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Detection of Diethylstilbestrol Contamination in Swine Feedstuff

Ten commercial preparations of pelleted swine feed associated with hyperestrogenism in swine were analyzed for the mycotoxin F-2 (zearalenone) in an effort to explain the estrogenic activity. None of the suspected fungal estrogen, F-2, produced by *Fusarium roseum* was found. Five of these samples were selected for more intensive investigation, and all five were found to contain diethylstilbestrol (DES) in concentrations of 240, 2400, 28, 90, and 1–10 ppb, respectively. All five samples, when incorporated into a nutritionally balanced rat diet and fed to 21-day-old female white rats, caused a significant increase in the uterine weight. As little as 0.8 to 1.0 mcg of authentic DES (total dose) increased the uterine weight of test rats to 150 mg, as compared to 32 mg for control rats. Final identification of DES was made by mass spectrometry and mass fragmentography.

One known cause of estrogenism in swine is F-2 (zearalenone), produced by *Fusarium roseum* Snyder & Hanson (and possibly by other species of *Fusarium*) growing in maize or in other ingredients of the feed (Christensen *et al.*, 1965; Mirocha *et al.*, 1967; Nelson *et al.*, 1970; Stob *et al.*, 1962). Over the past several years we have received numerous samples of feed from practicing veterinarians in Minnesota and other states suspected of being responsible for outbreaks of estrogenism in herds of swine. These were tested for F-2 (zearalenone), and also were fed to 21-day-old virgin female weanling white rats for 5–7 days, after which the rats were sacrificed and their uteri removed and weighed. In our experience this is a very reliable bioassay for estrogenic compounds in feedstuffs. Approximately 50% of the samples received did not contain detectable amounts of F-2, but caused an increase in the weight of uteri of rats to which they were fed. It was apparent that the hyperestrogenism was due to either an unidentified natural metabolite or to causes other than fungal contamination. We decided to look closely for the presence of minute quantities of diethylstilbestrol (DES) in such samples, although some of these samples had already been screened for DES using the standard colorimetric method as described in the AOAC manual (1971).

As judged by the increase in weight of the uteri of rats, DES is approximately 1000 times as potent as F-2. Since DES is added to many batches of feed compounded for beef steers, and since feeds for swine may be mixed and pelleted or otherwise processed in the same plant and in the same machines as those used to prepare feed for beef steers, it was thought that DES might occasionally get into swine feed as a contaminant or pollutant.

Amount of DES Necessary to Cause Enlargement of Uteri of Rats. Measured amounts of DES were incorporated into a nutritionally balanced ration developed for rats and were fed to rats. A total dose of 0.8–1.0 mcg, over a period of 5–7 days, resulted in an approximately fivefold increase in the weight of uteri of the rats (average of five controls, 32 mg; average of three rats given 0.8–1.0 mcg of DES, 150 mg). This amount is too small to be detected by the standard colorimetric method specified by the AOAC manual (1971).

Method Used to Detect Small Amounts of DES in Sample Feeds. The ground feed sample (6 kg) was moistened to about 15–20% water content and extracted with about 10 l. of ethyl acetate. The extract was dried over Na_2SO_4, concentrated under vacuum, and yielded 195.9 g of solids. The latter was partitioned between equal volumes of acetonitrile and petroleum ether (bp 30–60°) and the acetonitrile layer was extracted four times with petroleum ether. Each of the purification steps was checked for biological activity by incorporating an aliquot of the extract on a balanced ration and feeding to 21-day-old weanling female rats. The rats were sacrificed after 5 days and the degree of estrogenic activity was determined by weighing the uterus (Umberger *et al.*, 1958). The most active fraction was found in the acetonitrile (390 mg average uterine weight) as compared to the petroleum ether (75 mg).

The acetonitrile-soluble extract (19.1 g) was loaded onto a large column (55 mm o.d.) consisting of 850 g of Davison 923 silica gel. The column was developed with solvents of the eluotropic series in increasing order of polarity and starting with petroleum ether, 60–70°.

As shown in Table I, the estrogenic component was found in fractions 9 and 10 as evidenced by the rat uterus bioassay ($\frac{1}{120}$ of total fraction added to a balanced rat ration). Column fraction 9 was next chromatographed on

Table I. Purification by Column and Thin-Layer Chromatography (tlc) of the Estrogenic Component in the Extract of Swine Feed

Treatment	Solvent system	Total wt of solute	R_f	Average uterine wt,[a] mg
Column fraction 9	$CHCl_3$	00.30 g		229
Column fraction 10	$CHCl_3$–EtOAc (9:1)	01.97 g		237
Tlc-1, Band 3	$CHCl_3$–EtOH (97:3)	37.40 mg	0.14–0.22	
Tlc-1, Band 4	$CHCl_3$–EtOH (97:3)	24.70 mg	0.22–0.28	111
Tlc-2, Band 4	EtOAc–hexane (80:15)	9.43 mg	0.76–0.88	60
Tlc-3, Band 1	$CHCl_3$–EtOH (98:2)		0.08–0.12	39
Tlc-3, Band 2	$CHCl_3$–EtOH (98:2)		0.15–0.25	55.5
Tlc-3, Band 3	$CHCl_3$–EtOH (98:2)		0.25–0.30	163.5

[a] Average weight of the uterus of two rats.

silica gel G tlc (0.5 mm) and the estrogen was located in bands 3 and 4. The estrogenic activity in band 3 was determined from other tests, and in order to conserve solute, a bioassay in this experiment was not felt necessary. Activity was also found in band 4 of tlc-1. Band 3 of tlc-1 was again separated on silica gel G (tlc-2) and the estrogenic activity was found in band 4, which contained only 9.43 mg of solute. Only $\frac{1}{100}$ of this fraction was added to 150 g of ration for the rat uterus bioassay. Fraction 4 (9.43 mg) was rechromatographed on silica gel G using $CHCl_3$–EtOH (98:2) as the developing system and divided into three fractions. About $\frac{1}{20}$ of each fraction was used in the rat uterus bioassay. The fraction on the silica gel resolved into three distinct components and estrogenic activity was found between R_f 0.15 and 0.30 (tlc-3). Band 1 (tlc-3) corresponded to the R_f value of the cis isomer of diethylstilbestrol. Band 2 (tlc-3) appeared as one distinct component on the tlc plate but was resolved into five components when subjected to analysis by glc. Band 3 (tlc-3) corresponded to the R_f value of the trans isomer of diethylstilbestrol.

A less detailed and more direct method of DES analysis was developed employing the LKB-9000 gas chromatograph–mass spectrometer (gc–ms). The use of the mass spectrometer would confirm the identity of DES without any equivocation. The ground feed sample was mixed with 10% diatomaceous earth and extracted in a Soxhlet extractor overnight with chloroform–ethanol (93:7, v/v), followed by partitioning the extract first with 1 N H_2SO_4, and then with 1 N NaOH. The pH of the NaOH extract was then adjusted to 9.0 and extracted with $CHCl_3$. The $CHCl_3$ extract was dried with anhydrous Na_2SO_4 and concentrated to dryness. For quantitation by glc, a substantial amount of cleanup was necessary using multiple developments on tlc plates. The solute was taken up in acetone, spotted on plates of silica gel G (0.5 mm), and first developed in $CHCl_3$–EtOH (97:3, v/v). The area corresponding to DES, as based on standards, was eluted off the silica gel with acetone and respotted on another tlc plate and developed in ethyl acetate–hexane saturated with H_2O–ethanol (80:15:5, v/v). The same procedure was repeated and the solute was developed in $CHCl_3$–EtOH (98:2). The area corresponding to the R_f of both the cis and trans isomers of DES was eluted with acetone and concentrated, and TMS ether derivatives were made using *N,O*-bis(trimethylsilyl) acetamide. Quantitation was done on the basis of the trans isomer on a Varian gas chromatograph equipped with a flame ionization detector.

For qualitative identification, the TMS ether derivatives of the solute were prepared after a single separation on silica gel G using $CHCl_3$–EtOH (97:3), and these were injected directly into the LKB-9000 gc–ms, using 3% OV-1 column. The total ion current (TIC) chromatogram of the TMS ether derivatives can be seen in Figure 1. Because of the minute amount of DES present, it was impossible to detect DES on the total ion recorder because of interfering substances. Simultaneously, a mass fragmentograph of the sample was run on the oscilloscopic recorder with the ion monitor set at mass 412 (mass of TMS ether of DES). As shown in Figure 2, M/e^+ ions of both the cis and trans isomers of the TMS ether derivative of DES were found, buried among other components. After the location of the

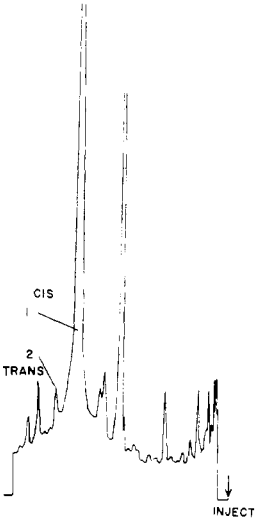

Figure 1. Total ion current chromatogram of the TMS ether derivatives of field sample 273B after injection and separation on the LKB-9000 gc–ms. Numeral 1 denotes the presence of the cis isomer of DES buried under a large peak and numeral 2 denotes the trans isomer.

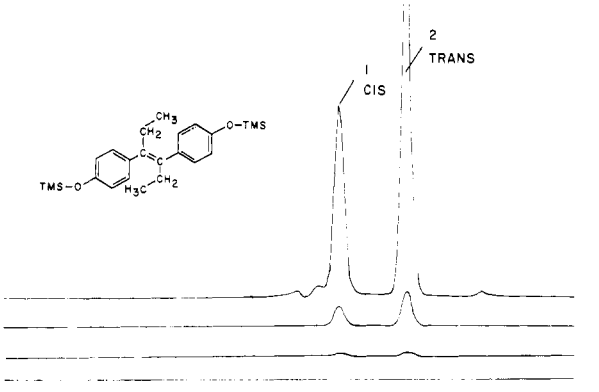

Figure 2. Mass fragmentogram of the TMS ether derivative of DES from field sample 273B monitored at mass 412 on the oscilloscopic uv recorder of the LKB-9000 gc–ms. Numerals 1 and 2 correspond to the location of the cis and trans isomers of DES as shown in Figure 1. Note that the cis isomer is only a minor component of the large peak shown.

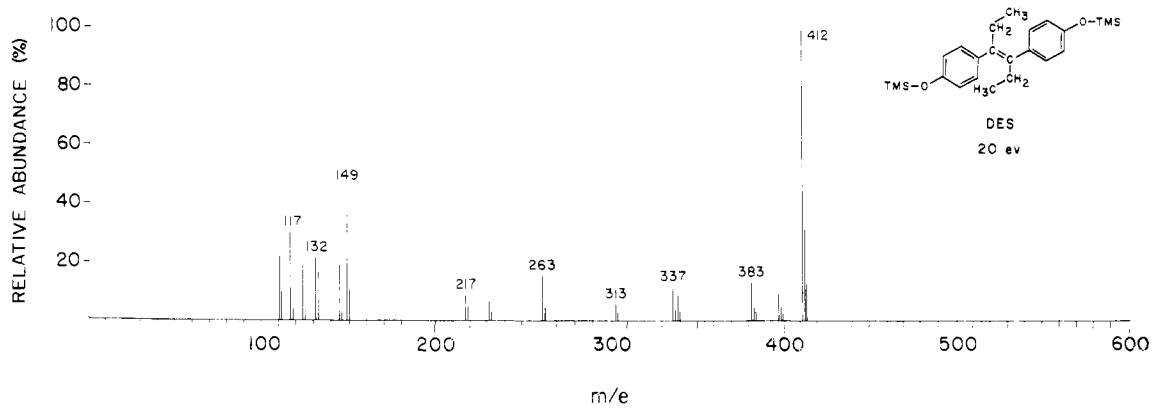

Figure 3. Mass spectrum of the TMS ether of the trans isomer of DES found in field sample 273B. The scan was taken at the time peak number 2 of Figure 1 emerged on the total ion current recorder.

trans isomer of DES on the TIC recording was determined (see no. 2 in Figure 1), the sample was injected once more into the gc–ms and this time a scan of the fragmentation pattern was taken in the area of interest. A plot of the fragmentation pattern obtained is shown in Figure 3. In this case, the scan was made at 20 eV instead of the usual 70 eV, resulting in a very intense M/e^+ which was the base peak. To confirm the presence of DES, the identical procedure was repeated but this time the methyl derivatives were made on column by injecting the sample together with Methelute (Pierce Chemical Corporation; trimethylanilinium hydroxide) and monitoring at mass 296. Identical results confirming the presence of the cis and trans isomers of DES were found as with the TMS derivatives; *i.e.*, the mass fragmentograph and the mass spectrum confirmed the presence of the dimethoxy derivative of DES.

Both the cis and trans isomers of DES were found in all five samples analyzed. The cis isomer is generally thought to be inactive, although Winkler *et al.* (1971) reported the the cis form has 45% of the activity of the trans.

Samples of Swine Feed Suspected of Having Been Responsible for Estrogenism in Swine, and in Which Diethylstilbestrol Was Detected. Feed sample coded 281, a commercially prepared pelleted sow ration, was sent to us from Iowa by the Food and Drug Administration. When fed to swine, this ration caused the estrogenic syndrome. When this feed was cultured for fungi, *Aspergillus glaucus* and *Penicillium* spp. were common, but no colonies of *Fusarium* were found. The organic portion of this foodstuff consisted of 63% maize, 17.5% soybean meal, and 10% wheat middlings. The pellets were placed on a screen of a 0.5-mm mesh and soaked in water until they disintegrated. Washing was continued until nearly all the particles smaller than 0.5 mm washed through. The fragments remaining were predominantly corn and wheat. The average weight of uteri of rats fed this fraction (25 g) was 144 mg, as compared to 41 mg for the control. When the whole ration (40 g) was fed to the rats in another test, the average weight of the uteri of two rats was 325 mg, as compared to 32 mg for the controls.

Feed sample 295 was also a commercially prepared pelleted feed brought to the Diagnostic Laboratory of the College of Veterinary Medicine from Aitkin, Minn. This was a swine ration thought to be responsible for vaginal prolapse in swine. As determined by plating on agar, *Absidia* was the predominant fungus, with a few colonies of *Rhizopus* and *Mucor*. The predominant cereal base of this feed was barley; no maize was used. When this ration (25 g) was tested in female rats, the uterine weight of four rats averaged 134 mg, whereas the uteri of the control rats averaged 39 mg.

Sample PV-1963 was the pelleted feed first reported to be estrogenic by Christensen *et al.* (1965), and which caused the estrogenic syndrome in a swine herd near St. Paul, Minn. Analysis of this feed in 1965 and 1966 revealed F-2 in a concentration of about 2000 ppm. When this same feed was analyzed for F-2 in 1971, F-2 was not found. However, when this ration (35 g) was fed to rats, the uterine weight of two rats averaged 228 mg and the controls averaged 40 mg.

Feed sample 273B was a pelleted, mixed feed suspected of being involved with an outbreak of vulvovaginitis in swine in Iowa. Analysis for fungi as determined by plating on agar revealed *Aspergillus glaucus* almost exclusively, with only a few colonies of *Alteraria* sp. and *Rhizopus* sp. appearing. The results of the uterotrophic test are recorded in Table II.

Feed sample 193B was a cubed sow ration sent to the University of Minnesota from Iowa. This feed was associated with signs in swine that included abortion, and at the time of farrowing small weak pigs were born. No appreciable amount of fungi could be found upon subculturing the ration on agar. Consult Table II for results of rat uterotrophic test.

The concentration of DES in each sample, expressed in mcg/g of feed, was determined by glc on the basis of the trans isomer. The results can be found in Table II.

Band 2, tlc-3 (Table I) found in field sample 281, showed a consistent uterotrophic response when fed to female rats. The five components detected within this band, TMS ethers by glc, had masses of 482, 492, 518, 520, and 548. The last four had the same base peak and fragmentation pattern and are thought to be derivatives. The possibility that there may have been minute amounts of the trans isomer of residual (from the silica gel) DES mixed in with band 2 remains and may account for the

Table II. Field Samples of Commercial Feeds and the Results of Chemical and Biological Analysis for DES

Sample no.	DES	Rat uterus wt, mg (experimental sample)	Rat uterus wt, mg (control)
PV pellets	240 ppb	228 ± 28[a]	40 ± 4
FS no. 281	2400 ppb	325 ± 42[a]	32 ± 4
FS no. 295	28 ppb	134 ± 35[b]	39 ± 12
FS no. 273B	90 ppb	442[b]	44 ± 6
FS no. 193B	1–10 ppb	276 ± 41[b]	42 ± 2

[a] 100% of the ration fed to rats consisted of suspect feed; average of two rats. [b] 50% of the ration (25 g/rat) consisted of suspect feed and the remainder was made up of a nutritionally balanced rodent diet. Average of two rats.

estrogenic activity. No further work with band 2 is contemplated.

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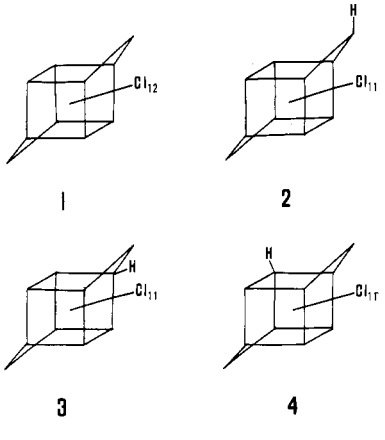
Photochemistry of Mirex

The photolysis of dodecachloropentacyclo[5.3.0.$0^{2,6}$.$0^{3,9}$.$0^{4,8}$]decane (Mirex) in hydrocarbon solvents yielded two major products. These photoproducts have been identified as a monohydro derivative and a dihydro derivative. Possible structures for the derivatives (based on nmr, ir, and mass spectra) are discussed.

The insecticide Mirex (dodecachloropentacyclo[5.3.0.$0^{2,6}$.$0^{3,9}$.$0^{4,8}$]decane), structure 1, has been used extensively in the southern United States to control the imported fire ant. This persistent chlorocarbon has been implicated as a possible environmental problem in several recent papers (Lowe *et al.*, 1971; Ludke *et al.*, 1971; Van Valin *et al.*, 1968). In this report, evidence of Mirex degradation by photolysis in hydrocarbon solvents is presented. In all cases, the elemental analyses, mass spectra, and nmr spectra indicated that chlorine atoms were replaced with hydrogen atoms.



In an investigation of some chemical reactions of Mirex and related compounds, Dilling *et al.* (1967) synthesized several hydrogen derivatives of Mirex. Each product was characterized by elemental analyses, infrared, and nmr spectra. One monohydro derivative was assigned structure **2** (1,2,3,4,5,5,6,7,8,9,10 - undecachloropentacyclo[5.3.0.$0^{2,6}$.$0^{3,9}$.$0^{4,8}$]decane), and another monohydro derivative was assigned either structure **3** (1,2,3,4,5,5,6,7,8,10,10-undecachloropentacyclo[5.3.0.$0^{2,6}$.$0^{3,9}$.$0^{4,8}$]decane) or **4** (1,2,3,4,5,5,6,7,9,10,10 - undecachloropentacyclo[5.3.0.$0^{2,6}$.$0^{3,9}$.$0^{4,8}$]decane). The mass spectra of these compounds (Dilling and Dilling, 1967) indicated that the predominant modes of fragmentation are dechlorination and cleavage of the pentacyclodecane skeleton in half.

EXPERIMENTAL SECTION

Technical Mirex, donated by the Allied Chemical Corp., was recrystallized three times from benzene and vacuum dried for 6 hr at 200°. Eastman technical isooctane was distilled twice through a five-ball Snyder column. After being washed with concentrated sulfuric acid, Eastman technical cyclohexane was passed through a silicic acid column, fractionally frozen, and then distilled. Gas chromatography and ultraviolet spectroscopy were used to establish the purity of all compounds used in the study. Spectral studies were performed with a Perkin-Elmer Model 270 mass spectrometer, a Perkin-Elmer Model 521 infrared spectrophotometer, and a JEOL Model MH-60II nuclear magnetic resonance spectrometer. Elemental analyses were performed by Galbraith Laboratories, Knoxville, Tenn.

Mirex in cyclohexane or isooctane (350 ml, 0.04 *M*) was placed in an Ace photochemical reactor and irradiated with a Hanovia 450 W, medium pressure, mercury lamp. No differences in the course of the reaction in these two solvents were observed. The Pyrex photochemical reactor and the quartz immersion well were water cooled in order to keep the reaction temperature below 32°. The solutions were stirred with a magnetic stirring bar and continuously aerated. The reactions were monitored with a Varian Aerograph Model 1400 gas chromatograph equipped with a flame ionization detector (column: 4 ft × 1 mm i.d.; 0.35% SE-30 on 100/120 mesh textured glass beads; 220°). Two major products were detected (retention times relative to Mirex were 0.70 and 0.55).

When 95% of the Mirex had disappeared (48 hr), the photoproducts (40% yield) were separated from the solution. Preliminary purification procedures involved evapor-