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INHIBITION OF PROTEIN SYNTHESIS IN MAIZE AND WHEAT BY TRICHOTHECENE MYCOTOXINS AND HYBRIDOMA-BASED ENZYME IMMUNOASSAY FOR DEOXYNIVALENOL

By

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ABSTRACT

INHIBITION OF PROTEIN SYNTHESIS IN MAIZE
AND WHEAT BY TRICHOTHECENE MYCOTOXINS
AND
HYBRIDOMA-BASED ENZYME IMMUNOASSAY
FOR DEOXYNIVALENOL

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The 12,13-epoxytrichothecene mycotoxins are responsible for toxicoses in humans and animals consuming contaminated food and feed. Although several 12,13-epoxytrichothecene-producing fungi are plant pathogens, studies on the phytotoxicity of these compounds has been limited to gross effects such as wilting, chlorosis, necrosis and reduced growth. The ability of these compounds to inhibit protein synthesis in animal and yeast cells, however, is well known. This study demonstrated that the inhibition of protein synthesis in host plants of trichothecene-producing Fusarium spp. is a specific phytotoxic mechanism. Protein synthesis in tissue (leaf discs and kernel sections) was measured by incorporation of ^{3}H-leucine into acetone:ethanol insoluble material. Cell-free translation systems were extracted from wheat embryos

and maize seedling plumules, and protein synthesis measured by incorporation of ^{3}H-leucine into trichloroacetic acid-insoluble material. The trichothecenes deoxynivalenol (DON, vomitoxin) and T-2 toxin inhibited protein synthesis in tissue and cell-free translation systems from wheat and maize. The toxin concentrations inhibiting 50% of ^{3}H-leucine incorporation (ID_{50}) by several maize varieties were 0.9 uM (T-2 toxin) and 9-22 uM (DON). ID_{50} values for wheat were 0.26 uM (T-2) and 4.5 uM (DON). Inhibitory levels of toxin were similar in cell-free systems and leaf discs, indicating a direct effect of the toxins on the protein synthetic mechanism. T-2 toxin reached near-maximum inhibitory levels in leaf discs within 5 min exposure to toxin. The toxin an/or inhibition of protein synthesis persisted at least 120 min after removal of leaf discs from toxin solutions. Generally, sensitivity to DON was not correlated with susceptibility to ear rot by a DON-producing strain of Gibberella zeae (anamorph=Fusarium graminearum) for six maize lines with a range of disease reactions from highly susceptible to highly resistant. However, the ID_{50} for one moderately resistant line (A509) was 2.3 times greater than the ID_{50} of the most susceptible line (B79). Protein synthesis in wheat and maize was inhibited by DON and T-2 toxin at concentrations occurring in infected tissue, suggesting the need for further evaluation of these compounds as plant disease determinants.

A hybridoma line was developed which secretes

monoclonal antibodies with affinity for DON and several analogs. To facilitate protein conjugation for production of immunogen and immunoassay reagents, DON was converted to 3-O-hemisuccinyl-DON after protection of C7 and C15 hydroxyls with a cyclic boronate ester. Derivatization was confirmed by thin-layer chromatography, mass spectrometry and proton magnetic resonance spectrometry. Direct- and indirect-competitive enzyme immunoassays utilizing monoclonal antibodies prepared against this conjugate detected deoxynivalenol at 0.2-5.0 ug/ml (0.2-5.0 ug/g grain) in extracts of qround maize kernels.

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PART I:

INHIBITION OF PROTEIN SYNTHESIS IN MAIZE AND WHEAT BY TRICHOTHECENE MYCOTOXINS

INTRODUCTION

The 12,13-epoxytrichothecenes are a group of related esters of sesquiterpene alcohols possessing the tricyclic trichothecane structure (15,36). These compounds are elaborated by plant-parasitic and saprophytic fungi in the genera Fusarium, Myrothecium, Trichoderma, Cephalosporium, Verticimonosporium and Stachybotrys (58,93). Trichothecenes are generally considered "secondary metabolites," since many are strain specific and apparently not essential for survival of the producing organism (8,85). The importance of trichothecenes as mycotoxins, fungal metabolites toxic to animals, has generated several general reviews (4,78,93,99). Some of the effects of trichothecenes on animals, including humans, will be discussed in Part II.

Several early studies were concerned with the toxicity of trichothecenes to fungi. Mycelial growth (9,25,27,70), sporulation and spore germination (75) of fungi in the classes Ascomycetes, Deuteromycetes and Zygomycetes (sensu Alexopoulos [1]) were inhibited by trichothecenes. Bacteria were insensitive to the trichothecenes tested (9,27).

Although several trichothecene-producing fungi are plant pathogens, studies on the phytotoxicity of these compounds have been limited. These have generally been concerned with gross effects such as wilting, chlorosis, necrosis or inhibited growth, of whole plants or plant organs. There have been no reports of the effects of

trichothecene mycotoxins on specific biochemical mechanisms in plants.

There are various responses of plants to trichothecenes, and sensitivities differ with species. Trichothecin, the first trichothecene to be isolated and characterized (26,27,28), was reported to cause large areas of necrosis on bean leaves at 50 ug/ml, but tobacco plants were not affected by doses as high as 200 ug/ml (5). Although growth of tobacco plants is inhibited by 10^{-4} M trichodermin, chlorosis and necrosis occur only at 10^{-2} M, and the symptoms do not appear until 5 days after treatment (21). At similar concentrations of trichodermin, bean plants have symptoms similar to those of virus infection, and corn plants are seriously damaged. Macrocyclic trichothecenes (e.g., roridan A and verrucarins) sprayed on intact bean, maize and tobacco plants caused chlorosis, necrosis, stunting and malformations (at 10^{-4} M for roridan A, the most toxic of the compounds tested on intact plants) (20). In contrast, Baccharis megapotamica Spreng (Compositae) can accumulate high levels of macrocyclic trichothecenes (0.02 to 0.03 percent by dry weight) with no obvious adverse effects (43,44,45).

Brian et al (7) reported severe scorching of foliage, retarded stem growth, and frequent death of pea seedlings sprayed with culture filtrates of Fusarium spp., especially F. equiseti. Diacetoxyscirpenol (DAS), a trichothecene isolated from the culture filtrates, caused a "just-detectable

inhibition" of stem elongation when pea seedlings were sprayed to run-off with solutions of DAS at 1 ug/ml. At 10 ug DAS/ml, stem extension was severely reduced, leaves were scorched, and many plants were killed. Similar damage was observed on lettuce and winter tares; scorching and stunting occurred on cress and tomato. Beetroot, carrots, mustard and wheat (var. "Victor") were unaffected by DAS at 10 ug/ml. Pea seedlings wilted and became necrotic when roots were immersed in T-2 toxin (10 ug/ml) or in suspensions of F. tricinctum strains which produced T-2 toxin, but not when suspensions of non-T-2 toxin-producing strains were used (59). Burmeister and Hesseltine (9) developed a bioassay for T-2 toxin, based on sensitivity of pea seedlings to these compounds. Germination of pea seeds was inhibited by 50, 64 and 90% when soaked overnight in water containing 0.5, 1.0 and 2.0 ug T-2 toxin/ml, respectively. Inhibition of cress seed germination by DAS has also been reported (76).

Joffe and Palti (48) also found variation among plant species with respect to phytotoxicity of Fusarium culture filtrates, although it is not clear that trichothecenes were reponsible for the toxicity. They compared the reaction of 10 different crop plant species to suspensions of 642 isolates (of 49 species and varieties) of Fusarium. They also reported the skin reaction of rabbits to 90% alcohol-extracts of the same fusaria grown on wheat grain. The severity of dermal lesions by Fusarium strains was propor-

tional to the number of plants killed and number of plant species affected. Wheat was rarely affected even by the most toxic strains, whereas tomato, eggplant and onion were affected by the greatest number of isolates. Cucumbers, pepper, cotton and maize were intermediate in severity.

Several researchers reported diminished growth of plants exposed to trichothecenes. Cress root elongation was inhibited by DAS concentrations greater than 1-2.5 ug/ml, but stimulated by lower concentrations (7,75). The fresh weight and average length of pea seedlings immersed in T-2 toxin (625 ng/ml) was significantly less than controls (59). T-2 toxin inhibited mitosis in onion root tip meristem cells by arresting cells at metaphase; the effect was similar to that of colchicine (51). If this effect on cell division by trichothecenes is a general phenomenon, it would obviously explain their effects on plant growth.

Some trichothecenes reduced growth regulator-enhanced expansion of plant tissue. In an early study, trichothecin apparently inhibited the action of indoleacetic acid (25). DAS inhibited indoleacetic acid-promoted extension of pea internode sections and gibberellic acid-promoted expansion of bean leaf discs (7). T-2 toxin was also found to inhibit auxin-promoted elongation in soybean hypocotyls; the response was similar to that of cycloheximide, a protein synthesis inhibitor (86). Since T-2 toxin and other 12,13-epoxytrichothecenes are potent inhibitors of protein synthesis (discussed below), the comparison with cycloheximide

is pertinent. T-2 toxin, trichodermin and neosolaniol monoacetateare inhibitory to wheat coleoptile growth at 10^{-6} M, and several other T-2 analogs are inhibitory at 10^{-5} M (16). Macrocyclic trichothecenes also inhibit wheat coleoptile growth: verrucarin A, verrucarin J and trichoverrin B at 10^{-7} M, isororidin E and baccharinol at 10^{-6} M and roridan A at 10^{-5} M (20). Verrucarin A was 100 times as inhibitory as was abscisic acid in this assay. Although these studies suggest an interaction between trichothecenes and plant growth regulators, no direct or competitive interaction has been demonstrated.

Several metabolites of plant-pathogenic fungi cause at least indirect damage to the host plasma membrane (23,31,32,79,98). There are conflicting reports on the effect of trichothecenes on the permeability of the plasma membrane; results show electrolyte-leakage to be unaffected (86) or increased (42) by T-2 toxin.

The 12,13-epoxytrichothecenes are among the most potent low molecular weight inhibitors of protein synthesis in eukaryotic cells (63). Ueno et al (92) first demonstrated the ability of a trichothecene, nivalenol, to inhibit protein synthesis in intact rabbit reticulocytes and a cell-free system extracted from rabbit reticulocytes. There has since been considerable interest in trichothecenes as inhibitors of eukaryotic protein synthesis. Inhibition of protein synthesis in cell cultures has even been developed as an assay to detect and quantify T-2 toxin (90).

Trichothecenes block peptidyl transferase activity (3,11,12, 13) and have been divided into two groups based on whether they inhibit protein synthesis at initiation (I-types, e.g., nivalenol, T-2 toxin and verrucarin A) or elongation/termination (E- or T- types, e.g., deoxynivalenol, trichodermin, and trichothecin) (10,13,18,19,24,83). Inhibitors of elongation or termination cause buildup of polyribosomes, whereas inhibitors of initiation cause polyribosome "run-off." Some of the differences between types may, however, be concentration dependent (19,95). Substituents at C3, C4 and C15 appear to be responsible for determining whether trichothecenes are I-, E- or T-types, and for their potency (19,24,63,91); substituents at C8 also affect potency (91). Trichothecenes bind to a single mutually exclusive site on eukaryotic ribosomes (3,97). Using trichodermin resistant mutants of Saccharomyces cerevisiae (46,47,84), the trichodermin binding site was mapped to the 60S ribosomal subunit (38). The S. cerevisiae gene for resistance to trichodermin was cloned and is contained in a 3.5 kb fragment that also codes for ribosomal protein L3 (29).

This study was undertaken to demonstrate the inhibition of protein synthesis by DON and T-2 toxin in wheat and maize, hosts for Fusarium spp. which produce these trichothecenes (58,88). Demonstration of a specific mechanism of phytotoxicity may contribute to an understanding of the role of trichothecenes in plant disease.

MATERIALS AND METHODS

Materials

Human placental ribonuclease inhibitor (RNasin) and rabbit globin mRNA were from Bethesda Research Laboratories, (Gaithersburg, MD 20877); glass fiber filters (GF-C) and micrococcal nuclease (Nuclease S7) from Boehringer Mannheim Biochemicals (Indianapolis, IN 46250); peptone and yeast extract from Difco Laboratories (Detroit, MI 48232); NZ-amine, type A, from Humko Sheffield Chemical Division of Kraft, Inc. (Lafayette, NJ);L-[3,4,5-^{3}H(N)]-leucine and Protosol from New England Nuclear (Boston, MA 02118); 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]-benzene (=dimethyl-POPOP) and 2,5-diphenyloxazole (=PPO) from Research Products International Corp. (Elk Grove Village, IL 60007); carboxymethylcellulose, cycloheximide, diethyl pyrocarbonate, penicillin-G, streptomycin sulfate, and T-2 toxin from Sigma Chemical Co. (St. Louis, MO 63178); and chloramphenicol from United States Biochemical Corp. (Cleveland, OH 44128). Fungal strains and sources were *Fusarium graminearum* (R6576) [=*Gibberella zeae* (U5373)] (L. Patrick Hart, Department of Botany and Plant Pathology, Michigan State University, East Lansing 48824), *F. graminearum* (type B) (John F. Leslie, Department of Plant Pathology, Kansas State University, Manhattan 66506), *F. sporotrichioides* (T-2) (Eugene B. Smalley, Department of Plant Pathology, University of Wisconsin, Madison 53706).

Incorporation of ^{3}H-leucine into leaf disc protein.

A modification of a procedure by Gardner et al (30) was used to monitor protein synthesis in plant tissues. Ten discs (5 mm) cut from leaves with a sharp cork borer, or five longitudinal sections (2 mm thick) of developing maize kernels were swirled briefly in 20 mM Tris buffer (pH 6.5) containing 1% Tween-20, blotted dry, weighed and placed in a 20 ml scintillation vial containing 950 ul of Tris buffer with chloramphenicol (50 ug/ml). After all samples were prepared, 50 ul Tris buffer containing 1 x 10^{6} DPM ^{3}H-leucine was added to each vial and the tissue incubated at room temperature for the appropriate time. Amino acid incorporation was stopped by placing the vials in an ice bath, immediately drawing off the liquid and rinsing the discs with 5 ml ice-cold Tris buffer. The rinse buffer was discarded and 10 ml ice-cold acetone:95% ethanol (1:1) was added to each vial which was then stored overnight at 4 C. The solvent was discarded and the tissue washed by incubating in 5 ml fresh acetone:ethanol for 3 hr at 4 C, twice. Residual water was removed from the samples by placing them in 3 ml diethyl ether:absolute ethanol (1:1) for 10 min, then in 3 ml diethyl ether for 10 min, at room temperature. After the solvent was drawn off and the tissue allowed to air-dry in open vials, 0.5 ml Protosol:toluene:water (45:50:5) was added and the sealed vials incubated at 55 C for 90 min. When the vials were cool, 5 ml scintillation cocktail (11 g PPO, 0.6 g dimethyl-POPOP, 1 L toluene, 1 L

ethylene glycol monomethyl ether [methyl cellusolve]) was added to each vial, and radioactivity measured in a Beta Trac 6895 liquid scintillation counter (Tm Analytic, Elk Grove Village, IL).

Optimization and evaluation of leaf disc assay.

Several preliminary experiments were performed to demonstrate the sensitivity of the leaf disc assay to alterations in net protein synthesis and to optimize the assay conditions.

Tritium emits β-particles of energy low enough to permit significant quenching by the plant tissue used in the experiments, resulting in an underestimation of ^{3}H-leucine incorporation. The extent of quenching was determined by solublizing leaf disc protein with Protosol, as described above, prior to counting. The apparent DPM of Protosol-treated discs was compared with that of equivalent discs without Protosol treatment.

Charging of tRNA with radiolabeled amino acid could lead to an overestimation of ^{3}H-leucine incorporation into protein. This source of error can easily be eliminated because aminoacyl tRNA is sensitive to alkaline hydrolysis. To determine the magnitude of the contribution by aminoacyl tRNA, leaf discs were labelled with ^{3}H-leucine for 3 hr. The vials were placed on ice, the buffer drawn off, discs rinsed with Tris buffer and placed in 10 ml acetone:ethanol overnight, as usual. After removal of the solvent, 5ml of

1.0 M NaOH (or acetone:ethanol for controls) was added to each vial and incubated for 5 or 15 min. The NaOH solution was drawn-off and the discs rinsed with 5 ml Tris buffer. Another 5 ml acetone:ethanol was added, the discs incubated overnight, then dryed with ether:ethanol and ether.

The contribution of ^{3}H-leucine incorporation from prokaryotic sources (bacteria, chloroplasts, mitochondria) was also examined. Prokaryotes apparently are insensitive to protein synthesis inhibition by 12,13-epoxytrichothecenes (9,19,27). Incorporation of radiolabeled amino acids by prokaryotes would mask the true level of inhibition of plant protein synthesis by these compounds. Chloramphenicol, a specific inhibitor of prokaryotic (including chloroplast and mitochondrial) protein synthesis (41,50,62,94), was included to determine the significance of prokaryotic protein synthesis in the leaf disc assay.

Two criteria were used to demonstrate that incorporation of ^{3}H-leucine into acetone:ethanol insoluble material was due to protein synthesis. First, incorporation of amino acids into protein should, under optimum conditions, be linear with respect to time. Second, protein synthesis by eukaryotes should be inhibited by cycloheximide (94). Twenty vials containing 10 leaf discs each were incubated in buffer containing ^{3}H-leucine. At 30 min intervals, incorporation of radiolabeled amino acid was stopped in two replicate vials and measured as described above. Incorporation by equivalent discs incubated in buffer containing

cycloheximide (60 ug/ml) was determined at 60 min intervals.

Inhibition of protein synthesis in vivo by trichothecenes.

The sensitivity of plant protein synthesis to inhibition by trichothecenes was tested. Leaf discs were incubated in 950 ul of buffer containing T-2 toxin or DON for 60 min prior to addition of ^{3}H-leucine (0.5 uCi in 50 ul buffer). T-2 toxin, which is less hydrophilic than DON, was dissolved in methanol and diluted so that the final buffer solution contained 0.1% methanol (controls without toxin also contained 0.1% methanol). After a 1-3 hr radiolabeling period, discs were washed and measured for incorporated radioactivity, as described above.

Inbred maize lines with disease reactions to ear rot by G. zeae from resistant to highly susceptible are available (35,39). Since virulent strains of G. zeae produce DON, the correlation between susceptibility to the fungus and sensitivity to trichothecenes was examined. The sensitivity of the most susceptible (B79) and most resistant (Pa347) maize lines to T-2 toxin was tested. Six maize varieties representing the range of disease reactions were screened for sensitivity to DON. No wheat lines resistant to head scab caused by G. zeae were available, so only a single variety of wheat was tested for sensitivity to T-2 and DON.

Dynamics of toxin movement into maize leaf tissue and cells.

T-2 toxin was selected to study the movement of trichothecenes into tissue and cells and the persistence of these compounds in plant tissue. The high potency of T-2 toxin made the effects of changes in concentration observable.

One experiment was designed to determine how quickly T-2 toxin reached inhibitory levels in tissue exposed to the toxin. Ten maize leaf discs per vial were incubated in 1 ml buffer containing T-2 toxin (10 ug/ml). At intervals from 0-60 min, the toxin solution in sample vials (3 replicates) was removed, and the leaf discs rinsed with 5 ml Tris buffer. Another 5 ml fresh buffer was added and after 10 min was removed. The leaf discs were rinsed with 5 ml buffer and incubated in 1 ml buffer containing ^{3}H-leucine for 120 min. The radiolabeled leaf discs were washed and radioactivity was determined as before.

There were two experiments to determine whether or not the toxin or its inhibitory effects persisted after removal of tissue from toxin solutions. In the first of these experiments, 10 leaf discs per vial were incubated in 1 ml buffer, or buffer containing T-2 toxin (10 ug/ml) for 60 min. The leaf discs were rinsed two times, each with 5 ml fresh buffer. Then 1 ml buffer containing 0.5 uCi ^{3}H-leucine, with or without T-2 toxin (10 ug/ml), was added to each vial. Incorporation of ^{3}H-leucine by leaf discs in sample vials (3 replicates) was stopped after 0-120 min.

Discs were washed and radioactivity measured as before.

In the second experiment, the duration of recovery time in fresh buffer was varied for toxin-treated leaf discs prior to radiolabeling. Leaf discs were exposed to T-2 toxin (10 ug/ml) for 60 min, and discs washed with 5 ml buffer, twice, as in the previous experiment. Control leaf discs were treated similarly, but incubated in buffer without T-2 toxin. Each group of 10 discs was then floated on 50 ml buffer. At intervals from 0 to 120 min, sample groups (3 replicates of 10 discs each) were rinsed with 5 ml buffer, twice, and transfered to 1 ml buffer containing 0.5 ml ^{3}H-leucine. Incorporation of ^{3}H-leucine was stopped after 120 min. Leaf discs were washed and radioactivity measured as usual.

Cell-free translation system from wheat.

A cell-free translation system was extracted from wheat embryos by the method of Marcus et al (61). Wheat embryos were obtained by grinding 250 g aliquots of Ionia wheat seed in a Waring blender for approximately 5 sec, just long enough to dislodge the embryos from the endosperm. The embryos were separated from larger particles by passing the ground seed through a #30 sieve. The material passing through the sieve was collected and resifted. Bran was removed from the embryos in an air classifier. Embryos were separated from residual endosperm by floating them on 20-25% cyclohexane in carbon tetrachloride. The exact ratio was

determined by placing the air-classified mixture in carbon tetrachloride and adding cyclohexane until only embryos floated, with pieces of endosperm remaining on the bottom of the vessel.

In order to avoid contaminating ribonuclease, all solutions used to prepare cell-free translation systems were treated with 0.1% diethyl pyrocarbonate for at least 12 hr, then autoclaved prior to use; all glassware and spatulas were baked at 220 C for at least 4 hr, generally overnight. Acid-washed sand (boiled in 1 N HCl for 30 min, then washed extensively with distilled water until runoff was neutral pH) was baked at 220 C overnight. In a mortar cooled on ice, 0.3 g wheat embryos were ground to a smooth paste with 0.3 g acid-washed sand and 1 ml CKCM buffer (90 mM potassium chloride, 2 mM calcium chloride, 1 mM magnesium acetate, 6 mM potassium carbonate, pH 6.6). An additional 0.5 ml grinding buffer was added and grinding continued for several minutes. Finally another 1.8 ml grinding buffer was added and the embryos ground to a liquid. The mixture was transfered to a 16 ml polyallomer tube (which had been washed with 50% sulfuric acid, rinsed extensively, then treated with 0.1% diethyl pyrocarbonate for 12 hr) and centrifuged in a SS-34 rotor at 23,000 xg for 10 min at 0 C. The central portion was recovered, avoiding the lipid pellicle, and stored in 0.5 ml aliquots at -70 C. This extract is refered to as S23. Protein was measured by the Bradford method (6).

Dialysis tubing (Spectra/Por 2, 12-14,000 MW cutoff) was cut into 30 cm lengths and boiled in 5 mM EDTA for 15 min, twice, then rinsed well with glass distilled water and stored in 50% ethanol at -4 C. Immediately before use, 0.5 ml S23 was dialyzed against 500 ml TKM buffer (50 mM potassium acetate, 2.0 mM magnesium acetate, 4.0 mM 2-mercaptoethanol, 25 mM Tris acetate, pH 7.6 [2-mercaptoethanol and Tris must be added after autoclaving the diethyl pyrocarbonate treated solution]) for 1.75 hr. The complete reaction mixture is shown in Table 1.

Cell-free translation system from maize.

Cell-free translation systems were extracted from both maize embryos (adapting the procedure described above for wheat embryos) and seedling plumules.

Maize embryos were dislodged from kernels by brief grinding in an Omnimixer. The embryos were collected, removing as much of the adhering fragments of endosperm and pericarp as possible. Gloves were worn during this operation to reduce contamination from RNases. In a mortar chilled on ice, 1 g aliquots of embryos were ground to a paste in 1.5 ml HKCM buffer (0.1 M potassium chloride, 2.0 mM calcium chloride, 1.0 mM magnesium acetate, 40 uM spermine, 20 mM Hepes, pH 7.6) with 1 g acid-washed sand. With continued grinding, an additional 3 ml grinding buffer was gradually added in 1 ml increments. When thoroughly ground to a liquid, the mixture was centrifuged at 30,000 x g for

10 min at 2 C. The central portion of the supernatant was recovered, avoiding the lipid pellicle. This extract is referred to as S30. Maize embryos were also extracted with TSM buffer (0.45 M sucrose, 0.5 mM magnesium acetate, 50 mM Tris acetate, pH 7.5) in a similar manner.

A cell-free translation system was extracted from maize seedling plumules by the method of Mans and Novelli (57). Maize kernels were washed thoroughly in distilled water, then soaked in 50% ethanol for 2 min. The kernels were soaked in distilled water for 6 hr, then spread out between moist paper towels and incubated in the dark for 48 hr. Plumules were excised from germinated kernels and placed in 25 mM Tris-acetate (pH 6.5) until all plumules were collected. In a mortar chilled on ice, 250-300 plumules were ground in 5 ml TSM buffer with 2 g acid-washed sand. The mixture was centrifuged and the supernatant collected as for the embryo preparation.

Some plumule extracts were treated with a calcium-dependent micrococcal nuclease to degrade endogenous mRNA (72). To 0.5 ml S30 was added 2.5 ul of 50 mM calcium carbonate (2 mM final concentration) and 3.3 ul micrococcal nuclease (15,000 units/ml in 50 mM glycine, 5 mM calcium carbonate, pH 9.2) (100 units/ml final concentration). The mixture was incubated at 20 C for 20 min. Then 10 ul of 100 mM EGTA (ethyleneglycol-bis-[β-aminoethyl ether]-N,N,N',N'-tetraacetic acid) (pH 7.0) (2 mM final concentration) was added to chelate Ca^{++}, thereby inactivating the nuclease.

Both embryo and plumule S30 fractions were dialyzed in TKM buffer prior to use. The reaction conditions for translation were similar to the wheat embryo cell-free system (Table 1), with optimization for K^+ and Mg^{++} discussed in the Results section.

Measurement of amino acid incorporation by cell-free translation systems.

Four methods were compared for efficiency in freeing precipitated protein from unincorporated ^{3}H-leucine. In method 1 (61), 100 ul ice-cold 0.5% bovine serum albumin containing 10 mM cold leucine was added to 50 ul reaction mixture and kept at 0 C for 10 min. Then 1 ml of 10 % trichloroacetic acid (TCA) was added and the mixture incubated at 90 C for 15 min, then at 0 C for 15 min prior to collection of precipitate on GF-C filters. The precipitate was washed with 250 ul of 10% TCA, 10 mM leucine 10 times, then 250 ul ether:ethanol (1:1) 2 times, and finally 250 ul ether 2 times. The filters were air dryed, placed in scintillation vials and prior to counting, digested with Protosol as described above for leaf discs.

For the second method (14), 0.5 ml ice-cold distilled water, followed by 0.5 ml 1.0 N NaOH was added to each 50 ul reaction mixture, which was then incubated at 37 C for 20 min. After adding 263 ul of 50% TCA (to a final concentration of 10% TCA), the mixture incubated at 0 C for 30 min prior to collection and washing of precipitate as in method

1.

For the filter disc method (14,56), 20 ul of the reaction mixtures were spotted on GF-C filters (method 3) or Whatman #1 filters (method 4) which were then swirled gently (10-20 discs together) in 400 ml of 10% TCA at 0 C for 15 min, then 100 ml of 10% TCA at 90 C for 15 min, and finally 500 ml of 10% TCA at room temperature for 15 min. The washed discs were dehydrated by swirling in 100 ml absolute ethanol for 1 min, then 100 ml acetone for 1 min. The discs were placed on aluminum foil to air-dry, then transfered to scintillation vials, treated with Protosol and counted.

Extraction of RNA from Fusarium graminearum.

Fusarium graminearum (R6576) was grown in 50 ml shake cultures in CMC medium modified by the substitution of NZ-amine for yeast extract (15 g carboxymethylcellulose, 1 g NZ-amine, 1 g ammonium nitrate, 1 g potassium phosphate-monobasic, 0.5 g magnesium sulfate-7 hydrate, 1000 ml distilled water) at room temperature. Macroconidia were harvested after 1 week by filtering the suspension through 4 layers of cheesecloth, and centrifuging at 1000 xg x 10 min at room temperature. The conidia were washed with one volume fresh distilled water and recentrifuged, three times. Macroconidia were germinated at 7 x 10^5 conidia/ml in 50 ml of YPG medium (10 g glucose, 5 g peptone, 5 g yeast extract, 1000 ml distilled water; penicillin and streptomycin [each at a final concentration of 100 ug/ml] were added after the

medium was autoclaved) in a 1000 ml Erlenmyer flask at 200 rpm on a rotary shaker (New Brunswick Scientific Co., New Brunswick, N.J.) at room temperature. There was nearly 100% germination after 5.5 hr, and the germ tubes were approximately the length of a macroconidium.

Germlings (5.5 hr old) were harvested by centrifuging and washed as described above. Germlings were ground to a powder in liquid N_2 with a mortar and pestle. Total RNA was extracted by a modification of published methods (52). RNA-extraction buffer (50 mM sodium acetate, pH 5.0, 1 mM EDTA [ethylenediamintetraacetic acid], 1% sodium dodecylsulfate) warmed to 65 C. The ground fungus was quickly suspended, and immediately 5 ml phenol (saturated with 50 mM sodium acetate, pH 5.0, 1 mM EDTA) warmed to 65 C. The resuspended fungus was extracted for 15 min at 65 C (temperature is critical) with vigorous shaking. The aqueous phase was recovered and reextracted with phenol. The phenolic phase (first extract) was re-extracted with RNA-extraction buffer, and all aqueous phases combined. The pooled aqueous phase was extracted with one volume phenol:chloroform:isoamyl alcohol (25:24:1) (saturated with 50 mM sodium acetate, pH 5.0, 1mM EDTA) at room temperature. The aqueous phase was recovered and extracted with one volume diethyl ether. This aqueous phase was removed and chilled in an ice bath, and 1/4 volume of 2 M sodium acetate (ph 5.0) added. The RNA was precipitated by the addition of 2.5 volumes absolute ethanol and incubation at -20 C, overnight. RNA was pel-

leted at 10,000 xg x 10 min at 4 C. The pellet was washed with 10 ml cold 70% ethanol, 0.4 M sodium acetate, pH 5.0, and recentrifuged. The pellet was dissolved in 1 ml sterile distilled water and dispensed in 50 ul aliquots into 1500 ul microcentrifuge (Eppendorf) tubes in a dry ice-acetone bath. The frozen aliquots were stored at -70 C. RNA was quantified spectrophotometrically at 260 nm (1 A_{260} unit=40 ug RNA/ml) (52).

Inhibition of cell-free translation systems by trichothecenes.

Cell-free translation systems from wheat and maize were used to demonstrate that inhibition of ^{3}H-leucine incorporation into TCA-precipitated material by trichothecenes was due to a direct effect upon the protein synthetic mechanism. Aqueous DON was added to translation systems and incubated for 45 min at 28 C; other reaction conditions were as described.

Effect of trichothecenes on protein synthesis in Fusarium germlings.

Fusarium graminearum (R6576), F. graminearum (type B) and F. sporotrichioides (T-2) were grown in CMC medium and harvested at described above. The washed, pelleted conidia were suspended in distilled water and counted with a hemacytometer (American Optical Corporation, Buffalo, NY 14215). Conidia were germinated at 2 x 10^{6} conidia /ml in

50 ml of GS medium (10 g glucose, 1 g ammonium nitrate, 1 g potassium phosphate-monobasic, 0.5 g magnesium sulfate-7 hydrate, 1000 ml distilled water, adjusted to pH 6.2 with potassium hydroxide; penicillin and streptomycin [each at a final concentration of 100 ug/ml] were added after the medium was autoclaved) in a 1000 ml Erlenmyer flask at 200 rpm on a rotary shaker at room temperature. After 5 hr, there was nearly 100% germination and germ tubes were approximately the length of a macroconidium. At this time, 1 ml aliquots of the germling suspension was dispensed to 20 ml scintillation vials. DON (aqueous), T-2 toxin (5.5% methanol), cycloheximide (aqueous) or the appropriate control solution (50 ul) was added to the germling suspensions. After 10 min, 50 ul ^{3}H-leucine was added to a final concentration of 1 uCi/ml. The suspensions were incubated on a rotary shaker for 15 min at room temperature.

After labelling, 400 ul of the germling suspension was placed in a 1500 ul microcentrifuge tube with 400 ul of 20% TCA, and heated at 90 C for 30 min. The tubes were then placed in an ice bath for 30 min. The germlings were collected on a GF-C filter and washed 15 times with 250 ul of ice-cold 10% TCA (the first 5 washed were also used to rinse the vial). The washed germlings were dried first with 2 x 250 ul diethyl ether:ethanol (1:1), then 2 x 250 ul diethyl ether. After air-drying, the discs were placed in clean scintillation vials with 0.5 ml Protosol mix (see leaf disc preparation). The vials were incubated at 55 C for 90 min,

and when cool, 10 ml scintillation cocktail added and the samples counted.

RESULTS

Incorporation of amino acids by leaf discs.

There was significant quenching of radioactivity by leaf tissue. Radiolabeled leaf discs in toluene-based scintillation cocktail had 3.2 times the apparent DPM for discs solubilized with Protosol (1491 DPM/mg fr wt) as for equivalent discs without Protosol treatment. Solubilization of tissue with Protosol was, therefore, routinely included in the preparation of leaf discs to increase the efficiency of counting.

The reduction in radioactivity of leaf discs by hydrolysis of aminoacyl-tRNA with 1.0 N NaOH was not statistically significant (Table 2). In addition, leaf discs treated with NaOH retained chlorophyll after washing, which added false counts due to chemiluminescence. Consequently, leaf discs were not treated with NaOH as part of the preparation for counting.

Chloramphenicol reduced ^{3}H-leucine incorporation up to 27% (Figure 1). In order to minimize the contribution to net protein synthesis from prokaryotic sources (bacterial contaminants and organelles), chloramphenicol (100 ug/ml) was routinely included in leaf disc labelling buffer.

Incorporation of ^{3}H-leucine into acetone:ethanol insoluble material by leaf discs was linear for at least 5 hr (Figure 2). Cycloheximide (60 ug/ml) reduced by 80% the amount of ^{3}H-leucine incorporated, demonstrating that a

major portion of the incorporation was due to plant protein synthesis.

Inhibition of protein synthesis in leaf discs by trichothecenes.

Protein synthesis in leaf discs was inhibited by both trichothecenes tested. The concentration of T-2 toxin required to inhibit 50% of ^{3}H-leucine incorporation (ID_{50}) was 261, 919 and 919 nM (1 nM=467 pg/ml) for Ionia wheat, Pa347 maize and B79 maize, respectively (Figure 3). ID_{50} values were determined from linear regressions from 2.1 nM to 21 uM T-2 toxin; r^2=.880 (Ionia), .919 (Pa347) and .952 (B79). There was no significant difference between B79 maize leaf discs and kernel sections in sensitivity of protein synthesis to T-2 toxin (Table 3).

DON was less effective than T-2 toxin at inhibiting protein synthesis. ID_{50} of DON was 9.2, 11.0, 15.1, 21.6, 10.3, and 14.8 uM (1 uM=296 ng/ml) for maize lines B79, Mo17, B73, A509, MS74, and Pa347, respectively (Figure 4). The only significantly different ID_{50} at α=.05 were for B79 and A509. ID_{50} values were determined from linear regressions from 1 to 34 uM DON; r2=.946 (B79), .994 (Mo17), .990 (B73), .946 (A509), .994 (MS74) and .983 (Pa347). Susceptibility to F. graminearum ear rot was not correlated with sensitivity to DON for all maize lines, but A509, a moderately resistant variety, was less sensitive to DON than were the other varieties (Figure 5). ID_{50} of DON for Ionia

wheat leaf discs was 4.5 uM (r^2=.931) (Figure 6).

Dynamics of toxin movement into maize tissue and cells.

T-2 toxin entered leaf tissue and cells quickly. Near-maximum inhibition of ^{3}H-leucine incorporation was achieved after 5 min exposure to T-2 toxin (Figure 7).

The rapid uptake of toxin was in contrast to the persistence of the toxin or toxic effects. Inhibition of ^{3}H-leucine incorporation in toxin-treated leaf discs was equal for discs radiolabeled in the presence of T-2 toxin and discs radiolabeled in the absence of T-2 toxin (Figure 8). Even when toxin-treated leaf discs were incubated in fresh buffer to remove toxin prior to radiolabeling, ^{3}H-leucine incorporation increased only 22% when leaf discs were removed from toxin solution for 60 min; there was no further increase for recovery periods up to 120 min (Figure 9).

Measurement of radiolabeled protein from cell-free translation systems.

The filter disc methods (methods 3 and 4) collected precipitated, radiolabeled protein more efficiently than the other two methods. The efficiency was determined by the difference in TCA-precipitated radioactivity of samples from reactions with and without added mRNA (Figure 10). High radioactivity in samples from reactions with added mRNA (+RNA) incicates high recovery of precipitated protein, and low radioactivity in samples from reactions without added

mRNA (-RNA) indicates efficient removal of unincorporated ^{3}H-leucine. Precipitation method 1 produced a flocculent precipitate of relatively large particle size during heating in 10% TCA at 90 C. The precipitate may have physically trapped unincorporated ^{3}H-leucine and caused the high level of radioactivity in the -mRNA treatment. This reduced the apparent increase in ^{3}H-leucine incorporation with the addition of exogenous mRNA (Figure 10).

Although the filter paper discs were more resistant to rough handling, glass fiber discs (GF-C) recovered slightly more precipitated protein. GF-C filters were used in all subsequent experiments, and survived washing treatments well if handled gently.

Cell-free translation system from wheat.

The yield of wheat embryos from whole seed was 0.6% by weight. From 3.6 g embryos, 32.5 ml of 23,000 xg supernatant (S23) containing 10.5 mg protein/ml was obtained. The concentrations of components for translation are listed in Table 1. The optimum concentrations of Mg^{++} and K^{+} were 3 mM and 140 mM, respectively (Figures 11 and 12).

Without added mRNA, the wheat cell-free system incorporated ^{3}H-leucine, and the incorporation was inhibited by cycloheximide (Table 4). This indicates the S23 preparation from wheat embryos contained low, but detectable, endogenous mRNA. However, the addition of rabbit globin mRNA (complete

system) increased the incorporation of ^{3}H-leucine (Table 4). There was an increase in ^{3}H-leucine incorporation upon addition of human placental ribonuclease inhibitor (RNasin) (80), demonstrating contamination of the wheat embryo system by ribonucleases (Table 4). RNasin was, therefore, routinely included in all remaining cell-free translation reactions to reduce degradation of mRNA.

RNA-dependent incorporation of ^{3}H-leucine was linear for 45 min, but then slowed (Figure 13). Subsequent reactions were, therefore, terminated after 45 min. The wheat embryo system also translated total RNA from Fusarium graminearum germlings, and the amount of ^{3}H-leucine incorporated was directly proportional to the amount of RNA added up to 93 ug/ml (Figure 14). Above this optimum concentration, ^{3}H-leucine incorporation decreased with increasing the concentration of Fusarium total RNA (to at least 350 ug/ml), suggesting the presence of an endogenous inhibitor of protein synthesis in the total RNA preparation. The yield from 2.8 x 10^{6} macroconidia germinated for 5.5 hr was 1.4 mg total RNA.

Cell-free translation system from maize.

The cell-free system extracted from maize embryos did not actively incorporate ^{3}H-leucine. However, a cell-free amino acid-incorporating system was prepared from the plumules of 2-day old maize seedlings. The yield from 250-300 plumules was 6 ml of 30,000 xg supernatant (S30) con-

taining 7.0 mg protein/ml. The reaction conditions were similar to the wheat embryo cell-free system (Table 1), but the optimum concentrations of Mg^{++} and K^{+} were different: 7.5 mM Mg^{++} and 200 mM K^{+} for maize inbred line Pa347, 7.5 mM Mg^{++} and 125 mM K^{+} for B79, 10 mM Mg^{++} and 150 mM K^{+} for A509 (Figures 15,16). Extracts from inbred lines B79 and A509 were not as active as Pa347 extracts, and the differences in the responses of B79 and A509 cell-free systems to varying concentrations of Mg^{++} and K^{+} were not as dramatic as for the Pa347 cell-free system. The concentration of S30 in the maize reaction mixture was 3.0 mg/ml. Unlike the wheat embryo system, the maize cell-free system was not only independent of exogenous mRNA, but added rabbit globin mRNA did not stimulate incorporation of ^{3}H-leucine. Maize S30 treated with micrococcal nuclease (to degrade endogenous mRNA) still did not respond to exogenous mRNA.

Inhibition of cell-free translation systems by trichothecenes.

Incorporation of ^{3}H-leucine by cell-free system from wheat and maize was inhibited by DON, indicating a direct effect on the protein synthesizing components. The cell-free translation system from Ionia wheat embryos was somewhat less sensitive to inhibition by DON (ID_{50}=12.3 uM) than were Ionia wheat leaf discs (ID_{50}=5.6 uM) (Figure 6). However, there was no significant difference in sensitivity to DON between Pa347 maize cell-free translation system

(ID_{50}=9.7) and Pa347 maize leaf discs (ID_{50}=14.8), r^2=.962 (Figure 17). Cell-free translation systems from B79 and A509 maize incorporated ^{3}H-leucine less actively than did Pa347 system, making precise determination of ID_{50} difficult. The B79 system incorporated 7.0 times more ^{3}H-leucine than did the preparation containing cycloheximide (384 ug/ml); ID_{50} of DON was 22.1 uM (from regression analysis, r^2=.847) (Figure 18). When DPM are adjusted by subtracting the DPM for cycloheximide reactions (assuming this represents background DPM due to ^{3}H-leucine not incorporated into protein), the ID_{50} is 10.7 uM, compared to 9.2 uM for B79 leaf discs. The A509 system incorporated only 2.9 times more ^{3}H-leucine than did the preparation containing cycloheximide; ID_{50} was 108 uM (r^2=.922) (Figure 19). When the DPM for the cell-free system are adjusted by subtracting DPM for reactions containing cycloheximide, the ID_{50}=3.4 uM, compared to 21.6 uM for A509 leaf discs.

Effect of trichothecenes on protein synthesis in Fusarium germlings.

Sensitivity of protein synthesis in 5 hr-old germlings varied with the Fusarium strain, and was correlated with the ability of the fungus to produce trichothecenes. F. graminearum (R6576), a DON-producer, was not sensitive to DON up to 50 ug/ml, whereas protein synthesis in F. graminearum (type B), a non-trichothecene-producer, was inhibited 31.5% and 41.0% by DON at 1 and 50 ug/ml, respec-

tively (Table 5).

There was a similar response to T-2 toxin. Protein synthesis in F. graminearum (R6576) (DON-producer) and F. sporotrichioides (T-2 toxin-producer) exposed to T-2 toxin (up to 50 ug/ml) was not significantly different than controls (Table 6). Protein synthesis by F. graminearum (type B), a non-trichothecene-producer, was inhibited 65.5 and 83.5%, respectively, by T-2 toxin at 1 and 50 ug/ml.

DISCUSSION

Several reviews deal with pathogen-produced compounds that are toxic to plants and are determinants of plant disease (22,68,82,100). Gaumann (34) stated: "Micro-organisms are pathogenic only if they are toxigenic; in other words, the agents responsible for diseases can damage their hosts only if they form toxins -- microbial poisons -- that penetrate into the host's tissues." However, Gaumann's broad definition of toxins would encompass all known or hypothetical molecular disease determinants of pathogen origin. A more specific and useful definition of toxin is given by Scheffer (81) and includes compounds that: a) are low-molecular weight products of microbial pathogens, b) cause obvious damage to plant tissues, and c) are known with confidence to be involved in disease development. A number of low-molecular weight compounds which are toxic to plants have been isolated from cultures of *Fusarium* spp.: naphthazarins (fusarubin, marticin, isomarticin, javanicin, norjavanicin, and novarubin) from *F. solani*, fusaric acid and lycomarasmin from *F. oxysporum*, enniatins from several *Fusarium* spp. (49,81), and trichothecenes. However, none of these phytotoxic compounds have yet been demonstrated to be involved in disease development (81). In experiments described herein, T-2 toxin and DON entered wheat and maize cells readily, and were potent inhibitors of protein synthesis in cell-free systems isolated from these plants.

They were effective at concentrations much lower than occur in infected tissue. However, their role in disease development remains uncertain.

There have been few attempts at demonstrating the involvment of trichothecenes in plant disease, and these have not clarified the situation. Brian et al (7) thought it probable that F. equiseti produces phytotoxic compounds in plant tissue, although they found "little suggestion that any of the characteristic symptoms of the diseases which it causes are toxigenically induced." Manka et al (53) attempted to show correlations between virulence and trichothecene production. Fusarium species highly virulent on wheat, rye and triticale (F. graminearum and F. culmorum) produced deoxynivalenol and/or 15-acetyl-deoxynivalenol on wheat kernels in culture, whereas avirulent or mildly virulent species did not produce the compounds. However, within species, some avirulent isolates produced the trichothecenes, and some virulent isolates did not. In another report (87) differences in virulence among 3 pathogenic isolates of F. acuminatum and F. avenaceum on red clover roots were not correlated with inhibition of lettuce seed germination or radicle elongation (lettuce and red clover) by culture filtrates of the Fusarium isolates. However, a fourth isolate (F. avenaceum) was not pathogenic, and its culture filtrates were not phytotoxic. No compounds were isolated from the culture filtrates, however, and only these four isolates were tested.

All of these studies have used different Fusarium isolates (or even species) to evaluate the role of trichothecenes in plant disease. Obviously, these isolates and species differ in characteristics other than trichothecene production. Evaluation of factors affecting virulence is based on quantitative data, and the results are less explicit than for pathogenicity factors. The use of genetically diverse isolates only adds to the difficulty of obtaining unequivocal results. The role of trichothecenes could most effectively be tested using near-isogenic mutants of toxigenic, pathogenic fusaria which have lost the ability to produce toxin. This approach has been successful with other toxins (17,71,89,101). Non-toxin-producing mutants should be of lower virulence than the toxin-producing wild-type, and toxin added to sites on host plants inoculated with these mutants should increase virulence of the fungus. Such toxin-less mutants of F. graminearum were unavailable at the time of this study. The available avirulent, non-toxin-producing strain (type B) was sensitive to protein synthesis inhibition by DON and T-2 toxin (Tables 5,6), and therefore unsuitable for this experiment. The experiment described should be performed when suitable near-isogenic, non-toxin-producing mutants become available.

Protein synthesis in wheat and maize, as measured by incorporation of ^{3}H-leucine, was inhibited by T-2 toxin (Figure 3) and DON (Figures 4,6,17,18,19). While leaf disc experiments showed that these compounds inhibited ^{3}H-leucine

incorporation by intact tissue (Figures 4,6), this approach was incapable of demonstrating a direct action on protein synthesis. Decreased incorporation of ^{3}H-leucine by leaf discs may have been due to reduced uptake of the radiolabeled amino acid, altered amino acid pools or toxic effects on other cellular components. The inhibition of cell-free translation systems, however, indicated that the toxins acted directly on the protein synthetic mechanism. The somewhat higher sensitivity of wheat leaf discs to DON compared with the cell-free system from wheat (Figure 6) may indicate an additional site of action in the cell, or perhaps DON was being concentrated within the intact cells by active transport. Leaf discs from maize inbred Pa347, however, did not show this increased sensitivity compared to the cell-free system from maize (Figure 18). Precise ID_{50} values for cell-free systems from maize inbreds B79 and A509 were difficult to obtain due to their lower activity compared to preparations from Pa347. However, assuming the true regression line for these inbreds lies somewhere between the "cell-free" and "cell-free (adjusted)" lines (Figures 17,19), the sensitivity of the cell-free translation systems to DON may be similar to that of protein synthesis in leaf discs.

Uptake of T-2 toxin by leaf tissue was rapid (Figure 7) and the toxin, or its effects, persisted in tissue removed from the toxin and placed in fresh buffer (Figures 8,9). This is also consistent with an active transport

mechanism, although further evaluation is required. There is no evidence against the rapid uptake of toxin being due to simple diffusion into the cell. The persistence of toxic effects in cells could be a result of tight binding of the toxin to the active site or by irreversible damage to the cell by the toxin.

The concentrations of T-2 toxin and DON that inhibit protein synthesis in wheat and maize were comparable or, in some cases, higher than concentrations reported for other systems. ID_{50} of T-2 toxin for wheat and maize leaf discs were 122 and 429 ng/ml, respectively (Figure 3). The ID_{40} of T-2 toxin for intact human HeLa cells was 100 ng/ml (19) and the ID_{45} for reticulocyte cell-free system was 100 ng/ml (95). The ID_{50} for intact rat spleen lymphocytes and Vero cells were 3.0 and 6.7 ng/ml, respectively (91). The ID_{50} of DON for wheat was 1.3 ug/ml (leaf discs) and 3.3 ug/ml (cell-free) (Figure 6), and for maize inbreds 2.7-6.4 ug/ml (leaf disc) and 2.9 ug/ml (cell-free) (Figures 17-19). The ID_{50} of DON for intact rat spleen lymphocytes and Vero cells were 252 and 444 ng/ml, respectively (91). It is notable that lymphocytes and Vero cells were most sensitive to T-2 toxin, as well (there is no available data on protein synthesis of other cell types for DON). Protein synthesis in Ionia wheat is inhibited by these trichothecenes, although there are previous reports of the relative insensitivity of wheat to trichothecenes (7,48). However, these previous studies examined gross effects (chlorosis, necrosis,

stunting) of trichothecenes, did not measure effects on protein synthesis, and used different wheat varieties.

Inhibition of protein sythesis could explain reduced growth and elongation of plants and plant organs (7,16,20,25,75). Normal growth could obviously not occur without active protein synthesis. There is one report (86) that cycloheximide, an inhibitor of protein synthesis, inhibited auxin-promoted elongation of soybean hypocotyls as did the trichothecenes tested. Perhaps the arresting of cells at metaphase (51) is the result of inhibition of tubulin synthesis, or synthesis of other proteins required for mitosis.

The concentrations of DON that inhibited protein synthesis in wheat and maize are well below those that occur in infected plants. Up to 370 ug DON/g grain have been reported in infected maize kernels (39,67), although the toxin may build up after colonization and death of the plant cells. Data on toxin concentrations adjacent to and in advance of invading hyphae is lacking. This region is important in evaluating trichothecenes as disease determinants, since it is here that resistance or susceptibility is expressed. Toxin concentrations in this localized area would be expected to be much higher than in the surrounding tissues.

If all maize inbreds are considered, there is no correlation between sensitivity to DON or T-2 toxin and susceptibility to a DON-producing Fusarium strain (Figures 3,5).

However, resistance in the most disease-resistant lines (Pa347 and MS74) may be independent of sensitivity to DON. Since the fungus grows very little, if at all, in these maize lines, the production of toxin may not become a factor in virulence. However, in less resistant inbreds (lacking the high-level resistance mechanism) DON may be a factor in disease development. If the highly resistant inbreds Pa347 and MS74 are ignored, there is apparently a good correlation between DON sensitivity and susceptibility to the fungus (Figure 5). However, the only significantly different ID_{50} of DON ($\alpha = .05$) were for B79 and A509 (Figure 4), so these may be the only two lines tested for which differences in susceptibility are based on differences in sensitivity to DON. Any of these explanations require multiple resistance mechanisms in the host.

Leaf tissue was used for *in vivo* experiments because it is easily produced in the greenhouse and because it readily takes up radiolabeled amino acids and incorporates them into protein. Although kernels are more important tissues for colonization of toxigenic *Fusarium* spp., it is more difficult to produce and maintain a supply of young kernels in the greenhouse. Also, kernels must be sectioned for uniform uptake of radiolabeled amino acids, and this probably damages the tissue more than does cutting leaf discs. It was shown, however, that protein synthesis in maize kernels was inhibited at the same toxin levels as in leaf tissue (Table 3). Also, since it is unlikely that leaf

ribosomes differ from kernel ribosomes, leaf tissue was more suitable for these studies on inhibition of protein synthesis. Incorporation of ^{3}H-leucine by leaf discs was linear as a function of time and was inhibited by cycloheximide (Figure 2), indicating that the system indeed measured protein synthesis. There was a significant contribution to overall protein synthesis from prokaryotic sources (bacteria, chloroplasts, mitochondria), but this was reduced by the inclusion of chloramphenicol (Figure 1).

Cell-free translation systems have been derived from pea seedlings (73,96), maize kernels (74), maize seedling plumules (55), tissue cultures of maize (37),wheat embryo (61), commercial wheat germ (60,77), rabbit reticulocytes (2), and Saccharomyces cerevisiae (33). Mans (54) reviewed the early studies on protein synthesis in plants, most of which utilized cell-free systems. In the studies described herein, cell-free extracts used for in vitro translation contained ribosomes and factors (69) required for protein synthesis; cofactors and an energy generating system were added exogenously. The wheat embryo translation system was low in endogenous mRNA, but was stimmulated by added mRNA (Figures 13,14). A cell-free translating system was successfully extracted from maize seedling plumules, but not from maize embryos. While embryos are expected to be low in mRNA, the young, actively growing seedling tip should contain high levels of mRNA. As expected, the cell-free system from maize plumules did not require exogenous mRNA for in-

corporation of ^{3}H-leucine into protein. In fact, the plumule system was not stimulated by added mRNA, perhaps because the system was saturated with endogenous mRNA. Treatment with micrococcal nuclease to degrade endogenous mRNA did not, however, make the system responsive to exogenous mRNA. This suggests some preferential utilization of endogenous mRNA. Alternatively, the nuclease treatment may not have sufficiently reduced endogenous mRNA levels. The abundant endogenous mRNA in the plumule system would, however, provide a way to examine inhibition of translation of specific plant mRNA's by comparing proteins synthesized in the presence and in the absence of inhibitors.

Based on present knowledge, one can speculate as to possible roles for trichothecenes in plant disease. Several proposed plant defense mechanisms involve the induction of enzymes through interaction with a pathogen. Inhibition of protein synthesis by trichothecenes released by the pathogen might prevent or delay these host defenses so as to facilitate infection by the pathogen. The action of trichothecenes need not be this specific, however. Protein synthesis is, of course, required for general maintainence of the cell. Disruption of protein synthesis would decrease viability of host cells and increase susceptibility to the fungus. Miller et al (64,65,66,67) suggested that fusarium head blight-resistant wheat cultivars suppressed the production of DON, or detoxified it, more effectively than susceptible cultivars. They offer this as a possible mechanism of

resistance, although strong evidence is lacking. It is not clear, for example, that the reported degradation of DON by plant extracts is actually occurring in the intact plant.

Toxins may be divided into host-selective (host-specific), those toxic only to hosts of the fungus that produced the toxin; and non-specific, those toxic to many plants whether or not they are hosts of the toxin-producing microorganism (82). Trichothecenes are clearly not host-selective, since they are toxic to animals, fungi and non-host plants. If trichothecenes are, in fact, disease determinants, they would be toxins of the non-specific type.

Toxins may also be classified as pathogenicity factors or virulence factors (100). Pathogenicity is the ability of a microorganism to induce disease; virulence is the relative severity of disease induced by a specific microorganism on a specified host (81,100). Trichothecenes do not appear to be pathogenicity factors, since non-trichothecene-producing strains can also infect and cause disease (53). If they are involved in disease, they would likely be virulence factors.

Finally, trichothecenes as inhibitors of protein synthesis in plants and animals may have no selective advantage or ecological role, but merely be the result of the conservation of ribosomal components among eukaryotes. Selection for trichothecene production in fusaria and other fungi may be based on competition with other fungi for substrate. These compounds have been shown to inhibit spore germination, growth and sporulation in many fungi studied

(9,25,27,70,75). Release of trichothecenes into the environment might give the producing fungus an advantage in substrate colonization by suppressing competing fungi. Two toxigenic fusaria were insensitive (or have much reduced sensitivity) to the DON and T-2 toxin, while a non-toxigenic strain was sensitive to both compounds (Tables 5,6). This phenomenon should be examined further to determine if, in general, toxigenic fusaria are resistant to trichothecenes while non-toxigenic fusaria are sensitive, and what the mechanism of the resistance is. Resistance to trichothecenes by a trichothecene-producing fungus has been reported previously: Myrothecium verrucaria has 60S ribosomal subunits which are not subject to inhibition by T-2 toxin (40). The toxigenic fusaria may also have resistant 60S ribosomal subunits, or, alternatively, exclude, compartmentalize or detoxify trichothecenes. Most interesting is that resistance may be species or even strain specific. Resistance to an antibiotic is obviously of great benefit to the producing organism in competition for substrate.

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Table 1. Reaction conditions for cell-free translation system from wheat embryos.

Component	Concentration
S23 (dialyzed)	3 mg protein/ml
Potassium acetate	140 mM
Magnesium acetate	3.0 mM
Amino acids[1]	100 uM
Creatine phosphate	11 mM
Creatine phosphokinase	55 ug/ml
ATP	1.4 mM
GTP	250 uM
Dithiothreitol	3 mM
^{3}H-leucine	50 uCi/ml
RNasin	150 units/ml
mRNA	0-5 ug/ml

[1]Includes 19 protein amino acids minus labeled amino acid.

Table 2. Hydrolysis of aminoacyl-tRNA in Pa347 maize leaf discs labelled with ^{3}H-leucine for 3 hr, treated with 1.0 N NaOH, then washed with acetone:ethanol (1:1) as described in text.

Duration of NaOH treatment (min)	DPM/mg fr wt
0	1348[1]
5	1202
15	1195

[1]Means not significantly different at α=.05 ($F_{(2,6)}$=2.69).

Table 3. Inhibition of protein synthesis in B79 maize leaf discs and kernel sections by T-2 toxin and cycloheximide.

	DPM (% of control)		
Tissue	T-2 toxin (2.1 uM)	T-2 toxin (21 uM)	Cycloheximide (178 uM)
Leaf Discs	35.6 a[1]	22.4 bc	16.2 c
Kernel Sections	28.0 ab	25.1 abc	20.4 bc

[1]Means with a common letter are not significantly different by Tuckey's procedure ($w_{.05}$=11.26).

Table 4. Effects of human placental ribonuclease inhibitor (RNasin) (190 units/ml) and cycloheximide (100 ug/ml) on wheat embryo translation system. The complete reaction mixture is shown in Table 1.

Reaction mixture	DPM
Complete	7,844 a[1]
- mRNA	3,072 b
+ cycloheximide (50 ug/ml)	1,294 c
+ RNasin (190 units/ml)	10,949 d
+ cycloheximide + RNasin	1,339 c

[1]Means with a common letter are not significantly different by Tuckey's procedure ($w_{.05}$=1689).

Table 5. Incorporation of ^{3}H-leucine into trichloroacetic acid-precipitated material by *Fusarium* germlings exposed to deoxynivalenol (DON).

	DPM (% of control)			
		DON (ug/ml)		Cycloheximide
Fungus	Control	1	50	(100 ug/ml)
F. graminearum (R6576)	100.0 a[1]	107.5 a	93.5 a	5.5 c
F. graminearum (type B)	100.0 a	68.5 b	59.0 b	6.0 c

[1]Means with a common letter are not significantly different at α=.05 by Duncan's Multiple Range Test.

Table 6. Incorporation of ^{3}H-leucine into trichloroacetic acid-precipitated material by *Fusarium* germlings exposed to T-2 toxin.

	DPM (% of control)			
		T-2 toxin (ug/ml)		Cycloheximide
Fungus	Control	1	50	(100 ug/ml)
F. graminearum (R6576)	100.0 a[1]	92.5 a	89.5 a	2.5 b
F. graminearum (type B)	100.0 a	34.5 b	16.5 b	5.0 b
F. sporotrichioides (T-2)	100.0 a	104.0 a	89.0 a	2.0 b

[1]Means with a common letter are not significantly different at α=.05 by Duncan's Multiple Range Test.

Figure 1. Inhibition of ^{3}H-leucine incorporation into acetone:ethanol insoluble material by chloramphenicol in leaf disc assay.

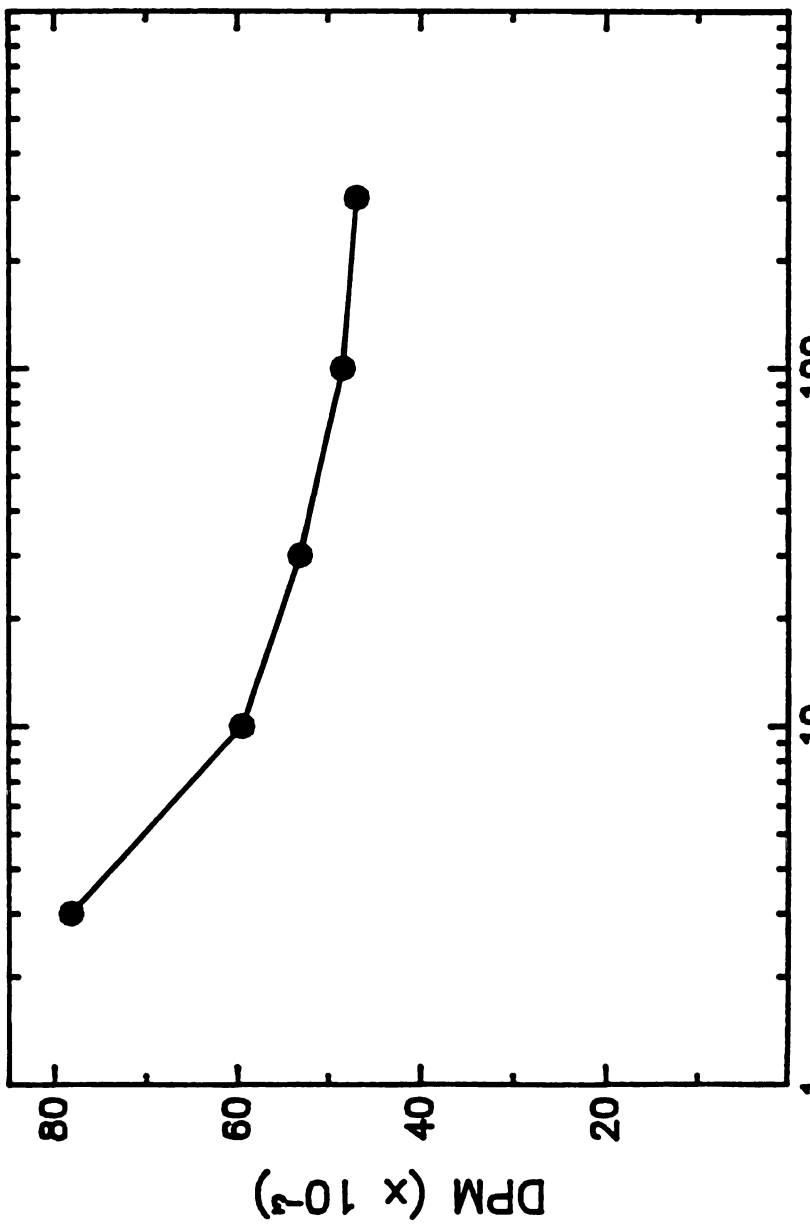
DPM (x 10⁻²)
80
60
40
20
1
10
100
Chloramphenicol concentration (μg/ml)

Figure 1.

Figure 2. Incorporation of ^{3}H-leucine into acetone:ethanol insoluble material by maize leaf discs. Some discs were incubated in the presence of cycloheximide (60 ug/ml).

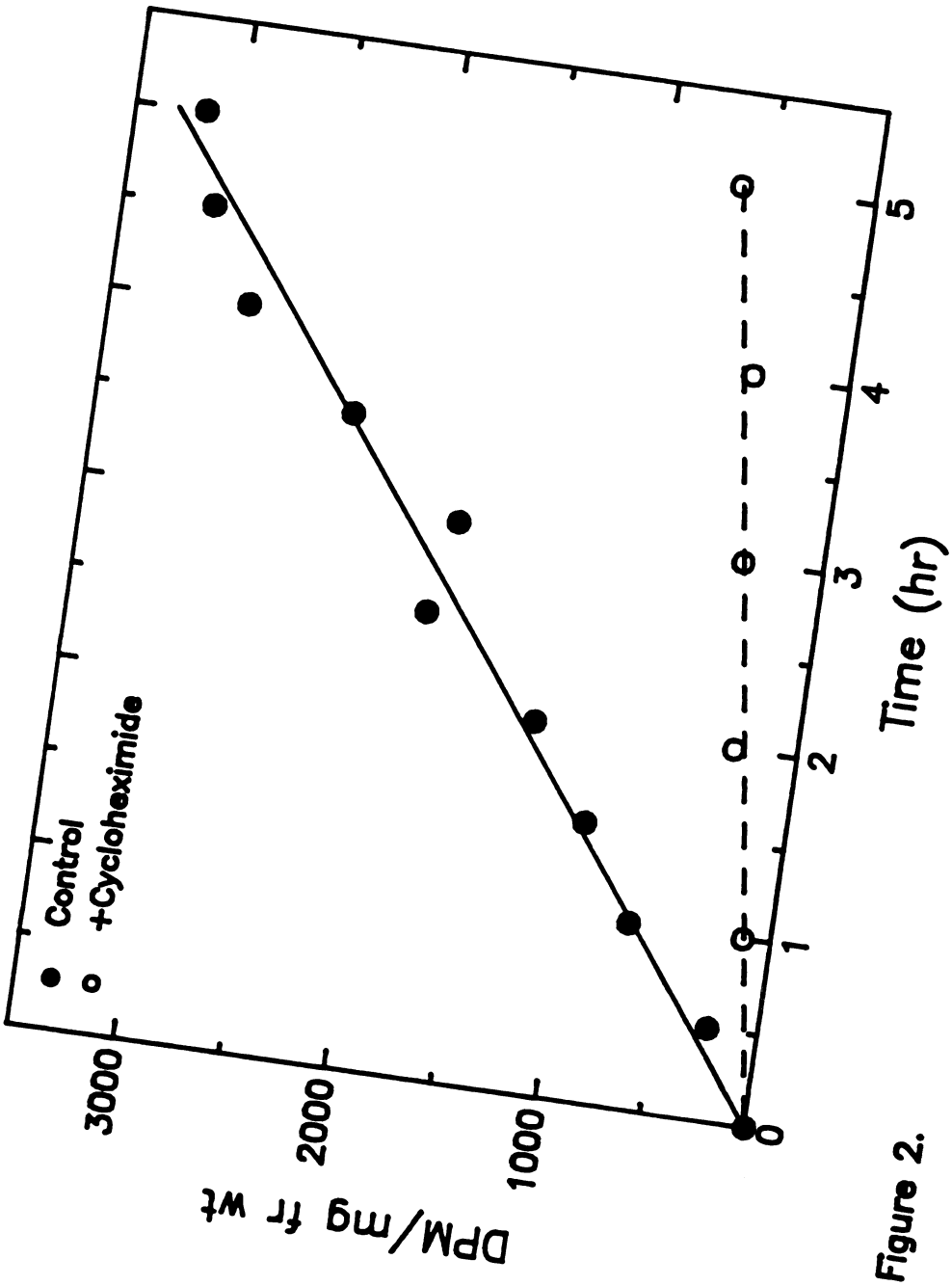
DPM/mg fr wt
3000
2000
1000
0
1
2
3
4
5
Time (hr)
Control
+Cycloheximide

Figure 2.

Figure 3. Sensitivity of two maize inbreds, Pa347 (———) and B79 (-----), and Ionia wheat (·····) to T-2 toxin, as determined by ^{3}H-leucine incorporation into acetone:ethanol insoluble material by leaf discs for 120 min (1 nM T-2 toxin=467 pg/ml).

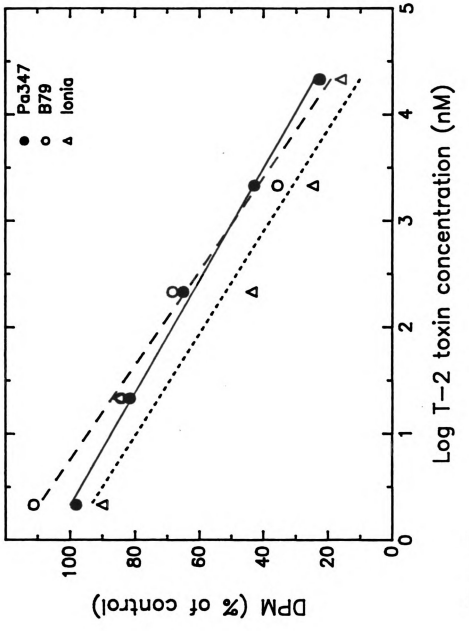
Pa347
B79
Ionia
DPM (% of control)
Log T-2 toxin concentration (nM)
0
20
40
60
80
100
0
1
2
3
4
5

Figure 3.

Figure 4. Sensitivity of six maize inbred lines to deoxynivalenol, as determined by ^{3}H-leucine incorporation into acetone:ethanol insoluble material by leaf discs for 180 min (1 uM deoxynivalenol=296 ng/ml).

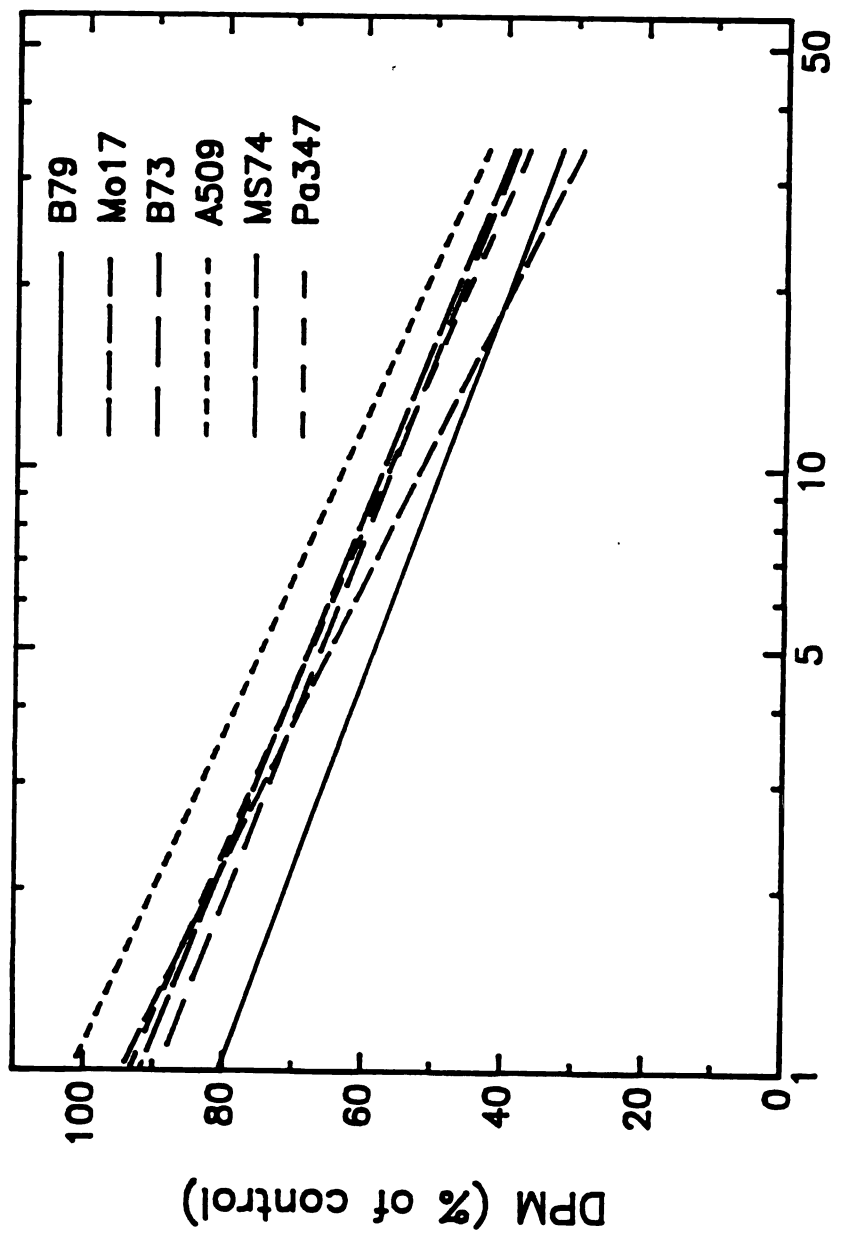
B79
Mo17
B73
A509
MS74
Pa347
100
80
60
40
20
0
DPM (% of control)
1
5
10
50
DON concentration (μM)

Figure 4.

Figure 5. Correlation of sensitivity of maize inbred lines to deoxynivalenol with susceptibility to ear rot caused by Gibberella zeae. Disease ratings are on a scale of increasing severity from 0 to 5. ID_{50} is the concentration of deoxynivalenol inhibiting 50% of ^{3}H-leucine based on regression analysis of data shown in Figure 4.

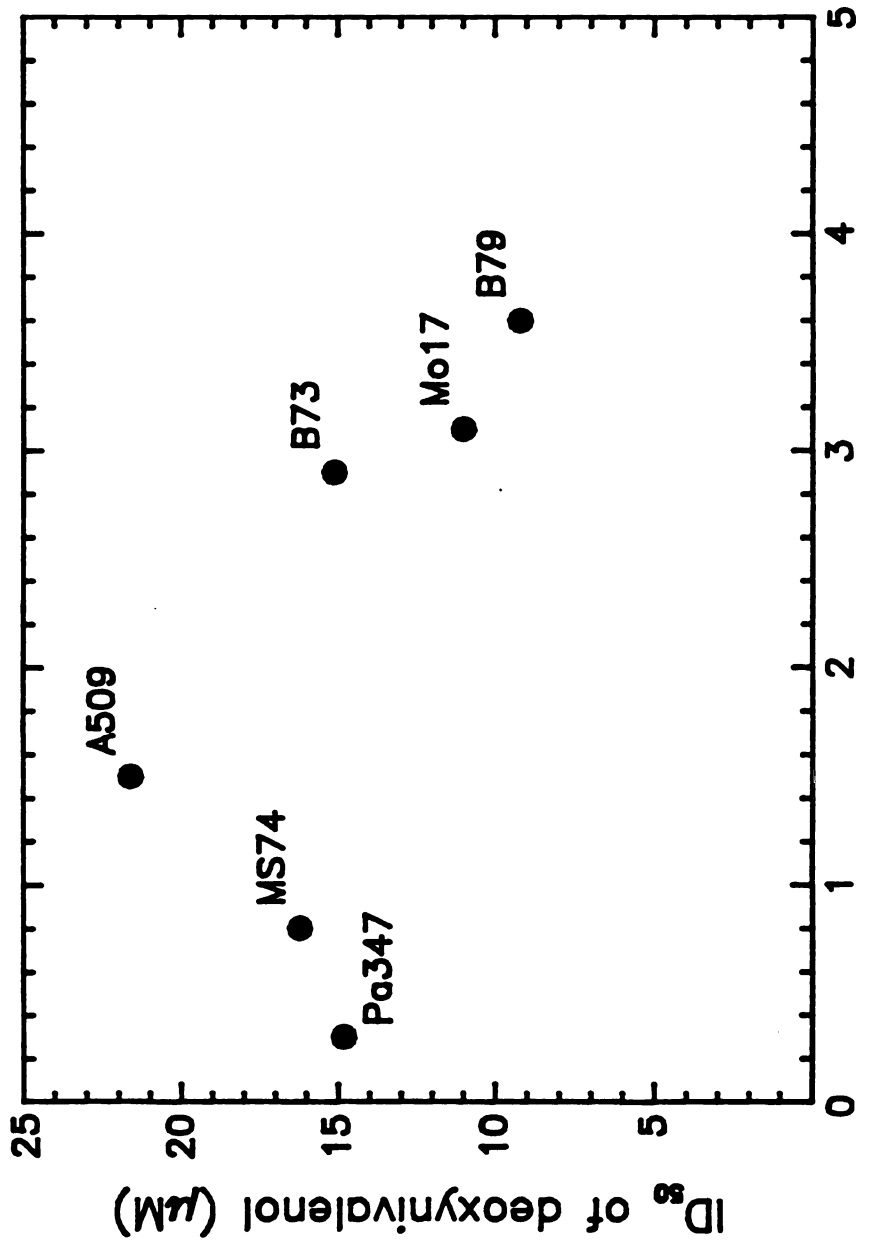
25
20
15
10
5
0
1
2
3
4
5
A509
MS74
Pa347
B73
Mo17
B79
Disease rating
ID50 of deoxynivalenol (μM)

Figure 5.

Figure 6. Inhibition of ^{3}H-leucine incorporation by deoxynivalenol for Ionia wheat discs and cell-free translation system.

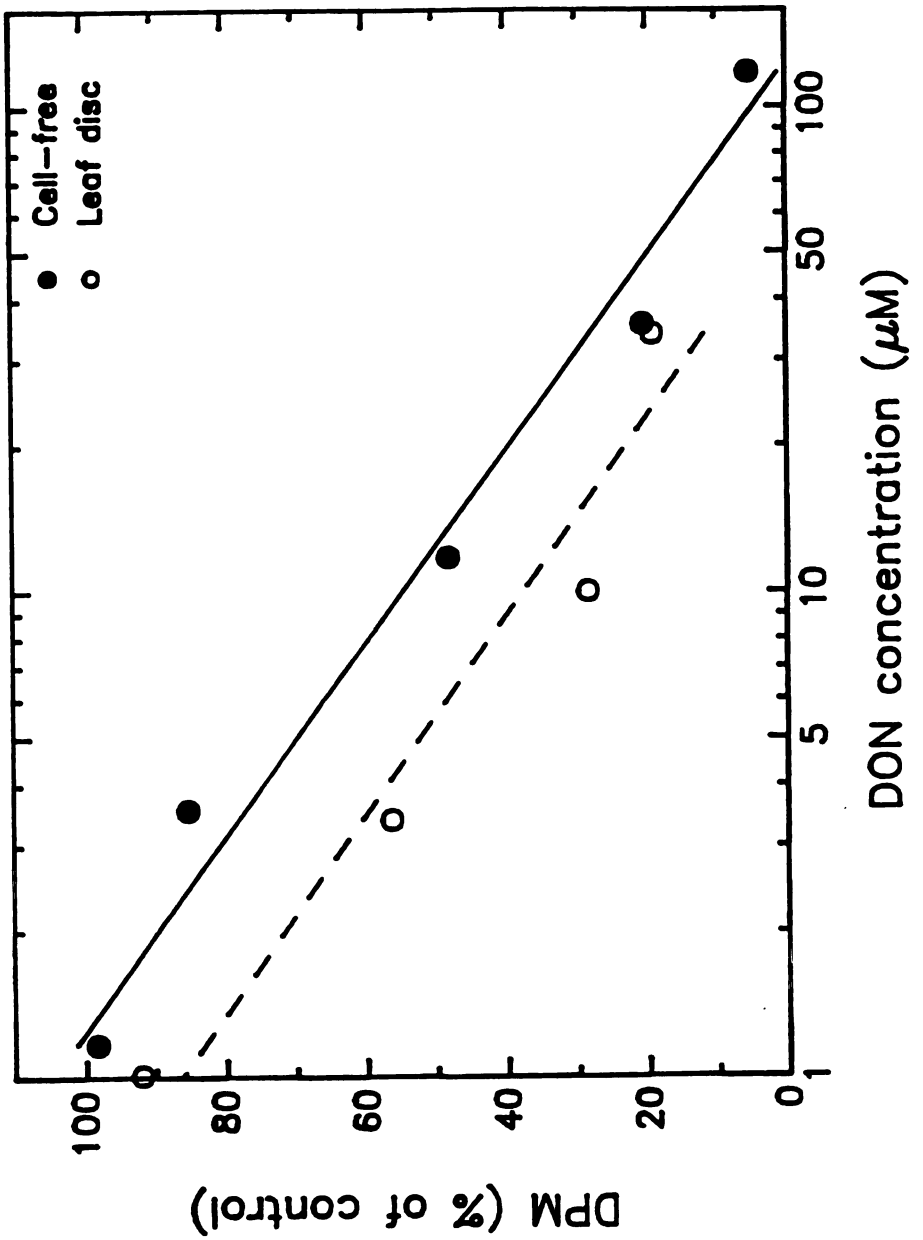
Cell-free
Leaf disc
DPM (% of control)
DON concentration (μM)
0
20
40
60
80
100
1
5
10
50
100

Figure 6.

Figure 7. Incorporation of ^{3}H-leucine into acetone:ethanol insoluble material by maize leaf discs as a function of the duration of exposure to T-2 toxin (10 ug/ml) prior to radiolabeling. Error bars represent 2 standard deviations.

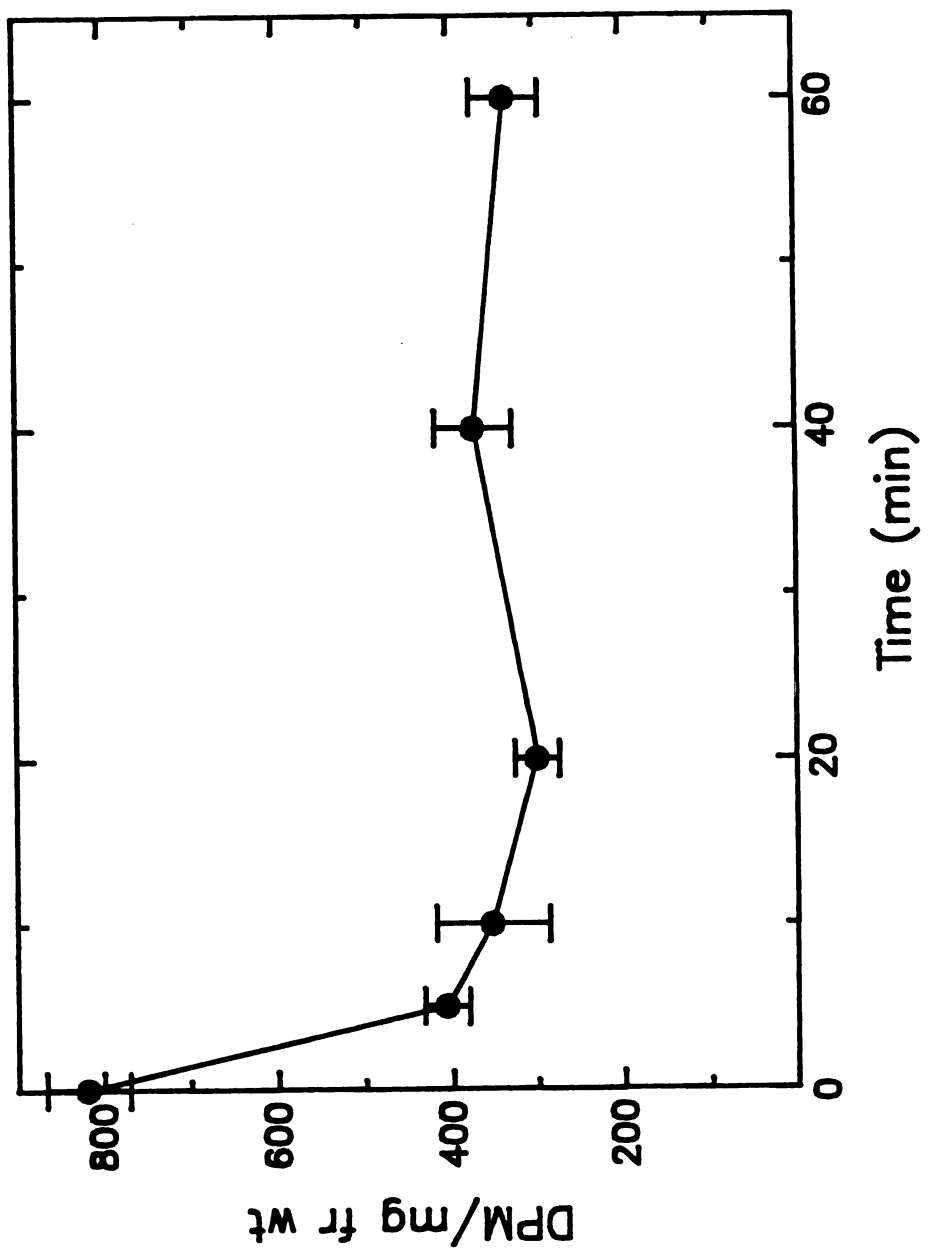

Figure 7.

Figure 8. Effect of T-2 toxin on incorporation of ^{3}H-leucine into acetone:ethanol insoluble material by maize leaf discs. Leaf discs were exposed to T-2 toxin (10 ug/ml) for 60 min prior to radio-labeling in the presence of toxin (+T2/+T2), prior to radio-labeling in the absence of toxin (+T2/-T2), or unexposed to toxin (-T2/-T2). Error bars represent 2 standard deviations.

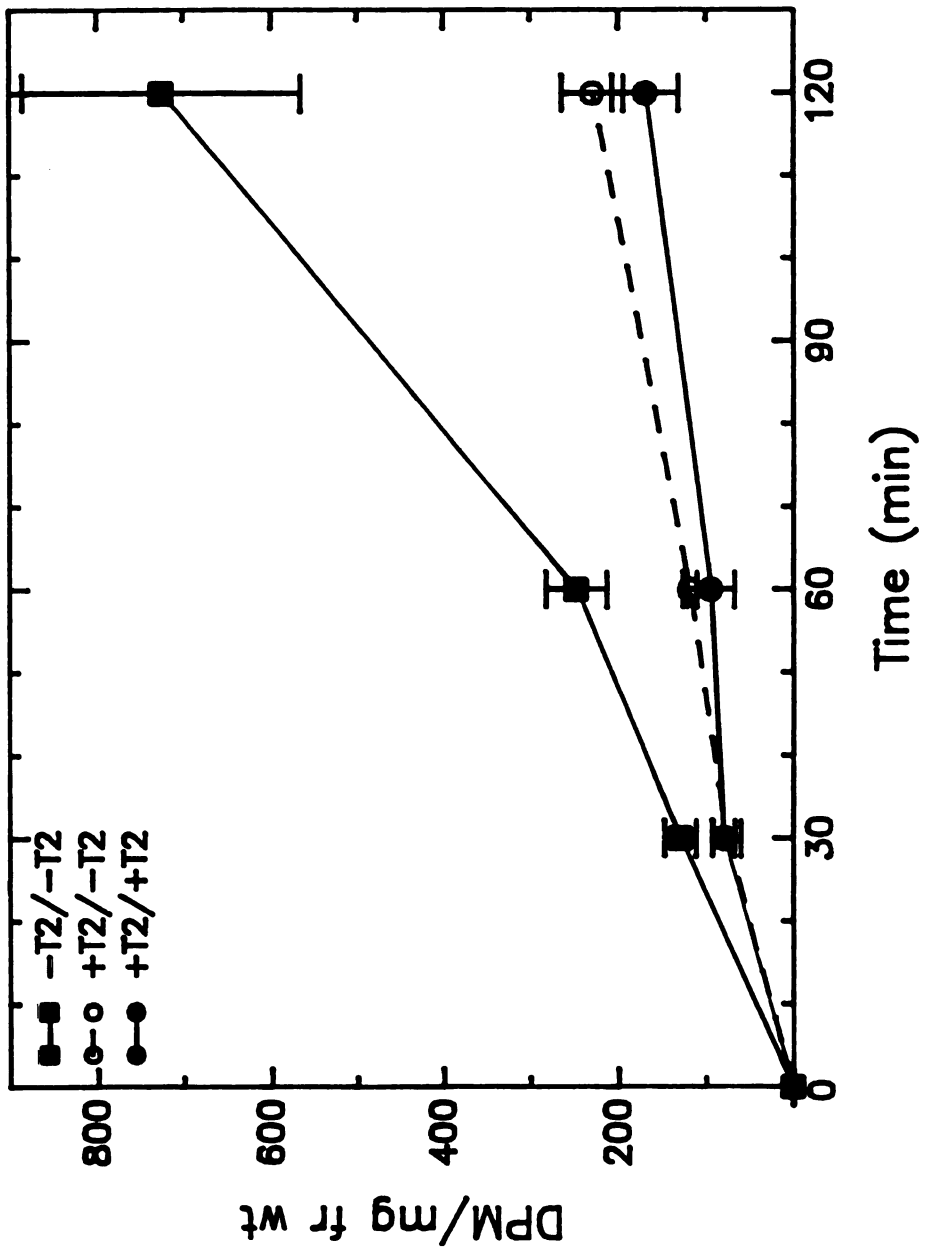
DPM/mg fr wt
800
600
400
200
0
30
60
90
120
Time (min)
-T2/-T2
+T2/-T2
+T2/+T2

Figure 8.

Figure 9. Incorporation of ^{3}H-leucine into acetone:ethanol insoluble material by maize leaf discs as a function of duration of recovery after T-2 toxin exposure, and prior to radio-labelling (120 min). Leaf discs were removed from T-2 toxin (10 ug/ml) after 60 min, washed and placed in fresh buffer for 0-120 min prior to transfer into buffer containing ^{3}H-leucine. Control leaf discs were treated similarly, but without exposure to T-2 toxin. Error bars represent 2 standard deviations.

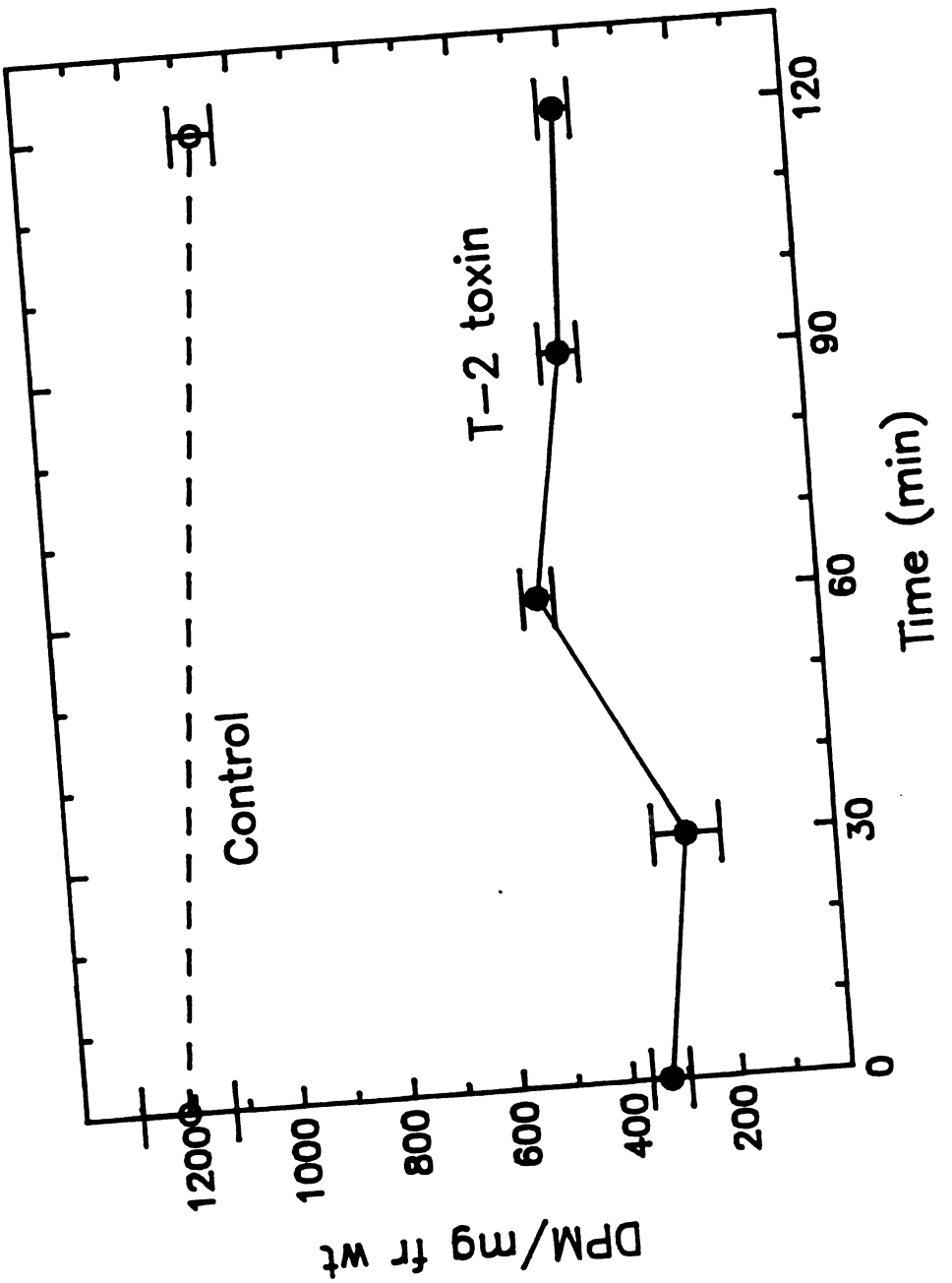
DPM/mg fr wt
1200
1000
800
600
400
200
0
30
60
90
120
Time (min)
T-2 toxin
Control

Figure 9.

Figure 10. Comparison of methods for precipitation of ^{3}H-leucine-labeled protein from wheat embryo cell-free translation reactions, with or without added rabbit globin mRNA (5 ug/ml): method 1, 10 % TCA at 90 C x 15 min, then 0 C for 15 min, and collection of precipitate on GFC filters; method 2, 0.5 N NaOH at 37 C x 20 min, then 10% TCA at 0 C x 30 min, and collection of precipitate on GFC filters; methods 3 and 4, reaction mixtures were spotted on GFC filters (method 3) or Whatman #1 filters (method 4) which were then placed sequentially in 10% TCA at 0 C x 15 min, 90 C x 15 min, and 23 C x 15 min. Error bars represent 2 standard deviations.

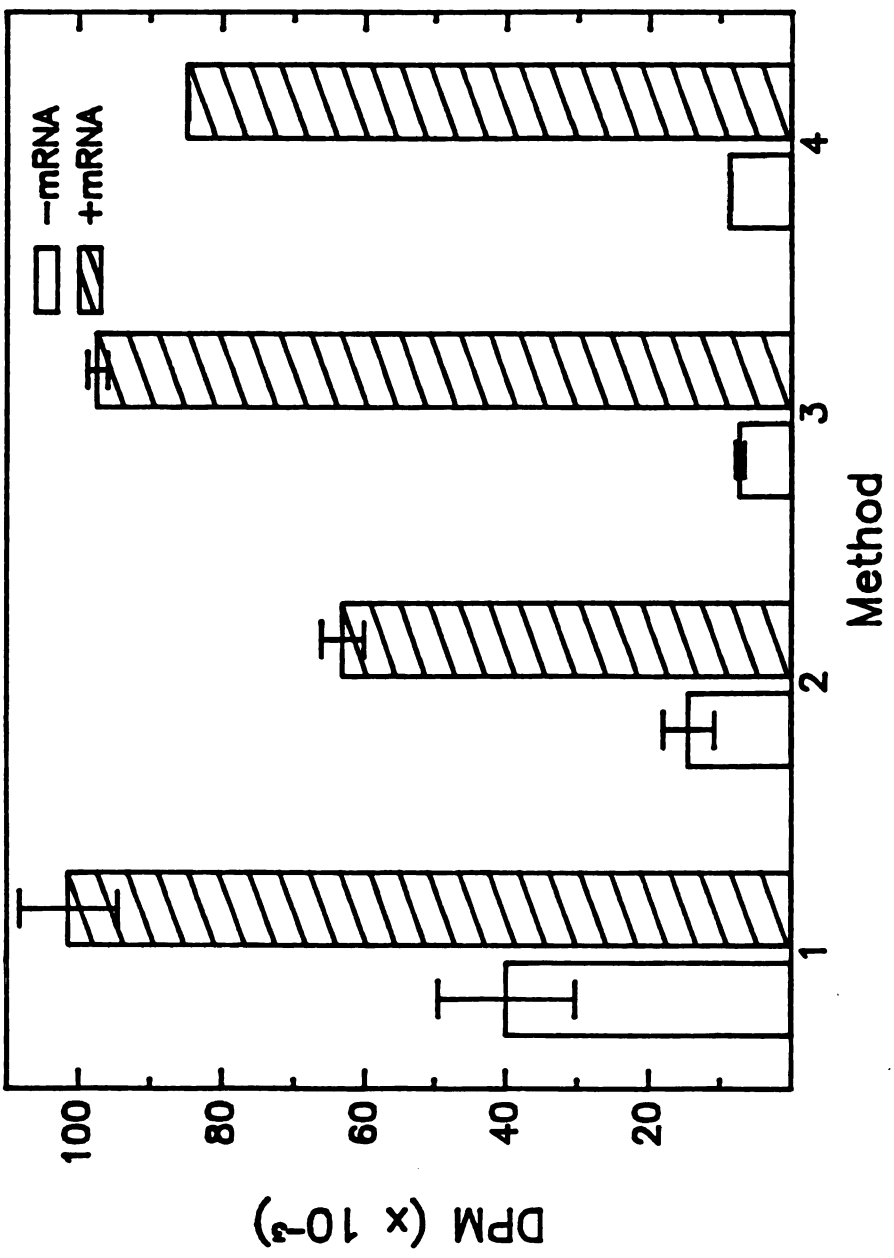

Figure 10.

Figure 11. Optimization of Mg^{++} concentration for wheat embryo cell-free translation system.

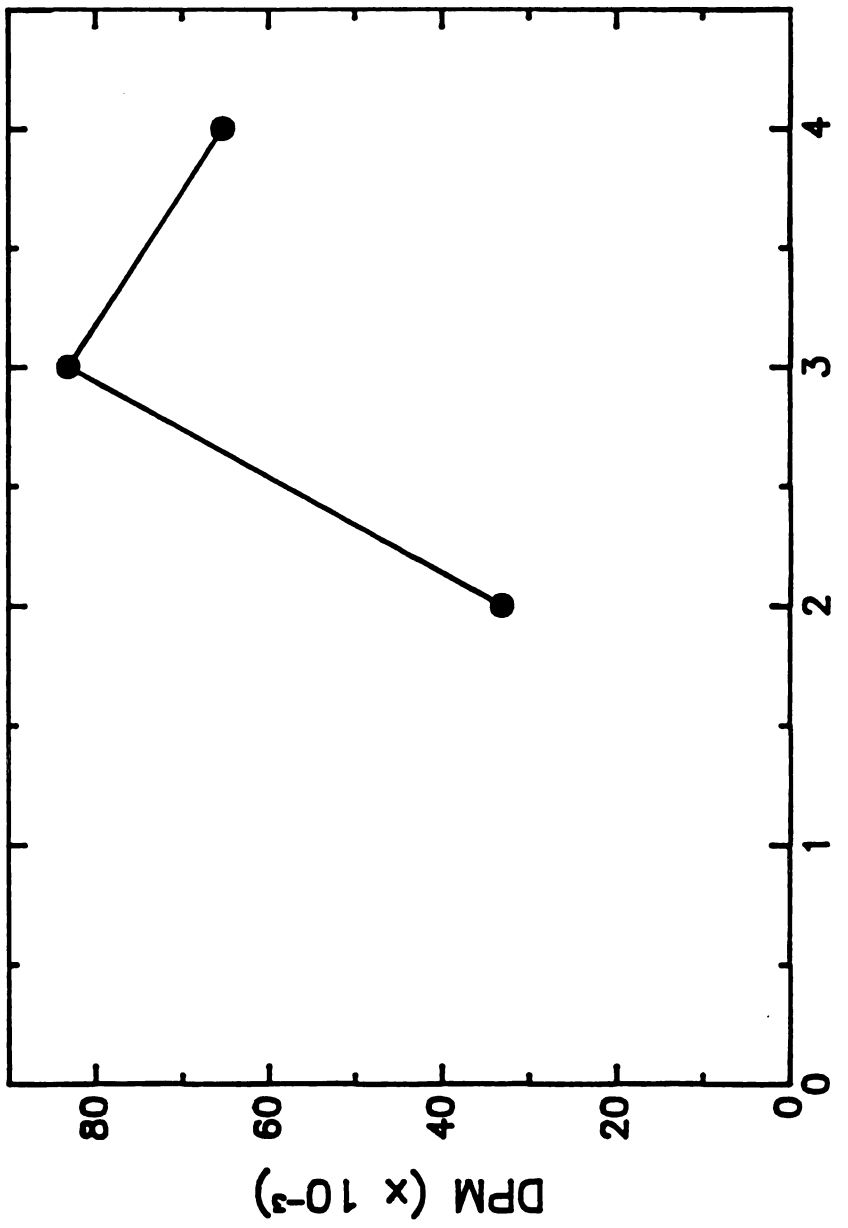

Figure 11.

Figure 12. Optimization of K^+ concentration for wheat embryo cell-free translation system.

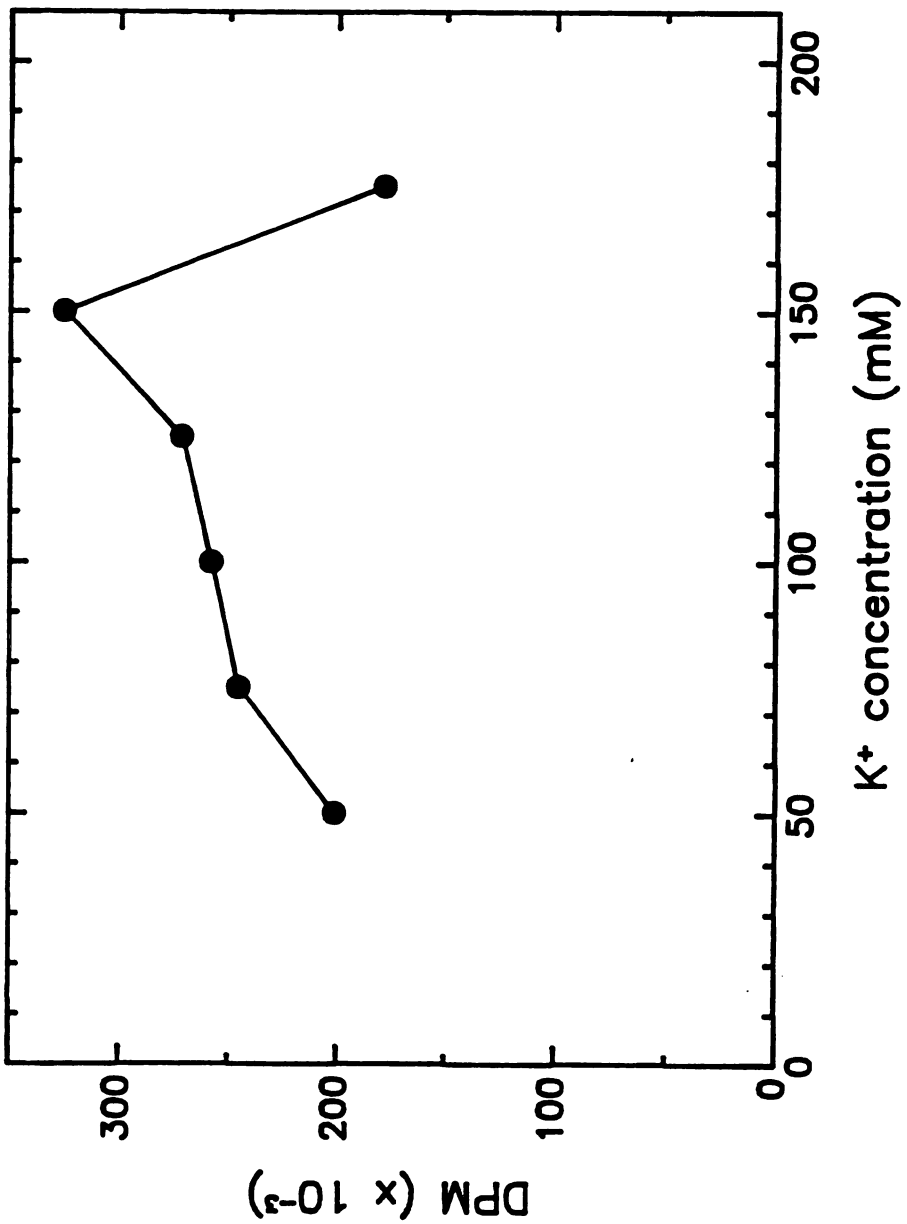

Figure 12.

Figure 13. Incorporation of ^{3}H-leucine into TCA-precipitated material by wheat embryo cell-free translation system. Treatments are with or without the addition of rabbit globin mRNA (5 ug/ml).

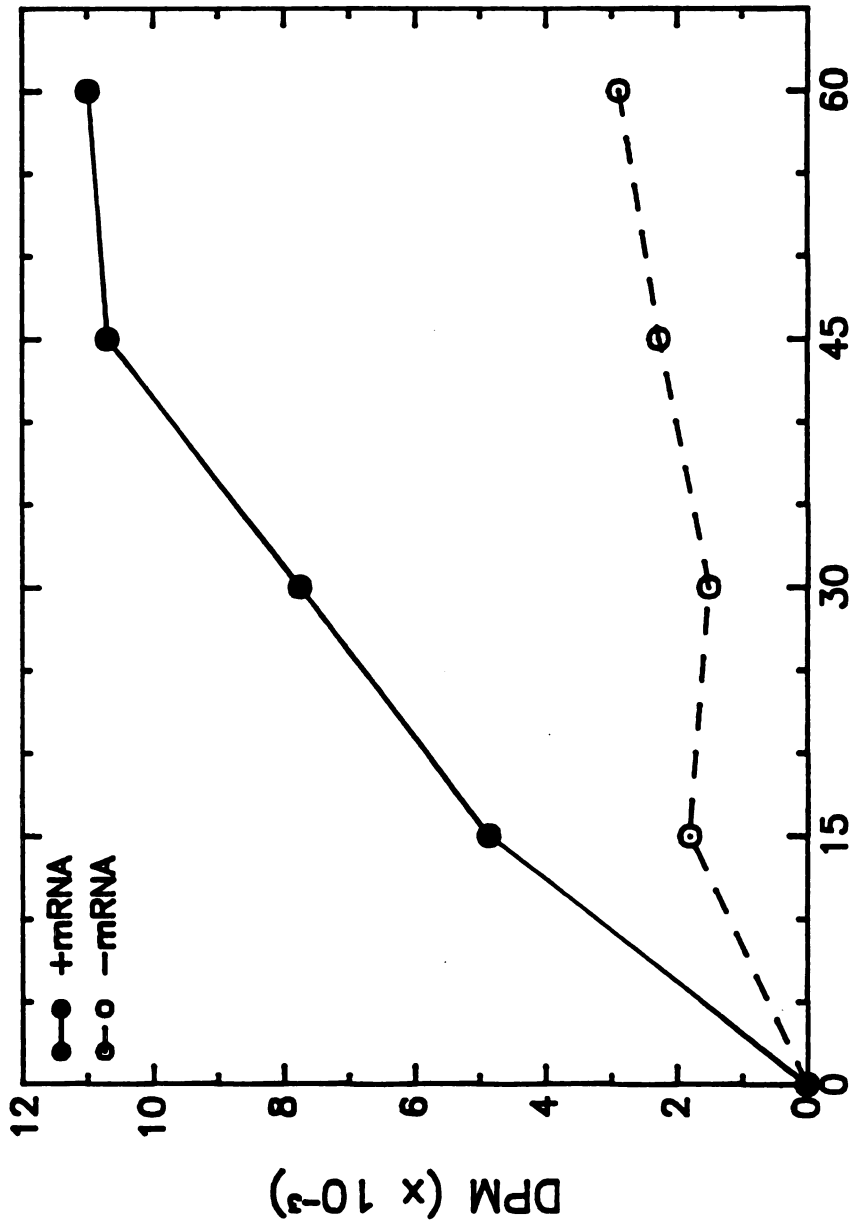

Figure 13.

Figure 14. Incorporation of ^{3}H-leucine into TCA-precipitated material by wheat embryo cell-free translation system in response to added total RNA extracted from Fusarium graminearum germlings.

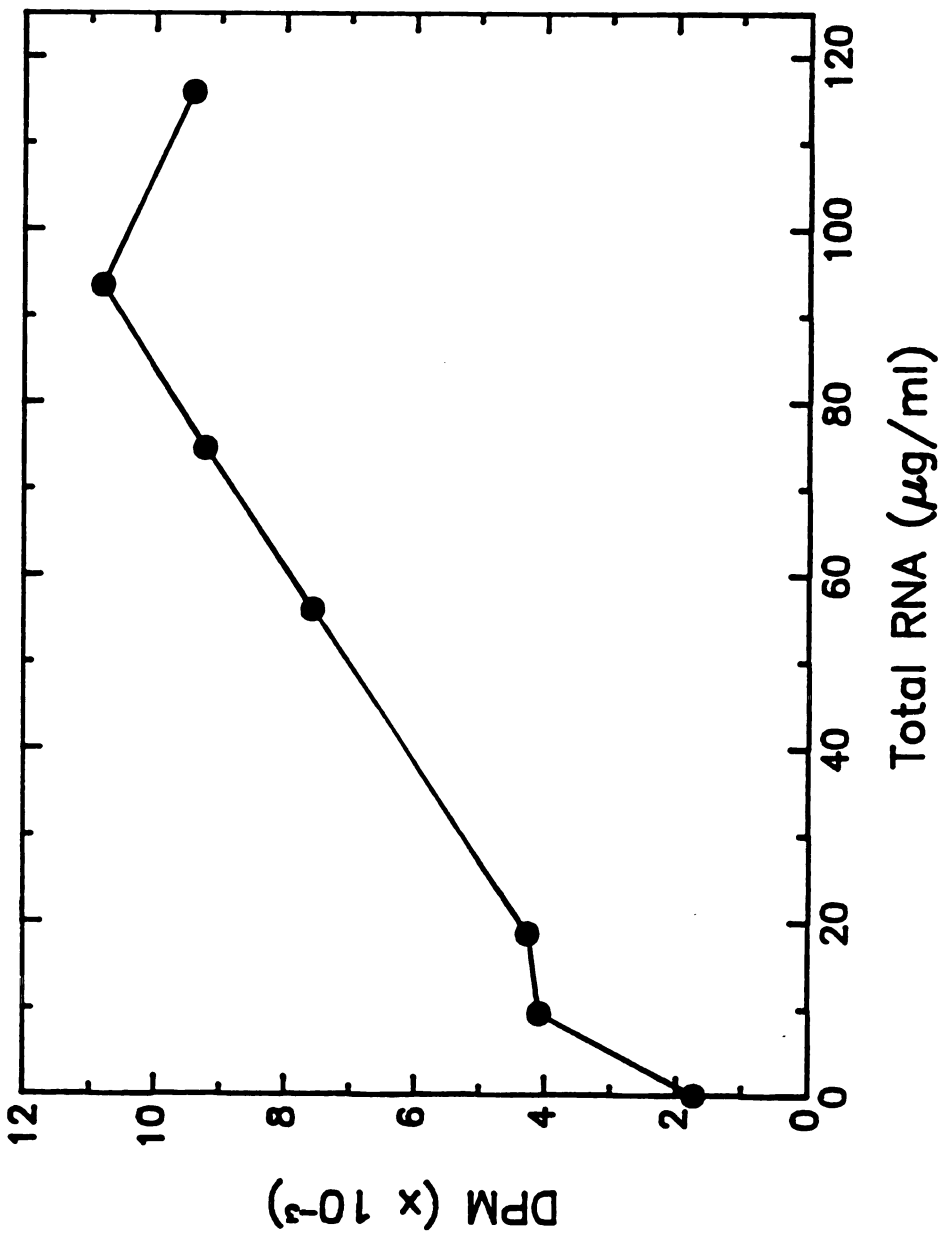

Figure 14.

Figure 15. Optimization of Mg^{++} concentration for Pa347, B79 and A509 maize plumule cell-free translation system.

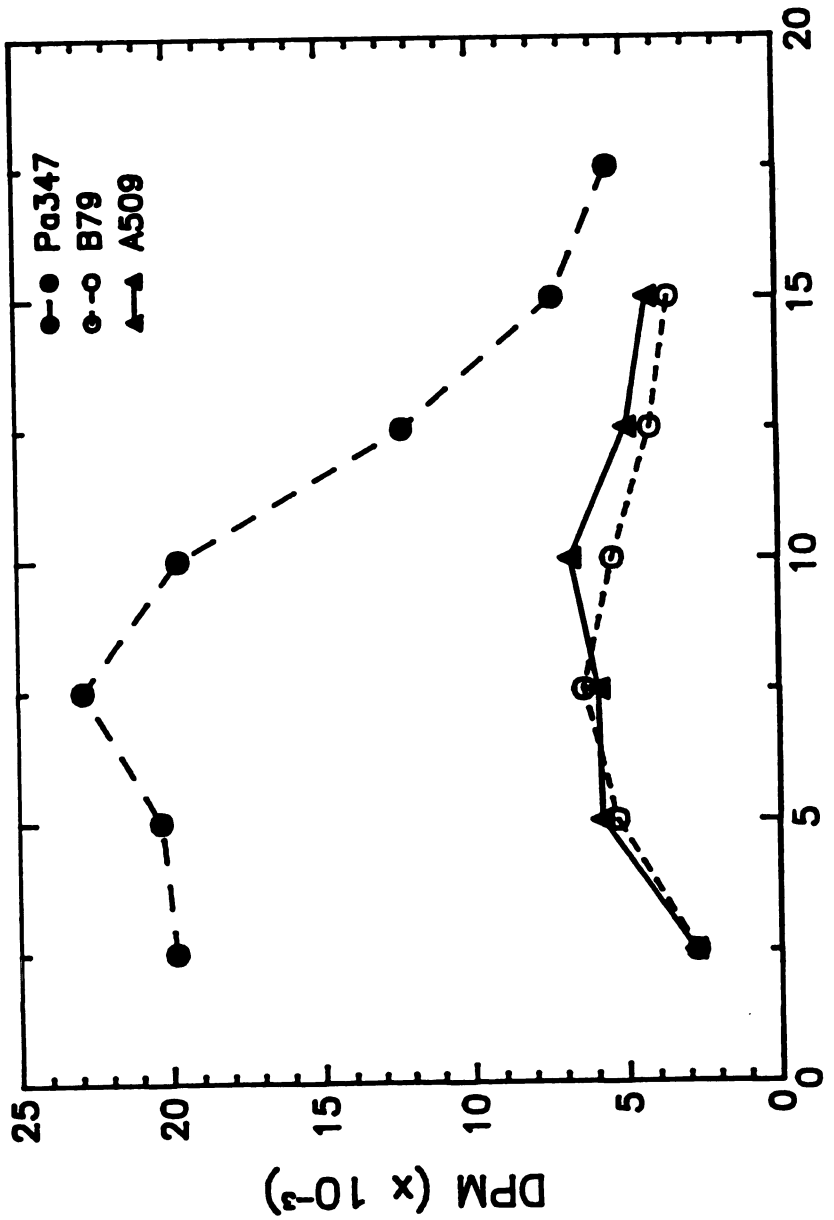
Pa347
B79
A509
DPM (x 10-3)
25
20
15
10
5
0
0
5
10
15
20
Mg++ concentration (mM)

Figure 15.

Figure 16. Optimization of K^+ concentration for Pa347, B79 and A509 maize plumule cell-free translation system.

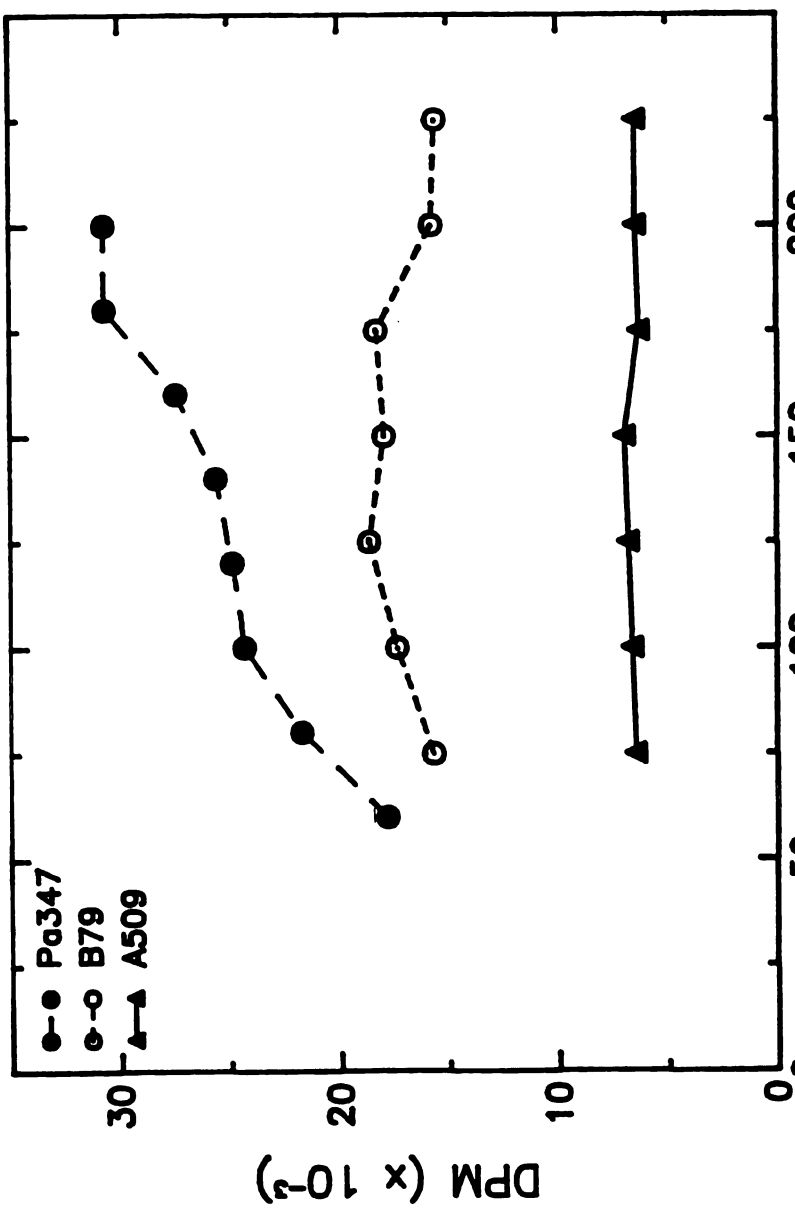
Pa347
B79
A509
DPM (x 10⁻²)
0
10
20
30
0
50
100
150
200
K⁺ concentration (mM)

Figure 16.

Figure 17. Inhibition of ^{3}H-leucine incorporation by deoxynivalenol for Pa347 maize leaf discs (-----) and cell-free translation system (———).

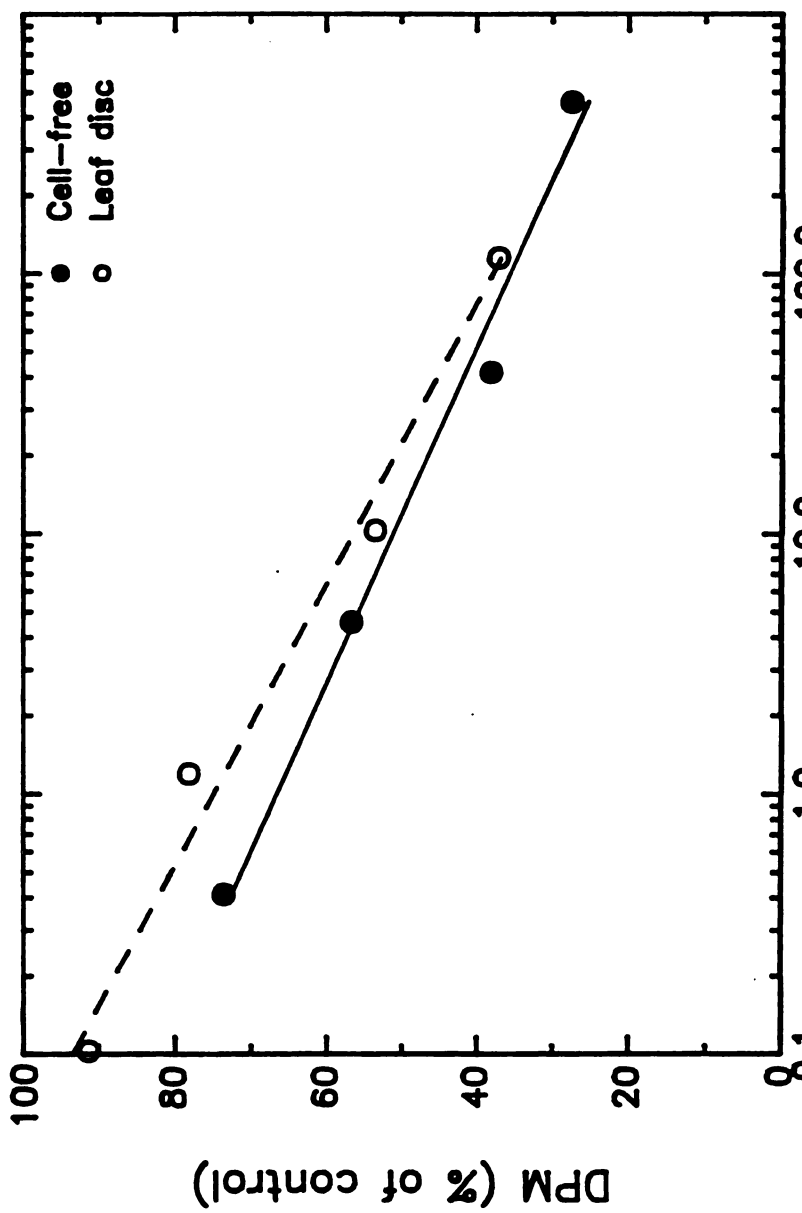
Cell-free
Leaf disc
DPM (% of control)
100
80
60
40
20
0
0.1
1.0
10.0
100.0
DON concentration (μM)

Figure 17.

Figure 18. Inhibition of ^{3}H-leucine incorporation by deoxynivalenol for B79 maize leaf discs (-----) and cell-free translation system (———). DPM of the cell-free system were also adjusted by subtracting DPM of reactions containing cycloheximide (384 ug/ml) (·····).

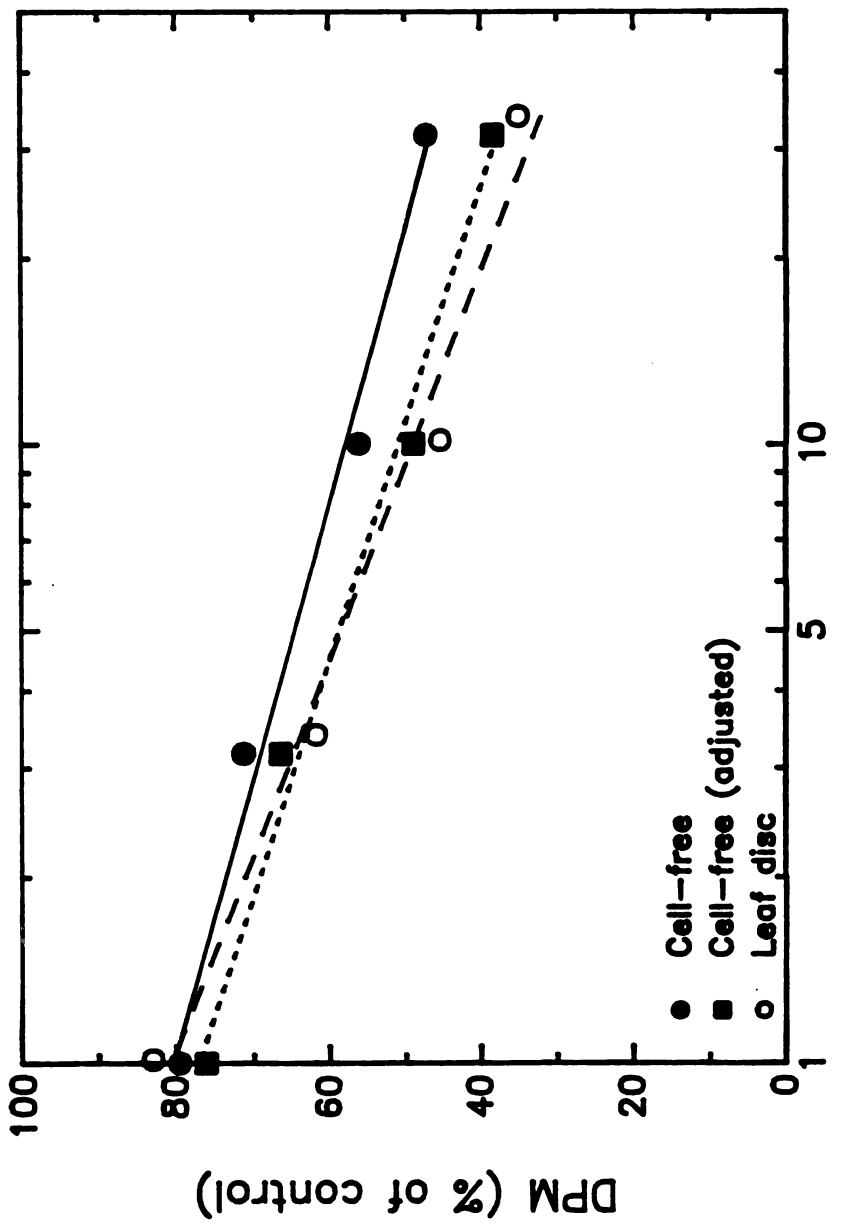
DPM (% of control)
100
80
60
40
20
0
1
5
10
DON concentration (μM)
Cell-free
Cell-free (adjusted)
Leaf disc

Figure 18.

Figure 19. Inhibition of ^{3}H-leucine incorporation by deoxynivalenol for A509 maize leaf discs (-----) and cell-free translation system (———). DPM of the cell-free system were also adjusted by subtracting DPM of reactions containing cycloheximide (384 ug/ml) (·····).

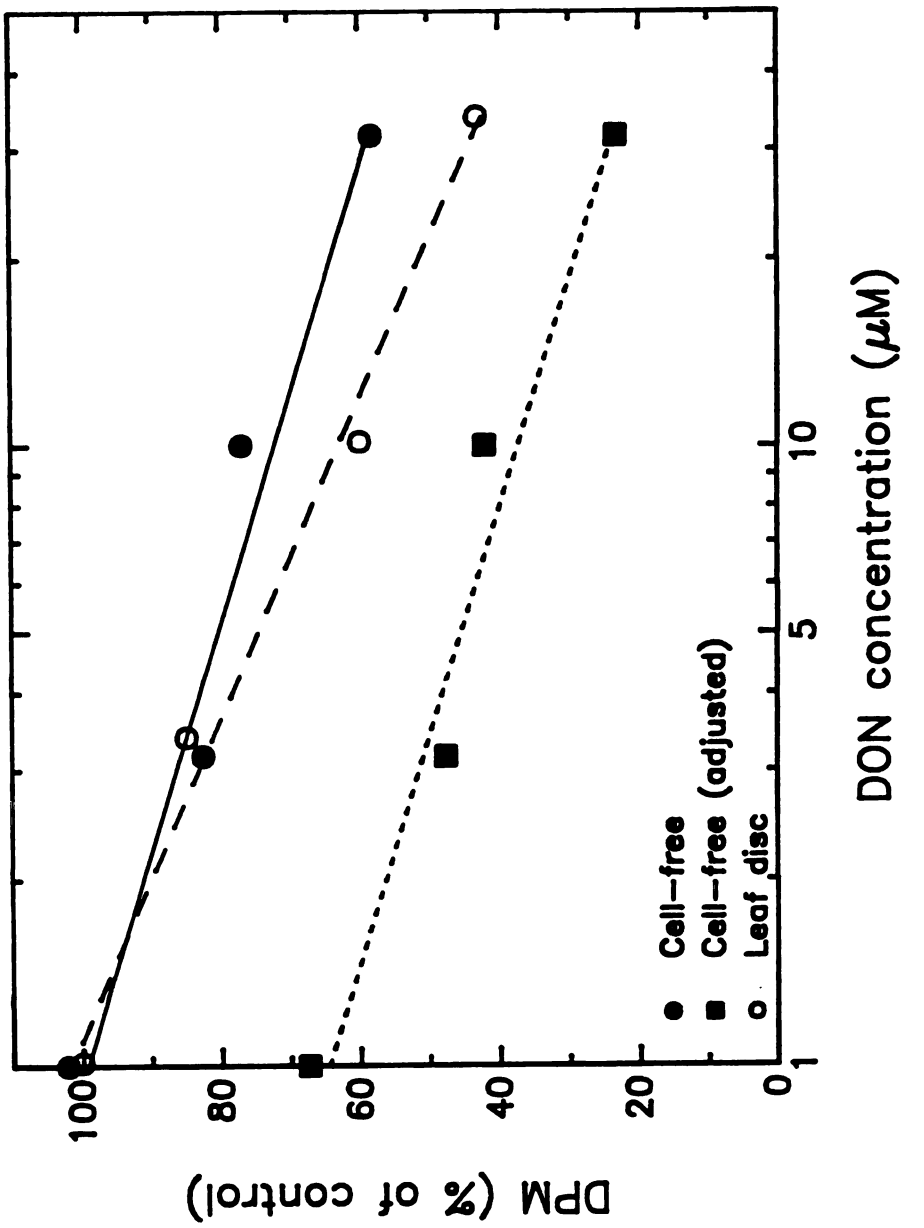
DPM (% of control)
100
80
60
40
20
0
1
5
10
DON concentration (μM)
Cell-free
Cell-free (adjusted)
Leaf disc

Figure 19.

PART II:

HYBRIDOMA-BASED ENZYME IMMUNOASSAY

FOR DEOXYNIVALENOL

INTRODUCTION

Deoxynivalenol (DON, vomitoxin) is one of a group of sesquiterpene mycotoxins classified as 12,13-epoxy-trichothecenes (140,157). The specific chemical designation is 3α,7α,15-trihydroxy-12,13-epoxytrichothec-9-ene-8-one (114). DON has been reported in corn (132,141,162), wheat (131,143,149), barley (143,149,166) and mixed feeds (141). DON is produced by Gibberella zeae (Schw.) Petch (anamorph=Fusarium graminearum Schw.) (139) and is associated with emesis and feed refusal in swine (124,141,161); it also causes skin irritation, hemorrhaging, hematological changes, radiomimetic effects and severe immunosuppression (123,160). It is an inhibitor of protein synthesis (158) and has been reported to impair human lymphocyte blastogenesis (122).

Current detection methods (145), including gas chromatography (148), gas chromatography-mass spectrometry (149), high-performance liquid chromatography (106,154), and thin-layer chromatography (104,131,156,159) involve considerable sample preparation, are time consuming, and require technical expertise. Enzyme immunoassays employing polyclonal (110,126,163) or monoclonal (125,129,136) antibodies have been developed to detect other mycotoxins. These assays offer several advantages over other methods. They require little sample preparation, are rapid and relatively simple to execute, yet are sensitive. In addition,

enzyme immunoassays lack the health hazards associated with radio-immunoassays. Monoclonal antibodies have important advantages over polyclonal antibodies. They are extremely specific, even if the immunizing antigen was not pure, and offer a theoretically unlimited supply of homogeneous reagent (128). For certain applications, however, monoclonal antibodies may be too specific, and unable to detect related members of a group of molecules. They may also have relatively low affinity or be unusually sensitive to environmental conditions (pH, salt concentrations, organic solvents). Finally, the production of monoclonal antibodies is an extremely labor-intensive project. Based on their advantages and the successes with other mycotoxins, an attempt was made to produce monoclonal antibodies specific for DON, and develop an enzyme immunoassay to detect these compounds in extracts of ground maize kernels. The problems associated with production of antibody specific for DON and approaches to overcoming these difficulties are discussed.

Immune response in higher animals is a complex interaction among a number of specialized cell types, one of them being the antibody-producing B-lymphocyte (B-cell). B-cell antibody response to most antigens is dependent on the presence of auxilliary cells, T-lymphocytes (T-cells) (112,127). For B-cell activation, an antigen must bind simultaneously to B-cell surface immunoglobulin and a T-cell surface receptor molecule (142); in addition, there must be recognition of allotypic B-cell antigen (class II major his-

tocompatibility antigen) by the corresponding T-cell receptor (113). In this interaction, B-cells are stimulated to divide and secrete large amounts of antigen-specific antibody (135,155). It has been shown that B- and T-cells may recognize different determinants on the antigen (144). The requirement for simultaneous binding of B- and T-cells explains the lack of an immune response to small molecules (haptens) too small to accommodate simultaneous binding by surface receptors of both lymphocytes. Elicitation of DON-specific antibody, therefore, requires conjugation of DON to a large, immunogenic molecule. Surface Ig (B-cell) binds DON, and T-cell receptor binds a portion of the "carrier" molecule, resulting in the complex required for stimulation of B-cells producing DON-specific antibody.

DON possesses no groups to directly link the molecule to a carrier protein. Conjugation can be facilitated by the introduction of a carboxyl group. This can most easily be accomplished at the C8-keto position by preparing DON-8-(O-carboxymethyl)oxime. This approach was used to produce antibodies against aflatoxin B_1 (111,138) and various steroids (117,118).

Another method for introduction of a carboxyl group onto DON involves esterification of a hydroxyl using a cyclic acid anhydride (117,118,151). The presence of three hydroxyls on the DON molecule might result in the addition of multiple carboxyls to DON using standard esterification methods, resulting in undesirable cross-linking of carrier

proteins and masking of antigenic determinants peculiar to DON. This problem can be solved by protecting two of the hydroxyls during derivatization to carboxylated DON. Sugihara and Bowman (153) synthesized cyclic phenylboronate esters of alkanediols, forming 5-, 6- or 7-membered rings, but not an 8-membered ring. Since they readily form from 1,2- and 1,3-diols and are easily removed by water or polyols, cyclic phenylboronates have been used for protecting glycoside hydroxyls during acetylation (108,120,121) or during 5'-O-derivatives of nucleosides (167), and protecting cis-1,3-diols during synthesis of monoesters of 14-membered macrolide aglycones (147). These studies suggested the possibility of protecting the C7- and C15-hydroxyls (a 1,3-diol) of DON by formation of a 6-membered cyclic boronate ester prior to esterification for introduction of a carboxyl (Figure 20). After carboxylation of DON and removal of the boronate protecting group, conjugation could proceed by amide linkage (102,103,116). This approach was ultimately successful in eliciting murine antibodies with affinity for DON.

MATERIALS AND METHODS

Materials.

Deoxynivalenol (DON) was produced and purified by the method of Witt et al (164). Fusarenone-X (FX), 15-acetyldeoxynivalenol (15Ac-DON), and nivalenol were supplied by James J. Pestka (Department of Food Science and Human Nutrition, Michigan State University, East Lansing 48824); 3-acetyldeoxynivalenol (3Ac-DON) and 12,13-deepoxydeoxynivalenol (DOM-1) by Steven P. Swanson (Department of Veterinary Biosciences, University of Illinois, Urbana 61810). N-hydroxysuccinimide and tetraethylenepentamine were from Aldrich Chemical Co., Inc. (Milwaukee, WI 53233); n-butylboronic acid from Alltech Associates, Inc. (Deerfield, IL 60015); Adsorbosil silica gel (200/435 mesh) from Anspec (Ann Arbor, MI 48107); Coomassie Brilliant Blue G-250 from J. T. Baker Chemical Co. (Phillipsburg, NJ 08865); goat-anti-mouse immunoglobulin conjugated to horseradish peroxidase (GAM-HRP) from Cooper Biomedical (Malvern, PA 19355); Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum, Freund's complete adjuvant, Freund's incomplete adjuvant, NCTC-135 medium, and penicillin-streptomycin solution from Gibco Laboratories, (Grand Island, NY 14072); 2,4,6-trinitrobenzenesulfonic acid (TNBS) from Eastman Kodak Co. (Rochester, NY 14650); 2,2'-azinobis(3-ethylbenz-thiazoline sulfonic acid) (ABTS), bovine insulin, bovine serum albumin (BSA), car-

boxymethoxylamine (aminooxyacetic acid), 1,3-dicyclohexylcarbodiimide, glutaric anhydride, horseradish peroxidase (HRP), 4-(p-nitrobenzyl)pyridine (NBP), ovalbumin (OA), polyethylene glycol (MW 1450), pristane, succinic anhydride, and T-2 toxin were obtained from Sigma Chemical Co. (St. Louis, MO 63178). Myeloma cell line P3/NS1/1-Ag4-1 (NS-1) was obtained from American Type Culture Collection, Rockville, MD 20852.

Derivatization of deoxynivalenol.

DON-8-(O-carboxymethyl)oxime (DON-CMO) was prepared by reacting DON with carboxymethoxylamine (111,117,118,138). DON (27 umoles [8 mg]) and carboxymethoxylamine (271 umoles [29.6 mg]) were refluxed in 0.8 ml pyridine for 2 hr. The reaction mixture was dried under N_2, redissolved in a small volume of methanol (0.5-1.0 ml) and air-dried onto 1 g silica gel. A glass column (25 x 300 mm) (Ace Glass Incorporated, Vineland, NJ 08360) was packed with 35 g silica gel added as a slurry in chloroform. The sample adsorbed to silica gel was carefully layered on top of the packed column as a slurry in chloroform, and the column then washed with 120 ml chloroform. DON-CMO was separated from unreacted DON and carboxymethoxylamine by elution with chloroform:methanol (3:1) at a flow rate of 7 ml/min. Fractions (8 ml) were collected and monitored by silica gel-thin-layer chromatography (TLC). The fractions were evaporated to dryness under a stream of N_2 and redissolved in 200 ul ethyl acetate. A 2

ul aliquot of each sample was spotted onto silica gel-G TLC plates (20 x 20 cm) and developed with chloroform:methanol (1:1). DON-CMO and unreacted DON were visualized by spraying with NBP reagent A (1 g 4-[p-nitrobenzyl]pyridine, 60 ml carbon tetrachloride, 40 ml chloroform), heating at 150 C for 30 min, then, when cool, spraying with NBP reagent B (50 ml NBP reagent A, 5.5 ml tetraethylenepentamine) (156). Unreacted carboxymethoxylamine was visualized by spraying duplicate TLC plates with 0.04% bromcresol purple in 50% aqueous ethanol. Fractions containing compounds with R_f=0.49 and 0.44, which were blue when treated with NBP, were pooled.

3-O-hemisuccinyl-DON (3-HS-DON) was prepared by protection of the C7 and C15 hydroxyls with a cyclic boronate ester, esterification at the C3 position, and then removal of the boronate ester. In a 2 ml reaction vial, 68 umol DON (20 mg) was dissolved in 200 ul pyridine. To this solution, 667 umol n-butylboronic acid (68 mg) was added and the mixture stirred at room temperature, overnight. Then 800 ul of 1.7 M succinic anhydride (in pyridine) was added while stirring. The reaction vial was sealed and the mixture stirred for 90 min in a boiling water bath. The pyridine was evaporated under a stream of N_2 at 100 C, and the residue dissolved in a small amount of methanol and air-dried onto 1 g silica gel. The reaction mixture adsorbed to silica gel was carefully layered on top of a silica gel column (25 x 300 mm) packed as a slurry in benzene. The

column was washed with 100 ml benzene, and excess succinic anhydride eluted with benzene:ethyl acetate:acetic acid (80:20:1) at a flow rate of 7 ml/min until no non-volatile material was present in the eluate (approximately 700 ml). The solvent was changed to benzene:ethyl acetate:acetic acid (70:30:1) and 8 ml fractions were collected and monitored by silica gel-TLC developed in chloroform:methanol (1:1) as was done for DON-CMO. Unreacted DON and hemi-succinyl derivatives were visualized with NBP. Fractions containing a compound at R_f=.61, which appeared blue after treatment with NBP, were pooled and evaporated to dryness under reduced pressure in a Buchi Model-R rotary evaporator (Brinkman Industries, Westbury, NY 11590) at 50 C. Additional benzene was added as needed to remove the last traces of acetic acid as an azeotrope. The residue was taken up in 5 ml water. To remove a yellow contaminant, 5 ml chloroform was added to the aqueous solution and shaken vigorously. The upper aqueous phase was drawn off, and the chloroform washed with 5 ml water, four additional times. The aqueous phases were pooled and evaporated to dryness under reduced pressure in a rotary evaporator at 50 C. The residue was dissolved in 1 ml ethyl acetate. The concentration of 3-HS-DON was estimated by TLC visualized with NBP and compared with DON standards. Some hemisuccinyl-DON was also prepared by direct reaction with succinic anhydride, without protection by the cyclic boronate ester (HS-DON mixture).

The 3-hemiglutaryl derivative of DON (3-HG-DON) was

prepared in a similar manner as 3-HS-DON, but substituting glutaric anhydride for succinic anhydride. The 3-hemiglutaryl derivative of 15-acetyl-DON (3-HG-15-Ac-DON) was prepared by direct reaction with glutaric anhydride, since the 1,3-diol (C7,15) was not available for protection by the cyclic boronate ester.

Preparation of deoxynivalenol-protein conjugates.

The derivatives DON-CMO, 3-HS-DON, 3-HG-DON, and 3-HG-15-Ac-DON were conjugated to bovine serum albumin (BSA), ovalbumin (OA) and horse radish peroxidase (HRP) through an activated N-hydroxysuccinimide ester (102). In 80 ul dimethylformamide (DMF) were dissolved 47 umol 1,3-dicyclohexyl-carbodiimide (9.6 mg) and 97 umol N-hydroxysuccinimide (11.2 mg). This solution was added to 48 umol DON derivative (=19 mg of 3-HS-DON) in 80 ul DMF, and stirred at room temperature for 30 min. Precipitated dicyclohexylurea was removed by centrifugation. The supernatant was slowly added dropwise to 40 mg protein in 1.6 ml of 0.1 N sodium bicarbonate, while stirring slowly. The reaction mixture was then placed at 4 C and stirred for 2 hr. The DON-protein conjugate was dialyzed against 2 changes of 50 mM sodium phosphate buffer, pH 8.0, aliquoted and stored at -20 C.

Conjugation ratios (moles of DON per mole of protein) were determined by comparing the number of amino groups free to react with trinitrobenzene sulfonic acid (TNBS), and

therefore not involved in amide linkage to the DON derivative, of conjugated and unconjugated protein (130). In a 16 x 125 mm screw cap culture tube, 1 ml of protein solution containing 0.1-1.0 mg protein, 1 ml of 4% sodium carbonate buffer (pH 8.5), and 1 ml of 0.1% aqueous TNBS was reacted at 40 C for 2 hr. Then 1 ml of 10% sodium dodecylsulfate (SDS) was added and the tube vortexed briefly. Finally, 0.5 ml of 1 N HCl was added and the absorbance at 335 nm determined with a Varian Series 634 spectrophotometer. Total protein was determined by mixing 0.1 ml protein solution containing 10-100 ug protein with 5 ml dye reagent and reading the resultant absorbance at 595 nm (109). The dye reagent was prepared by dissolving 10 mg Coomassie Brilliant Blue G-250 in 5 ml of 95% ethanol, adding 10 ml of 85% phosphoric acid, and bringing the volume to 100 ml with distilled water. Concentrations of conjugated protein by each method were determined by comparison with unconjugated standards, and the conjugation ratios calculated by Equation 1.

$$(TP - AP)\ (TP)^{-1}\ (FA) = \text{moles DON/mole protein} \qquad (1)$$

where TP is the total protein concentration determined by the Bradford method, AP is the protein concentration based on the free amino groups reacting with TNBS, and FA is the number of free amino groups in the native protein (BSA contains 61 free amino groups, OA contains 20 free amino groups

[130]).

Immunization of animals.

Several DON-protein conjugates and immunization schedules were followed. Each of three New Zealand white female rabbits (6 lbs) were inoculated intradermally with 500 ug DON-CMO-BSA in 1 ml saline:Freund's complete adjuvant (1:1) at approximately 20 sites on its shaved back (133,134). At 6 wk intervals, each rabbit was boosted with 250-500 ug conjugate in saline:Freund's incomplete adjuvant (1:1), administered intramuscularly. The rabbits were bled via the marginal ear vein 2 wks after each booster inoculation (133).

A total of 28 mice were inoculated intraperitoneally or subcutaneously with either DON-CMO-BSA, 3-HS-DON-BSA, 3-HG-DON-BSA, or 3-HG-15-Ac-DON-BSA. Initial intraperitoneal inoculations were 100-250 ug conjugate in 0.5 ml saline:Freund's complete adjuvant (1:1), followed 2 weeks later by 100-250 ug conjugate in 0.5 ml saline:Freund's incomplete adjuvant (1:1) (133,134). Subsequent intraperitoneal booster inoculations followed at 2-6 week intervals, and consisted of 100-250 ug conjugate in 0.5 ml saline. Subcutaneous inoculations were made in the shoulder region with 250-500 ug conjugate in 0.5 ml saline, boosted at 2-6 week intervals with a similar dosage. Mice were bled 1 week after each booster inoculation via the retro-orbital venous plexus (133).

Antiserum was prepared by allowing the blood collected from immunized animals to clot overnight at 4 C, then centrifuging and drawing-off the liquid serum. Antisera were screened for anti-DON activity by the indirect-competitive EIA described below.

Hybridoma production.

Hybridoma cell lines secreting DON-specific antibody were produced by modification of the method of Siriganian et al (150). A mouse exhibiting DON-specific antibody activity was sacrificed and immersed in 70% ethanol for 1 min. The spleen was excised and washed in a culture dish containing 5 ml of 2.5% fetal bovine serum in complete Dulbecco's Modified Eagle Medium (2.5% FBS-cDMEM). Complete DMEM (cDMEM) contains 1% NCTC, 10 mM sodium pyruvate, 1 mM oxaloacetate, insulin (0.002 units/ml), penicillin (100 ug/ml), and streptomycin (100 ug/ml). The spleen was cut into several pieces which were placed in a sterile tube containing 5 ml of 2.5% FBS-cDMEM and gently mashed with a teflon pestle. After allowing the debris to settle, the cell suspension was transfered to a 15 ml centrifuge tube with an additional 5 ml of 2.5% FBS-cDMEM and centrifuged at 450 xg x 8 min at room temperature. The pellet was resuspended in 10 ml fresh 2.5% FBS-cDMEM and recentrifuged. After resuspending the pellet in 10 ml of 2.5% FBS-cDMEM, the cells were stained with trypan blue for 5 min and unstained, living spleen cells counted using a

hemacytometer.

P3/NS1/1-Ag4-1 myeloma cells (NS-1) (a hypoxanthine-guanine phosphoribosyl-transferase [HGPRT] deficient line) were kept at log phase in 20% FBS-cDMEM at 37 C, 8% CO_2, for one week prior to fusion. Myeloma cells were pelleted by centrifugation and resuspended in 10 ml of 2.5% FBS-cDMEM.

For fusion, 2 x 10^7 myeloma cells and 2 x 10^8 spleen cells were placed together in a 50 ml conical tube and centrifuged at 300 xg x 8 min at room temperature. The pellet was resuspended in 0.4 ml of 50% polyethylene glycol (MW 1450) in cDMEM which had been vortexed until pH 8.0-8.5 (the solution will turn fuschia). The cells were centrifuged at 200 xg x 8 min at room temperature. Then 10 ml of 2.5% FBS-cDMEM was added dropwise to the cells, and after 2 min, another 10 ml of the same medium. The cells were centrifuged at 300 xg x 8 min, the supernatant removed, and the pellet resuspended in 10 ml of 20% FBS-cDMEM. After 20 min at room temperature, an additional 90 ml of the same medium was added.

The fused cells (100 ml) were added to 100 ml of 20% FBS-cDMEM containing 1 x 10^7 spleen cells (unfused) which were used as "feeder" cells to supply additional growth factors to the hybridomas. To each of the 60 inner wells of 96-well tissue culture plates was added 200 ul of the cell suspension; 200 ul of cDMEM was added to the outer wells. The cells were incubated at 37 C in an atmosphere of 8% CO_2. After 24 hr, 100 ul of the culture medium was removed from

each well and replaced with 100 ul of HAT medium (13.61 mg hypoxanthine, 3.875 mg thymidine, 167 ug aminopterin, 1000 ml 20% FBS-cDMEM). The growth of colonies was accompanied by a drop in the pH of the medium, indicated by the normally red medium turning yellow. As the medium turned yellow, the colonies were fed with HAT medium as before. After 10 days, unfused myeloma cells should have died, and colonies were fed with HT medium (HAT without aminopterin) instead of HAT medium.

Hybridoma colony supernatants were screened for anti-DON antibody activity in the indirect-EIA described below. Positive colonies were transfered to 24-well tissue culture plates and grown at a volume of 0.5 ml until confluent. Antibody activity was rescreened at this stage, and colonies which continued to show anti-DON activity were cloned by limiting dilution. The colony was suspended in the medium and the cell concentration determined. An aliquot was serially diluted in macrophage-conditioned HT medium (152), and 200 ul (containing an average of 1, 3 or 10 cells) were placed in each of the 60 innermost wells of a 96-well tissue culture plate (generally, 2 plates at each cell concentration). Selected colonies (from dilutions yielding less than 50% of the seeded wells containing dividing cells) testing positive for anti-DON antibody activity were recloned by the same dilution method until a high probability of monoclonality was achieved (115). Selected hybridomas with anti-DON activity were stored in

FBS:dimethylsulfoxide (9:1) in liquid N_2.

Precipitation of antibody from ascites.

Reagent anti-DON antibodies were precipitated from ascites fluid (137). BALB/c mice were injected interperitoneally with 0.5 ml pristane (2,6,10,14-tetramethylpentadecane) each. From one to nine weeks later, each mouse was injected interperitoneally with 1 x 10^7 hybridoma cells from a log phase culture which were suspended in 0.5 ml of 20% FBS-cDMEM. As the abdomens of the mice became swollen (after 7-10 days), ascites fluid was collected by inserting an 18-gauge hypodermic needle into the abdomen and allowing the viscous fluid to drain.

Ascites fluid was centrifuged at 5000 xg x 10 min, the supernatant diluted four-fold with ice-cold PBS and placed on ice. An equal volume of saturated ammonium sulfate solution (4 C) was slowly added with stirring (final concentration was 50% ammonium sulfate). The solution was left on ice for 60 min, then centrifuged at 5000 xg x 10 min. The pellet was dissolved in PBS and reprecipitated twice.

Indirect-competitive enzyme immunoassay.

Microtiter plates (96 well Immulon I, Dynatech Laboratories, Inc., Alexandria, VA 22314) were coated with the appropriate DON-OA conjugate by incubating 50 mM carbonate buffer (pH 9.6) containing DON-OA or OA control at 10 ug/ml (100 ul/well) at 4 C, overnight. After washing the

plates twice with 0.1 M sodium phosphate buffer, normal saline containing 0.05% Tween-20 (PBS-T) (300 ul/well), 300 ul of 1% OA in PBS was added to each well to block sites on the polystyrene not occupied by the DON-OA conjugate. The plates were incubated at 37 C for 60 min. After washing three times with PBS-T, 50 ul of 1% methanol in PBS with or without DON (10 ug/ml) was added to each well, followed by 50 ul of the appropriate antiserum diluted 1:50, 1:200, 1:800 or 1:3200 in PBS, or hybridoma supernatant. The plates were incubated at 37 C for 60 min. After washing three times with PBS-T, 100 ul of goat-anti-mouse immunoglobulin conjugated to horse radish peroxidase (GaM-HRP) diluted 1:500 in PBS containing 1% OA was added to each well and incubated for 30 min at 37 C. The plates were washed 8 times with PBS-T, and then 100 ul substrate (400 uM ABTS, 0.009% hydrogen peroxide in 45 mM sodium citrate buffer, pH 4.0) added to each well. When sufficient color developed, 100 ul stopping reagent (300 mM citric acid, 15 mM sodium azide) was added to each well, and the absorbance at 405 nm read on an EIA Reader Model EL 308 (Bio-Tek Instruments, Winooski, VT 05404). Antiserum was considered to contain DON-specific antibody (anti-DON) if binding of antibody to the solid-phase DON-OA (indicated by absorbance greater than for solid-phase OA control binding) was inhibited by free DON.

Direct-competitive enzyme immunoassay.

Extracts of maize were assayed using anti-DON antibody precipitated from ascites fluid. The optimum concentrations of antibody and DON-HRP were determined experimentally using serial dilutions of these reagents. Into each well (Immulon 2 Removawell strips, Dynatech) was dispensed 125 ul antibody (3 ug/ml PBS containing 0.0001% OA). The antibody was dried onto the wells in a forced air oven at 50 C. DON-HRP (diluted 1:10,000 in PBS containing 1% BSA) was mixed with an equal volume of maize extract (described below) or standards, and 100 ul of this mixture was immediately added to each well. Standards were prepared in water or in extracts of maize testing negative for DON by TLC analysis (131). After incubation at 37 C for 40 min, the wells were washed 8 times with PBS-Tween and 100 ul ABTS reagent added. The reaction was stopped as before, and absorbance at 405 nm determined for each well.

Detection of deoxynivalenol in spiked samples.

Maize kernels were ground in a Model-2A Romer Mill (Romer Laboratories, Washington, MO 63090). Methanolic DON (up to 0.5 ml) was added to 4 g ground grain to achieve the desired concentration, and the samples were air-dried, overnight. The spiked ground kernels were extracted with 20 ml methanol:water (7:3) for 60 min at room temperature with vigorous shaking in a 50 ml conical centrifuge tube. The samples were then centrifuged at 1000 xg for 10 min, and 10

ml of supernatant drawn off. The supernatant was reduced to between 1 and 2 ml in a rotary evaporator, and the volume adjusted to 2 ml with distilled water. The solution was centrifuged at 1000 xg for 10 min, and the supernatant poured off and assayed by the direct-competitive EIA. Standards were prepared by adding the appropriate amount of DON to final extracts (i.e., resuspended in water) of unspiked grain.

RESULTS

Confirmation of deoxynivalenol derivatization.

DON-CMO purified by silica gel liquid chromatography was analyzed by silica gel-TLC developed in chloroform:methanol (1:1) and visualized with NBP. Two spots with R_f=.49 and .44 had a blue color reaction with NBP. Underivatized DON reacted blue with NBP and had an R_f=.84. Since no useful antibodies were elicited by this derivative, no further structural determinations were performed.

The hemisuccinyl derivative of DON was synthesized by esterification with succinic anyhydride either directly, or after protection of C7 and C15 hydroxyls with a cyclic boronate ester (Figure 20). When analyzed by TLC (10 x 10 cm Whatman LHP-K silica gel TLC plates) developed in chloroform:methanol (1:1), direct esterification of DON yielded three spots (R_f=.33, .60 and .68) which turned blue when treated with NBP; the slowest migrating compound (R_f=.33) gave the most intense spot of the three (Figure 21,A). The mass spectrum (MS) contained peaks at m/z 297, 397 and 497 in the approximate ratio of 1:1:4 (Table 7). The mass spectrum of underivatized DON had the expected $M+H^+$ peak at m/z 297 (Figure 22).

TLC analysis of DON reacted with succinic anhydride after protection of C7 and C15 hydroxyls by a cyclic boronate ester (3HS-DON) showed a single spot (R_f=.61) that

turned blue when treated with NBP (Figure 21,A). This compound migrated more slowly than DON (R_f=.84). DOM-1 (12,13-deepoxy-DON) did not turn blue when treated with NBP.

When sprayed with aluminum chloride:methanol:water (15:85:15 [w/v/v]) and heated at 120 C for 6 min (104), DON and 3HS-DON fluoresced blue under long wave ultraviolet light (Figure 21,B). T-2 toxin, lacking the C8-ketone, did not fluoresce after aluminum chloride treatment.

The 3HS-DON turned yellow when sprayed with 0.04% bromcresol purple in 50% aqueous ethanol, as did the succinic acid control (Figure 21,C). DON did not turn yellow with bromcresol purple treatment.

Development of TLC plates in chloroform:methanol (75:25) yielded R_f values of .76 and .43 for DON and 3HS-DON, respectively (Figure 23,A). However, when the developing solvent was acidified, as chloroform:methanol:acetic acid (75:15:10), migration of 3HS-DON increased (R_f=.86), while the R_f of DON was unchanged (Figure 23,B).

In order to check for contaminants in the 3HS-DON preparation that would not have been detected by the other visualization procedures, TLC plates were also sprayed with 30% aqueous H_2SO_4 and heated to 100 C. This universal reagent detects many organic compounds (107). Only a single charred spot was observed for the 3HS-DON preparation.

The mass spectrum for 3HS-DON (Figure 24) had peaks at m/z 397, 419 and 489, to which were assigned 3HS-DON, 3HS-DON+sodium and 3HS-DON+glycerol, respectively. Other peaks

with assignments were m/z 115, glycerol+sodium; 185 $(glycerol)_2$; 207, $(glycerol)_2$+sodium. Unassigned peaks probably represent fragments.

The proton magnetic resonance (^{1}H-NMR) spectrum for DON (Figure 25) agrees with a previously published spectrum for DON (114). The ^{1}H-NMR spectrum for 3HS-DON (Figure 26) shows the expected shift of H3 (4.55 to 5.21 ppm), which is similar to the H3 peak in the spectrum of 3-acetyl-DON published previously (114). The protons at positions 7 and 15 were unchanged, indicating these positions did not undergo esterification.

The yield from 20 mg DON was 15 mg DON-equivalents of 3HS-DON. Conjugation ratios (moles of DON per mole of protein) were typically 5-10 for DON-OA conjugates and 20-30 for DON-BSA conjugates (conjugation ratios for DON-HRP were not determined).

Fusion results.

Although antisera produced in response to all DON-BSA conjugates did bind to solid phase DON-OA in an indirect EIA, only 3HS-DON-BSA elicited antisera in which antibody binding to solid phase DON-OA was inhibited by free DON. The hybridoma line secreting DON-specific antibody used in maize screening assays was derived from a mouse injected subcutaneously, with 500 ug DON-BSA in normal saline, boosted with 500 ug DON-BSA on week 4, and with 250 ug DON-BSA on weeks 10, 12, 21, and 29). Based on calculations

presented by Coller and Coller (115) the antibody-secreting hybridoma line selected for enzyme immunoassays had a probability of monoclonality of 0.95 after 2 subclonings (Table 8).

Characterization of monoclonal antibody.

The monoclonal antibody used for EIA (aDON-1) was IgG_1 subclass with k-light chain, determined using a mouse Ig subclass-identification kit (Boehringer Mannheim Biochemicals, Indianapolis, IN 46250).

The antibody had a higher affinity for 3-acylated DON (3-acetyl-DON and 3HS-DON) than for DON, while fusarenone-X and nivalenol (differing from DON at C4) reacted somewhat less than DON, and 15-acetyl-DON and T-2 toxin cross-reacted poorly (Figure 27, Table 9).

In both direct- and indirect-competitive EIA, aDON-1 had a sensitivity range of from 10 to 250 ng per assay (Figure 28). Although sensitivity of the direct-competitive EIA was only slightly greater for standards dissolved in water than for standards in maize kernel extract, the absorbance increased more rapidly for water-standards than for extract-standards (Figure 29). It was necessary for samples to be aqueous, as even 10% methanol interfered with the assay.

Detection of deoxynivalenol in spiked samples.

Triplicate samples (4 g each) of ground maize kernels were individually spiked with DON, extracted, and assayed by the direct-competitive EIA. The recovery (DON detected/DON added) was 97.3 and 66.3% for samples spiked with 0.3 and 3.0 ug/g (Table 10).

DISCUSSION

The reaction of DON with carboxymethoxylamine under conditions for oxime formation resulted in at least two compounds which migrated more slowly than DON on TLC. Since the carbon atom at position eight, involved with oxime formation, is part of a six-membered ring, two isomeric oximes are possible, i.e., the oxygen-nitrogen bond may lie above or below the plane of the ring. Pejković-Tadić et al (146) showed that α and β isomeric oximes could be separated by silica gel-TLC. The reaction of ketones with oxylamines to form oximes is a commonly used and well-characterized reaction. The decreased mobility of DON on silica gel-TLC after reaction with carboxymethoxylamine is expected for the addition of a carboxyl. Although no additional analyses were performed, it is likely that the two spots on TLC were α and β isomers of DON-CMO.

DON esterified directly with succinic anhydride yielded at least 3 compounds detected by TLC ("HS-DON mixture") (Figure 21,A). Since the addition of a hemisuccinate by esterification would contribute 100 to the molecular weight of DON (MW 296), the three peaks revealed by MS at m/z 297, 397 and 497 could represent $M+H^+$ of DON, HS-DON and $(HS)_2$-DON, respectively (Table 7). It is not clear, however, whether the lower MW peaks were actually present in the derivative mixture or were fragments formed in the mass spectrometer by sequential loss of hemisuc-

cinates. The highest MW fragment (m/z 497) was in greatest abundance, however, and this could correspond to the most intense spot on TLC (R_f=.33), the slower migration in TLC explained by the substitution of two hemisuccinates, with the two rapidly migrating spots being isomers of hemisuccinyl-DON. No peak for $(HS)_3$-DON ($M+H^+$=597) was seen in the mass spectrum, although this may indicate it was very unstable. We did not determine the specific positions of the hemisuccinates for the compounds in this mixture, although one compound had an R_f similar to 3HS-DON described below (Figure 21,A). Initial attempts at separating the several derivatives in the mixture were unsuccessful, and an alternate method of obtaining a single hemisuccinate derivative of DON was devised.

By protecting C7 and C15 during esterification with succinic anhydride, a single derivative, 3HS-DON, was synthesized (Figure 20). Although phenylboronate derivatives of 1,3-diols have been described previously (105,108,119,120,121,147,153,167), a phenylboronate derivative of DON could not be isolated. This may have been due to steric hindrance by the large, rigid ring, since the butylboronate ester formed readily. The butylboronate ester was easily hydrolyzed by addition of acetic acid to the elution solvent during liquid chromatography. The inclusion of benzene in the elution solvent allowed subsequent removal of acetic acid as an azeotrope.

The derivatization of DON to 3-O-hemisuccinyl-DON was

confirmed by TLC, FAB-MS and ^{1}H-NMR. In order to be useful in eliciting antibodies with affinity to DON, it was important that the DON molecule was unmodified, except for addition of the hemisuccinyl required for protein conjugation. Visualization of TLC plates with various reagents demonstrated the presence of specific groups on the 3HS-DON molecule. NBP reacts with the 12,13 epoxide of trichothecenes to give a blue color (156). The 3HS-DON, as well as DON, appeared blue when treated with NBP reagent, indicating that the 12,13-epoxide remained intact; DOM-1 (deepoxidated DON) did not turn blue, which demonstrated the specificity of the NBP reagent (Figure 21,A). Aluminum chloride is relatively specific for visualizing trichothecenes possessing the 8-keto moiety, the so-called Type-B trichothecenes (104). When treated with aluminum chloride, 3HS-DON fluoresced as did DON, suggesting that the ketone at C8 was still present in the derivative; included as a negative control, T-2 toxin, a Type-A trichothecene lacking the C8 ketone, did not fluoresce (Figure 21,B). Derivatization of DON to 3HS-DON was accompanied by an increase in acidity, which would be expected for addition of a carboxyl. 3HS-DON turned bromcresol purple (pK_{ind} 6.1) yellow, indicating increased acidity of the derivative compared to DON which did not cause the color change, while succinic acid, included as a positive control, also turned the indicator yellow (Figure 21,C). Additional evidence for the presence of a carboxylic acid moiety on the derivative came

from protonation of 3HS-DON when developed by TLC. Un-acidified solvent (chloroform;methanol, 75:25) resulted in an R_f of .43, but when acidified (chloroform:methanol:acetic acid, 75:15:10) the R_f increased to .86 (Figure 3). The increased migration rate is suggestive of an acid changing from an ionized to an unionized state. The migration rate of DON (R_f=.77), by comparison, was not altered by acidification of the solvent.

The mass spectrum of 3HS-DON confirmed the expected mass for $M+H^+$ of m/z 397, i.e., 297 for DON plus 100 for the hemisuccinate moiety (Figure 24). Comparison of ^{1}H-NMR spectra for DON and 3HS-DON shows the shifts expected for the 3-hemisuccinyl derivative (Figures 25,26). The ^{1}H-NMR spectrum of 3HS-DON is similar to the spectrum of 3-acetyl-DON reported previously (114).

The competitive enzyme immunoassays utilizing monoclonal antibody elicited by 3HS-DON-BSA detected DON at 0.2-5.0 ug/ml (direct) and 0.2-2.0 ug/ml (indirect). This range is useful as the Food and Drug Administration has issued a "level of concern" of 2 ug DON/g whole grain, and DON is regulated at these levels in Canada (1 ug/ml in these EIA represents 1 ug/g grain). While the direct-EIA had a somewhat greater range, the slope of the indirect-EIA was greater, therefore changes in DON concentration result in more dramatic changes in absorbance with the indirect-EIA, enhancing quantification. The direct-EIA is desirable, however, since incubation with the second (GaM-HRP)

antibody-conjugate is unnnecessary, and for routine screening is the method of choice. Standards dissolved in water result in a more rapid absorbance increase than standards in maize kernel extracts, although the sensitivity is not drastically different (Figure 29). Therefore, EIA results for sample extracts must be compared to standards dissolved in extracts from uncontaminated maize, although this is less convenient than if standards were in water.

In order to detect haptens in an EIA, antibody binding to solid phase (e.g., hapten-protein conjugates attached to polystyrene microtiter wells) must be competitively inhibited by free hapten in the sample. Previous attempts at producing such antibodies against DON have been unsuccessful (168). It is not clear why DON does not elicit useful antibodies as readily as, for example, T-2 toxin (110, 125,136). The reason may be that T-2 toxin is more highly substituted than DON (Table 9). Based on this possibility, 15-Ac-DON-BSA conjugates were used as antigens. No antibodies were detected which were useful in a competitive EIA against either DON or 15-Ac-DON. Increasing the spacer between DON and the carrier protein by using hemiglutaryl-DON derivatives was also unsuccessful. The 3HS-DON-BSA conjugates did, after many inoculations, finally elicit useful antibodies. However, while the antisera from two mice showed the presence of antibodies useful in a competitive EIA, no hybridomas derived from these mice secreted such antibodies. Hybridomas secreting useful anti-DON were, for-

tunately, obtained from a third mouse.

Many antisera and hybridoma supernatants contained antibody which bound DON-protein conjugates, but this binding was not competitively inhibited by free DON, and therefore not useful in a competitive EIA. The higher affinity of these antibodies for DON-protein conjugates than for free DON suggests the recognition of DON, the bridge region (e.g., succinyl) and possibly part of the protein (e.g., lysine side-chain) by these antibodies. Even the antibody which has affinity for free DON has an even higher affinity for derivatives containing esters at C3, i.e., 3HS-DON and 3-Ac-DON (Figure 27, Table 9). The low affinity of this antibody for 15-Ac-DON suggests that much of the DON molecule is recognized, since C15 is nearly on the opposite side of the molecule from C3 (Table 9).

An antibody has recently been developed against 3,7,15-triacetyl-DON in another laboratory (168). However, the radio-immunoassay requires acetylation of DON in contaminated grain samples (165). This acetylation reaction, including several subsequent clean-up steps, is unnecessary with the anti-DON antibody described here. The enzyme immunnoassay described here does not involve the health hazard involved in the radio-immunoassay. The antibody and EIA described herein will be useful in the rapid and simple routine screening for DON in contaminated grain samples. The antibody may also be useful for other laboratory detection of DON, for example, screening for DON-nonproducing

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Table 7. Relative abundance of selected ions in positive ion FAB-mass spectrum of hemisuccinyl-deoxynivalenol mixture (HS-DON mixture) run in glycerol, obtained on a JEOL HY-110 HF mass spectrometer.

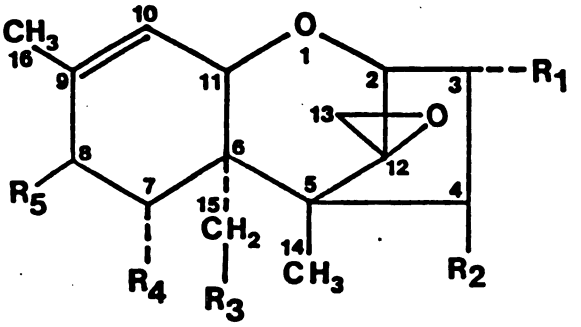
m/z	relative abundance	assignment
297	0.22	DON [1]
397	0.24	HS-DON
497	1.00	$(HS)_2$-DON

[1] DON = deoxynivalenol
HS-DON = hemisuccinyl-deoxynivalenol
$(HS)_2$-DON = dihemisuccinyl-deoxynivalenol

Table 8. Results of fusion of spleen cells from mouse with antiserum demonstrating anti-deoxynivalenol (DON) activity and NS-1 murine myeloma cells. The percentage of wells seeded with hybridoma suspension that developed colonies was used to determine the probability of monoclonality, P(1), after each cloning. Colonies expressed anti-DON activity when antibody binding to solid phase DON was inhibited by free DON in an indirect-competitive EIA.

	No. wells seeded	% wells with colonies	P(1)	% colonies with anti-DON activity
Fusion	827	100	---	3.6
First cloning	120	38	.77	10.8
Second cloning	120	63	.95	95.3

Table 9. Structures of deoxynivalenol (DON) analogs.



Compound	R1	R2	R3	R4	R5
Deoxynivalenol	OH	H	OH	OH	=O
Nivalenol	OH	OH	OH	OH	=O
Fusarenone-X	OH	OAc[1]	OH	OH	=O
3-Acetyl-DON	OAc	H	OH	OH	=O
DON-3-HS	OHS[2]	H	OH	OH	=O
15-Acetyl-DON	OH	H	OAc	OH	=O
T-2 toxin	OH	OAc	OAc	H	OIp[3]

[1] OAc = $-OOCCH_3$
[2] OHS = $-OOC(CH_2)_2COOH$
[3] OIp = $-OOCCH_2CH(CH_3)_2$

Table 10. Recovery of deoxynivalenol (DON) from spiked ground maize kernels. Samples were extracted and assayed by a direct-competitive enzyme immunoassay as described in the text.

DON added (ug/g)	DON detected[1] (ug/g)	Recovery (%)
0	.092 ± .023	---
0.3	.292 ± .023	97.3 ± 13.0
3.0	1.99 ± .344	66.3 ± 34.4

[1]Concentrations of DON for triplicate samples derived from regression analysis of standards in clean maize extract (r^2=.911).

Figure 20. Steps in derivatization of (A), deoxynivalenol: (B), 7,15-O-butylboronyl-deoxynivalenol; (C), 3-O-hemisuccinyl-7,15-O-butylboronyl-deoxynivalenol; (D), 3-O-hemisuccinyl-deoxynivalenol.

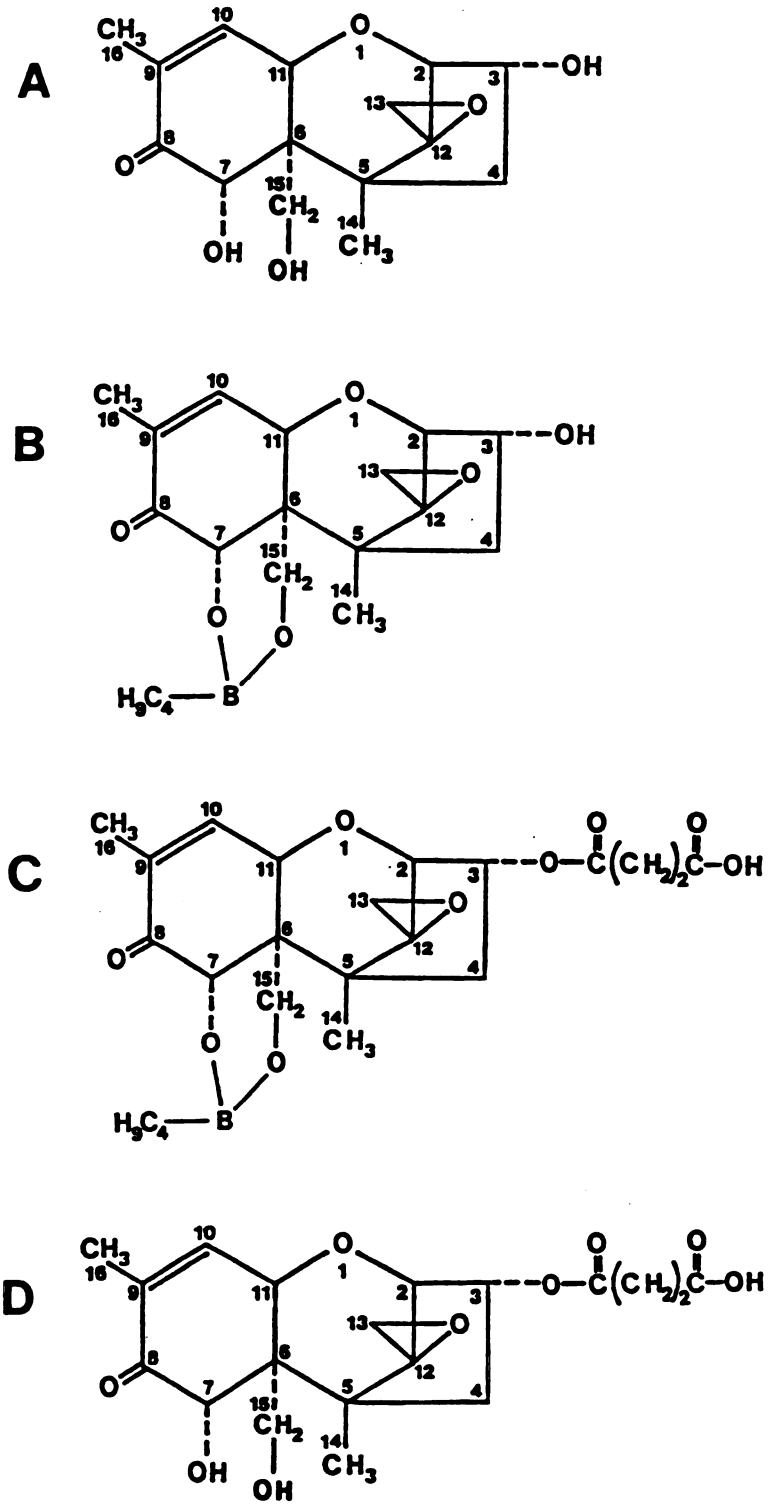


Figure 20.

Figure 21. Thin-layer chromatograhy of deoxynivalenol (DON) and derivatives developed in chloroform:methanol (1:1): (A) DON (lane 1), 3-O-hemisuccinyl-DON (lane 2), hemisuccinyl-DON mixture (lane 3), 15-acetyl-DON (lane 4), 3-O-hemiglutaryl-15-acetyl-DON (lane 5), and 12,13,deepoxy-DON (lane 6) visualized with nitrobenzylpyridine; (B) DON (lane 1), 3-O-hemisuccinyl-DON (lane 2) and T-2 toxin (lane 3) visualized with aluminum chloride; (C), DON (lane 1), 3-Ohemisuccinyl-DON (lane 2) and succinic acid (lane 3) visualized with bromcresol purple.

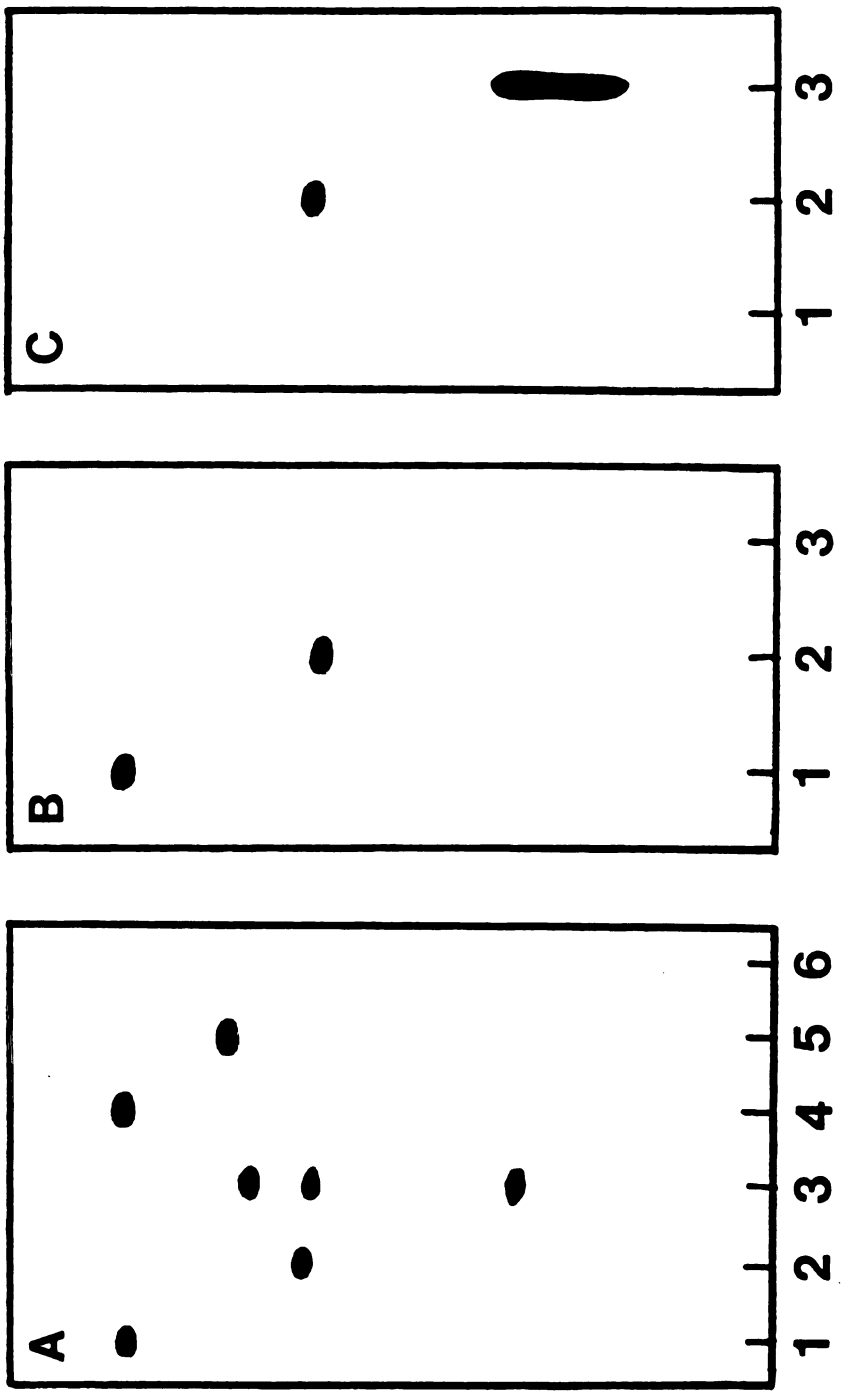


Figure 21.

Figure 22. Positive ion FAB-mass spectrum of deoxynivalenol (DON) run in glycerol, obtained on a JEOL HY-110 HF mass spectrometer. Assigned peaks: m/z 277, $(glycerol)_3$; 297, DON; 369, $(glycerol)_4$; 389, DON+glycerol.

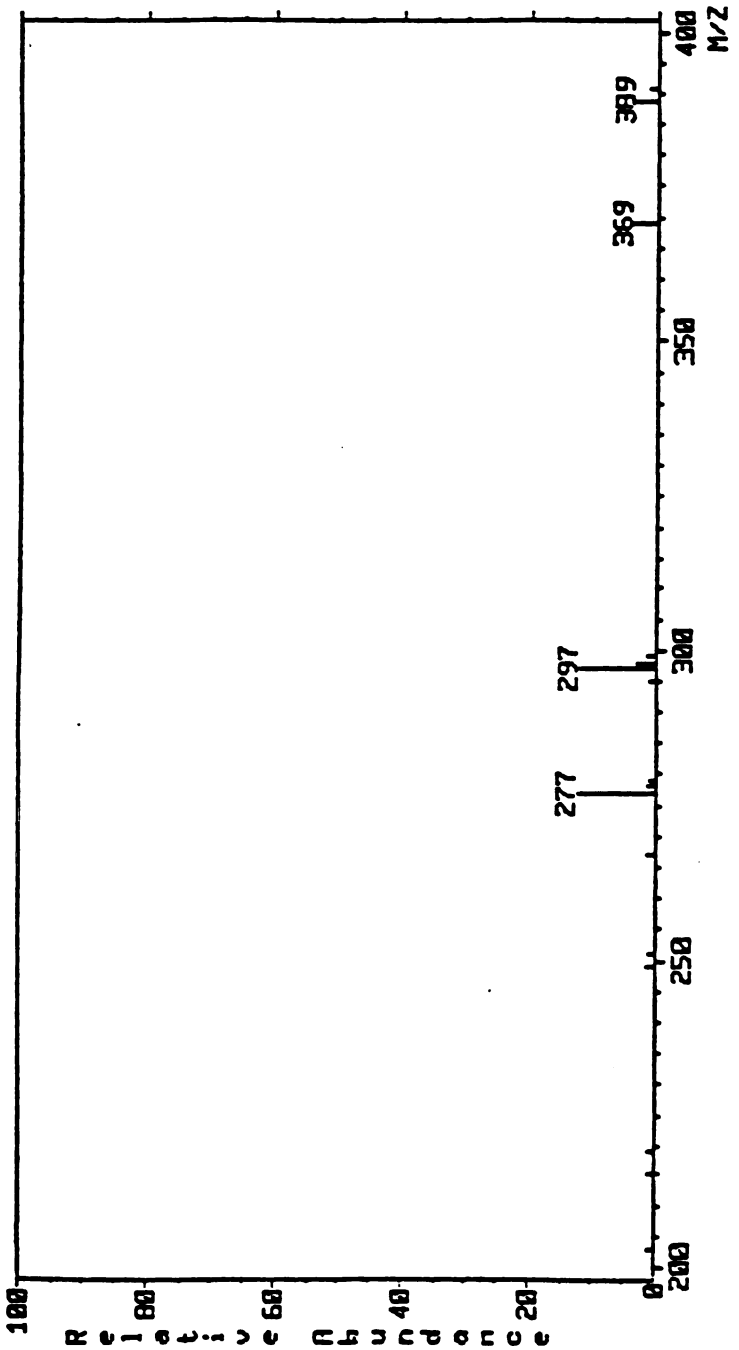

Relative Abundance
100
80
60
40
20
0
200
250
277
297
300
350
369
389
400
M/Z

Figure 22.

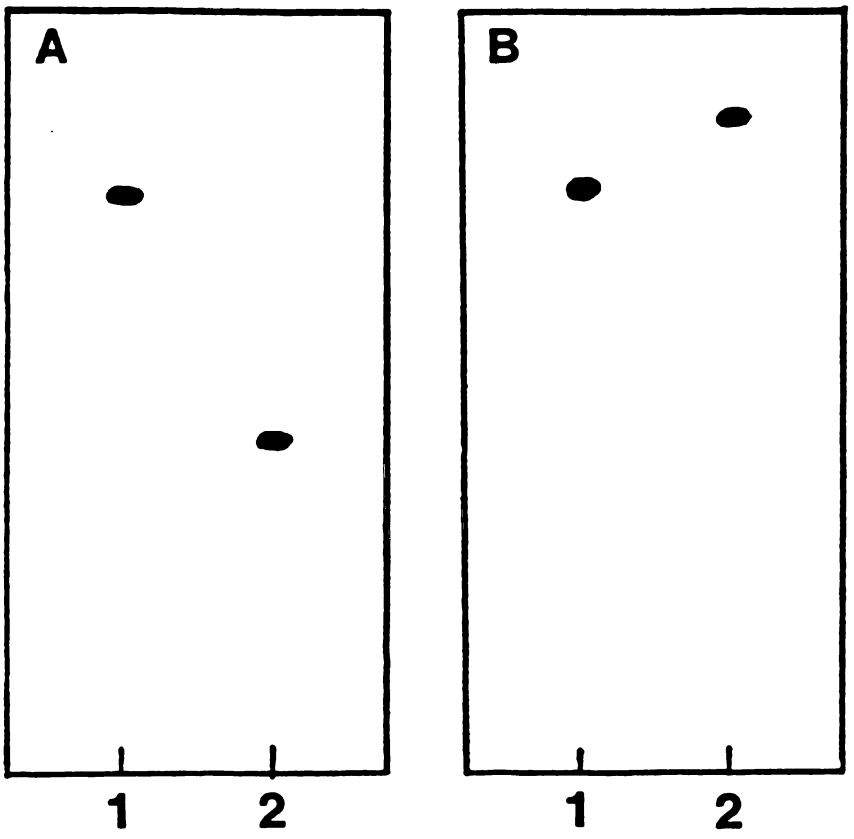


Figure 23. Thin-layer chromatography of deoxynivalenol (lane 1) and 3-hemisuccinyl-deoxynivalenol (lane 2) developed in (A), chloroform:methanol (75:25), or (B), chloroform:methanol:acetic acid (75:15:10), and visualized with nitrobenzylpyridine.

Figure 24. Positive ion FAB-mass spectrum of 3-O-hemisuccinyl-deoxynivalenol (3HS-DON) run in glycerol, obtained on a JEOL HY-110 HF mass spectrometer. Assigned peaks: m/z 115, glycerol+sodium; 185, $(glycerol)_2$; 207, $(glycerol)_2$+sodium; 397, 3HS-DON; 419, 3HS-DON+sodium; 489, 3HS-DON+glycerol.

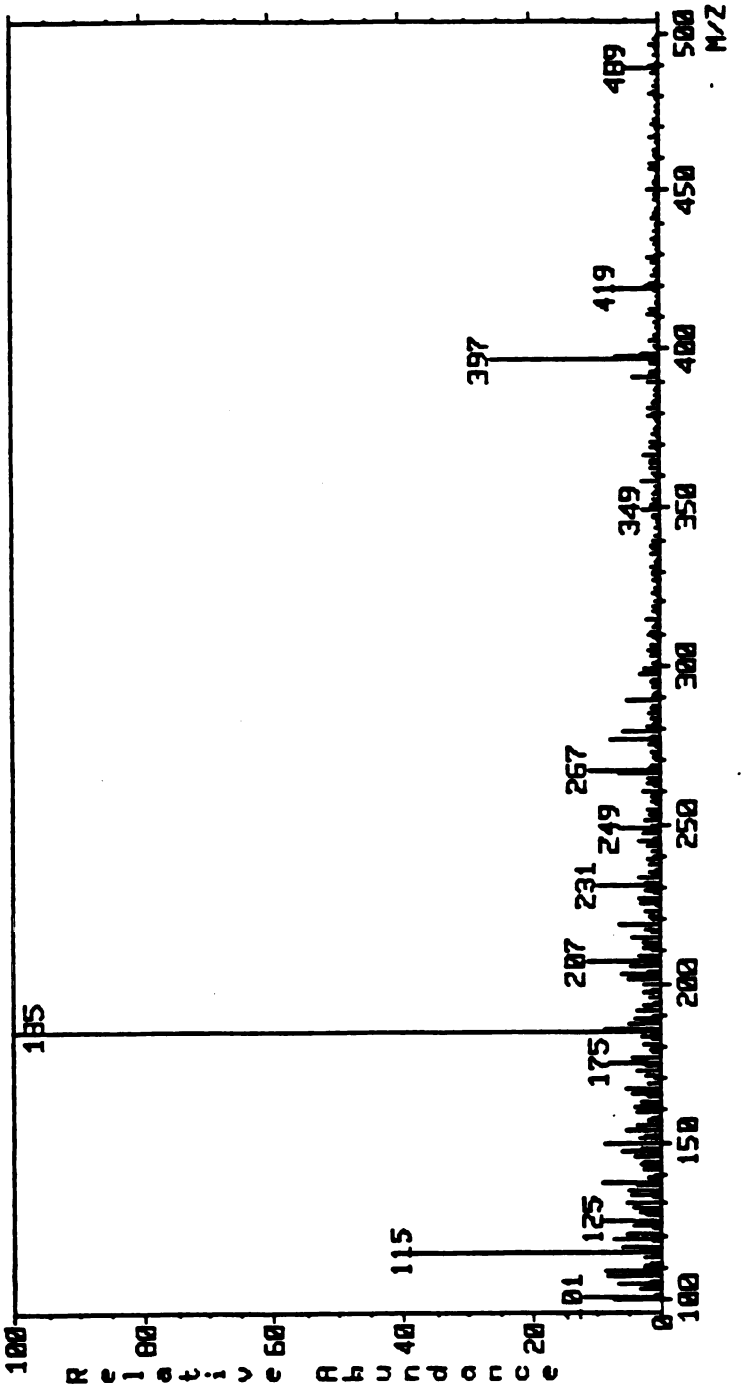

Relative Abundance
100
80
60
40
20
0
100
150
200
250
300
350
400
450
500
M/Z
101
115
125
135
175
207
231
249
267
349
397
419
489

Figure 24.

Figure 25. Proton magnetic resonance spectrum of deoxynivalenol (DON) in $CDCl_3$, obtained on a Bruker WM-250 (250 MHz) FT spectrometer. Assigned peaks (ppm): H2, 3.62; H3, 4.55; H4, 2.10; H7, 4.85; H10, 6.62; H11, 4.81; H13a, 3.09; H13b, 3.14; H14, 1.14; H15a, 3.77; 15b, 3.89; H16, 1.91.

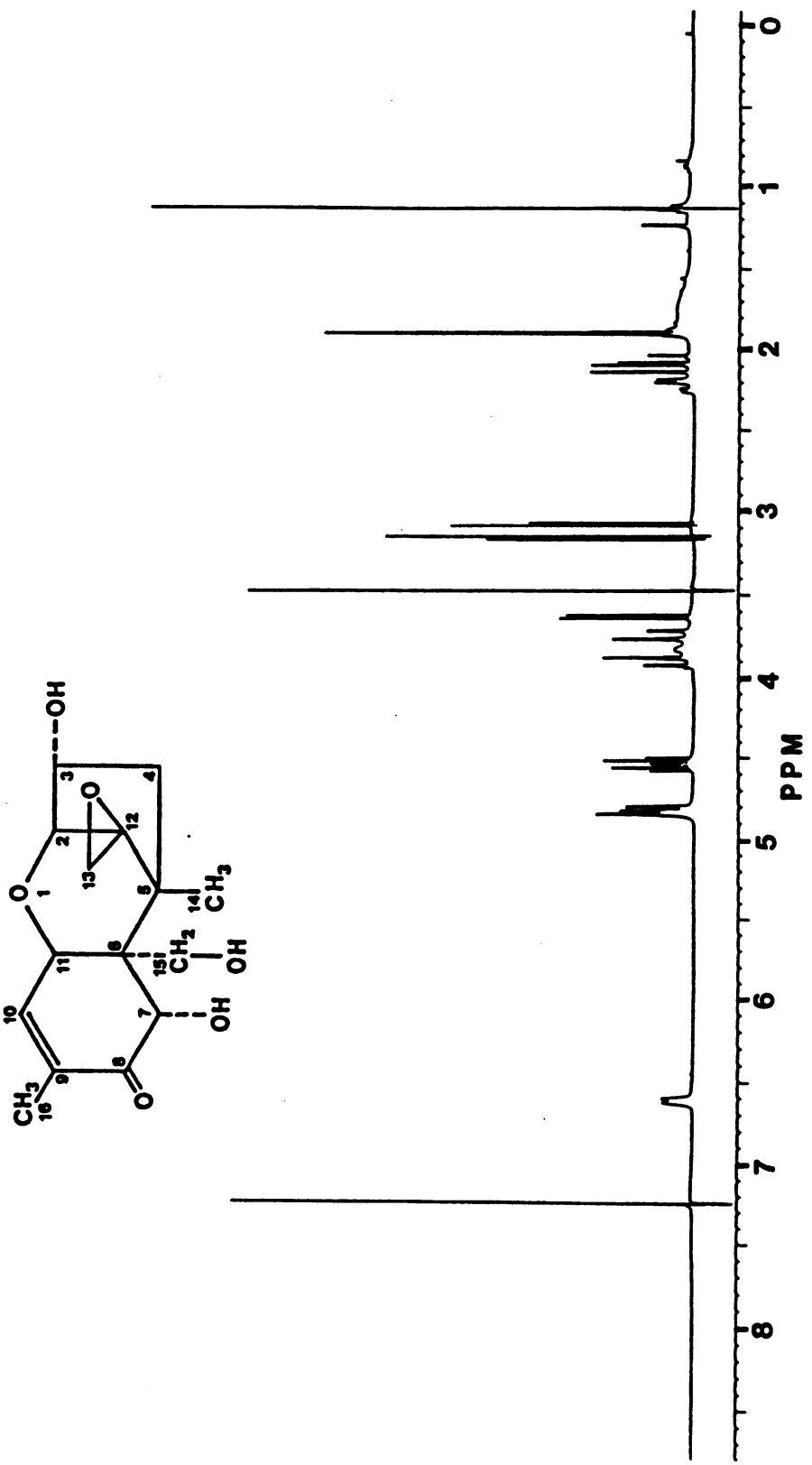


Figure 25.

Figure 26. Proton magnetic resonance spectrum of 3-O-hemisuccinyl-deoxynivalenol (3HS-DON) in $CDCl_3$, obtained on a Bruker WM-250 (250 MHz) FT spectrometer. Assigned peaks (ppm): H2, 3.49; H3, 5.21; H4, 2.20; H7, 4.83; H10, 6.61; H11, 4.68; H13a, 3.11; H13b, 3.17; H14, 1.15; H15a, 3.81; H15b, 3.90; H16, 1.89.

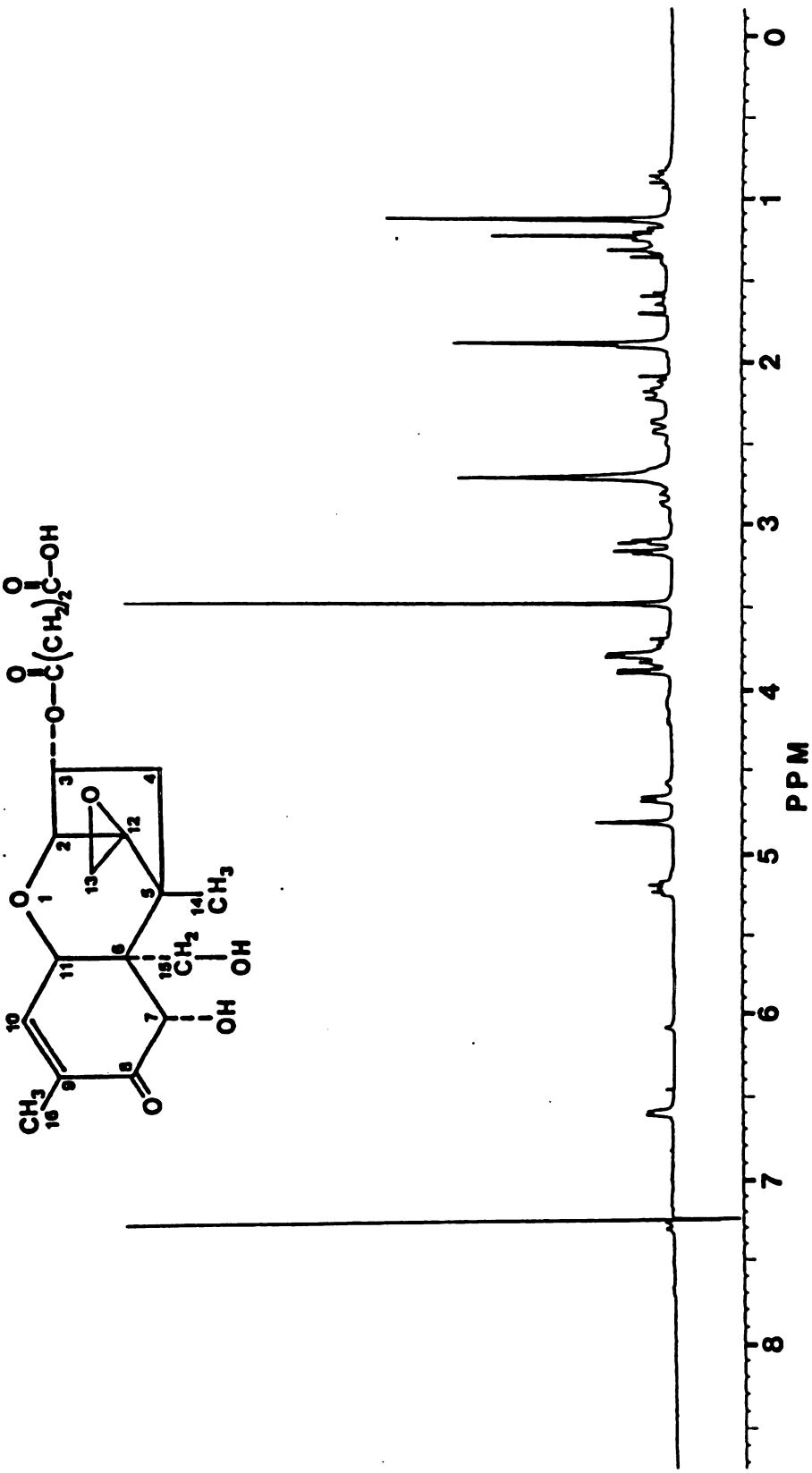


Figure 26.

Figure 27. Cross-reactivity of deoxynivalenol (DON) analogs in a direct-competitive enzyme immunoassay utilizing a monoclonal antibody elicited by 3-O-hemisuccinyl-deoxynivalenol (3HS-DON) conjugated to bovine serum albumin.

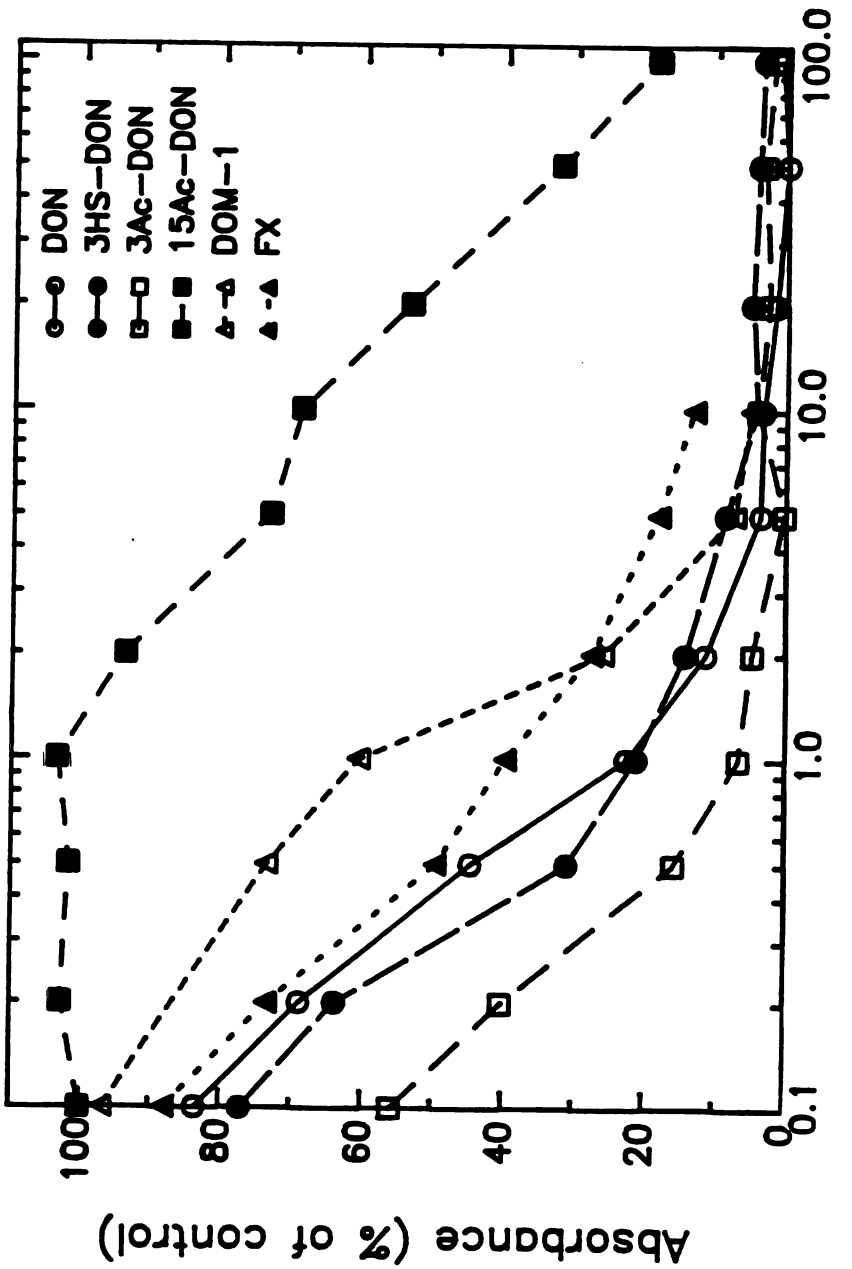

DON
3HS—DON
3Ac—DON
15Ac—DON
DOM—1
FX
100
80
60
40
20
0
Absorbance (% of control)
0.1
1.0
10.0
100.0
Concentration (μg/ml)

Figure 27.

Figure 28. Standard curves for deoxynivalenol (DON) dissolved in maize kernel extract by both direct- and indirect-competitive enzyme immunoassay. For regression lines of direct and indirect assay, r^2=.989 and .999, respectively.

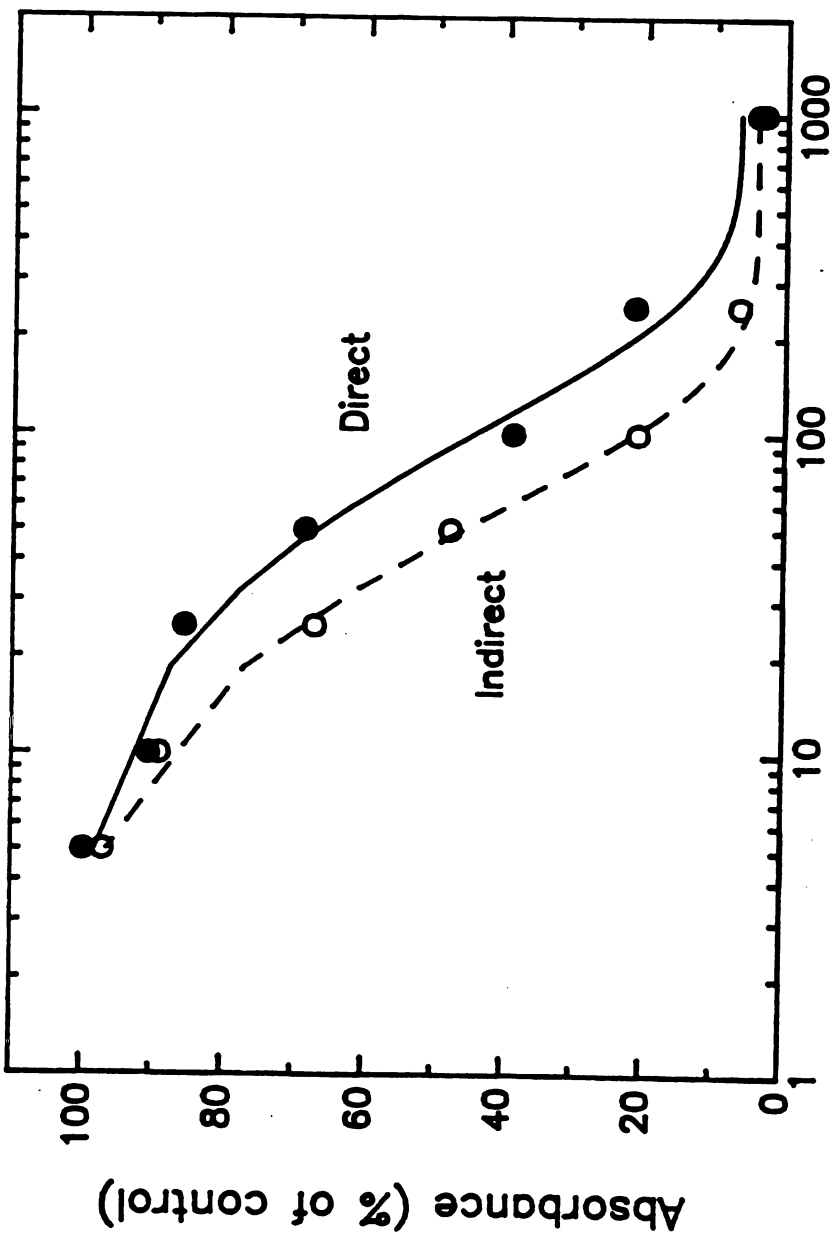

Absorbance (% of control)
100
80
60
40
20
0
1
10
100
1000
DON (ng/assay)
Direct
Indirect

Figure 28.

Figure 29. Direct-enzyme immunoassay of deoxynivalenol standards in maize kernel extract or water. After 90 min substrate incubation, the absorbances of water and extract controls were 1.082 and .839, respectively.

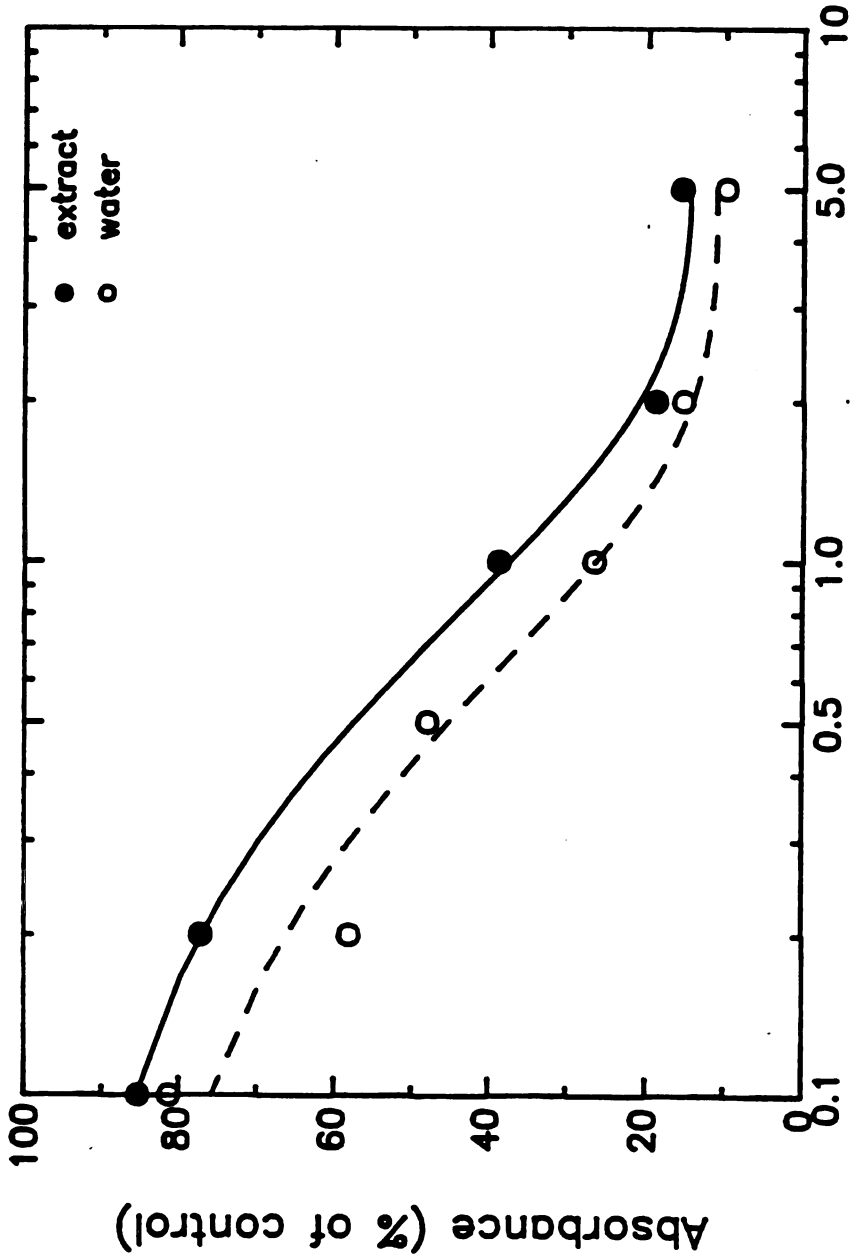

extract
water
100
80
60
40
20
0
0.1
0.5
1.0
5.0
10
Absorbance (% of control)
DON concentration (µg/ml)

Figure 29.

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