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Feed Refusal Factors in Pure Cultures of *Fusarium roseum 'graminearum'*

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Isolations from 1972 Wisconsin feed refusal corn yielded predominantly cultures of *Fusarium roseum 'graminearum.'* With one possible exception, none of the selected isolates of this fungus induced emesis in pigeons, whereas six of nine isolates produced feed refusal responses in all test animals. A single isolate of *F. roseum 'equiseti'* also induced a severe refusal response and possibly slight emesis. None of the other fungi isolated from this corn (*F. moniliforme, Acremoniella atra*) or controls caused either emesis or feed refusal. Zearalenone was detected in all isolates and was shown to be partially responsible for refusal activity. The remaining activity was ascribed to one or more nonvolatile, neutral, relatively polar molecules. T-2 toxin, although not detected in these isolates, was shown to have dramatic refusal activity in rats.

The 1972 growing season was particularly favorable for the development of *Gibberella* stalk and ear rot (*Gibberella roseum 'graminearum'* = *G. zeae* = *Fusarium roseum 'graminearum'*) on corn (*Zea mays*) in the north central United States (6). In most areas temperatures were generally cool, rainfall frequent and often heavy during much of the late summer and autumn. At crop maturity ears on a high percentage of field plants (as much as 25% in some Wisconsin fields) were invaded by *F. roseum*. The extent of invasion varied from minor tip rot to almost complete decay of the ear. The situation was further aggravated by a delayed harvest due to wet conditions and by a shortage of natural gas for artificial drying of the corn.

Heavily infested lots of this corn when fed to swine resulted in feed refusal and occasionally emesis (5). Refusal was evident with this corn even when it was later artificially dried or fermented as high moisture corn in sealed silos. Symptoms of vulvo-vaginitis (9) were generally absent. Cattle, horses, and poultry failed to develop any obvious problems after ingestion of similarly infested corn. Later development of other potentially toxic fungi in crib-stored, high-moisture corn made the overall field problem even more complex.

In previously reported epiphytotics of this kind, it has been suggested that ears of corn contain at least three different physiologically active factors which affect swine (3). One, zearalenone (6-[10-hydroxy-6-oxo-*trans*-1-undecenyl]-3-resorcylic acid lactone) (15), is anabolic and at appropriate concentrations causes uterine hypertrophy (7, 8; F. N. Andrews and M. Stob, U.S. patent 3,196,019, 1965). A second factor attributed to various tricothecenes (4, 13, 14, 16) induces vomiting (emesis), while a third is reported to cause feed refusal (3).

Metabolites responsible for swine feed refusal have not been identified, nor has really definitive evidence been presented to suggest that feed refusal is a separate phenomenon from either emesis or uterine hypertrophy as was suggested by Curtin and Tuite (3). In their feeding trials using refused feed samples naturally infested with *F. roseum*, however, uterine hypertrophy was not observed in test animals. Recently Roine et al. (10) observed typical refusal accompanied by a decrease in weight gain in rats fed a diet of pure cultures of *F. graminearum* (=*F. roseum*) grown on moist, sterilized oat-barley-wheat grains. This diet was shown to contain 9.55 μg of zearalenone per g. In these studies, *F. tricinctum* cultures similar to those known to produce trichothecenes, failed to cause either feed refusal or weight loss when compared to controls.

We report here the results of investigations which demonstrate the production of specific feed refusal factors by pure cultures of *F. roseum 'graminearum'* isolated from 1972-crop moldy corn. In addition, a practical laboratory

bioassay for feed refusal was developed and used to initiate purification and chemical characterization of these factors.

MATERIALS AND METHODS

Cultures. Representatives of the major components of the fungal flora in refusal corn were isolated in pure culture from Wisconsin field samples which were selected in November 1972 at the height of the feed refusal period (Table 1). Kernels from rotted ears of both field corn and sweet corn were surface disinfected for 10 min in a solution of 0.32% sodium hypochlorite, dried on clean paper towels, and incubated at room temperature on either potato dextrose agar or 2% water agar. Single conidial cultures were derived from pure cultures obtained as hyphal transfers from the original isolation plates. Mature perithecia present on corn husks of rotted ears were fastened to petri dish lids with masking tape, allowed to discharge ascospores onto water agar plates, and the resulting single germinating ascospores were transferred aseptically to potato dextrose agar tube slants. For long-term storage, fungus cultures were held on sterile soil in glass vials (12).

From several hundred fungal isolates derived from these samples, 12 strains representing the predominant fungi were selected for detailed animal feeding trials. Pure cultures of each were grown in 500-ml Erlenmeyer flasks on moistened sterilized corn for one week at room temperature followed by four weeks at 12 C, oven dried at 50 C for 2 days, ground in a Wiley mill, and then stored frozen. In addition, isolate no. 3 was also grown at room temperature for 5 weeks.

Feed refusal and weight change in pigs. Ten Chester White and 16 Hampshire pigs (average weight = 22.7 kg) in pens (1.2 by 2.4 m), two animals per pen, were fed a standard commercial swine ration for 2 days. After this, pigs in each pen received different ground, dry, fermented corn cultures (3.6 kg/day per pen) as their sole diet. The two control animals received similar amounts of unfermented ground corn from the same source of corn used to prepare the original cultures. The feed remaining each morning was weighed before the introduction of fresh feed. Animals were weighed immediately before confinement and every other day thereafter. The study was concluded on the 6th day after a 96-h exposure to the test diets (Table 2).

Feed refusal, weight change, and estrogenism in rats. Young (21 day old) female Sprague-Dawley rats (Holtzman strain), caged individually, were fed dried, ground corn cultures (five rats per isolate) ad libitum as their sole diet. An equal number of control rats were fed dried, ground, unfermented corn. The feeding period lasted 3 days (72 h) with the day length adjusted for 12 h. Trials were begun at 4:00 p.m. Friday and concluded at 10:00 p.m. Monday (66 h). Both rats and test diets were weighed initially and at the end of the study (Table 3). In all cases, the rats were starved for 8 h before initiating the assay.

To determine possible estrogenic effects of fermented corn cultures rats were killed using chloroform, and both the uterus and ovaries were removed, carefully separated from surrounding fatty tissue, and weighed (Table 3).

Statistical treatment of rat bioassays. Analysis of covariance on the control (C) and test (T) rat groups were performed in all assays. In each case, due to the relatively small in-group variances, a mean difference ($\bar{D} = \bar{T} - \bar{C}$) of 1.5 and 2.0 was found to be sufficient to reject the null hypothesis (H_0: $\bar{T} = \bar{C}$) at the 0.05 level of significance (using a two-tailed test) for percentage of body-weight change and feed consumption (g), respectively.

Emesis testing. All isolates were examined for emetic activity using an earlier procedure (5). Ground corn cultures (200 g/culture) were extracted with ethyl acetate (600 ml) by continuous stirring for 24 h. The solvent was removed in vacuo and the oily, yellow residue was force-fed (0.5 ml/dose) to adult pigeons. Control pigeons were fed similar extracts from unfermented corn. A positive response consisted of a distinct vomiting episode occurring within 35 min followed by further episodes over a 1-h period. Emetic activity in pigs, if any, was noted during refusal tests with the corn cultures.

Chemical fractionation. Ground corn (500 g) inoculated with isolate number 3 (the most active refusal isolate) was extracted with ether (200 ml) in a soxhlet apparatus (30 extractions per h) for 3 h. The resulting solution was washed two times with 100-ml portions of 5% sodium bicarbonate. The aqueous layer was washed twice with 100-ml portions of ether and the ether extracts combined to give fraction A.

The aqueous layer was acidified with 1 M hydrochloric acid (125 ml) and extracted three times with ether as before. The combined ether layers were washed with two 100-ml portions of saturated sodium chloride and dried over sodium sulfate to yield fraction B (strong acids). The aqueous layers were discarded.

Fraction A was shaken with two 100-ml portions of 0.1 M sodium hydroxide. The aqueous layers were backwashed with ether as before and the organic extracts were combined to yield fraction C. The aqueous phase was made acidic with 1 M hydrochloric acid and extracted with ether. The ether layers were backwashed with saturated sodium chloride to yield fraction D (weak acids).

Fraction C was shaken twice with 100-ml portions of 1 M hydrochloric acid, the aqueous phase was backwashed with ether, and the combined organic layers were washed with saturated sodium chloride to yield fraction E (neutral).

In a separate experiment, isolate number 3 (250 g) was extracted as above and the resulting ether solution was shaken with two 100-ml portions of 1 M hydrochloric acid. After washing with ether (100 ml) the aqueous layer was made basic with 1 M sodium hydroxide and extracted three times with 100-ml portions of ether to give fraction F (bases).

For feeding studies, the above ether solutions were added to ground, dried, unfermented corn, and

the ether was allowed to evaporate until no trace of solvent could be detected. This corn was used as feed for rats in the test groups. Rats used as controls against each test fraction were fed corn impregnated with the equivalent fraction from normal, unfermented corn which had been subjected to the same fractionation scheme. This procedure was adhered to in all subsequent assays.

Isolation of zearalenone. To isolate zearalenone from corn cultures of *F. roseum*, 200 g of ground, dried culture was extracted for 3 h with ethyl acetate using a soxhlet apparatus (30 extractions/h). Evaporation of the solvent yielded an oil (5.1 g) which was dissolved in Skelly B (500 ml) and extracted twice with 300 ml of 5% aqueous sodium bicarbonate-saturated sodium chloride (2:1, vol/vol). The aqueous layer was discarded and the organic layer was extracted three times with 5% aqueous sodium carbonate (200 ml). The aqueous layers were combined, adjusted to pH 6.0 with 1 M hydrochloric acid, and extracted five times with chloroform (200 ml). The combined extracts were dried over sodium sulfate and evaporated to yield a yellow residue (54 mg). The residue was purified by preparative thin-layer chromatography using chloroform-methanol-water (70:30:1, vol/vol. Zearalenone (8 mg) was recovered from a band having R_f = 0.67 to 0.77 and an intense blue-green fluorescence under ultraviolet light. The compound was characterized by mixed TLC in 2 solvent systems and comparison of its proton magnetic resonance spectrum and mass spectrum with an authentic sample of zearalenone.

Quantitation of zearalenone in cultures. A solution of zearlenone in chloroform was serially diluted and duplicate aliquots (2 μl) of each concentration were spotted on TLC plates (Silplate F22) every 1 cm with 2-cm margins at plate edges. Plates were developed to 12 cm with Skelly-B-chloroform-methanol-water (50:35:15:0.5, vol/vol) and examined under ultraviolet light (365 nm). The minimum detectable amount of zearalenone (0.25 $\pm$ 0.005 μg) was used to determine the concentration in the moldy corn samples. For this purpose, each feed sample (100 g) was soxhlet extracted with ethyl acetate as above. Two-microliter aliquots of serially diluted chloroform solutions of these extracts were spotted on plates alongside standards and the zearalenone concentration was determined by comparison (Table 3).

RESULTS

Fungal microflora. Isolations from surface-disinfected samples of moldy refusal corn kernels (field or sweet) yielded a rather restricted fungal flora. Over 90% of the mass hyphal transfers from isolation plates developed into *F. roseum 'graminearum'* cultures (Table 1). The remainder were predominantly strains of *F. moniliforme*, *F. roseum 'equiseti'*, or *Acremoniella atra*. Ascospores from mature *Gibberella* perithecia present on the outer husks of refusal corn yielded only pure cultures of *F. roseum 'graminearum'*. Older cultures of *F. roseum 'equiseti'*, however, formed perithecia apparently identical to that described for *G. intricans* (=*G. roseum 'intricans'*) originally used by Brian et al. (1) to produce the trichothecene diacetoxy-scirpenol (3-hydroxy-4,15-diacetoxy-12,13-epoxy-Δ^9-trichothecene).

Swine feed refusal. Of the nine isolates of *F. roseum 'graminearum'* from ascospore and conidial isolations tested for refusal activity, measured by either feed consumption or weight loss, all but one produced physiologically active quantities of the refusal factor(s) (Table 2). The isolate of *F. roseum 'equiseti'* (no. 9) was also highly active and produced a refusal response equal to the *F. roseum 'graminearum'* strains. *F. moniliforme* and *Acremoniella atra* were inactive.

With the exception of *F. roseum 'graminearum'* strains no. 10 and 11 which were fed only to Hampshires, each strain was fed to one Hampshire and one Chester White in the same pen. The Hampshires appeared more sensitive to the refusal factor. For purposes of statistical analysis, however, values obtained were tabu-

TABLE 1. *Sources of fungal cultures isolated from 1972 Wisconsin corn associated with feed refusal in swine*

Strain no.	Pure culture used as fermenting organism	Source
1	*Gibberella roseum 'graminearum'*	Field corn-single ascospore
2	*Gibberella roseum 'graminearum'*	Sweet corn-single ascospore
3	*Gibberella roseum 'graminearum'*	Sweet corn-mass ascospore
4	*Gibberella roseum 'graminearum'*	Sweet corn-single ascospore
5	*Fusarium roseum 'graminearum'*	Field corn-single conidium
6	*Fusarium roseum 'graminearum'*	Field corn-single conidium
7	*Fusarium moniliforme*	Field corn-single conidium
8	*Fusarium roseum 'graminearum'*	Field corn-single conidium
9	*Fusarium roseum 'equisiti'*[a]	Field corn-single conidium
10	*Fusarium roseum 'graminearum'*	Field corn-single conidium
11	*Fusarium roseum 'graminearum'*	Sweet corn-single conidium
12	*Acremoniella atra*	Sweet corn-single conidium
13	Uninoculated control	

[a] *Gibberella initricans* (= *G. roseum*) stage present in old cultures.

TABLE 2. *Percent body weight change and feed consumption of pigs*

Fungal strain[a]	% Wt change/pig[b, c]	Feed consumption/pig/4 days (kg)
1	−8.5	1.25
2	−11.3	1.86
3	−10.9	0.15
4	−5.9	2.27
5	1.7	4.38
6	−8.5	0.46
7	1.5	4.84
8	−8.5	1.31
9	−14.6	1.03
10	−14.5	0.40
11	−15.7	0.40
12	−1.7	4.66
Control	00.0	4.66

[a] See Table 1.

[b] Percent weight change after 4 days for test and control pigs calculated from initial and final weights of pigs.

[c] Pigs showing weight loss before addition of test feed were not included in these calculations.

lated as averages per pair. Body weight changes were expressed as percent change based on initial weights taken at the beginning of the test diet and strongly correlated (r_s = 0.81, L.S. < 0.01) (11) with corresponding reluctance to eat as measured by total feed consumed (Table 2). Two female Chester White showed distinct signs of hyperestrogenism induced by corn cultures of *F. roseum 'graminearum'* strains no. 3 and 4.

Rat feed refusal. The potential of rats for use as a more convenient assay animal was examined in a 3-day study. Groups of five rats were fed diets consisting of the same corn cultures used in the pig study. Feed refusal activity was measured as the percent body weight change and feed consumption as compared to controls. All test feeds in this trial resulted in weight loss, feed refusal, and, with the exception of strain no. 7 (*F. moniliforme*), increased fresh weight of the uterus plus ovaries.

Emesis. Using an earlier procedure (4), strains 1 to 12 were examined for emetic activity by feeding evaporated residues of ethyl acetate culture extracts to pigeons. None of the samples proved active with the possible exception of strains 8 (*F. roseum 'graminearum'*) and 9 (*F. roseum 'equiseti'*), which induced a limited, barely detectable vomiting response. Verification of these two isolates was complicated by the fact that the pigeons also vigorously refused these samples by expectoration. Emesis was never observed, however, in pigs during free-choice feeding studies.

Chemical fractionation. Feeding studies using the rat assay showed that although refusal activity was extracted by ethyl acetate, substantial activity still remained in the corn residue. Later work showed that improved yields of activity could be obtained by extraction with methanol, suggesting that the factor might be somewhat polar. Activity was retained in the methanol solution even after repeated washing with Skelly B. Evaporation of ethyl acetate yielded an oil which was active in both rat and pig assays. The refusal factor was presumed relatively nonvolatile after exposure of the oil to high vacuum distillation (0.2 μm, 2 h, 50 to 60 C) yielded an inactive distillate and an active residue in the rat assay. When the oil was heated at 110 to 115 C for 2.25 h under a nitrogen atmosphere, most activity was lost, suggesting that the refusal factor(s) may be thermally labile.

The gross chemical nature of the refusal factor(s) was explored by fractionation into base, neutral, strong, and weak acid fraction by conventional liquid-liquid partition. No activity could be found in either the base or strong acid fraction. Activity was found consistently in the weak acid (e.g., phenols) fraction although it was not sufficient to account for all of the activity of the feed. Activity was also found in the neutral fraction and it later became evident that the extent of activity depended upon the degree to which the aqueous phase was backwashed with ether during the extraction. The weak acid fraction consisted predominently of one active compound which was isolated by preparative TLC and shown to be zearalenone by comparison with an authentic sample.

Zearalenone content. Samples of ground corn cultures of *F. roseum 'graminearum'* (no. 2, 3, 5, and 11), *F. roseum 'equiseti'* (no. 9) and *F. moniliforme* (no. 7) were extracted with ethyl acetate and the concentration of zearalenone was determined by dilution to extinction on TLC plates. All samples examined contained zearalenone with concentrations ranging from 4.0 ± 1.6 to 42.8 ± 10.6 μg/g (Table 3). TLC analysis of a second extraction of the corn residue showed that complete extraction of the zearalenone was effected by the initial ethyl acetate procedure. Comparison of increased uterine and ovary weight over controls for each test rat gave a near perfect correlation (r_s = 0.96, level of significance [L. S.] < 0.01) with determined increased zearalenone consumption (vide infra).

Zearalenone and trichothecene feeding. Zearalenone and/or T-2 toxin (3-hydroxy-4,15-diacetoxy-8-isovaleroxy-12,13-epoxy-Δ^9-trichothecene) were examined in a rat feeding trial

TABLE 3. *Percent body weight change, feed consumption, and estrogenic activity in rats after 3-day feeding of isolates containing zearalenone*[a]

Strain no.[b]	Control	2	3	5	7	9	11
% Wt. change[c]	6.0	−5.0	−1.3[g]	−2.3	−0.2	−7.5	−1.8
Δ % Wt. change[d]		−11.0	−10.2	−8.3	−6.2	−13.5	−7.8
Feed consumption (g)	23.4	7.9	10.1	12.6	13.5	12.8	11.8
Δ Feed consumption (g)[e]		15.5	13.3	10.8	9.9	10.6	11.6
Uterus plus ovaries weight (mg)	50 (11)[f]	85 (27)	139 (37)	112 (33)	47 (27)	70 (21)	84 (21)
Zearalenone (μg/g)[h]	0.0	22.8 ± 6.7	47.8 ± 10.6	17.8 ± 6.0	4.0 ± 1.6	7.2 ± 2.4	13.8 ± 5.4

[a] Mean values of five rats.
[b] See Table 1.
[c] Weight change (%) calculated from initial and final weight of rats.
[d] Δ Weight change (%) = percent test weight change − percent control weight change.
[e] Δ Feed consumption = control feed consumption − test feed consumption.
[f] Standard deviations in brackets.
[g] Rat control for no. 3 was 8.9%.
[h] Zearalenone concentrations determined by dilution to extinction with errors propagated.

using a diet of normal ground corn impregnated with 50 or 200 μg of zearalenone per g alone, 5 or 50 μg of T-2 toxin per g alone, or in combinations. With zearalenone alone, both concentrations resulted in small but significant feed refusal activity (Table 4). Whereas no relative decrease in body weight was observed at 50 μg/g, at 200 μg/g test rats lost weight slightly as compared to controls. Both T-2 toxin concentrations resulted in considerable feed refusal and weight loss when compared to controls. In the presence of 50 μg of zearalenone per g (the approximate concentration present in corn cultures of isolate no. 3), 50 μg of T-2 toxin per g caused an enhancement of feed refusal and weight loss when compared to 50 μg of T-2 toxin per g alone. In the presence of 50 μg of zearalenone per g, 5 μg of T-2 toxin per g caused no significant change in feed refusal but a significantly smaller weight loss when compared to 5 μg of T-2 toxin per g alone. The smaller weight loss was presumably due to the anabolic activity of zearalenone. T-2 toxin (5 μg/g) plus zearalenone (50 μg/g) produced refusal activity comparably to isolate no. 3 in rats.

TABLE 4. *Effects of T-2 toxin and zearalenone on percent body weight change and feed consumption in rats after 3-day feeding*[a]

Contents of ground corn feed	Wt change per rat (g), (%)[b]	Feed consumption per rat (g)
Zearalenone (200 μg/g)	2.90 (4.6)	18.00[c]
Zearalenone (50 μg/g)	3.35 (6.2)	18.40[c]
T-2 Toxin (50 μg/g)	−11.00 (−19.6)	5.70
T-2 Toxin (5 μg/g)	−5.90 (−10.3)	10.30
Zearalenone (50 μg/g) − T-2 Toxin (50 μg/g)	−13.10 (−24.3)	2.86
Zearalenone (50 μg/g) − T-2 Toxin (5 μg/g)	−4.40 (−7.9)	10.70
Control corn	3.30 (5.9)	22.20

[a] Mean values of five rats.
[b] Weight change (%) calculated from initial and final weight of rats.
[c] Significantly different from controls at L.S. = 0.05.

DISCUSSION

Rejection of moldy corn can now be definitively associated with the low temperature growth of *F. roseum 'graminearum'* on parasitized corn. In pure corn culture studies, eight of nine isolates of this organism induced active feed refusal by pigs in ad libitum feeding studies. The strain of *F. roseum 'equiseti'* was also active, but none of the other fungi commonly present in the field samples induced refusal. These results are consistent with the reports of Roine et al. (10) who noted refusal by rats of pure cultures of *F. roseum 'graminearum'* grown in pure culture on moistened small grains. Refusal has previously been assumed to be associated with this organism (3), but corresponding feeding studies were only conducted with field-infested corn and thus failed to offer definitive proof-of-cause.

The fact that emesis was not evident in most of our *F. roseum 'graminearum'* corn cultures which clearly caused feed refusal provides evidence that these two phenomena are caused either by different metabolites, or by different concentrations of the same metabolite. The former possibility was suggested, but not clearly established, by earlier studies (3). The fact that

slight emetic activity may have been associated with one strain of *F. roseum 'graminearum'* (no. 8) and one strain of *F. roseum 'scirpi'* (no. 9) does not alter these conclusions. Burmeister et al. (2) reported that certain strains of *F. roseum 'graminearum'* in the NRRL culture collection produced moderate quantities of the emetic trichothecene T-2 toxin. Closely related diacetoxyscirpenol has been isolated from strains of *F. roseum 'scirpi'* (1). Careful analysis of crude extracts of our most active refusal isolates failed to detect known emetic trichothecenes such as T-2 toxin, HT-2 toxin, or 4-deoxynivalenol (3,7,15-trihydroxy-12,13-epoxy-trichothec-9-ene-8-one), although the presence of other trichothecenes is not excluded.

For practical purposes, in the chemical purification of refusal factors, laboratory rats were examined as possible assay animals. A 3-day feeding trial during the quiet weekend period provided the most consistent and least variable results. Under such conditions, although individual test diets showed statistically significant refusal activity relative to controls (Table 3), a weak correlation ($r_s = 0.32$, L.S. > 0.05) was obtained when comparing feed refusal activity against corresponding weight gain. Overall correlations between pig and rat data, either on the basis of feed refusal activity ($r_s = 0.54$, L.S. > 0.05) or weight gain ($r_s = 0.43$, L.S. > 0.05) was not strong.

The presence of varying concentrations of zearalenone with known anabolic activity appeared to be a factor in the overall variability of the feeding trials. The actual zearalenone consumption was determined from known concentrations in the feeds (Table 1) coupled with the actual feed consumption (Table 3). Comparison of zearalenone consumption with uterine weights (including ovaries) or rats fed these diets showed near perfect correlation ($r_s = 0.96$, L.S. < 0.01) in spite of possible errors in the fresh weight determinations (Table 3). Such concentrations of zearalenone are also within the range reported by Mirocha et al. (7) to result in whole body weight gain in rats. Thus it seems highly probable that the varying amounts of zearalenone consumed in the *Fusarium* corn culture diets contributed to the lack of good correlation between feed consumption and weight gain. However, in spite of the lack of strong correlation between the results of rat and pig feeding studies the rat assay remained a convenient assay tool for chemical fractionation studies when feed refusal was measured. However, periodic confirmation feeding studies using pigs were necessary, and for this purpose feed refusal (as distinct from body weight) was considered the most appropriate parameter for measurement. Thus, the stability of the refusal factor to the initial workup was verified by feeding the crude oil as a dietary component to both rats and pigs and noting significant refusal in each animal.

The isolation and characterization of zearalenone as an active fraction stimulated our interest in its role in feed refusal. By dilution to extinction, the concentration ($\mu g/\mu g$) of zearalenone was determined in six isolates (Table 3), and a strong correlation with refusal was found in rats ($r_s = 0.93$, L.S. < 0.01) and a moderate correlation in pigs ($r_s = 0.68$, L.S. $= 0.05$). However, further examination with pure zearalenone demonstrated that the magnitude of the refusal activity with pure corn cultures could not be accounted for by zearalenone alone. Compare, for example, the activity of isolate no. 3 (Table 3) with pure zearalenone (50 $\mu g/g$) (Table 4). In fact, additional factors, either through additive or synergistic effects, must have been present in order to explain the refusal syndrome. Since preliminary chemical fractionations indicated the presence of active nonvolatile, relatively polar, neutral molecule(s), like trichothecenes, and since various trichothecenes have been associated in one way or another with *Fusarium* infested corn intoxication, the possible role of trichothecenes in the refusal syndrome was investigated. To investigate the potential of toxic trichothecenes, readily available T-2 toxin was evaluated in the rat assay. A diet of as little as 5 μg of T-2 toxin per g was found sufficient to account for all of the refusal activity of infected feed. In addition, at higher T-2 toxin concentrations (50 $\mu g/g$), zearalenone was found to enhance its activity.

These results suggest that refusal activity may well be due to a combination of zearalenone and one or more of the trichothecenes. Further work to clarify this possibility is presently in progress.

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