THE UNIVERSITY OF MANITOBA

INFLUENCE OF TOXIN-FREE MOLD-CONTAMINATED BARLEY AND OCHRATOXIN A ON CHICK PERFORMANCE AND METHODS TO REDUCE THEIR EFFECTS

BY

 ROLAND G. ROTTER

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ABSTRACT

Fungal and mycotoxin contamination of stored grains can have serious detrimental effects on animals which ingest them. In western Canada, Penicillium and Aspergillus fungi and ochratoxin A (OA), a potent nephrotoxin, are the molds and toxin of concern. A method based on quantification of glucosamine (derived from the hydrolysis of chitin) using liquid chromatography was developed to estimate the degree of fungal material in a grain sample. The glucosamine content of a sample was estimated using a standard reference fungus or an average of mixture fungal species grown on liquid culture.

A series of experiments using Leghorn chicks was conducted to investigate the effects of a chemically characterized toxin-free mold-contaminated barley, with and without added OA, and several treatments to reduce OA toxicity. Chick performance and apparent nutrient digestibility, particularly fat, were significantly ($P<0.05$) reduced by the consumption of fungal contaminated barley. The reduction in feed consumption and body weight gain was directly related to the concentration of mold in the diet. The effects of the mold-contaminated barley were not reduced by high dietary tallow (10%) or starch supplementation. Birds fed barley moderately contaminated with mold also had performance values 50% lower than birds fed a diet in which half of the barley component of the diet was replaced by cellulose, a non-nutritive bulk substitute. The presence of 4 ppm OA in a mold-free diet greatly depressed chick performance, but the effects were not as dramatic as seen in chicks fed a diet containing only 15% of a 16% mold

contaminated barley. A combination of OA and mold caused reductions only slightly greatly than the mold alone. Supplementation of diets containing 4 ppm OA with activated charcoal (up to 10,000 ppm) or phenylalanine (to a total dietary concentration of 2.6%) did not result in any significant ($P>0.05$) lessening in OA toxicity. In vitro ensiling studies indicated that the concentration of OA in barley was reduced by 68% after 49 days. OA contaminated barley was also ensiled for 28 days for use in a feeding trial. Although the detectable concentration of OA decreased by almost 60%, there was no corresponding reduction in the toxic effects of OA as determined by changes in chick performance. Similarly, no improvement was seen after the ensiling of toxin-free mold-contaminated barley. These studies show that diets contaminated by toxin-free mold-contaminated barley or OA have serious detrimental effects on animals. The methods investigated for reducing the effects of mold and OA were not effective, and the causative agent(s) due to fungal contamination has (have) not yet been identified.

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This thesis is dedicated to my wife Barbara, and my sons Richard and Robert, for their love, support, encouragement and understanding during the pursuit of this degree. I also want to thank my parents, Rudi and Marianne Rotter, for without them, none of this would have been possible.

FOREWORD

This thesis is written in manuscript style. The first manuscript has been accepted for publication in the "Canadian Journal of Animal Science" and the second has been submitted to the same journal. The third manuscript will be submitted to "Poultry Science", the fourth to the "Canadian Journal of Veterinary Research", the fifth to "Nutrition Reports International" and the sixth to the "Journal of Agricultural and Food Chemistry".

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GENERAL INTRODUCTION

The practice of storing grain on the farm is widespread. As a consequence of improper drying of these grains prior to storage, or the seepage of water or snow into the storage bin, the grain may be subject to mold contamination. In western Canada, among the most common storage fungi are species belonging to the Penicillium and Aspergillus genera (Sinha et al. 1986). Under certain conditions, these fungi produce mycotoxins, such as ochratoxin A (OA). As a result of climatic conditions and the species found in the prairie provinces, OA is the most prominent toxin found in western Canada (Andrews et al. 1981). It has been reported in concentrations as high as 27 mg kg^{-1} of contaminated grain (Scott et al. 1972), and because of its potency and its occurrence in slaughter pigs (Marquardt et al. 1988), is of particular concern in western Canada.

While the toxic effects of OA on domestic animals have received considerable attention, very little is known of the effects caused by the contaminating mold itself. Most reports studying OA have involved either the addition of pure toxin to a mold-free basal diet or natural OA with mold, but not to toxin-free moldy grain. Toxin screening of feed samples is a recent procedure which earlier studies of mold-contaminated grain did not have the benefit of. Also, the degree of fungal contamination was not reported in the few recent reports of the effects of feeding moldy grain, mostly corn, to chicks (Bartov et al. 1982; Bartov 1983, 1985). Comparison of results between studies is therefore difficult. In addition, very few studies have been attempted to reduce the detrimental effects of OA (Chelkowski et al. 1981a,b, 1982; Madsen

et al. 1983) or moldy grain (Bartov 1983, 1985).

The objectives of the research presented in this thesis were to:

1) Derive a procedure to estimate the concentration of mold in a contaminated grain sample so as to permit comparisons within and between studies.
2) Determine the effect on chick performance of mold contaminated barley with and without added toxin (OA).
3) Examine the influence of low and high concentrations of dietary tallow and of starch supplementation on the effects of moldy barley in chicks.
4) Determine if the depression in chick performance is caused by a reduction in available nutrients or some other factor.
5) Examine the feasibility of reducing the toxicity of OA by supplementing OA contaminated diets with:
    a. activated charcoal to adsorb OA and prevent its absorption from the intestinal tract;
    b. phenylalanine to compete with OA for binding sites both in the gut, thereby reducing OA absorption, and within the body, reducing the interference of protein synthesis by OA.
6) Examine the feasibility of ensiling OA or mold-contaminated barley to reduce their detrimental effects in growing chicks.

LITERATURE REVIEW

1. Fungal contamination

1.1 Fungal species and occurrence in western Canada

Numerous species of storage fungi are found throughout western Canada, but many of these are localized (Mills and Abramson 1981). Among the more prevalent species are Aspergillus versicolor, A. flavus, some members of the A. glaucus group, Penicillium verrucosum var. cyclopium (P. cyclopium) and Fusarium spp. (Mills and Abramson 1981; Abramson et al. 1983, 1987; Sinha et al. 1986). The prominence of the individual species can vary each year and in different regions depending upon local environmental conditions. While each species has its particular requirements, growth is generally favored by wet and warm harvest and/or storage conditions.

Despite conditions which promote mold growth, not all fungi will produce mycotoxins. Factors which have been studied in regard to influencing toxin production include O_2 and CO_2 concentrations, moisture, temperature, substrate composition, incidence and abundance of microflora, fungal abundance, spore load invertebrate vectors and time (Abramson et al. 1987). It is suggested that toxin production is caused by a complex interaction of several of the above factors.

1.2 Detection of fungal material

Methods for the detection of the presence of fungal material include analysis for ergosterol (Seitz et al. 1979; Miller et al. 1983) and chitin, as determined by its hydrolysis product, glucosamine (GlcN; Ride and Drysdale 1972; Stahmann et al. 1975; Cochran and Vercellotti 1978). Chitin, a polymer of N-acetyl-D-glucosamine, is a major component

of the cell wall of many fungal species (Wu and Stahmann 1975).

Techniques used for chitin analysis generally involve either colorimetric methods (Ride and Drysdale 1972), gas chromatography (Hicks and Newell 1983) or ion exchange chromatography (Hubbard et al. 1979; Cochran and Vercellotti 1978; Zacharius 1976; Wu and Stahmann 1975). The colorimetric assay, though simple and rapid, is not specific to GlcN and galactosamine (GalN), a second amino-sugar found in hydrolyzates of some species of fungi (Schmit et al. 1975). The procedure is also affected by interfering substances in plant hydrolysates. While gas chromatography provides information on the individual hexosamine, it requires derivitization of the sample before analysis. Ion exchange chromatography, in contrast, is both a sensitive and specific method for the quantification of the hexosamines released during sample hydrolysis. Unfortunately, the latter method has not been standardized, and various authors have suggested different hydrolysis conditions. Wu and Stahmann (1975) and Stahmann et al. (1975) advocated that samples be hydrolyzed in 6 N HCl in an autoclave (110°C) for 2-4 h. Cochran and Vercellotti (1978) also suggested that hydrolysis be conducted for 2-3 h, but at 95°C in 8 N HCl. While ion exchange methods has been available for several years to detect the presence of mold, it has not been used to quantify the amount of mold.

Sharma et al. (1977) suggested that fungal mass can not be quantified based on a chitin assay. They claimed that the relationship between the dry weight of the fungal mass and increasing concentrations is not linear. This conclusion was supported by Cousen et al. (1984), who further noted that the chitin content varies between species. Both

these authors, however, used modifications of the colorimetric assay of Ride and Drysdale (1972). Plassard et al. (1982), also using a colorimetric procedure, and Cochran and Vercellotti (1978), using an ion exchange method, in contrast claimed that the relationship between mycelial biomass and GlcN is linear. Cochran and Vercellotti (1978) also noted this for GalN using four different species of fungi grown on liquid culture.

1.3 Effects on growing chicks

Over the years, various studies have purported to study the effects of feeding mold-contaminated grain to animals, but only in the last few years has the presence of numerous mycotoxins been recognized. Therefore, while the results of earlier studies may in fact be correct, many of the early reports, such as Jones et al. (1955) can not be considered reliable since the effects attributed to the mold may have been due to a toxin. The reports of the effects of mold contamination cited in this thesis all involved the screening of the samples for most of the major toxins. Unfortunately, no quantitative estimates as to the degree of mold contamination of these samples was given in any study.

Growing chicks fed different samples of toxin-free corn contaminated with either Aspergillus, Penicillium or Fusarium species of fungi have all demonstrated some degree of depressed weight gain and feed efficiency. Sharby et al. (1973) noted poor performance and a reduction in dry matter (DM) digestibility in chicks fed a diet containing corn contaminated with Aspergillus niger and two Fusarium species. Depression of chick performance was also noted by Bartov et al. (1982) and Bartov (1983, 1985). Fritz et al. (1973), in contrast,

claimed that A. niger, A. candidus, A. repens, P. cyclopium, Tricoderma viride and Fusarium moniliforme caused no serious adverse effects while A. ochraceus was toxic to chicks. Laying hens were fed a diet containing corn damaged by Gibberella zeae at a dietary concentration of 35% by Adams and Tuite (1976). They noted that egg production decreased by 8% and feed consumption by 16% compared to control birds, but saw no adverse effects on egg weight or mortality. Unfortunately, as there was no indication as to how contaminated the grain samples were, the results of these studies are not directly comparable. It appears likely that in some of the cases where no effect was noted, that the grains were only slightly contaminated with mold.

The reason(s) for the detrimental effects of toxin-free moldy grain have not been determined, but several factors have been proposed. While Bartov et al. (1982) and Bartov (1983, 1985) suggested the detrimental effect was due to a deficiency in energy, the presence of thiaminase (Fritz et al. 1973), reduced lysine concentrations (Bartov et al. 1982). However, the possibility of undetected mycotoxins or other antinutrients can not be disregarded. Other contributing factors may be palatability or odor problems associated with the mold. Filtrates of Fusarium and Penicillium fungi grown on liquid culture contained unknown factors which caused feed refusal in one-week-old chicks (Burditt et al. 1983). The presence of volatile compounds have also been reported. Kaminski et al. (1972, 1974), Abramson et al. (1980) and Sinha et al. (1988) identified 1-octanol, 3-methylbutanol and 3-octanone, among other compounds, in mold-contaminated grains. The concentrations of these compounds varied between reports, but this may have been due to

differences between fungal species and the growth media (Kaminski et al. 1974). Although not yet tested in feeding trials, the odors associated with these, or as yet unidentified compounds may act to reduce chick appetite or interest in the contaminated feed.

Other studies which have involved moldy grain also involved the presence of a toxin, and no attempts to separate the effects of the mold and toxins were made. They are therefore not considered in this review.

1.4 Dietary additions to counter the effect of mold

The use of cultured fungal material as a protein source in animal diets has received some interest. Crude protein values of fungal material are high. For example, protein values of 45% have been reported for Chaetomium cellulolyticum (Touchburn and Chavez 1986) and 61.8% for yeast (Slagle and Zimmerman 1979). Santos and Gomez (1983) fed fungal protein derived from A. fumigatus grown on cassava to rats. Although the body weights of rats fed diets containing the unsupplemented fungal proteins were lower, methionine supplementation improved performance similar to that of a casein diet. They concluded that, in general, the sulphur-containing amino acids are the main limiting constituents in fungal protein for non-ruminant animals. Slagle and Zimmerman (1983) and Touchburn and Chavez (1986) also demonstrated that incorporation of fungal protein in the diet, with some possible modifications, did not cause detrimental effects.

Fungi utilize fat contained in grains as a source of energy. It has therefore been suggested that this lower fat concentration is the main reason for the poorer performance of birds fed moldy grain (Bartov et al. 1982; Bartov 1983; 1985). In the one study (Bartov 1985), fungal

contamination decreased the fat concentration of corn from 4.1 to 2.3%. Bartov (1983) observed depressed performance in chicks fed toxin-free corn and sorghum contaminated with Penicillium and Aspergillus fungi. Moldy corn also caused a reduction in protein digestibility of approximately 13%, and a similar effect was observed with moldy ground sorghum. Bartov (1983) suggested that this decrease in protein digestibility might have been related to the lower energy content of the mold-containing diets. In a subsequent study, broiler chicks fed a diet containing moldy corn weighed 17% less than control birds after 21 days. When soybean oil was supplemented in the diet, chick body weights were additional 22% lower compared to mold alone. Chick performance was only 4% poorer than controls when propionic acid was added to the soybean oil supplemented diet which, to allow mold growth, was then stored for 25 days. Fat supplementation did not overcome the reduced nutrient digestibilities. Similar results were reported by Bartov (1985).

No other methods to reduce the influence of mold contamination have been tested. Some mold contaminated barley, however, may be removed from a grain sample by mechanical sorting. It is the nature of storage molds to form clumps in infected grain. While no studies have been reported, it would appear logical then that utilization of mechanical sorting to remove various toxins (Muller 1983), could also separate out the mold.

2. Ochratoxin A

Ochratoxin A (OA) is a secondary metabolite of various species of Aspergillus and Penicillium fungi (Table 1), but was first isolated from A. ochraceus by Van der Merwe et al. (1965). It occurs naturally in

Table 1. Fungal species known to produce ochratoxin A (modified from Roschenthaler et al. 1984).

Aspergillus species	*Penicillium* species
A. ochraceus	*P.variable*
A. ostianus	*P. cyclopium*
A. melleus	*P. commune*
A. petrakii	*P. palitans*
A. sclerotiorum	*P. purpurescens*
A. sulphureus	
A. alliaceus	

barley, wheat, corn, dried white beans, peanuts, heated grain and mixed feeds. There are also numerous reports of its occurrence in the tissues and blood of pigs (Hult et al. 1984; Golinski et al. 1984, 1985; Marquardt et al. 1988) and chickens (Prior and Sisodia 1978: Prior 1981) which have ingested OA contaminated grain.

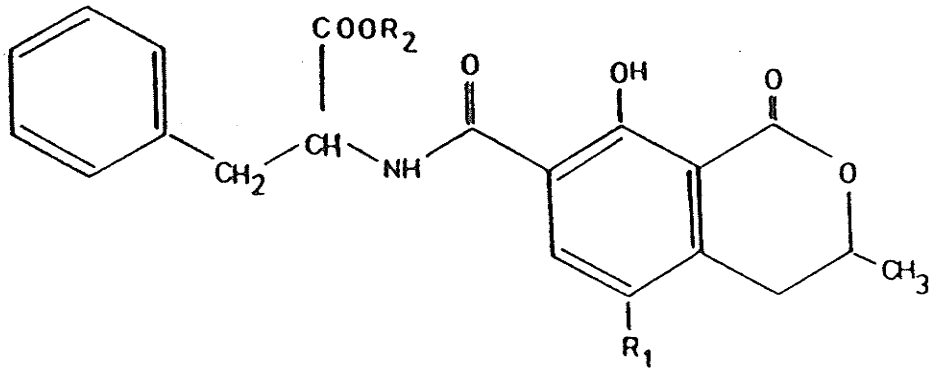
2.1 Structure and properties

The OA molecule (Fig. 1) consists of a 7-carboxy-5-chloro-8-hydroxy-3,4-dihydro-3R-methylisocoumarin moiety with the 7-carboxy group linked to an L-β-phenylalanine (Phe) by an α-amide bond (Huff et al. 1974; Roschenthaler et al. 1984). Pure OA is relatively insoluble in water, but its sodium and potassium salts are readily soluble in polar solvents. It has an UV-absorption maxima in aqueous solutions of 333 nm at pH 1.5, and 380 nm at pH 8.5 (Roschenthaler et al. 1984).

Several metabolites produced by the metabolic breakdown of OA have been described. Ochratoxin α (Oα), OA with the Phe group removed, is one of the most common and non-toxic of these. Another, (4R)-OH-OA is toxic (Creppy et al. 1983a,b), while others, such as (4S)-OH-OA, 10-OH-OA or the ester forms, have not been tested (Roschenthaler et al. 1984).

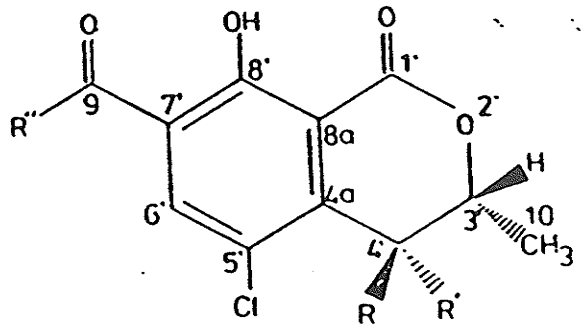
The small intestine appears to be the principal site of absorption of ingested OA (Kumagai and Aibara 1982). Once absorbed, it is bound tightly by albumin, which retards the elimination of OA, and so prolongs its half-life ($t_{\frac{1}{2}}$; Kumagi 1984). OA has a binding efficiency of 2.47 molecules per molecule of serum albumin *in vitro* and *in vivo*, with an equilibrium constant of 3.17×10^{-6} (Roschenthaler et al. 1984), but the affinity of albumin to bind OA varies between species. The affinity constant for swine, chickens and rats being 71100, 50700 and 40100 M^{-1},

Figure 1. Structures of ochratoxin A and its metabolites (Roschenthaler et al. 1984)



The Ochratoxins

$R_1 = Cl$, $R_2 = H$	Ochratoxin A
$R_1 = H$, $R_2 = H$	Ochratoxin B
$R_1 = Cl$, $R_2 = CH_3$	
$R_1 = Cl$, $R_2 = C_2H_5$	Esters of
$R_1 = H$, $R_2 = CH_3$	Ochratoxin A and B
$R_1 = H$, $R_2 = C_2H_5$	



R = R' = H , R'' = Phe [a]	Ochratoxin A
R = R' = H , R'' = OH	Ochratoxin
R = H, R' = OH , R'' = Phe	(4S) [b]
R = OH , R' = H , R'' = Phe	(4R) [b]

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[a] L — phenylalanine [b] epimers of 4— OH — ochratoxin A

respectively (Galtier et al. 1981). These authors reported that after intraperitoneal (i.p.) injection the respective $t_{\frac{1}{2}}$ values for OA in these three species were 84.5, 3.0 and 10.8 h, while after oral administration, the values were 88.8, 4.1 and 8.2 h. They also noted that only 65.7, 55.6 and 40%, respectively, of the oral dosage given was absorbed by each species. These results indicate the greater problem of OA residues in pork products than the other species.

2.2 Toxic effects

2.2.1 Pathological and biochemical actions

The effects of OA on an affected animal are cumulative, and, in all species tested, it is a potent nephrotoxin. OA causes lesions in the kidney, mainly in the renal proximal tubules (Stein et al. 1984, 1985), resulted in the loss of glucose and protein in the urine, and finally a malfunction of the kidneys. Endo (in Ueno 1984) observed the release of alanine aminotransferase followed by the release of leucine aminopeptide and g-glutamyl trans peptidase in OA treated isolated renal nephron preparations. He further noted that OA may disrupt membrane transport of parahippuric acid. These results suggest that OA may interfere specifically with the anion transport mechanism located on the surface of the brush border.

The mode of action of OA apparently is through competition with Phe for phenylalanine-t-RNA synthetase, resulting in the inhibition of protein synthesis in both eucaryotic (primarily kidney, but also liver and spleen) and procaryotic cell systems (Roschenthaler et al. 1984), primarily in the kidney, but also the liver and the spleen.

At low concentrations, OA can have a serious effect on the immune

system of affected animals. Prior and Sisodia (1982) and Creppy et al. (1983a) noted that OA depressed humoral antibody responses which suggest functional changes in the immune system. When Boorman et al. (1984) injected mice with OA concentrations that were mildly toxic to other organs, they observed that the bone marrow cells and macrophages were functionally altered. Phagocytic activities of heterophilic leucocytes were also impaired in broiler chicks fed 4 and 8 ug g^{-1} of feed (Chang and Hamilton 1980), but leucocytopenia was induced when the concentration of OA was as low as 0.5 ug g^{-1} (Chang et al. 1979). Regression of the thymus (Chang et al. 1981) and depletion of lymphoid cells in the bursa of Fabricius (Pekham et al. 1971; Huff et al. 1974; Dwividi and Burns 1984; 1985) have also been reported.

Although Ueno (1984) claimed that results using the Rec-assay and the Ames test showed OA was not genotoxic, Creppy et al. (1985a,b) noted that OA induced DNA damage in mice, both <u>in vitro</u> in spleen tissue and <u>in vivo</u> in spleen, liver and kidney. The appearance and extent of the damage varied between tissues, but the OA induced damage in the DNA was repaired over time. In addition, OA is also carcinogenic (Bendele et al. 1985), particularly in the kidney and liver (Ueno 1984), and can cause leukemia (Creppy et al. 1985b). Ueno (1984) suggested that although the mechanisms of OA carcinogenicity are hard to explain at this time, the immunosuppression effect of low concentrations of OA may lead to the promotion of endogenous tumor cells. While OA does not cross the placental barrier and is not teratogenic in pigs (Shreeve et al. 1977), it does both in mice (Appelgren and Arora 1983; Fukui et al. 1987). Burns and Dwividi (1986) have also shown OA to be teratogenic in quail

and in rats by Mayura et al. (1983, 1984a,b).

2.2.2 Effect on chicken and swine performance

In addition to the health effects of OA described above, animal performance is also affected. Marked decreases in performance have been reported in chickens (Peckham et al. 1971; Huff et al. 1974, 1975, 1984; Prior et al. 1980; Dwividi and Burns 1984a; Kubena et al. 1984, 1985, 1986; Harvey et al. 1987) and in swine (Krough et al. 1974; Madsen et al. 1982a,b; Mortensen et al. 1983; Lippold 1988). Reduced performance in both species is seen not only in lower feed consumption and weight gain values, but also poorer feed efficiencies. The magnitude of these effects depends on the amount of OA ingested, but within species, the results compare favorably between studies. In broiler chicks fed 4 ppm OA, the birds had almost 35% lower weight gains over a three week period than control birds (Dwivedi and Burns 1984a), while growing pigs fed the same OA concentration showed only 17% lower daily gain values (Krough et al. 1974).

Nelson et al. (1982) compared the effects of dietary OA (300 ppb), aflatoxin B_1 (AFB_1 3 ppm) and citrinin (800 ppb) on percent dry matter (DM) and amino acid (AA) digestibilities and metabolizable energy (ME; Kcal g^{-1} DM) values in growing chicks. While the respective values for DM, AA and ME for birds fed the basal diet were 87.2, 92.6 and 3.91, they decreased to 57.0, 54.1 and 2.53 for the OA fed birds. The values for citrinin and AFB_1 were basically only slightly lower or not different from the controls.

Additional studies have examined the interactive effects of other dietary toxins (eg. AFB_1, citrinin) with OA. Although OA can be found in

the presence of other toxins, these studies are beyond the scope of the current investigation.

2.3 Residues in animal tissues

In addition to the problems associated with the ingestion of OA in affected animals, is the concern that OA residues occur in animal tissues and products. The toxin is not completely destroyed during the processing and cooking of these products (Trenk et al. 1971; Josefsson and Moller 1980). The presence of OA has been demonstrated in the tissues (primarily kidney and liver) and also the carcasses of both pigs (Hult et al. 1980, 1984; Madsen et al. 1982a; Golinski et al. 1984,1985; Marquardt et al. 1988) and chickens (Prior and Sisodia 1978; Prior et al. 1980; Micco et al. 1987). All authors echoed concerns for human health as a consequence of the consumption of OA exposed animals.

3. Ochratoxin A degradation/decontamination

The breakdown of OA, or any toxin, not only depends on the nature of the molecule, but also the specific conditions under which degradation occurs. While the toxin may decrease to undetectable levels, each treatment must be tested separately to actually verify decontamination. Unfortunately, most studies which purported to lower OA concentrations in contaminated samples did not also perform biological tests with their 'decontaminated' sample.

3.1 Natural degradation

OA is subject to natural degradation in cereals. In wheat, barley and maize flour, Szebiotko et al. (in Muller 1984) observed that OA, added at a concentration of 500 ug kg^{-1}, decreased by 78, 80 and 71%, respectively after 6 months of storage. Similarly, Trenk et al. (1971)

noted OA concentrations of 1800 ug kg^{-1}, added to oatmeal and stored at a temperature of 28°C, decreased 38% in 3 weeks, and by 55% after 12. At 4°C, the rate of reduction in OA concentration was slightly slower. In another report, Krogh (in Schuh and Schweighardt 1981) was quoted as noting a decrease in the concentration of OA from 4 to 1.5 mg OA kg^{-1} of a barley sample over a 2 year period.

The method by which OA is naturally degraded is not known, but Szebiotko et al. (in Muller 1984) suggested that it may be caused by peroxides. Oxidation and the influence of light were ruled out as the cause by Trenk et al. (1971), but the role of microbial activity has not been explored.

3.2 Decontamination methods

Detoxification methods can be divided into three main categories: (1) separation of the contaminant from the grain by physical means; (2) removal of the contaminant by extraction (Jemmali 1979); and (3) chemical, biological or physical (heating) inactivation of the toxin(s) (Jemmali 1979; Doyle et al. 1982). Each method, as applied to a specific toxin, must not only be able reduce the effect(s) of the toxin below a specified level in an *in vivo* test, but also not reduce the physiological or nutritional quality of the feedstuff and be cost effective (Muller 1983).

The majority of mycotoxin decontamination research has centered on $_{A}FB_{1}$, but with the exception of treating it with NH_3, little is known of the effects of decontamination procedures in practice. Excellent reviews of mycotoxin decontamination studies covering a wide range of methods as applied to various toxins have been published by Doyle et al. (1982),

Muller (1983, 1984) and Scott (1984). Unfortunately, little attention has been given to OA, or mold itself, in this regard before or since these reviews.

3.2.1 Physical methods of OA degradation

The reduction of OA concentrations by physical methods, such as mechanical sorting, wet or dry milling, tend to be somewhat ineffective as OA is not confined to the bran portion of the seed. Chelkowski et al. (1981a) noted that the concentration of OA was similar in the meal and bran of wheat and barley after milling as the infecting fungus had penetrated into the grain endosperm. They claimed that only 10-50% of the OA present in whole grain can be extracted from a sample, but gave no indication of the method of extraction.

Studies using heat treatments to attempt to destroy OA have yielded variable results, but this may be due to varying water contents of the samples during treatment. Autoclaving oatmeal and rice cereal for 3 h reduced OA concentrations by 87.5 and 86%, respectively, in the absence of added water, but only 74 and 68.5%, respectively, in the presence of 50% water (Trenk et al. 1971). Similarly, Osborne (1979) noted no change in OA concentrations in bread baked for 25 min at 220°C, while during the making of biscuits, requiring higher moisture levels, OA was reduced 62% after only 5 min at 180°C. When raw, white beans containing OA concentrations of 255 and 960 ug g^{-1} were autoclaved for 1 h, only 13.3 and 9.5% of the OA was degraded (Harwig et al. 1974). Levi et al. (1974) roasted ground hard red winter wheat and ground green coffee beans containing added concentrations of OA of 1.4 and 1.8 mg kg^{-1}. After 5 min at a temperature of 198-210°C, they observed the destruction of 88

and 80%, respectively, of the OA. In contrast, Tsubouchi et al. (1987) roasted coffee beans containing an average of 135 ug OA g^{-1} coffee at 200°C for 10-20 min, but only reduced OA concentrations by a maximum of 12%. When white flour containing OA was heated to 250°C for 40 min, the OA concentration was apparently decreased by 76%, but 32% was actually absorbed by the flour (Tomova in Scott 1984). An equivalent loss (32%) was also seen in naturally contaminated grain similarly treated, but none was attributed to the heat treatment.

Cooking of faba beans containing OA by two different methods only reduced OA concentrations between 16 and 20%, while only 6% was destroyed after cooking polished wheat (El-Banna and Scott 1984). Studies conducted during the beer brewing process have shown that up to 28% of the OA contained in the barley sample may be found in the final product (Chu et al. 1975; Nip et al. 1975). These authors claimed that OA, and AFB_1, are heat stable and insensitive to cooker mash treatment, but both are partially removed in the mashing and brewing processes. In contrast, Krough et al. (1974) found no OA in the malt from barley containing 0.42 and 0.83 ppm OA, but up to 19% of the original OA in the wort of a heavily contaminated sample, claiming that a marked reduction occurs during the mashing process. Nip et al. (1975) suggested differences between the reports may be due to the binding of OA to grain components, and this hypothesis is supported by difficulties in extraction of OA from cooked wheat (El-Banna and Scott 1984).

Josefsson and Moller (1980) tested the heat stability of OA in contaminated pork products. After frying kidneys, muscle and fat tissue at 150-160°C for up to 12 min, on average only 20% of the OA was

destroyed, and they concluded that OA is heat-resistant.

OA, as is the case with some other mycotoxins including the aflatoxins, is sensitive to light (Neely and West 1972). Unfortunately, visible and ultraviolet radiation do not penetrate deeply into seeds, and so is not a very practical solution for detoxification of contaminated feedstuffs.

3.2.2 Chemical treatments

Ammoniation of contaminated grain can greatly reduce the concentration of OA. Madsen et al. (1983) observed a reduction in the concentration of OA from 2329 to 112 ug kg^{-1} when contaminated barley was treated with 5% NH_3 for 96 h at 70°C. Although the nephrotoxic effects of OA were eliminated, only slight improvements were seen in feed consumption, daily gain and feed efficiency compared to untreated OA diets (Madsen et al. 1983). These authors concluded that use of NH_3 under practical conditions is too risky. Chelkowski et al. (1981b) also treated OA in maize grain, wheat and barley with NH_3. They observed that NH_3 applied at a concentration of 2% at 20°C completely degraded 1.2 mg OA kg^{-1} of barley within 6 weeks, while 20 mg OA kg^{-1} took about 16 weeks, or 4 weeks at 45°C. They suggested that this method would be practical for farm use, but only up to an OA concentration of 50 mg kg^{-1}. In another study, Chelkowski et al. (1982) treated OA contaminated barley, wheat and corn until the OA was completely degraded (up to 8 weeks). Based on a chick embryo test and a broiler chick feeding trial, OA toxicity was determined to have been greatly reduced by both systems. The chicks, given the NH_3-treated grains, but only at a concentration of 5-10% of the diet, performed almost as well as the control birds.

Chelkowski et al. (1982) concluded that ammoniation must be continued until the OA concentration falls below detectable levels.

It has been shown that as the NH_3 concentration in the feed increases, feed consumption in pigs (Jensen et al. 1977) and laying hens (Brekke et al. 1977) decreased. One reason for this may be a residual stinging smell and taste of the NH_3 in the feed, but ammoniation also reportedly reduces the concentrations of lysine in grain up to 2% (Madsen et al. 1983) and that of cystine (Waldroup et al. 1976). The dietary concentration of ammoniated grain must therefore be restricted to avoid adverse effects.

Madsen et al. (1983) noted that barley samples contaminated with 1878 and 311 ug OA kg^{-1} had their respective concentrations reduced to 310 and 56 ug kg^{-1} after treatment with 0.5% NaOH and a temperature of 490°C. Despite the reduced OA concentrations, they considered the method to be too risky for use at present.

Ruminant animals are less susceptible to the effects of OA than monogastric species. Hult et al. (1976) observed that OA was quickly degraded to Oα and Phe in all stomach compartments of the cow except the abomasum, when OA was incubated with stomach contents. About 60% of the added OA was detected as Oα. Similarly, OA was effectively degraded to Oα and Phe in the rumen of sheep (Pettersson and Kiessling 1976; Kiessling et al. 1984). These authors suggested that the observed reduction may be due to rumen protozoa, and noted that the rate of OA degradation was reduced after feeding, but it increased gradually by the next feeding.

OA is susceptible to the activities of carboxypeptidase A, but also

has a lesser affinity for chymotrypsin (Pitout 1969).

3.3 Dietary additions and animal treatments

3.3.1 Activated charcoal

Ingested OA must be absorbed from the gastrointestinal tract (GIT) to cause its detrimental effects. It therefore follows that any method which prevents or reduces OA absorption would also decrease its effect. One such method may be the use of activated charcoal. Charcoal has long been considered to be an effective oral antidote in the treatment of poisonings. It is an insoluble carrier that non-specifically adsorbs molecules, preventing their absorption from the gut (Rodgers and Matyunas 1986).

There are no published reports of the use of charcoal for treating OA ingestion, but charcoal has been used with some success with other mycotoxins. Concentrations of the mycotoxin patulin were reduced to non-detectable levels in apple cider by the addition of 20 mg charcoal mL^{-1} (Sands et al. 1976). Most reports, however, have centered on reducing the effects of AFB_1. Hatch et al. (1982), in an acute exposure study, administered a lethal dose of AFB_1 (3 mg kg^{-1} body weight) intraruminally to goats, followed within 8 h by a charcoal slurry. The charcoal treatment was able to reduce the severity of the AFB_1 effect, but not its duration. In a series of feeding studies, Ademoyero and Dalvi (1983), Dalvi and Ademoyero (1984) and Dalvi and McGowan (1984) showed that the effects of AFB_1 in chicks were lessened by charcoal addition to the diet. Supplementation of 0.1% activated charcoal to a diet containing 10 ppm AFB_1 was able to improve chick performance by 10% compared to the AFB_1 fed birds, and it prevented toxin-induced liver

injury (Dalvi and McGowan 1984; Dalvi and Ademoyero 1984).

3.3.2 Phenylalanine

Several studies have reported an ability of Phe to overcome or reduce the effects of OA. In vitro, Creppy et al. (1979), using rat liver parenchymal cells, demonstrated that when 50 uM of Phe is present in cell cultures containing an OA concentration of 90 uM, the OA-induced inhibition of protein synthesis can be completely prevented. Reversal of the OA effect was even possible if Phe was only added to the culture 2 h after the OA. They concluded that Phe must compete with OA for cell receptor sites. These findings were later supported by Creppy et al. (1983a). Subsequent in vivo studies with mice have shown that Phe injected concurrently with OA, but at a concentration 10 times that of the OA, also prevented OA inhibition of protein synthesis (Creppy et al. 1984; Moroi et al. 1985), while Phe at twice the OA concentration prevented immunosuppression (Haubeck et al. 1981; Creppy et al. 1983b). In contrast, female rats injected on day 7 of gestation with either a single dose of 1.75 mg OA kg^{-1} in combination with a single or daily dose of 25 mg of Phe kg^{-1} provided only slight protection from the teratogenic effects of OA (Mayura et al.1984a,b).

In the only study of acute oral dosing of OA and Phe, the LD_{50} in mice increased from 46.0 to 71.0 mg kg^{-1} when the Phe concentration was twice that of OA (Moroi et al. 1985). Based on their results, the authors concluded that Phe and OA not only compete for phenylalanyl tRNA synthetase, but also for absorption sites in the gastrointestinal tract (GIT). Gibson et al. (1988) supplemented broiler chick diets containing 14% protein and 4 ppm OA with Phe up to a total dietary concentration of

3.2%. They claimed Phe supplementation improved chick performance, but that these birds never did as well as the controls. The most dramatic effect, which they attributed to a protective action by Phe, was a decrease in mortality from 42.5% in OA fed birds to 10% for those given OA plus a dietary Phe concentration of 3.2%. The protein content of the basal diet (14%), however, was low for the broilers, as NRC (1984) recommendations range from 23% protein (0-3 weeks) down to a low of 18% (6-9 weeks), so that the birds were under a protein deficiency stress situation. This stress would sensitize the birds to the effects of OA, resulting in the extremely high mortality seen for OA alone. The supplemental Phe would therefore have beneficial effects under such a situation.

One concern in using supplemental Phe in OA contaminated diets is the inclusion of excessive Phe to the point of Phe toxicity or amino acid imbalance. Information on Phe toxicity to chicks is sparse except for reports of reduced feed consumption and growth depression (Elkin and Rogler 1983). Other effects reported include poor feather development, swollen hocks and discoloration of the shanks, face and eyelids as well as reduced brain serotonin concentrations and lower cerebral energy utilization. It can also reduce egg production, egg weight, fertility, hatchability and day-old chick weight when excessive Phe is fed to laying or breeding hens. Elkin and Rogler (1983) observed that diets containing a total of 2.52% Phe reduced weight gain by 72%, but that tyrosine partially alleviated the Phe influence on growth.

4. High moisture (ensiled) grains

Ensiled or high moisture grains are generally not used in the

formulation of chicken diets, but their use is widespread for swine. The data concerning their feeding value is contradictory. The performance of growing pigs fed ensiled barley has been shown by some authors to be similar to control diet fed pigs (Jamieson 1968; Cole et al. 1970). In contrast, studies by Livingstone and Livingston (1970) and Livingstone et al. (1971) noted as much as 9% poorer growth and increased feed to gain ratios due to high moisture barley. They suggested that this may have been due to mold growth on the moist barley, but Goransson and Ogle (1985) also obtained similar results, but with no apparent mold problem. Dried distillers-spent-grain (barley) can be used in the diets of growing chicks with no adverse effects (Newman et al. 1985; Pettersson et al. 1987). These authors, however, only used up to 20% of the spent grain in the diet, which may not have been sufficient to see any detrimental effects. Livingstone and Livingston (1970) stated that in high moisture grain, the characteristic sweet-sour smell cannot be removed by drying the grain, and so this could affect feed consumption by chickens.

5. Summary

The problems associated with the direct ingestion of OA contaminated feed by animals are well documented, and the carryover of OA to the human population in animal products is also clear. While it has been shown the concentration of OA in infected grains can be reduced, only limited information is available and the methods tried are not necessarily practical for producer use. More emphasis must therefore be directed at examining methods of eliminating OA in contaminated grains, or at least reducing its toxicity in monogastric animals.

In addition to the detrimental effects of OA, very little is known of the effects of mold itself in grain, with the exception of corn. In studies which attempt the degradation of OA, or some other mycotoxin, the influence of a contaminating fungus on animal performance should be understood so as not to mistake its effect for a presumed destroyed toxin, or the treatment applied. Further complicating the question of the effects of molds themselves is the lack of a method to estimate the degree of mold contamination. Such a method would allow for the comparison of results between studies.

MANUSCRIPT I[1]

ESTIMATION OF FUNGAL CONTAMINATION OF CEREAL GRAINS AS DETERMINED BY MEASURING GLUCOSAMINE CONCENTRATION

[1]Accepted for publiction by Can. J. Anim. Sci.

ABSTRACT

The degree of fungal contamination of a grain or feed sample can be estimated by determination of its glucosamine (GlcN) content, using a modified amino acid analysis procedure. A sample (100mg) was hydrolyzed in 6 N HCl for 7 h at 121°C, evaporated to dryness, reconstituted in citrate buffer and applied to the ion exchange column of an amino acid analyzer. Hydrolysis conditions were optimized for the recovery of GlcN, released by the breakdown of chitin, a major fungal cell wall component. The GluN and GalN concentrations of several species of fungi indigenous to western Canada, grown on liquid culture, were determined to provide reference values for estimating fungal contamination. A fungal contamination index (FCI) is proposed based on a comparison of the GlcN concentration of a grain sample to the specific reference fungus or an average concentration from several species. Based on the fungal species tested, a suggested average for use with most naturally contaminated grain samples is 76 mg GlcN g^{-1} of fungal DM. This approximation would be valid for most cases of mixed or unknown fungal species. Knowing the fungal content of a grain sample will aid in feed formulation and thereby avoid costly losses to the livestock producer.

KEY WORDS: Fungal contamination, glucosamine, chitin, grain, amino acid analysis

INTRODUCTION

Fungal contamination of cereal grains and animal feed is a problem facing animal producers since the presence of mold, even in the absence of detectable toxins, can have serious detrimental effects on animal production (Rotter et al. 1986; Manuscripts II and III). Analyzing a given sample for fungal contamination may reduce or eliminate financial losses by allowing the producer to dilute contaminated feed. In addition, it would warn the producer that a toxin(s) may be present. Chitin, a major constituent of the cell walls of many fungal species, is a polymer of N-acetyl-D-glucosamine (Wu and Stahmann 1975). The quantification of chitin, as measured by its hydrolysis product glucosamine (GlcN), has been proposed as a method of estimating the degree of mold contamination (Cousin et al. 1984; Hubbard et al. 1979). Since GlcN is also found in bacterial and fungal spores, seed glycoproteins (Donald and Mirocha 1977) as well as insects (Hicks and Newell 1983), chitin from these sources could theoretically interfere with the quantification of fungal material. The actual interference however is negligable. The GlcN concentration Polymers of a second compound, galactosamine (GalN), have also been found in the walls of some species (Schmit et al. 1975).

The techniques currently used for chitin analysis generally involve colorimetric methods such as that of Ride and Drysdale (1972), gas chromatography (Hicks and Newell 1983) or liquid chromatography (Hubbard et al. 1979; Cochran and Vercellotti 1978; Wu and Stahmann 1975; Zacharius 1976), and recently near infra-red spectrophotometry (Roberts et al. 1987). The colorimetric procedure is not specific in the

determination of GlcN and GalN and is also affected by interfering substances in plant hydrolysates. Gas chromatography provides information on individual hexosamines, but is also undesirable as sample derivatization is required. In contrast, ion exchange chromatography provides a sensitive and specific method for the quantification of individual hexosamines released during the hydrolysis of a fungal mass. Although its use has been advocated by other authors (Hubbard et al. 1979; Wu and Stahmann 1975; Stahmann et al. 1975), the optimal conditions of hydrolysis, limitations and applications of the method have not been examined.

In western Canada, fungal contamination of stored grain usually involve Aspergillus or Penicillium species (Abramson et al. 1982). One purpose of the current study was to adapt and refine a method for the quantification of GlcN and GalN using a modified version of the standard amino acid analysis procedure followed in our department. An estimate of the degree of fungal contamination of local cereal grains was determined based on comparison to the GlcN content of pure cultures of several species of storage fungi found in western Canada. Estimates of the fungal mass of several experimentally and naturally contaminated grain samples are reported.

MATERIALS AND METHODS

Hydrolysis and analysis procedures

The procedure for sample hydrolysis and analysis is a modification of that by Wu and Stahmann (1975), Stahmann et al. (1975) and Hubbard et al. (1979), but it incorporates methods for amino acid analysis used in

our facility. In the current procedure, a 100 mg sample was transferred into a Pierce Vacuum Hydrolysis Tube (19 x 100 mm), (Chromatographic Specialties, Brockville, Ont.). Four mL 6 N HCl acid was added to each tube, the air evacuated (750 mm Hg) and the tube placed in a preheated blockheater (Temp-Blok Module Heater) at 121°C for the specified time. After hydrolysis, the contents of the tube were evaporated to dryness at 90°C in a Buchler Rotary Evapo-Mix with the vacuum at 700 mm Hg. The residue was dissolved in 10 mL sodium citrate buffer (0.2 N Na^+), pH 2.2, shaken for 25-30 sec, filtered through Whatman No.40 filter paper and stored at -70°C until analyzed. The sample was refiltered through an MSI 0.22 u nylon 66 membrane filter (Fisher Scientific) and 50 uL was applied to the column.

Optimization of hydrolysis conditions

The optimum time for hydrolysis was determined using mycelia from a strain of Aspergillus ochraceus. The fungus was grown on a modified liquid medium (Yamazaki et al. 1970) for 10 d. Fungal samples were hydrolyzed for 0 to 14 h (at 1 h intervals) and for 16, 24 and 48 h. All analyses were performed at least in duplicate.

The release and recovery of GlcN from purified crabshell chitin (Sigma Chem. Corp., St. Louis, MO) was also tested. Chitin, in amounts of 0, 4, 10, 20 and 40 mg, was mixed with ground mold-free barley to a total of 100 mg and then hydrolyzed for 7 h at 121°C in 6 N HCl.

Instrumentation, Standards and Test for Linearity

The amino acid analyzer (LKB Biochrom 4151 Alpha Plus Amino Acid Analyzer; LKB-Produkter AB, Bromme, Sweden) was equipped with a 200 x 4.6 mm ID stainless steel column packed with LKB Ultropac 8 cation

exchange resin (Na^+ form) having a particle size of 8.0 ± 0.5 um. The reaction coil temperature was 135°C for ninhydrin detection and the column temperature was 85°C. The buffer and ninhydrin flow rates were 35 and 25 mL h^{-1}, respectively. Sodium citrate buffers used were: buffer 1, pH 3.20, 0.2 N Na^+; buffer 2, pH 4.25, 0.2 N Na^+; and buffer 5, pH 6.45, 1.2 N Na^+. Buffer reservoir 6 contained 0.4 N NaOH. The buffer flow pattern for analysis was: buffer 1 for 2.00 min, buffer 2 for 5.00 min, buffer 5 for 14.00 min, buffer 6 for 4.00 min and buffer 1 for 16.00 min. Results were recorded on an LKB2220 (HP3390A) recording integrator. The sensitivity of the analyzer was better than 30 pmol, and reproducible within 1.5%.

Reference compounds used in this study were D-GlcN-HCl (Calbiochem, San Diego, CA), and D-GalN-HCl and purified chitin (poly-N-acetyl-GlcN) from crab shells (Sigma Chemical Co., St. Louis, MO). Values herein are expressed as the absolute weights. GlcN and GalN weights were corrected for HCl content. Amino Acid Standard H (Pierce Chem. Co., Rockford, IL) was used as the amino acid standard mixture.

Standard curves for both GlcN-HCl and GalN-HCl were obtained by applying each compound to the amino acid analyzer at concentrations ranging from 0.0 to 50 mg of hexosamine-HCl per 100 mL citrate buffer in 5 mg increments.

Fungi and culture conditions

Three species of fungi were used in this study. A. ochraceus NRRL3174 and A. versicolor NRRL573 were obtained courtesy of Dr. C. W. Hesseltine, U. S. Dept. of Agriculture, Northern Region Research Laboratory, Peoria, IL. Penicillium verrucosum var. cyclopium (Westling)

Samson, Stolk and Hadlock was isolated in our laboratory from Bedford barley (1984 crop) obtained from the University of Manitoba Glenlea Research Station, Glenlea, Man. All fungal inoculants were maintained on either a potato dextrose or Czapek agar (Difco Co.) with 20% sucrose and 0.5% yeast extract (BBL, Becton Dickenson and Co., Cockeysville, MD).

During the experimental period, the fungi were grown in triplicate on a liquid medium and on whole barley (Bedford) with either 25 or 30% moisture. Aspergillus ochraceus and P. cyclopium were grown on a liquid basal medium (Yamazaki et al. 1970) modified to contain 3% sucrose, 1% yeast (BBL), and 0.5% phenylalanine (Sigma Chem. Corp.). A. versicolor was grown on a liquid Czapek medium (Smith and Onions 1983).

The fungi grown in liquid media were cultured in 250 mL flasks containing 110 mL of sterilized medium. The media were inoculated with 1.0 mL of distilled water containing fungal spores obtained from potato dextrose agar slants incubated for 7 d at 28°C. Bedford barley (60 g) was placed in 250 mL flasks and distilled water was added to increase the moisture content of the barley to either 25 or 30%. Flasks were stoppered with foam plugs, covered with aluminum foil and kept at room temperature for 24 h to ensure uniform moisture concentrations throughout the barley. All barley samples were autoclaved at 121°C, and inoculated using the same procedures described above for the liquid media. Each culture was grown in triplicate.

After inoculation, the liquid and barley media were incubated at 28°C in a dark walk-in incubator for 10 or 20 d. Mycelia in the liquid media were mechanically harvested and separated from the media, rinsed three times with distilled water and freeze-dried using a Labconco Model

18 Freeze Drier. The barley samples were dried at 50°C to a moisture content of 8-10%. All samples were ground using a Tecator Cyclotec 1093 Sample Mill grinder with a 1 mm screen prior to subsamples being taken for GlcN and GalN determination.

Naturally contaminated samples

Four naturally contaminated barley samples were obtained for hexosamine determination. The Elkhorn Top and Bottom samples were obtained from the upper and lower sections, respectively, of the same grain bin at a farm in Elkhorn, Man. The other samples were obtained from Brandon, Man. and the University of Manitoba Glenlea Research Station, Glenlea, Man. The contaminating species and percentage of seeds affected by each species was determined by randomly taking 60 seeds from each sample. Fifteen seeds were then plated directly onto two plates each of potatoe dextrose agar and malt salt agar (Smith and Onions 1983). The plates were examined after 7 d incubation at 21°C and an analysis made of the fungi. Subcultures of the Penicillium species were made and sent to the National Identification Service, Biosytematic Research Institute, Agriculture Canada, Ottawa, Ont. for species determination.

The rye ergot samples from Meadowbank and North River, Prince Edward Island and Ottawa, Ont., were obtained from Dr. J. C. Young, Plant Research Centre, Agriculture Canada, Ottawa, Ont. The wheat ergot was obtained courtesy of Northern Sales Ltd., Winnipeg, Man. All ergot samples were previously characterized by Rotter et al. (1985).

Statistical analysis

The GlcN and GalN values of experimentally cultured fungi were subjected to split plot analysis with fungal species or fungal species and grain

moisture content as the main plot and culture time as the subplot. Analyses were conducted using the General Linear Models (GLM) procedure of the Statistical Analysis System (SAS Institute Inc. 1985), and differences between means determined using the Student-Newman-Keul's test (Snedecor and Cochran 1980).

RESULTS and DISCUSSION

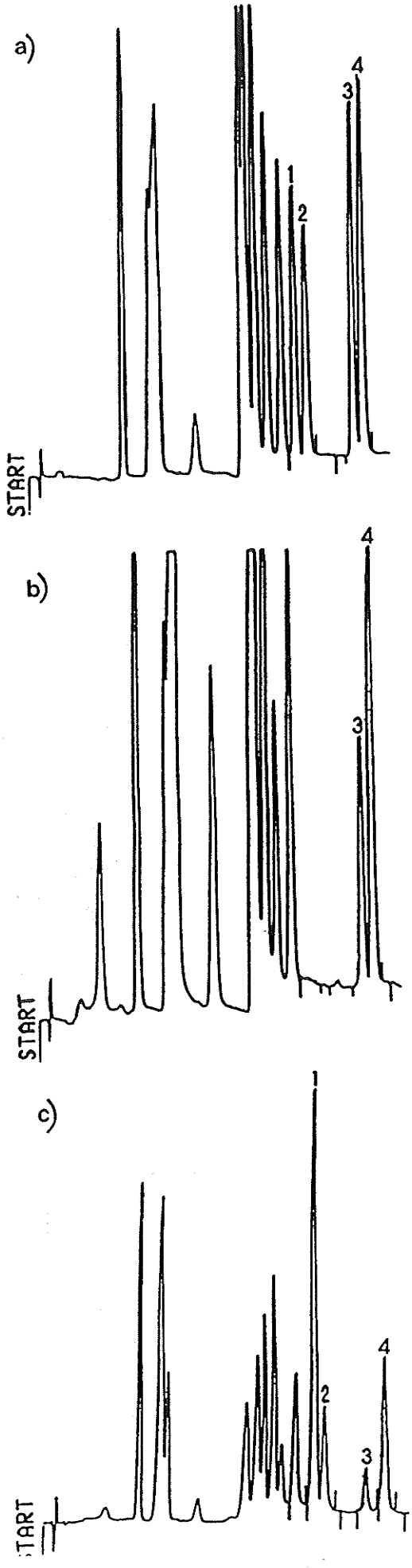
Standardization of analytical procedures

A standard mixture of the two hexosamines plus amino acids were clearly separated by ion exchange chromatography (Fig. 2a). Analysis of numerous barley samples, determined to be mold-free by visual examination using a stereomicroscope, indicates the absence of any significant peaks in the region where GlcN and GalN are eluted (Fig. 2b), while a chromatogram for barley contaminated with A. ochraceus demonstrates the presence of two new peaks corresponding with the hexosamines (Fig. 2c). These results confirm that mold-free barley contains only trace amounts of GlcN, while specific molds contain considerable quantities. They also show that GlcN and GalN can be resolved with out interference from coeluting compounds. The two hexosamines themselves were clearly separated under our conditions with a peak to valley separation of 98%.

A plot of the amount of hexosamine applied to the column versus the corresponding chromatographic peak area demonstrated a linear relationship between the two parameters from 0 to 10 mg hexosamine 10 mL^{-1} buffer. Samples with higher concentration were appropriately diluted.

A typical time-course pattern for the hydrolysis of A. ochraceus mycelia, which was used as a reference standard for subsequent studies,

Figure 2. Typical chromatographic representations of hydrolysates of: (a) standards; (b) clean Bedford barley; and (c) Bedford barley contaminated with Aspergillus ochraceus. Retention times for GluN (1), GalN (2), histidine (3) and lysine (4) were 19.9, 20.8, 23.6 and 24.9 min, respectively.

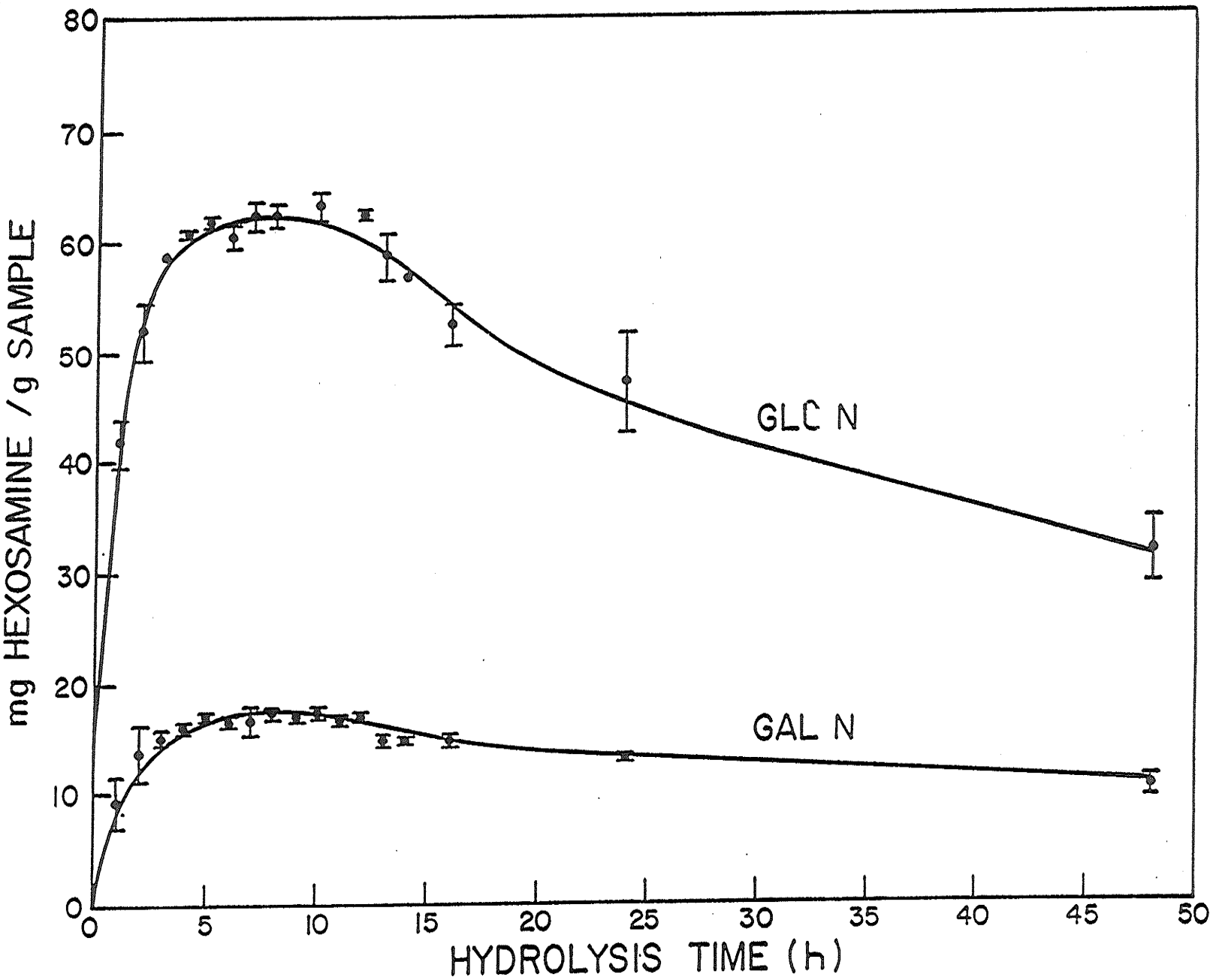

a)
START
1
2
3
4
b)
START
3
4
c)
START
1
2
3
4

shows a rapid release of GlcN and GalN during the first hour (Fig. 3). The highest values for each hexosamine were obtained between 5 and 9 h, with the peaks occurring at approximately 7 h. The optimum hydrolysis time was thus selected as being 7 h and was used for all subsequent studies. Other researchers using comparable methods have used different times and temperatures for hydrolysis. Stahmann et al. (1975) and Wu and Stahmann (1975) tested hydrolysis times of 1, 2, 4, 9 and 20 h and suggested a time of 2-4 h, while Hubbard et al.(1979) only used 3 h. The use of 8 N HCl at 95°C for 2-3 h was also advocated by Cochran and Vercellotti (1978). Other researchers using colorimetric methods have used either acid or alkaline hydrolysis with times varying from 15 min (Cousen et al. 1984) to 18 h (Schmit et al. 1975) and temperatures of 80°C (Plassard et al. 1982; Swift 1973) to 130°C (Sharma et al. 1977).

The rate of destruction of GlcN HCl and GalN HCl when hydrolyzed at 121°C in the presence of 6 N HCl acid for different lengths of time was approximately 5 and 2.4 % h^{-1}, respectively. This is in general agreement with Wu and Stahmann (1975) who obtained a degradation rate of about 3.3% h^{-1} for GlcN after 6 h under their conditions. An examination of the hydrolysis curve (Fig. 3) following 12 h of hydrolysis supports these observations. It must be noted that, among other things, this destruction is countered to a small degree by a continuing hydrolysis of chitin and other fungal material releasing more GlcN and GalN.

Hydrolysis of graded amounts of pure chitin resulted in a linear production of GlcN. After 7 h of hydrolysis, about 60% of the chitin added to mold-free barley was recovered as GlcN. This yield is reasonable considering that a GlcN molecule, less H_2O, accounts for 79%

Figure 3. Yield of GlcN and GalN from Aspergillus ochraceus hydrolyzed at 121°C in 6 N HCl for different times. The optimum hydrolysis time was determined to be 7 h. Points represent means of 4 samples ±SE.


80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
35
40
45
50
mg HEXOSAMINE /g SAMPLE
HYDROLYSIS TIME (h)
GLC N
GAL N

of the weight of a basic chitin subunit (acetylglucosamine) and that free GlcN released from chitin during the previous 7 h is undergoing destruction, and that not all the chitin may be hydrolyzed after 7 h. Overall, the yield was reasonable at 76% ((60/79)x100) of the theoretical yield.

Comparative data

Statistical analysis of the data demonstrated that there was variation in hexosamine concentrations between fungal species ($P<0.0001$), and that the hexosamine concentrations changed with the age of the culture (Table 2). It also indicated a species x time interaction ($P<0.0002$). This may be attributed to apparent increases in the concentrations of both hexosamines over time for both Aspergillus species, and no change in GlcN and a decrease in GalN for P. cyclopium. Fungal mass was similarly affected by fungal species ($P<0.0001$), time ($P<0.0009$) and a species x time interaction ($P<0.0008$). This time effect and interaction are explained by the decrease in fungal mass in the A. ochraceus cultures from day 10 to 20 but not for the other two species.

Sharma et al. (1977) and Cousin et al. (1984) claimed that there is no direct relationship between fungal mass and its chitin concentration. Their conclusions, however, were based on GlcN data derived using colorimetric methods. In contrast, Cochran and Vercellotti (1978), using ion exchange chromatography, and Plassard et al. (1982), using a colorimetric procedure, who stated that concentrations of GlcN are proportional to the amount of fungal biomass. The current liquid culture data are in general agreement with the latter conclusion, but also further indicates that differences do exist between species.

Table 2. Growth of fungi in liquid culture for 10 and 20 days and a comparison of GluN and GalN concentrations in the mycelia ±SE

Fungal species	Culture time (days)	GlcN (mg g^{-1} fungal DM)	GalN (mg g^{-1} fungal DM)
Aspergillus ochraceus	10	76.9^{c}±2.28	39.9^{b}±1.99
	20	$100.^{a}$±2.67	54.02^{a}±1.93
Aspergillus versicolor	10	72.2^{cd}±3.32	3.89^{c}±0.27
	20	90.2^{b}±1.02	4.62^{c}±0.08
Penicillium cyclopium	10	65.4^{d}±1.52	8.76^{c}±0.49
	20	66.8^{d}±1.12	5.73^{c}±0.26

a-d Means in the same column with different superscripts are significantly different ($P<0.05$) according to the Student-Newman-Keul's Test (Snedecor and Cochran 1980)

No differences were seen in the average freeze-dried weights of the A. versicolor and P. cyclopium liquid cultures between days 10 and 20, but the average weight of A. ochraceus decreased 35% from 1.43 to 1.05 g from 10 to 20 d. This decrease in weight could explain the observed 31% increase in the GlcN concentrations seen over this time period. Decreases in mycelial dry matter weights over time have been reported by others (Cousin et al. 1984; Cochran and Vercellotti 1978; Chu and Alexander 1972; Trinci and Righelato 1970) after the fungus reaches the stationary-culture phase. When the carbon source or some other nutrient is depleted, the fungus recycles cellular components of older cells. Chitin, however, once incorporated into the cell wall, is inert (Cochran and Vercellotti 1978).

The amount of chitin in the mycelium reportedly changes with age, growth rate and environmental conditions (Whipps and Lewis 1980; Sharma et al. 1977; Swift 1973; Ride and Drysdale 1972). The chemical composition of the carbon and nitrogen sources, moisture content, acidity and amount of the medium all influence the accumulation of chitin. Kostina et al. (1979) claimed that the chitin concentration in fungi grown on acidic media increase with the age of the culture while it decreases on alkaline media. Grains on which the fungi grow tend have near neutral or only slightly acidic pH values (Rotter, unpublished data), so the relative chitin concentration should remain somewhat constant over time under natural conditions on different grains.

The concentrations of hexosamines in the fungi grown on barley were considerably lower than those seen in liquid culture (Table 3). The values were higher, however, on the 35% moisture barley than on the 25%

Table 3. Comparison of fungal growth on high moisture barley at 10 and 20 days as determined by their GlcN and GalN concentrations ±SE†

		GlcN (mg g^{-1} barley)		GalN (mg g^{-1} barley)		Fungal contamination index (FCI)‡	
	Culture time (days)	10	20	10	20	10	20
Fungal species	Barley moisture content (%)						
Aspergillus ochraceus	25	0.37^{d}±0.02	0.48^{d}±0.02	ND§	ND	0.48	0.62
	35	5.20^{b}±0.22	9.18^{a}±0.85	ND	0.90±0.30	6.76	11.9
Aspergillus versicolor	25	0.27^{d}±0.05	0.34^{d}±0.07	ND	ND	0.32	0.41
	35	1.17^{d}±0.09	4.02^{c}±0.27	ND	ND	1.41	4.84
Penicillium cyclopium	25	0.46^{d}±0.02	0.36^{d}±0.13	ND	ND	0.70	0.54
	35	3.71^{c}±0.43	4.43^{bc}±0.24	ND	ND	5.61	6.70

†Individual GlcN and GalN values are the means of 4 analyses.

‡FCI values determined using the overall GlcN concentrations of the same fungal species grown in liquid culture (Table 2) with the exception of A. ochraceus. Only the 10 d GlcN values were used for this fungus as a high degree of autolysis had occurred in the growths between 10 and 20 d, resulting in 35% lower mass weights. Reference GlcN values used were (mg GlcN g^{-1} reference fungus): A. ochraceus, 76.9, A. versicolor, 83.0 and P. cyclopium, 66.1. The equation for determining FCI was:

$$\text{FCI (\%)} = \frac{(\text{mg GlcN } g^{-1} \text{ sample})}{(\text{mg GlcN } g^{-1} \text{ reference fungus})} \times 100\%$$

§ ND - not detected.

a-d Means with different superscripts are significantly different ($P<0.05$) according to the Student-Newman-Keul's Test (Snedecor and Cochran 1980)

moisture barley ($P<0.0001$), reflecting the influence of moisture on fungal growth. Donald and Mirocha (1977) stated that the lower the moisture content, the slower the fungal invasion and consequently the lower the chitin content would be.

Analysis of mold-free barley, as well as rye and wheat, indicated the presence of GlcN in all samples at concentrations of 0.4 mg g^{-1} or less (Table 4). GlcN values of less than 0.6 mg g^{-1} sample should be considered insignificant in terms of effects on animal performance unless a fungal growth is visually detectable (Manuscripts II and III).

Other researchers have reported GlcN in fungal contaminated grain, but none have attempted to establish a relationship between the amount of GlcN in a sample and the content of the fungi as estimated from a fungal reference standard. Donald and Mirocha (1977) compared soybean and corn seeds experimentally infected with mold using a modified Ride and Drysdale (1972) procedure to assay for GlcN and the percent of seed yielding fungal growth after surface disinfection procedure. Though they found a relation between the methods, they noted one can not be used to estimate the other. They pointed out that the plating method measures the number of seeds infected by mold, but not the extent, while chitin analysis directly estimates the degree of fungal colonization based on both viable and nonviable mycelium.

Due to differences in methodology or procedures, it is difficult to compare our results with those of others. Hubbard et al. (1979), using an amino acid analyzer, reported a GlcN concentration of 6.4 mg g^{-1} and GalN of less than 0.2 mg g^{-1} for A. flavus grown at 25°C on rice for 10 d with 30% moisture. The highest GlcN value they detected was 26 mg g^{-1}

Table 4. Fungal characterization of some naturally contaminated grain samples

Sample	Fungal species identified	GlcN concentration† (mg/g)	GalN concentration† (mg/g)	Total alkaloids (%)	Estimated fungal contamination ϕ (%)
Barley ∝					
Elkhorn Top	A. flavus P. cyclopium	10.7±0.12	1.79±0.07	ND∂	16.2a
Elkhorn Bottom	A. flavus P. cyclopium	2.12±0.40	-ǂ	ND	3.2a
Brandon	A. flavus P. cyclopium	0.41±0.02	-	ND	0.62a
Glenlea	P. cyclopium	1.07±0.39	-	ND	1.6a
Clean	None	0.4±0.04	-	ND	0.53
Rye					
Ottawa, Ont	C. purpurea	12.9±0.44	-	0.269*	17.0b
North River, PEI	C. purpurea	17.0±0.02	-	0.312*	22.4b
Meadowbank, PEI	C. purpurea	15.4±0.50	-	0.269*	20.3b
Clean	None	0.26±0.01	-	ND	0.34b
Wheat					
Manitoba	C. purpurea	15.1±0.27	0.17±0.04	0.308*	19.9b
Clean	None	0.28±0.03	-	ND	0.37b

†Means of 2 analyses ±SE.

ǂ -, not detected.

∂ ND, not determined.

*Determined by Rotter et al. (1985).

ϕ Reference GlcN values used for determinations were (mg GlcN g^{-1} reference fungus): a, 66; and b, 76. The barley samples where (a) was used were predominantly infected by P. cyclopium whereas (b) samples were infected by C. purpurea for which no reference values were determined so the overall average was used.

∝ The number of seeds affected by individual fungal species were (%): clean barley - Alternaria, 63, Penicillium, 6 and Fusarium, 10; Elkhorn (ET and EB) - P. cyclopium, 100 and A. flavus, 87; Brandon - P. cyclopium, 50 and A. flavus, 50; and Glenlea - P. cyclopium, 87.

of rice. Nandi (1978), using the method of Ride and Drysdale (1972), tested wheat samples experimentally infected with two Aspergillus sp. and a Penicillium sp. After 35 d of incubation, the highest reported GluN value was less than 4 mg g^{-1} of wheat. However, as the method used is not specific for GlcN, the accuracy of this value is questionable.

In the current study, A. ochraceus was again the most prolific in terms of growth, reaching a GlcN concentration of 9.19 mg g^{-1} of barley by day 20. While GalN was detected in all fungi grown on liquid culture (Table 2), it was not detected in the barley grown fungi except for A. ochraceus grown on 35% moisture barley (Table 3). The reason for this difference was not established, but it may be related to the relative stage of maturity of the fungi when harvested, or due to differences in nutrient availability between the liquid and barley media. Schmit and Brody (1975), however, noted in Neurospora crassa, that GalN maybe associated with enzyme production which occurs during the late log phase of growth, indicating that the growth must be mature.

Despite the variability in the chitin content between fungal samples, the current results indicate that the GlcN assay can be used to provide a fungal contamination index (FCI) or estimate of the fungal mass in a sample. This is important as, other than the ergosterol assay of (Seitz et al. 1979; Miller et al. 1983), there are no methods that provide reasonable estimates.

The FCI is calculated as follows:

$$\text{FCI(\%) of a sample} = \frac{\text{(mg GlcN/ g sample)}}{\text{mg GlcN/ g reference fungus}} \times 100$$

The concentration of GlcN per unit mass of reference fungus is

determined from the pure fungus grown in liquid culture. The accuracy of the calculation, as indicated above, can be increased if the fungal species in the sample is known and compared to the reference standard of the same species. Unfortunately, in most cases of natural contamination, more than one species is involved, they can not be readily identified and they usually are at different stages of growth, although virtually all immature. Therefore, a generalized FCI using the average of the GlcN concentrations of selected fungal strains grown in liquid culture is suggested. Excluding the values for A. ochraceus grown in liquid medium for 20 d because of the marked degree of autolysis (35% lower weight compared to 10 d), the range for the GlcN concentrations in the different species tested was 65-90 mg g^{-1} of immature fungus, ie. low concentrations of GalN. Based on this data, an appropriate median value would be 76 mg GlcN g^{-1} of reference fungus. This index should provide a reasonably good estimate of the amount of fungal mass in stored grain in western Canada as A. versicolor and P. cyclopium are two of the more prominent species (Abramson et al. 1982).

In a final study, fungal contamination of some naturally contaminated barley, wheat and rye samples was estimated (Table 3). The FCI values for the barley samples, other than the 'clean' sample, were calculated using P. cyclopium as the reference fungus as it was the predominant species found in these grains. Visual inspection of these samples clearly indicated that the Elkhorn Top sample contained a high degree of contamination compared to the Bottom sample and still lesser amounts were present in the Glenlea and Brandon barley samples. In the Brandon barley, the presence of mold was barely visible. These

observations are supported by hexosamine analysis and the FCI values. The proposal that GalN only occurs in the more mature fungal mass is also supported by these data.

Analysis of the samples of ergot (Claviceps purpurea) yielded values averaging about 15 mg GlcN g^{-1} ground sclerotia (Table 4). This is higher than previously reported. Schmauder and Groger (1978) stated that samples of a non-alkaloid producing strain of C. purpurea contained 3-5 mg g^{-1}, while an ergotoxine producing strain contained 7-9 mg g^{-1} of dry sclerotia. Unfortunately, they used the method of Ride and Drysdale (1972) and they did not report the concentrations of individual alkaloids nor the percent total alkaloid content of the ergot samples. Seitz and Pomeranz (1983), however, using the amino acid analysis method of Hubbard et al. (1979) reported 8.2-9.0 mg of chitin g^{-1} of ergot sclerotia. No indication of the conversion factor from GlcN to chitin was given. From the results of the current study, in contrast to Seitz and Pomeranz (1983), there does not appear to be a relationship between GlcN or chitin content and alkaloid concentration. More samples should be tested and over a much greater range of alkaloid concentrations than reported here before this question could be properly addressed. Although FCI values are presented for the ergot samples, it must be noted that they are based on the average GlcN value (76 mg g^{-1} fungus) for the Aspergillus and Penicillium species tested. To be more reliable, the Claviceps fungus should be grown under standard conditions to provide an accurate reference value.

The results of this study demonstrate that GlcN (chitin) can be used to provide a reasonable estimate of fungal contamination. Further,

using these values, a reasonable FCI can be determined which can be applied to mold contamination problems in animal feedstuffs. Although the FCI only provides an estimate of fungal mass, it is superior to estimates based on visual inspection, and it provides an alternate or additional method to the ergosterol assay (Seitz et al. 1979; Miller et al. 1983). Further refinement of the procedure, including obtaining reference GlcN values for more fungal species, is required for broader application of the technique. It does however have the advantage that samples can be easily tested for fungal contamination using slight modifications of the amino acid analysis procedure.

MANUSCRIPT II

COMPARISON OF THE EFFECTS OF TOXIN-FREE AND TOXIN-CONTAINING MOLD-CONTAMINATED BARLEY ON CHICK PERFORMANCE

ABSTRACT

A series of chick (Single Comb White Leghorn) growth trials were conducted using chemically characterized, toxin-free barley samples naturally contaminated with Penicillium cyclopium and Aspergillus flavus fungi. The concentrations of fungal (mold) material in the samples, estimated by chemical analysis, ranged from about 1 to 16%. Compared to a mold-free barley control, the mold-contaminated barleys were generally higher in percent protein, ash and ADF, but had much lower bushel weights and percent fat and starch. The first trial compared the effects of feeding mold-free barley and 30% (w/w) of the 16% mold-contaminated barley in combination with 0, 2 and 4 ppm ochratoxin A (OA) on chick performance and apparent nutrient digestibilities. Relative to controls, the average decreases in feed consumption (RFC) and weight gain (RWG) for birds fed the mold-contaminated barley were 50 and 80%, respectively. In contrast, 4 ppm of OA caused corresponding reductions of 22 and 30%, while in combination, mold and OA reduced performance slightly more than the mold alone. Similarly, apparent dry matter (DM), fat and protein digestibilities for birds fed mold-contaminated barley decreased 16.7, 37.7 and 13.7%, respectively, compared to controls, but only 10.7, 21.5 and 12.0% for those fed 4 ppm OA. A second trial compared mold-contaminated barley samples from different sources with different degrees of contamination. Decreases in RFC and RWG were directly related (R^2=0.96) to the mold concentration of the diet. The final study examined the effect on chick performance of incorporating increasing dietary concentrations of heavily contaminated (16% mold) moldcontaminated barley. As in trial 1, RFC and RWG were markedly

affected by increasing dietary mold-contaminated barley. For example, the presence of only 15% (w/w) of the heavily mold-contaminated barley in the diet caused reductions of 20 and 30% in RFC and RWG, respectively. Fat, DM and protein digestibilities again were all significantly ($P<0.0001$) reduced by the mold. The results indicate that mold contaminated barley can have a serious detrimental effect on chick performance, even when no toxins are detectable. Nutrient digestibility data suggest that antinutritive agent(s) in the mold-contaminated barley act to impair utilization of dietary nutrients rather than only those of the affected barley. Factors affecting diet palatability may also be involved.

KEY WORDS: Mold, toxin-free, mycotoxin, ochratoxin A, barley, chick performance, apparent nutrient digestibility

INTRODUCTION

The detrimental effects of feeding toxin-containing feeds to animals, particularly monogastric species, are well documented. While the effects observed due to the consumption of mold-contaminated grain may be due to the presence of various identified mycotoxins, additional effects may be due to other antinutritive compounds or toxins, or a reduction in the nutritional value of the grain. Unfortunately, very little has been reported concerning the effects of toxin-free fungal contamination alone on animal performance. Corn (Sharby et al. 1973; Fritz et al. 1973; Bartov et al. 1982; Bartov 1983, 1985), soybean (Richardson et al. 1962) and sorghum (Bartov et al. 1982), infected by several species of fungi have been shown to cause reduced weight gain and feed efficiency in growing chicks. Of these studies, only Bartov et al. (1982) and Bartov (1983, 1985) reported screening the contaminated grain for several toxins, none of which were found. None of these studies attempted to quantify the degree of fungal contamination of the grain and relate it to the effects seen in animals.

In western Canada, the presence of the potent nephrotoxin ochratoxin A (OA) in stored grains is of particular concern (Marquardt et al. 1988). The purpose of the present study was to determine the effects of feeding mold contaminated barley with and without OA at different concentrations on chick performance. In addition, the influence of different sources and increasing dietary concentrations of toxin-free mold-contaminated barley on chick performance were compared.

MATERIALS AND METHODS

Source and characterization of barley samples

Five sources of barley (Hordeum vulgare L. cv. Bedford) were obtained for use in this study and they are characterized in Table 5. The OA contaminated (referred to as Glenlea barley) and mold-free (control) samples (1984 crop) were obtained from the University of Manitoba Glenlea Research Station, Glenlea, Manitoba (Man). They had been stored in separate steel grain bins. The Brandon barley sample (1983 crop) was obtained from a farmer near Brandon, Man and had also been stored in a steel bin. The Elkhorn Top (ET) and Bottom (EB) samples (1983 crop) were obtained from the upper and lower sections, respectively, of the same wooden storage bin from a farm in Elkhorn, Man. The degree of fungal contamination was estimated according to the procedure outlined by in Manuscript I. The absence or presence of mold on the mold-free and Brandon samples was further verified by visual examination of three subsamples of each using a stereo-microscope. All barley samples were characterized in terms of fungal species present. A random sample of 60 seeds from each sample was taken. Fifteen seeds were then plated directly onto two plates each of potatoe dextrose agar and malt salt agar (Smith and Onions 1983). The plates were examined after 7 d incubation at 21°C and an analysis made of the fungi by Dr. G. Platford, Manitoba Department of Agriculture and the percentage of seeds affected by each species calculated. Subcultures of the Penicillium species were made and sent to the National Identification Service, Biosytematic Research Institute, Agriculture Canada, Ottawa, Ont. for species determination.

Table 5. Physical and chemical characterization of Bedford barley samples used in this study†

Sample/source‡	Bushel weight (kg)	DM (%)	Fat (%)	Protein (%)	Ash (%)	ADF (%)	Starch (%)	Estimated fungal concentration (%)	OA concentration
Mold-free (control)	23.62	88.9	2.24	11.7	2.39	6.57	52.5	0.6	ND
Elkhorn Top (ET)	13.24	88.0	1.85	20.4	4.07	14.9	30.3	16.1	<3 ppb
Elkhorn Bottom (EB)	15.40	88.3	1.25	13.6	2.42	9.38	49.3	3.2	<3 ppb
Brandon	18.50	88.0	1.71	9.63	2.73	7.26	56.0	0.6	<3 ppb
Glenlea	20.83	89.3	1.89	12.2	3.07	7.06	50.9	1.6	20.2 ppm

†Values are on a dry matter basis.

‡*Penicillium cyclopium* and *Aspergillus flavus* were identified in all barley samples except the mold-free and the sample from Glenlea. In the Glenlea sample, only *P. cyclopium* was found.

ʔ1 Bushel = 36.37 litres.

*Estimated according to the procedures outlined in Manuscript I

All samples were ground in a Viking Electric Hammer Mill Model C-H (Horvick Manufact. Inc., Moorhead, MN) prior to incorporation into the experimental diets.

Source of OA

The OA that was added to experimental diets 2,3,5 and 6 was contained in freeze-dried media (FDM) used in the liquid fermentation of Aspergillus ochraceus (NRRL 3174) for 10 - 12 d at 28°C (Davis et al. 1972; Yamazaki et al. 1970). The FDM contained an OA concentration of 1.9 g kg^{-1}.

Diet formulation

All diets were formulated to meet the minimum NRC (1984) requirements for Leghorn pullets (Table 6). All diets were fed as mash.

Chicks and management

One-day-old male Single Comb White Leghorn (Leghorn) chicks were purchased from a commercial hatchery and housed in electrically heated, thermostatically controlled Jamesway brooder batteries with raised wire floors and provided with continuous lighting for 7 d prior to the start of the experiment. All chicks were fed commercial chick starter crumbles containing a minimum of 21% protein during the pre-experiment period. The birds were managed and randomized as described in Rotter et al. (1985a). During the experimental periods the chicks were housed in Petersime Battery brooders and provided with continuous lighting and heat and free access to feed and water.

Experiments

Three experiments were run concurrently as some barley samples were available only in limited quantities and certain comparisons of interest could not be otherwise made. Each experiment involved six replications

per treatment and six birds per replicate, and they were conducted over two consecutive 7 d test periods.

Experiment 1. This experiment was designed to compare the effects of mold-free and mold-contaminated barley diets containing increasing concentrations of OA on chick performance and apparent dry matter (DM), fat and protein digestibilities. The experiment involved a 2 x 3 factorial arrangement of the treatments, using two dietary concentrations of ET barley (0 and 30%) in combination with three concentrations of added OA (0, 2 and 4 ppm, diets 1-6; Table 6). Additional performance comparisons using linear contrasts were made between the 2 and 4 ppm OA diets (diets 3-6, Table 5) and reference diets containing the Glenlea barley naturally contaminated with OA at 2 and 4 ppm (diets 7 and 8, Table 6), respectively.

Experiment 2. Four different samples of toxin-free barley (mold-free control, ET, EB and Brandon) were used as the sole barley component (60%) of the diet (diets 1 and 9-11, respectively; Table 6). Each barley had a different estimated degree of fungal contamination and the effects on chick performance were compared.

Experiment 3. The purpose of this experiment was to determine the influence of increasing dietary concentrations of a toxin-free fungal contaminated barley (ET) (0, 15, 30 and 60% barley, diets 1, 12,4 and 9, respectively, Table 6) on chick performance and apparent DM, fat and protein digestibilities.

Analyses

Nitrogen and fat were determined by the Kjeldahl procedure (AOAC 1984) and the method of Marchello et al. (1973), respectively. Excreta

Table 6. Dietary formulations used in Experiments 1, 2 and 3.†

Diet number	1-3‡	4-6‡	7‡	8‡	9	10	11	12
Ingredient (%)								
Barley								
Mold-free (control)	60	30	50	40	-	-	-	45
Elkhorn Top (ET)	-	30	-	-	60	-	-	15
Elkhorn Bottom (EB)	-	-	-	-	-	60	-	-
Brandon	-	-	-	-	-	-	60	-
Glenlea	-	-	10	20	-	-	-	-
Soybean meal								
90% Protein isolate	-	-	-	-	-	-	2.5	-
47.5% Protein	29.5	29.5	29.5	29.5	29.5	29.5	27.0	29.5
Other§	10.5	10.5	10.5	10.5	10.5	10.5	10.5	10.5

†The calculated crude protein and metabolizable energy values for all diets were 20.8% and 2800 kcal kg^{-1}, respectively, except for diet 11 whose values were 22.0% and 2830 kcal kg^{-1}

‡Ochratoxin A concentrations of completed diets were: diets 1 and 4, 0 ppm; diets 2 and 5, 2 ppm added as FDM OA; diets 3 and 6, 4 ppm added as FDM OA; and diets 7 and 8, 2 and 4 ppm of naturally present OA, respectively.

§Other ingredients consisted of (%): tallow, 6.55; $CaPO_4$, 1.4; Ca_2CO_3, 0.7; vitamin premix, 1.0; mineral premix, 0.5; methionine, 0.05; and Cr_2O_3, 0.3. The vitamin and mineral premixes were the same as those described by Young and Marquardt (1982). Ochratoxin A in the form of ground FDM was added to the vitamin/mineral premix in place of an equivalent weight of wheat middlings.

nitrogen values were adjusted for uric acid content (Marquardt 1983) prior to converting the values to excreta protein (Rotter et al. 1988). Chromic oxide in the diets and feces were analyzed by the method of Williams et al. (1962) on an atomic absorption spectrophotometer (Instrumentation Laboratory aa/ae spectrophotometer Model 551; Instrumentation Laboratory Inc., Wilmington, MA).

Prior to incorporation in the diets, all barley samples were analyzed for dry matter (DM), ash and acid detergent fibre (ADF) according to AOAC (1984) procedures. Diets and excreta samples were also analyzed for DM. Weights per unit volume (bushel weight) were obtained by taking the average weight of four 2.5 litre subsamples of each barley and multiplying the value by 14.55. Starch analysis of barley samples was determined using a combination of two methods. Starch was first hydrolyzed by amyloglucosidase (Boehringer Mannheim 1984) followed by the determination of glucose using the glucose oxidase method (Sigma Chem. Corp. 1978) and a Beckman DU-8 spectrophotometer.

Multitoxin analysis, which included analysis for aflatoxins, OA, penicillic acid, citrinin, sterigmatocystin, patulin and zearalenone, was conducted by the methods of Wilson et al. (1976) and Josefsson and Moller (1977). Actual OA concentrations of the experimental diets were confirmed by the method of Frohlich et al. (1988). The degree of fungal contamination was estimated by analysis for glucosamine (Manuscript I) and the fungal species were identified by Dr. G. Plattford, Manitoba Dept. of Agriculture, Winnipeg, Manitoba. All chemical analyses were performed in duplicate.

Chick performance was measured in terms of feed consumption, weight

gain and feed to gain ratio (F/G). Both feed consumption and weight gain are presented as values relative to control birds. The F/G data are the average of F/G values of pens on the same dietary treatment. In cases of mortalities occurring during this study, the correction method outlined in Rotter et al. (1985b) was followed. The apparent nutrient digestibilities values were calculated according to the standard formula given in Crampton and Harris (1969).

All chick performance data were analyzed by split plot analysis of variance using the General Linear Models (GLM) procedure of the Statistical Analysis System (SAS Institute, Inc. 1982). In Experiment 1, mold and OA concentrations were used as the main plot comparisons, while diet and mold concentration were used in Experiments 2 and 3, respectively. In all studies time was used as the subplot comparison. Nutrient retention and mortality data were analyzed using the appropriate competely randomized designs. In addition, for Experiments 2 and 3, chick performance and apparent nutrient digestibility data were subjected to regression analysis while linear contrasts were conducted for Experiment 1. The data in part was interpreted according to the idea of partitioning of sums of squares as outlined by Little (1981), and differences between means determined using the Student-Newman-Keul's test (Snedecor and Cochran 1980).

RESULTS

Barley samples

The DM content of all barley samples was similar (Table 5), but bushel weight and percent fat and starch in the fungal contaminated samples

were considerably lower than the mold-free barley control. In the most extreme case, the percent starch of the ET barley was only 58% of the control value. In contrast, ADF and percent protein and ash were higher in the mold-contaminated barleys, with the percent protein in ET being almost twice that of the mold-free barley. Only the Brandon barley had a protein concentration lower than that of the control.

The contaminated barley samples all visibly contained mold, but to varying degrees. The ET barley consisted of clumps or cakes of molded grain while the majority of the others contained surface mold. The degree of fungal contamination was chemically estimated to range from 0.6 to 16% (Table 5). The contamination estimate for the mold-free barley suggested a 0.6% contamination, but other glucosamine-containing contaminants such as bacterial spores and seed glycoproteins (Donald and Mirocha 1977) can be mistaken for mold growth at low glucosamine concentrations. Although visual examination of the mold-free barley indicated no observable mold, mold was seen on the Brandon barley. Penicillium verrucosum var. cyclopium (Westling) Samson, Stalk and Hadlock was the dominant fungal species in all of the contaminated samples. With the exception of the Glenlea barley, these samples also contained A. flavus Link ex Fries. Ochratoxin A was the only mycotoxin detected and only in appreciable concentrations in the Glenlea barley.

Experiment 1

Statistical analysis of performance of chicks fed the mold-free and 30% ET barley diets that contained increasing concentrations of added OA (diets 1-6) indicated significant interactions and main effects ($P<0.05$) for relative feed consumption (RFC) and relative weight gain (RWG)

(Table 7). Partitioning the sums of squares demonstrated that the mold concentration of the diet was responsible for 87 and 91% of the variation in RFC and RWG, respectively, but only 40% of that for the feed to gain ratio (F/G). Time and a mold x time interaction accounted for an additional 19 and 20% of the variance, respectively, for F/G. Another 5% of the variation in RFC and RWG was due to OA concentration, while all other effects in total accounted for less than 8% of the variation. The data indicate that the 30% ET diets reduced RFC and RWG values by 50 and 80%, respectively, compared to the control diet. When OA was present in the mold-free diet at the 2 and 4 ppm concentrations, it only reduced RFC by 10 and 22% and RWG by 11 and 30%, respectively. In contrast to the trend seen for OA, F/G for mold fed birds was higher in Week 1 than 2. These results clearly demonstrate that at the concentrations used, the mold infected barley affected chick performance to a much greater degree than the OA, and that these factors appeared to depress performance independently.

Analysis of variance of the nutrient digestion data (Table 8) demonstrated that mold-contaminated barley significantly ($P<0.0001$) reduced apparent DM, fat and protein digestibilities. Ochratoxin A only affected apparent fat and protein digestibilities. Although there was a mold x OA interaction for fat digestibility, partitioning of the sums of squares indicated it was only responsible for 1.5% of the variation. Compared to controls, the presence of OA at concentrations of 2 and 4 ppm, respectively, reduced digestibilities of fat by 20.4 and 21.5% ($P<0.0001$) and protein by 9.3 and 12.0% ($P<0.0002$), but did not significantly affect DM ($P>0.12$). The effect of mold-contaminated barley

Table 7. Effect of increasing concentrations of dietary OA in the absence and presence of fungal contaminated barley on chick performance (Experiment 1).

Diet	OA concentration (ppm)	Estimated mold content (%)	Week	Relative feed consumption	Relative weight gain	Feed to gain ratio (g/g)
Mold-free (1)†	0	0	1	1.00 (91.6 g)‡	1.00 (46.7 g)‡	1.96
			2	1.00 (145.0 g)‡	1.00 (70.3 g)‡	2.07
Mold-free + 2 ppm OA (2)†	2	0	1	0.91	0.89	2.03
			2	0.89	0.88	2.09
Mold-free + 4 ppm OA (3)†	4	0	1	0.81	0.72	2.23
			2	0.75	0.69	3.82
ET 30 (4)†	0	4.8	1	0.53	0.15	13.23
			2	0.44	0.29	3.16
ET 30 + 2 ppm OA (5)†	2	4.8	1	0.58	0.24	5.73
			2	0.50	0.32	3.82
ET 30 + 4 ppm OA (6)†	4	4.8	1	0.53	0.12	14.06
			2	0.41	0.20	4.17
SE				0.02	0.04	3.73

Continued .....

Table 7 (continued) Summary of analysis of variance (Experiment 1)

Source of variation	df	Relative feed consumption		Relative weight gain		Feed to gain ratio (g/g)	
		MS	Prob.	MS	Prob.	MS	Prob.
Mainplot comparisons							
Mold	1	2.71	0.0001	7.17	0.0001	481.2	0.002
OA concentration	2	0.089	0.0001	0.213	0.0001	33.1	0.47
Mold x OA	2	0.067	0.0001	0.083	0.003	29.6	0.50
Error a	29	0.006		0.012		42.2	
Subplot comparisons							
Time	1	0.070	0.0001	0.058	0.002	227.5	0.027
Mold x time	1	0.022	0.004	0.029	0.0016	235.7	0.025
OA x time	2	0.004	0.16	0.003	0.57	32.0	0.47
Mold x OA x time	2	0.0003	0.85	0.001	0.80	31.9	0.48
Error b	29	0.0022		0.005		41.8	

†Number in parentheses denotes diet number.

‡Values in parentheses denote actual values for controls (average per bird per 7 days).

ℓ Corresponding relative feed consumption, relative weight gain and feed to gain ratio values for the Glenlea barley with naturally present OA for weeks 1 and 2, respectively were: for 2 ppm OA, diet 7, 0.98 and 0.97 g, 0.96 and 0.95 g, and 2.02 and 2.12; and for 4 ppm OA, diet 8, 0.81 and 0.75 g, 0.73 and 0.69 g, and 2.18 and 2.26, respectively. See text for statistical comparison with above diets.

Table 8. Influence of increasing concentrations of dietary mold-contaminated barley with and without OA on apparent nutrient digestibilities (Experiment 1)†

	Apparent nutrient digestibility		
	DM	Fat‡	Protein§
OA concentration (ppm)			
0	62.2	71.7	81.8
2	60.1	67.8	77.7
4	59.2	66.9	75.4
SE	1.06	0.66	0.95
Mold-contaminated barley (%)			
0	66.2	85.7	83.0
30	55.2	53.1	74.0
SE	0.85	0.53	0.76

Summary of analyses of variance

		DM		Fat		Protein	
Source of variation	df	MS	Prob.	MS	Prob.	MS	Prob.
Mold-contaminated barley concentration (mold)	1	873.3	0.0001	7731.0	0.0001	577.5	0.0001
OA concentration (OA)	2	23.0	0.12	57.7	0.0001	102.5	0.0002
Mold x OA	2	19.0	0.17	59.7	0.0001	6.57	0.47
Error	23	10.0		3.94		8.19	

†Actual values for controls were (%): DM, 66.3; fat, 85.2 and protein, 85.7.

‡Values for fat digestibilities for each diet were (%): Diet 1, 85.2; Diet 2, 87.6; Diet 3, 84.7; Diet 4, 58.2; Diet 5, 52.0; and Diet 6, 49.1.

§Excreta protein values were corrected for uric acid content

was greater, however, as DM, fat and protein digestibilities were reduced by 16.7, 37.7 and 13.7%, respectively ($P<0.0001$).

In another comparison, linear contrasts of RFC and RWG suggest that the OA naturally present in the Glenlea barley was less potent than the lab cultured toxin when added to the mold-free diet at the 2 ppm concentration (diet 7 vs 2, $P<0.003$ and 0.04, respectively; see Table 7 footnote for data). At 4 ppm, however, the Glenlea barley OA produced a similar effect to that obtained with the OA in the mold-free diet (diet 8 vs 3, $P>0.97$ and 0.87, respectively). The F/G values were not different ($P<0.0001$).

Experiment 2

Split plot analysis of the four toxin-free barley samples (diets 1,9,10 and 11) indicated a diet x time interaction for RFC, RWG and F/G ($P<0.05$, 0.002 and 0.0001, respectively) in addition to a time effect ($P<0.0001$) for RWG and F/G and a diet effect for RFC and RWG (Table 9). Diet accounted for 99.7, 97.2 and 73.3% of the variation of RFC, RWG and F/G, respectively, with the diet x time interaction accounting for another 17.5% of the variation for F/G. The F/G values for the 60% ET, 60% EB and 60% Brandon barley diets for weeks 1 and 2 were 10.02 and 7.12, 2.42 and 2.37, and 2.08 and 2.09, respectively.

Regression analysis between mold content of the diet and RFC and RWG gave R^2 values of 0.96 ($P<0.022$ and 0.0001, respectively, for both parameters, but only 0.04 ($P>0.99$) for F/G. These results suggest there was a corresponding reduction in chick performance for a given degree of fungal contamination. Analysis of variance of the mortality data indicated a diet effect ($P<0.0001$). The percent weekly mortality for the

Table 9. Chick performance as affected by barley samples containing varying amounts of fungal mass (Experiment 2)

Diet	Estimated fungal contamination (%) of barley	Relative feed consumption	Relative weight gain	Feed to gain ratio (g/g)
Mold-free (1)†	0.6	1.00 (236.6 g)‡	1.00 (117.0 g)‡	2.02
ET 60 (9)†	16.1	0.34	-0.02	8.70
EB 60 (10)†	3.2	0.94	0.79	2.40
Brandon 60 (11)†	0.6	1.04	1.00	2.08
SE		0.01	0.02	0.54

Summary of analysis of variance (Experiment 2)

Source of variation	df	Relative feed consumption MS	Relative feed consumption Prob.	Relative weight gain MS	Relative weight gain Prob.	Feed to gain ratio (g/g) MS	Feed to gain ratio (g/g) Prob.
Mainplot comparison							
Diet	3	1.09	0.0001	2.31	0.0001	598.0	0.34
Error a	19	0.034		0.060		500.8	
Subplot comparison							
Time	1	0.0013	0.44	0.087	0.0002	150.5	0.0001
Diet x time	3	0.0028	0.27	0.025	0.004	140.3	0.0001
Error b	18	0.002		0.004		2.98	

†Numbers in parentheses denotes diet number.

‡Values in parentheses denote actual values for controls (average per bird per 14 days).

mold-free, 60% ET, 60% EB and 60% Brandon diets were 0.0, 21.8, 1.4 and 0.0, respectively.

Experiment 3

Diets containing increasing concentrations of ET barley (diets 1,12,4 and 9) caused significant mold ($P<0.0001$) and time main effects ($P<0.003$ and 0.0002, respectively) for RFC and RWG, and a mold x time interaction for RWG ($P<0.0012$) and F/G ($P<0.003$) (Tables 10). Mold concentration accounted for 98.9 and 96.9% of the variability for RFC and RWG. The mold x time interaction for F/G indicates the trend during the study differed over time. While the F/G for mold-free and 15% ET fed birds increased slightly from week 1 to 2, birds fed the 30 and 60% ET diets had dramatic reductions in F/G. Regression analysis between the amount of mold-contaminated grain in the diet and RFC, RWG and F/G gave R^2 values of 0.86 ($P<0.0001$), 0.86 ($P<0.0001$) and 0.03 ($P>0.27$), respectively. This suggests that there is a strong relationship between reduced performance and concentration of mold-contaminated grain in the diet.

The presence of mold-contaminated barley in the diet caused dramatic reductions in apparent nutrient digestibility compared to the mold-free diet (Table 11). Dry matter and fat digestibilities were severely reduced ($P<0.0001$), decreasing 56 and 50% for 60% ET compared to the control values. Protein digestibility was less dramatically affected ($P<0.05$), decreasing from 85.7 in the mold-free to 69.1% in the 60% ET barley fed birds, a relative decrease of 20%. The ET barley when included in the diet at only 15% depressed the digestibilities of fat by 21% and DM by 13%, but protein by less than 8%. Regression analysis

Table 10. Comparison of increasing quantities of mold-contaminated barley in the diet (Experiment 3)

Diet	Amount of mold contaminated barley added (%)	Week	Relative feed consumption	Relative weight gain	Feed to gain ratio (g/g)
Control (1)†	0	1	1.00 (91.60 g)‡	1.00 (46.73 g)‡	1.96
		2	1.00 (145.03 g)‡	1.00 (70.27 g)‡	2.07
ET 15 (12)†	15	1	0.82	0.69	2.35
		2	0.80	0.69	2.42
ET 30 (4)†	30	1	0.53	0.15	13.23
		2	0.44	0.29	3.16
ET 60 (9)†	60	1	0.38	-0.11	10.02
		2	0.30	0.10	7.12
SE			0.02	0.03	2.62

Summary of analysis of variance (Experiment 3)

Source of variation	df	Relative feed consumption		Relative weight gain		Feed to gain ratio (g/g)	
		MS	Prob.	MS	Prob.	MS	Prob.
Mainplot comparison							
Mold	3	0.935	0.0001	2.09	0.0001	800.2	0.42
Error a	19	0.004		0.008		536.7	
Subplot comparisons							
Time	1	0.019	0.003	0.095	0.0002	16.3	0.54
Mold x time	3	0.004	0.075	0.035	0.0012	281.9	0.003
Error b	18	0.002		0.004		41.1	

†Number in parentheses denotes diet number.

‡Values in parentheses denote actual values for controls (average per bird per 7 days).

Table 11. Influence of increasing dietary mold on apparent nutrient digestibilities (Experiment 3)

Mold-contaminated barley in diet (%)	Apparent nutrient digestibility (%)		
	Dry matter	Fat	Protein‡
0 (1)†	66.3a	75.2a	85.7a
15 (12)†	57.8b	67.2b	79.2b
30 (4)†	58.1b	58.2b	77.9b
60 (9)†	33.0c	37.3c	69.1c
SE	2.65	4.02	1.48

†Numbers in parentheses denote diet number.

‡Excreta protein values corrected for uric acid content

a-c Means in the same column with different letters are significantly different ($P<0.05$) according to the Student-Newman-Keul's test (Snedecor and Cochran 1980).

between mold concentration in the diet and DM, fat and protein digestibilities gave R^2 values of 0.77, 0.81 and 0.77, ($P<0.0001$), respectively.

Analysis of variance of the mortality data again indicated a significant diet effect ($P<0.0001$) due to increasing mold concentrations. The percent weekly mortality for the diets containing 0, 15, 30 and 60% ET barley (diets 1,12,4 and 9) were 0.0, 0.0, 2.8 and 21.8, respectively.

DISCUSSION

The fat and starch concentrations in the mold-contaminated barley samples tended to be lower than the control barley values. Similar results were previously reported in stored corn by Bartov et al.(1982) and Bartov (1983; 1985). In one study, the values dropped from 4.1 in the controls to 2.3% fat in the contaminated corn (Bartov 1985). This is consistent with the observation of the current study, and a similar situation appears to be true for starch. The decrease in both fat and starch could also account for the drop in bushel weight of mold-contaminated barley. The concentrations of protein, ash and ADF while lipids and carbohydrates were greater in contaminated than uncontaminated barley. The change is probably the result of the selective loss of starch and fat while protein and ash were conserved. The net effect would be seen as a decrease in bulk density of the barley and an apparent relative increase in the protein and ash concentrations, but with little change in the absolute quantities. Researchers examining the feasibility of using fungal protein in animal feeds have reported

high values of crude protein, such as 45% for Chaetomium cellulolyticum (Touchburn and Chavez 1986) and 61.8% for yeast (Slagle and Zimmerman 1979). Therefore, it is logical to assume that the greater the degree of fungal contamination, the higher the protein value that would be obtained from the sample. The higher ADF values for the mold-contaminated samples could also be a reflection of the amount of chitin present. Chitin is the major component of the fungal skeleton (Hicks and Newell 1983) and it is relatively indestructible, and so could erroneously show up in increased fiber readings.

It was not surprising that the fungi P. cyclopium and A. versicolor were the species found in the contaminated barley samples. These two species are among the most commonly found storage fungi in western Canada (Abramson et al. 1982). Considering the degree of contamination, it is noteable that no detectable toxins were found in most of these samples. Generally it is assumed that a mold-contaminated grain will likely contain toxin(s), and the greater the degree of contamination, the higher the toxin concentration will be. This is not necessarily true as toxins are produced by the fungi in response to a stress and not all fungi are capable of or do produce detectable toxins on certain substrates.

The results of the current studies demonstrate that diets containing a high degree of mold-contaminated barley had a serious effect on RFC, RWG and F/G. The apparent digestibilities of dry matter, protein and especially fat were also markedly reduced. The decreases in tha apparent digestibilities of these nutrients may be partially attributed to a decrease in the availability of the carbohydrate

fraction. The starch concentrations of the mold contaminated samples were all much lower than the mold-free barley. Of particular interest were the reduced apparent digestibilities of fat and protein, considering most of the dietary fat was derived from tallow and much of the protein from soybean meal. This suggests that not only were the nutrients in the mold-contaminated barley less available, but that digestion or uptake of dietary nutrients from all components are interfered with. Further studies are required to establish the precise effects and nature of the antinutritive agent(s).

A reduction in the apparent nutrient digestibilities of chicks fed mold-contaminated grain and toxin-added diets have been reported by other authors (Sharby et al. 1972; Bartov et al. 1982; Nelson et al. 1982). Nelson et al. (1982) fed corn diets contaminated naturally or artificially with a Penicillium species or A. ochraceus. The diets contained 3 ppm aflatoxin, 800 ppb citrinin and 300 ppb OA, respectively, but the greatest antinutritive effects seen were due to the OA. It caused a reduction in DM, amino acid and metabolizable energy digestibilities of 34.6, 41.6 and 35.3%, respectively, compared to a control diet. Aflatoxin caused no significant differences ($P>0.05$), while citrinin was intermediate in effect, but significantly different ($P<0.05$) from the control and aflatoxin diets and the OA diet. Sharby et al. (1972), in a series of studies fed mold-contaminated corn, containing one of several species of Fusarium or Aspergillus fungi, but apparently no toxins were tested for. In this case, DM digestibility reductions ranged from 4 to 8%, coincidently on an A. flavus infected sample. Similarly, a 13% decrease in protein digestibility was noted by

Bartov et al. (1982) in chicks given mold-contaminated corn, but no difference was seen in birds given mold-contaminated sorghum compared to the controls. No indication of the degree of mold contamination was given in any of these studies.

Other studies on the use of fungal protein in animal diets suggest that, with some possible modifications or additions, they can be used without detrimental effects (Santos and Gomez 1983; Touchburn and Chavez 1986). The same, as discussed above, is obviously not true for mold contaminated grain.

In western Canada, OA is the most commonly found toxin in contaminated stored grain (Abramson et al. 1982). Although it effects have been studied extensively, no studies have been reported on the effects of OA and storage fungi. Under the conditions of the current study, the results suggest that even moderately mold contaminated grain, apparently toxin free, has a greater effect than relatively high concentrations of OA. The concentrations of OA used in the current study (2 and 4 ppm) are not atypical under practical feeding conditions, however, as evidenced by the 20 ppm OA detected in the Glenlea barley. The results also indicated that the OA added to the mold-contaminated barley diet, only caused a slight additional decrease in chick performance compared to that of the mold alone.

The possible dangers of feeding a grain that is even slightly contaminated with mold is evident from examination of data from the Glenlea barley diets. Despite the low degree of fungal contamination (0.16% mold), the natural level of OA was high at 20 ppm. Incorporation of this barley as the sole barley component of the diet would have

caused serious problems in animals fed such a diet, especially since the mold contamination was barely visible.

The results clearly indicate that even when a sample is found to be relatively toxin-free, it may adversely affect animal performance, but that its effects may be partially alleviated by dilution of the mold-contaminated with mold-free grain. At moderately high concentrations of mold, the effects appear to be due to interference with nutrient utilization, but this does not exclude other factors which affect diet palatability. Both mold and toxin, either singly or in combination, can have serious detrimental effects on animal performance, but in light of the current studies more emphasis should be directed to the effects caused by the fungus alone.

MANUSCRIPT III

INFLUENCE OF OCHRATOXIN A, DIETARY TALLOW AND STARCH SUPPLEMENATION ON THE DETRIMENTAL EFFECTS OF TOXIN-FREE MOLD-CONTAMINATED BARLEY ON GROWING CHICKS

ABSTRACT

The effects of feeding toxin-free Penicillium cyclopium and Aspergillus flavus contaminated barley on Leghorn chicks were examined. In the first experiment, the detrimental effects of increasing dietary concentrations (0, 15 and 30%) of a mold-contaminated barley (16% mold) in combination with 0 or 4 ppm added ochratoxin A (OA) on chick performance and apparent nutrient digestibilities were studied. Relative to the control birds, feed consumption (RFC) and body weight gain (RWG) for chicks fed the 15% mold-contaminated barley diets were reduced 40 and 50%, respectively, and 53 and 79%, respectively, when given those with 30% mold-contaminated barley. OA alone caused respective reductions of 30 and 40%, while combined with mold, only reduced performance an additional 10% in relation to mold alone. Both mold and OA caused significant ($P<0.006$) depressions in apparent dry matter and protein digestibilities, but the effects were greatest when both were present in the diet, digestibilities decreasing to lows of 49 and 70%, respectively. While protein was more sensitive to the presence of OA, the apparent digestibility of fat was more affected by mold, decreasing to it to 45 and 20% that of birds given 15 and 30% mold-contaminated barley, respectively.

The second study examined the hypothesis that the effects of mold contaminated barley are due to a deficiency of readily digestible nutrients, or some other factor. Chicks were fed diets containing either mold-free barley, a non-nutritive bulk (cellulose) plus mold-free barley, or a mold-contaminated barley (7% mold), in combination with low (2%) or high (10%) dietary tallow and supplemental starch (0 and 15%).

High fat and starch supplementation both resulted in slightly higher RWG in birds fed the mold-contaminated barley and cellulose containing diets, but the maximal improvement values were still more than 60 and 20%, respectively, lower than birds given a 6.7% tallow reference diet. Neither fat nor starch affected RFC. The birds fed the cellulose diet performed about 100% better than those given mold-contaminated barley, despite a theoretically lesser concentration of available nutrients in the former. Results of both studies strongly suggest the presence of some unidentified factor(s) in mold-contaminated grain which interfere with nutrient digestibility/ absorption, in addition to reducing feed intake.

KEY WORDS: Toxin-free mold, ochratoxin A, mycotoxin, chick performance, nutrient, digestibility, starch, fat

INTRODUCTION

Stored grains are susceptible to contamination by various fungal species, most of which are capable of producing secondary metabolites. These metabolites, which include mycotoxins, can have serious detrimental effects when ingested by animals. In western Canada, ochratoxin A (OA), a potent nephrotoxin produced by Aspergillus and Penicillium species fungi, is increasingly being viewed with particular concern (Abramson et al. 1983; Sinha et al. 1988; Marquardt et al. 1988). While most research has dealt with the effects of mycotoxins, the possible toxic or antinutritional effects of the fungi themselves have received very little attention. Under many conditions, a fungus may not produce detectable concentrations of identified toxin(s), and so the contaminated grain may erroneously be considered safe for animal feed (Manuscript II). The inclusion of such a grain into animal feed could result in major economic losses to animal producers through poor animal performance.

The detrimental effects of feeding fungal contaminated barley (Manuscript II), corn (Sharby et al. 1973; Fritz et al. 1973; Bartov et al. 1982), sorghum (Bartov et al. 1982) and soybeans (Richardson et al. 1962) to growing chicks have been reported, but only Bartov et al. (1982), Bartov (1983) and Rotter et al. (Manuscript II) reported screening for toxins. Studying the effect of toxin-free mold-contaminated barley, Rotter et al. (Manuscript II) noted a dramatic reduction in chick performance and lower nutrient digestibilities, particularly for fat. Addition of 4 ppm OA to the mold-contaminated barley did not further reduce performance. Bartov et al. (1982) claimed

that the effect of mold contamination is due to a reduction in the fat content of the grain caused by the mold. He concluded that supplementation of a contaminated diet with fat to a level similar to that lost to the fungus would be sufficient to overcome the mold effect. In addition to a reduction in fat concentration, Rotter et al. (Manuscript II) demonstrated that mold contamination of barley can result in a decrease in the concentration of starch, thereby proportionaly increasing the apparent concentrations of other grain components, such as protein, minerals and fibre. The lower starch and fat concentrations would undoubtedly affect the metabolizable energy of the grain and be reflected in poorer chick performance.

The objective of the initial study was to compare the effects of increasing concentrations of a toxin-free mold-contaminated barley with and without added OA on chick performance and nutrient digestibilities. This study should aid in estabishing the effects of mold versus toxin and further examine the nature of the interaction between the two. The purpose of the second study was to determine if the growth depressing effects of mold-contaminated barley are attributable to a deficiency of readily digestible nutrients or some other factor. To test this hypothesis, the performance of chicks fed diets containing either a mold-free barley, a non-nutritive bulk (cellulose) plus mold-free barley or a mold-contaminated barley, and different concentrations of dietary tallow and supplemental starch were compared. These studies should provide a better understanding of the factor(s) responsible for the antinutritive properties of mold-contaminated grain.

MATERIALS AND METHODS

Source of barley samples

Barley (Hordeum vulgare L. cv. Bedford) from two sources, previously described in Manuscript II, was used in this study. Mold-free (control) barley (1984 crop) was obtained from the University of Manitoba Glenlea Research Station, Glenlea, Manitoba (Man). In Experiment 4, the mold-contaminated sample refered to as Elkhorn Top (ET) was used, while Experiment 5 involved a mixture (50:50) of ET and Elkhorn Bottom (EB) due to a limited supply of ET. These samples (1983 crop) were obtained from the top (ET) and bottom (EB) sections of the same storage bin from a farm in Elkhorn, Man. All samples were previously charcterized chemically and mycologically by Rotter et al. (Manuscripts I and II)

The barley samples were ground in a Viking Electric Hammar Mill Model C-H (Horvick Manufact. Inc., Moorhead, MN) before mixing experimental diets.

Source of OA

The OA that was added to diets 2, 4 and 6 in Experiment 4 was contained in freeze-dried media (FDM) used for the liquid fermentation of Aspergillus ochraceus (NRRL 3174) for 10-12 d at 28°C (Davis et al. 1972; Yamazaki et al. 1970). The FDM contained OA at a concentration of 1.9 g kg^{-1}.

Diet formulation

All basal diets were formulated to meet the minimum NRC (1984) requirements for Leghorn pullets (Tables 12 and 13), and fed as mash. The non-nutritive bulk compound, cellulose (Celufil), incorporated in diets 5-8 of Experiment 5 was purchased from United States Biochemicals

Table 12. Dietary Formulations used in Experiment 4.†

Dietary component (%)	Diet					
	1	2	3	4	5	6
Mold-free barley	59.0	59.0	44.0	44.0	29.0	29.0
Mold-contaminated barley	-	-	15.0	15.0	30.0	30.0
OA/wheat‡ middlings	1.00	1.00	1.00	1.00	1.00	1.00
Other§	40.0	40.0	40.0	40.0	40.0	40.0

† Protein and metabolizable energy values for all diets were calculated to be 21.1% and 2770 Kcal kg^{-1}, respectively.

‡ Diets 1, 3 and 5 incorporated only wheat middlings. Diets 2, 4 and 6 incorporated OA contained in FDM mixed with wheat middlings to produce a dietary concentration of 4 ppm OA.

§ Other ingredients consisted of (%): Soybean meal (47.5% protein), 29.5; Tallow, 6.55; $CaCO_3$, 1.4; Ca_2PO_4, 0.7; Vitamin premix, 1.0; Mineral premix, 0.5; Methionine, 0.05; and Cr_2O_3, 0.3. The vitamin and mineral premixes used are detailed in Young and Marquardt (1982).

Table 13. Dietary formulations used Experiment 5.

Dietary component	Diet 1	2	3	4	5	6	7	8	9	10	11	12	13‡
Mold-free barley	64.45	56.55	49.55	41.55	34.5	26.5	34.5	26.5	-	-	-	-	60.0
Celufil	-	-	-	-	30.0	30.0	15.0	15.0	-	-	-	-	-
Moldy barley	-	-	-	-	-	-	-	-	64.0	56.55	49.55	41.55	-
Starch	-	-	15.0	15.0	-	-	15.0	15.0	-	-	15.0	15.0	-
Soybean meal (47.5% protein)	29.5	29.5	27.75	25.0	20.9	20.9	20.9	20.9	25.0	28.5	27.75	25.0	29.5
Soybean protein concentrate (90% protein)	-	-	1.75	4.5	8.65	8.65	8.65	8.65	-	-	1.75	4.5	-
Tallow	2.0	10.0	2.0	10.0	2.0	10.0	2.0	10.0	2.0	10.0	2.0	10.0	6.55
Wheat middlings	-	-	-	-	-	-	-	-	5.05	1.00	-	-	-
Other†	3.95	3.95	3.95	3.95	3.95	3.95	3.95	3.95	3.95	3.95	3.95	3.95	3.95
Calculated values													
Protein (%)	22.4	21.4	21.1	21.0	21.7	20.6	21.7	20.6	21.0	21.0	21.1	21.0	20.8
Metabolizable energy (Kcal kg^{-1})	2570	2965	2745	3165	1875	2265	2420	2810	2590	2960	2745	3165	2800

† Other ingredients consisted of vitamin and mineral premixes, $CaCO_3$, Ca_2PO_4 and methionine in quantities as described in Table 12

‡ Diet 13 was fed to birds used as a reference. Data from these birds was not included in statistical analyses.

Ltd., Cleveland, OH.

Chicks and management

One-day-old male Single Comb White Leghorn (Leghorn) chicks were purchased from a commercial hatchery. For 7 d prior to the start of an experiment, the birds were housed in electrically heated, thermostatically controlled Jamesway brooder batteries with raised wire floors and continuous lighting and fed commercial chick starter crumbles (21% protein). The chicks were managed and randomized as outlined in Rotter et al. (1985a). They were housed in Petersime Battery Brooders and provided with continuous heat and lighting and free access to feed and water during the experimental periods.

Experiments

Experiment 4. The objective was to determine the effects of increasing concentrations of toxin-free mold-contaminated barley with and without 4 ppm added OA on chick performance, mortality and apparent nutrient digestibility. The treatments were set up as a 3 x 2 x 2 factorial arrangement, using three concentrations of mold-contaminated barley (0, 15 and 30% of the diet) incombination with two OA concentrations (0 and 4 ppm) over two consecutive 7 d test periods. Six replicates, with six birds each, were used per treatment.

Experiment 5. The influence of dietary tallow and supplemental starch on reducing the performance depressing effects of a toxin-free mold-contaminated barley on chicks was examined. In addition, a comparison of effects was made with a non-nutritive bulk (cellulose) substitute for mold-contaminated barley. This experiment was arranged as a 3 x 2 x 2 x 2 factorial, involving three types of diets [control barley, mold-

contaminated barley and control barley plus cellulose (cellufil)], and two concentrations each of tallow (2 and 10%) and of starch (0 and 15%) conducted over two consecutive 7 d test periods. Each treatment included six replicates with six birds each. Chick performance and mortality were used as measurement parameters.

Analyses

Multitoxin screening of the barley samples and fungal material used as the source of OA was conducted according to the methods of Wilson et al. (1976) and Josefsson and Moller (1977). The procedure included analysis for aflatoxins, penicillic acid, citrinin, sterigmatocystin, patulin, zearalenone as well as OA. All diets were also tested to verify the final dietary OA concentration using the method of Frohlich et al. (1988). Fungal species contained in the contaminated samples were identified by Dr. G. Platford, Manitoba Department of Agriculture, Winnipeg, Manitoba, (Manuscripts I and II) and the degree of contamination of all samples determined using the glucosamine assay procedure (Manuscript I). The control barley (three subsamples) was also subjected to visual examination for mold using a stereo microscope.

The starch concentrations of the barley samples were determined using a combination of two procedures. First, starch was hydrolyzed by amyloglucosidase (Boehringer Mannheim 1984) and then the glucose content was determined using the glucose oxidase method (Sigma Chem. Corp. 1978) and a Beckman DU-8 spectrophotometer. Prior to analysis, all barley, diet and excreta samples were finely ground in a Tecator Cyclotec 1093 Sample Mill grinder (Hoganas, Sweden) with a 1mm screen. All chemical analyses were conducted in duplicate.

Chick performance was measured in terms of feed consumption, weight gain, both presented as values relative to controls (1.00), and feed to gain ratio (F/G). F/G values presented are the average of F/G values of pens on the same dietary treatment. Correction of performance data for chick mortalities was done according to Rotter et al. (1985b).

In order to conduct the apparent nurient digestibility calculation, mixed feed and excreta samples (Experiment 4) were analyzed. Nitrogen was determined by the Kjeldahl procedure (AOAC 1984) and fat by the method of Marchello et al.(1973). Excreta nitrogen values were adjusted for uric acid content (Marquardt 1983) before conversion to excreta protein values (Rotter et al. 1988) and chromic oxide in the diets and excreta were determined by the method of Williams et al.(1962) on an Instrumentation Laboratory aa/ae spectrophotometer Model 551 atomic absorption spectrophotometer (Instrumentation Laboratory Inc., Wilmington, MA). All diets and excreta samples were also analyzed for dry matter (DM) content. The apparent nutrient digestibilities were then calculated according to the formula outlined in Crampton and Harris (1969).

All data were analyzed using the General Linear Models (GLM) procedure of the Statistical Analysis System (SAS Institute, Inc. 1982). Chick performance data were subjected to split-plot analysis, using mold and OA concentrations as the mainplot comparisons in Experiement 4. In Experiment 5, diet type and starch and fat concentations were used as the mainplot comparison, while time was used as the subplot comparison in both studies. Apparent nutrient digestibility and mortality data were analyzed using appropriate completely randomized design analyses. The

data were in part interpreted by partitioning of sums of squares as outlined by Little (1981). In Experiment 4, six F/G values, three each from the 30% mold-contaminated barley with and without OA, during week 1 were removed from the data for statistical analysis purposes. These F/G values were all negative and greatly biased the analysis to non-significance for all factors.

RESULTS

Barley samples

The mold-free control barley sample did not contain fungal material as determined by both chemical analysis (Manuscript I) and visual examination using a stereo microscope. The ET barley was estimated to contain 16.1% mold (w/w) and the ET/EB mixture 7.0% mold. The contaminating fungal species were identified primarily as Penicillium verrucosum var. cyclopium (Westling) Samson, Stalk and Hadlock and Aspergillus flavus Link ex Fries (Manuscripts I and II). None of the samples tested contained detectable toxins.

The starch concentration of the mold-free, ET and ET/EB barley samples were determined to be 52.5, 30.3 and 39.5%, respectively, and the corresponding fat concentrations were 2.24, 1.85 and 1.57%.

Experiment 4

Analysis of variance of the performance data (Table 14) indicated highly significant ($P<0.0001$) mold and OA concentration main effects for relative feed consumption (RFC) and relative weight gain (RWG) and a mold x OA interaction ($P<0.0001$ and $P<0.0005$) for both parameters,

Table 14. Effect of increasing the mold-contaminated barley content of the diet in the presence and absence of OA on relative feed consumption and relative weight gain (Experiment 4).

Amount of mold-contaminated barley (%)	OA concentration (ppm)	Relative feed consumption	Relative weight gain
0	0	1.00 (264.8 g)†	1.00(129.7 g)†
0	4	0.71	0.61
15	0	0.62	0.51
15	4	0.53	0.33
30	0	0.47	0.21
30	4	0.36	0.10
SE		0.014	0.057

Continued .....

Table 14 (continued) Summary of analysis of variance (Experiment 4).

Source of variation	df	Relative feed consumption MS	Relative feed consumption Probability	Relative weight gain MS	Relative weight gain Probability	Feed to gain‡ ratio (g/g) MS	Feed to gain‡ ratio (g/g) Probability
Mainplot comparisons							
Mold concentration	2	1.18	0.0001	2.56	0.0001	364.	0.0001
OA concentration	1	0.479	0.0001	0.93	0.0001	397.	0.0001
OA x mold	2	0.077	0.0001	0.27	0.0005	261.	0.0001
Error a	30	0.0057		0.014		16.9	
Subplot comparisons							
Time	1	0.0004	0.56	0.47	0.0001	464.	0.005
Time x mold	2	0.0021	0.17	0.099	0.012	301.	0.0001
Time x mold	1	0.0023	0.16	0.022	0.30	165.	0.0006
Time x mold x OA	2	0.008	0.002	0.031	0.22	260.	0.0002
Error b	30	0.0011		0.019		21.5 (24 df)	

† Values in parentheses are actual values per bird per 2 weeks.

‡ Negative F/G data were exempted from the statistical analysis as described in the Analysis section of Materials and Methods

respectively. In addition, there was a time effect ($P<0.0001$) and a time x mold interaction ($P<0.012$) for RWG and a time x mold x OA interaction ($P<0.0023$) for RFC. An initial analysis indicated that none of the factors were significant ($P>0.05$) for F/G. However, when the six negative F/G values (week 1, 30% mold-contaminated barley with and without OA) were excluded from the analysis (Table 14), all factors were shown to be significant ($P<0.006$).

Partitioning the sums of squares (Little 1981) showed that mold concentration accounted for 78.3 and 72.4% of the variation for RFC and RWG, respectively. OA concentration was responsible for another 15.8 and 13.1% of the variation, respectively, while the mold x OA interaction accounted for about 5% for each and time an additional 6.7% for RWG.

Mold-contaminated barley, when incorporated in the diet at the lower concentration (15%) produced a dramatic effect on chick performance; RFC was reduced by about 40%, and RWG decreased by 50% compared to the controls (Table 14). Doubling the concentration of mold-contaminated barley to 30% of the diet further reduced RFC by 15% and RWG by 30% in relation to that obtained with birds fed the 15% mold-contaminated barley diets. In comparison, the presence of 4 ppm OA in the mold-free diet caused reductions of 30 and 40% in RFC and RWG, respectively. OA, when added to the mold-contaminated barley diets, only reduced RFC and RWG an additional 10%.

The F/G values became progressively poorer as the dietary concentrations of mold and OA increased, especially for the chicks fed the mold-contaminated barley (Table 15). Incombination, mold and OA, in all but one case, further depressed F/G. In general, the effects on F/G

Table 15. Effect of OA and mold-contaminated barley concentrations on feed to gain ratio over time (Experiment 4)

Time (week)	OA concentration (ppm)	Mold-contaminated barley concentration (%)		
		0	15	30
1	0	2.07	3.31	-4.43
	4	2.55	3.91	11.47
2	0	2.03	2.25	2.87
	4	2.29	2.91	3.36
SE		1.89	1.89	1.89

were less dramatic during week 2 than week 1 of the trial. A complicating factor in F/G determination and interpretation occurred with birds given the 30% mold-contaminated barley diets during week 1. In each of the OA-free and OA-contaminated diets, three of the six pens had negative weight gains, but only birds which ingested the 30% mold with 0 ppm OA diet had negative F/G values. These negative F/G values were caused by a net loss in pen weight.

The presence of mold and of OA in the diet had significant effects on apparent dry matter (DM) and protein digestibilities ($P<0.006$), but only mold affected apparent fat digestibility ($P<0.0001$; Table 16). Mold x OA interactions were also noted for fat and protein ($P<0.02$), and approached significance for DM digestibility ($P=0.052$). Partitioning the sums of squares (Little 1981), indicated that the majority of the variations in apparent DM and fat digestibilities were due to mold concentration of the diet (69.6 and 93.6%, respectively). In contrast, mold accounted for only 35% of the variation in apparent protein digestibility, while 52% was due to OA concentration. OA was also responsible for 16% of the variation in DM digestibility. The mold x OA interaction in turn accounted for 7.1, 5.4 and 13.0% of the total variability for apparent DM, fat and protein digestibilities, respectively.

Both the apparent DM and protein digestibilities were depressed in chicks fed OA or mold-contaminated barley, but the effects were greater when they were both present (Table 16). DM digestibility decreased 13.7% in birds given 30% mold-contaminated barley compared to the controls, and OA reduced it by an additional 13.4%. Protein digestibility was more

Table 16. Effect of dietary mold-contaminated barley and OA on apparent nutrient digestibilities of chicks (Experiment 4)

Amount of mold-contaminated barley (%)	OA Concentration (ppm)	Dry matter (%)	Fat (%)	Protein† (%)
0	0	66.5	77.5	83.9
	4	61.8	63.3	78.3
15	0	62.9	45.1	80.9
	4	62.0	39.7	78.1
30	0	57.4	20.1	80.3
	4	49.1	28.1	69.5
SE		1.46	3.73	1.33

Summary of analysis of variance

		Dry matter		Fat		Protein	
Source of variation	df	MS	Prob	MS	Prob	MS	Prob
Mold-contaminated barley	2	408.9	0.0001	6514.	0.0001	123.7	0.0002
OA concentration	1	190.6	0.0006	137.5	0.21	372.4	0.0001
Mold x OA	2	83.42	0.052	377.9	0.019	47.39	0.020
Error	30	12.78		83.59		10.63	

† Fecal protein values were corrected for uric acid content before apparent digestibility was calculated (Rotter et al. 1988).

sensitive to OA than mold, 4 ppm OA alone reducing digestibility 6.7%, but in combination with 30% mold-contaminated barley it was decreased by 17.2%. The most dramatic effects were observed in the apparent fat digestibility data as it was reduced 41.8 and 74.1% due to incorporation of 15 and 30% mold-contaminated barley in the diet. Although OA alone decreased fat digestibility by 18.3%, incombination with 30% mold-contaminated barley, OA actually appeared to improve fat digestibility from 20.1 to 28.1% over the mold alone. The same effect, however, was not seen in the 15% mold with OA.

Mortalities were only observed in birds fed diets containing mold-contaminated barley ($P<0.0012$), but as indicated by the mold x time interaction ($P<0.0045$), also differed over time (Table 17). The average mortality was low (1.39%) and it was identical for both groups of mold-fed birds during week 1. During the second week, the average percent mortality for birds fed 30% mold-contaminated barley increased dramatically to 11.4%, while the group fed 15% mold-contaminated barley was 0%. Mortality was not significantly affected by OA concentration ($P>0.58$).

Experiment 5

Although diet type and starch concentration significantly affected RFC, and F/G and RWG ($P<0.003$), RFC and F/G were also significant for a diet x starch and diet x fat interactions ($P<0.02$; Table 18). Starch x fat and diet x starch x fat interactions were also significant for RWG ($P<0.04$; Table 18 and 19). Partitioning of the sums of squares (Little 1981) indicated that time and time interactions, even though some were significant, did not contribute much to the total variation.

Table 17. Weekly chick mortality as affected by increasing dietary mold-contaminated barley concentrations (Experiment 4)

Mold-contaminated barley dietary concentration (%)	Average pen mortality (%) Week 1	Week 2
0	0.00	0.00
15	1.39	0.00
30	1.39	11.4
SE	1.81	1.81

Summary of analysis of variance

Source of variation	df	MS	Probability
Mold concentration	2	295.	0.0012
OA concentration	1	12.5	0.58
Time (Week)	1	148.	0.057
Mold x OA	2	3.24	0.92
Mold x week	2	232.	0.0046
OA x week	1	18.7	0.49
Mold x OA x week	2	4.79	0.89
Error	60	39.4	

Table 18. Influence of supplemental starch and fat concentration on relative feed consumption and feed to gain ratio (Experiment 5)

Dietary component		Relative feed consumption†	Feed to gain ratio (g/g)†
Diet x Starch	Added starch concentration (%)		
Mold-free barley	0	1.05	1.99
	15	0.96	2.12
Mold-free barley plus celufil	0	0.93	3.10
	15	0.92	2.27
Mold-contaminated barley	0	0.54	4.14
	15	0.51	3.47
SE		0.01	0.14
Diet x fat	Added fat concentration (%)		
Mold-free barley	2	1.04	2.15
	10	0.96	1.96
Mold-free barley plus celufil	2	0.93	2.91
	10	0.92	2.47
Mold-contaminated barley	2	0.52	4.53
	10	0.53	3.08
SE		0.011	0.14

Continued .....

Table 18 (continued) Summary of analysis of variance (Experiment 5)

Source of variation	df	Relative feed consumption MS	Prob.	Relative weight gain MS	Prob.	Feed to gain ratio MS	Prob.
Mainplot comparisons							
Diet type	2	3.18	0.0001	5.34	0.0001	34.5	0.0001
Starch	1	0.063	0.002	0.087	0.0033	7.49	0.0027
Fat	1	0.017	0.098	0.213	0.0001	17.5	0.0001
Diet x starch	2	0.030	0.010	0.268	0.0001	3.18	0.021
Diet x fat	2	0.0315	0.008	0.021	0.12	5.28	0.002
Starch x fat	1	0.020	0.075	0.094	0.0023	0.945	0.27
Diet x starch x Fat	2	0.007	0.31	0.032	0.038	0.156	0.82
Error a	60	0.006		0.009		0.767	
Subplot comparisons							
Time	1	0.013	0.047	0.0002	0.85	2.39	0.030
Time x diet	2	0.014	0.016	0.0087	0.22	3.33	0.002
Time x starch	1	0.013	0.047	0.024	0.042	0.073	0.70
Time x fat	1	0.017	0.022	0.015	0.11	0.21	0.51
Time x diet x starch x fat	7	0.005	0.20	0.008	0.20	0.094	0.99
Error b	60	0.003		0.006		0.48	

† Actual total feed consumption for the 6.55% tallow reference diet fed birds was 248.6 g per bird per 2 weeks and the F/G was 1.94.

Table 19. Influence of fat concentration and supplemental starch on relative weight gain (Experiment 5)

Diet component	Diet number	Added starch concentration (%)	Added fat concentration (%)	Relative weight† gain
Mold-free barley	1	0	2	0.97
	2		10	1.10
	3	15	2	0.97
	4		10	0.90
Celufil	5	0	2	0.52
	6		10	0.66
	7	15	2	0.77
	8		10	0.81
Mold-contaminated barley	9	0	2	0.24
	10		10	0.35
	11	15	2	0.29
	12		10	0.40
SE				0.075

† Actual total weight gain for 6.55% tallow reference diet fed birds was 128.1 g per bird per 2 weeks.

Virtually all of the variation in the data (over 90% of the variation in RFC and RWG and about 60% for F/G) was due to diet type. This is also evident from an examination of the data in Tables 18 and 19, which shows that the performance of birds fed the mold-contaminated barley diets was markedly lower than that obtained with birds fed the celufil containing diets, which was still poorer than that of the mold-free barley diets. The effects of fat and starch supplementation on RFC were similar except in the mold-free barley fed birds. When either fat was increased to 10% or starch supplemented in the diet, RFC of chicks fed the mold-free barley diets decreased 8.6% compared to the average of the respective low fat or no added starch diets. No changes in RFC occurred in birds given the celufil-containing diets, but while starch supplementation reduced RFC in the mold-contaminated barley diets, a very slight increase was seen due to fat. In contrast to the above, increasing the fat concentration of the diet from 2 to 10% or adding starch improved F/G values in all but one treatment (Table 18). The exception was noted with chicks fed the mold-free diet supplemented with starch. Starch supplementation or increasing the fat concentration from 2 to 10% also caused RWG to increase slightly in birds fed the mold-contaminated barley diets and more dramatically in the celufil-containing diets (Table 19). A combination of both starch and fat had an almost additive effect. RWG of birds fed the mold-free diet was only increased when fat was increased from 2 to 10%, but not when starch was added.

Only two chicks died during the course of this experiment. Both died during week 1, one each on the 2 and 10% tallow mold-contaminated

barley diets containing no supplemental starch.

DISCUSSION

The detrimental effects of feeding toxin-free mold-contaminated barley with and without added OA to growing chicks observed in the current study were generally similar to results reported in a previous study (Manuscript II). Rotter et al. (Manuscript II) showed that ingestion of a diet containing 30% barley contaminated by 16% mold, in the absence of detectable toxin, reduced RFC and RWG by 50 and 80%, respectively. When 4 pm OA was added to the mold-contaminated barley diet, chick performance was reduced only slightly more, despite the fact that OA alone caused RFC and RWG decreases of 22 and 30%, respectively. In the current study (Experiment 4), RFC and RWG were decreased 53 and 79%, respectively on the 30% mold-contaminated barley alone, and another 10% when combined with 4 ppm OA. Surprisingly, the effect of the OA alone was more pronounced than in a previous study, causing reductions of 29 and 39% in RFC and RWG, respectively. The effects of mold and OA on the apparent DM, fat and protein digestibilities were also similar to Rotter et al. (Manuscript II), although the effect of mold and OA on fat digestibility seen here was more pronounced.

Of particular interest was the comparison of the effects of the mold-contaminated barley to those of the cellulose-containing diets, especially the high fat and starch diets. These results show that the mold-contaminated barley diets depressed chick performance to a greater degree than a diet containing 30% cellulose. Since fewer nutrients were theoretically available in the celufil diets, chick performance should

have been inferior to those given mold-contaminated barley. To compensate for the lower concentration of nutrients, the celufil fed birds maintained high feed intakes approximating that of the controls, and as a result, the F/G values were intermediate to the controls and mold-fed birds. Such a compensatory process was not evident in the mold-contaminated barley birds. These results strongly suggest that mold contaminated grain contains some unidentified factor(s) which interfere with nutrient digestion/ absorption, in addition to reducing feed intake. This hypothesis is further supported by performance and nutrient digestibility data (Experiment 4; Manuscript II).

The mold-contaminated barley used in this study (Experiment 4) was moderately high in fungal contamination (16%), but even dilution of the contaminated barley, using only 15% in the total diet, resulted in marked reductions in chick performance and apparent nutrient digestibilities. Some of the effects of feeding mold contaminated grains may be attributable to palatability or oder problems. Burditt et al. (1983) found that filtrates of fungi grown on liquid culture contained unknown factors which caused feed refusal in one week old chicks. Other authors (Kaminski et al. 1972, 1974; Abramson et al. 1980; Sinha et al. 1988) have reported the presence of several volatile compounds present in mold-contaminated grain. Though the concentration of each compound varied between reports, possibly due to fungal species and growth medium (Kaminski et al. 1974), they all identified the presence of 1-octanol, 3-methylbutanol and 3-octanone in mold-contaminated grain. The volatile oders and/ or taste associated with these or possibly other unidentified compounds may act to lessen chick interest in the feed or directly

reduce appetite. While this may occur to some extent, reflected in the lower RFC values, the increase in F/G and decreases in DM, and especially fat digestibilities for mold-fed birds clearly indicate that other biochemical/ physiological processes are being affected.

Rotter et al. (Manuscripts I and II) noted that barley moderately to heavily contaminated with mold had noticeably lower starch and fat concentrations and that protein, ash and fiber concentrations were increased. The starch concentrations of the mold-contaminated barley samples used in Experiments 4 and 5 were 42.3 and 24.8% lower, respectively, than the control barley while fat was reduced 39.0 and 29.9%, respectively. Supplementation of mold-containing diets with starch and increasing the fat to 10% should have been able to compensate for the amounts used by the mold during its growth. Data from Experiment 5, however, demonstrated that this did not occur. In addition, the reduction in chick performance and nutrient digestibility (Experiment 4) occurred despite the fact that much of the dietary protein and energy was available from other sources in the diet other than the contaminated barley. These observations further support the idea of factors other than energy or nutrient limitation were the causative agent(s).

Bartov et al. (1982) and Bartov (1983, 1985) suggested that since fungi use the fat in cereal grains as a source of energy, the observed mold-contaminated grain effects are due to a reduced fat content of the diet. Bartov (1983) observed that broiler chicks fed mold-contaminated corn gained 17% less weight than control birds after a three week trial. Supplementation of the diet with a fat source, soybean oil, further reduced chick weight gain by 21.7%, but it was only 4% less than

controls when the mold-contaminated diet was supplemented with propionic acid, an anti fungal agent. The propionic acid, however, may only have prevented mold growth in the diet before feeding as the diets were stored for 25 d before use. No indication as to the degree of mold contamination was given prior to mixing the diets nor at any point during the study. Bartov et al. (1982) and Bartov (1983) also reported that mold-contaminated corn reduced nutrient digestibilities, and that fat supplementation did not overcome this.

These results demonstrate that use of a toxin-free mold contaminated barley in a diet can seriously affect chick performance. While dilution of the barley or other grain may lessen the effects, the grain may need to be diluted several fold depending on the degree of contamination, and even this may not be sufficient. OA, in the presence of mold, was shown to further reduce chick performance and nutrient digestibility. Mold-contaminated grain not only seems to contain a factor which causes feed refusal or appetite depression, but also an antinutritive factor(s) that interferes with nutrient digesitibilty/ absorption, particularly fat. This effect can not be overcome by fat supplementation. Future studies should be designed to examine these possibilities in more detail and to identify the causative agent(s).

MANUSCRIPT IV

INFLUENCE OF DIETARY CHARCOAL ON OCHRATOXIN A TOXICITY IN LEGHORN CHICKS

ABSTRACT

The ability of activated charcoal to adsorb ochratoxin A (OA) *in vitro* and to reduce the toxic effects of OA *in vivo* when added to the diet of growing Leghorn chicks was studied. Activated charcoal (50 mg) was able to adsorb 90% of the OA (150 ug) contained in 10 mL of a citrate-phosphate buffer (pH 7.0). When 2.0 g of a complete chick diet was mixed with OA in buffer, it adsorbed 66% of the OA, while addition of 50 mg of charcoal to this mixture further reduced the concentration of OA an additional 11.8%, or 65% compared to the diet alone. In the first of two feeding studies, charcoal addition of up to 10,000 ppm to diets (6.7% tallow) containing 4 ppm OA had no effect on OA toxicity. Feed consumption and weight gain , however, were reduced 10 and 20%, respectively in chicks fed diets which contained 10,000 ppm of charcoal compared to those fed no charcoal. In the second study, reducing dietary tallow to 2% did not alter the effects of OA or charcoal on weight gain and feed to gain ratio, but birds fed 4 ppm OA with 10,000 ppm charcoal had an 8.5% increase in feed consumption. An additional management problem was associated with the propensity of charcoal to blacken the feed, the birds and their environment. Addition of charcoal to OA contaminated diets appears to be an ineffective method for reducing the toxic effects of OA in growing chicks.

KEY WORDS: Ochratoxin A, charcoal, Leghorn chicks, performance

INTRODUCTION

Ochratoxin A (OA) is a potent nephrotoxin produced in stored grains by certain Aspergillus and Penicillium species fungi. Several methods have been tested in attempts to inactivate this mycotoxin, including extraction, heat treatment (Josefsson and Moller 1980), ammoniation (Chelkowski et al. 1982a,b,c) and ensiling of contaminated grain (Manuscript I). While some of these methods show promise, they also have limitations which do not make them practical.

The use of activated charcoal as an oral antidote for the treatment of poisonings is well established. Charcoal is an insoluble carrier that nonspecifically adsorbs molecules, thereby preventing their absorption. Several authors have tested the efficacy of activated charcoal in binding some mycotoxins, such as patulin (Sands et al. 1976) and aflatoxin B_1 (AFB_1; Dalvi and McGowan 1983; Dalvi and Ademoyero 1984; Ademoyero and Dalvi 1983; Hatch et al. 1982) with limited success. No studies examining the effect of activated charcoal on OA have been reported.

The purpose of this study was to test the ability of activated charcoal to adsorb OA in vitro and, when incorporated in the diet, to reduce OA toxicity to growing chicks. To detect a possible influence of dietray fat on the charcoal, two separate feeding studies were conducted using either a low or moderate tallow concentration.

MATERIALS AND METHODS

Source of OA and charcoal

The OA used in both the in vitro and in vivo studies was derived by liquid fermentation of Aspergillus ochraceus (NRRL 3174) for 10-12 d at

28°C according to Davis et al. (1972) and Yamazaki et al. (1970). OA used in the in vitro studies was extracted from the fungal mycelia based on a procedure developed by Frohlich and Marquardt (1988). A stock solution of crystalized OA (300 ug OA mL^{-1}) was prepared in 0.1 M sodium bicarbonate. The OA used in the chick studies was contained in freeze-dried media (FDM) used to culture the fungus. It was finely ground using a Tecator Cyclotec 1093 Sample Mill (Hoganas, Sweden) equipped with a 1 mm screen and mixed with the vitamin/ mineral premix. The FDM contained an OA concentration of 1.9g kg^{-1}.

Activated charcoal (fine grind; Darco Activated Charcoal, No. OXL-5698, Atlas Chem. Indust., Wilmington, Delaware) was obtained courtesy of Dr. R. A. Gallop, Dept. of Food Science, University of Manitoba.

In vitro study

The ability of activated charcoal to adsorb OA in solution or in the presence of a complete mixed diet was tested. Increasing amounts of charcoal (0, 50, 100 and 200 mg) were mixed with OA (7.5 ug mL^{-1}) either in buffer or buffer plus diet in a 4 x 2 factorial arrangement of the treatments. The diet used in the in vitro study was of the same formulation as diet 1 in Experiment 7 (Table 20). Each treatment included two replicates, and two subsamples of each replicate were analyzed in duplicate using high pressure liquid chromatography (HPLC).

A given weight of activated charcoal was placed into a 25 mL nylon centrifuge tube with a twist cap with 9.5 mL of a 0.1 M citrate-0.2 M sodium phosphate buffer, pH 7.0 (Colowick and Kaplan 1965). This was combined with either 0.5 mL of the OA stock solution, 0.5 mL OA stock solution plus 2.0 g of the chick diet, or 0.5 mL of buffer plus 2.0 g of

Table 20. Diet formulations used for in vivo charcoal studies (Experiments 7 and 8)

Diet component (%)	Experiment 1	Experiment 2
Barley (Bedford)	60.0	62.5
Soybean meal (47.5% protein)	23.8	25.8
Soybean protein concentrate (90% protein)	2.6	2.71
Tallow	6.7	2.0
Ca_2PO_4	1.4	1.46
$CaCO_3$	1.1	1.15
Vitamin premix†	1.0	1.04
Mineral premix†	0.35	0.36
Methionine	0.05	0.05
OA/Charcoal/Wheat middlings‡	3.0	3.0
Calculated values:		
Protein (%)	21.8	23.1
Metabolizable energy (Kcal Kg^{-1})	2760	2525

† The vitamin and mineral premixes were the same mixtures as described by Young and Marquardt (1982).

‡ In Experiment 7, the charcoal concentrations were: diets 1 and 5, 0 ppm; diets 2 and 6, 100 ppm; diets 3 and 7, 1000 ppm; diets 4 and 8, 10,000 ppm. Diets 1-4 contained 0 ppm OA and 5-8 contained 4 ppm OA.
In Experiment 8, the charcoal concentrations were: diets 1 and 4, 0 ppm; diets 2 and 5, 100 ppm; and diets 3 and 6, 10,000 ppm. Diets 1-3 contained 0 ppm OA and 4-6 contained 4 ppm OA.
OA was added in the form of finely ground (1 mm) FDM. The activated charcoal was also finely ground.

the diet containing OA. The mixture was continuously shaken in an automatic shaker for 10 min, allowed to settle for 30 min and then centrifuged for 10 min at 14,000 G. A 3.0 mL subsample of the supernatant was transfered to another nylon centrifuge tube, acidified with 0.1 M phosphoric acid to pH 2.0 and then made up to 10 mL with distilled water. Six mL choloform was added to this mixture and then shaken for 10 minutes. After allowing the water and chloroform layers to separate, 3.8 mL of the choloform layer was transfered into a 15 mL test tube with a screw top. Distilled water (1.5 mL) was added, the tube was shaken and centrifuged at 14,000 G for 10 min, and a 3 mL portion of the chloroform layer transfered into a 5 mL glass screwcap vial. The sample was dried under N_2 gas and stored until readied for HPLC analysis.

OA analysis was conducted as described by Frohlich et al. (1988) using an LKB HPLC system (2152 HPLC controller, 2150 HPLC pump and a 2155 HPLC column oven; LKB-Produkter AB, Bromme Sweden) equiped with a Waters 712 auto-sampler (Millipore Corp., Milford, Mass.) and a Hewlett Packard HP 1046A fluoresence detector. The HPLC contained a 4.6 x 250 mm Ultrasphere column of 5 um C-18 ODS and a 4.6 x 50 mm precolumn packed with CO:PELL C-18 groups chemically bonded to 38 um glass beads.

Diet formulation

All diets were formulated to meet minimum NRC (1984) requirements for Leghorn pullets (Table 20). All diets were fed as mash.

Chicks and management

One-day-old male Single Comb White Leghorn (Leghorn) chicks were obtained from a commercial hatchery and managed as outlined in Rotter et al.(1985a). Both experiments used 7-day-old chicks housed in Petersime

Battery Brooders for a 7 d test period. Chicks were provided with continuous light and heat and free access to feed and water.

In vivo trials

Two experiments were conducted concurrently over a 7 d period. Chick performance was measured in terms of feed consumption and weight gain, both presented as values relative to the respective control diet, and feed to gain ratio (F/G). Performance data was corrected for chick mortality according to Rotter et al. (1985b).

Experiment 7. The ability of activated charcoal to reduce OA toxicity in chicks fed a standard (6.7% tallow) barley-based diet was examined (Table 20). Four concentrations of charcoal (0, 100, 1000 and 10,000 ppm) and two concentrations of OA (0 and 4 ppm) were combined in a 4 x 2 factorial arrangement of the treatments. There were 6 replications per treatment.

Experiment 8. The objectives of this study were the same as Experiment 7, except that the basal diet contained only 2% tallow (Table 20) rather than 6.7% as above. This was done to lessen any possible interference by dietary fat on the adsorption of OA by charcoal. Three concentrations of activated charcoal (0, 100 and 10,000 ppm) were combined with two concentrations of OA (0 and 4 ppm) in a 3 x 2 factorial arrangement of the treatments, with 4 replications per treatment.

Analyses

The barley (Hordeum vulgare L. cv. Bedford) used in the chick studies was subjected to a multitoxin screening analysis (Wilson et al. 1975; Josefsson and Moller 1977), for OA, aflatoxins, penicillic acid, citrinin, patulin, sterigmatocystin and zearalenone. The barley was determined to be mold-free according to analysis for glucosamine (Manuscript I) and confirmed by visual examination using a stereo microscope. All chemical analyses were conducted in duplicate.

Statistical analyses of the in vitro and in vivo data were performed using the General Linear Models (GLM) procedure of the Statistical Analysis System (SAS Institute Inc. 1982). All analyses were conductedas completely randomized designs.

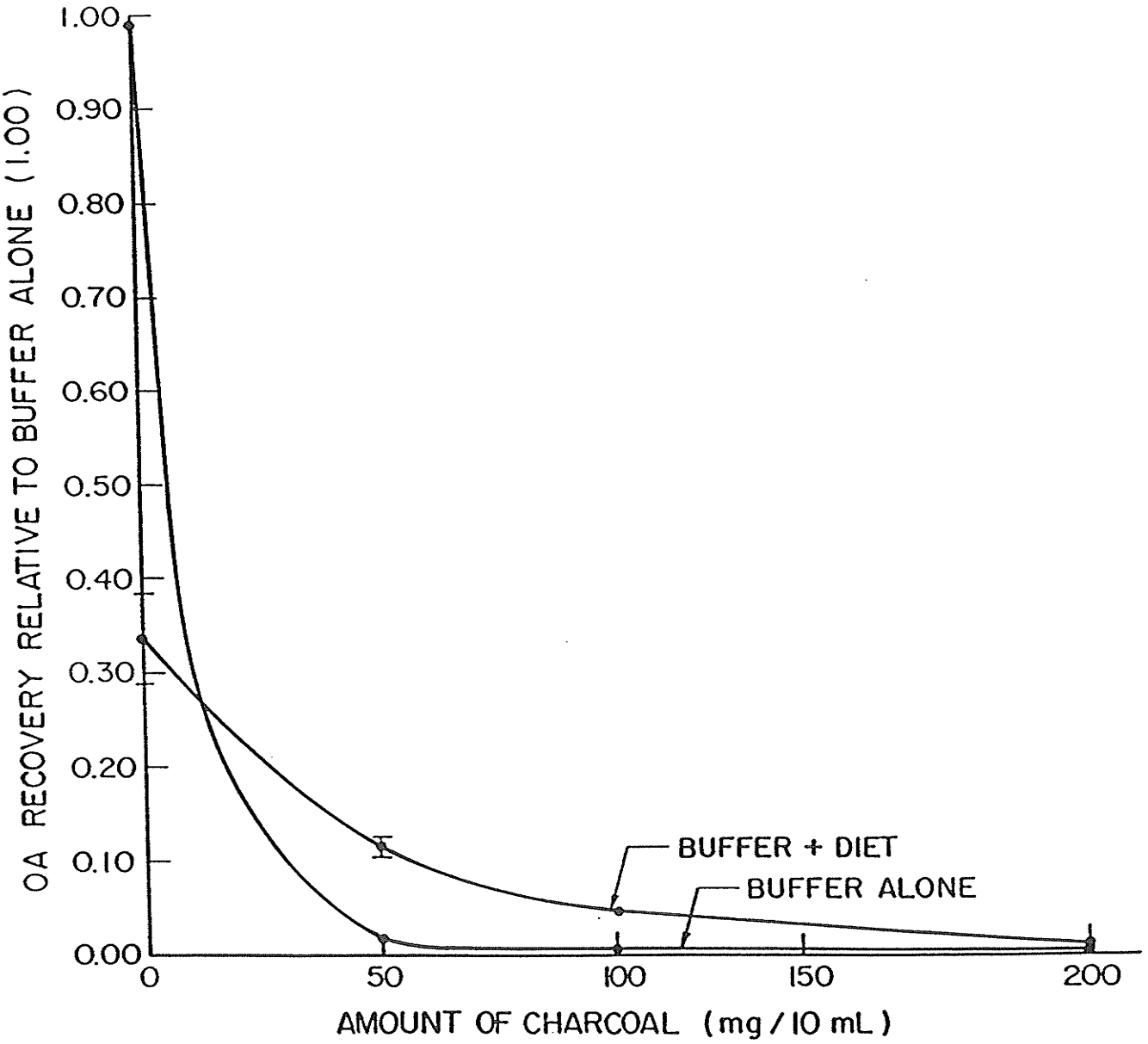
RESULTS

In vitro study

Prior to initiating the in vitro study, a series of preliminary tests were conducted to examine the effect of pH (3.0, 5.0 and 7.0) on the ability of charcoal to adsorb OA. The adsorption of OA by charcoal was not affected by the pH of the buffer. The pH 7.0 buffer was selected for the in vitro study as it approximated the pH of the duodenum (Scott 1982), the site of maximal OA absorption.

Activated charcoal, at moderately high concentrations, was able to almost totally remove OA from solution, but this ability was reduced in the presence of the diet (Fig. 4). In the charcoal-free mixtures, diet itself adsorbed approximately 66% of the added OA, while addition of 50 mg charcoal to the diet mixture reduced the concentration of soluble OA

Figure 4. Disappearance of ochratoxin A in a buffer (pH 7.0) and a buffer plus diet mixture due to increasing concentrations of activated charcoal. Points represent the average of 4 values ± SE.


1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
OA RECOVERY RELATIVE TO BUFFER ALONE (1.00)
BUFFER + DIET
BUFFER ALONE
0
50
100
150
200
AMOUNT OF CHARCOAL (mg / 10 mL)

to 11.8% of the control. This represented an additional decrease of65.2% relative to its concentration in the presence of the diet alone. Greater reductions were seen at the higher concentrations of charcoal. It is apparent that activated charcoal when present in a complete diet is able to adsorb OA, but that it is not as effective as in the diet-free system.

Feeding studies

Inclusion of charcoal in chick diets above the 100 ppm concentration created management problems due to the physical properties of the charcoal. The birds became coated with charcoal, and they tended to work it into their feathers during preening. In addition, the entire cage became covered in charcoal dust, including wire floor and sides. Although the concentrations of charcoal used in the feeding studies were much lower than those in the in vitro study, higher concentrations would likely have caused even greater management problems than were encountered.

Experiment 7

Incorporation of activated charcoal in diets containing 6.7% tallow affected relative feed consumption (RFC) and relative weight gain (RWG) ($P<0.021$), but the effects of OA on RFC, RWG and F/G ($P<0.0001$) were more pronounced (Table 21). Charcoal, as indicated by the lack of a significant ($P>0.45$) OA x charcoal interaction, had no effect on reducing the toxicity of OA (Table 21). Chick performance was basically not influenced by dietary charcoal except at the 10,000 ppm concentration. At this concentration, RFC and RWG decreased an average of 9.9 and 20.5%, respectively, compared to the non-charcoal fed birds,

Table 21. Effect of OA and activated charcoal on chick performance in a standard diet containing 6.55% tallow (Experiment 7).†

	Relative feed consumption	Relative weight gain	Feed to gain ratio (g/g)
OA concentration (ppm)			
0	0.98	0.95	2.02
4	0.62	0.42	3.17
SE	0.016	0.026	0.171
Charcoal concentration (ppm)			
0	0.81	0.73	2.37
100	0.82	0.71	2.64
1000	0.83	0.73	2.40
10000	0.73	0.58	2.97
SE	0.023	0.037	0.242

Summary of analysis of variance

		Relative Feed consumption		Relative weight gain		Feed to gain ratio	
Source of variation	df	MS	Probability	MS	Probability	MS	Probability
OA concentration	1	1.55	0.0001	3.36	0.0001	15.7	0.0001
Charcoal concentration	3	0.022	0.021	0.059	0.019	0.92	0.29
OA x charcoal	3	0.005	0.48	0.014	0.46	0.46	0.59
Error	40	0.0062		0.016		0.70	

† Actual values for control birds were (per bird per 7 d): feed consumption, 104.8 g; weight gain, 53.6 g; and F/G, 1.96 g/g.

and F/G increased from 2.37 to 2.97. The presence of OA in the diet reduced RFC values an average of 36.7% and RWG by 55.8%. F/G increased dramatically to 3.17 due to OA.

Experiment 8

Analysis of variance (Table 22) indicated an OA x charcoal interaction for RFC ($P<0.02$) in addition to a OA main effect on RFC and RWG ($P<0.0001$) and F/G ($P<0.0019$). The trend of decreasing RFC values with increasing dietary charcoal concentrations seen in OA-free diets (6 and 10% with 100 and 10,000 ppm charcoal, repectively) was opposite to that observed with OA containing diets (Table 22). These results indicate that charcoal was able to overcome some of the appetite depressing effects of OA. This apparent benefit due to the charcoal did not extend to RWG or F/G ($P>0.05$). OA reduced RWG an average of 48.9% and increased F/G from 2.19 to 3.12.

DISCUSSION

The activated charcoal used in the <u>in vitro</u> study was able to adsorb virtually all of the OA present in the buffer system, but it was less effective in the presence of a complete chick diet. Although the concentrations of charcoal used in the feeding studies was much less than in the <u>in vitro</u> trial, the amount of charcoal added to the diets, particularly the 10,000 ppm concentration, should have been adequate to adsorb most of the OA. Reducing the concentration of dietary tallow from 6.7 (Experiment 7) to 2.0% (Experiment 8) also did not result in any apparent improvement in chick performance due to the charcoal, suggesting that it may not have greatly interfered with the adsorption

Table 22. Effect of OA and activated charcoal on chick performance on a low fat diet (2%) (Experiment 8).†

OA concentration (ppm)	Relative feed consumption			Relative weight gain	Feed to gain ratio (g/g)
	Charcoal concentration (ppm)				
	0	100	10,000		
0	1.00	0.94	0.90	0.92	2.19
4	0.64	0.65	0.70	0.47	3.12
SE	0.026	0.026	0.026	0.032	0.181

Summary of analysis of variance

Source of variation	df	Relative feed consumption		Relative weight gain		Feed to gain ratio	
		MS	Probability	MS	Probability	MS	Probability
OA concentration	1	0.48	0.0001	1.21	0.0001	5.16	0.0019
Charcoal concentration	2	0.0016	0.56	0.0004	0.97	0.31	0.47
OA x charcoal	2	0.013	0.02	0.033	0.095	0.53	0.28
Error	18	0.0027		0.012		0.39	

† Actual values for control birds were (per bird per 7 d): feed consumption, 106.8 g; weight gain, 50.4 g; and F/G, 2.12 g/g.

of OA by coating the charcoal particles. Conversely, it is conceiveable that, even at the low concentration, the dietary tallow may have bound the OA and thereby prevented the charcoal from adsorbing OA. The disappearance of about 67% of the OA in the OA/buffer/diet mixture in the absence of charcoal in the in vitro trial supports the latter possibility. It is also possible that the charcoal would have been more effective if either a lower dietary concentration or only a single oral dose of OA had been given to the chicks. Based on the current results, it must be concluded that when charcoal added to OA contaminated diets, at best, it provides only limited protection against the effects of OA.

The highest charcoal concentration used in the chick studies, 10,000 ppm had a detrimental effect on RFC in both studies, but only reduced RWG and increased F/G in Experiment 7. The reduced feed consumption may, in part, be attributed to the ability of finely ground charcoal to blacken the diet, thereby reducing its visual appeal to the birds. In addition, the high charcoal concentration may have reduced the availabilities of certain essential nutrients in the diet through adsorption, decreasing RWG and, as smaller birds require less feed than larger ones, this may also have contributed to the lower RFC value.

The use of activated charcoal to eliminate or reduce the effects of OA has not been previously reported. Its usefulness in vitro has, however, been reported with patulin (Sands et al. 1976) and in animals treated with AFB_1 (Ademoyero and Dalvi 1983; Dalvi and Ademoyero 1984; Dalvi and McGowan 1984; Hatch et al. 1982).

Sands et al. (1976) attempted to remove patulin from apple juice and cider. They reported that a patulin concentration of 30 ug mL^{-1} was

reduced to undetectable levels by the addition of 5 mg activated charcoal mL^{-1}, and that 20 mg mL^{-1} completely removed [^{14}C]patulin. In an in vivo test, the severity but not the duration of the effects of patulin were decreased in goats dosed intraruminally with a lethal dose of AFB_1 (3 mg kg^{-1} body weight) followed by a charcoal slurry within 8 h (Hatch et al. 1982). The purpose of their study was conducted from the point of view of the practicing veterinarian, evaluating treatments for acute AFB_1 toxicity, not the prevention of their effects. Dalvi and Ademoyero (1984) and Dalvi and McGowan (1984) fed chick diets contaminated with AFB_1 and added charcoal. Inclusion of 0.1% charcoal reduced the performance depressing effect of 10 ppm AFB_1 in chicks by 10% compared to the AFB_1 fed birds and prevented toxin-induced liver injury (Dalvi and McGowan 1984). Similarly, Dalvi and Ademoyero (1984) also observed improved chick performance when 200 ppm charcoal was added to diets contaminated with 10 ppm AFB_1, and hepatic microsomal enzyme activity was restored. They concluded that AFB_1 is adsorbed by activated charcoal thereby preventing its absorption from the intestinal tract.

The decrease in RFC and RWG and increase in F/G caused by OA was similar to that reported elsewhere (Manuscripts II-VI). Chick performance in both feeding studies was virtually identical irrespective of the dietary tallow concentration, but average RWG for 4 ppm OA fed birds was actually better when the diets contained 2% (0.47) versus 6.7% tallow (0.42) diets. It is conceiveable that dietary tallow may have some influence on the absorption of OA.

Supplementation of activated charcoal in OA contaminated diets appears to be an impractical method of reducing OA toxicity to chicks

that are continuously consuming OA. Charcoal concentrations which may be beneficial would present other problems, including those associated with chick management. Results that differ from those seen in the current study may occur with charcoal that has been activated or treated by other methods. Also, charcoal may be effective at lower dietary concentrations of OA or when OA is administered as an acute dose.

MANUSCRIPT V

OCHRATOXIN A TOXICITY IN GROWING CHICKS: EFFECT OF SUPPLEMENTAL DIETARY PHENYLALANINE

ABSTRACT

The ability of supplemental dietary L-phenylalanine (Phe) to reduce the detrimental effects of ochratoxin A (OA), in the presence and absence of barley contaminated by Penicillium cyclopium and Aspergillus flavus, was studied. Changes in feed consumption (RFC) and body weight gain (RWG), relative to control birds, and mortality of seven-day-old Leghorn chicks were used as indicators of toxicity. Diets containing OA (0 and 4 ppm) in combination with mold-contaminated barley (0 and 30% of the diet) were supplemented with Phe (0, 0.75 and 1.75% of the diet) to give total determined dietary Phe concentrations of 0.9, 1.6 and 2.5%, respectively. Chick performance was reduced due to Phe supplementation ($P<0.0001$), but not to the same degree as by mold or OA. Decreases in RFC and RWG due to the supplemented Phe were not seen in the presence of OA. In fact, chicks fed OA diets supplemented with 0.75% Phe increased RFC and RWG values by 5 and 9%, respectively, compared to those given the non-supplemented OA-containing diets. These values were 70 and 60%, respectively, lower than the controls. Phe supplementation also was not beneficial in reducing the detrimental effects of the mold-contaminated barley. Chick mortality was only seen in chicks fed OA, particularly those given OA in combination with mold and supplemental Phe. Based on the concentrations tested, supplementation of Phe in diets known or suspected to contain OA is of no practical benefit to growing chicks. Phe supplementation itself could prove to be detrimental if added to a diet containing no or very low concentrations of OA.

KEY WORDS: Ochratoxin A, phenylalanine, diet, chick performance, mycotoxins

INTRODUCTION

Ochratoxin A (OA) is a potent nephrotoxin produced by various Aspergillus and Penicillium fungi. It consists of a chlorinated dihydroisocoumarin moiety linked to an L-β-phenylalanine group through an amide bond (Scott et al. 1972). Protein synthesis in bacterial and eukaryotic systems is inhibited by OA in vitro by competing with phenylalanine (Phe) in reactions catalyzed by phenylalanine-tRNA synthetase. Recent studies have shown that addition of Phe to cell cultures (Creppy et al. 1979; 1983b) or injected into mice concurrently with OA (Creppy et al. 1984; Moroi et al. 1985) reduced or prevented the inhibition of protein synthesis. Injection of Phe also reportedly prevents OA induced immunosuppression in mice (Haubeck et al. 1981; Creppy et al. 1983b) and partially reduced teratogenesis in rats (Mayura et al. 1985).

The studies indicate that the effects of acute exposure to OA may be eliminated or mediated by concurrent or slightly delayed injection of Phe to affected animals. On a practical basis, injection of large numbers of OA exposed animals, such as a broiler flock, is not feasible, but application of Phe via the diet may solve this problem. Gibson et al. (1988) observed reduced mortality in broiler chicks given supplemental Phe in diets containing 4 ppm OA, but it did not completely overcome the effects of OA on chick performance.

In cases of natural OA contamination of stored grains, the concentration of the toxin-producing fungus can range from very low to extremely high levels (Manuscripts I and II). The presence of mold does not, however, necessarily mean that toxin is present. Rotter et

al.(Manuscripts I and II) reported using one barley sample naturally contaminated with 16% mold, but another contained no detectable toxins and another sample with only 1.6% mold, but 20 ppm OA. The degree of mold contamination also directly affects chick performance and may interact with the toxin.

Although the effects of acute exposure to injected OA has been shown to be reduced by subsequent injection of Phe, the effects of longer term exposure of both OA and Phe in the diet has not received much attention. The purpose of the current study was to examine the effects of supplementing diets containing OA, toxin-free mold-contaminated barley, or both, with different concentrations of added Phe on chick performance.

MATERIALS AND METHODS

Source of barley samples

Two sources of barley (Hordeum vulgare L. cv. Bedford) were used in this study. Mold-free (control) barley (1985 crop) was obtained from the University of Manitoba Glenlea Research Station, Glenlea, Manitoba (Man) and a mold-contaminated sample (Elkhorn, 1983 crop) from a farm in Elkhorn, Man. These samples were previously characterized chemically and mycologically by Rotter et al. (Manuscripts I and II). They had been stored in a walk-in cooler (4^{o}C) for about 13 months prior to use in the study. Both samples were ground prior to mixing of the experimental diets using a Viking Electric Hammer Mill Model C-H (Horvick Manufact. Inc., Moorhead, MN).

Diet formulation

All diets were formulated to meet the minimum NRC (1984) requirements for Leghorn pullets (Table 23) and fed as mash.

Source of OA and phenylalanine

The OA used in diets 4-6 and 10-12 was contained in freeze-dried fungal media (FDM) which was used to culture Aspergillus ochraceus (NRRL 3174) for 10 - 12 d at 28°C (Davis et al. 1972; Yamazaki et al. 1970). The OA concentration of the FDM was 1.9 g kg^{-1}.

The Phe (L-phenylalanine) added to selected diets was purchased from Sigma Chemical Corp. (St. Louis, MO).

Chicks and management

Male Single Comb White Leghorn (Leghorn) chicks were purchased from a commercial hatchery at one-day of age. They were housed for 7 d prior to the start of the experiment in electrically heated, thermostatically controlled Jamesway brooder batteries with raised wire floors. They were provided with continuous lighting and fed a commercial chick stater crumbles containing 21% protein. At 7 days-old, the birds were managed and randomized as described in Rotter et al. (1985a). During the experimental period the birds were housed in Petersime Battery Brooders, and provided with continuous lighting and heat and free access to food and water.

Experimental design

The experiment was set up as a split plot design. Two concentrations of OA (0 and 4 ppm) and two concentrations of mold contaminated barley (0 and 30% barley w/w) were mixed incombination with three concentrations of supplemental Phe (0, 0.75 and 1.75% w/w) in a 2 x 2 x 3 factorial

Table 23. Dietary formulations used in Experiment 9†

Ingredient (%)	Diet number											
	1	2	3	4[2]	5[2]	6[2]	7	8	9	10	11	12
Mold-free barley (control)	60	60	60	60	60	60	40	40	40	40	40	40
Mold-contaminated barley (ET)	-	-	-	-	-	-	20	20	20	20	20	20
Phenylalanine (supplemental)	0	0.75	1.75	0	0.75	1.75	0	0.75	1.75	0	0.75	1.75
OA/Wheat middlings	2.0	1.25	0.25	2.0	1.25	0.25	2.0	1.25	0.25	2.0	1.25	0.25
Other‡	38.0	38.0	38.0	38.0	38.0	38.0	38.0	38.0	38.0	38.0	38.0	38.0

† The average determined dietary protein concentration was 20.8% and the calculated metabolizable energy was 2725 Kcal kg^{-1}

‡ Other ingredients consisted of (%): Soybean meal (47.5% protein), 24.5; Soybean protein concentrate (90.0% protein), 2.60; tallow, 6.70; Ca_2PO_4, 1.40; $CaCO_3$, 1.10; Vitamin premix, 1.00; mineral premix, 0.35; methionine, 0.05; and Cr_2O_3, 0.30. The vitamin and mineral premixes were the same mixtures as described by Young and Marquardt (1982).

[2] Completed diets contained an OA concentration of 4 ppm. The OA was added in the form of ground FDM and mixed with wheat middlings.

arrangement of the treatments. The supplemental Phe concentrations correspond to actual analyzed dietary concentrations of 0.9, 1.6 and 2.5%, respectively. Each treatment involved six replicates with six birds per relicate. This experiment was conducted over two consecutive 7 d test periods. The mold, OA and Phe concentrations were used as the mainplot comparisons and time as the subplot comparison.

Chick performance was measured in terms of feed consumption, weight gain, both presented as values relative to control birds (1.00), and feed to gain ratio (F/G). The F/G data are the average of F/G values of pens of the same dietary treatment. Performance data were adjusted for mortalities following the method of Rotter et al. (1985b).

Analyses

Multitoxin screening was conducted on the barley samples following the methods of Wilson et al. (1976) and Josefsson and Moller (1977). It included analysis for aflatoxins, OA, penicillic acid, citrinin, sterigmatocystin, patulin and zearalenone. Diets containing OA were analyzed to verify the OA concentration using the method outlined in Frohlich et al. (1988). The degree of fungal contamination was estimated according to Rotter et al. (Manuscript I) and the contaminating fungal species in the Elkhorn barley sample previously identified as Penicillium verrucosum var. cyclopium (Westling) Samson, Stalk and Hadlock and A. flavus Link ex Fries (Manuscripts I and II) by Dr. G. Platford, Manitoba Dept. of Agriculture, Winnipeg, Man.

Total Phe concentrations in complete diets were determined by amino acid analysis using an LKB Biochrom 4151 Alpha Plus Amino Acid Analyzer after hydrolyzing 100 mg of sample in 6 N HCl for 16 h (Manuscript I).

Chick performance and mortality data were analyzed by split plot analysis of variance using the General Linear Models (GLM) procedure of the Statistical Analysis System (SAS Institute, Inc. 1982). The data was in part interpreted according to the principle of partitioning of sums of squares outlined by Little (1981).

RESULTS AND DISCUSSION

Analysis of the performance data (Table 24) indicated significant effects due to Phe, OA and mold concentrations, as well as a time and an OA x time interaction for relative feed consumption (RFC), relative weight gain (RWG) and F/G ($P<0.002$). Interactions were also significant for OA x mold ($P<0.0001$) and Phe x OA ($P<0.002$) for RFC and RWG, and Phe x time ($P<0.03$) and Phe x mold ($P<0.045$) for RFC.

The effect of increasing concentrations of Phe on chick performance differed in the OA-free and OA-containing diets. Addition of 0.75 and 1.75% Phe to OA-free diets reduced RFC and RWG an average of 4.3 and 7.9, and 15.1 and 12.6%, respectively, in comparison to the OA-free basal diets. The Phe depression of chick performance was not as evident in the presence of 4 ppm OA, and in fact, addition of 0.75% Phe improved RFC and RWG by 5 and 9%, respectively. OA, by decreasing RFC, also reduced the amount of Phe ingested by the chicks. This, in turn, would have lessened any additional depression of chick performance due to the added Phe. Based on the concentrations tested, it is concluded that Phe supplementation is of little practical value in overcoming the effects of OA.

Creppy et al. (1979, 1983a,b, 1984, 1985), Haubeck et al. (1981),

Table 24. Effect of dietary OA, phenylalanine and mold-contaminated barley concentrations over time on chick performance.

	Relative feed consumption		Relative weight gain		Feed to gain ratio (g/g)	
OA Concentration	0	4	0	4	0	4
OA x Phe						
Total dietary Phe concentration (%)						
0.9	0.93	0.59	0.89	0.44	2.19	2.92
1.6	0.89	0.62	0.82	0.48	2.27	2.67
2.5	0.79	0.56	0.68	0.39	2.41	3.09
SE	0.006		0.01		0.06	
OA x mold						
Mold-contaminated barley concentration (%)						
0	0.94	0.61	0.91	0.47	2.14	2.75
30	0.80	0.58	0.68	0.40	2.44	3.04
SE	0.005		0.009		0.05	
OA x time						
Time (wk)						
1	0.89	0.62	0.81	0.41	2.22	3.15
2	0.85	0.56	0.78	0.46	2.36	2.63
SE	0.005		0.009		0.05	

Continued .....

Table 24 (Continued)

Summary of analysis of variance

Source of variation	df	Relative feed consumption MS	Prob	Relative weight gain Ms	Prob	Feed to gain ratio (g/g) Ms	Prob
Main plot comparisons							
Mold concentration	1	0.27	0.0001	0.80	0.0001	3.17	0.0001
OA concentration	1	2.81	0.0001	4.71	0.0001	13.02	0.0001
Phe concentration	2	0.11	0.0001	0.24	0.0001	1.00	0.002
OA x mold	1	0.12	0.0001	0.25	0.0001	0.0028	0.89
Mold x Phe	2	0.016	0.045	0.015	0.27	0.15	0.38
OA x Phe	2	0.036	0.0014	0.074	0.002	0.36	0.63
OA x mold x Phe	2	0.002	0.67	0.0079	0.49	0.069	0.63
Error a	60	0.005		0.011		0.15	
Subplot comparisons							
Week (Wk)	1	0.096	0.001	0.0067	0.15	1.29	0.0001
Wk x mold	1	0.0007	0.38	0.0051	0.20	0.002	0.87
Wk x Phe	2	0.0036	0.028	0.003	0.38	0.0075	0.91
Wk x mold x OA x Phe	7	0.0011	0.67	0.0032	0.43	0.31	0.36
Error b	60	0.0009		0.0031		0.078	

† Actual control values for feed consumption and weight gain per bird were 108.4 and 54.7 g, respectively, for week 1 and 156.8 and 74.0 g, respectively for week 2.

Mayura et al. (1984) and Moroi et al. (1985) previously reported that the acute effects of injected OA can be prevented or reduced by injection of Phe. Creppy et al. (1979, 1983a,b, 1984, 1985) examined the influence of Phe on the effects of OA on cellular functions, such as protein synthesis in liver, kidney or spleen tissue shortly after injection. They noted that inhibition of phenylalanine tRNA transferase was reversed by Phe addition. OA depression of the immune response was also prevented by Phe injected at a concentration twice that of the OA (Haubeck et al. 1981), but Phe at a dose 11 to 14 times the minimum teratogenic dose of OA was able to only slightly reduce the effect. In the only report of oral application of Phe given acutely to mice, the concentration used was twice that of the OA given concurrently, and it resulted in an increase in the LD_{50} from 46.0 to 71.0 mg kg^{-1} (Moroi et al. 1985). From this, Moroi et al. (1985) suggested that Phe and OA not only compete for phenylalanyl tRNA synthetase but also the absorption sites in the intestinal tract. Since they only considered the LD_{50}, no biochemical or physiological effect of the OA was reported.

Differences in magnitude of the effect of the Phe supplementation in the current study compared to the results obtained by other researchers may be due to the route or length of exposure or due to species used in the study. The in vivo studies involved i.p. injection of Phe from one to 20 times the injected OA concentration. The influence of intestinal enzymes and microflora on OA and Phe was therefore eliminated, as well as the respective absorption processes. In these acute exposure studies, test animals were generally exsanguinated within hours or a few days after exposure. They may have been able to bind or

eliminate a major portion of the injected OA to reduce its 'active' concentration, while the injected Phe further diluted the free OA around binding sites. During chronic exposure studies, including the current one, the rate of absorption of OA could surpass the binding and elimination processes. The rate of Phe absorption, in contrast, may have been reduced when the concentrations of Phe in the diet were increased and, at the same time, the rate of Phe metabolism/elimination may have increased so as to maintain a homeostatic concentration of Phe. This would result in a reduced effective intracellular Phe concentration in relation to that contained in the diet, whereas the OA concentration may not have been affected to the same degree. The net effect would be that orally administered Phe provided only limited protection against OA toxicity, especially when the results are compared to those when both OA and Phe are injected.Finally, all the above studies involved either mice or rats, or their tissues in culture. Chickens may treat OA and Phe in the intestinal tract differently than mammals. Although Gibson et al. (1988) claimed that supplemental Phe improved chick performance and reduced the effect of OA on certain biochemical parameters, they also reported that OA fed birds never did as well as the controls.

Very little information has been reported on Phe toxicity to birds, with the exception of growth depression. Elkin and Rogler (1983) noted affected birds had reduced brain serotonin concentration and lower cerebral energy utilization, while external effects include poor feather development, swollen hocks and discolouration of the shanks,face and eyelids. In breeding or laying hens, excessive Phe also causes a decrease in egg production, egg weight, fertility, hatchability and day-

old chick weight. Chicks fed diets containing 2.52% Phe and 0.47% tyrosine (Tyr) had markedly decreased weight gains (72.3%) and feed efficiencies (32.4%), although addition of Tyr partially alleviated the Phe induced growth depression. The Tyr concentrations of the basal mold-free and mold-containing diet in the current study were 0.69 and 0.66%, respectively, and the Phe concentration seen in the diets with 1.75% added Phe was about 2.68%, which would fall into or border the toxic level for Phe stated by Elkin and Rogler (1983). In the abscence of OA, supplemental Phe tended to reduce chick performance, especially the 1.75% concentration, but the effect was much less than that reported by Elkin and Rogler (1983).

The effects of OA and mold on chick performance (Table 24) are comparable with previous reports (Manuscripts II and III). Birds given 4 ppm OA were severely affected, the average RFC and RWG values being only about 35 and 50% of that seen in the toxin-free diets, but the trend of the effects differed between weeks compared to the OA-free diets. The average reduction of 0.06 in RFC for OA fed birds was more dramatic from week 1 to 2 than for those not given OA. In contrast to the OA-free diets, OA fed birds actually showed improved RWG and F/G values during week 2. The magnitude of the mold effect was not as great as that for OA, but this was due to the lower degree of fungal contamination of the Elkhorn barley used in the current study (6.4% mold) compared to that used in Rotter et al.(Manuscripts II and III) (16% mold w/w). The current findings provide further evidence for the need to be cautious when feeding fungal contaminated grains. Also, incorporation of OA in the mold-contaminated diets resulted in further reductions in chick

performance compared to OA or mold alone, supporting the findings of Rotter et al. (Manuscripts II and III). Phe, however, did not modify the negative effects of the mold-contaminated grain, other than to decrease RFC slightly.

Chick mortality was affected by OA concentration ($P<0.0001$), and an OA x Phe x mold x time interaction ($P<0.01$; Table 25)This interaction accounted for 40.7% of the variation according to the partitioning of the sums of squares (Little 1981). Birds fed OA in combination with Phe had mortalities much higher than OA alone, particularly during the first week of exposure. The highest percentage of mortalities in this study occurred during the second week in birds fed OA in combination with mold and Phe. Neither mold nor Phe, alone or in combination with each other, contributed to mortality. The reason for the different mortality patterns is not clear, but the combination of OA with the added Phe may induce more stress on the birds than with OA or Phe alone. The birds, being stressed, could have been more susceptible to the effects of OA alone during the first week. It may be hypothesized that since the birds consumed less of the mold- containing than the mold-free diets (Table 24), the quantity of OA consumed during week 1 was reduced, thereby delaying the OA/Phe induced death of these birds to the second week.

The observed mortality pattern is in contrast to Gibson et al. (1988) who noted that supplementing broiler chick diets containing 14% protein and 4 ppm OA with Phe reduced chick mortality. It decreased from a high of 42.5% in OA fed control birds to 7.5 and 10% for those given OA-containing diets having total dietary Phe concentrations of 1.6 and 3.2%, respectively. Although these results appear to contradict those

Table 25. Average percent weekly pen mortality of Leghorn chicks (Experiment 9)

Mold concentration	OA concentration	Total dietary Phe concentration	Week 1	Week 2
0	0	0.9	0.00	0.00
		1.6	0.00	0.00
		2.5	0.00	0.00
	4	0.9	0.00	2.78
		1.6	5.56	0.00
		2.5	8.33	2.78
30	0	0.9	0.00	2.78
		1.6	0.00	0.00
		2.5	2.78	0.00
	4	0.9	2.78	0.00
		1.6	0.00	11.1
		2.5	2.78	8.89
SE			2.2	2.2

Continued .....

Table 25 (Continued) Summary of analysis of variance (mortality)

Source	df	MS	Probability
Main plot comparisons			
Mold concentration	1	34.03	0.30
OA concentration	1	389.0	0.0007
Phe concentration	2	55.6	0.17
Mold x OA	1	0.077	0.96
Mold x Phe	2	1.62	0.95
OA x Phe	2	62.6	0.14
Mold x OA x Phe	2	16.3	0.59
Error a	60	30.63	
Subplot comparisons			
Time (Wk)	1	9.34	0.57
Wk x mold	1	130.0	0.04
Wk x OA	1	9.33	0.57
Wk x Phe	2	11.7	0.67
Wk x mold x OA x Phe	7	84.1	0.01
Error b	60	29.1	

observed here, it should be noted that the protein content of the current diets averaged 22.7%, almost 9% higher than that reported by Gibson et al. (1988). It would appear that the low protein concentration and also low Phe, sensitized the broiler chicks to OA. Phe supplementation in such a case presumably then has some beneficial effects, especially on mortality.

It may be concluded that Phe supplementation of diets contaminated with a moderately high concentration of OA is of little benefit in reducing the effects of OA. Although administration of Phe in cases of acute exposure may be able to lessen or overcome the toxic effects of OA, its abilities as a protective agent do not justify its addition to animal feeds. The effect may be different if much lower concentrations of OA are involved, or if treatment conditions are altered. Never-the-less caution should be taken to avoid Phe toxicity or amino acid imbalances.

MANUSCRIPT VI

ENSILING AS A MEANS OF REDUCING OF OCHRATOXIN A CONCENTRATIONS IN CONTAMINATED BARLEY

ABSTRACT

The feasability of ensiling ochratoxin A (OA) contaminated barley as a practical method of inactivating OA was examined in a set of *in vitro* and *in vivo* studies. In two *in vitro* studies, OA concentrations in barley were decreased by approximately 68% after after being ensiled for either 49 (trial 1) or 56 (trial 2) days. To verify the destruction of the OA, a chick feeding trial was conducted. Samples of a toxin- and mold-free barley, mold-contaminated barley, a barley sample naturally contaminated with OA or a barley with added OA were each divided into two equal portions, one of which was ensiled for a period of 28 days. The mold- contaminated barley mold was naturally contaminated with *Penicillium cyclopium* and *Aspergillus flavus*. Diets containing either the ensiled or non-ensiled barley samples were then fed to 7-day-old Leghorn chicks for two 7 d periods. Changes in feed consumption (RFC) and body weight gain (RWG) relative to control birds, feed to gain ratio and mortality were monitored. The OA concentration in the two OA-containing barley samples decreased by 58% after 28 d of ensiling, but there was no improvement ($P>0.05$) in chick performance or mortality compared to the non-ensiled diets. The average RFC values for chicks fed the mold- and OA-containing diets were less than 50% of the controls while RWG generally less than 30%. In absolute terms, chicks fed the ensiled barley diets also performed about 6% poorer than those given the non-ensiled barley. Ensiling does not appear to be a practical method of reducing the toxic effect(s) of OA, despite the apparent decrease in OA concentration. Grain samples should be tested for the presence of a toxin prior to ensiling to avoid possible toxin analysis problems later.

KEY WORDS: Ochratoxin A, mycotoxin, degradation, ensiling barley, mold, chicks

INTRODUCTION

Ochratoxin A (OA) is a secondary metabolite produced by several species of Aspergillus and Penicillium fungi. It is composed of a chlorinated dihydroisocoumarin molecule linked by an amide bond to L-β-phenylalanine (Scott et al. 1972). OA is a potent nephrotoxin and depressor of the immune system, acting primarily by inhibiting protein synthesis.

Despite the relative stability of the OA molecule, it is degradable as indicated by its decrease in concentration under natural conditions (Szebiotko in Muller 1984; Krogh in Schuh and Schweighardt 1981). During the beer making process, OA is reportedly degraded, but not totally eliminated from the final product. Krogh et al. (1974) claimed that moderate concentrations of OA are completely eliminated during the malting process, but that even if heavily contaminated samples are used, 11-19% of the original OA may be found in the wort, but only 2-7% (6-20 ug L^{-1}) will be detected in the final product. Chu et al. (1975) found that OA is only partially lost during brewing and that both are stable in the cooker mash. They found both toxins to be more sensitive to later treatments, including protein hydrolysis, wort boiling and final fermentation, noting that up to 28% of the initial OA concentration may be found in the beer.

Several methods to degrade OA or reduce its toxicity have been reported, but with varying degrees of success. Charcoal addition to the diet (Manuscript IV) and supplementation of phenylalanine (Phe; Manuscript V) were not able to reduce the effect of OA in growing chicks. Ammoniation (Chelkowski et al. 1981a,b,c; Madsen et al. 1983), heat plus NaOH and autoclaving have all proven useful in reducing OA

concentrations in grains, but, based on animal trials, practical use of these methods is too risky (Madsen et al. 1983). Ruminant animals are less susceptible to the effects of OA, and this is due to the actions of either rumen protozoa or bacteria (Hult et al. 1976; Kiessling et al. 1984) in breaking OA down into relatively non-toxiv Oα and Phe. In vitro studies have shown OA can be degraded to Oα and Phe by carboxypeptidase A and α-chymotrypsin (Pitout 1969) and other enzymes in various rat tissue extracts (Doster and Sinnhuber 1972) and to two hydrogenated metabolites by alcohol dehydrogenase (Syvertsen and Stormer 1983). The use of these enzymes as an additive to OA contaminated chick diets to act within the bird's gastrointestinal tract does not yet appear to be practical (Rotter, unpublished data).

The objectives of the current report were to examine the feasability of ensiling OA contaminated barley as a method for inactivating the toxin. Based on the findings of the in vitro studies, a chick feeding trial was conducted to confirm a reduction in OA toxicity in the ensiled barley and verify the reduced risk of feeding it. The benefits of ensiling a moderately heavily mold-contaminated barley was also studied.

MATERIALS AND METHODS

Source of barley samples

Three sources of barley (Hordeum vulgare L. cv. Bedford) were used in this study. The mold-free (control) barley (1986 crop) and the naturally OA contaminated barley (Glenlea; 1983 crop) were obtained from the University of Manitoba Glenlea Research Station, Glenlea, Manitoba

(Man). The Glenlea barley was previously characterized (Manuscript II). Toxin-free mold contaminated barley (Elkhorn; 1983 crop), previously descibed (Manuscripts I and II) was obtained from a farm in Elkhorn, Man and was a mixture from the top and bottom sections of the same grain bin.

Prior to use for the in vitro and the feeding trial, all samples were ground in a Viking Electric Hammer Mill Model C-H (Horvick Manufact. Inc, Moorhead, MN).

Source of OA

The OA used in the in vitro studies and 75% of that in diets 5 and 6 of the feeding trial was naturally present in the undiluted Glenlea barley sample at a concentration of 20 ppm (Manuscript II). OA that was added to diets 5-8 was contained in freeze-dried media (FDM) used in the liquid fermentation of Aspergillus ochraceus (NRRL 3174) for 10-12 d at 28°c (Davis et al. 1972; Yamazaki et al. 1970). The OA concentration of the FDM was 1.9 g kg^{-1}. The OA standard was obtained from Sigma Chemical Co., St. Louis MO.

In vitro experiments

Two in vitro studies were conducted to examine the feasibility of the ensiling process to eliminate or reduce the concentration of OA in contaminated barley. A commercial ensiling additive, Sil-Add (Alltec, Lexington, KY), containing encapsulated Lactobacillus species bacteria, was added to all samples at 4 times the suggested dosage at the start of each trial in an attempt to ensure rapid onset of the ensiling process.

Trial 1

Glenlea barley was combined with the control barley which was both mold-

and toxin-free to give approximate starting OA concentrations of 0, 2, 4 and 8 ppm (mixes 1-4, respectively). Tap water (1225.5 mL) was added to the barley samples (3500 g) to bring each mixture to up to a moisture content of 35%, and then they were mixed in a Hobart Mixer. The moisture content was confirmed by dry matter analysis and then 81.2 g of each sample were placed in separate Whirl-Pac 20 cm x 10 cm bags (Fisher Sci., Pittsburgh, PA). The bags were individaully sealed after the moistened barley was compressed to remove as much air as possible.

The high moisture barley samples were stored in a walk-in incubator set at 28°C and removed in sets of three bags per OA concentration on days 6, 14, 21, 28, 35 and 49 of the incubation period. The contents of each bag were air-dried on aluminum foil plates (21 cm in diameter) to for 3 d, but first a 2.0 g subsample was taken and transfered into a 25 mL nylon centrifuge tube. The subsamples were immediately covered with 10 mL of distilled water, homogenized with a Polytron PT 10 OD homogenizer (Brinkmann Instruments, Rexdale, Ontario) for 2 min, and then centrifuged in a Beckman J2-21M centrifuge (Beckman Instruments Inc., Irvine, CA) at 14,000 G for 15 min. The sample was allowed to equilibrate to room temperature and the pH determined. The dried sample was analyzed to determine the OA concentration after ensiling.

Trial 2

Glenlea barley was mixed with an equivalent weight of the control barley to yield an OA concentration of approximately 4 ppm. The procedure was the same as outlined for trial 1, except that small wide mouthed glass preserving jars, with a capacity of approximately 150 mL, were used as the ensiling vessel. The 81.2 g of 35% moisture barley, when compressed,

almost completely filled the jar. The jar was sealed with a No. 13 rubber stopper through which passed a small stainless steel tube (6 mm x 5 cm). The tube was connected to polyethylene tubing (4 mm x 1.2 m). Air in the jar and tubing was removed using a large syringe, afterwhich the free end of the tubing was placed in a container of water about 1 meter below the ensiling jar. This system allowed any gases produced during fermentation to be released into the water, but prevented air from getting into the artificial silo. The jars were stored under the same conditions as described above.

Three jars were removed at random on days 7, 14, 21, 28, 42 and 56 after ensiling. The pH determination and drying method were as outlined above. Dried samples were analyzed to determine their OA concentration after ensiling.

In vivo study - feeding trial

Treatment of barley samples

Four basic mixtures were prepared:(1) mold- and toxin-free control barley (CB); (2) mold-contaminated Elkhorn barley (Man); (3) Glenlea barley naturally contaminated with OA (75%) mixed with control barley (25%) and freeze-dried fungal mycelia containing OA to elevate the OA concentration (NAT); and (4) control barley with added freeze-dried fungal mycelia containing OA to approximate the OA concentration in the NAT sample (ADD). Each mixture was the divided into two equal portions (8 kg), one stored dry at 4°C (CB, MB, NAT and ADD) and the other ensiled in 5-litre plastic pails with air tight lids (CBF, MBF, NATF and ADDF, respectively).

The ensiling process was similar to that used in in vitro trial 2.

Each barley mixture (8 kg) was moistened with tap water (2.8 L) containing Sil-Add at 4 times the recommended dosage and mixed for 15 min. The mixture was then transfered into a 10 L pail, taking care to compress the barley to remove as much air as possible. A small stainless steel tube (as used above) was placed in a 1 cm deep hole in the centre of the barley surface and barley was packed around it. The surface was then covered with liquid paraffin wax to a depth of 2 cm as only 85% of the pail's capacity was filled with barley. The steel tube pertruded approximately 2 cm above the wax and was connected to polyethylene tubing. The tubing was passed through an opening in the container lid with the free end placed into a container of water 1 meter below the top of the barley. As above, any air in the system was removed. The artificial silos were placed in a walk-in incubator (28°C) for a period of 28 d. Subsamples were taken from each pail for the analysis of OA, pH, percent moisture, lactic acid and mold content as determined by glucosamine analysis.

Diet formulation

All diets were formulated to meet or exceed the minimum NRC (1984) requirements for Leghorn pullets (Table 26). Diets were fed as mash.

Chicks and management

Male Single Comb White Leghorn (Leghorn) chicks were purchased from a commercial hatchery at one day of age. They were housed in electrically heated, thermostatically controlled Jamesway brooder batteries for 7 d prior to the start of the experiment. They were fed a commercial chick starter crumbles (21% protein) and provided with continous lighting. For the experiment, they were randomized and managed as outlined in Rotter

Table 26. Diet formulations used in Experiment 10

Ingredient	Percentage of total diet
Barley†	60.0
Soybean meal (47.5% protein)	29.5
Tallow	6.55
Ca_2PO_4	1.40
$CaCO_3$	0.70
Vitamin premix‡	1.00
Mineral premix‡	0.50
Methionine	0.05
Cr_2O_3	0.30
Calculated values	
Protein (%)	21.8
Metabolizable energy (Kcal kg^{-1})	2735

† Barley used in the respective diets were: toxin and mold-free barley, not ensiled - CB, ensiled - CBF; moldy barley, not ensiled - MB, ensiled - MBF; barley naturally contaminated with OA, not ensiled - NAT, ensiled-NATF; barley with added OA, not ensiled - ADD, ensiled - ADDF.

‡ The vitamin and mineral premixes were the same as those described by Young and Marquardt (1982).

et al. (1985a). They were housed in Petersime Battery Brooders and provided with continuous lighting and heat and given free access to feed and water.

Experimental design

The feeding trial was set up in a 4 x 2 factorial arrangement consisting of 4 barley mixtures with two different treatments, non- and ensiled, and conducted over two 7 d experimental periods. The experiment was analyzed as a splitplot design, using the barley mixtures and ensiling treatment as the mainplot comparison and time as the subplot comparison. Six replicates with six birds each were used per treatment. Chick performance was measured in terms of feed consumption, weight gain and feed to gain ratio (F/G). Feed consumption and weight gain values are presented relative to the controls (1.00).

Analyses

Barley samples were subjected to a multitoxin screening analysis (Wilson et al. 1975; Josefsson and Moller 1977), which included screening for OA, aflatoxins, penicillic acid, citrinin, patulin, sterigmatocystin and zearalenone. The degree of fungal contamination was determined according to Rotter et al. (Manuscript I), and stereomicroscope was used to confirm the mold-free condition of the control barley and, in the mold-contaminated samples, the contaminating species characterized by Dr. G. Platford, Manitoba Department of Agriculture, as outlined in Rotter et al. (Manuscripts I and II). OA was quantified following the procedure of Frohlich et al. (1988), using an LKB HPLC system (2152 HPLC controller, 2150 HPLC pump and a 2155 HPLC column oven; LKB-Produkter AB, Bromme, Sweden) equipped with a Waters 712 automatic sampling device (Millipore

Corp., Milford, MA) and a Hewlett Packard HP 1046A flouresence detector. The column used was a 4.6 x 250 mm Ultrasphere column with 5 um C-18 ODS preceeded by a 4.6 x 50 mm precolumn packed with CO:PELL C-18.

The moisture content of barley samples was calculated by subtracting the determined dry matter percentage of the sample (AOAC 1984) from 100%. Lactic acid concentrations of the barley samples used in the feeding trial were determined according to (Barker and Summerson 1941).

Statistical analyses of the *in vitro* and *in vivo* data were performed using General Linear Models (GLM) procedures of the Statistical Analysis System (SAS Institute Inc. 1982). Chick performance was analysed using split-plot analysis.

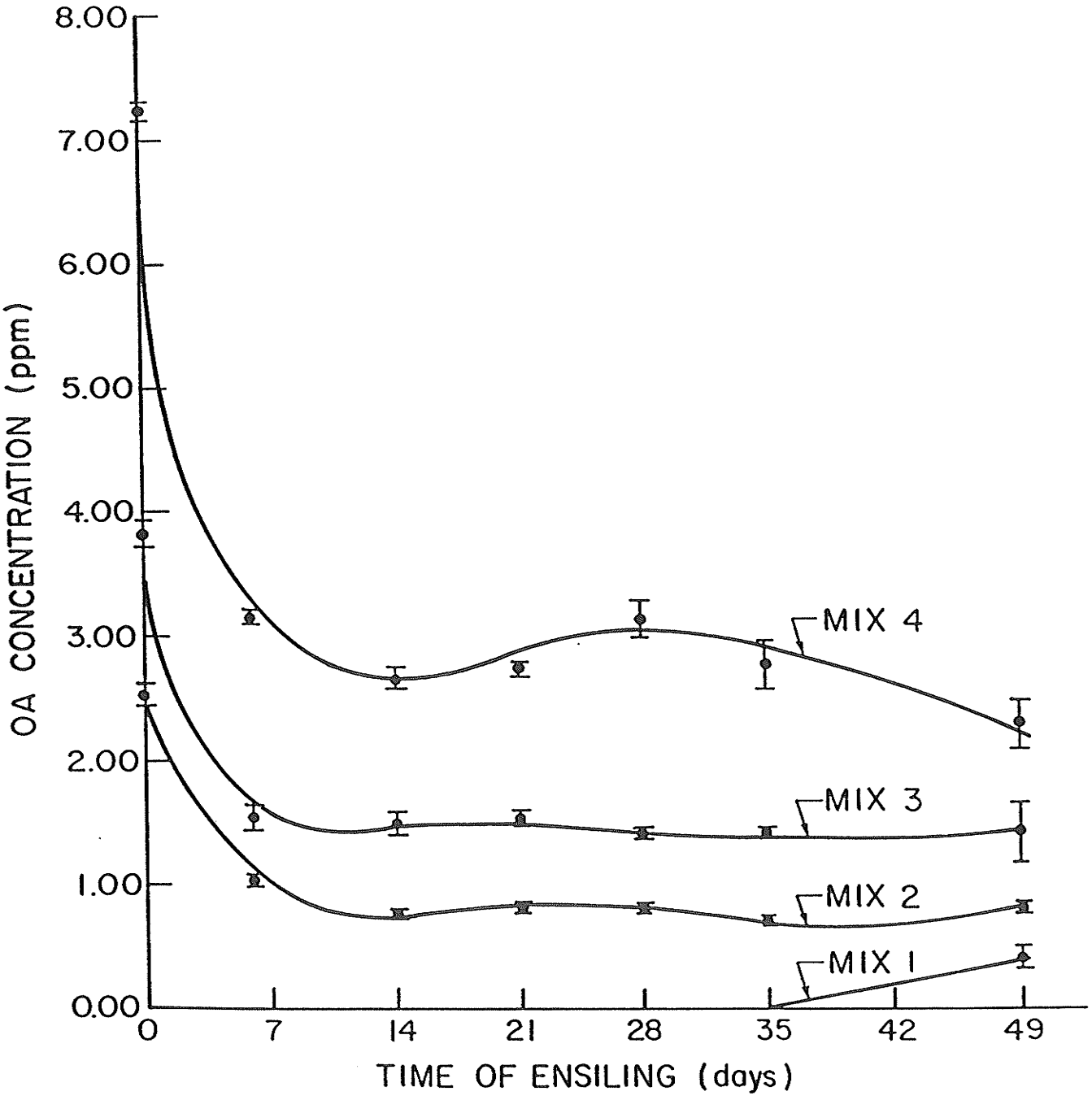
RESULTS

In vitro studies

Trial 1

In trial 1, the concentrations of OA in mixtures 2, 3 and 4 had decreased 68.1, 63.3 and 68.3%, respectively, after 49 days of ensiling (Fig. 5). On about day 21, the OA concentrations tended to increase in these three mixtures, most noticeably in No. 4. The increase in OA was presumably caused by isolated mold growths in most of the sample bags. This growth appeared to occur only along the sides of the bags, presumably forming where small, non-visible holes were located in the polyethylene. These holes presumably permitted the localized growth of small fungal colonies, some of which apparently produced OA. This additional OA production was greater in those samples containing the

Fig. 5. Effect of ensiling of contaminated barley on different concentrations of ochratoxin A over time. Points represent average of three values analyzed in duplicate ± SE.


8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
OA CONCENTRATION (ppm)
0
7
14
21
28
35
42
49
TIME OF ENSILING (days)
MIX 4
MIX 3
MIX 2
MIX 1

higher concentrations of the Glenlea barley, but the production during the study was short lived, lasting at most 14 d, as the fungus grew around the holes in the bag, essentially sealing it, preventing air from entering the bag, and thus preventing more fungal growth and toxin production. This study, despite the failure of the bags to act as a completely air-tight container, demonstrated that the ensiling process is an effective means of reducing OA concentrations in contaminated grain.

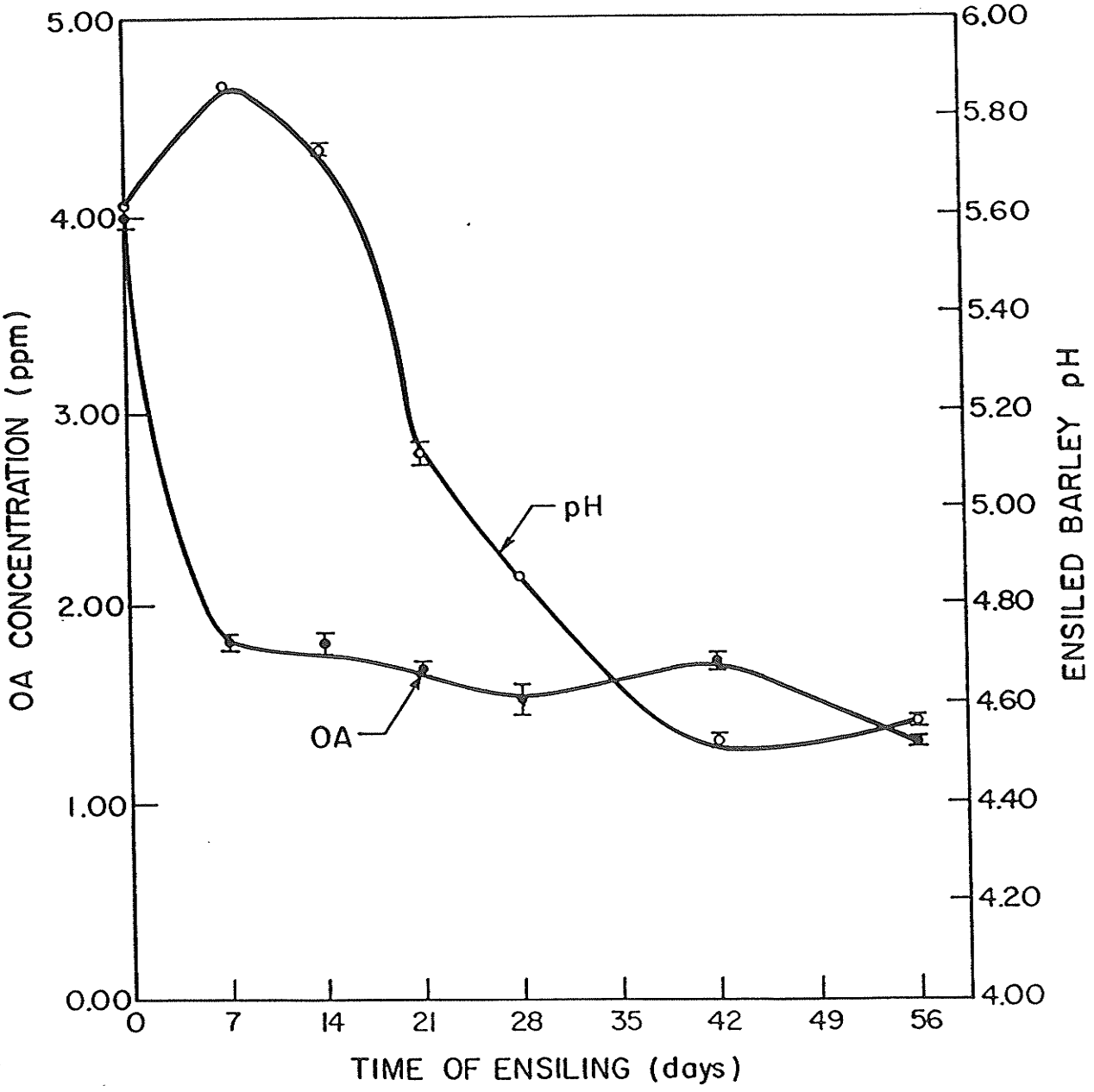
One complicating factor which occurred during this and the subsequent studies with ensiled barley samples was that HPLC analysis indicated the presence of a compound which eluted before Oα, but interfered with the quantification of Oα. The peak area (PA) of this compound increased over time, but it was not identified. Although the PA associated with this compound was greater in samples containing mold or OA, it was also prominent in the mold- and toxin-free barley. This suggests that the unidentified compound was not a breakdown product of OA. As a result, Oα could not be reliably quantified in this study.

The pH profiles over time differed between the ensiled mixtures. Although all pH values ± SE on day 0 were approximately 5.64 ± 0.016, they decreased rapidly in mix 1 (clean barley) to a low of 4.55 ± 0.020 by day 21, and increasing slightly thereafter. The pH of Mixture 2 also decreased initially, but less dramatically to a low on day 21 of 4.90 ± 0.015, and again increasing slightly over time. Mixtures 3 and 4 did not decrease until day 14, and they never decreased below pH 5.20.

Trial 2

The concentration of OA after 56 days of ensiling had decreased to a

Fig. 6. Changes in ochratoxin A concentration and barley pH values over time during ensiling (trial 2). Points represent average values ± SE.


5.00
4.00
3.00
2.00
1.00
0.00
OA CONCENTRATION (ppm)
6.00
5.80
5.60
5.40
5.20
5.00
4.80
4.60
4.40
4.20
4.00
ENSILED BARLEY pH
pH
OA
0
7
14
21
28
35
42
49
56
TIME OF ENSILING (days)

level 67.4% lower than that on day 0 (Fig. 6). The greatest absolute reduction in the concentration of OA occurred between days 0 and 7 (the first sampling day), suggesting that the maximal effect of ensiling occurred within the first few days of the start of the study. Thereafter, the OA concentration began to decrease slowly over time. The interfering peak seen in the HPLC analysis in trial 1 was evident in this trial, again increasing in area over time. No mold growth was visible in any of the sealed jars, indicating the integrity of the air-tight system. The pH profile over time resembled that seen in mix 1 of trial 1, except that the pH initially increased after 7 d, but then decreased to a low of about pH 4.5 after 42 d.

In vivo study

After 28 d of ensiling, detectable OA concentrations in both NATF and ADDF decreased by 58.3% compared to that in NAT and ADD (Table 27). No OA was detected in the other samples. HPLC analysis of the ensiled barley samples again demonstrated the presence of the unidentified compound, increasing with time. It was not seen in the non-ensiled version of the same barley sample mixture. The drop in pH values was similar in all the ensiled samples, decreasing from an initial value of about 5.63 to an average of 4.33 at the end of the fermentation period. The production of lactic acid, an indicator of the presence of Lactobacillus species bacteria, was also present in substantially higher concentrations than in the non-ensiled samples, but the values differed between mixtures. The estimated mold concentrations were similar in all samples, with the exception of CB and CBF, which were lower, and surprisingly the values decreased when the barley samples were ensiled,

Table 27. Characteristics of non-ensiled and ensiled barley samples used in the chick feeding trial after ensiling. Values given are ± SE.

Barley sample		OA concentration (ppm)	pH of† extract	Lactic acid concentration(%)	Estimated Mold‡ concentration (%)
Mold and-toxin-free	non-ensiled (CB)	0.00 ± 0.00	5.61	0.09 ± 0.02	1.12 ± 0.10
	ensiled (CBF)	0.00 ± 0.00	4.40	1.59 ± 0.07	0.89 ± 0.01
Mold-contaminated	non-ensiled (MB)	0.00 ± 0.00	5.64	0.09 ± 0.02	7.01 ± 0.04
	ensiled (MBF)	0.00 ± 0.00	4.35	2.42 ± 0.05	6.28 ± 0.18
Natural OA	non-ensiled (NAT)	6.36 ± 0.17	5.64	0.09 ± 0.02	5.45 ± 0.18
	ensiled (NATF)	2.65 ± 0.09	4.31	2.62 ± 0.00	4.55 ± 0.05
Added OA	non-ensiled (ADD)	8.50 ± 0.16	5.62	0.09 ± 0.02	6.55 ± 0.08
	ensiled (ADDF)	3.54 ± 0.07	4.27	2.07 ± 0.09	7.36 ± 0.21

† Duplicate readings were the same, so no SE values are given.

‡ Estimate calculated using reference standard values of : 76 mg glucosamine g^{-1} mixed fungus for CB and CBF; 66.1 mg glucosamine g^{-1} P. cyclopium for MB and MBF; and 76.9 g glucosamine g^{-1} A. ochraceus for NAT, NATF, ADD and ADDF. References based on fungal species either known to be present (or OA contained in its mycelium) or for unknown mixture as deemed appropriate (Manuscript I).

except for ADDF where the mold concentration increased by 1%.

Split plot analysis of the performance data (Table 28) indicated significant (P<.041) diet type and ensiling main effects for RFC, RWG and F/G and a time effect for RWG and F/G. The presence of mold-contaminated barley or OA in the basal diet resulted in reductions in RFC of more than 50%, while RWG was affected to a greater degree, but generally was less than 30% of the controls. F/G values were similarly affected. While the OA concentrations of ensiled diets were reduced by almost 60%, no differences were observed in the performance of birds fed the non-ensiled and ensiled treatments of the same basal diet. In addition, ensiling reduced RFC an average of about 10% and RWG by 12%, and caused an increase in F/G from 3.14 to 3.77 compared to those obtained with the non-ensiled diets.

The diet x ensiling inertaction was significant for RFC (P<0.037), but it only contributed 0.026% to the total variation according to the partitioning of the sums of squares (Little 1981), and was of little biological significance. The time x diet interaction was also significant (P<0.0005) for RWG and F/G. Over time, RWG and F/G did not change for the toxin- and mold-free basal diet fed chicks, but for those given diets containing either mold-contaminated barley or OA, RWG and F/G both showed marked improvements over time. In contrast, only the mold- and toxin-free basal diet fed birds showed no change in RWG and F/G. Although the time x diet interaction for RFC approached significance (P=0.066), examination of the data indicates no real differences between week 1 and 2 values.

Table 28. Effect of diet type and ensiling on chick performance.

	Relative feed consumption		Relative weight gain		Feed to gain ratio (g/g)	
Time x diet						
Basal diet type	Week 1	Week 2	Week 1	Week 2	Week 1	Week 2
Mold-free barley	0.97	0.97	0.99	0.99	1.97	2.04
Mold-contaminated barley	0.50	0.48	0.23	0.30	5.86	3.47
Natural OA	0.47	0.48	0.28	0.43	3.46	2.34
Added OA	0.40	0.39	0.15	0.30	5.66	2.83
SE	0.006		0.013		0.35	
Ensiling						
Non-ensiled	0.61		0.49		3.14	
Ensiled	0.55		0.43		3.77	
SE	0.0029		0.0065		0.17	

Continued .....

Table 28 (Continued)

Summary of analysis of variance

Source of variation	df	Relative feed consumption MS	Relative feed consumption Prob.	Relative weight gain MS	Relative weight gain Prob.	Feed to gain ratio MS	Feed to gain ratio Prob.
Main plot							
Diet	3	1.62	0.0001	3.08	0.0001	36.1	0.0001
Ensiling	1	0.09	0.0001	0.065	0.0034	9.45	0.041
Diet x ensiling	3	0.013	0.037	0.013	0.15	3.39	0.20
Error a	40	0.004		0.0067		2.12	
Subplot							
Time	1	0.0008	0.20	0.210	0.0001	59.0	0.0001
Time x diet	3	0.0012	0.066	0.030	0.0001	10.4	0.0005
Time x ensiling	1	0.0006	0.27	0.000008	0.95	5.25	0.063
Time x diet x ensiling	3	0.0006	0.25	0.0022	0.36	1.06	0.54
Error b	40	0.0004		0.0020		1.43	

† Actual values for feed consumption and weight gain control birds were (g per bird per 7 d): week 1, 114.9 and 57.2; and week 2, 158.8 and 76.1, respectively.

DISCUSSION

Muller (1983) stated that no matter what mycotoxin is considered, decontamination is not easy. Any method must truly eliminate the toxin *in vitro* and in a biological test, reduce the toxic effect below a specific tolerance level. In addition, the physical and nutritional quality of the feedstuff must not be greatly affected. Ensiling of toxin contaminated grains has been proposed as one method of reducing or eliminating various mycotoxins, and generally appears to fit the necessary criteria. Muller (1984) noted that aflatoxin B_1 (AFB_1) is susceptible to breakdown, depending on pH, temperature and length of storage under ensiling conditions. He noted one study in which AFB_1 was decreased 95% at pH 3.0 at 20°C after 300 d. Another study reported no breakdown of AFB_1 at pH 4.4 after 26 d, but concentrations were reduced 60% when 0.1 M HCl was added to the system (Lindenfelser and Ciegler 1970). Some toxins, such as zearalenone and some of the tricothecenes, are not degraded under ensiling conditions as they are stable at low pH (Muller 1984). Under the conditions used in the current study, OA is apparently degraded when the contaminated grain is ensiled, but its toxic effect remains.

Other researchers have reported that the concentration of OA decreases naturally over time during storage. Szebiotko (*in* Muller 1984) noted that a concentration of 500 ug OA kg^{-1} added to barley, wheat and maize flour decreased 78, 80 and 71%, respectively, over a 6 month storage period. Similarly, Krogh (*in* Schuh and Schweighardt 1981) observed a 62.5% decrease in barley (4 to 1.5 ppm) after 2 years. The mechanism was not known, but microbial activity is a possibility. In

addition, no *in vivo* toxicity studies were conducted by any of these authors.

Chelkowski et al. (1982c) claimed that to detoxify OA, its concentration must be reduced to nondetectable levels. It would seem logical however that a true reduction in the concentration of OA due to any treatment, especially the apparent 50% reduction seen here, should also lead to at least some decrease in the observed toxicity. Despite the much lower OA concentrations in the diets containing the ensiled barley samples, there was no reduction in the apparent OA effects on chick performance. Ensiling also had no effect on reducing the negative effects of mold contaminated barley or improving its nutritional value. For both the OA and the mold-contaminated barley, the effects were similar to previous reports (Manuscripts II and III).

Using thin-layer chromatography plates, Krogh et al. (1974) noted spots of a compound present in the wort but absent in the beer which differed from OA and Oα. Although they did not identify this compound, it may correspond to the unidentified HPLC peak in the ensiled barley samples observed in the current study.

Evidence concerning the feeding value of ensiled cereals is contradictory. While Jamieson (1968) and Cole et al. (1970) reported no differences in performance of growing pigs fed high moisture barley, Livingstone and Livingston (1970), Livingstone et al. (1971) noted poorer growth, as much as 9% lower, and increased F/G due to ensiled barley, but they attributed this to mold growth on the moist barley. Goransson and Ogle (1985) also observed poorer performance in pigs and rats fed high moisture grain, but indicated that mold contamination was

not a problem until late in the study. The reduced RFC and RWG and increased F/G of chicks fed diets containing ensiled barley agrees with the results of these studies, but the reason may be an undesirable residual oder or taste, production of appetite depressing compounds or to the utilization of nutrients by bacteria during the fermentation process. Livingstone and Livingston (1970) reported that the characteristic sour-sweet smell and taste of ensiled grains is not removed by drying. Other authors, including Newman et al. (1985) and Pettersson et al. (1987) have reported the use of distillers-spent-grain (barley) in the diets of growing chicks with no adverse effects, but they only utilized up to 20% of this grain in the diet.

Inspite of the apparent reduction in OA concentrations during ensiling, its toxic effects were not affected. The toxin may be converted into an undetected or unidentified derivative with a similar toxicity.The practice of ensiling OA or mold contaminated grain is therefore not recommended. Testing of grain samples before ensiling is suggested to avoid problems in identifying a toxin which may not be readily identified later.

GENERAL DISCUSSION

The feeding of fungal and mycotoxin contaminated grains can result in serious detrimental effects on the animals which ingest them. Although some species of fungi infect the grain in the field (eg. Claviceps purpurea), the reported research focused primarily on several major storage molds common to western Canada, P. cyclopium, and some Aspergillus species (Sinha et al. 1986), and OA, the toxin they produce.

The detrimental effects of feeding OA to chickens and swine are well documented, but the majority of the studies involved the addition of pure OA to a mold-free diet. Few studies have studied the effect of OA in the presence of mold (Nelson et al. 1982), primarily due to the difficulty in separating out the effects of each. Further, a limited number of studies have considered the influence of toxin-free mold contamination of grains (Sharby et al. 1973; Bartov et al. 1982; Bartov 1983, 1985). Unfortunately, these authors did not attempt to quantify the degree of mold contamination in their samples to relate this to the effects observed. These limitations in the knowledge of the effects of mold and OA guided the described research, because without a better understanding of these factors and their interactions, data from studies dealing with the detoxification of OA (and/or mold) could not be properly interpreted.

A modified version of the liquid chromatography procedure used by Stahmann et al. (1975), Wu and Stahmann (1975) and Hubbard et al. (1979) was developed for estimating the degree of fungal contamination of a grain sample using GlcN analysis. No other procedure for the quantification of fungal material in a naturally contaminated sample was

available. The commonly used percent of seed yielding fungi procedure was considered, but this only estimates the number of seeds infected, not the extent (Donald and Mirocha 1977). The GlcN assay, in contrast, directly measures the degree of fungal colonization based on both viable and nonviable mycelium. The procedure was used in the subsequent studies to estimate mold contamination of barley samples fed to chicks.

Reductions in RFC and RWG values in chicks fed toxin-free diets containing naturally contaminated barleys with differing concentrations of mold (0.6 to 16% mold) or increasing dietary concentrations of a moderately heavily contaminated sample, were directly related to the mold concentration in the diet. Dry matter and especially fat digestibilities were also markedly reduced by the presence of moldy barley, and, as with chick performance, the magnitude of the effects in the current studies was much greater than previously reported (Bartov et al. 1982; Bartov 1983, 1985; Sharby et al. 1972; Fritz et al. 1973). No direct comparison can be made to these studies, but it is conceivable that different fungi may produce different effects in animals.

The detrimental effects of dietary mold in chicks have been attributed to a palatability or odor problem, an energy deficiency (Bartov et al. 1982; Bartov 1983; 1985), the presence of thiaminase (Fritz et al. 1973) and low concentrations of lysine (Bartov et al. 1982) and methionine (Santos and Gomez 1983; Slagle and Zimmerman 1983). Neither a high dietary tallow concentration (10%) nor supplementation of the diet with starch (15%) was able to significantly ($P<0.05$) overcome the effects of mold in this study. Additionally, the performance of chicks fed moldy barley was about 50% of that seen in birds given diets

where up to half of the barley component of the diet was composed of cellulose. Since the cellulose contained no nutrients, the performance of these birds should have been similar to or worse than the moldy barley fed birds. These data strongly indicate that the moldy grain not only contains factors which cause feed refusal, but also antinutritive factors which interfere with nutrient digestion/absorption and utilization.

The combined effect of mold and OA on chick performance is slightly greater than each alone. OA also affects fat digestibility, but its influence on protein digestibility is much greater. This was also noted by Nelson et al. (1982). As was evidenced in one naturally contaminated barley sample from Glenlea, MB, even in the absence of large quantities of visible mold, OA concentrations can reach extremely high levels. Though not as high as the 27 mg OA kg^{-1} of barley reported by Scott et al. (1972), the 20 mg kg^{-1} seen in this sample should cause concern as the estimated mold content was only 1.6%.

Other authors have shown that OA concentrations are reduced naturally (Szebiotko et al. 1980; Trenk et al. 1971), by light (Neely and West 1972) and can be reduced by high temperatures in the presence of water (Osborne 1979; Chu et al. 1975; Krough et al. 1974), and ammoniation (Madsen et al. 1980, 1983; Chelkowski et al. 1981b, 1982). Unfortunately, only approximately 50% of the OA contained in a grain sample is located in the bran portion of a cereal, the rest in the endosperm (Chelkowski et al. 1981a). Some methods that could degrade OA would also cause other effects such as reducing the nutritional value of the grain. In feeding trials with ammoniated grain containing OA, for

example, slight improvements in chick (Chelkowski et al. 1982) and swine (Madsen et al. 1983) performance were observed, but only 5-10% of the treated barley was used in the diets. Madsen et al. (1983) concluded that use of ammoniation, among other more drastic treatments (0.5% NaOH at 490°C) are too risky for current consideration.

In vitro studies with Phe (Creppy et al. 1979, 1983), activated charcoal and ensiling of contaminated barley (current study) indicated the possibility that OA toxicity could be reduced. Additional in vivo studies with Phe also demonstrated that when Phe is injected concurrently with OA, that the effects of OA can be overcome or partially reduced (Creppy et al. 1984a,b; Moroi et al. 1985; Haubeck et al. 1984; Mayura et al. 1985). Unfortunately, no significant ($P>0.05$) benefits were seen in chick performance when either Phe or activated charcoal was added to OA contaminated diets or when contaminated barley was ensiled. While the use of Phe and charcoal as dietary additions to reduce the toxicity of OA contaminated grain is not feasible, their possible use in cases of acute exposure can not be ruled out. Broiler chicks fed AFB_1 with charcoal performed 10% better compared to AFB_1 fed birds, but goats injected intraruminally with a lethal dose of AFB_1 showed less severe effects when they were given a slurry containing activated charcoal (Hatch et al. 1982). Ensiling did result in an apparent reduction of the concentration of OA by almost 60%, but the toxicity associated with the original OA concentration was unaffected. This finding suggests that samples of grains which are to be ensiled should be tested for toxins before ensiling as it may not be possible to identify a toxin which could cause problems later. In addition, ensiling

did not reduce the detrimental influence of mold contaminated barley.

SUMMARY AND CONCLUSIONS

A method to estimate the degree of mold present in a grain sample based on ion exchange chromatographic analysis for GlcN was derived. Using this procedure, a series of experiments were conducted to investigate the effects of feeding chicks a chemically characterized toxin-free mold contaminated barley with and without added OA, and several possible treatments to reduce the detrimental effects of both mold and OA on chick performance. Chick performance and nutrient digestibility were markedly reduced by the presence of mold contaminated barley, the degree of the response was highly correlated with the concentration of fungal material. Tallow and starch supplementation of mold contaminated diets was not able to reduce the effects of the mold. In addition, birds fed diets in which 50% of the mold-free dietary barley had been replaced by cellulose, a non-nutritive bulk substitute, performed 100% better than those fed a moldy barley diet. While the detrimental effects of a dietary OA concentration of 4 ppm were less dramatic than a diet containing either 15 or 30% of a moderately contaminated moldy barley (16%), a combination of the OA and mold caused reductions only slightly more than the mold alone. Supplementation of diets containing 4 ppm OA with activated charcoal up to 10,000 ppm or Phe to a total dietary concentration of 2.6% were not able to overcome the effects of OA. Similarly, ensiling OA contaminated barley, although reducing the concentration of OA by about 60% after 28 days, did not lessen its toxic effects to growing chicks. Also, no improvement in chick performance was seen due to the ensiling of mold contaminated barley.

Based on this series of experiments, I conclude:

1. The degree of fungal contamination of a grain sample can be estimated from its GlcN content, as determined by liquid chromatography analysis, by relating it to that of a reference standard of the same species.

2. As the concentration of mold contaminated barley increases in the diet, there is a corresponding reduction in chick performance.

3. Mold contaminated barley contains an unidentified antinutritive factor(s) which interferes with nutrient digestibility/absorption, most notably fat. In addition, moldy grain also has a reduced content of readily digestible nutrients. These effects are not overcome by the supplementation of dietary tallow or starch.

4. Activated charcoal or phenylalanine supplementation of OA contaminated diets are not feasible dietary treatments to overcome or reduce the effects of OA.

5. Ensiling of OA contaminated barley causes a marked reduction in detectable OA concentrations, but does not lessen the effects of OA. Ensiling does not lessen the detrimental effects of moldy grain, but in itself slightly reduces chick performance.

Future research suggestions:

1. A greater variety of fungal species should be grown on liquid culture and their GlcN values determined for use as reference standards. Also, differences in GlcN (and GalN) concentrations during fungal growth should be more closely examined.

2. Additional research with more toxin-free mold-contaminated grains and preferably a variety of fungal species must be conducted to better establish the nature of the specific effects that mold has on animal performance. This research should also involve other species, in addition to chickens.

3. The antinutritive factor(s) in mold contaminated grain should be identified and treatments derived to overcome its (their) effects.

4. Research should be conducted to develop a practical, safe and preferably inexpensive treatment to counteract the effects of dietary OA. Work should also include moldy grain in the diet to simulate the natural situation.

5. The reason for the apparent decrease of OA concentrations during ensiling, but the failure to decrease its toxicity should be examined.

6. A simple and inexpensive method for the detection of OA, and other major toxins, in grain samples should be developed for use at the farm level. All grain samples should be tested periodically before using them in animal feeds.

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