was added individually to the substrate in anhydrous pyridine solution instead of in a prepared mixture of the reagents.

Silica gel used as the adsorbent in column chromatography was of reagent grade, 100 to 200 mesh (Fisher Certified, Fisher Scientific Co., Pittsburgh, Pa.).

Analyses by elemental combustion, mass spectrum, fluorescence emission, and infrared spectroscopy were carried out in the Chemistry Department of the University of Minnesota.

In experiments involving the effect of F-2 on the uterus of the rat, the estrogen was administered by first dissolving it in propylene glycol and then injecting intramuscularly every 2nd day for 7 days. As far as possible, aseptic conditions were used in the preparation and administration of the estrogen.

Studies involving the biosynthesis of F-2 were carried out by seeding sterile, autoclaved corn with spores of *F. graminearum*, incubating at room temperature (25 to 28 C) for 2 weeks, and then transferring to a chamber held at 12 C for the duration of the experiment. The fungus was stored on sterilized soil until ready for seeding of the culture flasks. The fungus retains its viability for months or years in such soil and undergoes no genetic change.

The lability of the estrogenic compound when subjected to ultraviolet irradiation was determined by irradiating the compound in a solution of ethyl alcohol with a G.E. blacklight lamp which has a peak of emission around 365 mμ and with a fluorescent sun lamp which emits at 312 mμ. The sample was kept in a sealed quartz cuvette during the irradiation period, and the absorption spectrum was monitored at appropriate time intervals. The total energy incident upon the sample illuminated with the G.E. blacklight lamp was 3.7×10^3 ergs per cm^2 per sec. The fluorescent sun lamp was used in combination with a Corning CS-54 ultraviolet transmitting and visible absorbing filter. The energy incident upon the sample was 1.5×10^3 ergs per cm^2 per sec.

RESULTS

Estrogenic response in rats. When the purified F-2 solution of crystal was injected into white weanling virgin female rats, a typical estrogenic response such as that reflected in the increase in fresh weight of the uterus was found. A dosage response curve (Fig. 1A) was obtained when a cumulative dose ranging from 20 to 650 μg was administered over a period of 7 days. There was a linear response in the increase in the weight of the uterus with increase of F-2 concentration. The opposite was true, however, when the body weight of the animals was averaged. Those rats treated with the highest concentration of F-2 (Fig. 1B) showed no increase in body weight when compared with the control rats, whereas the lowest concentrations caused a relative increase in weight. This appears to be a typical hormonal,

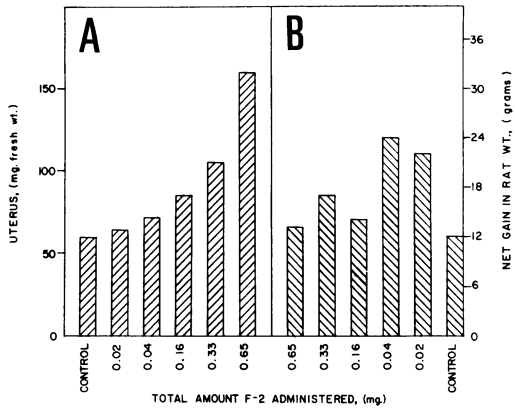


FIG. 1. *(A) Effect of different dosages of F-2 on the weight of the rat uterus when administered by intramuscular injection. (B) Effect of the same concentrations of F-2 on the body weight of the rat.*

concentration-dependent response which may suggest that caution should be taken if this compound were to be used as a feed additive to promote growth in animals.

Biosynthesis of F-2 by Fusarium. Best production of the estrogen F-2 can be obtained by growing the organism on moist, autoclaved corn for 2 weeks at 25 to 28 C followed by a temperature of 12 C for different amounts of time. Yields from two estrogen-producing isolates of *F. graminearum* designated as numbers 9 and 10 are shown in Fig. 2. As much as 3,500 ppm of F-2, calculated on the basis of the weight of the finely ground substrate after drying under an infrared lamp for 12 hr, was found after 8 weeks at 12 C. Production of F-2 in a defined, liquid medium has been attempted but yields as yet are not satisfactory.

Method of extraction from biological material and quantitation. The following method of chemical extraction from biological material has been worked out by use of corn and commercial pelleted feed products as test material.

Of dried, finely ground biological material, 20 g was adjusted to 30% moisture content and extracted in a Soxhlet Extractor overnight or for 16 hr with 120 ml of methylene chloride. When a highly pigmented extract resulted, the methylene chloride was re-extracted with acetonitrile by concentrating the methylene chloride extract on a flash evaporator until the consistency was syrup-like but not completely dry. The concentrate was transferred into a separatory funnel by rinsing it with petroleum ether (bp 30 to 60 C). Of acetonitrile, 50 ml was added; the mixture was shaken and the phases were allowed to separate. The bottom phase (acetonitrile) contained the F-2, and the top phase (petroleum ether) contained

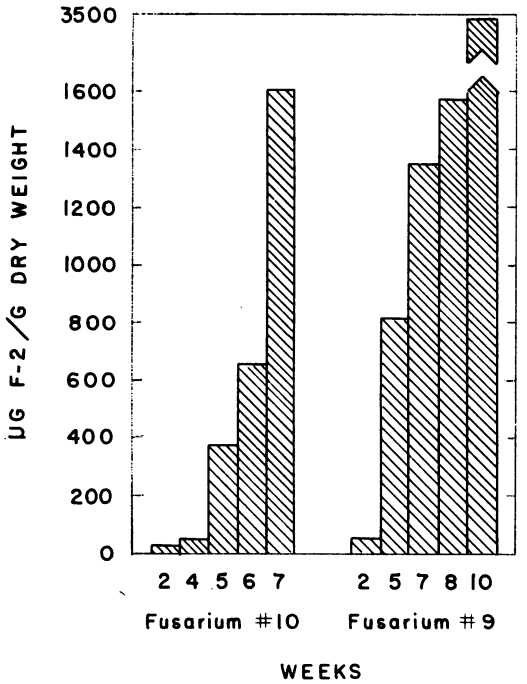


FIG. 2. *Production of F-2 by two isolates of Fusarium graminearum when grown on a solid corn medium at 12 C for various lengths of time, after 2 weeks of growth at 25 C.*

most of the pigments. When the phases were inverted or an emulsion formed, a small amount of the petroleum ether (bp 30 to 60 C) was added to the funnel for correction. The bottom layer was harvested and the top phase was re-extracted with 25 ml of acetonitrile in the same manner. The combined acetonitrile extracts (or the methylene chloride extract above) were concentrated to about 3 ml, then transferred to a small graduated vial, and brought up to 4 ml with chloroform or methylene chloride. Analyses at this point were made by the following methods.

Thin-layer chromatography. Thin-layer plates of Silica Gel (E. Merck AG, Darmstadt, Germany) were prepared in the conventional manner and then activated at 100 C for 1 hr; 50 µliter of the above extract, a known F-2 solution, and a combination F-2 and the unknown were applied. The plate was developed in a solution of 5% ethyl alcohol in chloroform, and fluorescence was checked at the same R_F value (0.5) as the standard after illumination with an ultraviolet lamp. An authentic sample of F-2 emits light at 450 mµ when excited by a wavelength of 310 mµ.

Ultraviolet spectrophotometry. Of the extract, 5 µliters was placed into 2 ml of absolute ethyl alcohol in a cuvette. The wavelength of the spectrophotometer was set at 376 mµ; the sample was diluted until the percentage of transmission registered between 30 and 50, and the sample was then scanned between 340 and 200 mµ. Maximal peaks of absorption were found at 314, 274, and 236 mµ when F-2 was present. The amount of F-2 present was computed from a standard curve calculated at any one of the three wavelengths (Fig. 3). Best quantitative results were obtained with 274 mµ.

Gas-liquid chromatography. Advantage was taken of the fact that F-2 is a phenolic compound with two hydroxy groups which can be converted to their corresponding tri- or dimethylsilyl ether and directly injected into the gas chromatograph.

The following conditions and equipment were used: Aerograph HI-FY, Model 600C, hydrogen flame; column, 5 ft (152.4 cm) stainless steel by one-eighth inch (0.32 cm), packed with 5% SE-30 and 60/80 Chromosorb W; column temperature, 238 C; injection block temperature, 280 C; helium carrier gas flow rate; 25 ml/min; hydrogen flow rate, 25 ml/min; Honeywell Brown Recorder speed, Slow-2 (6.4 mm/min).

The trimethylsilylether derivatives of F-2 were prepared by adding the following to 0.1 ml of the extract: 0.1 ml of pyridine, 0.1 ml of chlorotrimethylsilane, and 0.2 ml of hexamethyldisilazane.

Caution was taken to add the reagents only in the order listed. They were added in 1-dr vials

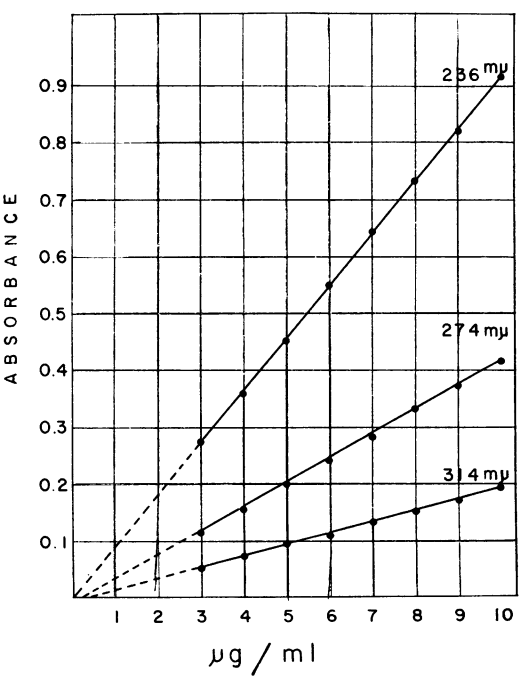


FIG. 3. *Straight line relationships between different concentrations of F-2 in ethyl alcohol when measured at 236, 274, and 314 mµ.*

which were then stoppered tightly; 30 min lapsed before injection. The retention time on the column at 238 C was about 12 min, and at 261 C, 2.5 min (Fig. 4A). The limit of sensitivity of this method was 0.4 μg (Fig. 4B).

To confirm the identification of F-2 present on a thin-layer chromatoplate by gas chromatography, the following procedure was used.

The fluorescent area on the Silica Gel G chromatoplate was scraped into a small centrifuge tube; 2 ml of methylenechloride was added with stirring, followed by centrifugation at 27,000 × *g* for 15 min. The supernatant solution was poured into a 1-dr vial and evaporated under nitrogen until the bottom of the vial was almost dry but still moist. The trimethysilylether was made as described above and was injected into the gas chromatograph. At least 10 μg of F-2 on the plate was necessary to make the derivatives for analysis by gas-liquid chromatography.

Preparation by column chromatography and countercurrent distribution. To obtain yields of F-2 from culture in amounts sufficient for crystallization, the extract was first fractionated on a Silica Gel column and then purified further on a countercurrent distribution apparatus. These two processes eliminated the pigments which created the most difficulty in purification procedures.

The following procedure was used when approximately 400 to 500 g of biological material was extracted. The sample was extracted as already described by use of the acetonitrile clean-up procedure. The acetonitrile was concentrated to a syrup-like consistency in a flash evaporator and brought into solution with a minimal amount of chloroform. A column of silica gel (Fisher Certified, 100 to 200 mesh), 2 by 25 cm, was prepared by slurring the activated silica gel into the column with chloroform. The column was loaded with the extract by using chloroform and then eluted with 500 ml of petroleum ether (bp 30 to 60 C) and approximately 3,000 ml of methylene chloride. The methylene chloride effluent was collected in 100-ml fractions and was monitored for the presence of F-2 in each container with the spectrophotometer. The best F-2 preparation was

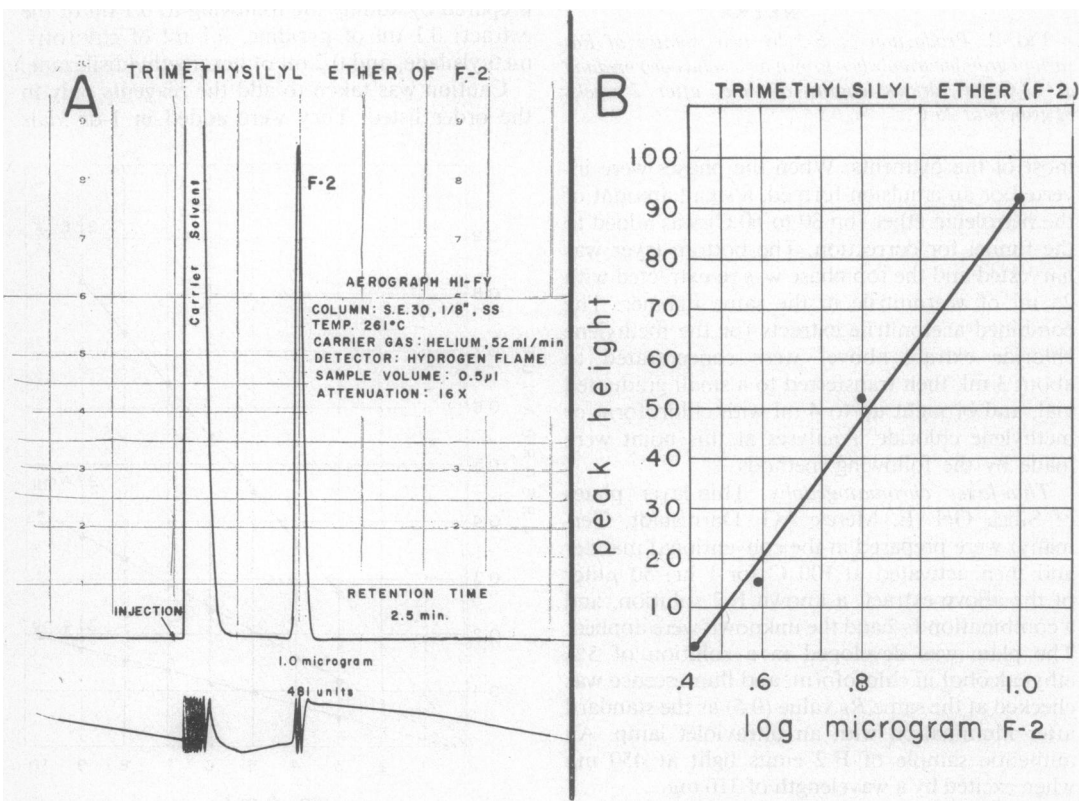


FIG. 4. *(A) Separation of the trimethylsilylether of F-2 by gas-liquid chromatography. (B) Straight line relationship between the peak height of the trimethylsilylether of F-2 and concentration between 0.4 and 1.0 μg when separated by gas-liquid chromatography.*

combined, concentrated, and saved for countercurrent distribution.

The effluent containing the F-2 was concentrated to a convenient volume (10 to 25 ml) for use on a 200-tube countercurrent distribution apparatus, each transfer tube of which holds 10 ml of top and 10 ml of bottom phase. The phase system which proved to be best was made up of petroleum ether (bp 30 to 60 C)–water–methanol –diethyl ether (2:1:3:5). In this solvent system, F-2 had a partition ratio (*K*) of 1.2. The equilibration time of this system was 1.5 min, and the F-2 was found in tubes 50 through 76 with the best preparation in tubes 62 and 63. These were combined and saved for crystallization.

Crystallization. The combined fractions obtained from the countercurrent distribution apparatus were concentrated. Enough chloroform was added so that all the material was in solution, and then petroleum ether (bp 60 to 70 C) was added drop by drop until a precipitate formed. The material was chilled in a refrigerator and then centrifuged, and the crystals were saved. The crystals were placed in solution again with chloroform and were reprecipitated with petroleum ether (bp 30 to 60 C). The cycle was repeated until white crystals devoid of any pigment were obtained. The purity of the compound was determined by separating by thin-layer chromatography and then charring with a methanolic solution of H_2SO_4.

Physical and chemical properties. The estrogenic metabolite herein referred to as F-2 has characteristic maxima of absorption in the ultraviolet at wavelengths of 314, 274, and 236 mμ (Fig. 5). Its melting point lies between 163 and 165 C. We have determined the molecular weight as 318 by both elemental and mass spectrometer analyses. Mass spectrometer analyses were made immediately after separation by gas-liquid chromatography and by conventional means with identical results. The proposed structure of the compound as reported by Andrews and Stob (Belgian Patent 611630 and U.S. Patent 3,196,019) and Urry et al. (8) is shown in Fig. 6.

Compound F-2 fluoresces blue-green when excited by ultraviolet radiation. Figure 7 depicts an emission spectrum obtained after irradiation at 314 and 274 mμ. As expected, the intensity of fluorescence was greater when excited with radiation at the higher wavelength. The fluorescence at 450 mμ is useful for the detection of the compound on thin-layer chromatoplates.

When treated with base in an ethyl alcohol solution, F-2 exhibited a characteristic bathochromic shift towards the higher wavelength. The band at 236 mμ shifted to 256 mμ, the weak band at 314 mμ became broad and strong, and the band of absorption at 276 mμ disappeared (Fig. 5). This bathochromic shift reversed immediately upon acidification.

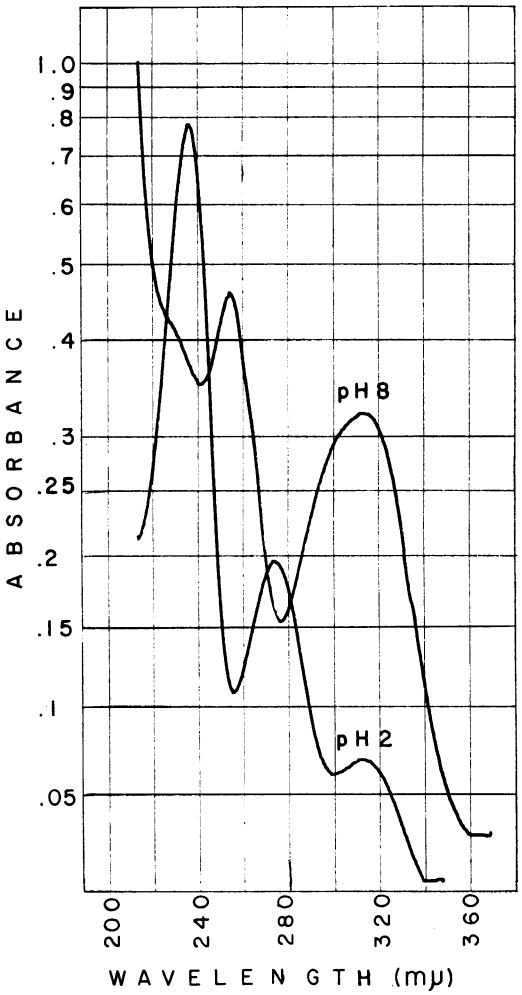
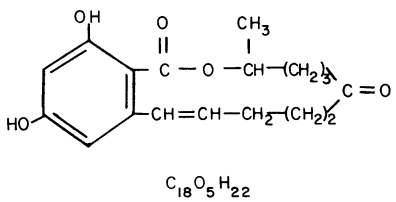


FIG. 5. *Absorbance of F-2 in the ultraviolet when measured at pH 2 in ethyl alcohol. Upon addition of base, a bathochromic shift to the higher wavelength results as can be seen by the curve labeled pH 8.*



$C_{18}O_5H_{22}$

FIG. 6. *Structural formula of the estrogenic factor, F-2.*

The estrogen gave a positive response for a

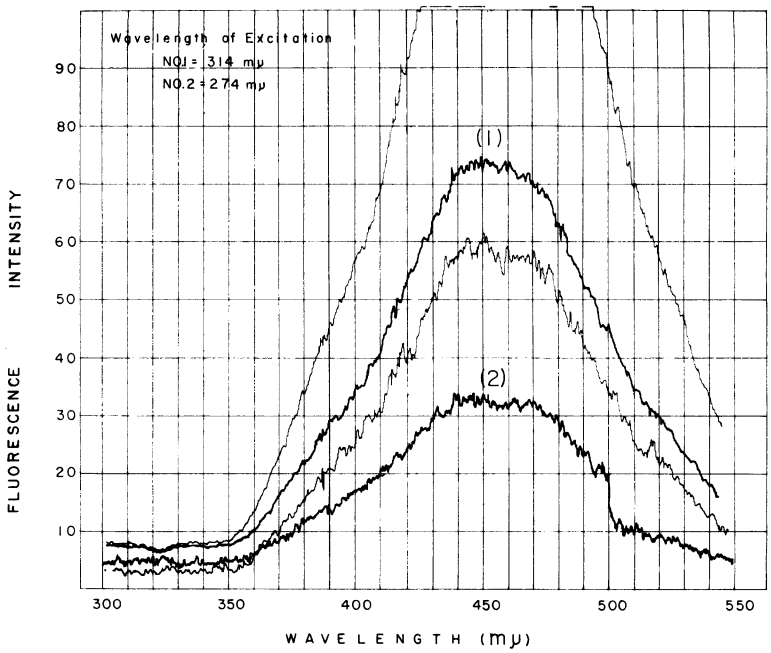


FIG. 7. *Fluorescence emission spectrum of F-2 in ethyl alcohol when irradiated at wavelengths of 314 and 274 mμ.*

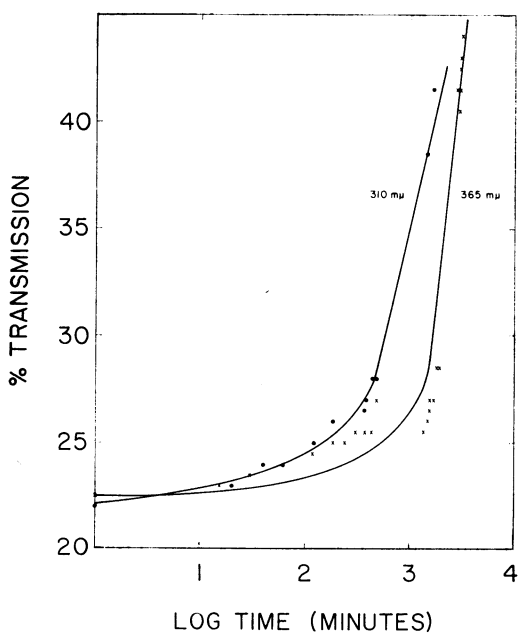


FIG. 8. *Sensitivity of F-2 in ethyl alcohol when subjected to continuous irradiation with ultraviolet lamps which emit strongly at wavelengths of 310 and 365 mμ.*

phenol with $FeCl_3$, and the Keto group reacted with 2,4-dinitrophenylhydrazine to form the corresponding hydrazone.

When the purified compound as separated on a thin-layer chromatoplate was sprayed with 50% H_2SO_4 in methanol, it immediately turned light-green and then quickly turned yellow; after charring at 100 C for 10 min, it turned a yellowish-brown.

The results involving the lability of F-2 when irradiated with ultraviolet radiation are in Fig. 8. The ordinate represents the percentage of transmission of F-2 as monitored at 274 mμ, although the entire spectrum was examined each time of sampling for any change. Similar curves were obtained when F-2 was irradiated at 312 and 365 mμ although F-2 broke down more quickly at the lower wavelength. The greatest change in its absorption spectrum took place between 8 and 24 hr when irradiation occurred at 310 mμ and between 31 and 47 hr at 365 mμ. It is concluded that the estrogen is relatively stable to ultraviolet irradiation when contained in ethyl alcohol. It would appear that this region of the spectrum does not afford a good means of degradation of F-2.

Heat stability of F-2. Tests were conducted in both absolute and 50% ethyl alcohol to determine the stability of the estrogenic factor. The absorption maximum at 276 mμ was monitored to determine whether any change had occurred over the period of heating.

When heated in absolute ethyl alcohol for 30 min at 60 C, no change occurred. When heated in 50% water-ethyl alcohol solution for 30 min at 60 C, no change occurred but at 111 C there was

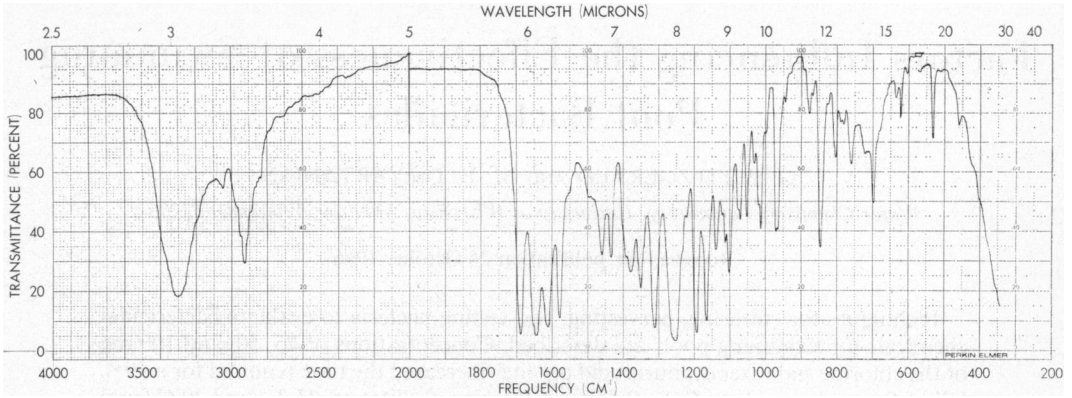


FIG. 9. *Infrared absorption spectrum of a KBr pellet preparation of F-2.*

a slow decomposition. Care should be taken to note that the change in the absorption spectrum was used as the criterion of evaluation and not of biological activity.

Infrared analyses. Analyses of the infrared absorption spectrum of F-2 were made in chloroform, Nujol mull, and KBr pellet. Best results were obtained by using the latter (Fig. 9). The strongest absorption maxima were found at the following frequencies expressed as cm^{-1}: 3,300, 2,925, 1,688, 1,645, 1,612, 1,578, 1,460, 1,435, 1,380, 1,350, 1,312, 1,254, 1,194, 1,166, 1,102, 1,070, 1,053, 1,013, 968, 845, 760, and 697. The greatest attention in the infrared study was paid to the presence or absence of the ketone and lactone function. The strong absorption at 1,688 cm^{-1} suggested a ketone or lactone function. To distinguish between the two, the keto group was reduced mildly with sodium borohydride and the resulting derivative was analyzed by infrared. The strong absorption band at 1,688 cm^{-1} disappeared, whereas the band at 1,645 remained. It is assumed that the latter absorption maximum represents the lactone function. Reduction of the ketone did not change the ultraviolet absorption spectrum.

The estrogenic factor herein described appears to be identical to the compound described by Andrews and Stob (Belgian Patent 611630 and U.S. Patent 3,196,019) and Urry et al. (8). It has been called by various trivial names such as "F-2" by the Minnesota group (1), "RAL" by Commercial Solvents Corporation, and more recently "Zearalenone" (8).

We have isolated this compound from samples of feed submitted to our laboratory and suspected to be involved in inciting the estrogenic syndrome in swine.

Studies are currently in progress concerning the biosynthesis of F-2 by *Fusarium* on various media as well as the metabolic pathway of its production by the fungus.

ACKNOWLEDGMENTS

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