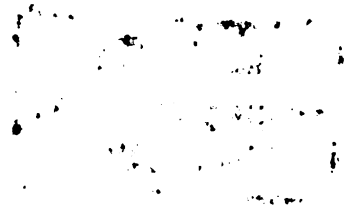

# PURIFICATION, IMMUNOTOXIC EFFECTS, AND CELLULAR UPTAKE OF TRICHOTHECENE MYCOTOXINS

By

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## ABSTRACT

# PURIFICATION, IMMUNOTOXIC EFFECTS, AND CELLULAR UPTAKE OF TRICHOTHECENE MYCOTOXINS

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Studies were carried out to better understand how the trichothecenes alter immune function in animals and humans. Deoxynivalenol (DON) was purified for use in animal feeding studies. Dietary exposure to DON for 8 weeks altered the serum immunoglobulin profile in mice and decreased the splenic plaque-forming cell response to the antigen sheep red blood cells. The uptake of [$^3$H]T-2 toxin by a murine B-cell hybridoma was studied in order to learn more about the way in which trichothecenes interact with immune cells.

A simple procedure was developed for the laboratory production and purification of gram quantities of crystalline DON. When Fusarium graminearum R6576 was grown on rice, concentrations of 600 to 700 ppm DON accumulated after 13 to 18 days of incubation. A DON derivative, 15-acetylDON, was also found at concentrations of 100 to 300 ppm after 7 to 10 days. DON was purified from crude culture extracts by water-saturated silica gel chromatography.

Alpha-[$^3$H]T-2 toxin of 99% chemical and radiochemical purity was prepared for use in uptake studies. Both the

rate of uptake of [$^3$H]T-2 toxin by hybridomas and the time required for accumulation of [$^3$H]T-2 to reach equilibrium were proportional to the concentration of [$^3$H]T-2. [$^3$H]T-2 toxin accumulated by hybridomas was proportional to the concentration of [$^3$H]T-2 between $10^{-8}$ and $10^{-3}$ M. The rate of uptake of [$^3$H]T-2 toxin by hybridomas was inhibited by the trichothecenes T-2 toxin, DON, verrucarin A, and roridin A, as well as the antibiotic anisomycin. Both the uptake of alpha-[$^3$H]T-2 by murine splenic lymphocytes and the uptake of beta-[$^3$H]T-2 by hybridomas were qualitatively similar to the uptake of alpha-[$^3$H]T-2 by hybridomas. The kinetics and concentration dependence of accumulation, along with the inhibition patterns, suggest that uptake of [$^3$H]T-2 toxin by hybridomas is mediated by binding of toxin to ribosomes.

## ACKNOWLEDGMENTS

I greatly appreciate the patient guidance and support of my major professor, Dr. James Pestka. I thank Dr. Deborah Dixon-Holland and Dr. Mohammed Abouzied for advice concerning cell culture; Dr. James Forsell for guidance with toxicity studies; Abdalla El-Bahrawy for help with fungal cultures; and Roscoe Warner for invaluable technical assistance.

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# INTRODUCTION

Fungi are capable of both primary and secondary metabolism. In contrast to primary metabolites, secondary metabolites are restricted in taxonomic distribution and are not essential for growth of the fungus. Furthermore, the physiological role of secondary metabolites in the producing organisms is unknown. Mycotoxins are one type of secondary fungal metabolite. These compounds cause toxic effects in mammals upon ingestion. One fungal genus that contains species capable of mycotoxin production is Fusarium. Among the mycotoxins produced by various Fusarium species is a group called the trichothecenes. The toxicology of the trichothecenes is currently an area of much interest, as these toxins have been identified in Fusarium-infected grains and so are likely to contaminate both the animal and human food supplies. This introductory section will provide the reader with a overview of the trichothecene mycotoxins and the rationale for the research contained in this thesis.

## Overview of trichothecenes

The trichothecenes are a group of structurally related mycotoxins produced by toxigenic strains of Fusarium, and, less importantly, Myrothecium, Trichoderma, Trichothecium, and Stachybotrys. These fungi are

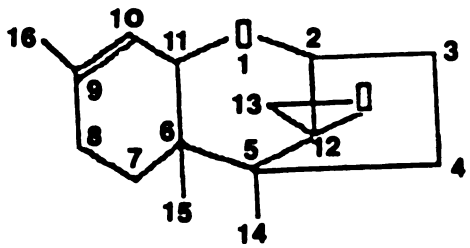
ubiquitous in air and soil; toxigenic strains have been isolated from moldy grains, fruits, and soil. Fusarium infection, growth, and toxin production can occur on grain crops in the field, at harvest, and during storage. Since weather conditions of high moisture and cool temperatures favor mold growth with concurrent trichothecene production, contamination in the United States is particularly a problem in the midwestern states.

The trichothecenes are sesquiterpenoid compounds that have in common the 12,13-epoxytrichothec-9-en (trichothecene) nucleus (Figure 1a). The epoxide and double bond are necessary for the biological activity of these compounds. The trichothecenes are biosynthesized from three molecules of mevalonate via the pathway of lipid biosynthesis, involving cyclization and isomerization of farnesyl pyrophosphate (Ciegler, 1979). Little is known about the genetic and environmental factors that affect trichothecene biosynthesis. The trichothecenes are typically classified into 3 types (A, B, and C) (Figure 1b); members of each group are distinguished by substitutions around the basic ring structure, mainly at carbons 3, 4, 7, 8, 14, and 15. Substituents are typically H, OH, or acyloxy groups, especially acetoxy groups. The trichothecenes most often identified in naturally contaminated grains are T-2 toxin, diacetoxyscirpenol, nivalenol, deoxynivalenol, and metabolites of these toxins.

Trichothecenes have been linked to acute toxicoses of farm animals and humans. Residents of the Soviet Union are

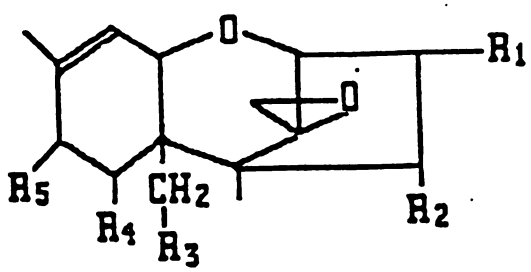
Figure 1. The trichothecene group of mycotoxins.
(a) the trichothecene nucleus
(b) Type A, B, and C trichothecenes.
Listed below are representative
trichothecenes.

a.



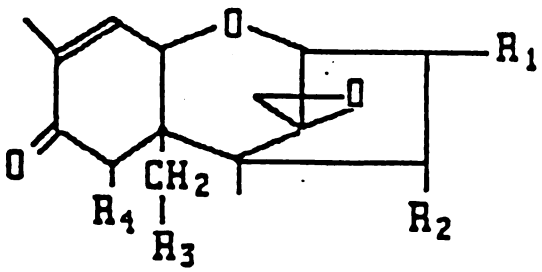

trichothecene nucleus

b.



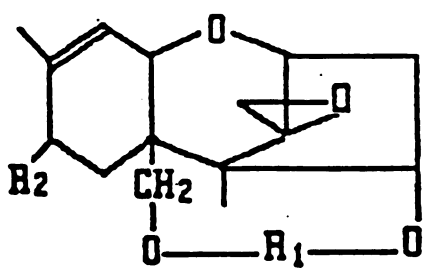

Type A

T-2 toxin (T-2)
diacetoxyscirpenol (DAS)




Type B

nivalenol (NIV)
deoxynivalenol (DON)
fusarenone X (FSX)




Type C

verrucarins
roridins
satratoxins

**FIGURE 1**

believed to have suffered alimentary toxic aleukia after eating overwintered grain contaminated with trichothecenes (Yagen and Joffe, 1976), and in Japan, red-mold disease in animals and humans has been linked to trichothecenes (Yoshizawa, 1984). In the United States, moldy corn toxicosis as a result of trichothecene ingestion has been documented in farm animals (Hsu et al., 1972). Studies have shown that trichothecenes survive processing operations such as milling and baking (Young et al., 1984; Abbas et al., 1985), and thus probably contaminate the human food supply as well as animal feeds (Trucksess et al., 1986).

The toxic effects of trichothecenes can be divided into two broad categories. First, there are acute effects which have been documented in laboratory and domestic animals, and which include diarrhea, vomiting, intestinal hemorrhaging, neurological disorders, skin irritation, and cardiopulmonary disorders culminating in death (Sato et al., 1975; Weaver et al., 1978; Bamburg, 1983; Ueno, 1984). Second are chronic effects, which differ greatly from acute effects. Chronic exposure of animals most often results in feed refusal and decreased feed efficiency (Hayes et al., 1980; Friend et al., 1982; Hoerr et al., 1982; Young et al., 1983; Trenholm et al., 1984; Morrissey et al., 1985; Pollman et al., 1985; Forsell et al., 1986). Another chronic effect, which is perhaps most significant as regards human exposure, is alteration of normal immune

function. Effects on immune function are summarized in Table 1.

At the cellular level, trichothecenes are potent inhibitors of eucaryotic protein synthesis (Ueno et al., 1969, 1973; Wei and McLaughlin, 1974). Trichothecenes were initially classified as inhibitors of either initiation, elongation, or termination of protein synthesis (Jimenez and Vazquez, 1979). More recent research, however, indicates that all trichothecenes probably act in the same intrinsic way (Cannon et al., 1976b; Carter and Cannon, 1978.), through inhibition of peptidyl transferase. The concentration of the trichothecene, the size and nature of ribosome bound nascent polypeptide chains, the functional state of a given ribosome, and the substituent groups on the trichothecene molecule are likely to be important factors affecting the ability of a trichothecene to exert its effect (Cannon et al., 1976a,b; Carter and Cannon, 1978).

## Rationale

While much is known about the toxicology of the trichothecenes, significant questions remain concerning the hazard of these toxins to both animals and humans. Before responsible regulatory decisions can be made concerning the

Table 1. Immune alterations caused by trichothecenes

| Effect | Reference |
|---|---|
| decreased host resistance | Boonchuvit et al., 1975<br>Salazar et al., 1980<br>Fromentin et al., 1981.<br>Kanai and Kondo, 1984<br>Tryphonas et al., 1986<br>Pestka et al., 1987 |
| alteration in humoral immunity | Lafarge-Frayssisnet et al., 1979<br>Rosenstein et al., 1979, 1981<br>Jagadeesan et al., 1982<br>Mann et al., 1982<br>Masuda et al., 1982a,b<br>Tryphonas et al., 1984<br>Forsell et al., 1986<br>Pestka et al., 1987 |
| alteration in cellular immunity | Otokawa et al., 1979<br>Rosenstein et al., 1979 |
| decreased macrophage function | Gerberick and Sorensen, 1983<br>Gerberick et al., 1984 |
| decreased neutrophil function | Buening et al., 1982<br>Jagadeesan et al., 1982<br>Mann et al., 1984<br>Yarom et al., 1984 |
| decreased lymphocyte proliferation in response to mitogen stimulation | Lafarge-Fraysssinet et al., 1979<br>Rosenstein et al., 1981<br>Buening et al., 1982<br>Jagadeesan et al., 1982<br>Masuda et al., 1982<br>Friend et al., 1983<br>Rosenstein and Lafarge-Frayssinet, 1983<br>Atkinson and Miller, 1984<br>Cooray, 1984<br>Mann et al., 1984<br>Forsell et al., 1985<br>Forsell and Pestka, 1985<br>Pestka and Forsell, 1988 |

presence of these compounds in foods and feeds, it is imperative that these questions be addressed. The chronic effect of trichothecenes which is perhaps most significant in terms of human exposure, and which our laboratory has studied for several years, is alteration of normal immune function. The research in this thesis was undertaken as a contribution to this effort. Three studies were carried out in sequence:

1. purification of the trichothecene deoxynivalenol, as a necessary first step in assessing its immunotoxicity,

2. investigation of the effects of dietary exposure to deoxynivalenol on murine humoral immune function, and

3. characterization of the uptake of the trichothecene T-2 toxin by a B-cell hybridoma, in order to better understand the interactions of the trichothecenes with immune cells at the cellular level.

The first and last studies are described in detail in separate chapters of this thesis. The second study was conducted within a larger study designed to assess the chronic and immunotoxic effects of deoxynivalenol. This work has been published and is not discussed in detail here. Rather, copies of the publications and a brief discussion of my contribution to the work described in them are included in an Appendix to this thesis.

# PART I

# PURIFICATION OF DEOXYNIVALENOL

## ABSTRACT

## PURIFICATION OF DEOXYNIVALENOL

By

Mary Frances Witt

The toxicological evaluation of the trichothecene mycotoxin deoxynivalenol (DON) has been hindered by the lack of gram quantities of DON. A simple procedure was developed for the laboratory production and purification of gram quantities of crystalline DON. When Fusarium graminearum R6576 was grown on rice, concentrations of 600 to 700 ppm DON accumulated after 13 to 18 days of incubation. A DON derivative, 15-acetylDON, was also found at concentrations of 100 to 300 ppm after 7 to 10 days. Crude culture extracts were purified by low pressure liquid chromatography using a column of water-saturated silica gel. This column selectively extracted DON from the extracts when methylene chloride was the mobile phase. After elution of DON with water and subsequent extraction with ethyl acetate, DON crystallized readily. Purity of crystalline DON was determined by high performance liquid chromatography. Identity was confirmed by melting point determination, UV absorption spectrum and mass spectrometry.

# INTRODUCTION

## Rationale

The trichothecene deoxynivalenol (DON), initially referred to as Rd toxin because of its association with red-pigmented Fusarium, was first isolated in Japan from Fusarium-infected barley (Morooka et al., 1972). The chemical structure of DON was determined to be 3α, 7α, 15-trihydroxy-12,13-epoxytrichothec-9-en-8-one (Figure 1) (Yoshizawa and Morooka, 1973). DON was also isolated in the United States from Fusarium-infected corn as the compound causing feed refusal and emesis in swine, hence the name vomitoxin (Vesonder et al., 1973, 1976).

DON has subsequently been found throughout the world (Table 1) and is the trichothecene most often identified in Fusarium-infected grains and cereals. Fusarium infection and attendant DON contamination of grains occurs in temperate climates, during periods of prolonged wet weather before harvest (Mills, 1982). In North America, the areas most often affected are the eastern provinces of Canada (Seaman,1982) and the midwestern region of the United States (Vesonder, 1983). Of recent concern was a 1980 fusarium head blight epidemic affecting winter and spring wheat in Ontario, Quebec, and the Maritime Provinces

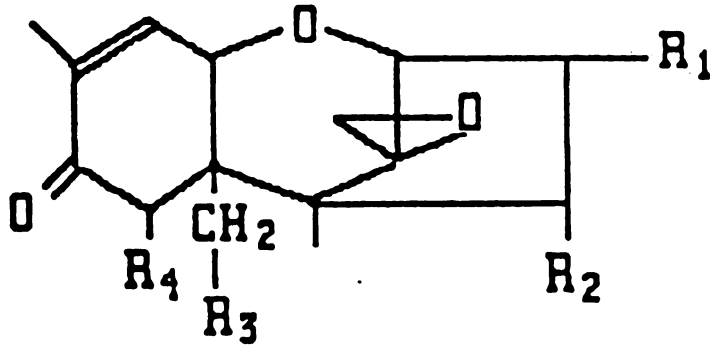



**FIGURE 1. DEOXYNIVALENOL**

**$R_1$=$R_3$=$R_4$=OH**

**$R_2$=H**

Table 1. Natural occurrence of DON in grains[a]

| Location | Year | DON concentration[b] (ppm) | Reference |
|---|---|---|---|
| United Kingdom | 1980-82 | 0.02-0.4 | Osborne and Willis, 1984 |
| | 1984 | 31 | Tanaka et al., 1986 |
| Korea | 1983-84 | 117 | Lee et al., 1985, 1986 |
| Sweden | 1984 | 0.05-1.18 | Petersson et al., 1986 |
| Poland | 1985 | 116 | Ueno et al., 1985 |
| Japan | 1976-84 | 0.3->5 | Yoshizawa, 1984 |
| Canada | 1979-80 | 0.05-6.3 | Neish et al., 1983 |
| | 1980-82 | 0.3-3.0 | Scott, 1984 |
| | 1985 | <1.4->3 | Abramson et al., 1987 |
| United States | 1977 | 0.5-10 | Vesonder et al., 1978 |
| | 1982 | 3.6 | Eppley et al., 1984 |
| | 1982 | 1.8 | Hagler et al., 1984 |
| Taiwan, China, USSR | 1984-85 | 0.01-2.5 | Ueno et al., 1986 |
| South Africa | 1976-77 | 0.25-4.0 | Marasas et al., 1979 |

[a]Grains surveyed include wheat, barley, corn, and oats.

[b]Concentrations reported are either averages (single values) or ranges.

(Sutton, 1982). Analysis of infected wheat revealed the presence of DON, at levels up to 8 ppm, in winter wheat (Trenholm et al., 1983) and in Quebec red spring wheat (Scott, 1983).

DON in grains is likely to contaminate both the animal and human food supplies since the toxin is not removed during processing. Cleaning only slightly reduces DON concentrations in wheat (Scott et al., 1983; Abbas et al., 1985; Seitz et al., 1985). After milling, DON is distributed throughout all fractions, with higher concentrations found in the outer layer of the wheat kernel (bran, shorts) than in the inner layer (break and reduction flours) (Hart and Braselton, 1983; Scott et al., 1983; Young et al., 1984; Abbas et al., 1985; Seitz et al., 1985; Lee et al., 1987). The concentration of DON in bran and shorts, fractions intended for animal feed, is increased 1 to 2-fold compared to raw wheat. The concentration of DON in flour fractions intended for human consumption is 15 to 40% lower than the concentration in raw wheat. However, foods made from these flours are likely to contain DON, since baking does not destroy the toxin (El-Banna et al., 1983; Scott et al., 1983; Young et al., 1984; Abbas et al., 1985). Indeed, DON has been identified in commercial rye bread (39-45 ppb) (Abbas et al., 1985) and in commercial breakfast cereals (100 ppb) (Trucksess et al., 1986). In addition, an isomer of DON (iso-DON, 3,8,15-trihydroxy-12,13-epoxytrichothec-8-en-7-one), apparently formed during

thermal processing, has been identified in processed wheat-based breakfast cereals and bread baked from grain naturally contaminated with DON (Greenhalgh et al., 1984a)

The presence of DON in animal and human food supplies is a concern because trichothecenes are known to cause toxic effects in laboratory and domestic animals (Mirocha, 1983; Ohtsubo, 1983; Ueno, 1983). Furthermore, corn and mixed feeds contaminated with DON have been associated with health problems, including vomiting, diarrhea, feed rejection, decreased weight gain, and alteration of immune function in cattle and swine (Mirocha et al., 1976; Vesonder et al., 1979; Cote' et al., 1984). Thus, the recognition of the widespread occurrence of DON in grains and of the hazard its presence may represent to animal and human health has raised questions about the need for regulation of DON levels in grains and foods (Elliot, 1982). In response to DON contamination of wheat during the 1980 fusarium head blight, Canadian officials set action levels of 2 ppm DON in uncleaned soft wheat intended for human consumption (1 ppm for wheat to be used in infant foods) and 1.2 ppm DON (flour or bran basis) in derived, non-staple food products. The United States Food and Drug Administration, meanwhile, has recommended levels of concern (2 ppm DON in whole grain and 1 ppm DON in finished products), but is waiting to establish action levels until there is suitable analytical methodology, better information on the extent of contamination, and more toxicological information (Anon., 1982).

Notably lacking is information about the chronic toxic effects -- particularly the immunotoxic effects -- of DON to animals and humans. This information is crucial in making informed regulatory decisions concerning contamination of the food supply by DON. Questions regarding dietary exposure to DON in naturally contaminated grains are best addressed through animal feeding studies. These types of studies, though, require gram quantities of pure DON which are not available commercially. Therefore, researchers must produce and purify DON for their own use.

## Review of Literature

The process of obtaining gram quantities of DON can be conveniently separated into two steps: development of (1) a concentrated source of DON and (2) a method for purifying the crude toxin. The literature pertaining to each of these steps will be reviewed.

### Source of DON.

Potential sources of DON include chemical synthesis, naturally contaminated grains, field-inoculated grains, and laboratory production. An acceptable source should provide a convenient, reliable, and concentrated supply of DON.

The chemical synthesis of the trichothecenes, including DON, presents a challenge to the organic chemist since regio- and stereo-selective chemical reactions are

involved. While Colvin and Thom (1986) have synthesized an advanced intermediate of DON, the complete synthesis has not been achieved. Thus, chemical synthesis of DON is not a practical source of DON.

The use of either naturally contaminated or field-inoculated grains is also impractical. Concentrations of DON in naturally contaminated grains are too low (Table 1) to justify their use as a source of DON. Field-corn inoculated with Fusarium strains known to produce DON can be a concentrated source of DON. Corn inoculated with F. graminearum contained 580 ppm DON 6 to 7 weeks after inoculation (Miller et al., 1983b). Scott et al. (1984a) purified over 6 grams of DON from field-inoculated corn that contained 400 to 500 ppm DON. Drawbacks, however, exist to the use of field-inoculated grains as a routine source of DON. Toxin production in the field is susceptible to unpredictable and uncontrollable weather conditions. Furthermore, optimization of growing and harvest conditions, and identification of key factors in DON production are hindered by time, land, and labor requirements.

Laboratory production of DON offers several advantages over the former three methods as a useful source of DON for purification. Optimization of parameters important for DON production is convenient since environmental conditions can be controlled and work can be performed year-round in the laboratory. Both liquid and solid media have been used for DON production.

Liquid media is generally preferred over solid media for biosynthetic studies due to its defined composition and ease of handling. DON yields in liquid culture, however, have generally been low. F. graminearum isolates produced maximum DON concentrations of 32 mg/liter in glucose-yeast extract-peptone (GYEP) medium (Miller et al., 1983a) and 16.5 mg/liter in modified Fries medium supplemented with 4% corn steep liquor (Pestka et al., 1985). Undoubtedly these low yields can be attributed, in part, to the lack of information available about nutrient requirements and regulation of DON production.

DON yields on solid substrates have been higher than in liquid media (Table 2). Rice appears to be the best substrate for DON production. Concentrations of DON exceeding 500 ppm have been achieved in rice culture (Greenhalgh et al., 1983; Neish et al., 1983). Under similar incubation conditions, DON production on rice was higher than on either corn or wheat (Megalla et al., 1987; Greenhalgh et al., 1983; Neish and Cohen, 1981).

While little is known about the regulation of DON biosynthesis, empirical work has identified several incubation and environmental parameters important in DON production. These experiments have been conducted using fungal strains, isolated from Fusarium infected grains, that produce DON on solid substrates in the laboratory. One corn isolate frequently used is F. graminearum NRRL 5883. This strain was the predominant one isolated from

Table 2. Production of DON on solid substrates

| Strain | Incubation conditions[a] | Conc. DON[b] | Reference |
|---|---|---|---|
| F. graminearum NRRL 5883 | 30%, 21, 28 rice | 450 | Ehrlich and Lillehoj 1984 |
| F. graminearum isolates | 40, 7, 25[c] 14, 15 corn | 211 | Megalla et al., 1987 |
| F. graminearum NRRL 5883 | 40, 21, 25 rice | 200 | Megalla et al., 1987 |
| Fusarium isolates | 40, 7, 25[c] 21, 12 rice | 300 | Pathre and Mirocha 1978 |
| F. graminearum NRRL 5883 | 30, 40, 30 corn | 362 | Vesonder et al., 1982 |
| F. graminearum DAOM 180379 | 40, 24, 28 rice | 515 | Greenhalgh et al. 1983 |
| F. graminearum DAOM 180379 | 30, 22, 28 rice | 542 | Neish et al., 1983 |

[a]Percent initial moisture content, days of incubation, temperature of incubation, and substrate.

[b]Concentrations (ppm) of DON are maximum yields reported.

[c]Two time/temperature combinations used in succession.

the contaminated corn in which DON was originally identified in the U.S. (Vesonder et al., 1973), and consistently produces DON in concentrations of 200 to 400 ppm on corn (Vesonder et al., 1982) and rice (Ehrlich and Lillehoj, 1984; Megalla et al., 1987). A set of isolates from Canadian corn, F. graminearum DAOM 180377, 180378 and 180379, has also been used frequently to examine DON production. Of these isolates, 180379 is the best, producing >500 ppm DON on rice (Neish et al., 1983; Greenhalgh et al., 1983). Optimization studies, carried out mainly with the Fusarium strains listed above, have shown that the initial moisture content of the substrate, incubation temperature, and incubation time all affect DON production on solid substrates.

Greenhalgh et al. (1983) determined the optimum initial moisture content of rice to be 40%. Vesonder et al (1982) found the optimum incubation temperature to be 26 to 32 C, while Greenhalgh et al. (1983) determined 28 C to be optimal. Some investigators have employed a temperature change during incubation. Megalla et al. (1987) incubated cultures at 25 C, then 15 C, and Pathre and Mirocha (1978) shifted incubation temperature from 25 C to 12 C. Neither of these temperature combinations, however, improved DON yields over incubation of cultures at temperatures close to 28 C for the entire incubation period.

Under conditions of optimum initial moisture (40%) and incubation temperature (28 C), Greenhalgh et al. (1983) reported 24 days to be the optimum incubation time for DON

production. Vesonder et al. (1982) observed maximum DON production after 40 days when cultures were incubated at the optimum temperature. The initial moisture content in these experiments, however, was 30%, rather than the optimal 40%, which may account for the longer incubation time. Megalla et al. (1987) reported maximum DON production after 4 weeks incubation when a temperature shift (25 C for 1 week, then 15 C for 3 weeks) was used.

In summary, regardless of the strain used, DON production is generally optimal under conditions of 40% initial substrate moisture and incubation at 28 C for 20 to 28 days. These conditions are routinely used when testing field isolates for their ability to produce DON.

Both optimization studies and testing of field isolates for toxigenicity are conveniently performed using 50 g corn or rice in 500-ml flasks, which gives adequate yields of DON. However, efforts to reproduce these fermentations on a larger scale for mass production of DON have been disappointing. Although Vesonder et al. (1982) predicted yields of 250 ppm DON, based on experiments carried out in 500 ml flasks (50 g corn), less than 1 ppm DON accumulated during fermentation of 1 kg batches of corn. Greenhalgh et al. (1983) were also unable to scale up their fermentation on rice. DON was not detected in culture flasks (2800 ml) containing 350 g rice despite abundant fungal growth. This inability to increase the scale of the fermentation greatly hinders the laboratory

production of gram quantities of DON on solid substrates.

Purification of DON.

Several schemes for purifying DON have been reported (Table 3). The extraction procedure (aqueous methanol followed by partitioning into ethyl acetate) is routine for isolation of the relatively polar Type B trichothecenes from contaminated grains. DON in the crude extract is generally purified by chromatography. Various combinations of chromatography, including liquid-liquid partitioning, column chromatography, preparative TLC, and preparative HPLC, have been developed largely through trial and error. Although crystalline DON of high purity has been obtained (Vesonder et al., 1982; Scott et al., 1984a), the steps required are lengthy and inefficient. Furthermore, these multi-step procedures result in significant losses of DON, with recoveries from grain ranging from 20 to 60%. Losses are especially heavy when silica gel chromatography is used.

Ehrlich and Lillehoj (1984) developed a method that reduced losses of DON during silica gel chromatography. DON extracted from rice culture was acetylated to triacetyl DON. This derivative was recovered essentially quantitatively from a silica gel column while polar impurities were retained by the column. Triacetyl DON was then hydrolyzed to DON, and nonpolar impurities were removed using a charcoal-alumina column. Finally, Sephadex LH-20 chromatography produced crystalline DON of greater

Table 3. Purification of DON

| Purification[a] | Purity (%)/ Recovery (%) | Reference |
|---|---|---|
| rice culture<br>liquid/liquid partitioning<br>prep silica gel TLC | 95/55 | Pathre and Mirocha (1979) |
| field-inoculated corn<br>liquid/liquid partitioning<br>prep silica gel HPLC<br>prep reverse phase HPLC | --[b]/20 | Bennett et al. (1981) |
| corn culture<br>silica gel chromatography<br>silica gel chromatography<br>prep silica gel HPLC<br>crystallization | 96/20 | Vesonder et al. (1982) |
| field-inoculated corn<br>liquid/liquid partitioning<br>Florisil chromatography<br>liquid/liquid partitioning<br>semi-prep reverse phase HPLC<br>crystallization | 95/60 | Scott et al. (1984b) |

[a]All methods include a routine extraction of DON from contaminated grain involving extraction with aqueous methanol, followed by partitioning into ethyl acetate.

[b]Not reported.

than 90% purity. Total DON recovered by this procedure, including mono- and di-acetylated derivatives present in the rice culture, was estimated to be 450 mg/kg rice.

Another approach has been to produce 3-acetyl DON (3-ADON) by fermentation of F. culmorum in liquid culture (Greenhalgh et al., 1984b), then hydrolyze this derivative to DON. 3-ADON (730 mg/L) was produced, purified, crystallized, and hydrolyzed to DON using an $OH^-$ ion-exchange resin in methanol (Greenhalgh et al., 1986). DON was recovered with an 87% yield. A drawback to this approach is the large volumes of solvents required for extraction and purification of 3-ADON.

## Research Objectives.

The research described here was aimed at obtaining crystalline DON in quantities and purity sufficient for toxicological studies. Part of this research has been published (Witt et al., 1985) (see Appendix). The experimental approach consisted of two steps: production of DON and purification of DON. For the first step, the research objective was to develop a method for large-scale laboratory production of DON on rice by F. graminearum. While the primary aim was to maximize DON yields, experiments performed to optimize production were expected to provide information about factors influencing DON biosynthesis. For the purification step, the goal was to

develop a practical method, based on silica gel chromatography, for obtaining crystalline DON from rice cultures.

# MATERIALS AND METHODS

## Inoculum Preparation

Potato dextrose agar (Difco Laboratories, Detroit, MI) plates were inoculated with stock soil cultures of Fusarium graminearum R6576 (Gibberella zeae U5373) (Hart et al., 1982) or Fusarium graminearum Schw. NRRL 5883 (Vesonder et al., 1973). Plates were incubated at 25 C for 7 days in a 12-hr. light/dark cycle. In initial experiments, agar plugs (4 mm) taken from the growing edge of colonies were added directly to rice for DON production (agar plug inoculum). Later experiments employed a spore suspension inoculum, prepared by adding agar plugs (3 to 4 per flask) to Erlenmeyer flasks (500-ml) containing (carboxymethyl)-cellulose (CMC, Sigma, St. Louis, MO) medium (90 ml) (Cappellini and Peterson, 1965). CMC medium contained per liter: $NH_4NO_3$, 1.0 g; $KH_2PO_4$, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; yeast extract (Difco), 1.0 g; and CMC, 15 g. These flasks were agitated on a rotary shaker (250 rpm) at 25 C for 3-5 days. The resulting spore suspension was filtered by gravity through sterile cheesecloth, and the concentration of macroconidia in it was determined by

counting in a hemacytometer (American Optical, Buffalo, NY).

## DON Production

Fusarium strains were cultured on commercially available enriched, white rice in either Erlenmeyer flasks (500-ml) containing 50 g rice, or Fernbach culture flasks (2800-ml) containing 350 g rice, referred to hereafter as small and large flasks, respectively. The volume of distilled water required to produce the desired initial moisture content (weight basis) was added to each flask. Flasks were either covered with aluminum foil or stoppered with cotton plugs before being autoclaved at 121 C for 30 min. The autoclaved rice was inoculated with Fusarium using either agar plugs or spore suspension and then incubated in the dark at 28 C. Fungal growth was stopped at the desired time by beginning the extraction procedure.

## DON Extraction

The extraction procedure was adapted from Pathre and Mirocha (1978). DON was extracted from rice into 60% aqueous methanol (150 and 1400 ml for small and large flasks, respectively). The rice and solvent were blended in a Waring blendor and then held overnight to allow complete extraction of DON. The slurry was then filtered

by vacuum filtration through Whatman No. 4 filter paper (Whatman, Inc., Clifton, NJ) and the filtrate reduced in volume by approximately one-half by evaporation on a steam bath. The concentrated aqueous extract was saturated with sodium chloride (Mallinckrodt, Paris, KY), then filtered as above. Saturated solutions were held overnight to allow complete precipitation. The filtered aqueous extract was extracted three times with ethyl acetate using a volume ratio of 1:2 (ethyl acetate:water). This extraction procedure was used whenever it was necessary to extract DON from an aqueous solution. Emulsions formed frequently and were best handled by allowing the emulsion to dissipate over time. Extracts were combined and ethyl acetate removed under reduced pressure in a Buchi R110 rotavapor (Brinkmann Instruments, Inc., Westbury, NY) at 45 C. Finally, the residue was dissolved in either ethyl acetate for quantitation by TLC or methylene chloride for silica gel chromatography.

## Liquid Chromatography

Low pressure silica gel chromatography was performed using Michael-Miller glass chromatographic columns (22 mmID x 300 mm, 37 mmID x 350 mm, and 50 mmID x 600 mm) fitted with safety shields and Teflon end fittings, connectors, and tubing (2 mmID) (Ace Glass, Inc., Vineland, NJ). Columns were dry packed with silica gel (Adsorbosil, Anspec

Co., Inc., Ann Arbor, MI; 10 um diameter silica gel for 22 mmID column and 200/450 mesh silica gel for 37 and 50 mmID columns. Water-saturated silica gel columns were prepared by pumping distilled water through the packed column, then allowing excess water to drain from the column by gravity. Three solvent systems were used: (1) a step gradient of ethyl acetate (0, 15, 30, 45, 60%) in hexane; (2) various step gradient systems of methanol (2 to 100%) in methylene chloride; and (3) a sequential solvent system consisting of methylene chloride followed by distilled water. A pump (Model RP-SY-1CSC; Fluid Metering, Inc., Oyster Bay, NY) adapted with low flow fittings and coupled to a pulse dampener maintained a flow rate of about 5 ml/min. The sample was dissolved in a minimum of the starting solvent and applied manually to the top of the column packing. Fractions (10-20 ml) were collected with an LKB Ultrorac Type 7000 fraction collector (Stockholm, Sweden) and monitored by TLC (see Analytical Procedures). Used silica gel was regenerated by removing it from the column, washing it with methanol and water, then removing the wash solvent by vacuum filtration. The washed silica gel was air dried and reused for chromatography.

Gel filtration was performed using a Sephadex LH-20 100 (Pharmacia Inc., Piscataway, NJ) column ( 2.6 x 45 cm; Pharmacia Inc.) prepared by the standard slurry packing method. The column was eluted with distilled water at a flow rate of 1 ml/min. Fractions (5 ml) were collected

with the LKB Ultrorac fraction collector and monitored by TLC.

A countercurrent distribution was performed using a series of eight separatory funnels (50 ml). The upper phase was ethyl acetate (10 ml) and the lower phase was distilled water (20 ml). Each separatory funnel originally contained fresh upper phase, with the first funnel also containing lower phase. The sample was put into the first funnel. The distribution consisted of eight equilibrations, each one involving all funnels containing lower phase. After each equilibration, all lower phases were transferred to the next funnel in the sequence. The first funnel always received fresh lower phase. Relative concentrations of DON in both phases were determined by TLC.

## DON Crystallization

Crystallization of DON by standard recrystallization techniques was not successful, so a modified procedure was used. The extract was dried under $N_2$ in a beaker (50-ml) and then redissolved in a minimum of solvent. This solution was seeded with a few crystals of DON. The beaker was covered tightly with aluminum foil and held at 4 C until a precipitate formed. The solvent was removed and saved, and the solids were dissolved in the appropriate solvent. In some cases a decolorization step using activated charcoal (Darco G-60, Matheson Coleman and Bell,

Norwood, OH) was performed before the solution was filtered by gravity through Whatman No. 4 filter paper. DON was crystallized from the filtrate as before and the solvent again removed and saved. Residual pigment contaminating DON was removed by washing the crystals repeatedly at 4 C with methanol or ethyl acetate (1 to 3 ml). The entire crystallization scheme was repeated with combined supernatants and wash solvents. White crystalline DON was stored at 4 C.

## Analytical Procedures

Semiquantitative TLC was performed on precoated 20 cm x 10 cm silica gel G plates (Redi-Plates, Fisher Scientific Co., Fair Lawn, NJ) using the solvent system toluene-ethyl acetate (1:3). Developed plates were sprayed with a 15% aluminum chloride (Fisher Scientific Co.) solution (15 g $AlCl_3 \bullet 6H_2O$ in 85 mL ethanol and 15 mL of water) and then heated for 5 min at 110 C (Baxter et al., 1983). Under longwave (365 nm) ultraviolet (UV) light, trichothecenes possessing the $\alpha,\beta$-enone function, including DON and 15-acetylDON (15-ADON), appeared as blue fluorescent spots. Plates to be photographed were visualized by spraying with 30% $H_2SO_4$ and heating for 5 min at 110 C. DON and 15-ADON appeared as dark red spots. 15-ADON in culture extracts was identified by comparison of its TLC $R_f$ value with that of a qualitative 15-ADON standard that was prepared in this

laboratory and had been confirmed by mass spectrometry performed by Dr. C.J. Mirocha (University of Minnesota). Zearalenone (ZEA) standard was donated by M. Bachman (International Minerals and Chemical Corp., Terre Haute, IN). TLC plates were placed in a UV viewing cabinet (UV Viewing Products, Inc., San Gabriel, CA) for quantitation. The concentration of DON and 15-ADON in culture extracts was estimated by visually comparing the fluorescent intensity of sample spots to the intensity of a set of graded standards of DON (Myco-Lab Co., Chesterfield, MO) dissolved in ethyl acetate. DON and 15-ADON concentrations in rice were calculated on a dry rice weight basis. The limit of detection for DON and 15-ADON was 1 ppm.

High performance liquid chromatography (HPLC) was performed at 25 C using a Model 2300 HPLC pump and a $V^4$ variable wavelength absorbance detector (5-mm flow cell) (Isco, Inc., Lincoln, NE). The system was equipped with RP-18 Spheri-10 MPLC analytical (22 cm x 4.6 mm ID) and guard (3 cm x 4.6 mm ID) cartridges (Brownlee Labs, Inc., Santa Clara, CA). Mobile phase was 7-33% (vol/vol) methanol (HPLC grade, Fisher Scientific Co.) in water (HPLC grade, Fisher Scientific Co.) with a flow rate of 1 ml/min. Samples were dissolved in mobile phase, filtered through Prep-Disc Sample Filters (Bio-Rad Laboratories, Richmond, CA) and injected through a Valco C6U sample injector (Valco Instruments Co., Inc. Houston, TX) fitted with a 10 ul sample loop. Absorbance was monitored at 224 nm. A

standard of 3,15-dihydroxy-12,13-epoxytrichothec-9-en-8-one (7-deoxy-DON) was supplied by Dr. G.A. Bennett (Northern Regional Research Center, Peoria, IL).

The mass spectrum of DON was obtained by Dr. W.E. Braselton, Jr. (Michigan State University) at 70 eV, using a direct insertion probe, on a Finnigan 3200 gas chromatograph-mass spectrometer coupled to a Riber SADR data system. The UV absorption spectrum of DON in ethanol (25 to 50 ug/ml) was determined on a Beckman Model 35 spectrophotometer (Beckman Instruments, Inc., Irvine, CA). Absorbance was monitored between 300 and 180 nm at a scan speed of 60 nm/min. The melting point of DON was estimated by observing the temperature range over which crystals of DON in a capillary tube melted in a hot oil bath.

Distribution coefficients were determined by adding DON or 15-ADON (both prepared in this laboratory) to separatory funnels (50-ml) containing a mixture of water and methylene chloride (5 ml each). Equilibrium concentrations of toxin in each phase were determined by HPLC, performed as described above using 33% methanol in water as the solvent system. DON and 15-ADON were quantitated with a Hewlett Packard 3392A Integrator (Hewlett-Packard Co., Avondale, PA). The distribution coefficient was defined as toxin concentration in water/toxin concentration in methylene chloride.

## Solvents

All solvents used (with the exception of HPLC solvents) were analytical grade or better and were purchased from Mallinckrodt, Inc., Fisher Scientific Co., or J.T. Baker Chemical Co. (Phillipsburg, NJ). These included methanol, ethyl acetate, toluene, hexane, and methylene chloride.

## Safety Precautions

Vinyl gloves were worn to avoid dermal contact with DON and crude extracts. Contaminated glassware was soaked overnight in a 10% bleach solution before being washed (Thompson and Wannemacher, 1984). Safety shields were used with Michael-Miller glass columns and operating pressure did not exceed 300 psi.

# RESULTS

## DON Production

Rice that was inoculated with F. graminearum NRRL 5883 by the agar plug method contained unacceptably low levels (4 to 10 ppm) of DON after incubation despite abundant fungal growth. Manipulation of environmental and incubation parameters including moisture content of the rice (20 to 40%), length of incubation period (2 to 4 weeks), temperature (10, 25, 28 C), light/dark cycle, and scale of fermentation (large or small flasks) did not appreciably increase yields (data not shown). Furthermore, DON concentration among flasks was variable. In preliminary experiments, use of a spore inoculum improved the reproducibility among replicate culture flasks, and this approach was used for subsequent experiments.

Using a spore inoculum, F. graminearum R6576 consistently accumulated higher concentrations of DON than did F. graminearum NRRL 5883 under the same conditions (Table 4). During this experiment, flasks were shaken daily to prevent clumping and matting of the rice. One flask inoculated with NRRL 5883, however, was not shaken during incubation, and rice in this flask contained 5 to 10

Table 4. Accumulation of DON in rice inoculated with *F. graminearum*[a]

| *F. graminearum* | DON concentration (ppm)[b] |
|---|---|
| NRRL 5883 | 29, 29, 143[c], 14, 14, 23 |
| R6576 | 143, 143, 143, 143, 143, 51 |

[a]Flasks (2800-ml) containing 350 g rice (30% moisture) were inoculated with $10^6$ spores/flask and stoppered with cotton plugs. Flasks were incubated in the dark at 28 C for 25 days and shaken daily.

[b]DON was quantitated by TLC (see Methods) and concentrations are based on dry rice weight. Values are for individual flasks.

[c]Flask not shaken during incubation.

times as much DON as that in shaken flasks (Table 4). Because of this observation and the inconvenience of shaking flasks, flasks in remaining experiments were not shaken. During incubation, rice typically developed the pink-red color characteristic of Fusarium with abundant fluffy white growth on the surface of the rice.

Moisture content of the rice and length of incubation period affected DON production by both F. graminearum NRRL 5883 and R6576 (Table 5). NRRL 5883 exhibited a slight trend toward greater production at 35 to 40% moisture. R6576 consistently produced higher concentrations of DON than NRRL 5883, with accumulation being maximum at 35 to 40% moisture and 3 weeks of incubation. In view of the relative success of R6576 over NRRL 5883, the former strain was selected for further experiments. The concentration of DON in rice (37% moisture) inoculated with R6576 depended on the incubation time (Figure 2), and was maximum at 18 days. Concentrations of 15-ADON, in contrast to DON, decreased steadily over the incubation period.

Attempts to reproduce the fermentation on a larger scale under similar conditions were unsuccessful (Figure 3). Maximum DON concentrations of rice in large flasks were approximately ten times less than in small flasks (Figure 2), although qualitatively the time courses of accumulation for both DON and 15-ADON were similar in both sizes of flasks. Preliminary experiments substituting cotton plugs for the foil caps routinely used yielded

Table 5. Effect of moisture content of substrate and length of incubation on accumulation of DON in rice inoculated with *F. graminearum*[a]

| Moisture content (%, w/w) | Length of incubation (wks) | DON concentration (ppm)[b] NRRL 5883 | DON concentration (ppm)[b] R6576 |
|---|---|---|---|
| 30 | 2 | 20, 20 | 120, 120 |
| | 3 | 20, 20 | 240, 240 |
| | 4 | 16, <16 | 66, 100 |
| | 5 | --[c] | 100 |
| 35 | 2 | 20, 40 | 240, 300 |
| | 3 | 20, 40 | 400, 400 |
| | 4 | 26, 34 | 66, 266 |
| 40 | 2 | 40, 20 | 300, 360 |
| | 3 | 40, <20 | 400 |
| | 4 | <16, <16 | 66 |
| | 5 | -- | ND[d] |

[a]Flasks (500-ml) containing 50 g rice were inoculated with 5 x $10^5$ spores/flask and capped with aluminum foil. Flasks were incubated in the dark at 28 C without shaking.

[b]DON was quantitated by TLC (see Methods) and concentrations are based on dry rice weight. Values are for individual flasks.

[c]Not determined.

[d]DON not detected (1 ppm detection limit).

Figure 2. Time course of accumulation of DON (O) and 15-ADON (●) in rice inoculated with F. graminearum R6576: accumulation in flasks (500-ml) capped with aluminum foil. Flasks containing 50 g rice (37% moisture) were inoculated with $10^6$ spores/flask and incubated in the dark at 28 C without shaking. Values are averages, with standard error bars, of 3 flasks, except for values at days 12 and 20 (2 flasks). DON and 15-ADON were quantitated by TLC (see Methods) and concentrations are based on dry rice weight.

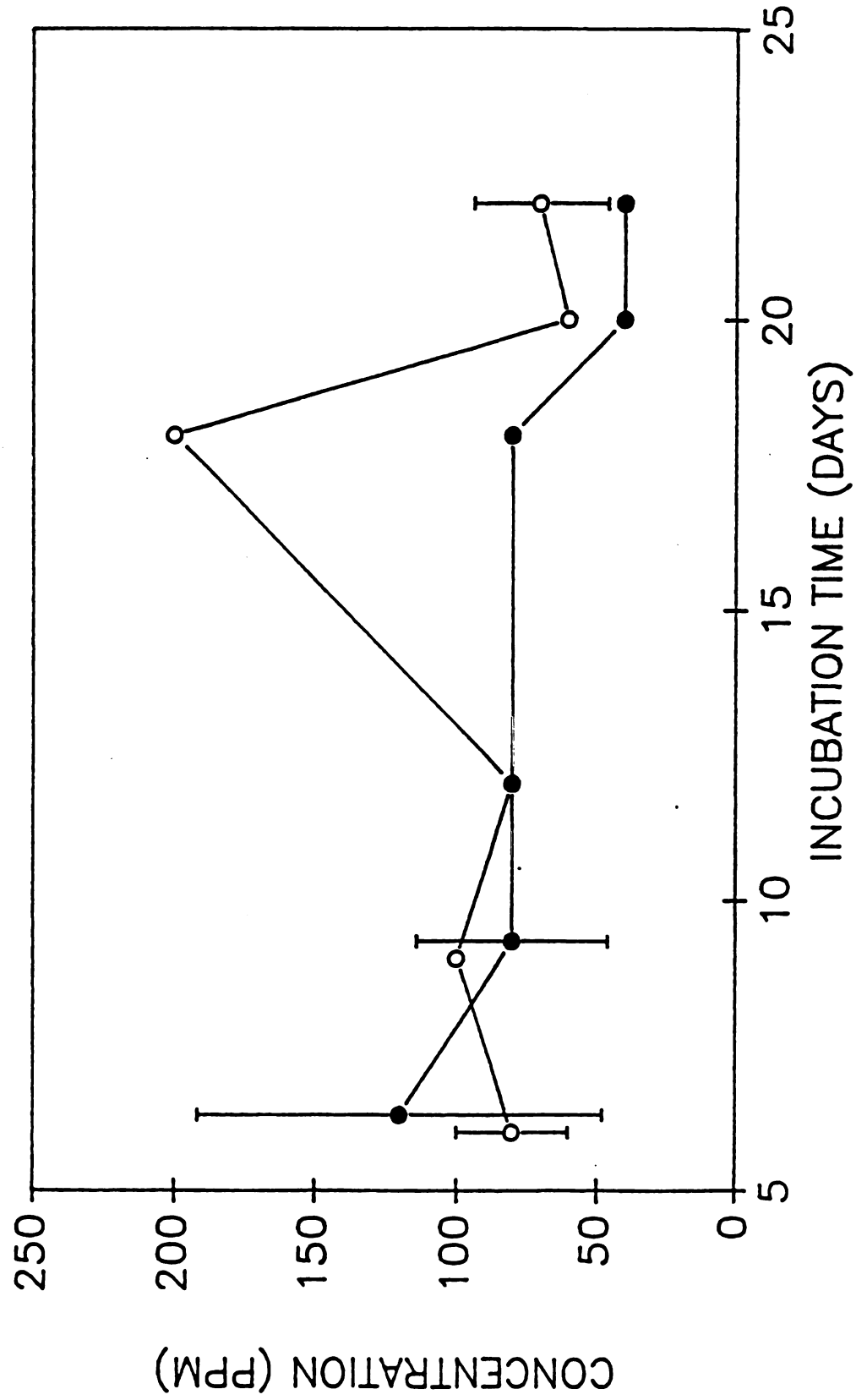

250
200
150
100
50
0
5
10
15
20
25
INCUBATION TIME (DAYS)
CONCENTRATION (PPM)

**FIGURE 2**

Figure 3. Time course of accumulation of DON (O) and 15-ADON (●) in rice inoculated with F. graminearum R6576: accumulation in flasks (2800-ml) capped with aluminum foil. Flasks containing 350 g rice (37% moisture) were inoculated with $10^6$ spores/flask and incubated in the dark at 28 C without shaking. Values are averages, with standard error bars, of 3 flasks. DON and 15-ADON were quantitated by TLC (see Methods) and concentrations are based on dry rice weight.

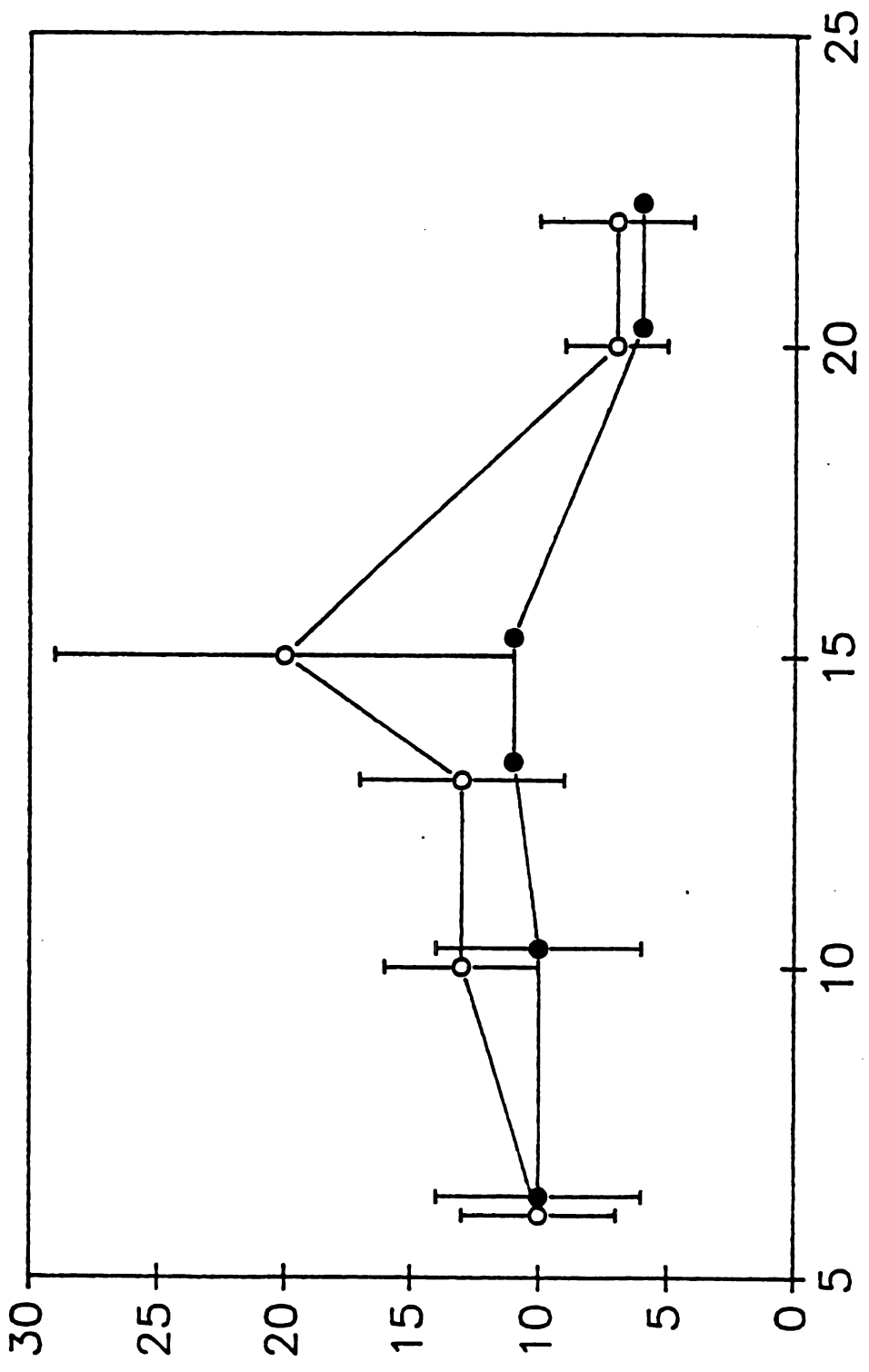

CONCENTRATION (PPM)
30
25
20
15
10
5
0
5
10
15
20
25
INCUBATION TIME (DAYS)

**FIGURE 3**

maximum DON concentrations of 300 to 600 ppm under conditions similar to those described in Figure 2. A time course study of DON production in large flasks confirmed the superiority of cotton plugs over foil caps (Figure 4). DON concentrations of 600 to 700 ppm in rice incubated for 14 to 17 days were consistently observed in similar experiments. 15-ADON concentrations were maximum earlier in the incubation period (7 to 9 days) and decreased steadily over time as DON accumulated. These optimal conditions for production of gram quantities of DON involved use of rice (350 g, 37% moisture) in large flasks, inoculation with $10^6$ spores of F. graminearum R6576/flask, use of cotton plugs, and incubation in the dark, without shaking, for 13 to 18 days.

## DON Purification

The thin layer chromatogram of the viscous black-brown residue extracted from rice included four major bands: DON ($R_f$ 0.3); 15-ADON ($R_f$ 0.5); an unidentified fluorescent compound ($R_f$ 0.8); and ZEA ($R_f$ 0.95). While the latter two compounds fluoresced in the absence of $AlCl_3$, DON and 15-ADON appeared as blue fluorescent spots under longwave UV light only after the developed plate had been sprayed with $AlCl_3$. Several minor, poorly separated, pigmented compounds were also present in the crude extract.

Two purification schemes leading to crystalline DON

Figure 4. Time course of accumulation of DON (O) and 15-ADON (●) in rice inoculated with F. graminearum R6576: accumulation in flasks (2800-ml) stoppered with cotton plugs. Flasks containing 350 g rice (37% moisture) were inoculated with $10^6$ spores/flask and incubated in the dark at 28 C without shaking. Values are averages, with standard error bars, of 3 flasks, except for values at days 17 and 21 (2 flasks) and day 23 (1 flask). DON and 15-ADON were quantitated by TLC (see Methods) and concentrations are based on dry rice weight. 15-ADON was not detected on days 21 and 23 (1 ppm detection limit).

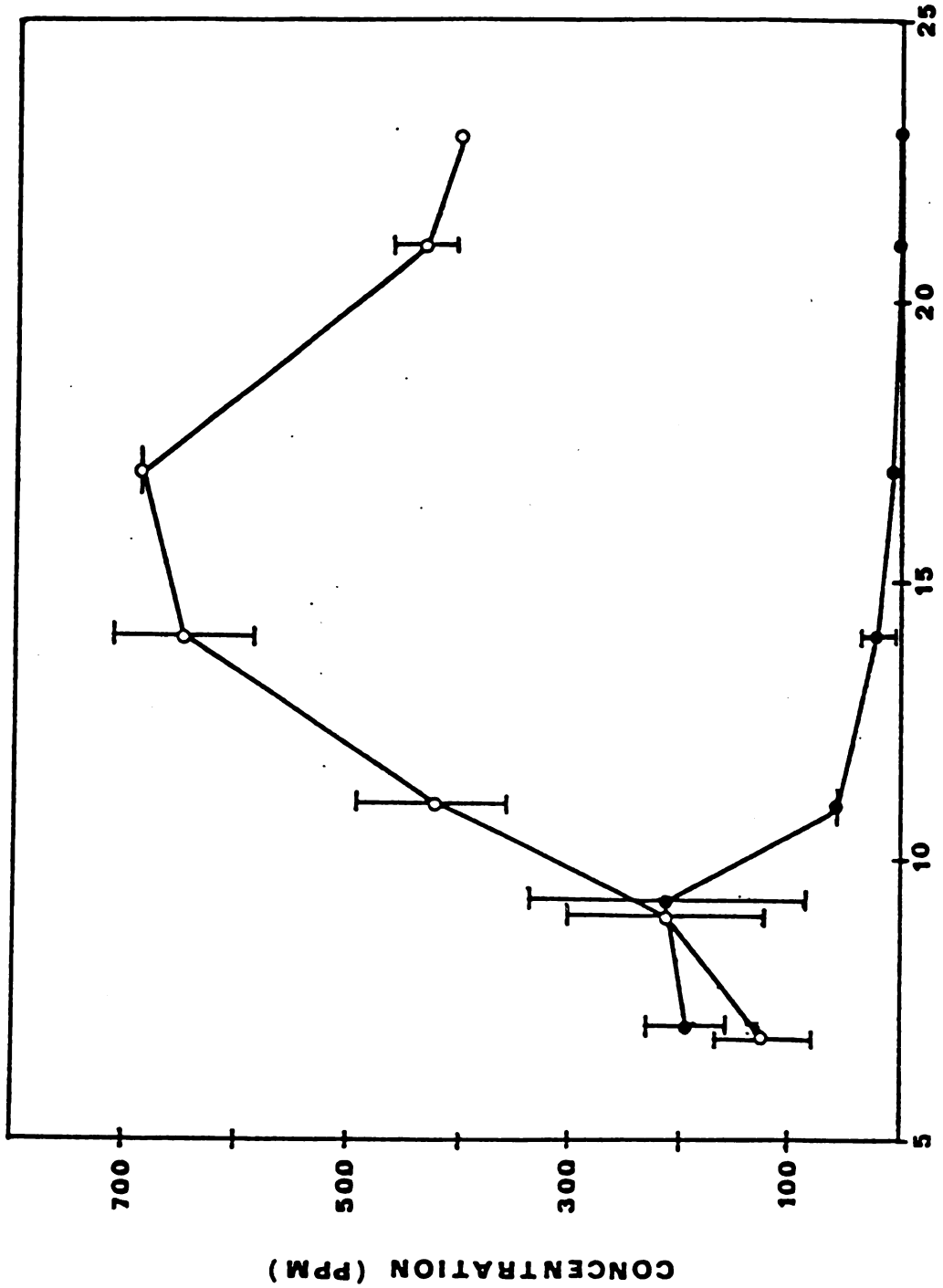

CONCENTRATION (PPM)
700
500
300
100
INCUBATION TIME (DAYS)
5
10
15
20
25

FIGURE 4

were developed. The first one was a lengthy process involving several chromatographic steps. Serendipitous use of a column packed with washed silica gel led to the development of an improved method based on water-saturated silica gel chromatography.

Multi-step chromatography method

The crude extract was partially purified by silica gel chromatography (50 mm ID column), using a solvent system of methanol (increasing from 2 to 10%, in steps of 2%) in methylene chloride. ZEA and the fluorescent compound at TLC $R_f$ 0.8 eluted from the column with 2% methanol, with 10% methanol being required to elute DON and 15-ADON. DON and 15-ADON were not separated in this chromatographic system. The DON-containing residue recovered from the column was still colored (orange-brown) and viscous, although less so than the crude extract.

Attempts to crystallize DON from this extract were unsuccessful; "oiling out" of DON occurred in several solvent systems, including ethyl acetate/hexane, toluene, and ethyl acetate/toluene. Decolorizing the solution with activated charcoal did not enable DON to crystallize. Since this step resulted in substantial loss of DON by adsorption of DON to the charcoal, it was not used again.

The DON residue recovered from the silica gel column was rechromatographed on a similar column, using a stepwise gradient of ethyl acetate/hexane. DON and 15-ADON eluted with 60% ethyl acetate and were partially separated in this

system (Figure 5). DON did not crystallize from the orange yellow DON-containing fractions.

Partial separation of DON and contaminating pigments was achieved by a countercurrent extraction using ethyl acetate and water. DON partitioned selectively into the water, while pigments were largely retained in the ethyl acetate layer. Although crystallization of DON recovered from the water layer was unsuccessful, DON from this layer was crystallized from ethyl acetate following Sephadex LH-20 chromatography. DON remaining in the ethyl acetate layer, however, did not crystallize after the same treatment.

Although the multi-step method described here was lengthy and inconvenient, it did yield crystalline DON. Therefore, this method was considered acceptable for purification of gram quantities of DON. A major expense associated with the procedure was the silica gel (approximately 600 g) required to pack the 50 mm ID column used in the first silica gel chromatographic step. As this step functioned primarily to remove gross impurities from the crude extract, it was decided to reuse the silica gel after washing it in methanol followed by water. During chromatography of the crude extract using this washed silica gel, an observation was made that led to the development of the water-saturated silica gel method, a much improved procedure for purifying DON.

Figure 5. Chromatogram from silica gel chromatography of DON extract. The extract, containing DON (——), 15-ADON (---), and the compound of TLC $R_f$ 0.8 (•••), was recovered from a silica gel column eluted stepwise with 2 to 10% methanol in methylene chloride. The column (22 mmID) from which the chromatogram above was obtained was eluted stepwise (5 ml/min) with 300 ml each of 0, 15, 30, and 45% ethyl acetate in hexane, then 60% ethyl acetate in hexane. Fractions (20 ml) were monitored by TLC ($AlCl_3$ visualization, see Methods) and relative fluorescent intensity of the three compounds was estimated visually under longwave UV light. Fraction numbers refer to elution with 60% ethyl acetate in hexane.

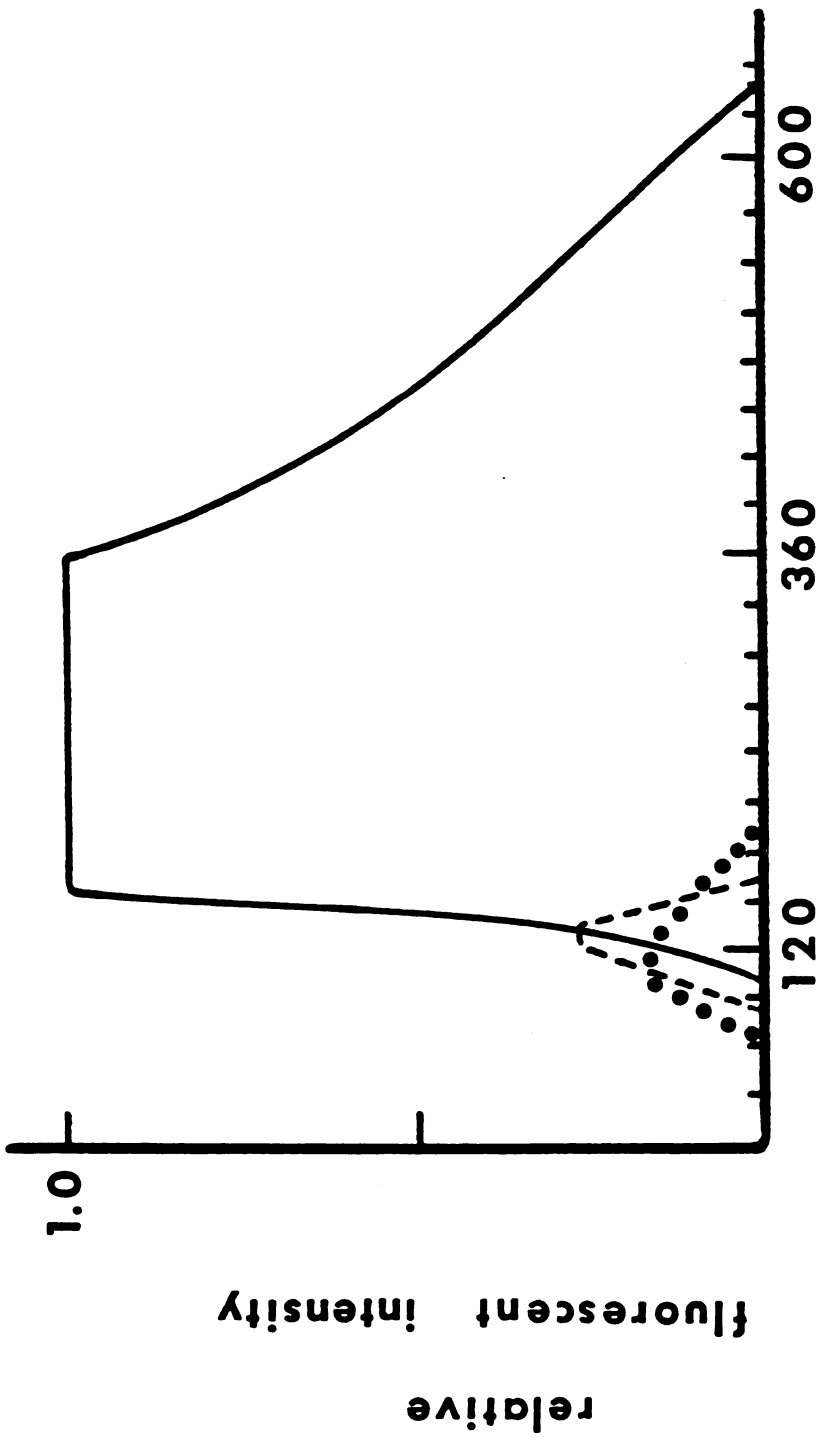

relative
fluorescent intensity
1.0
120
360
600
fraction number

FIGURE 5

Water-saturated silica gel chromatography

Crude extract was chromatographed on a column (50 mm ID) packed with washed silica gel and equilibrated with 2% methanol in methylene chloride. ZEA, the fluorescent compound at TLC $R_f$ 0.8 and 15-ADON eluted with 2% methanol and were not separated. The solvent was changed to 20% methanol, and fractions reflecting this solvent change consisted of two layers: the top one, water/methanol and the bottom one, methylene chloride/methanol. Both layers contained DON, the concentration in the aqueous layer being approximately four times greater than that in the organic layer. This observation suggested that the use of water in the separation system may be exploited in the purification of DON. Subsequent modification of this system resulted in an efficient procedure for purifying DON.

A silica gel column saturated with water, then equilibrated with 2% methanol in methylene chloride provided starting conditions that were reproducible. The crude extract was eluted with the equilibration solvent until ZEA, the compound of TLC $R_f$ 0.8, and 15-ADON appeared in the eluate. The solvent was then changed to methanol. Fractions composed of two solvent layers (water/methanol and methanol/methylene chloride) contained DON. Again, the concentration of DON in the aqueous layer was greater than that in the organic layer. Subsequent fractions composed of methanol (or methanol/water) contained DON in gradually decreasing concentrations.

Stepwise elution of the water-saturated column with

two immiscible solvents - methylene chloride followed by water - eliminated the inconvenience of extracting DON from both aqueous and organic solvent mixtures. ZEA, the compound of TLC $R_f$ 0.8, and 15-ADON eluted from the column with methylene chloride (Figure 6). After the mobile phase was changed to water, only one fraction contained both solvents. DON was detected only in the water layer of this fraction. The concentration of DON was maximum in the first few water fractions and then decreased rapidly (Figure 6). DON extracted from the water fractions crystallized readily from ethyl acetate. This chromatographic system was reproducible using both new and washed silica gel. Crude extracts weighing 30 g and containing as much as 2 g DON could be purified in a single run using the 37 mm ID column.

The distribution coefficients (water/methylene chloride) of both DON and 15-ADON were determined in order to provide some information about the mechanism of separation during water-saturated silica gel chromatography. The distribution coefficient of DON was between two and three orders of magnitude greater than that of 15-ADON (Table 6).

Analysis of crystalline DON

HPLC analysis of DON purified by either the multi-step or water-saturated silica gel method showed a single peak at 4.60 min using methanol:water (1:2, 1 ml/min). Use of a more polar eluent revealed a shoulder on the DON peak

Figure 6. TLC analysis of fractions obtained from water-saturated silica gel chromatography of crude extract. The water-saturated column was prepared and eluted as described in Methods. The crude extract (10.8 g) contained approximately 1.3 g of DON. The first spot, left to right, is crude extract before chromatography. Remaining spots, left to right, are fraction (10 ml) numbers 15, 25, 35, 45, 55, 65, 75, 82, 88, 92, 98, 102, 108, 112, 118, 125, 135, and 145. Fractions 1 to 70 were eluted with methylene chloride and the rest with water. Fractions 90 to 110 yielded 0.62 g of crystalline DON. TLC was carried out as described in Methods using $H_2SO_4$.

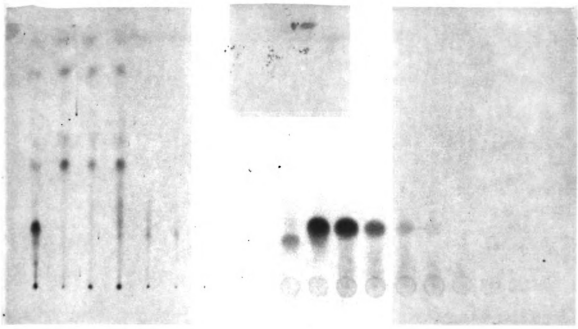


**FIGURE 6**

Figure 6. TLC analysis of fractions obtained from water-saturated silica gel chromatography of crude extract. The water-saturated column was prepared and eluted as described in Methods. The crude extract (10.8 g) contained approximately 1.3 g of DON. The first spot, left to right, is crude extract before chromatography. Remaining spots, left to right, are fraction (10 ml) numbers 15, 25, 35, 45, 55, 65, 75, 82, 88, 92, 98, 102, 108, 112, 118, 125, 135, and 145. Fractions 1 to 70 were eluted with methylene chloride and the rest with water. Fractions 90 to 110 yielded 0.62 g of crystalline DON. TLC was carried out as described in Methods using $H_2SO_4$.

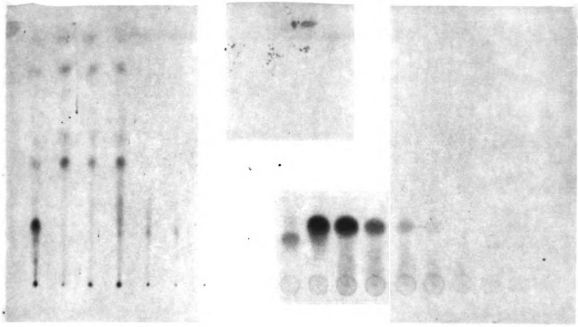


**FIGURE 6**

Table 6. Distribution coefficients of DON and 15-ADON between water and methylene chloride[a]

| Total toxin (mg) | Distribution coefficient[b] | |
|---|---|---|
| | DON | 15-ADON |
| 5.0 | 21.98 | 0.05 |
| 1.0 | 28.29 | 0.04 |
| 0.5 | 20.55 | 0.02 |
| 0.1 | 18.88 | --[c] |

[a]Either DON or 15-ADON was added to separatory funnels containing water and methylene chloride (5 ml each) and allowed to equilibrate between the two phases.

[b]The distribution coefficient was defined as toxin concentration in water/toxin concentration in methylene chloride. Toxins were quantitated by HPLC (see Methods).

[c]Not determined.

(Figure 7). This shoulder was also seen in commercial DON standard. The retention time of this shoulder did not correspond to that of 7-deoxy-DON. The melting point range of crystalline DON was 150-152 C. The UV absorption spectrum of DON (Figure 8) was characterized by an absorption maximum of 218 nm and an E of 6805 (25 ug/ml).

Figure 7. HPLC chromatogram of DON. Reverse phase HPLC was performed as described in Methods using a mobile phase (1 ml/min) of methanol:water (1:3). Absorbance was monitored at 224 nm. The arrow indicates the shoulder peak observed upon changing the mobile phase from 1:2 to 1:3 methanol:water.

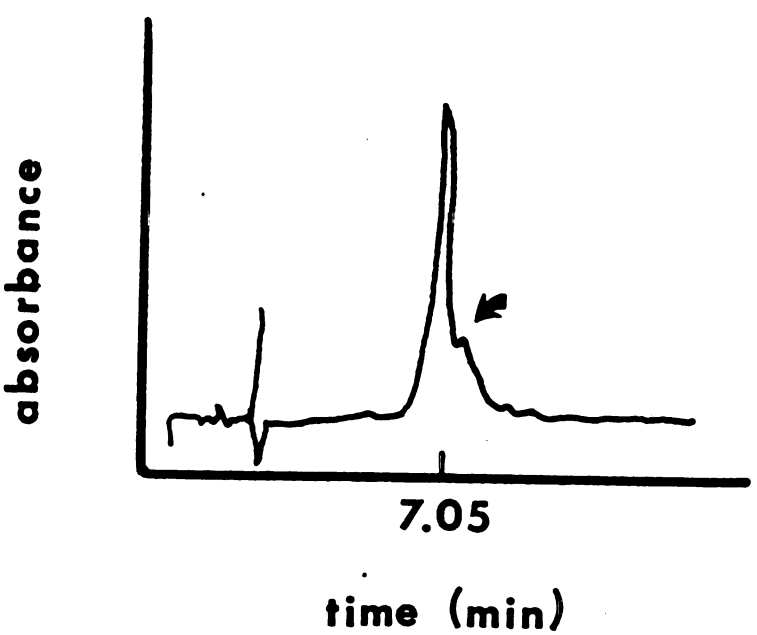



FIGURE 7

Figure 8. UV absorption spectrum of DON in ethanol (50 ug/ml).

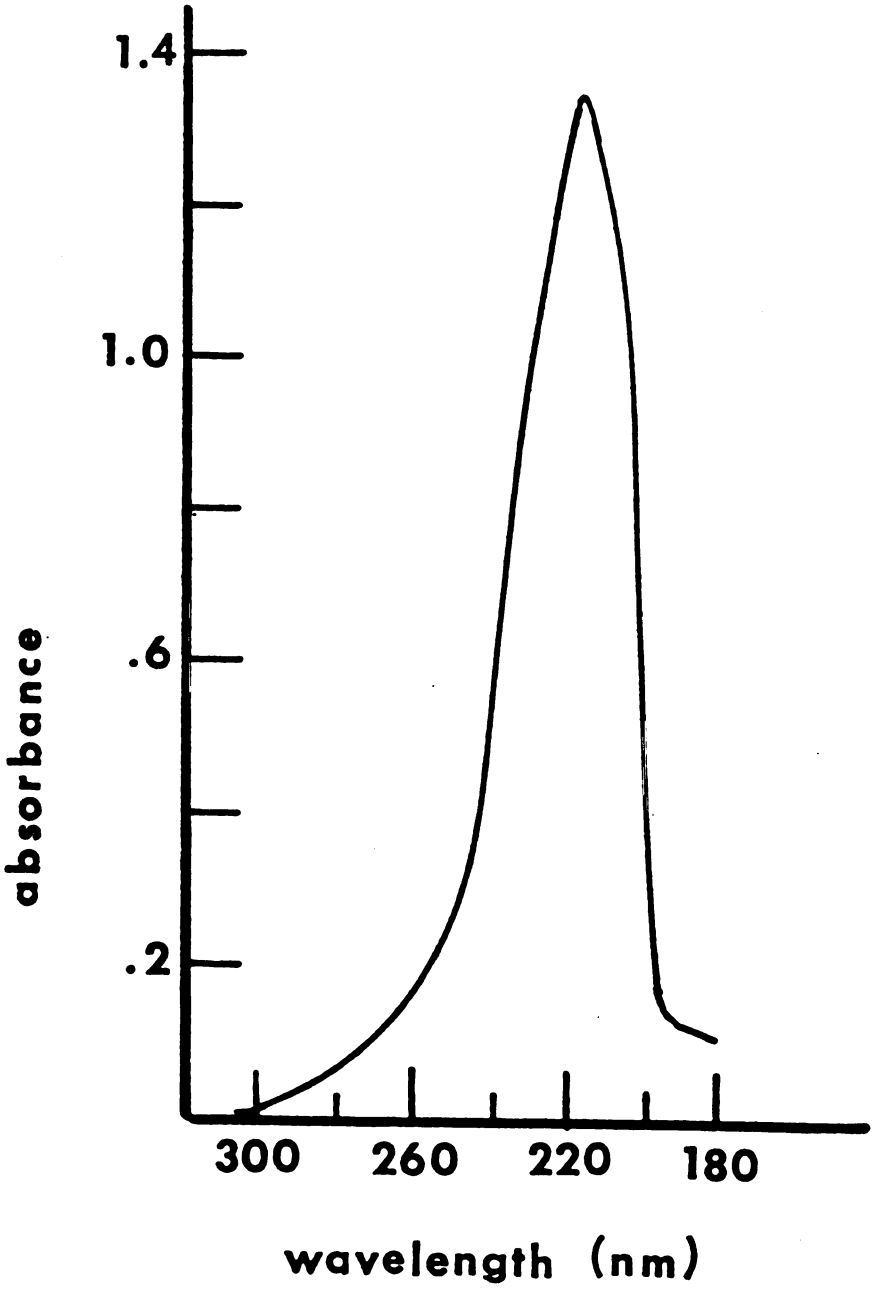



FIGURE 8

Figure 8. UV absorption spectrum of DON in ethanol (50 ug/ml).

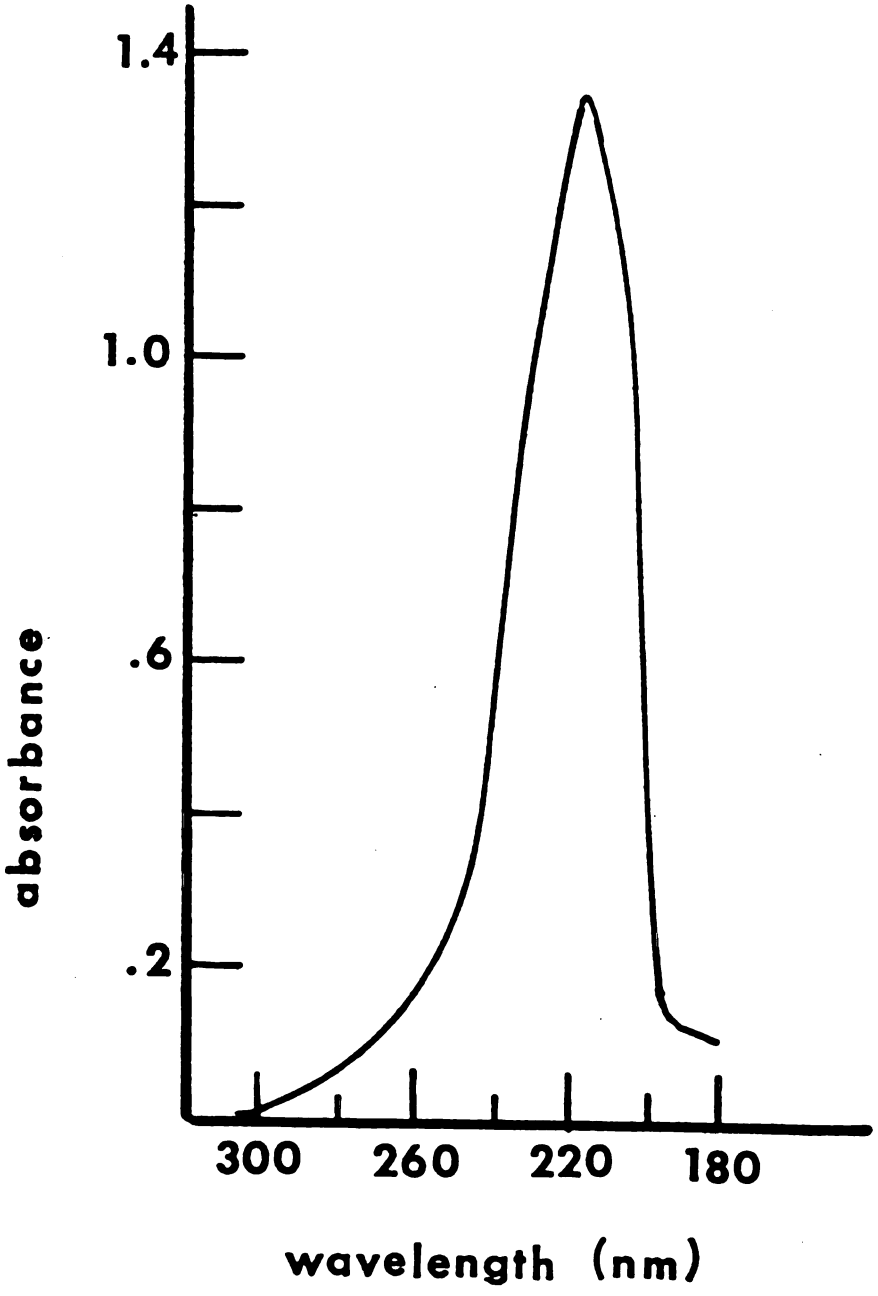



FIGURE 8

# DISCUSSION

## DON Production

The toxicological evaluation of DON has been hindered by the lack of gram quantities of DON needed for animal feeding studies. A major obstacle to the laboratory production of DON on solid substrates has been the inability to reproduce on a larger, practical scale the levels of DON accumulated during fermentations carried out in small culture flasks. Thus, the method described here for large scale production of DON can provide researchers with the quantities of DON needed for toxicological studies. Several toxicity studies have been performed in our laboratory (see Appendix) using DON purified by water-saturated silica gel chromatography.

An important factor in scaling up the fermentation was the use of cotton plugs rather than aluminum foil for covering the large culture flasks. The inability of Greenhalgh et al. (1983) to scale up their fermentation may also have been related to their use of aluminum foil caps. Because the head space of small flasks supporting DON biosynthesis had lowered oxygen and raised carbon dioxide levels compared to large flasks not containing DON, these

authors suggest that the gaseous environment is important in DON biosynthesis. Supporting this idea are experiments by Paster et al. (1986) and Paster and Menasherov (1988) that indicate the gaseous environment is important in the biosynthesis of T-2 toxin by F. tricinctum NRRL 3299 on solid substrates. There appeared to be a need for the presence of carbon dioxide, although high (>50%) levels were inhibitory to T-2 production. Low concentrations of oxygen were apparently not a factor in T-2 production. The lack of carbon dioxide accumulation in the large flasks of Greenhalgh et al. (1983) could indicate that these flasks need to be incubated longer in order to achieve the nutrient depletion required for commencement of secondary metabolism. The results reported here, however, suggest that large flasks do not inherently require a longer incubation time, since the time course of accumulation of DON was similar in both sizes of flasks. In these experiments, foil capped flasks remained relatively dry throughout the incubation period, while flasks stoppered with cotton plugs contained standing water. Foil caps on larger flasks may not have fit tightly enough, thereby allowing increased water loss and gas exchange. The relative humidity inside the flask may be an important factor in DON production.

While the optimum initial moisture content of the rice determined here agreed with that reported by Greenhalgh et al. (1983), the optimum incubation time was

somewhat shorter than that reported by Greenhalgh et al. (1983) and Vesonder et al. (1982). The shorter incubation time could be a characteristic of the isolate, F. graminearum R6576, used in these experiments. This strain was isolated in Michigan by Hart et al. (1982) from fusarium infected wheat. Although production of DON by R6576 has been studied in field inoculated corn (Hart et al., 1982,1984) and in liquid culture (El-Bahrawy et al., 1985; Pestka et al., 1985), it has not previously been studied in solid culture.

The time course of accumulation of DON in rice inoculated with F. graminearum R6576 was similar to that in liquid culture (Pestka et al., 1985) where maximum accumulation, coinciding with exhaustion of carbohydrate in the medium, occurred at 20 days. Qualitiatively similar time courses of accumulation have been observed after inoculation of field corn (Miller et al., 1983b) and winter wheat (Miller and Young, 1985) with other F. graminearum isolates: DON concentrations in corn and wheat peaked at about 7 weeks and then decreased over the next few weeks. A decline in DON concentration in the 2 to 3 weeks before harvest was also observed in winter wheat naturally infected with head blight (Scott et al., 1984b). The cause of this decrease in DON accumulation with time is not known. Miller et al. (1983b) suggested that the decrease in DON in field corn inoculated with F. graminearum was due to degradation by corn enzymes, rather than fungal enzymes, since the decrease was associated with a decrease in viable

fungal counts. However, the use of autoclaved rice as described here and the use of liquid culture as described by Pestka et al. (1985) indicate that either fungal enzymes or chemical reactions are likely to be responsible for degradation of DON in fermentations by F. graminearum R6576.

Fungal enzymes are also likely to responsible for the apparent conversion of 15-ADON to DON in these rice cultures. The time course of 15-ADON accumulation in rice in relation to that of DON suggests that 15-ADON is a precursor of DON, and studies in liquid culture support this idea. In liquid culture, DON accumulation began as 15-ADON concentrations peaked (Miller et al., 1983a; El-Bahrawy et al., 1985; Pestka et al., 1985). A similar relationship between the accumulation of 3-ADON and DON was observed in the fermentation of a F. roseum isolate (Yoshizawa and Morooka, 1975). Japanese isolates of G. zea showed similar patterns of accumulation for nivalenol and its acetylated derivative fusarenon X in rice and liquid culture, suggesting that this relationship is typical in trichothecene biosynthesis (Abouzied and Pestka, 1986). In liquid culture, both the fungal strain (El-Bahrawy et al., 1985) and the composition of the medium (Pestka et al., 1985) are important in determining the level and ratio of DON and 15-ADON accumulation by F. graminearum over time. Although the effects of medium composition on trichothecene production are not clear, both the carbon and nitrogen

sources have been shown to affect the yields and ratios of DON, 3-ADON, and 15-ADON by F. graminearum (Miller and Greenhalgh, 1985). Further studies are needed to elucidate the complex interactions between genetic components of trichothecene biosynthesis and its regulation and environmental factors. However, either chemical hydrolysis or exogenous enzymes (plant or microbial) could play a role in conversion of 15-ADON (or 3-ADON) to DON in the field. The deacetylation of trichothecenes by Fusarium strains (Yoshizawa and Morooka, 1975; Yoshizawa et al., 1980) and by bacteria (Claridge and Schmitz, 1978; Ueno et al., 1983) has been demonstrated.

Surprisingly, F. graminearum NRRL 5883, a strain frequently used in DON production studies (Table 2), was a poor DON producer in the present experiments. Likewise, this NRRL 5883 isolate produced mainly 15-ADON (12.4 mg/L) rather than DON (0.5 mg/L) in liquid culture (El-Bahrawy et al., 1985). Although NRRL 5883 (Vesonder et al., 1982) -- and other Fusarium strains (Pathre and Mirocha, 1978) -- failed to produce DON in certain liquid media despite production on rice, NRRL 5883 has previously been shown to produce DON on rice under incubation conditions similar to those used here. Thus, the NRRL 5883 culture used in our laboratory may have mutated during laboratory passage, losing its ability to produce DON.

The success of a spore inoculum in decreasing the variability of DON accumulation in liquid culture (Pestka et al., 1985) led to its use here instead of the PDA plug

inoculum used by Greenhalgh et al. (1983). Although sporulation of fungi is frequently induced on solid media, for example, the hay agar slants used by Vesonder et al. (1982) and Megalla et al. (1987) for DON production, preparation of a spore inoculum in liquid culture is generally more convenient and gives an inoculum that is readily quantitated (Hockenhull, 1980). CMC has been shown to stimulate sporulation in some fungi in submerged culture (Cappellini and Peterson, 1965), including F. graminearum used in the present study.

It is not clear whether or not shaking flasks during incubation affected the level of DON production. The unshaken NRRL 5883 flask exhibiting higher DON accumulation may have merely been an "outlier", or perhaps a flask that was mislabeled since an abnormally low accumulation was observed for one R6576 flask in the same experiment. The increased DON accumulation seen in the single flask was not reproduced in subsequent experiments where flasks inoculated with NRRL 5883 were not shaken. Furthermore, whether or not flasks were shaken did not affect DON accumulation by R6576, when other incubation conditions were held constant. Although several procedures in the literature recommend shaking flasks during mycotoxin production on solid substrates in order to break up mycelial masses (Lindenfelser et al., 1978; Lee and Mirocha, 1984; Hagler et al., 1981), it is doubtful that this practice has much effect since after only a few days

of incubation shaking the flask is no longer effective in breaking up clumps of rice.

## DON Purification

Although crystalline DON was obtained using the multi-step chromatography method, this time-consuming procedure offered no advantages over other published methods. Silica gel chromatography was useful for the removal of gross impurities from the crude extract, but did not produce DON pure enough for crystallization. Water-saturated silica gel chromatography, however, which purified DON in a single chromatographic step, was a marked improvement over the lengthy multi-step procedure. This simple procedure also avoided the acetylation/hydrolysis and hydrolysis steps of Ehrlich and Lillehoj (1984) and Greenhalgh et al. (1986), respectively.

Deactivation of silica gel with water apparently resulted in a separation based on liquid-liquid partitioning rather than the usual adsorption mechanism of silica gel. Here, silica gel functioned as a support for a layer of adsorbed water. This water layer selectively extracted the hydrophilic DON from the crude rice extract. DON purified by water-saturated silica gel chromatography crystallized readily, although standard recrystallization techniques were unsuccessful. The method used here, however, has also been used for crystallization of other

trichothecene mycotoxins: nivalenol and fusarenone-X (Lee and Mirocha, 1984) and 15-ADON (Pestka et al., 1986).

Unexpectedly, 15-ADON was not retained on the column even though it differs from DON only in the replacement of a single hydroxyl with an acetyl group. The difference in distribution coefficients of DON and 15-ADON between water and methylene chloride, however, appears to account for this result. Although Type B trichothecenes, including DON, are routinely extracted from grains using aqueous methanol, water has occasionally been used to achieve a somewhat selective extraction of DON from grains for analysis by gas chromatography (Stahr et al., 1983; Terhune et al., 1984).

The water-saturated silica gel column is analogous to the solid phase extraction systems commonly used to extract trace quantities of organic compounds from complex sample matrices (Tippins, 1987). In these systems, the use of a bonded silica sorbent selective for the compound of interest results in high recoveries of the purified compound. Deactivated silica gel can be viewed as a polar sorbent used on a preparative scale. Like solid phase extractions, the water-saturated silica gel procedure is rapid and much less costly (for example, in solvent requirements) than alternative separation techniques. Scott et al. (1984a) used a solid phase extraction in the purification of gram quantities of DON. An aqueous extract of DON was adsorbed onto a ChemTube containing a

hydrophilic matrix, then DON was eluted with ethyl acetate. Several other steps, however, including columnn chromatography and semi-preparative liquid chromatography, were required to obtain crystalline DON.

The identity of DON purified by water saturated silica gel chromatography was confirmed by several analytical techniques. Both the melting point range and UV absorption maximum determined for DON purified here were the same at those reported in the literature (Windholz, 1983). There was a discrepancy between the E value at 218 nm determined here (6805) and that reported by Windholz (1983) for DON (4500), although a value of 6384 was also determined by Scott et al. (1984a). A mass spectrum of the purified DON was identical to that of a DON standard.

Crystalline DON was of high chromatographic purity, although manipulation of chromatographic conditions did reveal a trace contaminant. The shoulder peak seen during HPLC of the DON purified in the present study could be a closely related trichothecene of similar polarity. Bennett et al. (1981) reported a similar situation where both DON purified from field inoculated corn and an analytical standard were contaminated by a trichothecene identified as 7-deoxyDON. The fact that commercial DON standards contain these contaminants suggests that they are essentially unavoidable in DON prepared from natural sources. This is not unexpected considering the large number of trichothecenes already identified and the many sites available for structural modification around the

trichothecene nucleus.

Acute toxicity studies confirmed the biological activity of DON purified by water-saturated silica gel chromatography (Forsell et al., 1987; see Appendix). The $LD_{50}$ of DON in female B6C3F1 mice was 78 mg DON/kg body weight by the oral route of administration and 49 mg/kg by intraperitoneal (ip) exposure. These values are similar to those reported by Yoshizawa and Morooka (1977) in male mice: 46 mg/kg (oral) and 70 mg/kg (ip). Strain, sex, and age differences among the animals used may account for the different $LD_{50}$ values.

## Conclusions

The methods developed in the present study for the laboratory production and purification of DON can provide the gram quantities of crystalline DON needed for toxicological studies. Furthermore, these methods improve upon existing ones. High yields of DON were achieved in rice culture on a scale practical for mass production of DON. The maintainence of a crucial gaseous environment in the large flasks used may have been a key factor in scaling up the fermentation. Purification of DON by water-saturated silica gel chromatography was simple, rapid, and economical. In addition, DON purified by this method crystallized readily.

The time course of accumulation of DON and 15-ADON in rice by F. graminearum R6576 was consistent with that observed previously for Fusarium strains in general. Fungal enzymes or chemical reactions are likely to be responsible for both the decline in DON concentrations and the apparent conversion of 15-ADON to DON in these rice cultures.

The work described here was required in order to conduct feeding studies in mice to assess the chronic toxicity and immunotoxicity of DON. Dietary exposure to DON was a key feature of these toxicity studies, since this is the route of exposure expected for animals and humans. From these toxicity studies, in turn, developed both the experiments described in Part II of this thesis and in vitro experiments being conducted by others in this laboratory.

# PART II

# CELLULAR UPTAKE OF [$^{3}$H]T-2 TOXIN

ABSTRACT

CELLULAR UPTAKE OF [$^3$H]T-2 TOXIN

By

Mary Frances Witt

The uptake of [$^3$H]T-2 toxin by a murine B-cell hybridoma was studied in order to learn more about the way in which trichothecenes interact with immune cells. Alpha-[$^3$H]T-2 toxin (specific activity 508 mCi/mmol) of 99% chemical and radiochemical purity was prepared for use in the uptake studies. Both the rate of uptake and the time required for accumulation of alpha-[$^3$H]T-2 by hybridomas to reach equilibrium were proportional to the concentration of [$^3$H]T-2 over the range of $1 \times 10^{-8}$ to $4 \times 10^{-7}$ M. The total cell associated [$^3$H]T-2 was proportional to the concentration of [$^3$H]T-2 from $10^{-8}$ to $10^{-3}$ M. Cells accumulated 20 to 40% of added radioactivity in the presence of $10^{-8}$ to $10^{-7}$ M [$^3$H]T-2, but only 2 to 3% in the presence of $10^{-5}$ to $10^{-3}$ M. The rate of uptake of [$^3$H]T-2 by hybridomas was inhibited by the trichothecenes T-2 toxin, deoxynivalenol, verrucarin A, and roridin A, as well as the structurally unrelated antibiotic anisomycin. Both the uptake of alpha-[$^3$H]T-2 by murine splenic lymphocytes and the uptake of beta-[$^3$H]T-2 by hybridomas were qualitatively similar to the uptake of alpha-[$^3$H]T-2 by hybridomas. It was suggested that the uptake of [$^3$H]T-2 by hybridomas is mediated by binding of toxin to an intracellular site, namely, ribosomes.

# INTRODUCTION

## Rationale

In order to understand how trichothecenes cause their toxic effects -- particularly their immunotoxic effects -- in animals and humans, current research has focused on the action of the trichothecenes at the cellular level. One important aspect of cell-toxin interaction that has not been studied in detail for the trichothecenes is how these toxins are taken up by cells.

The entry of trichothecenes into cells plays a key role in both the pharmacokinetic and pharmacodynamic interactions of these toxins. Pharmacokinetic processes such as absorption, distribution, accumulation, and elimination of toxin in animals and humans all involve movement of toxin across cell membranes. The dose-response relationship for a toxin is a function of both the concentration of the toxin at its active site and the intrinsic ability of the toxin to exert its effect. The pharmacodynamics of the trichothecenes relate to their intracellular concentration, since the primary action of these toxins is the inhibition of eucaryotic protein synthesis through interaction with ribosomal peptidyl

transferase (Ueno et al., 1973; Wei and McLaughlin, 1974; Cundliffe et al., 1974).

Thus, by contributing to an understanding of their pharmacokinetics and pharmacodynamics, knowledge of how trichothecenes are taken up by cells will help assess the hazard trichothecenes present to animal and human health. This information may also be useful in developing preventive and therapeutic measures for trichothecene toxicity.

## Terminology

The terminology used here will be that of Wohlhueter and Plagemann (1980), which is based on the use of radioisotope techniques to monitor cellular uptake. Uptake, or accumulation, is defined operationally as the appearance within the cell of radioactivity derived from an exogenous substrate and is due to processes including permeation, binding of substrate to intracellular material, and the metabolic conversion of isotopic substrate within the cell. Permeation is defined as the transfer of substrate from one side of the membrane to the other in chemically unaltered form and is either mediated or nonmediated. Mediated permeation, also called transport, refers to structurally specific, saturable mechanisms of transfer. In contrast, neither specificity nor

saturability can be demonstrated experimentally for nonmediated permeation, or simple diffusion. Mediated mechanisms of permeation include passive and active transport. Passive transport, for example facilitated diffusion, is a nonconcentrative process. Active transport is characterized by the capacity to establish an electrochemical gradient across the membrane.

## Uptake of Molecules

The movement of molecules across cell membranes is fundamental to cell biology. The survival and optimal functioning of individual cells depend on the function of the membrane as a semi-permeable barrier that maintains the cell's selective intracellular environment while allowing the exchange of nutrients and waste products and the passage of certain regulatory molecules. The effects of many xenobiotics - both therapeutic agents and toxins - on cells are closely linked to their ability to cross cell membranes. Because the mechanism by which molecules cross cell membranes determines factors influencing uptake, it is desirable to be able to distinguish experimentally among the different types of permeation. In general, mediated and nonmediated systems can be distinguished by their kinetics using radioisotope techniques to monitor uptake.

Nonmediated permeation, or simple diffusion, is governed by a modification of Fick's Law (Stein, 1986).

The net flux of a permeant crossing a biological membrane by diffusion is proportional to the concentration gradient of the permeant across the membrane. The proportionality constant relating the flux to the concentration gradient, the permeability coefficient, depends on properties of both the permeant and the membrane. For a given membrane, the membrane permeability is proportional to the lipid solvent solubility of the permeant and inversely related to the molecular weight of the permeant.

The kinetics of mediated permeation are equivalent to Michaelis-Menten enzyme kinetics (Christensen, 1975), that is, the rate of transport is saturable with respect to the concentration of permeant. This is a consequence of the finite number of "acceptor" sites on the membrane that facilitate transport of substrate. Another hallmark of transport is its chemical specificity, which can be demonstrated by the competitive inhibition of transport by compounds structurally related to the permeant.

The uptake of molecules by cells, as measured by the accumulation of substrate-derived radioactivity within the cell may involve more than permeation. Substrate may accumulate in the cell due to binding to intracellular sites, for instance, the binding of steroid hormones to their cytoplasmic receptors. The uptake of metabolizable substrates results in the accumulation of radioactive, nonpermeable metabolites. For example, the uptake of sugars and nucleosides is followed by their phosphorylation

by intracellular kinases. Both receptor-ligand binding and enzyme kinetics exhibit the saturation and specificity characteristic of transport systems. Thus, the demonstration of saturability and specificity for an uptake system must be interpretated with caution, and within the context of the whole cell.

## Reveiw of Literature

Because they are low molecular weight, uncharged, and relatively nonpolar compounds, trichothecenes are expected to cross biological membranes by simple diffusion. A review of the literature, however, suggests that the cellular uptake of at least one trichothecene, T-2 toxin, may be more complicated.

Trusal and Watiwat (1983) and Trusal and Martin (1987) observed a dose-dependent accumulation and rate of uptake of [$^3$H]T-2 toxin by both African green monkey kidney (Vero) cells and Chinese hamster ovary (CHO) cells. Accumulation and rate of uptake increased with temperature, over a temperature range of 0 to 37 C. Between the two studies, the dependence of accumulation of [$^3$H]T-2 by cells on dose and temperature was observed for concentrations of [$^3$H]T-2 ranging from $2.15 \times 10^{-9}$ to $2.15 \times 10^{-7}$ M.

The time required for accumulation of [$^3$H]T-2 by Vero and CHO cells to reach equilibrium may depend on the dose of toxin. Trusal and Martin (1987) reported that

accumulation of [$^3$H]T-2 by Vero and CHO cells in the presence of 2.15 x $10^{-9}$ to 2.15 x $10^{-8}$ M [$^3$H]T-2 increased with time for at least 3 hr, after which the experiment was terminated. Trusal and Watiwat (1983) found that accumulation in the presence of 2.15 x $10^{-8}$ to 2.15 x $10^{-7}$ M [$^3$H]T-2 reached equilibrium after 2 to 3 hr, at both 22 and 37 C. Thompson and Wannemacher (1983), however, reported that equilibrium levels in Vero cells were reached in only 10 to 15 min. Unfortunately, the authors of this abstract described the uptake of T-2 only in qualitative terms and did not include the concentration of [$^3$H]T-2 used in uptake assays.

Vero cells exhibited greater accumulation and a higher rate of uptake than CHO cells (Trusal and Watiwat, 1983; Trusal and Martin, 1987). Over the range of [$^3$H]T-2 concentrations (2.15 x $10^{-8}$ to 2.15 x $10^{-7}$ M) and temperatures (0, 22, 37 C) tested, Vero cells accumulated 3 to 4 times as much [$^3$H]T-2 as CHO cells (Trusal and Watiwat, 1983). The authors hypothesized that differences in uptake between cell types may have reflected differences in either the number or the affinity of T-2 binding sites associated with the cells. Furthermore, it was suggested that since the differences in uptake were most pronounced at lower toxin concentrations, these binding sites may be saturated at high toxin concentrations, thereby masking differences between cell types.

In an effort to discern the basis for differences in

uptake parameters between the two cell types, Trusal and Martin (1987) measured cell volume and total cellular RNA content of Vero and CHO cells. Total cellular RNA represented the ribosomal content of the cells and, as such, was assumed to be related to the number of trichothecene ribosomal binding sites. The RNA content of CHO cells was 3.8 times greater than that of Vero cells. Thus, differences in the number of ribosomal binding sites did not appear to be responsible for the observed increased uptake by Vero cells compared to CHO cells. The mean cell volume of Vero cells, however, was 3.4 times larger than that of CHO cells, raising the possibility that differences in cell volume may play a role in uptake differences.

Sodium fluoride (NaF) increased the accumulation and rate of uptake of [$^3$H]T-2 toxin by both CHO and Vero cells (Trusal and Martin, 1987). The relative increase in toxin uptake caused by NaF was greater for Vero cells than for CHO cells. Since NaF affects the glycolytic pathway, the authors speculated that this action, for example, a disruption of the cell's energy supply, may be important in the observed increased uptake of [$^3$H]T-2 by cultured cells.

Gyongyossy-Issa and Khachatourians (1984) studied the uptake of [$^3$H]T-2 toxin by murine splenic lymphocytes. Lymphocytes accumulated [$^3$H]T-2 in a dose-dependent manner and equilibrium levels were reached after 10 to 15 min of incubation. The accumulation appeared to be saturable with respect to [$^3$H]T-2 concentration over a 10-fold

concentration range. The accumulation of [$^3$H]T-2 was inhibited by unlabeled T-2 toxin, over a 1000-fold range of T-2 concentration, with a maximum of 60% inhibition in the presence of 2.7 x $10^{-7}$ M T-2. Both the accumulation and the rate of uptake of [$^3$H]T-2 by lymphocytes increased with temperature from 19 to 37 C. Accumulation was not affected by the presence of energy inhibitors when added simultaneously with toxin. Preincubation of cells, however, with energy inhibitors reduced accumulation of [$^3$H]T-2 by approximately 25%. Thus, at least part of the accumulation of [$^3$H]T-2 by lymphocytes appeared to be dependent on an available energy supply. A mean affinity constant of 1.6 x $10^7$ $M^{-1}$ for the interaction of [$^3$H]T-2 with lymphocytes was determined by Scatchard analysis of the accumulation data. The total binding capacity of lymphocytes was estimated to be 1.11 x $10^5$ molecules T-2/cell.

Further work by Gyongyossy-Issa and Khachatourians (1985) demonstrated a possible biological significance for the binding capacity of lymphocytes for T-2 toxin. The authors observed a threshold dose for inhibition of protein, DNA, and RNA syntheses by T-2 in both resting and mitogen-stimulated murine splenic lymphocytes. Below the threshold dose, no inhibition of macromolecular synthesis was detected compared to control cultures, while above the threshold dose, the time required for essentially complete inhibition depended on the concentration of T-2. The

threshold dose, 1.1 ng T-2/ml, corresponded to 5 x $10^5$ molecules T-2/cell, which was the same order of magnitude as the binding capacity (1.11 x $10^5$ molecules T-2/cell) estimated earlier (Gyongyossy-Issa and Khachatourians, 1984).

While questions remain, the studies reviewed above suggest that the uptake of [$^3$H]T-2 toxin by cultured cells and murine lymphocytes is more complicated than simple diffusion. Although Gyongyossy-Issa and Khachatourians (1984) speculated that a T-2 toxin membrane interaction may be responsible for the association of T-2 with lymphocytes, no direct evidence for this exists. It brings up the possibility, however, that T-2 toxin may enter cells via a mediated permeation process. This possibility can be explored through more detailed kinetic studies designed to test for chemical specificity and saturability of uptake.

It is also possible that the interaction of the trichothecenes with eucaryotic ribosomes plays a role in the uptake of these toxins by cells. The radiolabeled trichothecene [acetyl-$^{14}$C]trichodermin has been shown to bind to ribosomes from human tonsils and yeast with dissociation constants of 6.7 x $10^{-7}$ and 1.8 x $10^{-6}$ M, respectively (Barbacid and Vazquez, 1974). The kinetics of binding may depend not only on the source of the ribosomes, but also their form, since the dissociation constants for binding of [acetyl-$^{14}$C]trichodermin to yeast monoribosomes and polyribosomes were 2.10 x $10^{-6}$ and 7.2 x $10^{-7}$ M, respectively (Cannon et al., 1976a). In both of the above

studies, Scatchard analysis of the binding data indicated that one molecule of [acetyl-$^{14}$C]trichodermin bound per ribosome and in each case the binding was of a single affinity.

The binding of [acetyl-$^{14}$C]trichodermin was competitively inhibited by the trichothecenes trichothecin, trichodermol, T-2 toxin, fusarenon X, and verrucarin A (Barbacid and Vazquez, 1974a; Cannon et al., 1976a). Again, the form of the ribosomes seemed to influence binding since all the trichothecenes above competed to a similar degree with monoribosomes, but to varying degrees with polyribosomes (Cannon et al., 1976a). It was suggested that the presence of nascent polypeptidyl-tRNA chains on the polyribosomes exclude binding of trichothecenes to varying degrees. This is consistent with the observation that T-2 toxin failed to inhibit poly (U)-directed polyphenylalanine synthesis in cell-free systems once the ribosomes carried nascent polyphenylalanine chains above a certain critical chain length (Cannon et al., 1976b).

The binding site for trichocenes appears to be located on the 60S ribosomal subunit. The dissociation constant for binding of [acetyl-$^{14}$C]trichodermin to yeast 60S ribosomal subunits was $1.4 \times 10^{-6}$ M, similar to the $1.8 \times 10^{-6}$ M for 80S ribosomes (Barbacid and Vazquez, 1974a). A mutant of Saccharomyces cerevisiae containing an altered 60S subunit was resistant to trichodermin (Schindler et

al., 1974). A single recessive nuclear gene, tcm1, was responsible for the resistance (Grant et al., 1976) and mutations at this locus also determined resistance to the trichothecenes nivalenol and trichothecin. A plasmid carrying tcm1 transformed sensitive cells to resistant cells (Fried and Warner, 1981). Polyribosomes from transformed cells were resistant to trichodermin in an in vitro protein synthesis assay. Since this plasmid also carried the gene for the 60S ribosomal protein L3, the authors proposed that the gene for trichodermin resistance in yeast, tcm1, specifies the ribosomal protein L3.

The mutation conferring resistance against trichodermin appears to affect the ribosomal binding site for trichothecenes. 80S and 60S ribosomes of a S. cerevisiae mutant resistant to trichodermin and cross-resistant to fusarenon X, trichothecin, and verrucarin A (Jiminez et al., 1975) showed decreased binding of [acetyl-$^{14}$C]trichodermin (Jimenez and Vazquez, 1975). Dissociation constants for ribosomes from sensitive and resistant strains were $9.9 \times 10^{-7}$ and $1.54 \times 10^{-5}$ M, respectively. Gupta and Siminovitch (1978) isolated mutant CHO cells resistant to trichodermin and cross-resistant to trichodermol and verrucarin A. Binding of [acetyl-$^{14}$C]trichodermin to 60S subunits from these mutants was decreased compared to 60S subunits from sensitive cells.

Anisomycin, an antibiotic structurally unrelated to the trichothecenes that also inhibits eucaryotic protein synthesis, inhibited the binding of [acetyl-

[$^{14}$C]trichodermin to yeast ribosomes (Barbacid and Vazquez, 1974a). Likewise, binding of [$^{3}$H]anisomycin to yeast ribosomes was inhibited by trichodermin, trichodermol, fusarenon X, trichothecin, and verrucarin A (Barbacid and Vazquez, 1974b). It appears, however, that although the binding sites of anisomycin and trichothecenes are mutually exclusive, they are not identical. Two S. cerevisiae mutants resistant to trichodermin (Grant et al., 1976; Jimenez et al., 1975) were also resistant to anisomycin, but ribosomes isolated from the S. cerevisiae mutants did not exhibit altered binding of [$^{3}$H]anisomycin compared to sensitive strains (Jimenez et al., 1975; Jimenez and Vazquez, 1975). Thus, the resistance of these mutants to anisomycin, unlike that to trichodermin, did not appear to be due to a lowered binding affinity to ribosomes. Rather, it could be related to a conformational or structural alteration in the ribosome related to the change that decreases trichodermin binding. Such an alteration could affect the way anisomycin interacts with the ribosome to inhibit peptidyl transferase. Inhibition of protein synthesis by these antibiotics may involve more than mere occupancy of a single binding site. For instance, proteins of both the large and small ribosomal subunits of Drosophila were affinity labeled with [$^{14}$C](bromoacetyl)trichodermin (Gilly et al., 1985), suggesting that the interaction of trichodermin with the ribosome is complex.

## Research Objectives

The intent of this research was to study the cellular uptake of a representative trichothecene, T-2 toxin. The uptake was monitored using tritium-labeled T-2 toxin. Because certain quantitative aspects of the uptake of T-2 may depend on the type of cell (Trusal and Watiwat, 1983; Trusal and Martin, 1987), uptake was studied in a homogeneous population of cells -- a murine B-cell hybridoma. The B-cell hybridoma was chosen because of its relation to the immune system.

In order to determine if the uptake of [$^3$H]T-2 toxin by hybridomas occurs by a mediated or nonmediated process, experiments designed to detect saturability and specificity of uptake were performed. The possibility that the ribosomes are involved in the uptake of [$^3$H]T-2 was investigated.

Preparation of [$^3$H]T-2 toxin by the method of Wallace et al. (1977) was expected to require substantial effort. Two isomers of [$^3$H]T-2 toxin -- 3-alpha-hydroxy- and 3-beta-hydroxy-3-[$^3$H]T-2 toxin -- are produced in the labeling reaction. The alpha isomer is preferred for uptake studies since it is the naturally occurring one. Thus, it was necessary to prepare alpha-[$^3$H]T-2 toxin of high chemical and radiochemical purity. Notably, information about the configuration and purity of the [$^3$H]T-2 used in the studies reviewed earlier is absent.

# MATERIALS AND METHODS

## Preparation of [$^3$H]T-2 Toxin

Oxidation of T-2 toxin.

T-2 toxin was oxidized to 3-dehydro T-2 toxin by the method of Wallace et al. (1977) using N-chlorosuccinimide, and by a method recommended by G. Zhang (University of Wisconsin, personal communication) using pyridinium chlorochromate. T-2 toxin was provided by Dr. J.J. Pestka. During both oxidation reactions, the conversion of T-2 toxin to 3-dehydro T-2 toxin was followed using thin layer chromatography as described below.

N-cholorosuccinimide method. This reaction was carried out in an atmosphere of dry $N_2$. Dimethylsulfide (4.8 ul) (Aldrich Chemical Co., Inc., Milwaukee, WI) was added to a stirred suspension of N-chlorosuccinimide (4.5 mg) (Sigma Chemical Co., St. Louis, MO) in dry toluene (0.5 ml). The temperature of the reaction mixture was maintained at 0 C by an ice water bath. A white precipitate appeared upon addition of dimethylsulfide. The mixture was then cooled to -25 C in a carbon tetrachloride-dry ice bath and a

solution of T-2 toxin (10 mg) in toluene (0.5 ml) was added dropwise. The reaction proceeded at -25 C for 2 hr, then a solution of triethylamine (3.4 mg, 4.7 ul) (99%, Aldrich Chemical Co., Inc.) in toluene (0.25 ml) was added dropwise. The cooling bath was removed and, after 5 min, diethyl ether (20 ml) added. The organic layer was washed with 0.1 N HCl (1 x 10 ml), then water (2 x 15 ml). Finally, the organic layer was dried with $Na_2SO_4$ and the ether evaporated under $N_2$, leaving a residue of 3-dehydro T-2 toxin.

Pyridinium chlorochromate method. T-2 toxin (10 mg) dissolved in dry methylene chloride (0.25 ml) was added to a stirred suspension of pyridinium chlorochromate (PCC) (50 mg) (98%, Aldrich Chemical Co., Inc.) in dry methylene chloride (1.0 ml). The reaction proceeded at room temperature, marked by precipitation of the reduced (black) reagent. After 12-15 hr., more PCC (10 mg) was added. The reaction was terminated after a total of 24 hr by washing the reaction mixture with 1 N HCl (4 x 10 ml) to remove unreacted (orange) PCC. The organic layer was then dried with $Na_2SO_4$ and the methylene chloride evaporated under $N_2$, leaving a residue of 3-dehydro T-2 toxin.

Reduction of 3-dehydro T-2 toxin.

3-dehydro T-2 toxin was reduced by the method of Wallace et al. (1977). The reduction was carried out at

room temperature in an atmosphere of dry $N_2$. Tritiated sodium borohydride (25 mCi, specific activity 10 Ci/mM) (Research Products International, Corp., Mt. Prospect, IL) or sodium borohydride (0.2 mg) (Sigma Chemical Co.) dissolved in isopropanol (5 ml) was added to a stirred solution of 3-dehydro T-2 toxin (6 to 10 mg) in isopropanol (5 ml). After 5 hr, the reaction mixture was diluted with chloroform (4 ml) and washed with 0.3 N HCl (3 ml). The aqueous layer was extracted with chloroform (5 x 4 ml), then the chloroform layers combined, dried with $Na_2SO_4$, and concentrated under $N_2$ to approximately 6 ml.

Thin layer chromatography (TLC).

TLC was performed on precoated 10 cm x 10 cm silica gel high performance TLC (HPTLC) plates with preadsorbent strips (LHP-K Linear K plates, Whatman Chemical Separation, Inc., Clifton, NJ). The solvent system was toluene:ethyl acetate (1:3). T-2 and 3-dehydro T-2 toxins were visualized by 4-(p-nitrobenzyl)pyridine (NBP), a reagent specific for compounds possessing an epoxide function (Takitani et al., 1979). NBP (Sigma Chemical Co.) (3%, wt/wt) dissolved in chloroform:carbon tetrachloride (2:3) was sprayed on the developed TLC plate, the solvent evaporated, and the plate heated at 150 C for 30 min. After the plate had cooled, it was sprayed with 10% (v/v) tetraethylene pentamine (TEPA) (Aldrich Chemical Company, Inc.) in chloroform:carbon tetrachloride (2:3). T-2 and 3-

dehydro T-2 toxins appeared as dark blue spots. TEPA solution was prepared every 2 days. 3-Alpha-hydroxy T-2 toxin ($R_f$ = 0.59) was identified by comparison with a standard of naturally occurring T-2 toxin (Sigma Chemical Co.). 3-Beta-hydroxy T-2 toxin ($R_f$ = 0.50) was identified by its relation to alpha T-2 (Zhang, personal communication) and its reaction with NBP. 3-dehydro T-2 toxin was identified by its characteristic appearance upon TLC: the compound appeared as two separate spots, each exhibiting extensive tailing, immediately above and below T-2 toxin (Zhang, personal communication).

High performance liquid chromatography (HPLC).

Analytical and reverse phase semi-preparative HPLC were performed at 25 C using a Model 2300 HPLC pump and $V^4$ variable wavelength absorbance detector (5-mm flow cell) (Isco, Inc., Lincoln, NE). The system was equipped with either RP-18 Spheri-10 MPLC analytical (22 cm x 4.6 mm ID) and guard (3 cm x 4.6 mm ID) cartridges (Brownlee Laboratories, Inc., Santa Clara, CA) or a Partisil 10 ODS Magnum 9/25 HPLC prepacked column (semi-preparative) (Whatman Chemical Separations, Inc.) connected to a guard column (CSK I, Whatman) packed with Pellicular ODS (Whatman). The mobile phase used with both columns was 50% (vol/vol) methanol in water and the flow rate was 1 to 2 ml/min for analytical HPLC and 3 ml/min for semi-preparative HPLC. Samples were dissolved in ethanol,

filtered through Prep-Disc Sample Filters (Bio-Rad Laboratories, Richmond, CA), and injected through a Valco C6U sample loop injector (Valco Instruments Co., Inc., Houston, TX) fitted with either a 10 ul (analytical) or 500 ul (semi-preparative) sample loop. Absorbance was monitored at 210 nm and peaks of interest were collected manually at the detector outlet. Peak areas were determined by a Hewlett-Packard 3392A Integrator (Hewlett-Packard Co., Avondale, PA). Relative amounts of alpha and beta T-2 toxin were estimated by comparison of peak areas.

Normal phase semi-preparative HPLC was performed at 25 C using a Model 396-89 mini-pump (Laboratory Data Control, Milton Roy Corp., Riviera Beach, FL) and Partisil 10 Magnum 9/25 HPLC prepacked column (Whatman Chemical Separations, Inc.) connected to a guard column (CSK I, Whatman) packed with HC Pellosil (Whatman). The mobile phase was a gradient ranging from 100% hexane to ethyl acetate:hexane (1:1), at a flow rate of 2 ml/min. Samples were dissolved in hexane, filtered through Prep-Disc Sample Filters, and injected through a Valco C6U sample loop injector fitted with a 2 ml sample loop. Fractions (5 to 20 ml) were collected using a Retriever III fraction collector (Isco, Inc.) and monitored by either TLC (see TLC above) or, for radiolabeled T-2, liquid scintillation counting (see Radiochromatography below).

To recover [$^{3}$H]T-2 toxin from aqueous HPLC solvents, fractions were extracted with chloroform (6 times), using a

volume ratio of 1:2, aqueous:chloroform. The combined chloroform extracts were dried with $Na_2SO_4$ and concentrated under reduced pressure in a Buchii R110 rotavapor (Brinkmann Instruments, Inc., Westbury, NY). [$^3$H]T-2 toxin was transferred to ethanol (200 proof, Midwest Solvents Company of Illinois, Pekin, IL) by dilution of chloroform into ethanol and evaporation of the chloroform under $N_2$. This extraction procedure recovered 99% of [$^3$H]T-2 from aqueous solutions, and the transfer from chloroform to ethanol was essentially quantitative.

The following solvents were used for HPLC: methanol and water (HPLC grade, Fisher Scientific Company, Fairlawn, NJ); hexane (ChromAR HPLC, Mallinckrodt, Inc., Paris, KY); ethyl acetate (Baker Resi-Analyzed, J. T. Baker Chemical Company, Phillipsburg, NJ).

Radiochromatography.

Radiochromatography was performed using either TLC or analytical HPLC. For TLC, plates were developed as described above, then silica gel was removed from sample lanes in 0.5 cm wide sections, from the origin to the solvent front, by vacuum aspiration. Silica gel was rinsed with methanol into scintillation vials and scintillation cocktail (5 ml) (Safety-Solve, Research Products International Corp.) added. Radioactivity was determined by liquid scintillation counting using a Packard TRI-CARB 4430 Liquid Scintillation System (Packard Instrument Co.,

Inc., Downers Grove, IL). An equivalent section of adsorbent from a non-sample lane was counted for background radioactivity.

For analytical HPLC (performed as described above), eluant was collected at the detector outlet, from 0 to 30 min in 1 min intervals (1 ml), directly into scintillation vials. Counting cocktail (5 ml) was added and radioactivity determined by liquid scintillation counting. Background was monitored by collecting 1 min fractions prior to sample injection.

Enzyme-linked immunosorbent assay (ELISA)

[$^3$H]T-2 toxin was quantitated using a direct competitive ELISA based on the procedure described by Pestka et al. (1981). Monoclonal anti-T-2 antibody, prepared by Gendloff et al. (1987), was diluted (1:200) in 0.15 M phosphate-buffered saline (pH 7.2; PBS) containing 0.0001% ovalbumin (Grade II, Sigma) and added to Immulon II Removawell Microtiter Strips (Dynatech Laboratories, Alexandria, VA) (125 ul/well). The microtiter plates were incubated overnight at 40 C in a Model 338F Isotemp forced air oven (Fisher Scientific Co.). The coated plates were then washed 3 times with PBS containing 0.05% Tween 20 (Sigma) (PBS-Tween). T-2 standard (Sigma) and [$^3$H]T-2 samples were diluted in PBS containing 1% ethanol. T-2-horseradish peroxidase conjugate (T-2-HRP) prepared by Gendloff et al. (1984) was diluted (1:200) in PBS

containing 1% ovalbumin. T-2-HRP and either standard or sample solutions were mixed in a 1:1 ratio, and 100 ul added to each of 3 wells. Plates were incubated for 30 min at 37 C, then washed 6 times with PBS-Tween. Bound horseradish peroxidase activity was determined using 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS, Sigma) as described by Pestka et al. (1980): 100 ul of substrate solution (0.4 mM ABTS, 1.2 mM hydrogen peroxide in 50 mM citrate buffer, pH 4.0) were added to each well. Plates were incubated at 37 C for 15 min, then the reaction stopped by adding 100 ul of stopping solution (300 mM citric acid containing 15 mM sodium azide) to each well. Absorbance was measured at 405 nm on an ELISA plate reader (Dynatech Laboratories), and T-2 was quantitated by extrapolation from a standard curve. A standard curve (0 to 100 ng T-2/ml) was included on each microtiter plate. The limit of detection for the assay was 1 ng T-2/ml.

Both alpha and beta [$^3$H]T-2 toxin were quantitated by this procedure, with the assumption that the affinity of the anti-T-2 antibody was the same for both isomers. Thus, T-2 values obtained are assumed to represent total T-2 (alpha and beta). It was also assumed that the amount of 3-dehydro T-2 toxin that may still be present in the [$^3$H]T-2 samples was negligible, and so did not contribute to T-2 determination, even if cross-reactivity with the antibody was significant.

Analysis of [$^3$H]T-2 toxin

The chemical purity of the [$^3$H]T-2 preparation was assessed by analytical HPLC (as described above). The radiochemical purity, determined by radiochromatography using HPLC (described above) was estimated by the percent of total radioactivity associated with [$^3$H]T-2 toxins (alpha and beta). The percent of total [$^3$H]T-2 represented by either alpha or beta [$^3$H]T-2 was estimated by the percent of total [$^3$H]T-2 radioactivity associated with either alpha or beta. Background counts were 3 to 4 cpm and were considered negligible in calculations. The specific activity (mCi/mMol) was calculated from ELISA quantitation of T-2 and liquid scintillation counting.

Chemicals

All chemicals not specifically identified above were purchased from either J.T. Baker Chemical Company, Sigma Chemical Co., or Mallinckrodt, Inc. and were analytical grade or better.

Safety precautions

Glassware and plasticware contaminated with T-2 toxin were decontaminated by soaking in 10% bleach solution before washing or disposal. Safety precautions outlined by the Michigan State University Office of Radiation, Chemical and Biological Safety (ORCBS) were observed when handling radioisotopes. The reduction of 3-dehydro T-2 toxin

generated $^3H_2$ and precautions (reviewed and approved by ORCBS) were taken to prevent release of this gas into the atmosphere. $^3H_2$ produced during the reaction was captured in a vacuum sampler (Alltech Associates, Arlington Heights, IL) designed specifically for sampling gases. The capacity of the cannister was 280 cc, more than adequate to hold the volume of $H_2$ produced from the complete hydrolysis of 2.5 umole of sodium borohydride (0.25 $cm^3$) under initial reaction conditions (25 C, atmospheric pressure). After the reaction, the amount of radioactivity in the cannister was estimated by subtracting the radioactivity recovered in the reaction product from the original 25 mCi. The cannister was delivered to ORCBS for disposal.

The reduction reaction was carried out in a fume hood using a glass vial sealed with a rubber septum. The vial remained sealed throughout the reaction. The vial and cannister were connected by Teflon tubing fitted with hypodermic needles. Before addition of the reactants, the vial was flushed with $N_2$ to provide an inert atmosphere. A glass syringe was used to add reactants to the vial through the septum. When the reaction was complete, chloroform and HCl were added through the septum. The vacuum sampler was attached to capture any $H_2$ produced.

# Uptake of [$^3$H]T-2 Toxin

## Toxins

[$^3$H]T-2 toxins (alpha and beta) of 99% radiochemical and chemical purity were prepared as described above. Stock solutions (3.54 x $10^{-6}$ M; 508 mCi/mMol) in ethanol were stored at 4 C. For concentrations of [$^3$H]T-2 toxin greater than $10^{-7}$ M, [$^3$H]T-2 stock was diluted with unlabeled T-2 toxin (Sigma) and the specific activity calculated. Stock solutions (1 x $10^{-2}$ M) of T-2 toxin and deoxynivalenol (DON) (Witt et al., 1985) in ethanol (200 proof) were stored at 4 C. Stock solutions (1 x $10^{-3}$ M) of verrucarin A (VER A) and roridin A (ROR A) (Sigma) in ethanol (200 proof) were stored at -20 C. A stock solution (1.9 x $10^{-2}$ M) of anisomycin (Sigma) was prepared in dilute HCl. All toxins were diluted to the appropriate concentration in RPMI 1640 medium with 25 mM HEPES (RPMI 1640, pH 7.0; prepared as directed with the addition of sodium bicarbonate, 2 g/L) (Sigma) for uptake assays.

## Cells

The uptake of [$^3$H]T-2 toxin was studied using three types of cells: hybridomas, spleen cells, and erythrocytes. Cell counts were performed using a hemacytometer (American Optical, Buffalo, NY) and viability was assessed by trypan blue dye (Sigma; 0.4% in 0.85%

sodium chloride) exclusion. Viability was always greater than 95%.

Hybridomas. A murine B-cell hybridoma cell line produced by Dixon et al. (1987) was used. Cells of this line secrete anti-zearalenone monoclonal antibodies. Polystyrene tissue culture flasks (150 $cm^2$, Corning Glass Works, Corning, NY) containing hybridoma cells in hypoxanthine-thymidine (HT) medium (Littlefield, 1964) were incubated at 37 C in an atmosphere of 8% carbon dioxide. HT stock solution contained 1 x $10^{-2}$ M hypoxanthine (Sigma) and 1.6 x $10^{-3}$ M thymidine (Sigma) in distilled water, dissolved by adding 0.1 N NaOH dropwise. Stock solution was filter sterilized and stored at -20 C. HT medium was prepared by diluting HT stock solution 100-fold in Dulbecco's modified Eagle's medium (DMEM, with L-glutamine and 4.5 g glucose/L, pH 7.0; Gibco Laboratories, Grand Island, NY). DMEM was supplemented with 1% (v/v) NCTC (Medium NCTC 135 with L-glutamine, Gibco) and 1 mM sodium pyruvate (MEM sodium pyruvate solution, 100 mM; Gibco). Fetal bovine serum (FBS; Gibco) was added to a final concentration of 5 to 10% (v/v), and 1% (v/v) penicillin/streptomycin solution (Pen/Strep, 100,000 U/ml; Gibco) and 0.1% (v/v) amphotericin B solution (Fungizone, 250 ug/ml; Gibco) were added to minimize bacterial and fungal contamination, respectively. HT medium was filtered through a 2 um membrane filter and stored at 4 C. Cells

were maintained in log phase, at a concentration of 1 to 2 x $10^5$ cells/ml. Stock cultures were frozen in FBS containing dimethylsulfoxide (Sigma) (9:1, v/v) and stored in liquid nitrogen.

Cells were harvested by centrifuging cultures at 450 x g for 8 min. The resulting pellet was washed in RPMI 1640 and centrifuged as before. The final cell pellet was resuspended in RPMI 1640 to the desired cell concentration for uptake assays.

Spleen cells. Spleen cell suspensions were obtained from female B6C3F1 mice (Harlan/Sprague-Dawley, Inc., Indianapolis, IN), 12 to 20 weeks old. Mice were sacrificed by carbon dioxide asphyxiation and their spleens removed and rinsed in RPMI 1640. Each spleen was cut using scissors into several pieces which were homogenized using a Teflon coated pestle in a test tube containing RPMI 1640 (5 ml). The volume of medium was adjusted to give the desired cell concentration for uptake assays.

Erythrocytes. Erythrocytes were obtained from female B6C3F1 mice (Harlan/Sprague-Dawley, Inc.). Blood was collected from the retro orbital sinus into Alsever's solution (trisodium citrate, 8.0 g; sodium chloride, 4.2 g; glucose, 20.5 g; citric acid, 0.55 g in 1 liter distilled water; Tucker and Ellory, 1982), in a ratio of 1:1 (vol/vol). This suspension was stored at 4 C for up to 3 weeks.

Cells were prepared for uptake assays by centrifugation at 450 x g for 8 min. The buffy coat containing lymphocytes was removed from the surface of the pellet with a Pasteur pipette. The pellet was then washed three times in Dulbecco's phosphate-buffered saline (PBS, pH 7.0) (Adams, 1980). The final pellet was suspended in RPMI 1640 at a concentration of 1 x $10^8$ cells/ml.

Uptake assay

Uptake of [$^3$H]T-2 toxin by cells was monitored in RPMI 1640, at 25 C. The assay mixture was composed of: cell suspension, unlabeled toxin or RPMI 1640, and [$^3$H]T-2 toxin, in a ratio of 2:1:1, respectively. All concentrations of toxin refer to final concentrations in the assay mixture. Uptake was determined using a zero-trans experimental arrangement (Stein, 1986). Cis and trans refer to the two compartments separated by the cell membrane (i.e., intracellular and extracellular). The zero-trans arrangement enables the experimenter to measure a unidirectional flux, in the cis to trans direction. At time = 0, the concentration of substrate on the trans side is zero. Here, trans refers to the intracellular compartment; the unidirectional flux being measured is the influx. An appropriate volume of cell suspension was added to the stirred solution of toxin. Aliquots (100 ul) of the assay mixture were taken at the appropriate times and diluted in ice cold PBS (1 ml). After the mixture was

centrifuged in an Eppendorf microcentrifuge for 8 sec, the supernatant was removed by vacuum aspiration and the pellet washed two times with ice-cold PBS (1 ml/wash). The final pellet was solubilized with 1 N NaOH (100 ul), then neutralized with 1 N HCl (200 ul). Counting cocktail (Safety-Solve) (5 ml) was added to the solubilized pellet and radioactivity in the pellet determined by liquid scintillation counting using a Packard TRI-CARB 4430 Liquid Scintillation System (Packard Instrument Co., Downers Grove, IL). DPM calculations were based on the external standard technique of determining counting efficiency.

The number of viable cells/100 ul aliquot did not change over the 60 min assay period. Incubation of cells in the presence of $10^{-9}$ to $10^{-3}$ M T-2 or 10% (v/v) ethanol for up to 60 min did not decrease viability.

Extracellular contamination by radiolabel was assessed using inulin-carboxy-$^{14}$C ($^{14}$C-inulin) (2.6 mCi/mMol; Sigma) as an extracellular marker. Substitution of $^{14}$C-inulin (5 x $10^{-2}$ uCi/ml) for [$^{3}$H]T-2 in the assay mixture consistently resulted in contamination of the washed cell pellet by less than 1% of the extracellular label added, so uptake experiments were routinely performed without $^{14}$C-inulin. A value corresponding to 1% of added radioactivity was subtracted from the radioactivity associated with the cell pellet to account for extracellular contamination.

DPM values obtained for cell pellets were converted to pg [$^{3}$H]T-2 toxin based on the specific activity of

[$^3$H]T-2. Uptake rates (pg [$^3$H]T-2 accumulated/cell-min) were estimated by the slope of the linear portion (up to 10 min) of the accumulation curve (pg [$^3$H]T-2 accumulated/cell versus time). The slope was determined by linear regression (methods of least squares) using an HP-15C calculator (Hewlett-Packard Co., Corvallis, OR). The zero-time uptake was estimated by the y-intercept of the regression line.

# RESULTS

## Preparation of [$^3$H]T-2 Toxin

The sequence of reactions involved in labeling carbon 3 of T-2 toxin with tritium is outlined in Figure 1. The two isomers formed, 3-alpha-hydroxy and 3-beta-hydroxy T-2 toxin, were purified using HPLC.

### Oxidation of T-2 toxin.

Oxidation of T-2 toxin by the N-chlorosuccinimide method described by Wallace et al. (1977) was unsuccessful. T-2 remained unchanged even after reaction for 6 hr. T-2 toxin, however, was oxidized by PCC. Approximately 30% of starting T-2 was oxidized after 12 hr of reaction. Addition of more PCC completed the conversion of T-2 toxin to 3-dehydro T-2 toxin, with approximately 90% of starting T-2 being oxidized after 24 hr. The oxidized product exhibited extensive tailing on TLC due to enolization of the carbonyl function (Wallace et al., 1977).

Figure 1. Preparation of tritium labeled T-2 toxin.
$R_2 = R_3 = OCOCH_3$;
$R_5 = OCOCH_2CH(CH_3)_2$.
Carbon 3 of T-2 toxin was oxidized (a) using either N-chlorosuccinimide or pyridinium chlorochromate, then reduced (b) using tritiated sodium borohydride.

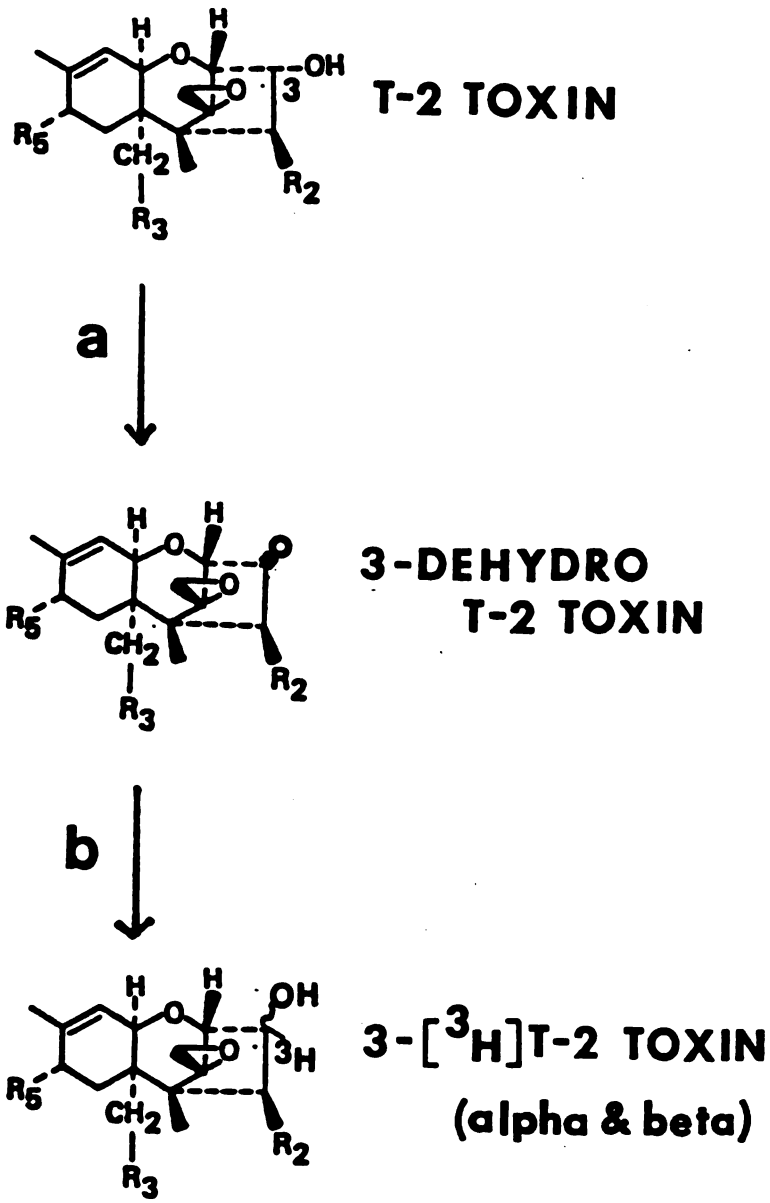



FIGURE 1

Reduction of 3-dehydro T-2.

Reduction of 3-dehydro T-2 with sodium borohydride yielded the expected isomeric mixture of T-2 toxin, 3-alpha-hydroxy (alpha) and 3-beta-hydroxy (beta), in a ratio of 1 to 9, respectively. The reduction reaction was carried out twice using tritiated sodium borohydride. The first labeling reaction yielded approximately 6 mg [$^3$H]T-2 (alpha and beta) from the same amount of 3-dehydro T-2, as estimated by TLC. The ratio of alpha to beta isomers was 1 to 9. The amount of radioactivity incorporated into the crude reaction product was 6.6 mCi. In contrast, the second labeling reaction produced, from approximately 10 mg 3-dehydro T-2 toxin, 4.4 mg [$^3$H]T-2 (alpha and beta), as quantitated by ELISA. Analysis of the product by TLC immediately after completion of the reaction indicated the ratio of alpha to beta isomers was 2 to 1. The amount of radioactivity incorporated into the crude reaction product was 0.84 mCi.

Purification of [$^3$H]T-2 toxin.

[$^3$H]T-2 toxin produced in the first labeling reaction was initially purified by normal phase semi-preparative HPLC ( method of G. Zhang) using a step gradient consisting of 0, 25, and 50% ethyl acetate in hexane. Both isomers eluted with 50% ethyl acetate, with beta eluting first (Figure 2). The radioactive peak corresponding to T-2 toxin was divided into three fractions, A, B, and C.

Figure 2. Chromatogram from normal phase semi-preparative HPLC of [$^3$H]T-2 toxin: first and second separations. Column was eluted (2 ml/min) stepwise (arrows indicate solvent changes) with: 0, 25, 50 (first separation) or 40% (second separation) ethyl acetate in hexane. Fractions (5 ml) were collected and radioactivity determined by liquid scintillation counting. The peak corresponding to [$^3$H]T-2 toxin was split into three fractions (A, B, C) and the ratio of alpha to beta isomers in each determined by thin layer radiochromatography (see Methods). The first separation was chromatography of the crude reaction product; the second was chromatography of fractions B and C recovered from the first separation. The radioactive peak between fractions 0 and 25 occurred only in the first separation.

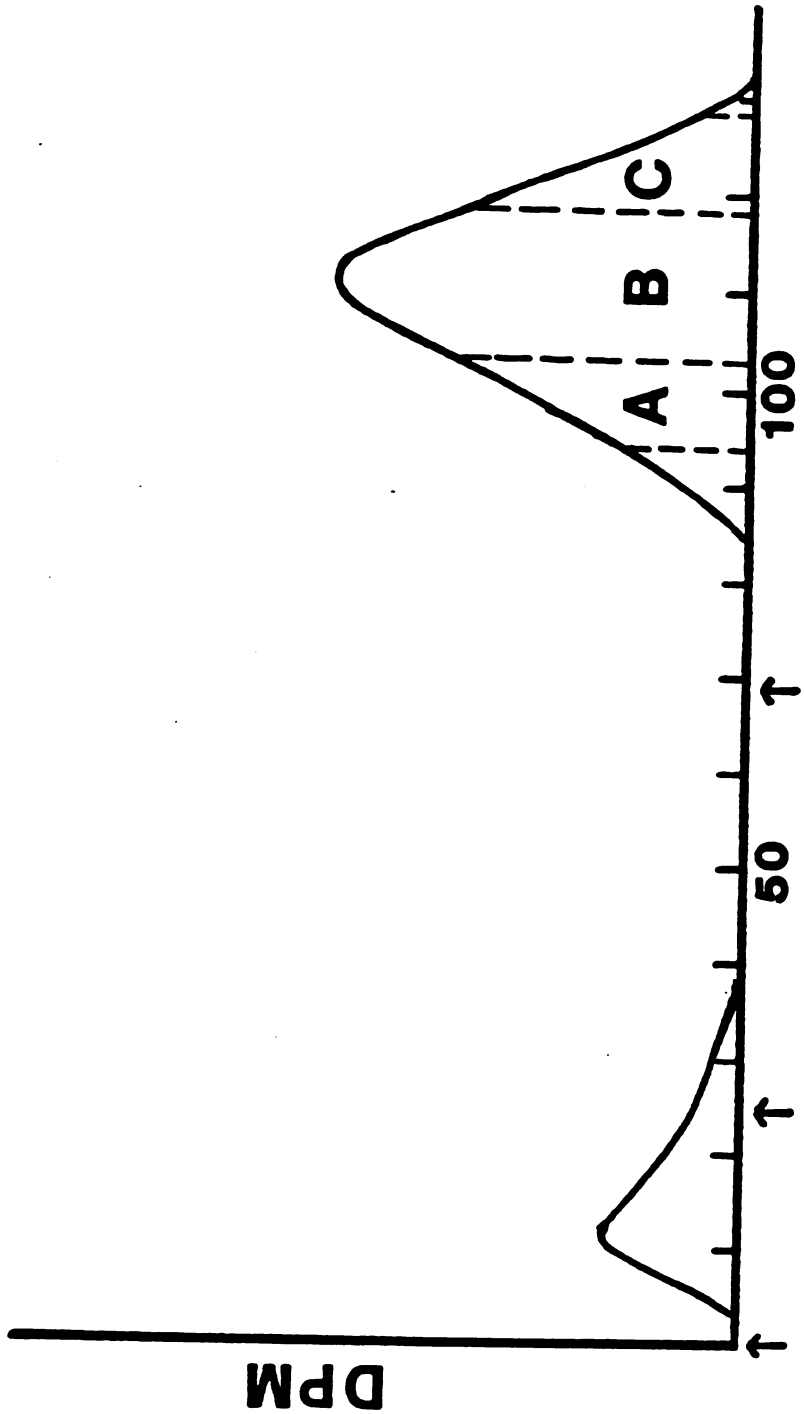



FIGURE 2

Ninety-eight percent of the radioactivity in fraction A was associated with the beta isomer. Fraction B contained both alpha and beta isomers, in a ratio of 2 to 3, respectively, and fraction C contained alpha and beta in a ratio of 2 to 1.

Further chromatography of fractions B and C recovered from the first separation, but using a gradient of 0, 25, and 40% ethyl acetate in hexane, gave similar results (Figure 2). Both alpha and beta isomers eluted with 40% ethyl acetate. Fraction A of the [$^3$H]T-2 peak again contained relatively pure beta (98%), fraction B, both alpha and beta (ratio 2 to 3), and fraction C, alpha and beta (ratio 1 to 9).

Fractions B and C recovered from the second separation were subjected to chromatography for a third time, using a more gradual gradient: 0, 25, 30, 35, and 40% ethyl acetate in hexane. Beta eluted with 35% ethyl acetate (Figure 3). The radioactive peak was split into four fractions, a, b, c, and d, containing 2, 14, 34, and 68 percent alpha [$^3$H]T-2, respectively.

Further purification of fraction d (Figure 3) was achieved using reverse phase analytical HPLC (Figure 4). This separation yielded two fractions: a chromatographically pure beta fraction (Figure 5a), and a fraction composed of 90% alpha (Figure 5b). Rechromatography of the alpha fraction increased its purity, but beta was still detectable. A third chromatographic step gave [$^3$H]T-2 toxin of 99%

Figure 3. Chromatogram from normal phase semi-preparative HPLC of [$^3$H]T-2 toxin: third separation. Column was eluted (2 ml/min) stepwise (arrows indicate solvent changes) with: 0, 25, 30, 35, 40% ethyl acetate in hexane. Fractions (5 ml) were collected and radioactivity determined by liquid scintillation counting. The peak corresponding to [$^3$H]T-2 toxin was split into four fractions (a, b, c, d) and the ratio of alpha to beta isomers in each determined by thin layer radiochromatography (see Methods). This separation was obtained from chromatography of fractions B and C from the second chromatographic separation (see Figure 2).

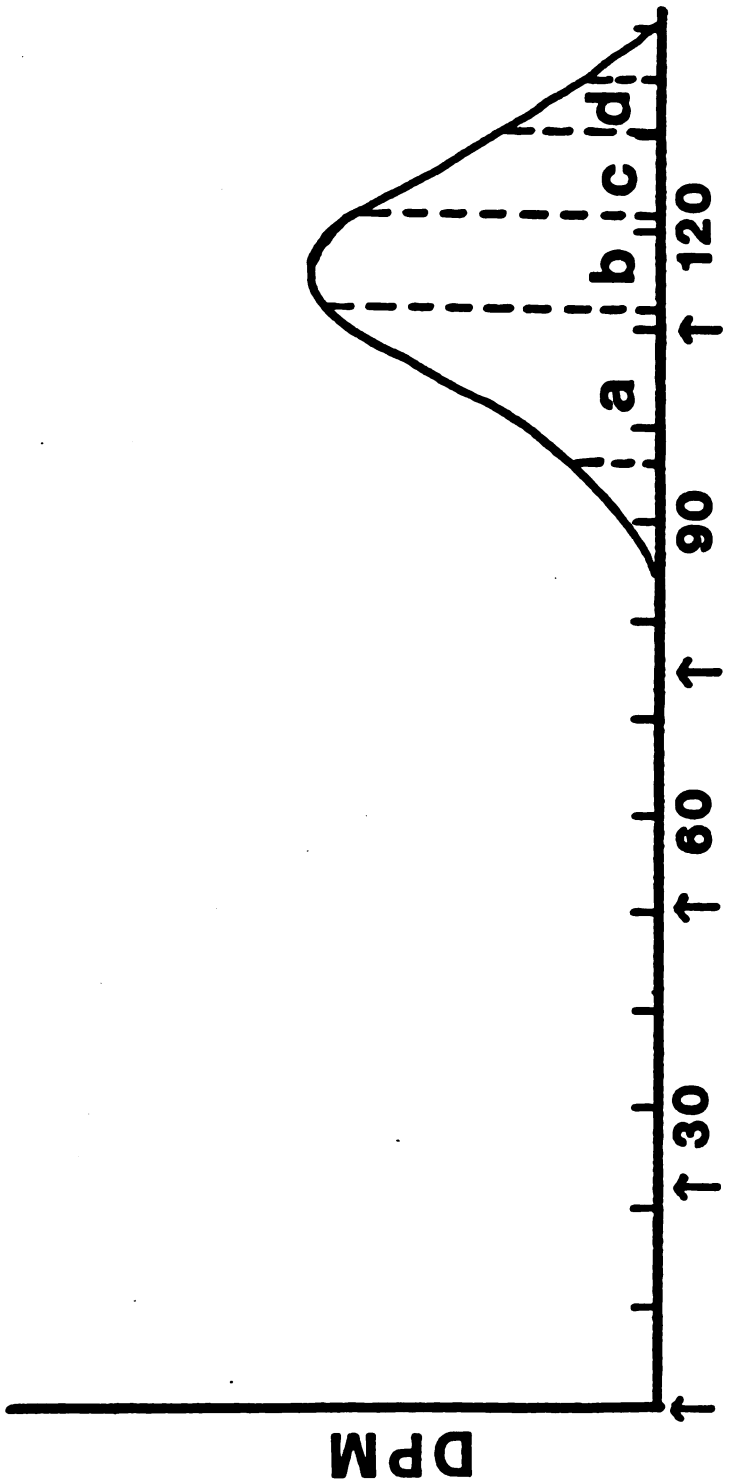



FIGURE 3

Figure 4. Chromatogram from analytical reverse phase HPLC of [$^{3}$H]T-2 toxin: chromatography of fraction obtained after three successive semi-preparative separations. HPLC was performed as described in Methods using a mobile phase (2 ml/min) of methanol:water (1:1). Absorbance was monitored at 210 nm. The sample was fraction d from the third semi-preparative separation (see Figure 3). Retention times (min) are indicated above peaks for beta (8.96) and alpha (12.09).

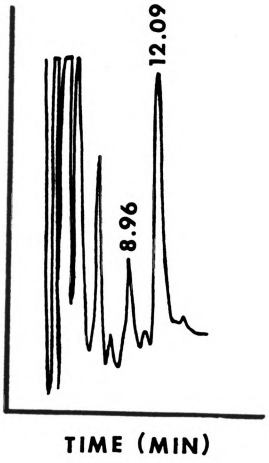



FIGURE 4

Figure 5. Chromatogram from analytical reverse phase HPLC of [$^{3}$H]T-2 toxin: alpha and beta peaks obtained from analytical HPLC. HPLC was performed as described in Methods using a mobile phase (2 ml/min) of methanol:water (1:1). Absorbance was monitored at 210 nm. Samples were (a) beta and (b) alpha peaks obtained from analytical HPLC (see Figure 4). Retention times (min) are indicated above peaks for beta (9.06) and alpha (13.26).

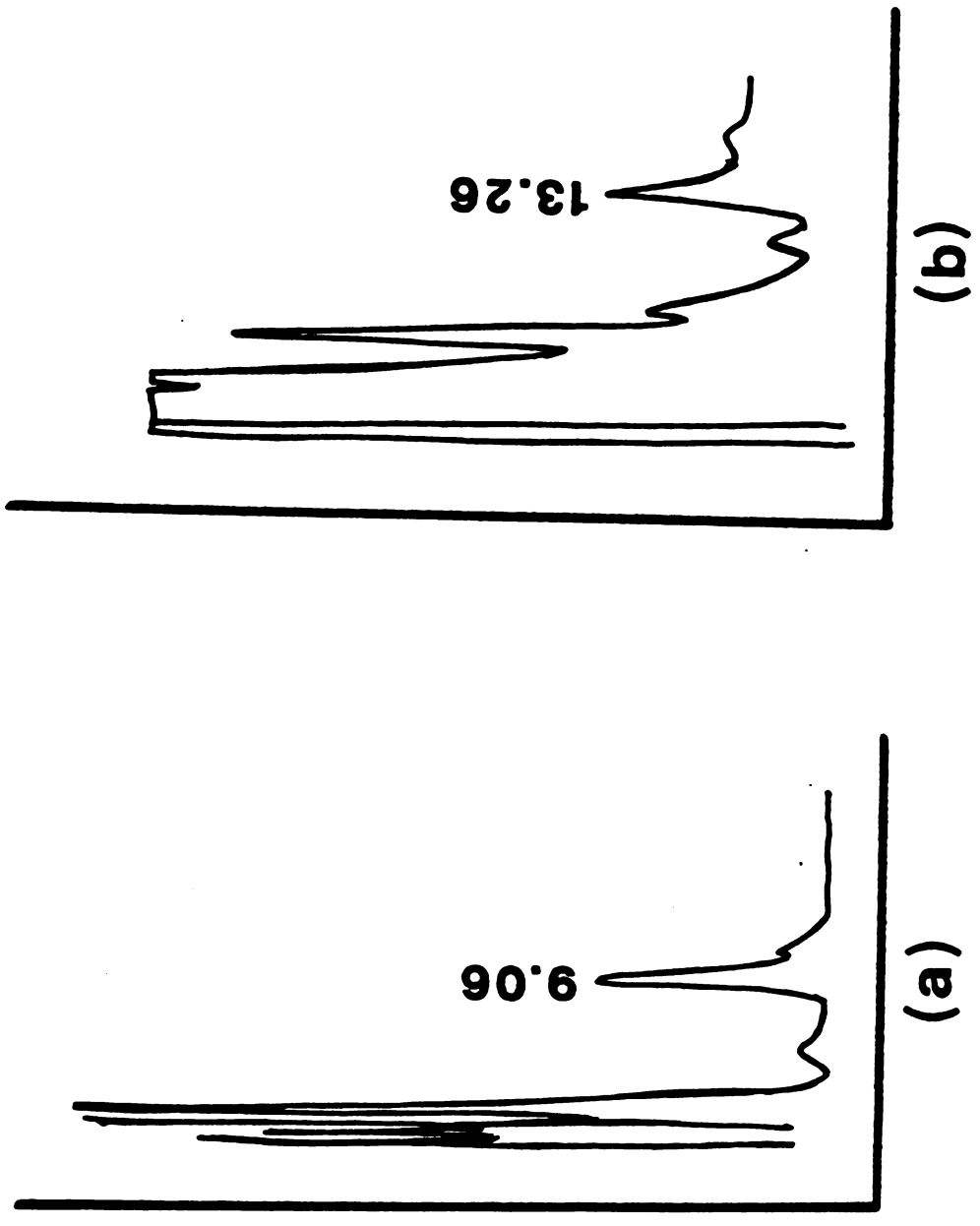



FIGURE 5

radiochemical purity, with 99% of the radioactivity associated with T-2 being in the form of alpha (Figure 6). The beta fraction purified was of similar radiochemical purity (Figure 7).

The remaining [$^3$H]T-2 fractions obtained from normal phase semi-preparative HPLC were similarly purified by successive reverse phase analytical HPLC to yield alpha and beta of high purity. The specific activity of alpha [$^3$H]T-2 was 508 mCi/mMol and approximately 2 ug (2.2 uCi) was obtained. Approximately 32 ug of beta [$^3$H]T-2, having a specific activity of 127 mCi/mMol was obtained (8.7 uCi).

Since the reverse phase chromatographic system was superior to the normal phase system for separation of alpha and beta isomers, reverse phase HPLC alone was used to purify [$^3$H]T-2 toxin from the second labeling reaction. HPLC analysis of the crude reaction product 24 hr after labeling showed the ratio of alpha to beta to be 1 to 2, in contrast to the ratio of 2 to 1 estimated by TLC immediately after labeling. Unexpectedly, both the alpha and beta peaks collected after reverse phase semi-preparative chromatography (Figure 8) contained alpha and beta isomers in a ratio of approximately 1 to 1 (Figures 9, 10).

Further purification of the "alpha" fraction (Figure 8) collected from semi-preparative HPLC was attempted by analytical HPLC. The peak corresponding to alpha was collected and found to contain both alpha and beta, in a

Figure 6. Radiochromatogram of alpha-[$^{3}$H]T-2 toxin. Radiochromatography was performed using analytical reverse phase HPLC (see Methods) with a mobile phase (2 ml/min) of methanol:water (1:1). Alpha-[$^{3}$H]T-2 was obtained after successive normal phase semi-preparative (three) and reverse phase analytical (three) chromatographic separations. Retention times were 8.68 and 12.8 min for beta and alpha, respectively.

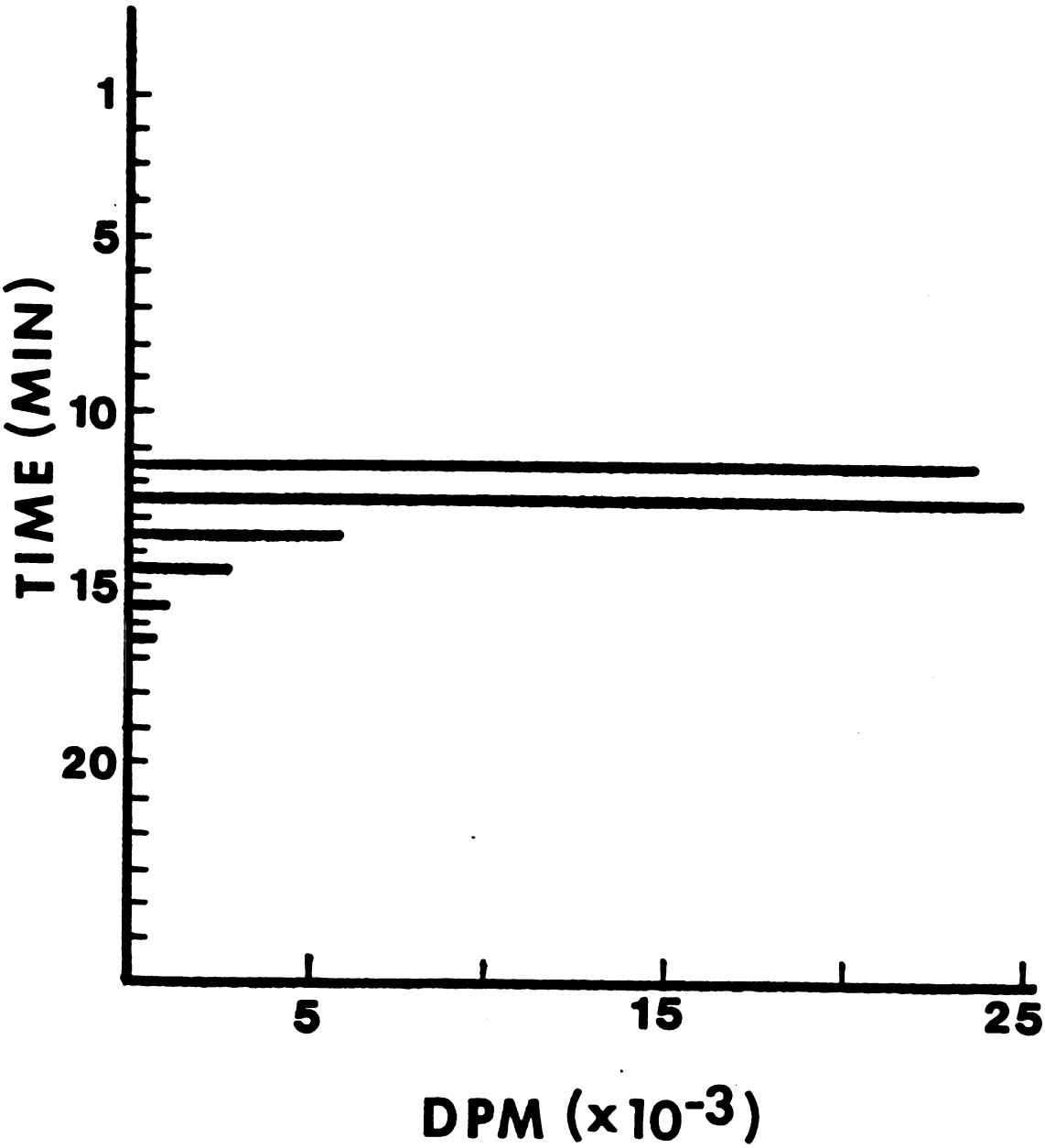



FIGURE 6

Figure 7. Radiochromatogram of beta-[$^{3}$H]T-2 toxin. Radiochromatography was performed using analytical reverse phase HPLC (see Methods) with a mobile phase (1 ml/min) of methanol:water (1:1). Beta-[$^{3}$H]T-2 was obtained after successive normal phase semi-preparative (three) and reverse phase analytical (three) chromatographic separations. Retention times were 15.1 and 20.3 min for beta and alpha, respectively.

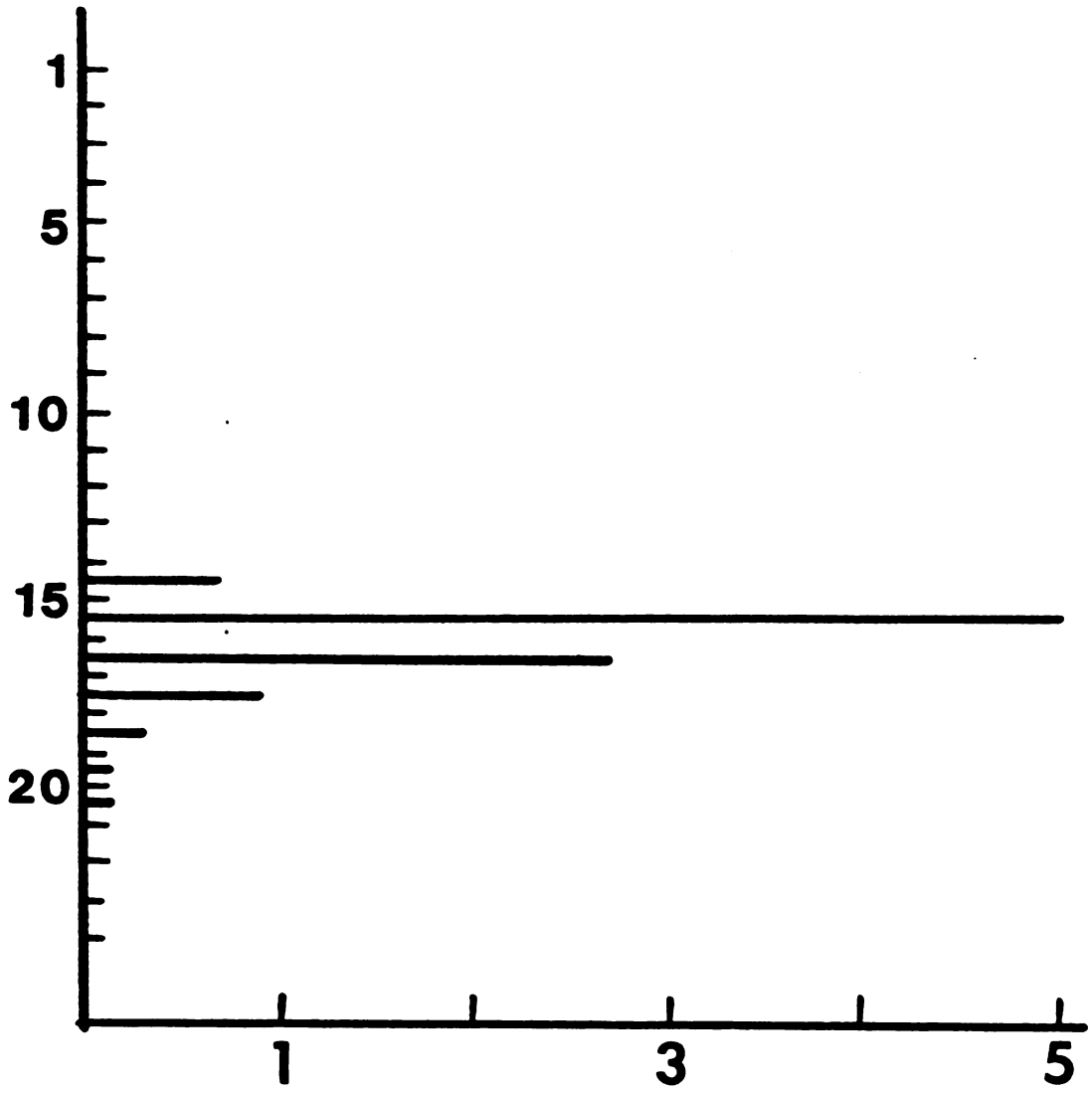



FIGURE 7

Figure 8. Chromatogram from semi-preparative reverse phase HPLC of [$^{3}$H]T-2 toxin: crude reaction product from second labeling. HPLC was performed as described in Methods using a mobile phase (3 ml/min) of methanol:water (1:1). Absorbance was monitored at 210 nm. Retention times (min) are indicated above peaks for beta (12.84) and alpha (14.65).

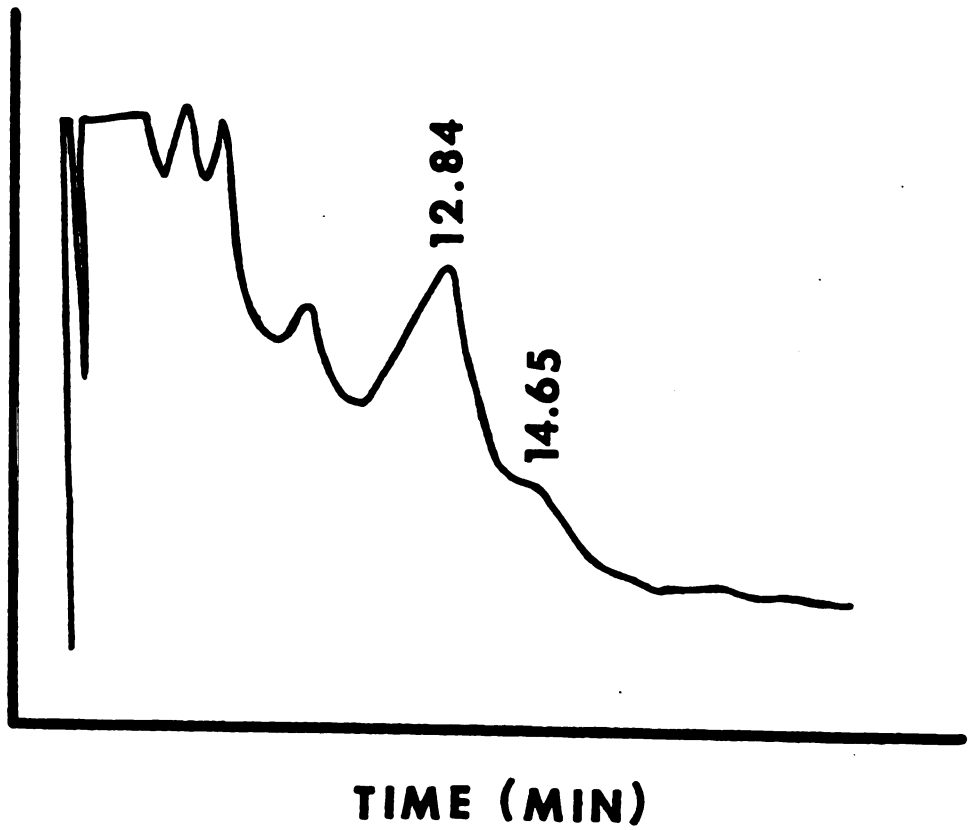



FIGURE 8

Figure 9. Chromatogram from analytical reverse phase HPLC of [$^{3}$H]T-2 toxin (second labeling): alpha peak obtained from semi-preparative reverse phase HPLC. HPLC was performed as described in Methods using a mobile phase (1 ml/min) of methanol:water (1:1). Absorbance was monitored at 210 nm. Sample was the alpha peak obtained from semi-preparative reverse phase HPLC of crude reaction product (see Figure 8). Retention times (min) are indicated above peaks for beta (14.71) and alpha (19.78).

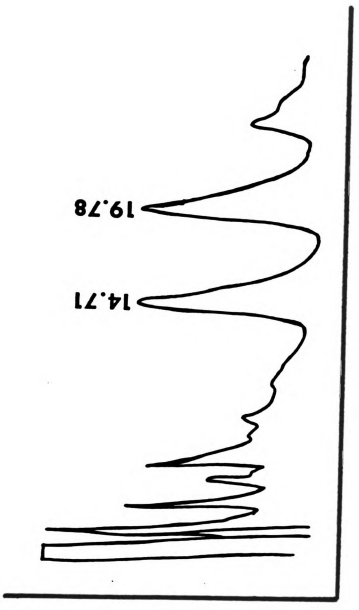



FIGURE 9

Figure 10. Radiochromatogram of beta-[$^{3}$H]T-2 toxin (second labeling). Radiochromatography was performed using analytical reverse phase HPLC (see Methods) using a mobile phase (1 ml/min) of methanol:water (1:1). Sample was the beta peak obtained from semi-preparative reverse phase HPLC (see Figure 8). Retention times were 15.1 and 20.3 min for beta and alpha, respectively.

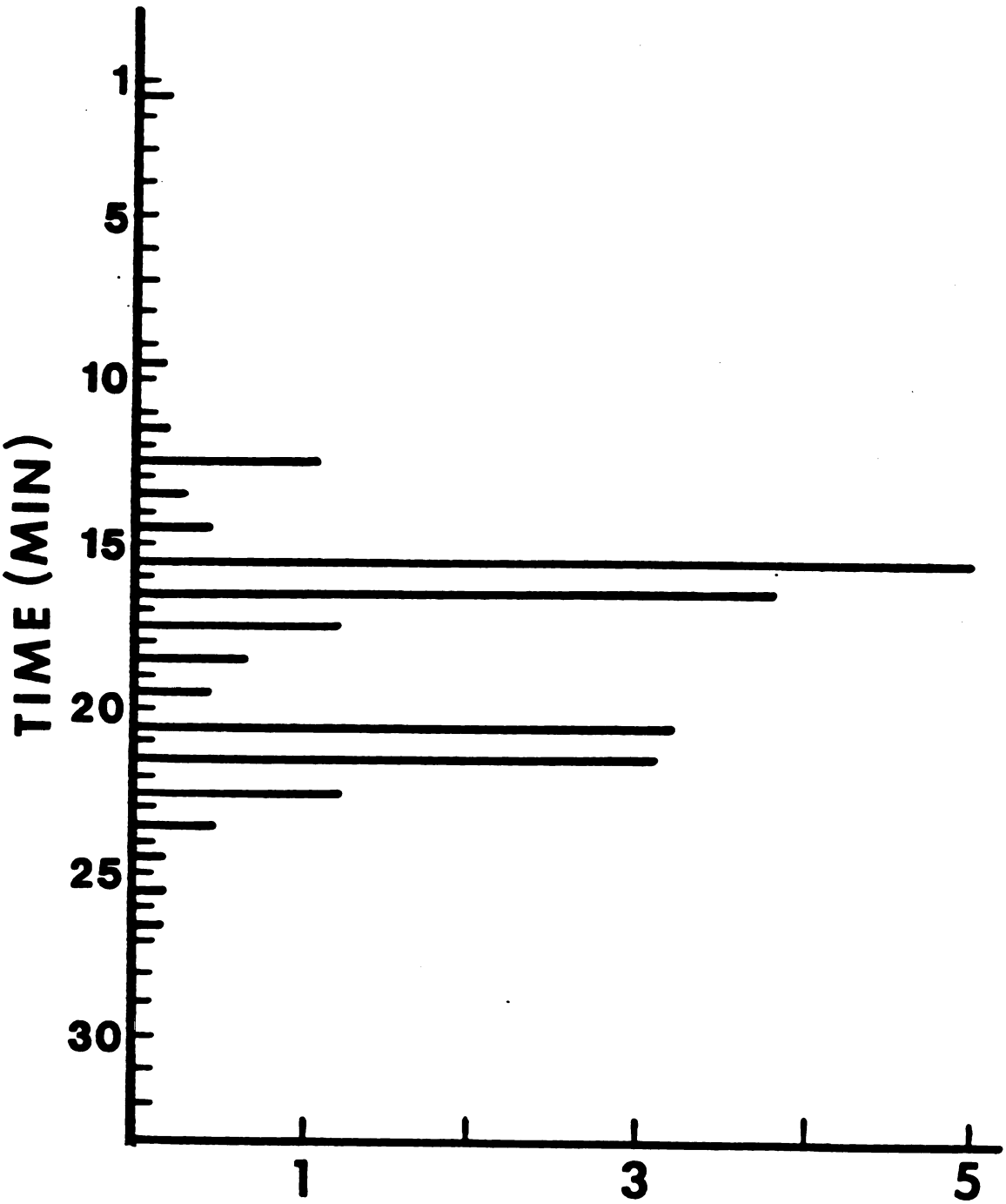



FIGURE 10

ratio of 1 to 1.5, respectively (Figure 11). Rechromatography of this alpha fraction again yielded a peak eluting as alpha, but containing, upon extraction, concentration, and HPLC analysis, both alpha and beta in approximately equal amounts (Figure 12). Thus, repeated chromatography did not produce isomerically pure [$^3$H]T-2 toxin, in contrast to results obtained with [$^3$H]T-2 produced in the first labeling reaction. The specific activity of [$^3$H]T-2 (alpha and beta mixture) obtained from the second labeling reaction was 40 mCi/mMol.

Figure 11. Chromatogram from analytical reverse phase HPLC of [$^{3}$H]T-2 toxin (second labeling): alpha peak obtained from analytical HPLC. HPLC was performed as described in Methods using a mobile phase (1 ml/min) of methanol:water (1:1). Absorbance was monitored at 210 nm. Sample was the alpha peak resulting from semi-preparative (see Figure 8) followed by analytical (see Figure 9) reverse phase HPLC. Retention times (min) are indicated above peaks for beta (15.16) and alpha (20.79).

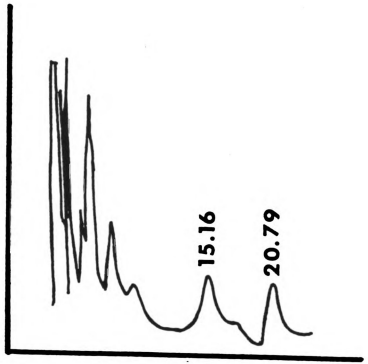



FIGURE 11

Figure 12. Radiochromatogram of alpha-[$^{3}$H]T-2 toxin (second labeling). Radiochromatography was performed using analytical reverse phase HPLC (see Methods) using a mobile phase (1 ml/min) of methanol:water (1:1). Absorbance was monitored at 210 nm. Sample was the alpha peak resulting from semi-preparative (see Figure 8) followed by analytical (see Figures 9 and 11) reverse phase HPLC. Retention times were 16.1 and 21.3 min for beta and alpha, respectively.

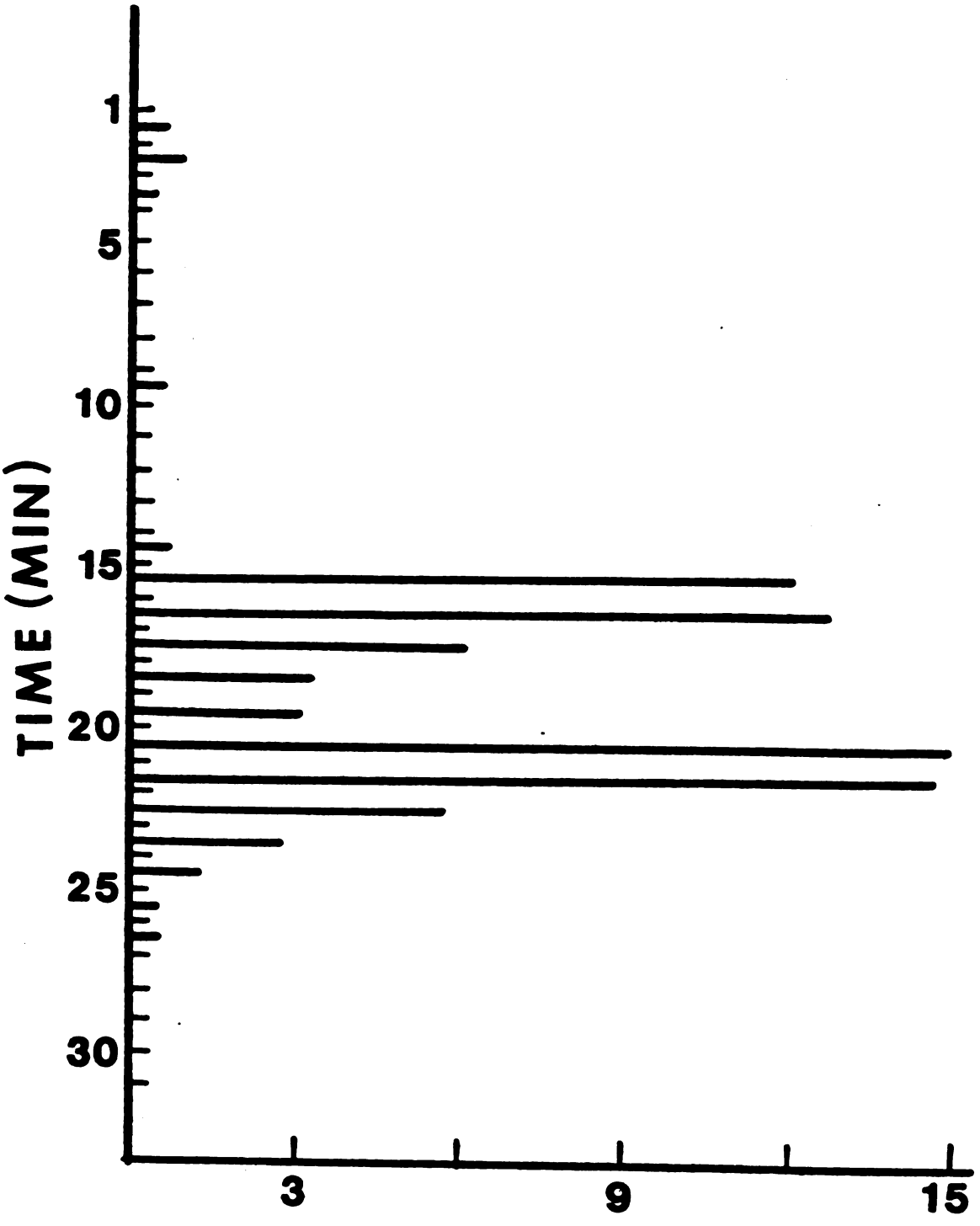



FIGURE 12

## Uptake of [$^3$H]T-2 Toxin

### Uptake of alpha-[$^3$H]T-2 by hybridomas

Hybridoma cells exposed to 5 x $10^{-8}$ M [$^3$H]T-2 toxin accumulated [$^3$H]T-2 in a linear fashion for the first 20 min of incubation (Figure 13). By approximately 45 min, the equilibrium level of accumulation, 1.65 x $10^{-3}$ pg [$^3$H]T-2/cell was reached. At equilibrium, about 75% of the radioactivity was associated with the cell pellet. The rate of uptake, estimated by the slope of the regression line determined from uptake data collected during the first 10 min of incubation (Figure 14), was 76 pg [$^3$H]T-2/1 x $10^6$ cells-min. The zero time uptake, estimated by extrapolation of the regression line, was 81 pg [$^3$H]T-2/1 x $10^6$ cells. By 10 min, approximately 35% of the radioactivity was associated with the cell pellet.

The time during which the rate of uptake was constant decreased as the concentration of [$^3$H]T-2 increased (Figure 15). The time for accumulation to reach equilibrium was also inversely related to concentration (data not shown). Equilibrium was reached in 40 to 45 min at $10^{-8}$ and $10^{-7}$ M [$^3$H]T-2, less than 30 min at $10^{-6}$ and $10^{-5}$ M [$^3$H]T-2, and less than 1 min at $10^{-4}$ and $10^{-3}$ M [$^3$H]T-2.

Using the data in Figure 15, uptake rates and zero time uptake values were estimated over a concentration range of $10^{-8}$ to 4 x $10^{-7}$ M [$^3$H]T-2 (Table 1). At concentrations greater than 4 x $10^{-7}$ M, rates could not be

Figure 13. Time course of accumulation of alpha-[$^{3}$H]T-2 by hybridomas: 0 to 60 min. Uptake was determined as described in Methods in the presence of 5 x $10^{-8}$ M [$^{3}$H]T-2. Values are averages of two determinations.

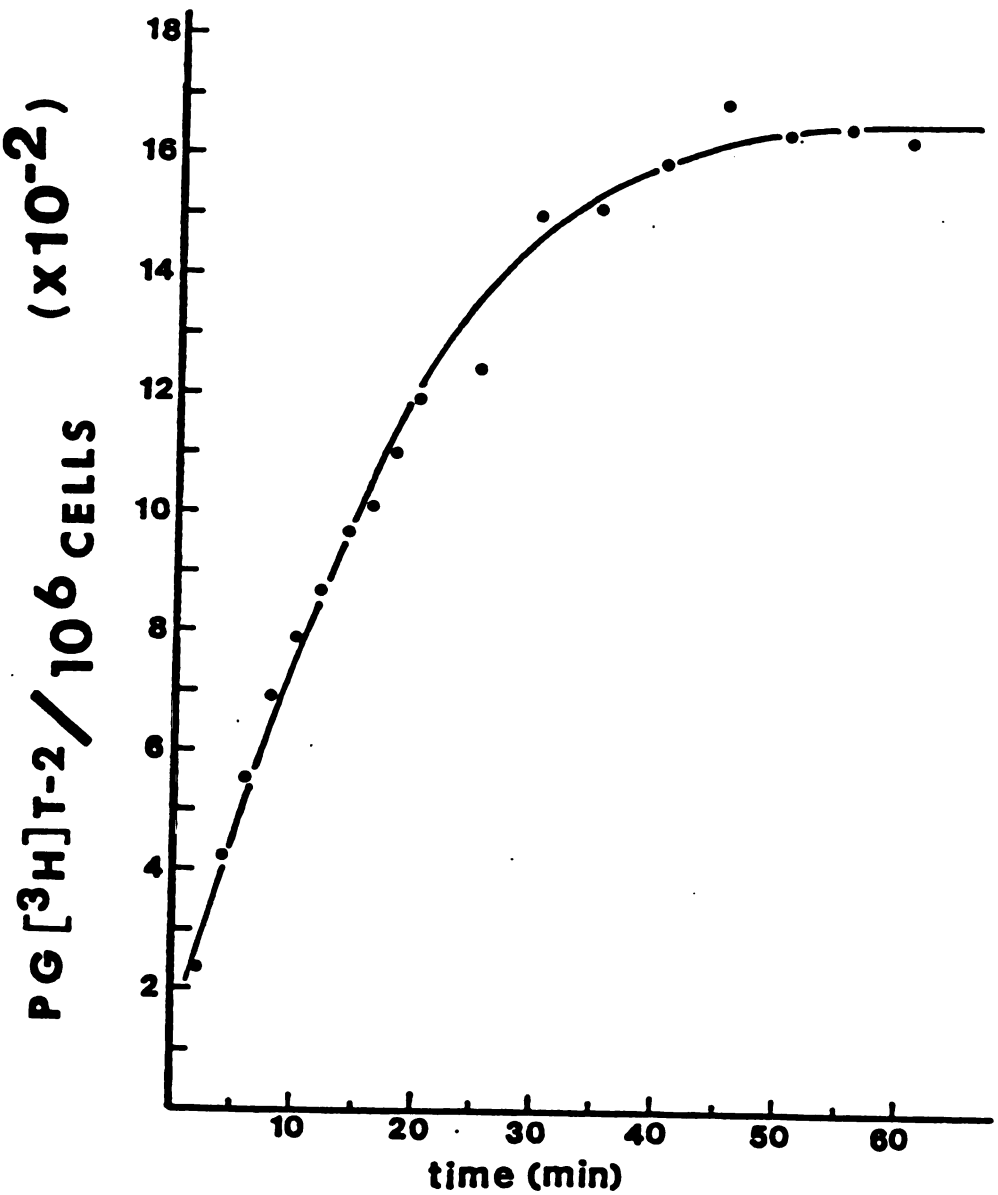

PG [3H]T-2 / 10^6 CELLS (x10^-2)
18
16
14
12
10
8
6
4
2
10
20
30
40
50
60
time (min)

FIGURE 13

Figure 14. Time course of accumulation of alpha-[$^{3}$H]T-2 by hybridomas: 0 to 10 min. Uptake was determined as described in Methods in the presence of $5 \times 10^{-8}$ M [$^{3}$H]T-2. Values are averages with standard error bars of 4 determinations. The rate of uptake and zero-time uptake were estimated from the slope and y-intercept, respectively, of the regression line ($r = 0.9864$) drawn.

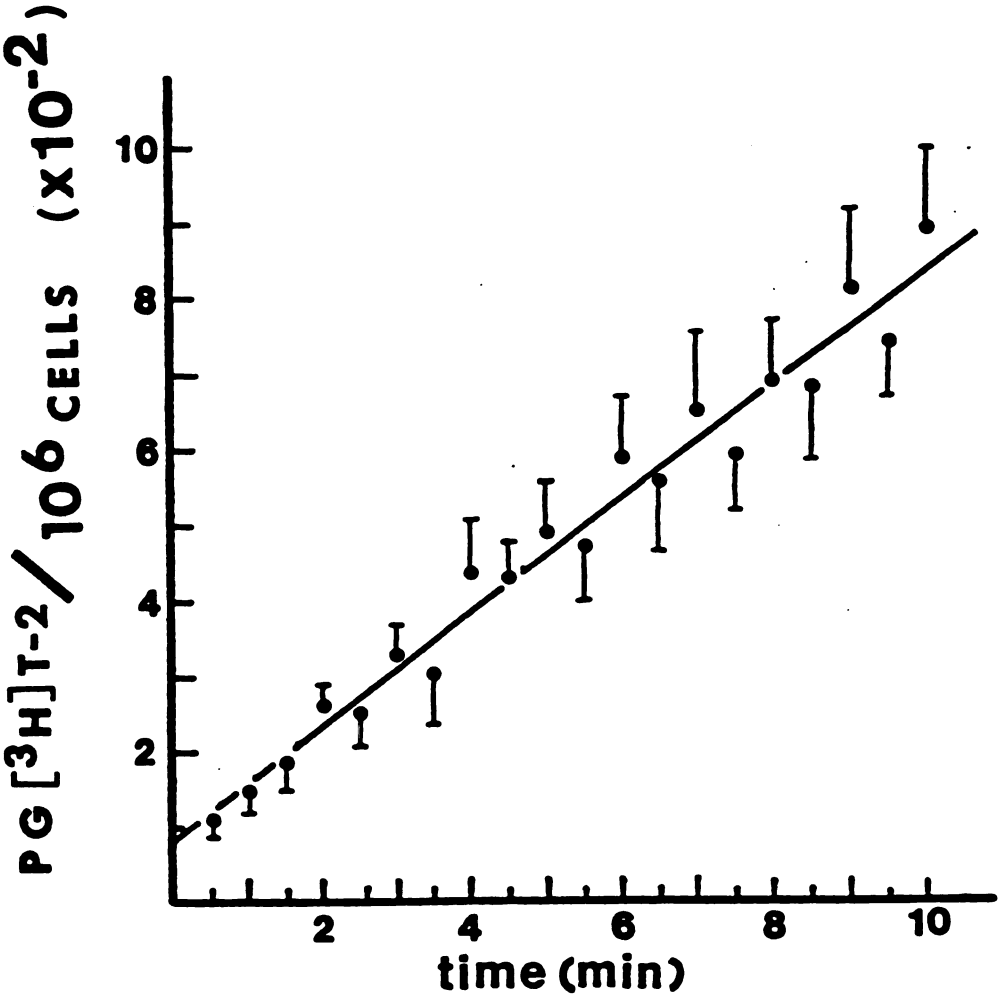



FIGURE 14

Figure 15. Concentration dependence of accumulation of alpha-[$^3$H]T-2 by hybridomas. Uptake was determined as described in Methods in the presence of (a) 1 x $10^{-8}$, (b) 2 x $10^{-8}$, (c) 4 x $10^{-8}$, (d) 1 x $10^{-7}$, (e) 2 x $10^{-7}$, (f) 4 x $10^{-7}$, (g) 1 x $10^{-6}$, (h) 2 x $10^{-6}$, (i) 4 x $10^{-6}$, and (k) 1 x $10^{-5}$ M [$^3$H]T-2. Values are averages of 2 determinations, except for $10^{-8}$, $10^{-7}$, $10^{-6}$, and $10^{-5}$ M (4 determinations and standard error bars)

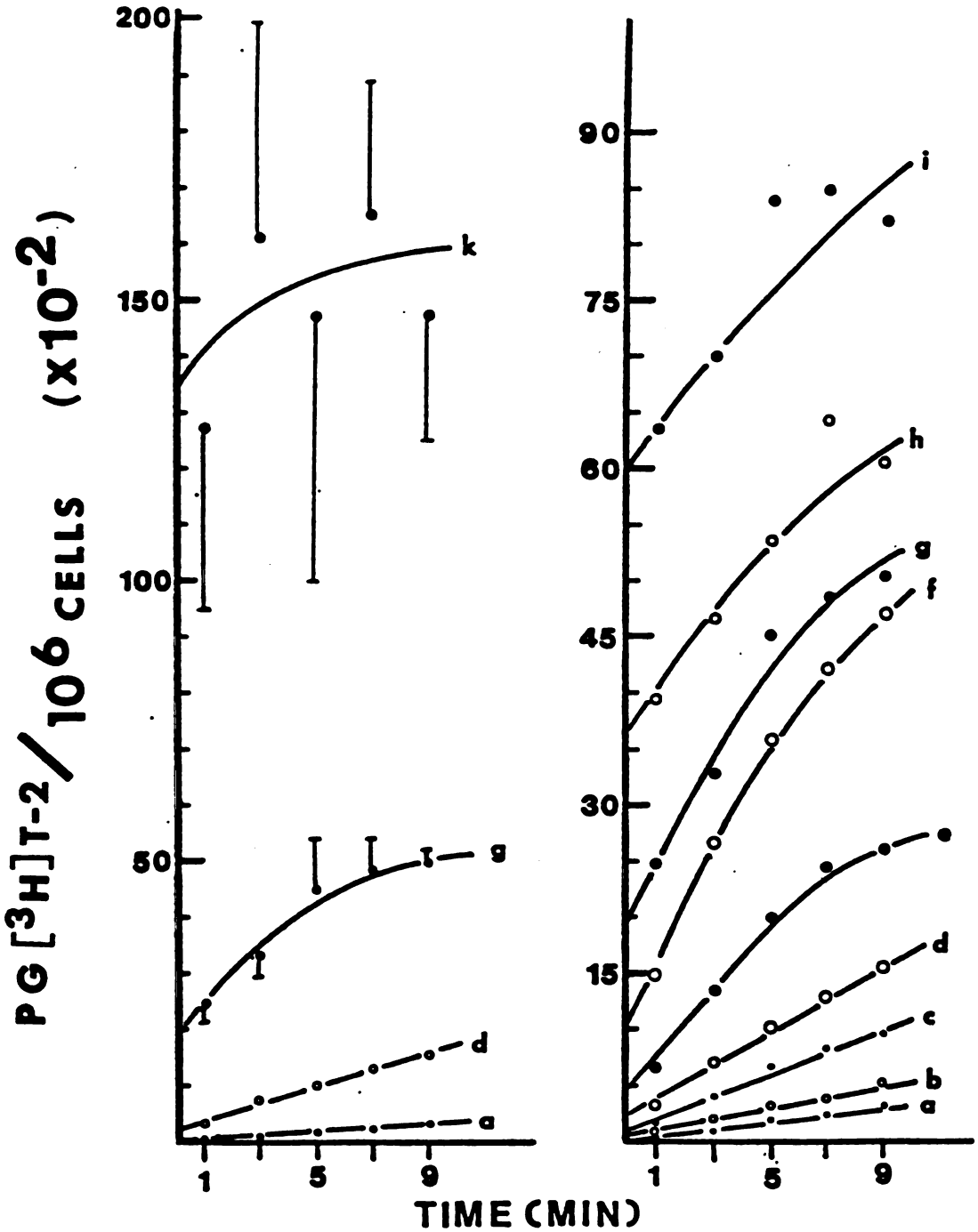

PG [3H]T-2/10^6 CELLS (X10^-2)
200
150
100
50
90
75
60
45
30
15
k
i
h
g
f
d
c
b
a
1
5
9
TIME (MIN)

FIGURE 15

Table 1. Concentration dependence of rate of uptake of alpha-[$^3$H]T-2 by hybridomas[a]

| [$^3$H]T-2 concentration (M) | Uptake rate[b] | Zero-time uptake[c] |
|---|---|---|
| 1 x $10^{-8}$ d | 31 | 13 |
| 2 x $10^{-8}$ d | 52 | 52 |
| 4 x $10^{-8}$ d | 102 | 90 |
| 1 x $10^{-7}$ d | 152 | 222 |
| 2 x $10^{-7}$ e | 300 | 405 |
| 4 x $10^{-7}$ f | 525 | 1005 |

[a]Uptake, as described in Methods, was monitored at 1, 3, 5, 7, and 9 min, 2 determinations per time point.

[b]The uptake rate was estimated as the slope of the regression line for uptake and represents pg [$^3$H]T-2/1 x $10^6$ cells-min.

[c]Zero-time uptake was estimated by the y-intercept of the regression line for uptake and represents pg [$^3$H]T-2/1 x $10^6$ cells.

[d]Regression performed using data from 1, 3, 5, 7, and 9 min.

[e]Regression performed using data from 1, 3, 5, and 7 min.

[f]Regression performed using data from 1, 3, and 5 min.

estimated with confidence since uptake curves were not clearly linear over the time period monitored. Both the rates of uptake and the zero-time uptake values were proportional to concentration of [$^3$H]T-2 (Table 1). The amount of [$^3$H]T-2 accumulated per cell was proportional to the concentration of [$^3$H]T-2 over a range of $10^{-8}$ to $10^{-3}$ M (Table 2). The percent of total [$^3$H]T-2 accumulated at equilibrium, however, decreased as the concentration of [$^3$H]T-2 increased, reaching a constant value of about 2%.

The accumulation of [$^3$H]T-2 by hybridomas was decreased in the presence of unlabeled T-2, DON, or anisomycin (Figure 16). At equilibrium, T-2, anisomycin, and DON decreased accumulation of [$^3$H]T-2 by 97, 86, and 76%, respectively. T-2, DON, VER A, ROR A, and anisomycin inhibited the rate of uptake of [$^3$H]T-2 by hybridomas in a concentration-dependent manner (Figure 17). The concentrations of toxins required to reduce the uptake rate of [$^3$H]T-2 ($10^{-7}$ M) by 50% were VER A, 2 x $10^{-7}$ M; ROR A, 3 x $10^{-7}$ M; T-2, 4 x $10^{-7}$ M; anisomycin, 8 x $10^{-7}$ M; and DON, 6 x $10^{-6}$ M. The zero-time uptake values of [$^3$H]T-2 estimated by extrapolation of competition curves, were similar for all inhibitors at all concentrations. The rate of uptake of [$^3$H]T-2 toxin ($10^{-7}$ M) by hybridomas preincubated with anisomycin ($10^{-4}$ M) was 4 pg [$^3$H]T-2/1.5 x $10^5$ cells-min over the 60 min incubation period, compared to 38 pg [$^3$H]T-2/1.5 x $10^5$ cells-min for cells preincubated without anisomycin (Figure 18). Zero-time uptake values were 243 and 223 pg [$^3$H]T-2/1.5 x $10^5$ cells for cells

Table 2. Concentration dependence of accumulation of alpha-[$^3$H]T-2 by hybridomas[a]

| [$^3$H]T-2 concentration (M) | Pg [$^3$H]T-2 accumulated/cell[b] | Percent [$^3$H]T-2 accumulated[c] |
|---|---|---|
| $10^{-8}$ | $1.4 \times 10^{-3}$ | 43 |
| $10^{-7}$ | $5.4 \times 10^{-3}$ | 21 |
| $10^{-6}$ | $8.0 \times 10^{-3}$ | 5 |
| $10^{-5}$ | $6.0 \times 10^{-2}$ | 3 |
| $10^{-4}$ | $4.4 \times 10^{-1}$ | 2 |
| $10^{-3}$ | 3.7 | 2 |

[a]Uptake, as described in Methods, was determined from 1 to 60 min, 2 determinations per time point.

[b]Total cell associated [$^3$H]T-2 at equilibrium.

[c]Total cell associated [$^3$H]T-2 at equilibrium/total [$^3$H]T-2 in reaction mixture.

Figure 16. Inhibition of accumulation of alpha- [$^3$H]T-2 by hybridomas: T-2, DON, and anisomycin. Uptake was determined as described in Methods in the presence of 5 x $10^{-8}$ M [$^3$H]T-2 either alone (●) or in combination with $10^{-5}$ M T-2 (○), DON (□), or anisomycin (■). Values are averages of 2 determinations.

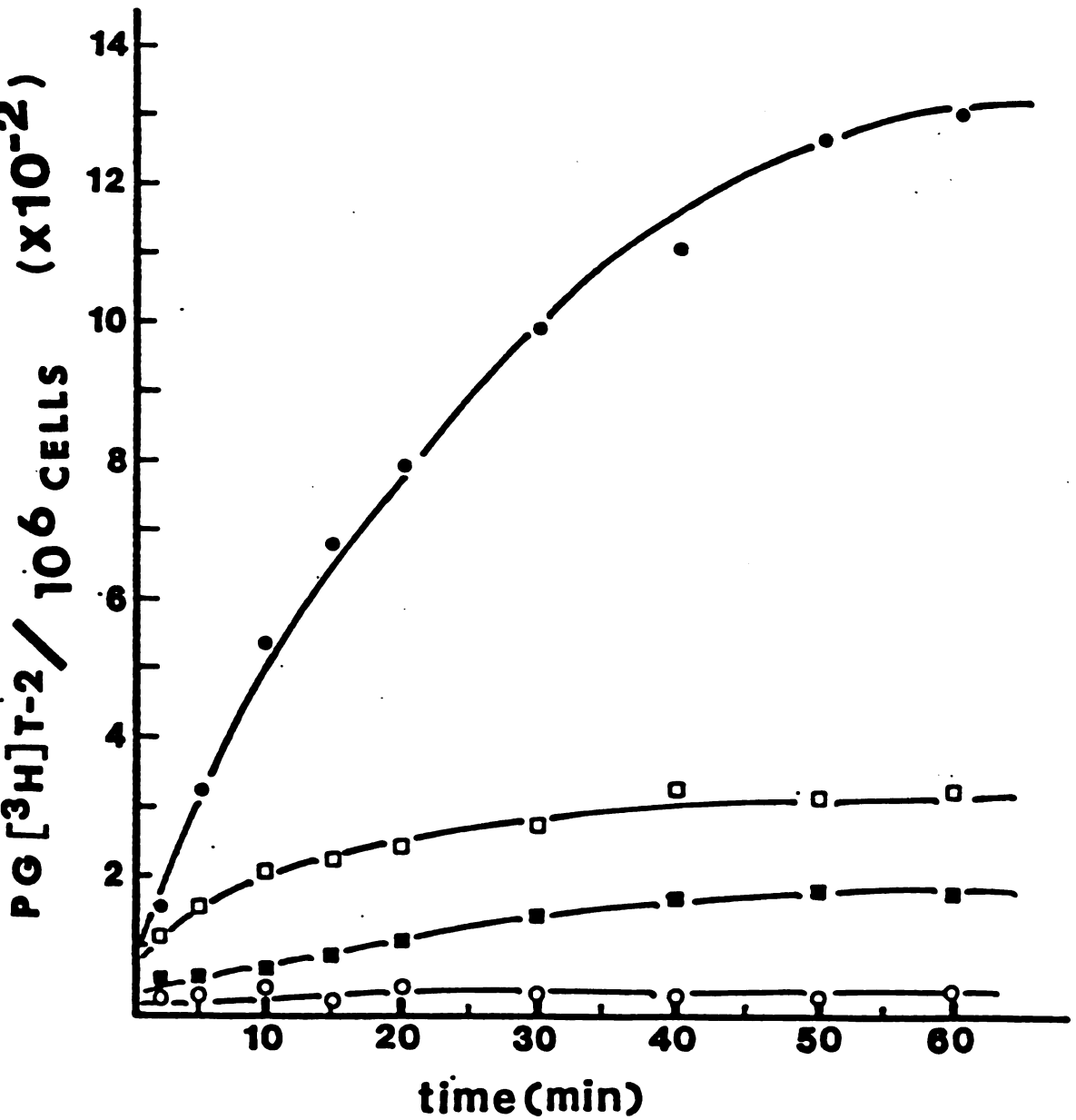



FIGURE 16

Figure 17. Inhibition of rate of uptake of alpha-[$^3$H]T-2 by hybridomas: T-2, DON, VER A, ROR A, and anisomycin. Uptake was determined as described in Methods in the presence of 1 x $10^{-7}$ M [$^3$H]T-2 and either T-2 ( ● ), DON ( ▲ ), VER A ( ■ ), ROR A ( □ ) or anisomycin ( ○ ). Uptake rates were estimated by the slope of the regression line determined from accumulation data obtained at 1, 3, and 5 min ($r > 0.95$) (2 determinations per time point) and compared to the rate of uptake of [$^3$H]T-2 (1 x $10^{-7}$) alone. 1.0 = 246 pg [$^3$H]T-2/$10^6$ cells-min.

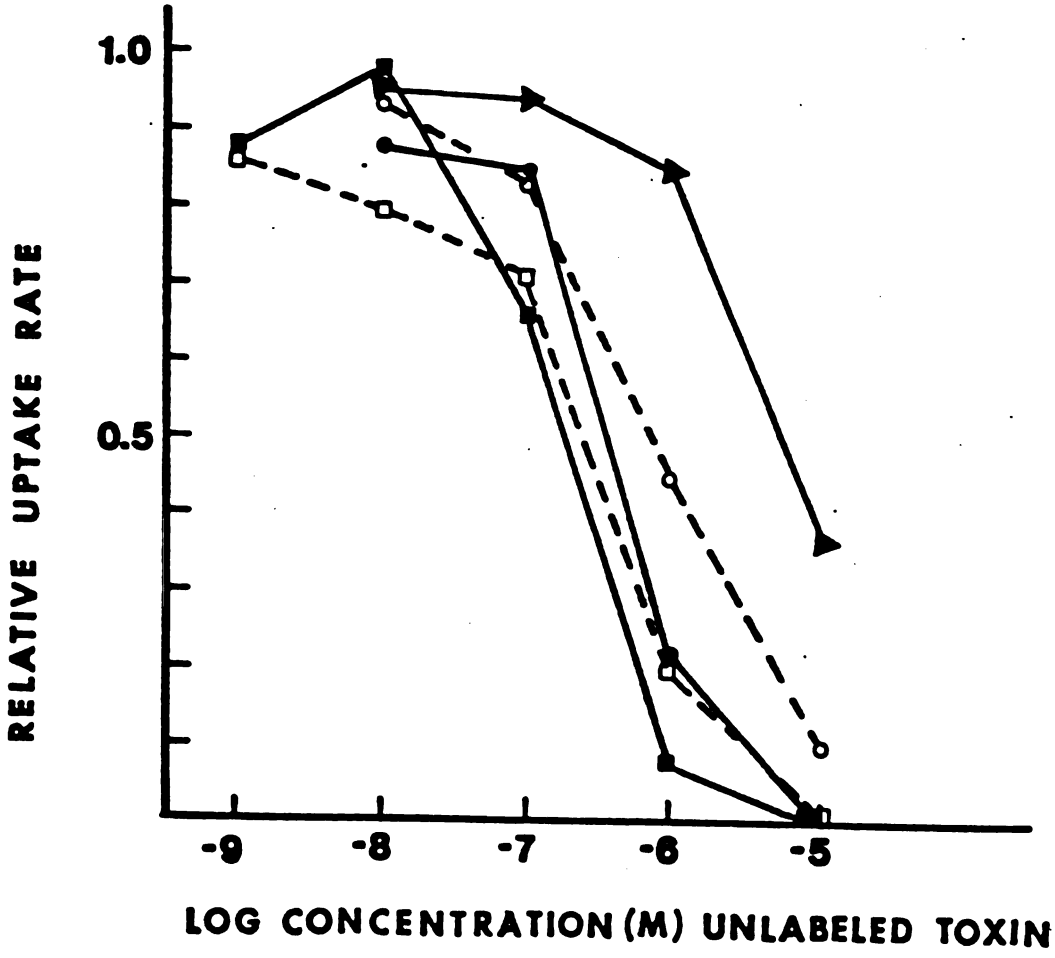

1.0
0.5
RELATIVE UPTAKE RATE
-9
-8
-7
-6
-5
LOG CONCENTRATION (M) UNLABELED TOXIN

FIGURE 17

Figure 18. Time course of accumulation of alpha-[$^3$H]T-2 by hybridomas preincubated with anisomycin. Cells were incubated for 60 min either in the presence (O) or absence (●) of anisomycin ($10^{-4}$ M), washed twice in RPMI, and resuspended in $10^{-7}$ M [$^3$H]T-2 for determination of uptake as described in Methods. Values are averages of 2 (●) or 4 (O) determinations. The uptake curve for cells preincubated with anisomycin was estimated by linear regression ($r = 0.9134$) using all time points.

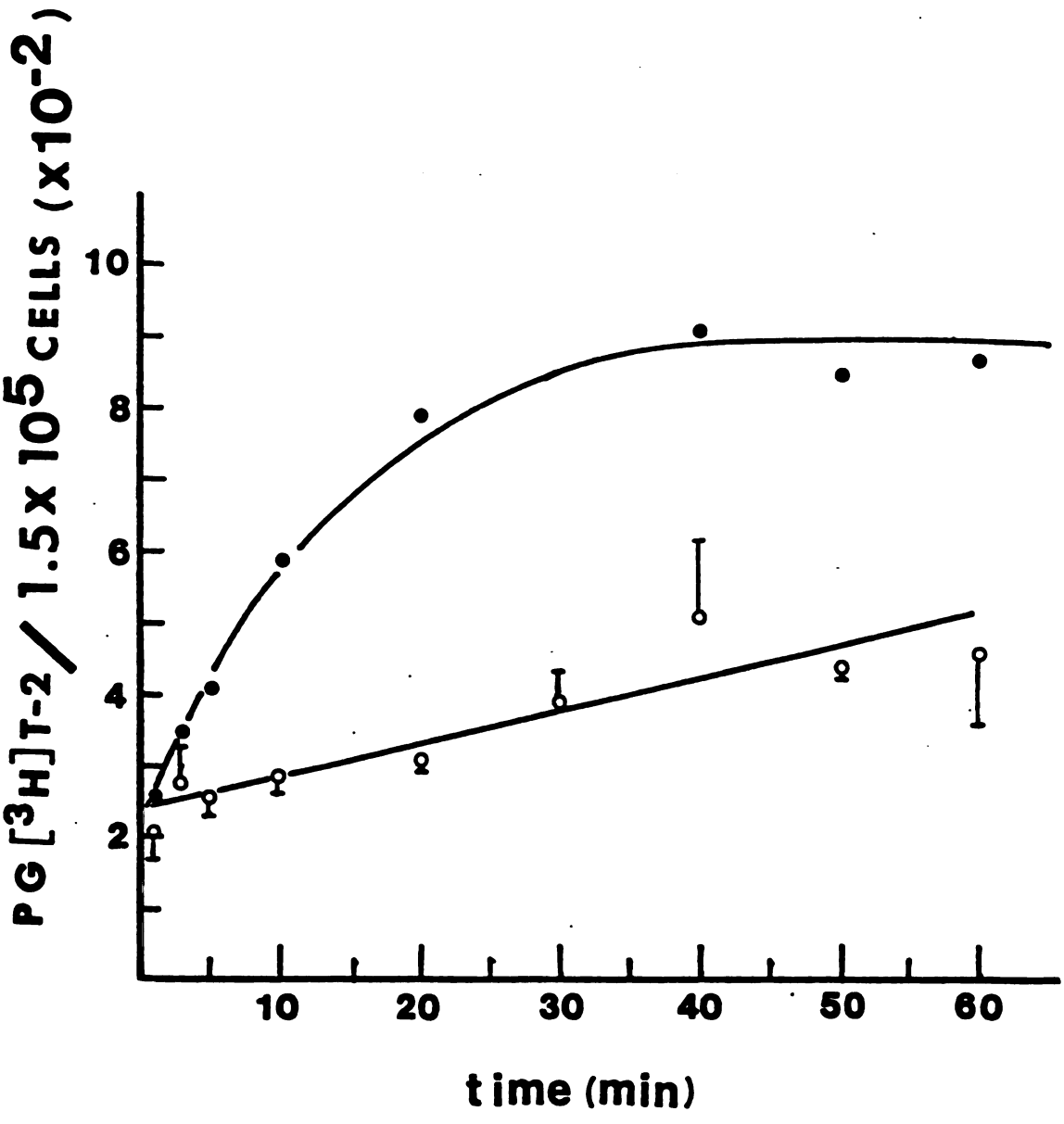



FIGURE 18

preincubated with and without anisomycin, respectively.

Uptake of alpha-[$^3$H]T-2 toxin by spleen cells

The uptake of [$^3$H]T-2 toxin by murine spleen cells was qualitatively similar to that by hybridomas. Uptake in the presence of $3.5 \times 10^{-8}$ M [$^3$H]T-2 was linear for about 30 min with equilibrium reached after about 45 min (Figure 19). Both the rate of uptake and zero-time uptake were proportional to the concentration of [$^3$H]T-2 over the concentration range $1.8 \times 10^{-8}$ to $7.3 \times 10^{-8}$ M (data not shown). The presence of T-2, DON, or anisomycin decreased the equilibrium accumulation of [$^3$H]T-2 in a dose-dependent manner (Figure 20).

Uptake of alpha-[$^3$H]T-2 toxin by erythrocytes

The accumulation of [$^3$H]T-2 by erythrocytes differed markedly from that by hybridomas. Accumulation in the presence of $5 \times 10^{-8}$ M [$^3$H]T-2 remained essentially constant, around $9 \times 10^{-6}$ pg/cell, from 0.5 to 60 min of incubation and this represented 2% of the total radioactivity (data not shown). The presence of unlabeled T-2 ($10^{-5}$ M) did not affect the accumulation of [$^3$H]T-2 by erythrocytes (data not shown).

Uptake of beta-[$^3$H]T-2 toxin by hybridomas

Qualitatively, the time course of accumulation of

Figure 19. Time course of accumulation of alpha-[$^{3}$H]T-2 by murine spleen cells. Uptake was determined as described in Methods in the presence of $3.5 \times 10^{-8}$ M [$^{3}$H]T-2. Values are averages of 2 determinations.

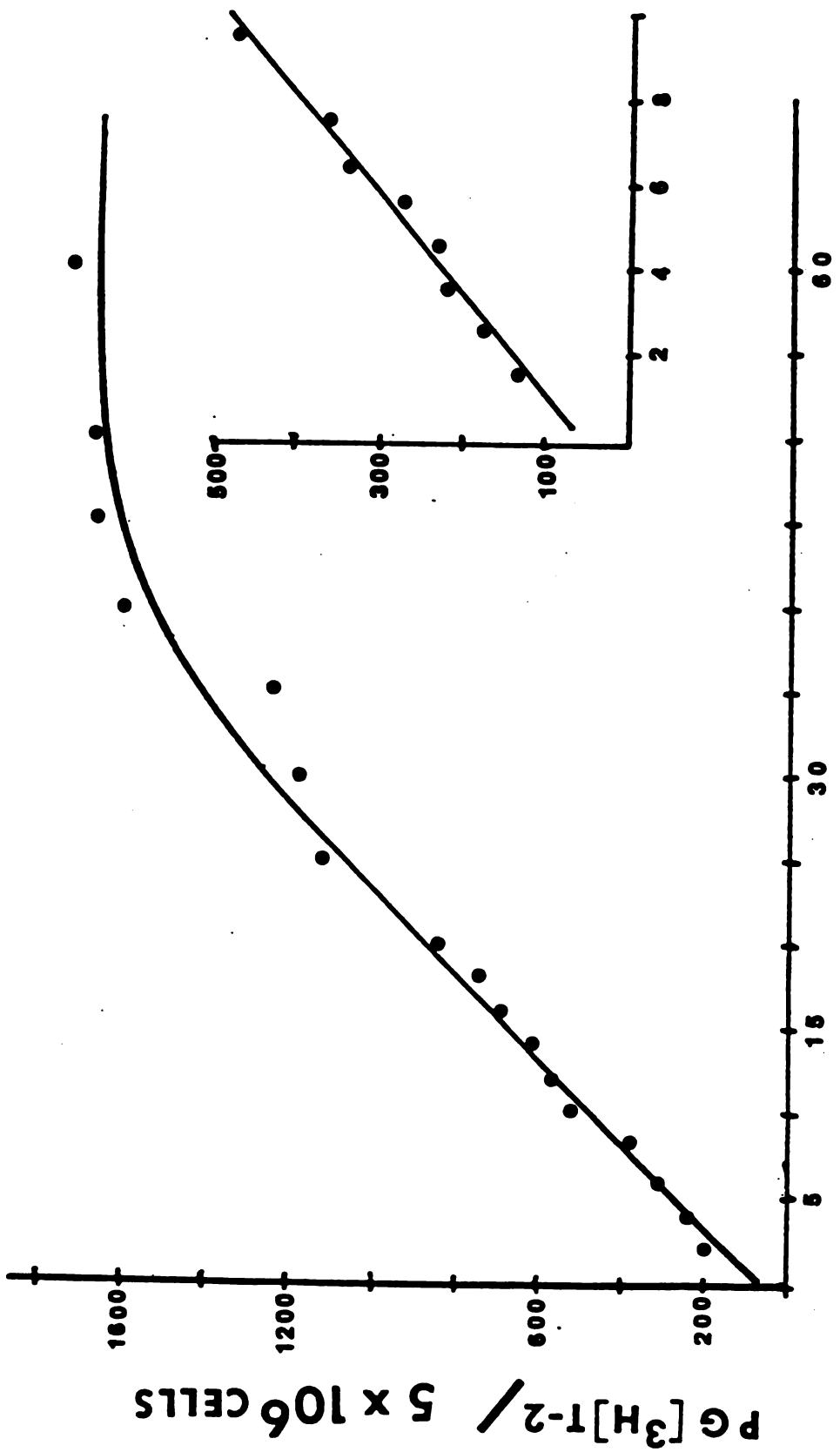

PG [3H]T-2/ 5 x 10^6 CELLS
1600
1200
800
400
5
15
30
60
TIME (MIN)
800
300
100
2
4
8

FIGURE 19

Figure 20. Inhibition of accumulation of alpha-[$^3$H]T-2 by spleen cells: T-2, DON, and anisomycin. Cells were exposed to 5 x $10^{-7}$ [$^3$H]T-2 in combination with either T-2 (●), DON (▲), or anisomycin (○). Accumulation was determined after 60 min incubation and compared to the accumulation of cells exposed to 5 x $10^{-7}$ M [$^3$H]T-2 alone. Values are averages of 2 determinations.

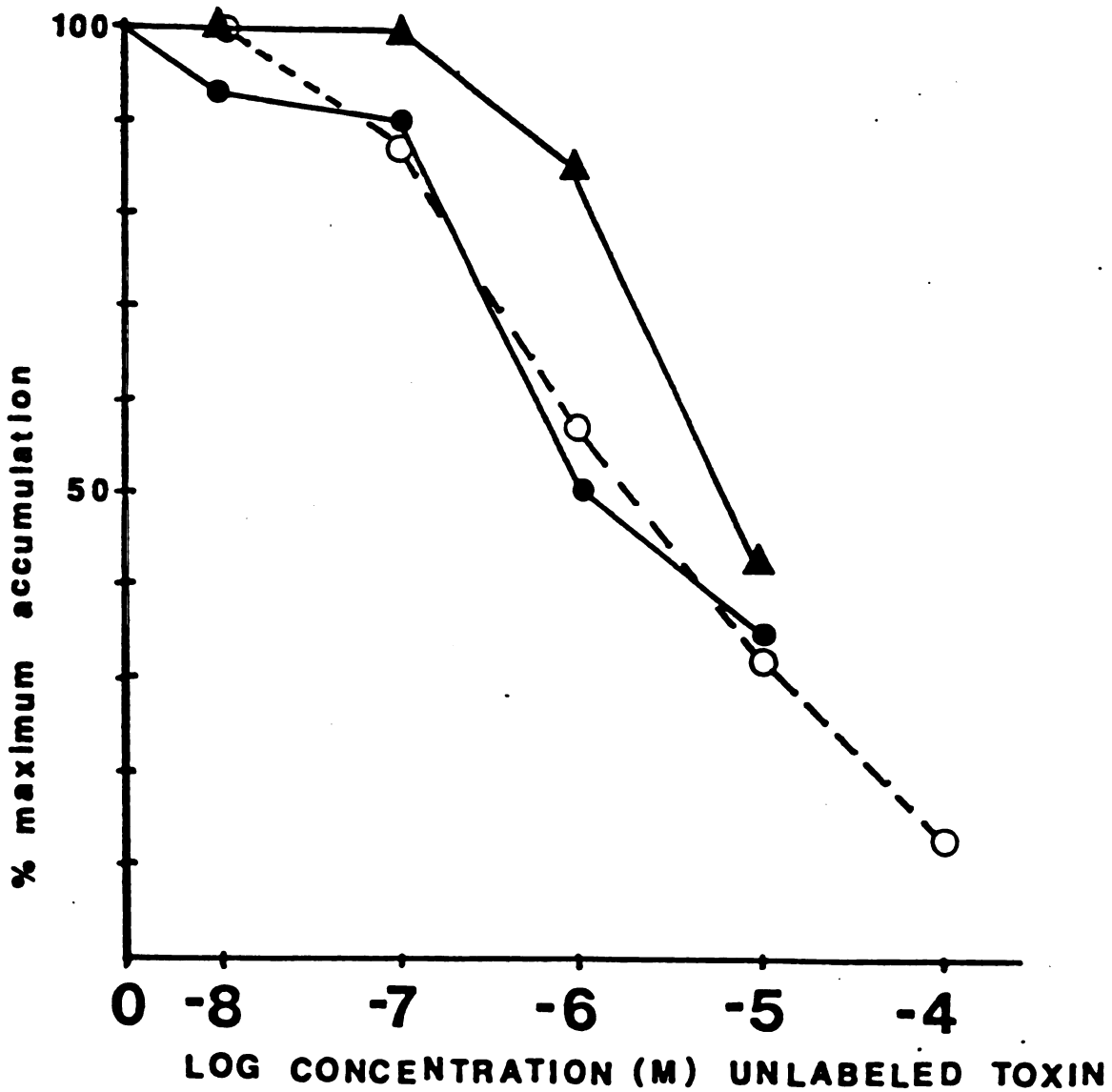

100
50
% maximum accumulation
0
-8
-7
-6
-5
-4
LOG CONCENTRATION (M) UNLABELED TOXIN

FIGURE 20

beta-[$^{3}$H]T-2 toxin by hybridomas was similar to that of alpha-[$^{3}$H]T-2 (Figure 21). Accumulation at equilibrium, in the presence of 1 x $10^{-7}$ M [$^{3}$H]T-2, was 3.9 x $10^{-4}$ pg [$^{3}$H]T-2/cell. The rate of uptake of beta-[$^{3}$H]T-2 by hybridomas, estimated by the slope of the regression line determined from accumulation data collected during the first 10 min of incubation (data not shown), was 10 pg [$^{3}$H]T-2/1 x $10^{6}$ cells-min. The zero-time uptake, estimated by extrapolation of this uptake curve, was 127 pg [$^{3}$H]T-2/1 x $10^{6}$ cells. The accumulation of beta-[$^{3}$H]T-2 was inhibited by unlabeled alpha-T-2 (Figure 21).

Figure 21. Time course of accumulation of beta-[$^{3}$H]T-2 by hybridomas. Uptake was determined as described in Methods in the presence of $10^{-7}$ M beta-[$^{3}$H]T-2 either alone (●) or in combination with $10^{-5}$ M alpha-T-2 (○). Values are averages of 2 determinations.

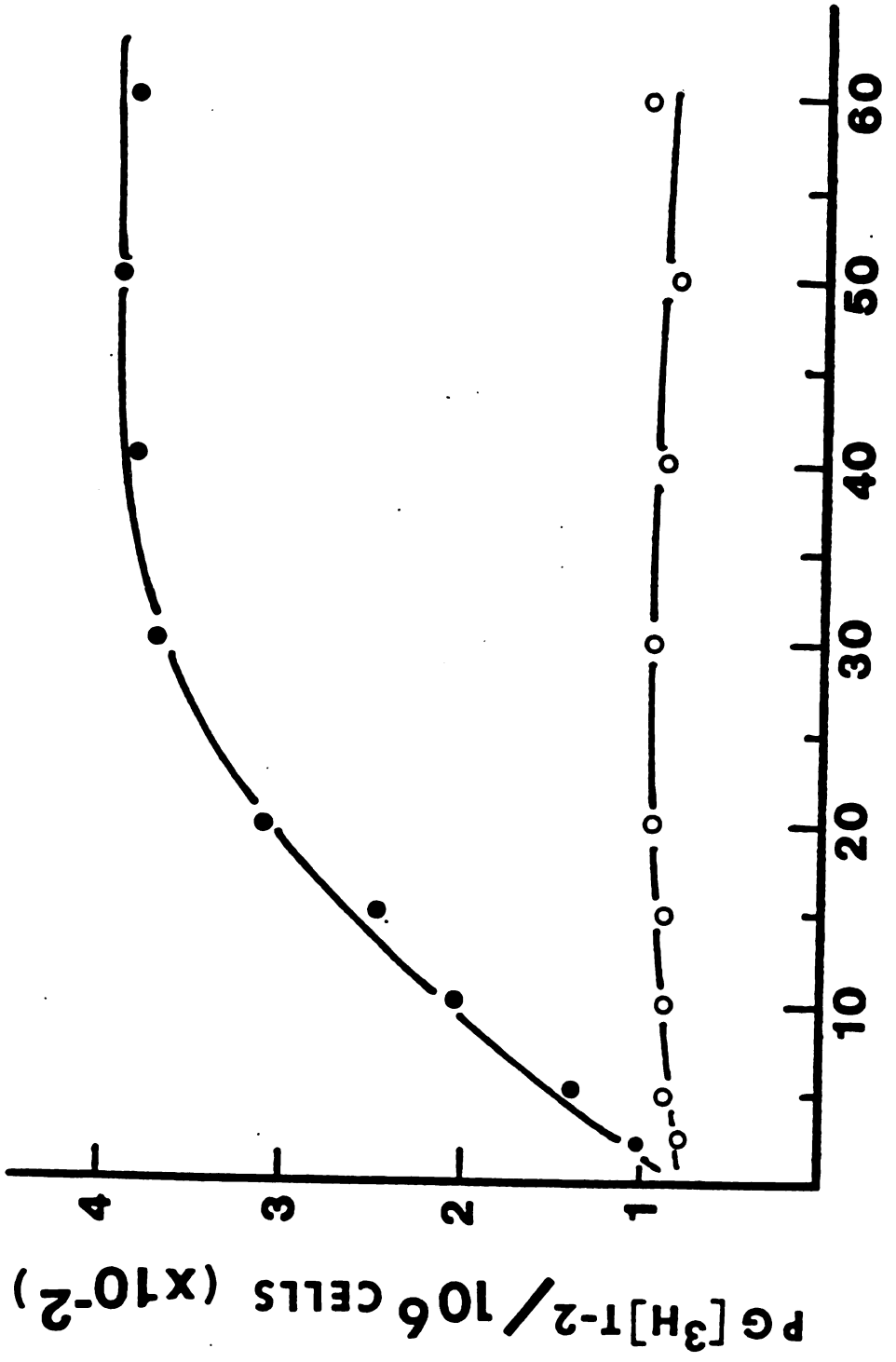



FIGURE 21

# DISCUSSION

## Preparation of [$^3$H]T-2 Toxin

Although the method of Wallace et al. (1977) has been cited frequently for the preparation of tritium labeled T-2 toxin, several difficulties were encountered using the procedure. Alpha-[$^3$H]T-2 toxin of purity sufficient for uptake studies was obtained, however, after modification of the Wallace procedure.

The first difficulty was the oxidation of T-2 toxin to 3-dehydro T-2 toxin. The success here with PCC over N-chlorosuccinimide as an oxidizing agent for T-2 is in contrast to that of Wallace et al. (1977), who reported N-chlorosuccinimide to be the most suitable oxidant of several tested, including PCC. Reaction with N-chlorosuccinimide for 2 hr produced 3-dehydro T-2 in 94% yield, but only 50% of T-2 was oxidized by PCC, and this yield could not be improved by increasing the reaction time. Others have found N-chlorosuccinimide to be unreliable as an oxidant for T-2 (G. Zhang, personal communication). Both PCC (Corey and Suggs, 1975) and the N-chlorosuccinimide/dimethyl sulfide complex (Corey and

Kim, 1972) are used to oxidize alcohols to carbonyl compounds. Due to the mild reaction conditions of N-chlorosuccinimide, this reagent has been particularly useful for the oxidation of complex or polyfunctional compounds. The poor results here with N-chlorosuccinimide may have been related to the difficulty in maintaining the temperature at -25 C during the reaction.

Although the specific activity of the [$^3$H]T-2 prepared here (508 mCi/mMol) was comparable to that of Wallace et al. (1977) (790 mCi/mMol), the relative yield of the desired alpha isomer (10%) was disappointing in light of the 23% yield of Wallace et al. (1977). These workers carried out the reduction at 38 C, rather than room temperature, and this may have contributed to the differences in relative yields of the two isomers. Future radiolabeling experiments would benefit greatly from the ability to increase the yield of alpha relative to beta. One approach would be to develop an asymmetric synthesis (Mosher and Morrison, 1983). During this type of reaction, a prochiral molecule is converted into a chiral one by reaction involving a chiral reagent; stereoisomeric products are produced in unequal yields. Asymmetric reductions of ketones to secondary alcohols have been carried out using chiral reducing agents of the metal hydride type.

A major objective was the separation of the two isomers produced in the radiolabeling reaction. [$^3$H]T-2

has reportedly been purified by Wallace et al. (1977), Chu et al. (1979), and Kemppainen et al. (1984) using successive TLC, although the latter two groups did not mention separating the alpha and beta isomers. However, although the two isomers were separated by HPTLC here, the small difference in $R_f$ values (<0.1) in conjunction with the length of the plate (10 cm) made recovery of the pure compounds after HPTLC separation impractical. Thus, an HPLC method was developed to separate the isomers. Adequate separation of alpha and beta was achieved with analytical reverse phase HPLC, which has been used for the analysis of T-2 toxin (Schmidt et al., 1981; Maycock and Utley, 1985; Martin et al., 1986). Despite the tedious nature of repeated chromatography (and the inevitable losses), this method was useful in the present study considering the small amounts of toxin to be purified.

The lower specific activity of beta compared to alpha in the first labeling experiment could be related to the use of an ELISA to quantitate [$^3$H]T-2. The ELISA used a monoclonal antibody obtained after immunization of mice with T-2-hemisuccinate-BSA (Gendloff et al., 1987), the conjugation site being carbon 3 of T-2 toxin. The reactivity of an antibody toward a hapten may be affected by the configuration of the hapten at the conjugation site of the hapten-protein immunogen used to elicit the antibody. For example, an anti-zearalenone monoclonal antibody obtained after immunization of mice with 6′-(carboxymethyl)zearalenone oxime-BSA (Dixon et al., 1987)

reacted as well with the metabolite alpha-zearalen-6′-ol as with zearalenone itself, but exhibited less reactivity with beta-zearalen-6′-ol. Thus, it is possible that the amount of beta-[$^{3}$H]T-2 was overestimated by ELISA due to a greater reactivity of beta with the antibody used in the assay compared to alpha.

The cause of the apparent interconversion of the alpha and beta isomers produced in the second labeling is not known. The labeling procedure used in that experiment was thought to be identical to the one used for the first labeling. It is possible that the purification procedures used affected the equilibrium alpha/beta ratios. One difference was the use of normal phase chromatography prior to reverse phase for purification of the first preparation. Whether or not the ommission of this step could result in equal amounts of the two isomers at equilibrium is not clear. Results of Colvin and Thom (1986) would indicate the opposite situation might exist: an unequal mixture of epimeric alcohols was equilibrated to a 1:1 mixture by stirring over chromatographic silica gel. Further experiments are required to determine the factors that affect the equilibrium ratio of alpha to beta T-2.

## Uptake of [$^3$H]T-2 Toxin

Interpretation of the uptake rates determined in zero-trans experiments as unidirectional transport rates (or initial rates) requires the existence of zero-trans conditions during the time of measurement (Stein, 1986). Although the demonstration of a constant rate of uptake is generally accepted as proof that zero-trans conditions are being closely approximated during the measurement, in certain situations this conclusion may be incorrect (Stein, 1986; Wohlhueter and Plagemann, 1980). Indeed, for the uptake of [$^3$H]T-2 toxin by hybridomas, two observations suggest that, despite an uptake curve that is clearly linear over the early time points, zero-trans conditions do not exist, and thus, rates estimated from these curves do not represent initial rates. First, during the time of linearity, a significant amount of [$^3$H]T-2 has accumulated within the cell -- up to 35% by 10 min at an initial concentration of $5 \times 10^{-8}$ M. Thus, not only is the intracellular concentration of toxin no longer close to zero, but the extracellular concentration has also decreased significantly. Secondly, the uptake curves extrapolate to zero time values greater than zero, contrary to that expected for initial rates.

The ability of the trichothecenes DON, VER A and ROR A to inhibit the accumulation and rate of uptake of [$^3$H]T-2 suggests that the uptake observed is proceeding by a

mechanism that is specific for this group of compounds. The inhibition of uptake of [$^3$H]T-2 by other trichothecenes has not previously been shown. Furthermore, any model proposed for the uptake of [$^3$H]T-2 toxin by hybridomas must be consistent with the observation that [$^3$H]T-2 is concentrated within the cell at extracellular toxin concentrations around $10^{-8}$ to $10^{-7}$ M, but not at higher [$^3$H]T-2 concentrations.

The ability of the structurally unrelated antibiotic anisomycin to inhibit the accumulation and rate of uptake of [$^3$H]T-2 by hybridomas suggests that ribosomal binding may be involved in the uptake, since binding studies have shown various trichothecenes and anisomycin to have mutually exclusive binding sites on eucaryotic ribosomes (Barbacid and Vazquez, 1974a,b). The lack of accumulation of [$^3$H]T-2 by erythrocytes, cells lacking ribosomes, also suggests a role for ribosomes in the uptake.

The simplest situation envisioned for ribosomal involvement is one where [$^3$H]T-2 enters the cell by simple diffusion, so that free toxin equilibrates rapidly across the membrane, then interacts with ribosomal binding sites according to the law of mass action describing receptor/ligand interactions (Limbird, 1986). T-2 toxin would in fact be expected to enter cells by simple diffusion because of its size and hydrophobic nature (Gyongyossy-Issa et al., 1984). Use of the mass action law to describe the kinetics of binding is also reasonable since [acetyl-$^{14}$C]trichodermin binds to ribosomes with a

single affinity and in the ratio of 1 molecule toxin/ribosome (Barbacid and Vazquez, 1974a; Cannon et al., 1976a).

Representing the binding of T-2 to its ribosomal receptor site (R) by :

$$T\text{-}2 + R \underset{k_2}{\overset{k_1}{\rightleftharpoons}} T\text{-}2/R$$

where T-2/R = T-2/ribosome complex and $k_1$ and $k_2$ are association and dissociation rate constants, respectively,

it follows that the equilibrium binding of T-2 to R is saturable with respect to [T-2]: [T-2/R] at equilibrium will approach [R] as [T-2] increases. Also, the binding of T-2 to R will be decreased in the presence of compounds that can compete with T-2 for its binding site on R.

The rate of binding can be described by a differential equation composed of the rate equations for association and dissociation:

$$d[T\text{-}2/R]/dt = k_1[T\text{-}2]\,[R] - k_2[T\text{-}2/R]$$

The rate of association, $k_1[T\text{-}2][R]$, is second order, but can be approximated by the pseudo-first order equation $k_1'[R]$, where $k_1' = k_1[T\text{-}2]$, if [T-2] >>> [R] and [T-2] remains essentially constant over the time of measurement. Integrating the simplified equation $d[T\text{-}2/R]/dt = k_1'[R] - k_2[T\text{-}2/R]$ over time with the constraint that [T-2/R] = 0 at time = 0 results in the integrated rate equation:

$$\ln\left( [T\text{-}2/R]_{eq} / ([T\text{-}2/R]_{eq} - [T\text{-}2/R]_t) \right) = k_{obs}t$$

where $k_{obs} = k_1' + k_2$ and $[T\text{-}2/R]_{eq}$ and $[T\text{-}2/R]_t$ are concentrations of the T-2/R complex at equilibrium and at time = t, respectively,

that describes the time course of binding of T-2 to R.

At early time points, the rate of binding can be approximated by the rate of association:

$$d[T\text{-}2/R]/dt = k_1 [T\text{-}2] [R]$$

and, for a given [R], will be constant and proportional to [T-2] over the time when [R] has not changed significantly. Over this time, the association curve will be linear. Thus, for a T-2/ribosome interaction described by the law of mass action, the initial rate of binding will increase and the time required for binding to reach equilibrium will decrease as the concentration of T-2 is increased.

Determination of the kinetic parameters of binding in the analysis above depend on the measurement of bound T-2, that is, the T-2/R complex. In the uptake experiments here however, total T-2 associated with cells was measured. This value includes T-2 bound specifically (i.e., that which can be inhibited by an excess of unlabeled T-2) to intracellular sites (for example, ribosomes), free T-2 in the intracellular space, and T-2 associated nonspecifically with the cell. Assuming permeation occurs by a nonconcentrative process, such as diffusion, the magnitude of the latter two components would be proportional to the extracellular concentration of T-2. Thus, the amount of T-2 specifically associated with cells would be saturable with concentration, but the amount of total T-2 associated would increase with concentration.

The results reported here are consistent with

predictions based on the binding of T-2 to an intracellular site -- presumably ribosomes. Specifically:

1. The time to reach equilibrium decreased as the concentration of [$^3$H]T-2 was increased. The range of times (min to hrs) to reach equilibrium reported in the literature (Trusal and Martin, 1987; Trusal and Watiwat, 1983; Thompson and Wannemacher, 1983; Gyongyossy-Issa and Khachatourians, 1984) may also be related to the concentration dependence of this parameter.

2. The rate of uptake increased as [$^3$H]T-2 concentration was increased. It is not certain if this relationship holds for concentrations greater than 4 x $10^{-7}$ M, since it was not possible to estimate rates in this range. A dose-dependency of rate of uptake over the concentration range 2.15 x $10^{-9}$ to 2.15 x $10^{-7}$ M has been observed previously in cultured cells (Trusal and Martin, 1987; Trusal and Watiwat, 1983). The zero time uptake values greater than zero and proportional to concentration may represent the essentially instantaneous equilibration of [$^3$H]T-2 across the membrane (along with nonspecific association of [$^3$H]T-2).

3. The total [$^3$H]T-2 associated with cells increased with the concentration of [$^3$H]T-2. Notably, Gyongyossy-Issa and Khachatourians (1984) observed a saturation of specific accumulation of [$^3$H]T-2 over a 10-fold concentration range.

4. The concentration dependence of percent accumulation can be explained by this model. As the

concentration of [$^3$H]T-2 is increased, and ribosomal binding becomes saturated, this component of cell associated [$^3$H]T-2 will become negligible compared to the ever increasing intracellular free and nonspecifically associated [$^3$H]T-2. Thus, the percent of T-2 associated with the cell will approach that value expected for diffusion equilibrium, here, 2%. This is consistent with the percent of [$^3$H]T-2 associated with erythrocytes in these experiments and others by DeLoach et al. (1987). The particular concentration where ribosomal binding becomes insignificant would depend on the size and ribosomal content of the cell.

The essentially complete inhibition of accumulation beyond zero time uptake of [$^3$H]T-2 by trichothecenes and anisomycin indicates that the time dependent accumulation of [$^3$H]T-2 is entirely due to the specific intracellular binding of [$^3$H]T-2 suggested here. Monitoring the uptake of [$^3$H]T-2 by hybridomas preincubated with a saturating concentration of anisomycin was intended to create a situation where binding sites were masked, thereby eliminating their contribution to the uptake of [$^3$H]T-2. The greatly decreased -- but still measurable -- rate of uptake of [$^3$H]T-2 by cells preincubated with anisomycin compared to those preincubated without reflects the binding of [$^3$H]T-2 to sites that become available as anisomycin dissociates. Thus, the uptake of [$^3$H]T-2 in these cells depends on both the dissociation of anisomycin and the

association of [$^{3}$H]T-2 with binding sites.

Correlation between quantitative ribosomal binding data and the uptake data presented here would provide direct evidence for the involvement of ribosomes in uptake. Unfortunately, given the available data, this comparison is limited for several reasons. First, in vitro ribosomal binding studies have used [acetyl-$^{14}$C]trichodermin, so a direct comparison with the uptake of [$^{3}$H]T-2 is not possible. Secondly, although [acetyl-$^{14}$C]trichodermin binds to a a given ribosome preparation, with a single affinity, the kinetics of binding appear to depend on both the source of the ribosome (Barbacid and Vazquez, 1974,a) and the form of the ribosomal units (Cannon et al., 1976a). Therefore, comparison of quantitative binding data obtained using yeast ribosomes and [acetyl-$^{14}$C]trichodermin with data from uptake studies using hybridomas and [$^{3}$H]T-2 may not be valid. Finally, use of the equations derived from the mass action law to calculate rate constants requires knowledge of some parameters that are not known for this system, including the volume and ribosomal content (more specifically, the number of T-2 binding sites) of the cell.

The time course of uptake and its dependence on concentration of [$^{3}$H]T-2 correlate with these parameters for inhibition of protein synthesis by trichothecenes. The time of exposure to T-2 required to inhibit protein synthesis in cultured cells by 50% compared to control cells decreased as the concentration of T-2 increased (Thompson and Wannemacher, 1984). Exposure to 2 x $10^{-7}$ M T-

2 for 5 min resulted in 50% inhibition, as did exposure to 1 x $10^{-7}$ M for 10 min and 7 x $10^{-8}$ for 15 min. The concentrations required to inhibit protein synthesis by 50% after exposure for 30, 45, or 60 min were similar: 2 to 3 x $10^{-8}$ M. These results are consistent with observations that maximum accumulation of [$^3$H]T-2 by hybridomas occurs by about 45 min in the presence of $10^{-8}$ M [$^3$H]T-2. Gyongyossy-Issa and Khachatourians (1985) observed a similar relationship, for concentrations of T-2 above a threshold dose of 2.4 x $10^{-9}$ M, between time and dose for inhibition of protein, DNA, and RNA syntheses. The time/concentration relationship of protein synthesis inhibition in a reticulocyte cell-free system (Carter and Cannon, 1977) also correlated with the uptake of [$^3$H]T-2 by hybridomas. In the presence of 4 x $10^{-7}$ M T-2, maximum inhibition of protein synthesis occurred by 15 to 20 min, while in the presence of 2 x $10^{-6}$ to 3 x $10^{-4}$ M, by 5 to 10 min. These studies in whole cells and cell-free systems suggest that the uptake of [$^3$H]T-2 by hybridomas is biologically relevant to the pharmacodynamic interactions of T-2.

Another test of biological relevance is comparing the order of potency for inhibition of uptake rate among the trichothecenes to the order of potency for inhibition of protein synthesis in whole cells. In Vero cells and rat spleen lymphocytes, VER A and ROR A were slightly more active than T-2 in inhibiting protein synthesis, while DON

was 100 times less active (Thompson and Wannemacher, 1986). This is the same order of potency for inhibition of rate of uptake of [$^3$H]T-2 by hybridomas. That the uptake may be mediated by binding to ribosomes is supported by the observation that, for the trichothecenes tested, the inhibition of [$^3$H]trichodermin binding to ribosomes correlates with the degree of inhibition of protein synthesis in a cell-free system (McLaughlin et al., 1977).

In summary, several lines of indirect evidence support the idea that the binding of [$^3$H]T-2 to ribosomes plays a key role in the uptake of this toxin by hybridomas: the kinetics of uptake, the patterns of inhibition, and the relationship of uptake to protein synthesis inhibition. However, it is possible that permeation of [$^3$H]T-2 occurs by a mechanism other than simple diffusion. It has been suggested that the permeation -- or transport -- of trichothecenes is important in the expression of their biological activity, since the polar trichothecence nivalenol was six times more potent in inhibiting protein synthesis in a cell-free system compared to whole cells (Ueno et al., 1973). On the other hand, the fact that diacetoxyscirpenol and T-2 both displayed greater activity in whole cells compared to cell-free systems cannot be explained by a permeation effect alone. In order to resolve the question of how trichothecenes cross the cell membrane, it is necessary to separate the permeation event from intracellular events such as ribosomal binding or metabolism (Wohlhueter and Plagemann, 1980; Heichalet et

al., 1979). The time scale of the uptake assay used to examine permeation must be sufficiently sensitive to measure initial rates.

A role for ribosomes in the uptake of [$^3$H]T-2 by hybridomas could be confirmed by additional experiments. Future uptake studies should include determinations of cell volume and ribosomal content, along with measurements of specific, in addition to total, accumulation, so that kinetic parameters based on ribosomal binding can be calculated and compared to ribosomal binding studies. Quantitative binding data are needed for the binding of [$^3$H]T-2 to ribosomes isolated from hybridomas. Another approach would be to compare uptake in trichothecene-sensitive and -resistant yeast strains, where the resistant strains have ribosomes that show decreased binding of trichothecenes (Jimenez and Vazquez, 1975). Additional studies on the uptake of [$^3$H]T-3 by erythrocytes, for instance, concentration dependence of uptake, would also be useful.

The similarity of the uptake of [$^3$H]T-2 by murine spleen cells with that by hybridomas suggests that the hybridoma has retained enough of its B-cell character to serve as a useful model for studying the uptake of trichothecenes by immune cells. Quantitative differences in uptake between hybridomas and spleen cells are undoubtedly due to both intrinsic differences between the cell types and the heterogeniety of the spleen cell

preparation. It is not clear whether or not the trichothecene-specific accumulation seen here in hybridomas is unique to immune cells and if it is, whether or not it is important in the immunotoxicity of the trichothecenes. However, spleen cells were more sensitive than Vero cells to the inhibition of protein synthesis by T-2 and other trichothecenes (Thompson and Wannemacher, 1986). Also, after intra-aortal administration of T-2 to swine, the concentration of T-2 in tissue was highest in the lymphoid organs (Beasley et al., 1986). Both of these observations could be related to the uptake of trichothecenes by immune cells.

Although the results here strongly suggest that binding to ribosomes may be a major factor in the uptake of [$^3$H]T-2 by hybridomas, other factors may be important in both these cells and in other types of cells. For example, the greater accumulation and rate of uptake of [$^3$H]T-2 by Vero cells compared to CHO cells (Trusal and Martin, 1987) cannot be explained by differences in RNA content of these cells. A possibility, however, is that differences in the metabolism of T-2 -- and subsequent accumulation of labeled metabolites -- exist between these cells. In uptake experiments, radioactivity continued to accumulate in cells even after 3 hr (Trusal and Martin, 1987). This is in contrast to the equilibrium level of accumulation observed for hybridomas. Metabolism of [$^3$H]T-2 could account for this steady accumulation of radioactivity. Indeed, metabolites of [$^3$H]T-2 were detected in the culture

medium of both CHO and VERO cells 1 hr after exposure to [$^3$H]T-2 (Trusal, 1986). It is possible that metabolites are also retained in the cell.

Finally, interpretation of uptake experiments using beta-[$^3$H]T-2 is difficult due to questions discussed earlier concerning the determination of the specific activity of beta-[$^3$H]T-2. Thus, quantitative comparisons between uptake of alpha and beta cannot be made. However, the similarity of the time course of accumulation for the two isomers, along with the ability of alpha to inhibit accumulation of beta, suggests that uptake of these two isomers occurs by a similar mechanism.

## Conclusions

Modification of the Wallace et al. (1977) method for radiolabeling T-2 toxin -- especially the purification parts -- was required to prepare alpha-[$^3$H]T-2 toxin of adequate purity for cellular uptake studies. Uptake experiments revealed a specific uptake of [$^3$H]T-2 by a murine B-cell hybridoma. Other trichothecenes are likely to be taken up in a similar manner. Several lines of evidence suggest that the accumulation observed here involves binding of [$^3$H]T-2 to ribosomes. Further experiments, however, are required to confirm this hypothesis.

# APPENDIX

# APPENDIX

This appendix contains copies of the publications resulting from DON toxicity studies, in addition to the publication based on the work described in Part 1. DON purified by water-saturated silica gel chromatography was used to conduct three toxicity studies in mice:

(1) the acute toxicity by oral and peritoneal routes,

(2) chronic toxicity of dietary exposure, and

(3) immunotoxicity of dietary exposure.

These studies were a group effort, and I participated in several general aspects of them, including toxin and diet preparation, care of animals, and monitoring of feed intake and body weight of the animals. During the assessment of the immunotoxic effects of dietary exposure to DON on mice, I focused my efforts on the alterations in humoral immunity. These included:

(1) determination of the serum immunoglobin profile in mice consuming 0, 0.5, 2.0, 5.0, 10.0, or 25.0 ppm DON for 8 weeks. Serum concentrations of IgG, IgM, and IgA were determined using an enzyme-linked immunosorbent assay (ELISA). In mice consuming 2 to 10 ppm DON, the concentration of IgM was decreased and that of IgA increased compared to mice consuming toxin free diet. The

unexpected observation that the concentration of IgA was increased formed the basis for further, ongoing research into the effects of DON on regulation of IgA status.

(2) determination of the plaque-forming cell (PFC) response to the T-dependent antigen sheep red blood cells in mice consuming 0, 5, or 25 ppm DON for either 2 or 8 weeks. This experiment also included a group of mice receiving a restricted amount of food to compensate for the feed refusal caused by DON. The PFC response was determined by a modification of the standard Jerne assay (Jerne and Nordin, 1963). The PFC response was decreased in mice consuming 25 ppm DON for 8 weeks compared to the response of mice on the corresponding restricted feeding regimen.

The alterations in humoral immunity observed here led to experiments focusing on the cellular interactions among trichothecenes and immune cells that may be responsible for the immunotoxic effects of DON. One group of experiments was the cellular uptake studies described in Part II.

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# Purification of Deoxynivalenol (Vomitoxin) by Water-Saturated Silica Gel Chromatography

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A simple procedure was developed for the laboratory production and purification of gram quantities of crystalline deoxynivalenol (DON). When *Fusarium graminearum* R6576 was grown on rice, concentrations of 600–700 ppm DON were obtained after 13–18 days of incubation. A DON derivative, 15-acetyl-DON (15-ADON), was also found at concentrations of 100–300 ppm after 7–10 days. Crude culture extracts were purified by low pressure liquid chromatography on a column of water-saturated silica gel which selectively extracted DON when methylene chloride was used as the mobile phase. After elution of DON with water and subsequent reextraction with ethyl acetate, DON could be readily crystallized. Purity of crystallized DON was verified by thin layer and high performance liquid chromatography.

Deoxynivalenol (3,7,15-trihydroxy-12,13-epoxytrichothec-9-en-8-one, DON), also known as vomitoxin, is a trichothecene mycotoxin produced by *Fusarium graminearum*, which has been associated with vomiting and feed refusal in swine (Vesonder et al., 1976; Forsyth et al., 1977). Recent examination of *Fusarium* infected grains, particularly in Canada and the Midwest, has confirmed the natural occurrence of DON (Scott et al., 1981; Eppley et al., 1984; Trenholm et al., 1983). Assessment of the hazards associated with exposure to DON has been hampered by a lack of the gram quantities of pure DON required for toxicological studies.

An efficient method for DON production requires a convenient, concentrated source of DON. Whereas concentrations found in naturally contaminated grains are not high enough for use of this material as a source, inoculation of growing corn with DON-producing *Fusarium* strains has provided a concentrated source of DON (Miller et al., 1983; Scott et al., 1984). DON production in lab culture provides a more convenient source of crude DON and both solid and liquid substrates have been investigated (Vesonder et al., 1982; Greenhalgh et al., 1983; Greenhalgh et al., 1984). Although several multistep schemes for purifying crude DON extracts involving solvent–solvent partitioning, column chromatography, preparative thin layer chromatography (TLC), and high pressure liquid chromatography (HPLC) have been reported (Pathre and Mirocha, 1978; Bennett et al., 1981; Ehrlich and Lillehoj, 1984; Scott et al., 1984), these have the disadvantage of requiring numerous, time consuming chromatography steps.

The results reported here describe an efficient method for producing and purifying gram quantities of crystalline DON on a laboratory scale. DON was produced at very high levels by *Fusarium graminearum* R6576 on rice and was purified in a single step by low pressure liquid chromatography using a water-saturated silica gel column that is selective for DON.

## MATERIALS AND METHODS

**Inoculum Preparation.** Potato dextrose agar plates were inoculated from stock soil cultures of *Fusarium graminearum* R6576 (*Gibberella zeae* U5373), a Michigan wheat isolate previously designated as W-8 (Hart et al., 1982), and incubated at 25 °C for 7 days in a 12-h light/dark cycle. Agar plugs (4 mm) removed from the growing edge of colonies were added to 500-mL Erlenmeyer flasks (3–4 plugs per flask) containing (carboxymethyl)-cellulose (CMC) medium (90 mL) (Cappellini and Peterson, 1965). These flasks were agitated on a rotary shaker (250 rpm) at 25 °C for 3–5 days. The suspension was

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filtered through sterile cheesecloth, and the macroconidia concentration was determined by counting on a hemacytometer.

**DON Production.** Fernbach culture flasks (2800 mL) containing dry white rice (350 g) and distilled water (150 mL) were stoppered with cotton plugs (foil caps were unsuitable) and autoclaved at 121 °C for 30 min. Flasks were inoculated with $10^6$ macroconidia and the cultures were incubated in the dark at 28 °C, without shaking. Cultures were extracted after 7, 9, 11, 14, 17, 21, and 23 days of incubation to monitor the time course of DON production. An incubation period of 13–18 days was used for the routine production of gram quantities of DON.

**DON Extraction.** The extraction procedure was adapted from Pathre and Mirocha (1978). At the end of the incubation period, the contents of each flask were blended with 60% aqueous methanol (1400 mL). After soaking overnight, the mixture was filtered through Whatman No. 4 filter paper and methanol removed on a steam bath. The aqueous extract was saturated with sodium chloride and filtered to remove any precipitate. Saturated solutions were sometimes held overnight to allow complete precipitation. The aqueous solution was extracted 3 times with ethyl acetate with a volume ratio of 1:2 (ethyl acetate–water). Ethyl acetate from combined extracts was removed on a rotatary evaporator and the residue dissolved in either ethyl acetate for TLC quantitation or methylene chloride for silica gel chromatography.

**Silica Gel Chromatography.** Low pressure liquid chromatography was performed on silica gel (Adsorbosil, 200/425 mesh, Anspec) by using the following equipment (Ace Glass, Inc., Vineland, NJ): 37-mm i.d. Michel-Miller glass chromatographic column fitted with a safety shield, Teflon end fittings, connectors, and tubing (2-mm i.d.). A pump (Model RP-SY-1CSC; Fluid Metering, Inc., Oyster Bay, NY), adapted with low flow fittings and coupled to a pulse dampener, maintained a flow rate of about 5 mL/min (10–40 psi). Fractions (10 mL) were collected and monitored by TLC (see Analytical Procedures). The column was prepared by dry packing with silica gel (170 g). The packed column was equilibrated with distilled water (500–600 mL) and allowed to drain. Methylene chloride (400–500 mL) was pumped through the column until water no longer appeared in the effluent. A sample of crude extract (combined extracts from 10–20 flasks) in a minimum volume of methylene chloride was applied to the column and eluted with methylene chloride until metabolites (excluding DON) ceased to appear in the effluent, as indicated by TLC monitoring. The column was then eluted with distilled water to remove DON. Water fractions (including water layers in tubes containing both solvents) containing DON were combined and extracted 5–10 times with ethyl acetate (volume ratio 1:1). The water layer was analyzed by TLC to ensure removal of DON. Combined ethyl acetate extracts were concentrated on a rotary evaporator. After each run, the silica gel column was washed with water for reuse in DON purification.

**DON Crystallization.** Concentrated extract was dried under $N_2$ in a beaker (50 mL) and dissolved in a minimum volume of ethyl acetate. The solution was seeded with a few crystals of DON, and the beaker was covered tightly with aluminum foil and refrigerated (4 °C). When a precipitate had formed (several days were sometimes required), the supernatant was removed and saved. Solids were dissolved in ethyl acetate and filtered through Whatman No. 4 filter paper. DON was recrystallized in ethyl acetate as before, again reserving the supernatant. Methanol (0.5–1.0 mL) was mixed with the crystals to extract residual pigment. The mixture was refrigerated (4 C) for 8 h, then methanol removed and saved. This washing procedure was repeated with ethyl acetate (0.5–1.0 mL). The entire crystallization scheme was repeated with combined methanol and ethyl acetate supernatants. After residual solvent had been evaporated, purity of the white crystalline DON was checked by HPLC.

**Analytical Procedures.** Semiquantitative TLC was performed on precoated 20 × 10 cm silica gel G plates (Redi-Plates, Fisher Scientific Co.) with toluene–ethyl acetate (1:3). DON was detected by spraying the developed plate with a 15% aluminum chloride solution (15 g $AlCl_3$-$6H_2O$ in 85 mL of ethanol + 15 mL of water) and then heating it for 5 min at 110 °C (Baxter et al., 1983). DON produces a blue fluorescent spot under longwave (365 nm) UV light at $R_f$ 0.3. The location of 15-acetyl-DON (15-ADON) was identified by comparison with that of a qualitative 15-ADON standard ($R_f$ 0.5) that had been previously prepared in this laboratory and confirmed with mass spectrometry by Dr. C. J. Mirocha (University of Minnesota). Both DON and 15-ADON were quantitated by visual estimation in a UV viewing cabinet using DON standard (Myco-Lab Co., Chesterfield, MO) dissolved in ethyl acetate.

Purity of crystalline DON was assessed with HPLC using a Model 2300 HPLC pump and $V^4$ variable wavelength absorbance detector (5-mm flow cell) (ISCO, Lincoln, NE). The system was equipped with RP-18 Spheri-10 MPLC analytical (22 cm × 4.6 mm i.d.) and guard (3 cm × 4.6 mm i.d.) cartridges (Brownlee Lab, Inc., Santa Clara, CA). Mobile phase was 7% (vol/vol) methanol in water with a flow rate of 2mL/min. Purity of DON dissolved in mobile phase was determined at 224 nm and 0.05 a.u.f.s. DON standard had a retention time of 16.9 min at 25 °C. A standard of 3,15-dihydroxy-12,13-epoxytrichotec-9-en-8-one (7-deoxy-DON) supplied by G. A. Bennet (Northern Regional Research Center, Peoria) had a retention time of 18.2 min in this solvent system.

Identity of crystalline DON was confirmed by comparison of its mass spectrum with that of the DON standard. Both spectra were obtained at 70 eV by using a direct probe, on a Finnigan 3200 gas chromatograph–mass spectrometer coupled to a Riber SADR data system. Ultraviolet (UV) spectrum of DON in ethanol (50 μg/mL) was determined on a Beckman Model 35 spectrophotometer. Partition coefficients were determined by adding either DON or 15-ADON to a mixture of water and methylene chloride. Equilibrium concentrations of toxins were determined by using the above HPLC procedure. Partition coefficient was defined as: toxin concentration in water/toxin concentration in methylene chloride.

Safety Note. Contact with DON and crude extracts was always avoided. Contaminated glassware was soaked in 10% bleach solution overnight before being washed (Thompson and Wannemacher, 1984). Safety shields were used with Michel-Miller glass columns and operating pressure did not exceed 300 psi.

## RESULTS AND DISCUSSION

**DON Production.** Preliminary experiments indicated that optimal incubation conditions for DON production on rice by our strain were consistent with those reported by Vesonder et al. (1982) and Greenhalgh et al. (1983); that is, an incubation temperature of 28 °C and a initial substrate moisture content of 35–40%. *F. graminearum* R6576, however, required a shorter incubation time for maximum DON production. Maximum concentrations (600–700 ppm, dry rice basis) of DON occurred between

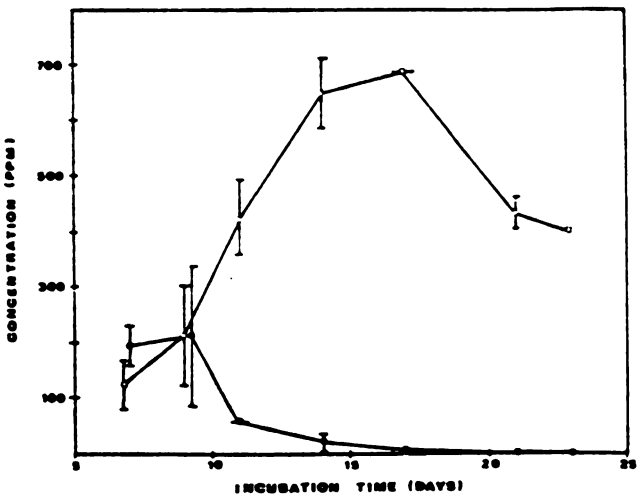


Figure 1. Time course of DON (○) and 15-ADON (●) production by *F. graminearum* R6576 on rice. Values are averages, with standard error bars, of 3 flasks, except for values at days 17 and 21 (2 flasks) and day 23 (1 flask). 15-ADON was not detected on days 21 and 23 (1 ppm detection limit). Concentrations are based on dry rice weights.

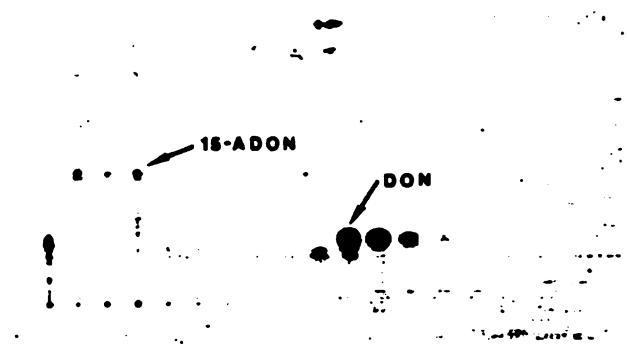


Figure 2. Elution pattern of crude extract on water-saturated silica gel column. The TLC plate was developed in toluene–ethyl acetate (1:3), then sprayed with 30% $H_2SO_4$, and heated for 5 min at 110 °C for photographic visualization. The first spot, left to right, is the crude extract before chromatography. Remaining spots, left to right, are fractions 15, 25, 35, 45, 55, 65, 75, 82, 88, 92, 98, 102, 108, 112, 118, 125, 135, and 145. Fractions 1–70 were eluted with methylene chloride and the remaining fractions with water.

greater than 3 weeks for the above two studies. Shorter incubation time could be a result of our inoculum procedure or a characteristic of the isolate. A derivative of DON, acetylated on carbon 15 (15-ADON), was also produced; maximum concentrations (100–300 ppm) occurred between 7 and 10 days of incubation (Figure 1).

The order of appearance of DON and 15-ADON by *F. graminearum* R6576 on rice was qualitatively similar to that found in liquid culture in our laboratory (Pestka et al., 1985) where maximum DON levels occur at day 20. Field inoculation experiments by Miller et al. (1983) and Hart et al. (1984) in corn and wheat, respectively, also describe qualitatively similar behavior for both DON and 15-ADON production. Yoshizawa et al. (1975) have previously found that disappearance of 3-ADON was concurrent with DON production in the fermentation by *F. roseum* and it has been suggested that 3-ADON deacetylation is a step in the biosynthesis of DON. Miller et al. (1983) indicate that plant enzymes may be responsible for conversion of 15-ADON to DON and subsequent DON degradation. Our results suggest that these changes also may be caused by enzymatic or chemical reactions associated with the fungal culture.

**DON Purification.** Figure 2 shows the elution pattern, as visualized by TLC, for a typical chromatographic separation of crude DON on water-saturated silica gel. Crude extract (10.8 g) used in this run contained approximately 1.3 g of DON. Methylene chloride eluted most of the pigments and metabolites other than DON in the first 70 fractions while DON was retained on the column. When the solvent was changed to water at fraction 71, water first appeared in the effluent in fraction 82 and DON apeared in fraction 90. DON concentration peaked in the first few DON-containing fractions and then decreased rapidly. Fractions 90–110 were combined for DON crystallization, yielding 0.62 g of crystalline DON. Mass spectrometry confirmed the identity of the compound crystallized by this procedure. The UV spectrum showed an absorption maximum at 218 nm (ε 6805). Purity of the crystalline DON (mp 150–152 °C), as determined by HPLC, was equal to that of the analytical standard; a single peak was seen at 16.9 min. 7-Deoxy-DON was not detected in the purified DON.

We believe that DON separation on water-saturated silica gel was based on liquid-liquid partitioning rather than the usual adsorption mechanism of silica gel. Here, silica gel functioned as a support for a layer of adsorbed water. DON was extracted from crude preparations by partitioning into the water layer. Other metabolites, including most pigments, were eluted from the column with little or no retention. Elution with water quickly removed the water layer containing DON. 15-ADON was not retained with DON on the column, even though it differs from DON only in the replacement of a single hydroxyl group with an acetyl group. The difference in partition coefficients of DON and 15-ADON, 20 and 0.03, respectively, between the two liquid phases (water to methylene chloride) appears to account for the efficient separation of these two trichothecenes. The high solubility of DON in water has also been used by Terhune et al. (1984) in a procedure for quantitation of DON in grains by gas chromatography where samples were extracted with water instead of aqueous methanol. The aqueous extract was adsorbed onto a Clin Elut column and DON eluted with ethyl acetate. Scott et al. (1984) used an analogous step in the purification of gram quantities of DON. An aqueous extract of DON was adsorbed onto a ChemTube containing a hydrophilic matrix and DON was eluted with ethyl acetate. Several other steps, however including column chromatography and semipreparative liquid chromatography, were required to obtain crystalline DON.

In summary, *F. graminearum* R6576, when grown on rice, produced a yield of DON which was equivalent or better than reported levels produced in rice by other strains of this fungus (Vesonder et al., 1982; Greenhalgh et al., 1983). The crude DON extract prepared from this culture was rapidly purified by a single chromatographic step on water-saturated silica gel. Besides reduced chromatography requirements, the purification procedure described herein had significant advantages over other reported methods because it had minimal solvent requirements and did not require acetylation or hydrolysis steps (Pathre and Mirocha, 1978; Bennett et al., 1981; Ehrlich and Lillehoj, 1984; and Scott et al., 1984). Water-saturated silica gel might also be used as a simple cleanup step for the analytical determination of DON.

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Registry No. DON, 51481-10-8; 15-ADON, 88337-96-6.

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# COMPARISON OF ACUTE TOXICITIES OF DEOXYNIVALENOL (VOMITOXIN) AND 15-ACETYLDEOXYNIVALENOL IN THE B6C3F$_1$ MOUSE

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**Abstract**—The acute toxic effects of deoxynivalenol (DON) and 15-acetyldeoxynivalenol (15-ADON) were compared in the B6C3F$_1$ female mouse after oral and intraperitoneal exposure. Using the abbreviated procedure of Lorke (*Archs Toxicol.* 1983, 54, 275), $LD_{50}$ values for DON were estimated to be 78 mg/kg (oral) and 49 mg/kg (ip) whereas the $LD_{50}$ values for 15-ADON were 34 mg/kg (oral) and 113 mg/kg (ip). Acute doses of these toxins resulted in extensive necrosis of the gastro-intestinal tract, bone marrow and lymphoid tissues, and focal lesions in kidney and cardiac tissue. The minimum doses required for these histopathological effects were consistent with $LD_{50}$ estimations. The results indicate that 15-ADON was more or less toxic than DON depending on the route of administration. Risk assessments for DON should therefore consider the potential for 15-ADON occurrence and toxicity in food and feed.

## INTRODUCTION

The trichothecene mycotoxin, deoxynivalenol (DON or vomitoxin) (Fig. 1), is a common contaminant of cereal grains worldwide (Ichinoe *et al.* 1983; Mirocha *et al.* 1976; Trenholm *et al.* 1981; Vesonder & Ciegler, 1979). Produced by *Fusarium graminearum*, DON has been associated with serious swine health problems (Coté *et al.* 1984) and can experimentally cause a variety of toxic effects that are characteristic of the trichothecene mycotoxin group including feed refusal, reduced body-weight gain, vomiting, and lymphopenia (Forsell *et al.* 1986; Ueno, 1983; Vesonder *et al.* 1976). Recently it has been determined that North American strains of *F. graminearum* produce 15-acetyldeoxynivalenol (15-ADON) (Fig. 1) prior to the appearance of DON in both laboratory culture (Miller *et al.* 1983a; Pestka *et al.* 1985; Witt *et al.* 1985) and experimentally infected wheat and corn (Hart *et al.* 1984; Miller *et al.* 1983b). Because this biosynthetic precursor is likely to contaminate cereal grains concurrently with DON, we have sought to evaluate its toxicity relative to that of DON. Dietary exposure to 5.0 ppm 15-ADON causes feed refusal and reduced weight gain in the B6C3F$_1$ female mouse (Pestka, Lin & Forsell, 1986) indicating that 15-ADON has approximately the same chronic toxicity as DON (Forsell *et al.* 1986). 15-ADON is approximately one half as toxic as DON in the mitogen-induced human lymphocyte blastogenesis assay (Forsell & Pestka, 1985). In this study, we compared the acute toxic effects of 15-ADON and DON in the B6C3F$_1$ female mouse after oral and intraperitoneal exposure. The relative toxicity of these two toxins varied with the route of exposure, suggesting that subtle differences may exist in absorption, metabolism and distribution of DON and 15-ADON.

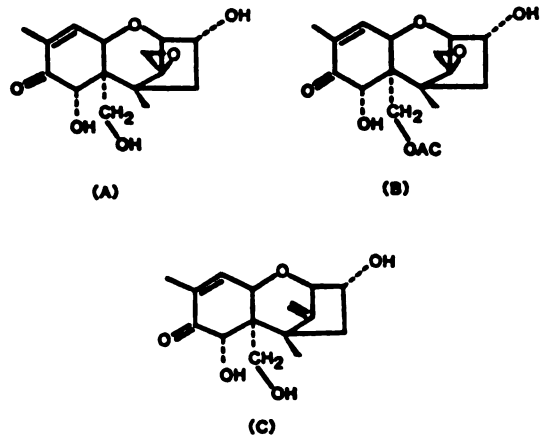


Fig. 1. Chemical structures of deoxynivalenol (A), 15-acetyldeoxynivalenol (B) and 3,7,15-trihydroxytrichothec-9,12-dien-8-one (C).

## MATERIALS AND METHODS

*Mycotoxins.* Deoxynivalenol was produced in *Fusarium graminearum* R6576 cultures and purified by water-saturated silica gel chromatography (Witt *et al.* 1985). 15-Acetyldeoxynivalenol was produced and purified on silica gel as described by Pestka *et al.* (1986).

*Animals.* B6C3F$_1$ weanling female mice were purchased from Harlan/Sprague–Dawley, Inc. (Indianapolis, IN). The mice were housed individually in protected environment cages (Nalgene, Rochester, NY). Each cage unit included a transparent polycarbonate body with filter cover, stainless-steel wire lid, and raised floor. Heat-treated hardwood chips covered the cage floor under the raised floor. Mice were fed pelleted Wayne Breeder Blox *ad lib.* (Wayne Research Animal Diet, Chicago, IL).

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*Experimental Design.* The procedure used for determining the $LD_{50}$ of DON and 15-ADON was that of Lorke (1983). Briefly, nine mice were used in a range-finding experiment in which three animals each received 10, 100 or 1000 mg DON/kg body weight. The same regimen was used for 15-ADON except the highest toxin dose was 200 mg/kg because of limited solubility. Based on the results obtained from the range-finding procedure, four dose levels were chosen for the second test, a single mouse being required for each dose level. Calculation of the $LD_{50}$ was based on the results of both the range-finding procedure and the second test as described by Lorke (1983). Using this protocol, only 13 experimental animals were necessary to provide adequate information for determination of the $LD_{50}$ for each toxin by each route of exposure.

Two routes of exposure were used for each of the mycotoxins: gavage and injection. A single exposure to mycotoxin was given in sterile saline in a total volume that ranged from 0.15 to 0.19 ml. The mice were observed for 14 days and then killed. Mice that died during the 14-day period were autopsied within 4 hr. The brain, thymus, spleen, kidney and liver were removed and weighed. Tissues from the gastro-intestinal tract, spleen, thymus, kidney, bone marrow, lung, liver and pancreas were fixed in 10% buffered formalin, embedded in paraffin, sectioned at 6 $\mu$m and stained with haematoxylin and eosin for histological evaluation. All tissue sections were evaluated without knowledge of treatment regimens.

## RESULTS

### $LD_{50}$

All mice that succumbed to DON or 15-ADON did so within 48 hr. The $LD_{50}$ values for DON were calculated to be 78 mg/kg by gavage and 49 mg/kg ip. The $LD_{50}$ values for 15-ADON were 34 mg/kg by gavage and 113 mg/kg ip. $LD_{50}$ values for both DON and 15-ADON were reproducible in replicate trials of the Lorke (1983) procedure.

### *Gastro-intestinal tract*

Gastro-intestinal lesions observed in mice exposed to DON or 15-ADON dying within 21 hr of exposure were similar. The most dramatic lesion was severe necrosis of the crypt epithelial cells in the small (Fig. 2) and large (Fig. 3) intestine. The lumens of the crypts were dilated and filled with necrotic cellular debris. The distal portion of the villi in the small intestine was lined by epithelial cells that appeared normal. The lamina propria of the villi was oedematous and congested. The lumens of the small and large intestine were distended with cellular debris and inflammatory cells. These lesions occurred with 15-ADON at oral doses of 100 mg/kg and above and at ip doses of 200 mg/kg and greater. Doses of DON causing these lesions were 100 mg/kg by either the oral or ip route.

Mice exposed to DON and 15-ADON that survived longer than 24 hr had a regenerative response of the epithelial cells lining the crypts. The crypts were lined by hyperplastic epithelial cells arranged in a disorganized pattern. The nuclei of the crypt epithelial cells were large and hyperchromatic with prominent nucleoli. The villi were short and occasionally coalesced. This response was seen in mice given 15-ADON at doses of 40 mg/kg orally or 160 mg/kg ip. Minimum DON levels required to cause these changes occurred at 100 mg/kg (oral) and 60 mg/kg (ip).

### *Lymphoid tissue*

All mice exposed to a minimum dose of 15-ADON, 60 mg/kg orally or 160 mg/kg ip, as well as mice given 100 mg/kg DON oral or 60 mg/kg ip had extensive lymphoid necrosis in the spleen and thymus (Figs 4 & 5). In the spleen, there was a dramatic decrease in the number of lymphocytes, primarily around the periarterial lymphatic sheaths. The red and white pulp had extensive amounts of free nuclear debris and pyknotic nuclei. The medulla of the thymus had a decreased density of lymphocytes and epithelial cells. Many of the lymphocytes present had karyolysis of nuclei.

### *Bone marrow*

The bone marrow from mice exposed to DON and 15-ADON exhibited severe necrosis of the haematopoietic and myelopoietic elements. There was a tremendous reduction in the number of intact cells and an abundance of nuclear and cellular debris. The threshold dose of DON and 15-ADON causing a response in the bone marrow was the same as for spleen and thymus.

### *Kidney*

Mice exposed to 15-ADON (100 mg/kg orally or 160 mg/kg ip) had focal tubular necrosis involving primarily the loop of Henle and the distal tubules (Fig. 6). The tubular epithelial cells of affected tubules had pyknotic nuclei, indistinct cell boundaries and granular cytoplasm. The kidneys from mice receiving the highest doses had nuclear debris within the glomeruli without evidence of glomerular degeneration or necrosis. The kidney lesions did not become apparent in DON-exposed mice until the dose reached 1000 mg/kg.

### *Heart*

The hearts from all mice given 100 mg/kg or more of DON, by either route of administration, had focal to locally extensive areas of myocardial necrosis (Fig. 7). Individual myocardial cell necrosis was often present. The necrosis was characterized by lysis of the sarcoplasma and sarcolemma with no inflammatory

Table 1. Minimum doses required to cause lesions in tissues of female B6C3F$_1$ mice after a single exposure to deoxynivalenol or 15-acetyldeoxynivalenol

| | Dose (mg/kg) | | | |
|---|---|---|---|---|
| | Deoxynivalenol | | 15-Acetyldeoxynivalenol | |
| Tissue | Oral | ip | Oral | ip |
| Intestine | 100 | 60 | 40 | 160 |
| Spleen | 100 | 60 | 60 | 160 |
| Thymus | 100 | 60 | 60 | 160 |
| Bone marrow | 100 | 60 | 60 | 160 |
| Kidney | 1000 | 1000 | 100 | 160 |
| Heart | 100 | 100 | ND | ND |

ND = Not detectable at 200 mg/kg


Fig. 2. (A) Section of small intestine from control mouse (H&E × 540); (B) Section of small intestine from a mouse given an ip dose of 200 mg 15-ADON/kg body weight. There is severe necrosis of the crypt epithelium (C) whereas the epithelium of the villi (V) is still intact (H&E ×370).

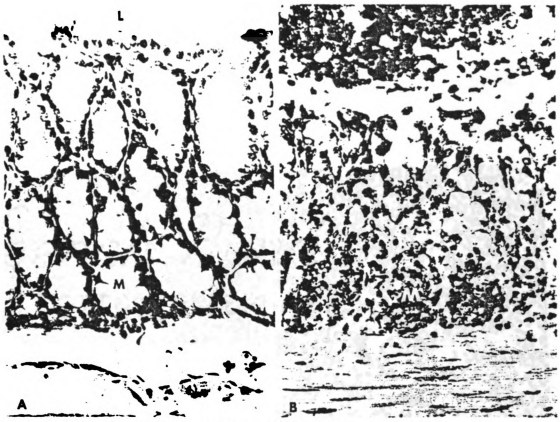


Fig. 3. (A) Section of colon from a control mouse (H&E × 370); (B) section of colon from a mouse given an oral dose of 1000 mg DON/kg body weight. The crypts of the colonic mucosa (M) are necrotic and the lumen of the colon is filled with necrotic debris (L) (H&E × 370).

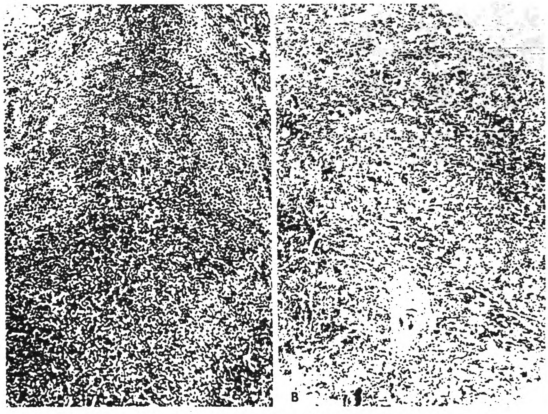


Fig. 4. (A) Section of spleen from a control mouse (H&E × 170); (B) section of spleen from a mouse given an oral dose of 200 mg 15-ADON/kg body weight. Notice the decreased density of lymphocytes particularly in the periarterial lymphatic sheaths (S) (H&E × 170).

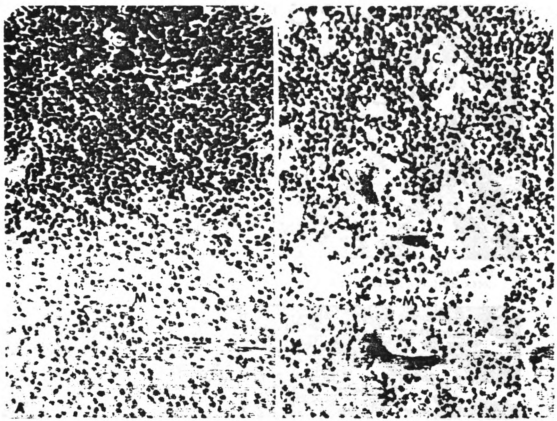

Fig. 5. (A) Section of thymus at the cortical-medullary junction from a control mouse (H&E ×370); (B) section of thymus from a mouse given an oral dose of 200 mg 15-ADON/kg body weight. Notice the karyolysis of cells within the medulla (M) and the decreased density of lymphocytes within the cortex (C) (H&E ×370).

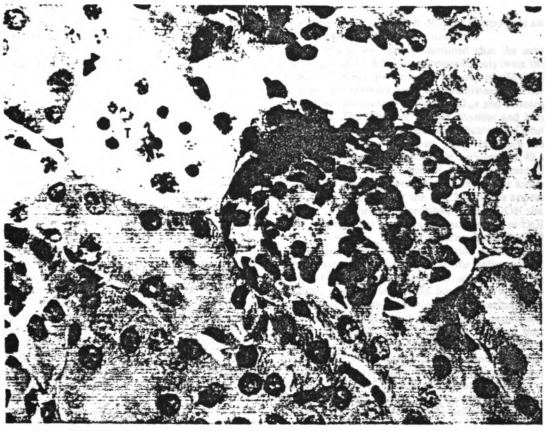


Fig. 6. Kidney from a mouse given an oral dose of 1000 mg DON/kg body weight. The epithelium of a distal tubule (T) is necrotic and characterized by pyknotic nuclei and cytoplasmic lysis (H&E ×850).

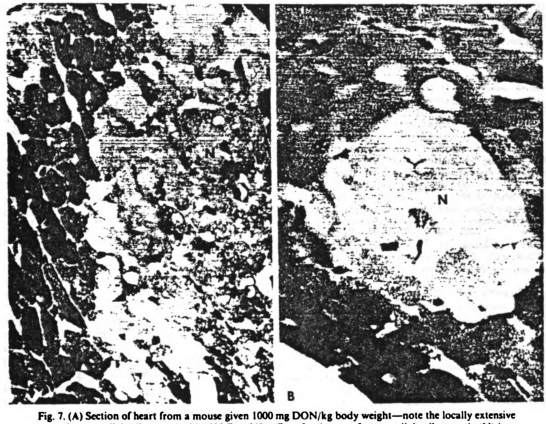


Fig. 7. (A) Section of heart from a mouse given 1000 mg DON/kg body weight—note the locally extensive area of myocardial cell necrosis (N) (H&E ×560); (B) a focal area of myocardial cell necrosis (N) in a section of heart from a mouse given 1000 mg DON/kg body weight (H&E ×1020).

cell infiltrate. 15-ADON exposure caused no lesions in the heart when tested up to 200 mg/kg.

All other organs were found to be histologically normal. Table 1 shows the minimum dose of either DON or 15-ADON required to cause the effects described above.

## DISCUSSION

The histopathological effects of DON and 15-ADON after oral and ip exposure were consistent with those described for trichothecenes in rodents (Carlton & Szczech, 1978). The $LD_{50}$ values were similarly consistent with the reported low order of toxicity for DON and its derivatives relative to other trichothecenes (Ueno, 1983). The method used for estimating acute toxicity has been shown to be highly reliable in assessing the toxicity of 42 structurally diverse model compounds ranging from highly toxic to non-toxic (Lorke, 1983). A major advantage of the procedure is that $LD_{50}$ estimates can be made using a minimum of experimental animals, making this method especially convenient for comparing structure–activity relationships and the effects of route administration for various compounds.

$LD_{50}$ estimates and minimum doses required for pathological effects suggest that DON was slightly more toxic when administered ip (49 mg/kg) as compared to orally (78 mg/kg) (Table 1). These data contrast somewhat with those reported for male mice by Yoshizawa & Morooka (1977), who found $LD_{50}$ values of 70 and 46 mg/kg for ip and oral administration, respectively. It is likely that strain, sex and age differences exist in DON absorption and organ distribution among test animals. Recently, King *et al.* (1984) reported that rumen micro-organisms were capable of reducing the epoxide of DON to form 3α,7α,15-trihydroxy-trichothec-9,12-dien-8-one (Fig. 1). Since the epoxide is required for trichothecene, cytotoxicity (Mirocha *et al.* 1977), such a transformation would ultimately detoxify DON. Functionally equivalent micro-organisms exist among the gut flora of the mouse and these may play a role in DON detoxification (Yoshizawa *et al.* 1986).

In contrast to DON, we found 15-ADON to be approximately three times more toxic when presented orally ($LD_{50}$, 34 mg/kg) as compared to ip dosing ($LD_{50}$, 113 mg/kg). Similar observations were made upon comparing minimal doses required for histopathological lesions (Table 1), suggesting that 15-ADON absorption and distribution to target tissues was greater via the oral route than via the ip route. Although we have previously found that chronic toxic effects of 15-ADON (feed refusal and reduced body-weight gain) were similar to those of DON (Pestka *et al.* 1986), 15-ADON had twice the acute oral toxicity (34 mg/kg) of DON (78 mg/kg). King *et al.* (1984) have noted that another DON derivative, 3-acetyldeoxynivalenol (3-ADON), is recalcitrant to de-epoxidation by rumen micro-organisms unless first deacetylated to DON. 3-ADON is slightly more toxic orally (34 mg/kg) than by ip administration (49 mg/kg) (Yoshizawa & Morooka, 1977). It is tempting to further speculate that microflora can modify the half-life of 15-ADON, 3-ADON and DON in the intestine and thus alter absorption and distribution of these compounds and ultimately their comparative toxicities.

In summary, we have determined that the acute oral toxicity of 15-ADON is approximately twice that of DON whereas the order of toxicity of these two compounds is reversed upon ip administration. There was a close relationship between $LD_{50}$s and histological lesions. The intestine, spleen, thymus and bone marrow were the most sensitive indicators of acute exposure. Recovery from an acute exposure was apparently complete if the mouse survived for 48 hr after exposure. The profile of organs affected by DON and 15-ADON were similar except for kidney and heart. The kidney of the $B6C3F_1$ mouse appears to be 5 to 10 times more sensitive to 15-ADON than DON, whereas, the heart was more sensitive to DON than to 15-ADON. The heart lesions caused by DON are interesting in that they do not appear to be stress-related and they occur at the same minimal dose (100 mg/kg oral exposure) as lesions to the intestine, spleen, thymus and bone marrow. Understanding the mechanisms involved in acute toxicity of DON and 15-ADON will require both quantitative studies on uptake and distribution of these compounds and careful elucidation of the role of gastrointestinal micro-organisms in these processes. These data nevertheless suggest that in those cases where DON is suspected in animal toxicoses (Coté *et al.* 1984), subsequent sample analyses for DON should include a concurrent 15-ADON determination.

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# EFFECTS OF 8-WEEK EXPOSURE OF THE B6C3F1 MOUSE TO DIETARY DEOXYNIVALENOL (VOMITOXIN) AND ZEARALENONE

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**Abstract**—Weanling female B6C3F1 mice were fed semi-purified diets containing 0, 0.5, 2.0, 5.0, 10.0 or 25.0 ppm (mg/kg) deoxynivalenol (DON) over 8 wk and were assessed for effects on feed intake, body-weight gain, terminal organ weights, histopathology, haematology and serum immunoglobulin levels. To determine whether DON effects were potentiated by the oestrogen zearalenone (ZEA), a mycotoxin frequently found to occur with DON in cereals, two additional groups of mice were fed diets containing either 10 ppm ZEA or 10 ppm ZEA plus 5 ppm DON. The rate of body-weight gain was significantly reduced ($P < 0.01$) for all mice consuming feed containing 2.0 ppm or more of DON, whereas only the mice ingesting the diet containing 25 ppm DON showed a significantly decreased ($P < 0.01$) rate of feed consumption. Gross and histopathological evaluation of thymus, spleen, liver, kidney, uterus, small intestine, colon, heart, brain, lungs and bone marrow from control and all mycotoxin-exposed mice revealed that these tissues were normal in appearance and in histological architecture. DON-amended diets did however, cause dose-dependent decreases in the terminal organ weights recorded (thymus, spleen, liver, kidney and brain). In the DON-treated groups, statistically significant dose-dependent decreases in the counts of total circulating white blood cells were associated with an increase in polymorphonuclear neutrophils and a decrease in lymphocytes and monocytes. Dietary DON caused a dose-dependent decrease in serum IgM but, in contrast, a dose-dependent increase in serum IgA. In none of the above instances was 10 ppm ZEA shown to act synergistically or antagonistically with 5 ppm DON. Since dietary DON at levels as low as 2.0 ppm exerted significant effects on the growing B6C3F1 female mouse, future approaches should include studies of the mechanisms by which this mycotoxin affects nutrient utilization and modifies the normal immune response.

## INTRODUCTION

Deoxynivalenol (DON or vomitoxin) is a trichothecene mycotoxin. It is produced by *Fusarium graminearum* (sexual stage, *Gibberella zeae*) and has been associated with feed refusal and emesis in swine (Forsyth, Yoshizawa, Morooka & Tuite, 1977; Vesonder, Ciegler, Jensen *et al.* 1976). DON occurs naturally in corn, wheat and barley produced or stored under cool and wet conditions. Numerous incidents of *F. graminearum* infection with concurrent DON contamination of cereal grains have been reported during the past two decades (Coté, Reynolds, Vesonder *et al.* 1984). In 1981, 80% of the feed samples submitted for diagnostic testing in Illinois because of possible association with animal health problems (primarily in swine) were shown to be contaminated by DON (Coté *et al.* 1984). In the 174 positive samples, DON levels ranged from 0.1 to 42 ppm, with a mean of 3.1 ppm. Feed refusal and reduced weight gain occurs in swine ingesting feed containing 1–2 ppm DON (Pollman, Koch, Seitz *et al.* 1985; Trenholm, Hamilton, Friend *et al.* 1984), while the minimum oral emesis-producing dose in swine is 100 μg/kg body weight (Forsyth *et al.* 1977). At levels of 40 ppm in the diet or 7.2 ppm in water, DON has induced feed refusal in rats and water refusal in mice, respectively (Burmeister, Vesonder & Kwolek, 1980; Vesonder, Ciegler, Burmeister & Jensen, 1979).

In addition to feed refusal and emesis, trichothecenes elicit numerous toxic manifestations, including skin irritation, haemorrhaging, haematological changes, radiomimetic effects and severe immunosuppression (Ueno, 1983). The high degree of resistance of DON to the conditions occurring during milling and processing (Greenhalgh, Gilbert, King *et al.* 1984; Hart & Braselton, 1983) suggests that this mycotoxin is likely to be encountered in the food chain. However, definitive data on the subtle toxic effects of chronic dietary exposure to DON in animal

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*Abbreviations:* BSA = bovine serum albumin; DON = deoxynivalenol (vomitoxin); MCH = mean corpuscular haemoglobin; MCHC = mean corpuscular haemoglobin concentration; MCV = mean corpuscular volume; PBS = phosphate-buffered saline; RBC = red blood cells; WBC = white blood cells; ZEA = zearalenone.

models are not available for meaningful risk assessment analysis. This paper reports the effects of naturally-occurring dietary levels of DON on feed refusal, weight gain, organ weights, histopathology, haematology and serum immunoglobulin levels in the growing B6C3F1 female mouse. Since the oestrogenic mycotoxin zearalenone (ZEA) is frequently co-produced by *F. graminearum* (Ichinoe, Kurata, Sugiura & Ueno, 1983), the potential for this mycotoxin to modify the effects of DON has also been evaluated.

## EXPERIMENTAL

*Mycotoxins.* DON was produced in *F. graminearum* R6576 cultures and purified by silica-gel chromatography (Witt, Hart & Pestka, 1985). ZEA was donated by Dr M. Bachman (International Minerals and Chemical Corp., Terre Haute, IN).

*Animals.* B6C3F1 weanling female mice were purchased from Harlan/Sprague–Dawley, Inc. (Indianapolis, IN). The mice were housed singly in protected-environment cages (Nalgene, Rochester, NY). Each cage unit included a transparent polycarbonate body with filter cover, stainless-steel wire lid and raised floor above a layer of heat-treated hardwood chips. Distilled water was provided *ad lib.* and was changed every 3–4 days. The mice were adapted to their housing and feed, a 12-hr light/dark cycle and the negative-pressure ventilated area for 7 days before trials began.

*Diet.* The mice were fed powdered AIN-76A semi-purified diet (ICN Nutritional Biochemicals, Cleveland, OH). For incorporation into the diet, DON and ZEA were first dissolved in ethanol and added to a small portion of the powdered diet (1000 ppm concentrate). After mechanical mixing and drying, the concentrate was mixed with more clean feed to produce the following concentrations: 0.5, 2.0, 5.0, 10.0 and 25.0 ppm DON, 10.0 ppm ZEA, and 5.0 ppm DON plus 10.0 ppm ZEA. Sufficient feed was prepared at one time to last for the duration of the experiment. During the experiment, all diets were kept in airtight containers in the dark at 4°C.

*Experimental design.* Eight mice were used in each treatment group and 26 mice served as controls. Mice were given a tared diet in individual feeders, which were designed to minimize scattering (Unifab, Kalamazoo, MI). During the first 24 days, feed consumption and body-weight changes were monitored at 3-day intervals. From day 24 to day 56, measurements were made at 4-day intervals. Food in the feeders was replaced with fresh diet weekly.

*Histopathology.* At day 56, feed was withdrawn and 12 hr later the mice were killed by ether exposure and necropsied. The body, brain, liver, kidney, thymus and spleen were weighed and tissues were fixed in 10% buffered formalin, embedded in paraffin and stained with haematoxylin and eosin. Gross and histopathological evaluations were made on the thymus, spleen, liver, kidney, uterus, small intestine, colon, heart, brain, lungs and bone marrow.

*Haematology.* At wk 6 of the feeding trial, blood was taken from the orbital sinus of all mice. Red blood cell (RBC) count, white blood cell (WBC) count, haemoglobin (Hb) content and packed cell volume (PCV) were determined on heparinized blood. A differential WBC analysis was performed on Wright-stained blood smears. Cell counts were determined with a Coulter particle counter (ZBI), and Hb with a Coulter Hemaglobinometer (Coulter Electronics, Inc., Hialeah, FL). PCVs were determined by micro-haematocrit centrifugation.

*Serum immunoglobulin (Ig) analysis.* At wk 6 of the feeding trial and before the animals were killed, serum was obtained from the orbital sinus for estimation of serum IgG, IgM and IgA by enzyme-linked-immunosorbent assay (ELISA). For this assay, Immulon II Removawell Microtiter Strips (Dynatech Laboratories, Alexandria, VA) were coated by overnight incubation with 100 $\mu$l goat antimouse IgG, IgM or IgA (Cooper Biomedical, Malvern, PA) diluted in 0.1 M-bicarbonate buffer (pH 9.6). Dilutions for anti-IgM ($\mu$-chain specific), anti-IgG (Fc-fragment specific) and anti-IgA ($\alpha$-chain specific) were 1:1000, 1:1000 and 1:250, respectively. Coated plates were washed three times with 0.15 M-phosphate-buffered saline (pH 7.2; PBS) containing 0.2% Tween 20) (PBS-Tween). To reduce non-specific protein binding, 0.3 ml 1% (w/v) bovine serum albumin (BSA) in PBS was added to each well. The plates were then incubated at 37°C for 30 min and washed three times with the PBS-Tween. For Ig determination, immunoglobulin reference serum (Miles Laboratories, Naperville, IL) or serum samples were diluted in 1% BSA-PBS, and 100 $\mu$l was added to appropriate wells. Plates were sealed and incubated at 37°C for 1 hr and were then washed five more times, after which 100 $\mu$l goat anti-mouse IgG peroxidase (heavy- and light-chain specific, Cooper Biomedical), diluted 1:5000 in 1% BSA-PBS, was added to each well. The specificity of this conjugate was such that it cross-reacted with murine IgG, IgA and IgM. Plates were then incubated at 37°C for an additional 30 min. Bound peroxidase was determined as described by Pestka, Gaur & Chu (1980). Absorbance was measured at 405 nm on an ELISA plate reader (Bio-tek Instruments, Inc., Burlington, VT) and immunoglobulins were quantitated by extrapolation from reference standard curves.

*Statistical analysis.* Data were analysed with a split plot design over time by analysis of variance with the Genstat V system at Michigan State University (East Lansing, MI). For some analyses, Dunnett's test and the Bonferroni $t$ statistic were used (Gill, 1978).

## RESULTS

### *Weight gain and feed intake*

Mean body-weight gains in each treatment group during the 8-wk feeding trial are illustrated in Fig. 1a. The rate of body-weight gain during this period was significantly reduced ($P < 0.01$) for all mice eating feed containing 2 ppm or more of DON. Body-weight gain in mice consuming the diet containing 0.5 ppm DON did not differ significantly from that of control animals. The numbers of days required to detect a significant effect of the various DON levels on body weight and the total quantity of mycotoxin consumed per mouse by that time are summarized in Table 1.

Cumulative feed intakes by the experimental groups during the 8-wk feeding period are shown in

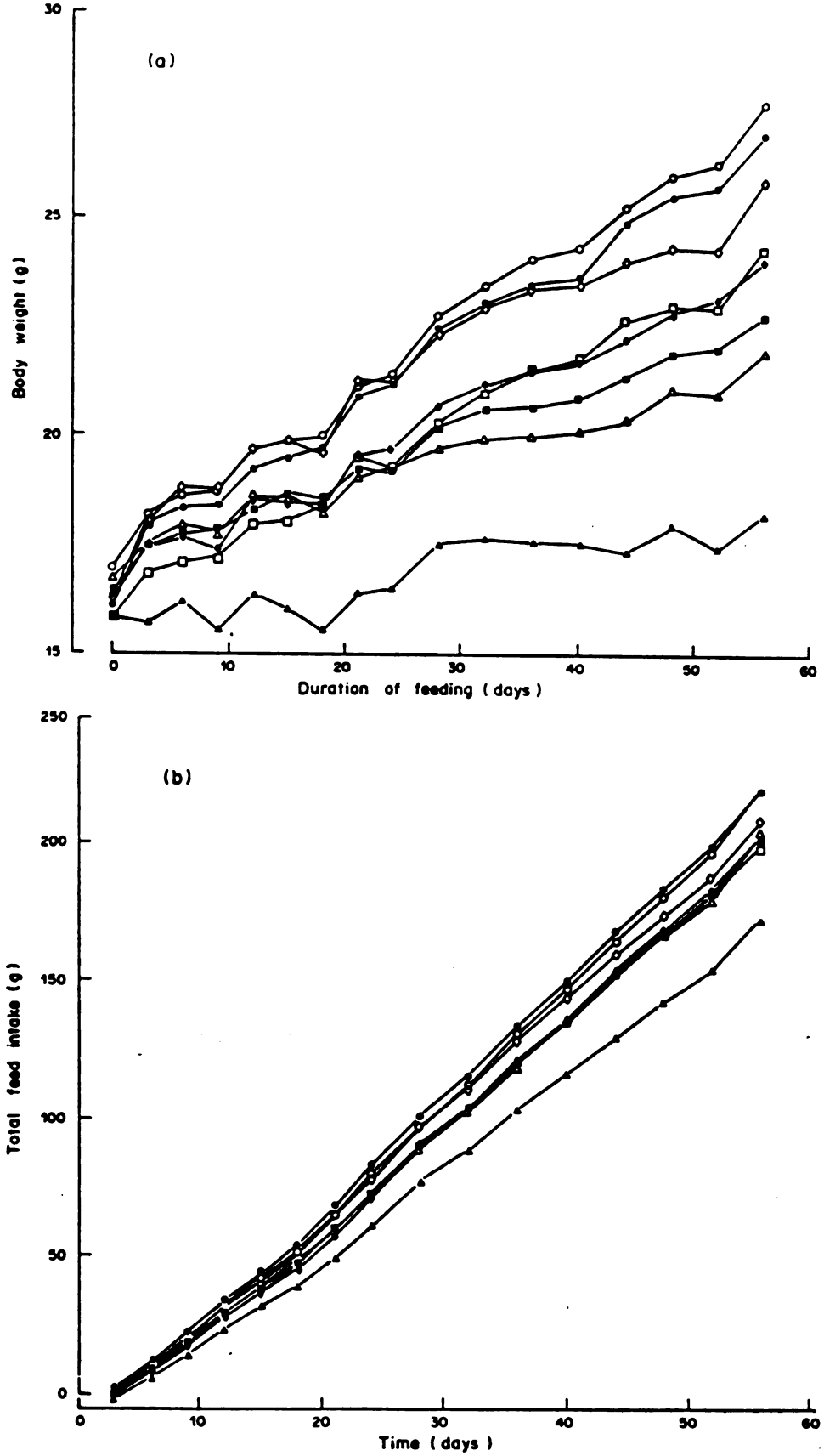


Fig. 1. Cumulative weight gains (a) and cumulative feed intake (b) in B6C3F1 mice fed 0 (○), 0.5 (●), 2 (□), 5 (■), 10 (△) or 25 (▲) ppm deoxynivalenol (DON), 10 ppm zearalenone (ZEA: ◇) or 5 ppm DON plus 10 ppm ZEA (◆). Points are means for groups of 26 controls and eight treated mice. Five lower curves in (a) and bottom curve in (b) differ significantly from the control: $P < 0.01$ by Dunnett's $t$ test.

Table 1. Time and dose required for deoxynivalenol (DON) and/or zearalenone (ZEA) to affect feed intake and body-weight gain of B6C3F1 mice

| Dietary treatment (ppm) | Time (days) to first effect on: | | DON or ZEA consumption (mg mouse) | |
|---|---|---|---|---|
| | Body weight | Feed intake | At appearance of body-weight effect | Total over 8 wk |
| DON 0.5 | ND | ND | — | 0.113 |
| 2 | 24 ($P < 0.05$) | SFR | 0.163 | 0.411 |
| 5 | 21 ($P < 0.01$) | ND | 0.342 | 1.043 |
| 10 | 10 ($P < 0.05$) | ND | 0.377 | 2.094 |
| 25 | 6 ($P < 0.01$) | 6 ($P < 0.01$) | 0.375 | 4.481 |
| ZEA 10 | ND | ND | — | 2.149 |
| DON 5/ZEA 10 | 21 ($P < 0.05$) | 6 ($P < 0.05$)* | 0.330/0.660 | 1.040 2.080 |

ND = Not detected SFR = Sporadic occurrences of feed refusal
*The significant effect on feed intake was not detectable after day 24.

Fig. 1b. Although some feed refusal appeared to occur with diets containing 2 ppm DON (Table 1), only those mice receiving 25 ppm showed a significant reduction ($P < 0.01$) in feed consumption. Between day 6 and day 21, mice in the group fed 5 ppm DON plus 10 ppm ZEA showed significant feed refusal ($P < 0.01$). This feed refusal ended about the time body-weight changes were detected (Table 1). Treatment with 5 ppm DON supplemented with 10 ppm ZEA appeared to increase the rate of weight gain slightly compared with that in the group treated only with 5 ppm DON, but this observation was not statistically significant.

*Organ weights and histopathology*

The effects of dietary DON and ZEA on the organ-weight profile are summarized in Table 2. DON caused dose-dependent decreases in the thymus, spleen, liver, kidney, brain and body weights. The organ-weight profile of mice consuming 5 ppm DON supplemented with 10 ppm ZEA did not differ significantly from that of mice consuming only 5 ppm DON. Threshold DON effect levels for spleen, liver, kidney, brain and body weights were 25 ppm ($P < 0.01$), 10 ppm ($P < 0.01$), 5 ppm ($P < 0.05$), 25 ppm ($P < 0.01$) and 2 ppm ($P < 0.01$), respectively. When organ weights were expressed as a percentage of brain weight, dietary DON caused a significant decrease only in liver and kidney values, the threshold effect levels being 10 ppm ($P < 0.01$) for the liver and 5 ppm ($P < 0.05$) for the kidney. A trend towards decreases in thymus and spleen weights was also evident. When organ weights were expressed as a percentage of terminal body weight, dietary DON caused a significant dose-dependent increase in liver and kidney weights, with threshold effect levels of 5 ppm ($P < 0.05$) and 25 ppm ($P < 0.01$), respectively.

Gross examination and histopathological studies showed that the thymus, spleen, liver, kidney, uterus, small intestine, colon, heart, brain, lungs and bone marrow from the control and all mycotoxin-exposed mice were normal in appearance and histological architecture.

*Haematology*

The effects of dietary DON and ZEA on haematological profiles are summarized in Table 3. DON exposure caused a significant dose-dependent decrease ($P < 0.05$) in circulating WBC with a threshold effect level of 10 ppm ($P < 0.01$). The WBC levels decreased despite a significant dose-dependent increase in polymorphonuclear neutrophil number ($P < 0.01$). Significant dose-dependent decreases in lymphocytes ($P < 0.01$) and monocytes ($P < 0.05$) apparently accounted for the overall decrease in WBC numbers. Eosinophil number also appeared to decrease with increasing doses of DON, but this decrease was not statistically significant. Dietary ZEA at the 10 ppm level did not affect WBC or the differential count. The WBC and differential counts of mice consuming 5 ppm DON supplemented with 10 ppm ZEA did not differ from those of mice consuming diets containing only 5 ppm DON.

Dietary DON caused a significant dose-dependent increase ($P < 0.01$) in RBC number and dietary ZEA also caused a significant increase ($P < 0.01$) in this count. There was also a trend toward a smaller red cell size (mean corpuscular volume; MCV) and less haemoglobin per RBC (mean corpuscular haemoglobin; MCH) in both DON- and ZEA-treated mice. However, since the RBC number was also increased in these mice, the net result was a normal value for total haemoglobin and for packed cell volume. The concentration of haemoglobin in the RBC (MCHC) was also normal because of a similar reduction in both MCV and MCH.

*Serum immunoglobulin levels*

Mean serum IgM, IgG and IgA levels found in mice after ingestion of DON- and ZEA-treated diets are summarized in Table 4. DON caused a dose-dependent decrease in serum IgM ($P < 0.01$) but, in contrast, a significant dose-dependent increase in serum IgA ($P < 0.01$). The threshold DON level for this increase was 2 ppm ($P < 0.05$). IgA concentration was maximal at the 10 ppm DON level (4625 $\mu$g/ml), decreasing to 2818 $\mu$g/ml at the 25 ppm level. ZEA at 10 ppm had no effect on IgG, IgM or IgA. Serum immunoglobulin levels in mice consuming the 5 ppm DON diet supplemented with 10 ppm ZEA did not differ from those in mice consuming the 5 ppm DON only diet.

## DISCUSSION

The B6C3F1 mouse has been previously used as a model for both the carcinogenicity (Haseman, Crawford, Huff *et al.* 1984) and immunotoxicity (Dean, Luster, Boorman & Laver, 1982) of xenobiotic chemicals. The major toxic manifestations observed in the growing B6C3F1 female mouse after chronic dietary

Table 2. Organ weights of B6C3F1 mice fed diets containing deoxynivalenol (DON) and/or zearalenone (ZEA) for 8 wk

| Organ | Weight units | Dietary level (ppm)... | Mean value ± SEM for mice treated with: DON 0 | DON 0.5 | DON 2 | DON 5 | DON 10 | DON 25 | ZEA 10 | DON/ZEA 5/10 |
|---|---|---|---|---|---|---|---|---|---|---|
| Thymus | mg† | | 56 ± 4 | 64 ± 6 | 68 ± 4 | 50 ± 5 | 45 ± 5 | 38 ± 7 | 56 ± 7 | 50 ± 5 |
| | % Br | | 13.8 | 13.6 | 17.5 | 10.5 | 9.0 | 10.1 | 12.9 | 9.6 |
| | % Bo | | 0.21 | 0.24 | 0.28 | 0.22 | 0.21 | 0.22 | 0.22 | 0.21 |
| Spleen | mg†† | | 58 ± 3 | 69 ± 3 | 56 ± 5 | 51 ± 9 | 49 ± 6 | 34 ± 6** | 61 ± 6 | 59 ± 6 |
| | % Br | | 13.2 | 15.0 | 11.1 | 12.4 | 10.4 | 8.8 | 15.0 | 13.4 |
| | % Bo | | 0.21 | 0.26 | 0.25 | 0.26 | 0.24 | 0.19 | 0.25 | 0.25 |
| Liver | g†† | | 1.06 ± 0.02 | 1.08 ± 0.05 | 0.95 ± 0.07 | 0.97 ± 0.05 | 0.88 ± 0.03** | 0.78 ± 0.04** | 1.05 ± 0.03 | 0.92 ± 0.02* |
| | % Br†† | | 241 | 236 | 218 | 220 | 202** | 191** | 251 | 210* |
| | % Bo†† | | 3.8 | 4.0 | 3.9 | 4.2* | 4.1 | 4.5** | 4.0 | 3.8 |
| Kidney | mg†† | | 322 ± 5 | 300 ± 13 | 295 ± 6 | 291 ± 13* | 261 ± 10** | 259 ± 6** | 315 ± 12 | 280 ± 9** |
| | % Br†† | | 73.5 | 66.2 | 68.2 | 65.9* | 59.9** | 63.8** | 74.9 | 63.6** |
| | % Bo†† | | 1.2 | 1.1 | 1.2 | 1.2 | 1.2 | 1.5** | 1.2 | 1.2 |
| Brain | mg† | | 440 ± 5 | 452 ± 9 | 432 ± 8 | 441 ± 8 | 436 ± 12 | 406 ± 6** | 422 ± 11 | 440 ± 10 |
| Terminal body weight | g†† | | 28.0 ± 0.4 | 27.7 ± 1.2 | 24.0 ± 1.0** | 23.1 ± 0.7** | 21.8 ± 0.8** | 17.4 ± 0.7** | 26.3 ± 0.9 | 24.6 ± 0.7** |

%Br = g/100 g brain weight %Bo = g/100 g body weight

Values are means for groups of 24 control and eight treated mice and those marked with asterisks differ significantly (by Dunnett's $t$ test) from the control value: *$P < 0.05$; **$P < 0.01$. Arrows indicate weights showing a significant DON dose response (by analysis of variance): †$P < 0.05$; ††$P < 0.01$.

Table 3. Haematological profile of B6C3F1 mice fed diets containing deoxynivalenol (DON) and/or zearalenone (ZEA) for 6 wk

| Measurement/calculation | Dietary level (ppm)... | Mean value ± SEM for mice treated with: DON 0 | DON 0.5 | DON 2 | DON 5 | DON 10 | DON 25 | ZEA 10 | DON/ZEA 5/10 |
|---|---|---|---|---|---|---|---|---|---|
| WBC (no./μl)†† | | 7966 ± 393 | 9222 ± 496 | 7425 ± 656 | 8431 ± 380 | 6155 ± 530** | 6140 ± 575** | 8594 ± 1373 | 7112 ± 472 |
| Lymphocytes (no./μl)†† | | 5937 ± 308 | 6732 ± 618 | 5261 ± 450 | 5659 ± 395 | 4249 ± 329 | 3299 ± 465** | 6737 ± 1387 | 5653 ± 389 |
| PMN (no./μl)†† | | 1803 ± 121 | 2276 ± 268 | 2017 ± 295 | 2564 ± 486 | 1872 ± 262 | 2754 ± 294* | 1536 ± 373 | 1340 ± 153 |
| Monocytes (no./μl)† | | 223 ± 39 | 133 ± 37 | 101 ± 24 | 175 ± 66 | 34 ± 14* | 77 ± 28 | 213 ± 67 | 99 ± 34 |
| Eosinophils (no./μl) | | 101 ± 33 | 70 ± 25 | 26 ± 14 | 31 ± 16 | 0 ± 0 | 0 ± 0 | 108 ± 30 | 9 ± 9 |
| RBC ($10^6$/μl)†† | | 7.06 ± 0.15 | 7.09 ± 0.20 | 7.40 ± 0.31 | 7.69 ± 0.26 | 8.03 ± 0.42 | 7.73 ± 0.46 | 8.06 ± 0.49** | 6.38 ± 0.29 |
| Hb (g/100 ml) | | 16.3 ± 0.3 | 16.2 ± 0.4 | 16.9 ± 0.5 | 16.0 ± 0.4 | 15.7 ± 0.5 | 16.0 ± 0.5 | 16.3 ± 0.8 | 16.3 ± 0.8 |
| PCV (%) | | 51.3 ± 0.6 | 50.4 ± 0.5 | 51.6 ± 0.7 | 49.3 ± 1.5 | 50.0 ± 0.5 | 47.8 ± 0.7 | 51.2 ± 0.3 | 49.1 ± 0.9 |
| MCV (fl)†† | | 73.2 ± 1.8 | 70.0 ± 1.7 | 70.6 ± 3.3 | 64.5 ± 2.5 | 63.5 ± 3.5 | 63.6 ± 4.5 | 65.4 ± 4.3 | 81.6 ± 2.7 |
| MCH (pg)† | | 23.9 ± 0.6 | 22.9 ± 0.8 | 23.1 ± 1.1 | 20.9 ± 0.7 | 20.0 ± 1.5 | 21.4 ± 2.1 | 20.5 ± 1.1 | 25.9 ± 1.7 |
| MCHC (g/100 ml) | | 32.3 ± 0.9 | 31.9 ± 0.8 | 32.8 ± 1.1 | 32.7 ± 1.4 | 31.8 ± 1.0 | 33.5 ± 1.1 | 31.9 ± 1.7 | 33.7 ± 1.6 |

WBC = White blood cells PMN = Polymorphonuclear neutrophils RBC = Red blood cells Hb = Haemoglobin PCV = Packed cell volume MCV = Mean corpuscular volume MCH = Mean corpuscular haemoglobin MCHC = Mean corpuscular haemoglobin concentration

Values are means for groups of 25 control and eight treated mice and those marked with asterisks differ significantly (by Dunnett's $t$ test) from the control value: *$P < 0.05$; **$P < 0.01$. Arrows indicate parameters showing a significant DON dose response (by analysis of variance): †$P < 0.05$; ††$P < 0.01$.

Table 4. Serum immunoglobulin levels in B6C3F1 mice fed diets containing deoxynivalenol (DON) and or zearalenone (ZEA) for 6 wk

| Dietary treatment (ppm) | Serum levels of immunoglobulins (μg ml) | | |
|---|---|---|---|
| | IgG | IgM†† | IgA†† |
| 0 (control) | 6157 ± 818 | 137 ± 12 | 1514 ± 136 |
| DON 0.5 | 5173 ± 1692 | 138 ± 23 | 1949 ± 449 |
| 2 | 5383 ± 1272 | 178 ± 30 | 3237 ± 509* |
| 5 | 6584 ± 452 | 109 ± 14 | 3657 ± 637** |
| 10 | 7117 ± 2043 | 136 ± 23 | 4625 ± 835** |
| 25 | 3556 ± 1084 | 71 ± 15* | 2818 ± 353 |
| ZEA 10 | 6552 ± 760 | 190 ± 17 | 2364 ± 502 |
| DON 5/ZEA 10 | 4424 ± 631 | 116 ± 15 | 2349 ± 579 |

Values are means ± SEM for groups of 24 control and eight treated mice. Those marked with asterisks differ significantly (by Dunnett's *t* test) from the control value: *$P < 0.05$; **$P < 0.01$. Arrows indicate parameters showing a significant DON dose response: ††$< 0.01$ by analysis of variance.

exposure to DON were reduced weight gain, reduced organ weights, lymphopenia and disruption of the normal serum immunoglobulin profile. The minimum DON threshold (2.0 ppm) required to reduce weight gain in these mice was comparable to that found in swine (Pollman *et al.* 1985; Schuh, Leibetseder & Glowischnig, 1982; Trenholm *et al.* 1984). However, we did not observe a consistent pattern of feed refusal concurrent with body weight effects until the DON content in the diet was increased to 25 ppm. In contrast, the threshold level for feed refusal in swine is 1 ppm DON. The amount of DON consumed before an effect on body weight was detected ranged from 0.163 to 0.377 mg (Table 1), a very small range considering the differences in time required to consume this quantity of toxin (6–24 days). This suggests that the body-weight effects of DON may be cumulative.

Comparison of the total feed consumed with the total weight gain over the 8-wk period indicates that feed/gain ratios in our model for the 0, 0.5, 2, 5, 10 and 25 ppm DON diets were 18, 19, 25, 28, 35 and 83, respectively. These results suggest that the decreased weight gain may be caused by reduced feed conversion efficiency as well as feed refusal. Although the latter interpretation may be limited, in part, by feed scattering encountered in the mouse model, we did not observe this to be a major problem in our protocol. Furthermore, reduced feed efficiency has been similarly observed in Sprague–Dawley rats (Morissey & Vesonder, 1985) and swine (Pollman *et al.* 1985).

In the mouse, over the 8-wk feeding period, DON, did not significantly decrease thymus, spleen, liver or kidney weights when expressed as a percentage of body weight. However, organ weight is directly related to the nutritional status of animals, and organ masses change in proportion to changes in body weight. Thus we observed that DON caused dose-dependent decreases in terminal spleen, liver and kidney weights. Similarly Pollman *et al.* (1985) found decreases in the liver, heart, spleen and kidney weights in growing pigs. A better comparison can be made by relating organ weights to brain weight, since the brain does not vary readily in mass as a result of differences in nutritional status. Thus, on comparing organ/brain weight profiles, it appeared that liver and kidney lost mass to a greater extent than could be attributed to losses of body weight in the different treatments. The greater effects on liver and kidney than on spleen and thymus contrast with the expected radiomimetic effects of trichothecenes on organ systems (Ueno, 1983).

In a recent acute toxicity study, we determined that the B6C3F1 female mouse developed necrotic lesions in the small intestine, colon, kidney, bone marrow, thymus, spleen and heart when dosed orally with high levels of DON (authors' unpublished data). However, after more prolonged (8-wk) exposure to DON, histopathological changes did not accompany the changes in the organ weight profiles. The acute dose necessary to cause the pathological changes was in the range of 1.0–2.0 mg/mouse. In comparison, the total dose consumed per mouse in this 8-wk study ranged from 0.11 mg for the diet containing 0.5 ppm DON to 4.48 mg for the diet containing 25 ppm DON. It appears that the target tissues affected by repeated exposure to DON, and possibly to other trichothecenes, differ from those affected by higher acute exposures.

Low levels of dietary DON also appear to cause subtle haematological and immune effects. As has been found for other trichothecenes (Ueno, 1983), DON exerted a dose-dependent lymphopenic effect. Decreased WBC counts were predominantly associated with a decrease in lymphocyte number. *In vitro*, the lymphocyte is the cell type that is most sensitive to trichothecenes (Forsell, Kately, Yoshizawa & Pestka, 1985; Lafarge-Frayssinet, Decloitre, Mousset *et al.* 1981; Rosenstein & Lafarge-Frayssinet, 1983), an effect that is possibly modulated by a receptor mechanism (Gyongyossy-Issa & Khachatourians, 1984). Concurrent with the WBC decrease, polymorphonuclear neutrophil numbers increased with increasing DON challenge. This rise could indicate an antigenic challenge to which neutrophils are responding. Since oestrogens inhibit erythropoiesis (Krantz & Jacobson, 1970), the observed increase in RBC number after ZEA exposure seems likely to be due to some factor other than ZEA's oestrogenic effect.

Other trichothecenes are immunotoxic and have been shown to reduce responses to antigens and to increase a host's susceptibility to infection. The observation that DON decreased IgM levels suggests either that this mycotoxin may have an immunosuppressive effect or that it could elicit this effect through malnutrition. Although increased IgA levels

may be a direct effect of malnutrition (Neumann, Lawlor, Stiehm *et al.* 1975). IgA changes may also be a manifestation of increased exposure to antigens in the intestine. DON causes extensive histopathological effects in the intestine at higher doses (authors' unpublished data). At the lower levels associated with more prolonged exposure, DON might elicit more subtle effects on the intestine, causing increased permeability to food antigens and microflora. This hypothesis would be consistent with the observed increase in neutrophil number.

In summary, we have demonstrated that dietary DON at levels as low as 2.0 ppm has significant effects on the growing B6C3F1 female mouse. Co-administration of ZEA did not potentiate the effects of DON. Future consideration of the role of DON in human and animal health should include an understanding of the mechanisms by which this mycotoxin affects nutrient utilization and modifies normal immune response.

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# SUPPRESSION OF IMMUNE RESPONSE IN THE B6C3F$_1$ MOUSE AFTER DIETARY EXPOSURE TO THE FUSARIUM MYCOTOXINS DEOXYNIVALENOL (VOMITOXIN) AND ZEARALENONE

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**Abstract**—The effect that dietary exposure to the naturally-occurring *Fusarium graminearum* toxins deoxynivalenol (DON) and zearalenone (ZEA) may have on immune function was assessed in the B6C3F$_1$ mouse. Dietary DON depressed the plaque-forming response to sheep red blood cells, the delayed hypersensitivity response to keyhole limpet haemocyanin and the ability to resist *Listeria monocytogenes*. Listerial resistance was similarly decreased in control mice fed restricted diets comparable to the dietary restriction caused by DON-induced feed refusal, whereas equivalent food restriction did not decrease the plaque or delayed hypersensitivity responses. ZEA ingestion decreased resistance to *L. monocytogenes* but did not affect splenic plaque-forming or delayed hypersensitivity responses. Resistance to Listeria was reduced to a greater extent by co-administration of DON and ZEA than by DON alone, whereas the ability of DON to inhibit the delayed hypersensitivity response was significantly lessened in the presence of ZEA. While effects on resistance to Listeria and delayed hypersensitivity were detectable in mice ingesting the mycotoxins for 2–3 wk, these effects disappeared upon extension of the feeding period to 8 wk. In contrast, some effect on the plaque-forming response was detectable with both the 2- and the 8-wk period of mycotoxin ingestion. Immunosuppression can thus result from ingestion of *F. graminearum*-infected agricultural staples, the suppression being attributable to interactions between direct immunotoxic effects of DON and ZEA and nutritional effects associated with DON-induced food refusal.

## INTRODUCTION

The trichothecenes and zearalenone are mycotoxins produced by members of the genus Fusarium and are of immense concern because of their frequent presence in agricultural staples such as wheat, corn, barley and oats (Ichinoe *et al.* 1983; Mirocha *et al.* 1976). The trichothecenes, a group of sesquiterpenoids characterized by a trichothecane nucleus, include some of the most potent inhibitors of protein synthesis known (McLaughlin *et al.* 1977; Ueno, 1983b). Acute exposure to these compounds results in severe damage to actively dividing cells in tissues such as bone marrow, lymph nodes, spleen, thymus and intestinal mucosa. Trichothecenes have been implicated as causative agents in numerous episodes of fatal human and animal toxicoses (Côté *et al.* 1984; Joffe, 1978; Ueno, 1983a). Although over 50 trichothecenes have been identified, deoxynivalenol (DON or vomitoxin) appears most commonly in cereal grains produced in North America (Mirocha *et al.* 1976; Vesonder *et al.* 1979). In 1981, 80% of midwestern feed samples submitted for diagnostic testing in Illinois were shown to be contaminated by DON (Côté *et al.* 1984). Zearalenone (ZEA) is a $\beta$-resorcyclic lactone that has a low order of toxicity but can exert oestrogenic effects in various mammalian species (Mirocha *et al.* 1977). Numerous field outbreaks of ZEA-associated hyperoestrogenism have been recorded in livestock. Both DON and ZEA are produced by *Fusarium graminearum* (perfect stage *Gibberella zeae*) and frequently occur simultaneously in cereal grains (Ichinoe *et al.* 1983; Marasas *et al.* 1977).

While the acute primary effects of the trichothecenes and ZEA are well characterized, the secondary effects are less clear. Immunosuppression is a frequently observed effect in field cases of low-level mycotoxin exposure in livestock (Pier *et al.* 1980). Experimentally, repeated injection of animals with the trichothecenes, T-2 toxin and diacetoxyscirpenol results in markedly increased susceptibility to candidiasis (Salazar *et al.* 1980) and cryptococcosis (Fromentin *et al.* 1981), decreased humoral response to T-dependent antigens and increased response to T-independent antigens (Rosenstein *et al.* 1981) and increased skin-graft injection times (Rosenstein *et al.* 1979). Similarly, lymphocytes from T-2 toxin-treated

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*Abbreviations*: CFU = colony-forming units; DON = deoxynivalenol; HBSS = Hank's balanced salt solution; KLH = keyhole limpet haemocyanin; PBS = phosphate-buffered saline; PFC = plaque-forming cell: SRBC = sheep red blood cells; ZEA = zearalenone.

animals have decreased B- and T-cell mitogen responses (Friend *et al.* 1983; Lafarge-Frayssinet *et al.* 1979). T-2 toxin induces DNA single-strand breakage in lymphoid tissue *in vitro* and *in vivo* but not in hepatic tissue (Lafarge-Frayssinet *et al.* 1981). Tryphonas *et al.* (1984) demonstrated that repeated gavage with DON results in reduced serum antibody titres and plaque-forming cell (PFC) responses to sheep red blood cells. Although no investigations have been specifically directed to assessing the immunosuppressive potential of ZEA, other oestrogens such as diethylstilboestrol decrease host resistance to pathogens (Pung *et al.* 1984 & 1985), increase tumour susceptibility (Dean *et al.* 1980), alter macrophage function (Boorman *et al.* 1980), reduce natural killer-cell activity (Kalland & Campbell, 1984) and depress the delayed hypersensitivity response (Luster *et al.* 1980).

Massive outbreaks of Fusarium infection in US and Canadian cereals in recent years have resulted in large-scale contamination of the food chain by DON and ZEA. These mycotoxins are resistant to inactivation during milling and processing, and this facilitates their entry into foods and feeds. *In vitro*, DON and ZEA inhibit human lymphocyte blastogenesis (Atkinson & Miller, 1984; Cooray, 1984; Forsell & Pestka, 1985). Although it is well established that injections of high doses of trichothecenes and oestrogens result in immunotoxicity *in vivo*, little comparable information exists on the immune effects of dietary administration of naturally occurring levels of DON and ZEA, the most likely exposure route for these mycotoxins. We have recently determined that dietary DON causes dose-dependent decreases in serum IgM, in circulating lymphocyte and monocyte numbers, in spleen weight and in thymus weight but dose-dependent increases in serum IgA (Forsell *et al.* 1986). Concurrently, we observed DON-associated feed refusal and reduced body-weight gain. The purpose of the study reported here was to evaluate the effects of dietary exposure to DON and ZEA at naturally-occurring levels on immune function over a short (2–3-wk) and longer (8-wk) feeding period, to relate observed immune effects caused by DON or ZEA exposure to possible nutritional effects caused by reduced food intake, and to evaluate whether DON and ZEA in combination affect various immune parameters in an antagonistic or potentiating manner.

## MATERIALS AND METHODS

*Mycotoxins.* DON was produced in *Fusarium graminearum* R6576 cultures and purified by silica-gel chromatography (Witt *et al.* 1985). ZEA was donated by M. Bachman (International Minerals and Chemical Corp., Terre Haute, IN).

*Animals.* B6C3F$_1$ (C57BL/6 X C3HeN) weanling female mice, weighing 15–18 g, were purchased from Harlan/Sprague–Dawley, Inc. (Indianapolis, IN). The mice were randomized and housed individually in protected-environment cages (Nalgene, Rochester, NY). Each cage unit included a transparent polycarbonate body with filter cover, stainless-steel wire lid and a raised floor above a layer of heat-treated hardwood chips. Distilled water was provided *ad lib.* and was changed every 3–4 days. The mice were adapted to their housing, feed, 12-hr light/dark cycle and negative-pressure ventilated area for 7 days prior to experimental treatments. The temperature of the animal room was thermostatically maintained at 72°C.

*Diet.* The mice were fed powdered AIN-76A semi-purified diet (ICN Nutritional Biochemicals, Cleveland, OH) to minimize non-specific immune effects caused by nutrient variability. Individual lots of diet were analysed and verified as being free of naturally-occurring DON and ZEA (Warner *et al.* 1986; Witt *et al.* 1985). DON and ZEA were incorporated into the diet by first dissolving the mycotoxins in ethanol and adding them to a small portion of the powdered diet (1000 ppm concentrate). After being mechanically mixed and dried, the concentrate was mixed further with uncontaminated diet to obtain the desired mycotoxin levels. Sufficient diet was prepared at one time to last for the duration of the experiment. During the experiment, all diets were stored in air-tight containers in the dark at 10°C.

### *Experimental design*

The general experimental approach consisted of feeding treatment groups with control or mycotoxin-contaminated diets before and during the course of an immune-function assay. The immune assays included resistance to Listeria, PFC response to sheep red blood cells, and delayed hypersensitivity response. Two feeding periods (either 2–3 or 8 wk) were used for each immune assessment. Mice were given diet in individual feeders designed to minimize feed scattering (Unifab, Kalamazoo, MI). Because dietary levels of DON exceeding 2 ppm cause feed refusal and reduced body-weight gain in the B6C3F$_1$ mouse (Forsell *et al.* 1986), additional control groups were required to compensate for reduced food intake. Initial Listeria experiments used a pair-fed design, whereby the consumption of diet containing 5 ppm DON by each mouse was monitored daily and a matched control mouse was fed the same amount of uncontaminated feed. To minimize the risk of human exposure to mycotoxins during the handling of the powdered diet and to increase the numbers of experimental animals, later experiments used a restricted feed design. In this design, average consumption of 5 and 25 ppm DON diets was estimated, and each mouse in two control groups, identified as Restricted Control I and Restricted Control II, respectively, was fed the same average amount of uncontaminated feed at 3-day intervals. The mean body weights of mice fed the restricted diets were not significantly different (taking $P < 0.05$ as the level of significance) from the matched DON treatment groups after a 2- or 8-wk feeding period. Neither ZEA at 10 ppm nor DON at 0.5 ppm alters food intake or body-weight gain (Forsell *et al.* 1986) and therefore these concentrations did not require additional matched dietary controls.

### *Resistance to* Listeria monocytogenes

*L. monocytogenes* strain 29303, previously used in immunotoxicity studies in the B6C3F$_1$ mouse (Bradley & Morahan, 1982), was kindly provided by P. S. Morahan (Medical College of Pennsylvania, Philadelphia, PA). Virulence was maintained by con-

tinuous mouse passage. For challenge studies, the bacteria were grown in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, MD) for 18 hr. Cells were washed in 0.1 M-phosphate-buffered saline (PBS, pH 7.3) three times by centrifugation and resuspended in PBS containing 15% (v/v) glycerol. Stocks of 0.5 ml were kept frozen at −80°C. Estimates of viable bacteria were made on Trypticase soy agar (BBL Microbiology Systems) after incubation for 24 hr at 37°C.

For infectivity experiments, treatment groups were challenged iv with *L. monocytogenes* ($1 \times 10^4$ or $5 \times 10^4$ CFU/mouse). Spleen and liver counts were performed on designated days by homogenizing the organs in sterile PBS with a Stomacher lab blender 400 (Tekmar Co., Cincinnati, OH) for 5 min and plating on Trypticase soy agar. Counts were expressed as mean log CFU/organ.

*PFC assay*

Mice were immunized on day 14 or day 56 of the 2- or 8-wk feeding regimens, respectively, by ip injection with 0.2 ml of a 10% suspension of sheep red blood cells (SRBC). The mice were killed 5 days later and a modification of the Jerne plaque assay (Jerne & Nordin, 1963) was performed. Spleen cells were suspended in Hank's balanced salt solution (HBSS; Gibco Laboratories, Chagrin Falls, OH) and the suspensions were diluted to $10^7$ cells/ml in HBSS for the PFC assay. Cells were counted with a haemocytometer; viability, assessed by trypan blue exclusion, was greater than 90% for all treatment groups.

SRBC lawns were prepared as described by Kennedy & Axelrad (1971). Briefly, 0.25 ml of poly-L-lysine (mol wt 55,000; Sigma Chemical Co., St Louis, MO), diluted to 25 μg/ml in PBS, was added to each well of a 24-well tissue-culture plate (Costar, Cambridge, MA). After incubation for 15 min at room temperature, the wells were rinsed twice with PBS and 0.3 ml of a 1% suspension of SRBC was added to each well. Plates were incubated at room temperature for 15 min, agitated to resuspend the SRBC and then incubated for an additional 15 min. After a second agitation, the supernatant fraction was removed by gentle aspiration. Wells were filled with PBS and stored overnight at 4°C before use.

For each mouse, the following were mixed together: 20 μl of a $10^7$/ml spleen cell suspension, 0.1 ml guinea-pig complement (Cappel, Malvern, PA), and 2 ml RPMI 1640 with 25 mM-HEPES (Microbiological Associates, Walkersville, MD). Plates (24-well) that had been precoated with SRBC were inverted and shaken to remove PBS and 0.3 ml of the spleen cell mixture was immediately added to each of three wells. The plates were incubated at 37°C for 45 min, after which the supernatant fluid was removed and the wells were refilled with PBS. PBS was removed from each well immediately before the plaques were counted on an inverted microscope. Data were expressed as the arithmetic means of PFC/$10^6$ spleen cells and PFC/spleen.

*Delayed hypersensitivity responses*

The delayed hypersensitivity response to keyhole limpet haemocyanin (KLH; Luster *et al.* 1982) was measured in individual mice by the degree of footpad swelling. Mice received in the back an initial sc injection of KLH (100 μg) emulsified in incomplete Freund's adjuvant on day 3 or day 38 of the 3- and 8-wk feeding regimens, respectively. An identical injection followed 9 days later, and 10 days after the second injection, the mice received a third sc injection of 30 μg KLH in 0.1 ml saline in the left hind foot pad. Swelling was measured at 24 hr with calipers and the percentage swelling was determined relative to the right footpad control. Statistically significant differences ($P < 0.01$) between the mean diameters of experimental and control hind feet were observed for all treatment groups 24 hr after the final footpad injection in both the 3- and 8-wk dietary studies.

*Safety*

Face masks and vinyl gloves were used by personnel handling mycotoxin preparations. Concentrated mycotoxin solutions were handled in a fume hood. Mycotoxin-contaminated labware was detoxified by soaking overnight in 10% sodium hypochlorite (Thompson & Wannemacher, 1984).

*Statistical analyses*

Data were analysed by analysis of variance with the Genstat V System at Michigan State University (East Lansing, MI). Comparisons with *ad lib.* controls were made by Dunnett's *t* test. Intergroup comparisons were made by the improved Bonferroni *t* test, which is a conservative approach for making multiple comparisons of non-independent data (Gill, 1978).

## RESULTS

*L. monocytogenes* challenge studies were conducted to assess the potential of DON and ZEA to alter host resistance. When mice were injected iv with $1 \times 10^4$ CFU *L. monocytogenes*/animal during the course of a 2-wk mycotoxin feeding period a general trend towards increased splenic counts on days 1 and 4 was observed in the groups consuming DON or ZEA (Table 1). The day 1 and day 4 splenic counts of mice ingesting 5 ppm DON with 10 ppm ZEA were significantly ($P < 0.05$) greater than the count for the corresponding *ad lib.* control but did not differ from the splenic counts for either the pair-fed control group or the 5 ppm DON treatment group. The day 4 splenic count of mice ingesting 0.5 ppm DON with 10 ppm ZEA was significantly ($P < 0.05$) higher than that of mice consuming 0.5 ppm DON but did not differ from counts for the *ad lib.* control. When mice were each injected iv with $1 \times 10^4$ CFU *L. monocytogenes* on day 52 of the 8-wk mycotoxin exposure period, no significant differences were shown to exist between the day 1 or day 4 spleen counts of mycotoxin-exposed and control groups (Table 1). Day 1 and day 4 liver counts of mycotoxin-treated mice did not differ significantly from the control groups after either the 2- or the 8-wk mycotoxin exposure (data not shown).

The effect on Listeria resistance of 2-wk exposure of mice to DON and ZEA was examined in a second experiment, in which the maximum DON level in the diet was increased to 25 ppm and animal numbers were also increased, to improve statistical sensitivity

Table 1. Splenic clearance of Listeria monocytogenes in mice fed deoxynivalenol (DON) and/or zearalenone (ZEA) for 2 or 8 wk

| Treatment (ppm mycotoxin in diet) | Day of count... | Mean log CFU/organ after mycotoxin feeding for: 2 wk | | 8 wks | |
|---|---|---|---|---|---|
| | | 1 | 4 | 1 | 4 |
| *Ad lib.* control (O) | | 2.35 ± 0.12[b] | 4.55 ± 0.16[c] | 3.27 ± 0.18 | 5.20 ± 0.13 |
| DON (0.5) | | 3.11 ± 0.45 | 4.41 ± 0.17[d] | 3.39 ± 0.21 | 5.26 ± 0.15 |
| (5.0) | | 3.11 ± 0.53 | 4.71 ± 0.12 | 3.39 ± 0.13 | 5.32 ± 0.15 |
| Pair-fed control* | | 3.12 ± 0.31 | 4.81 ± 0.07 | 3.65 ± 0.25 | 4.87 ± 0.15 |
| ZEA (10) | | 3.60 ± 0.35 | 4.97 ± 0.07 | 3.43 ± 0.18 | 4.98 ± 0.09 |
| DON (0.5) + ZEA (10) | | 2.59 ± 0.59 | 4.96 ± 0.23[d] | 3.84 ± 0.38 | 5.36 ± 0.18 |
| DON (5.0) + ZEA (10) | | 4.02 ± 0.60[b] | 5.17 ± 0.06[c] | 3.53 ± 0.28 | 5.05 ± 0.17 |

*Pair-fed with group fed 5.0 ppm DON.
Values are means ± SEM for groups of five mice. Those with the same superscript differ significantly ($P < 0.05$) from each other—[b] and [c] by Dunnett's $t$ test and [d] by the Bonferroni $t$ test.

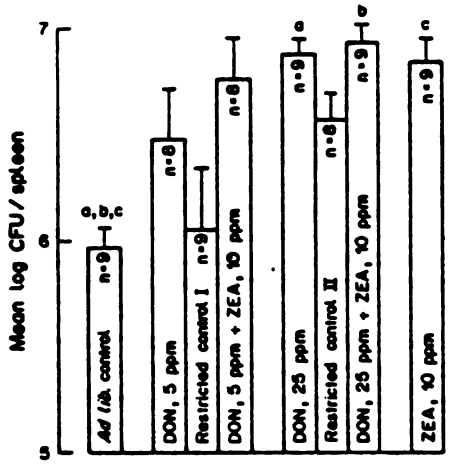


Fig. 1. Spleen clearance of *Listeria monocytogenes* following 2-wk dietary exposure of mice to deoxynivalenol (DON) and/or zearalenone (ZEA). The mice were challenged with $5 \times 10^4$ *L. monocytogenes* on day 10 and spleen counts were performed 4 days later. Means (± SEM) marked with the same letter (a, b or c) differ significantly ($P < 0.01$) from each other by Dunnett's $t$ test.

(Fig. 1). When mice were injected with $5 \times 10^4$ CFU *L. monocytogenes*, day 4 spleen counts were significantly ($P < 0.01$) greater in the treatment groups ingesting 25 ppm DON, 10 ppm ZEA or 25 ppm DON with 10 ppm ZEA than in the *ad lib.* control. However, spleen counts of the treatment groups ingesting 25 ppm DON or 25 ppm DON with 10 ppm ZEA were not significantly different from those of restricted controls. Differences in day 4 liver counts were again undetectable among treatment and control groups in the same experiment (data not shown).

The PFC response to SRBC challenge after mycotoxin exposure for 2 or 8 wk was used to assess the effects of DON and ZEA on humoral immunity. The direct PFC responses of mice receiving 25 ppm DON for 2 wk were 37 and 38% of the responses of the *ad lib.* and restricted controls, respectively, when expressed as PFC/1 $\times 10^6$ spleen cells (Table 2). On a PFC/spleen basis, the 25 ppm DON treatment group response was 28 and 54% of the *ad lib.* and restricted control responses, respectively, but these differences were not statistically significant. Other treatment and restricted control groups in the 2-wk experiment did not differ significantly from the *ad lib.* control.
detectable after dietary exposure to the toxin for 8 wk (Table 2). Expressed as SRBC PFC/1 $\times 10^6$ spleen cells, the response of mice receiving 25 ppm DON was significantly ($P < 0.01$) lower than that of the restricted controls but did not differ from that of the *ad lib.* controls. Both restricted control groups exhibited significantly greater PFC responses than did the *ad lib.* controls.

Cutaneous delayed hypersensitivity to KLH was evaluated in mice exposed to dietary DON and ZEA prior to and during antigen sensitization. The delayed hypersensitivity response of mice receiving 25 ppm DON for 3 wk was approximately half that found for

Table 2. Dietary exposure of mice for 2 or 8 wk to deoxynivalenol (DON) and/or zearalenone (ZEA) and its effect on the splenic plaque-forming cell (PFC) response to sheep red blood cells (SRBC)

| Treatment (ppm mycotoxin in diet) | PFC response at: 2 wk: SRBC PFC/$10^6$ spleen cells | 2 wk: SRBC PFC/spleen ($\times 10^6$) | 2 wk: Total spleen cells ($\times 10^7$) | 8 wk: SRBC PFC/$10^6$ spleen cells | 8 wk: SRBC PFC/spleen ($\times 10^6$) | 8 wk: Total spleen cells ($\times 10^7$) |
|---|---|---|---|---|---|---|
| *Ad lib.* control (0) | 696 ± 141 | 7.04 ± 0.90[b] | 14[c] | 346 ± 62[a,e] | 9.04 ± 1.55 | 27[a,b] |
| DON (5.0) | 1050 ± 538 | 5.83 ± 1.51 | 9.9 | 438 ± 61 | 11.18 ± 2.01 | 24[e] |
| Restricted control I* | 597 ± 123 | 4.64 ± 1.46 | 9.2 | 676 ± 117[d] | 16.12 ± 2.63 | 24 |
| DON (5.0) + ZEA (10) | 533 ± 144 | 4.77 ± 0.72 | 11 | 477 ± 86 | 14.32 ± 1.81 | 32[d] |
| DON (25) | 257 ± 105 | 1.96 ± 0.62[b] | 9.1 | 223 ± 59[f] | 4.08 ± 1.34 | 17[g] |
| Restricted control II | 672 ± 250 | 3.63 ± 0.83 | 6.0[c] | 788 ± 132[e,f] | 12.62 ± 2.39 | 17[h] |
| ZEA (10) | 696 ± 153 | 7.17 ± 1.69 | 10 | 346 ± 122 | 10.60 ± 3.89 | 28[f] |

*Pair fed with group fed 5.0 ppm DON.
†Pair-fed with group fed 25 ppm DON.
Values are means ± SEM for 24 mice in the *ad lib.* control group and for ten mice in all other groups. Those with the same superscript differ significantly from each other—by Dunnett's $t$ test at $P < 0.01$ ([a,b,c,d] and [b]) or at $P < 0.05$ ([e]) or by the Bonferroni $t$ test at $P < 0.01$ ([a] and [f]).

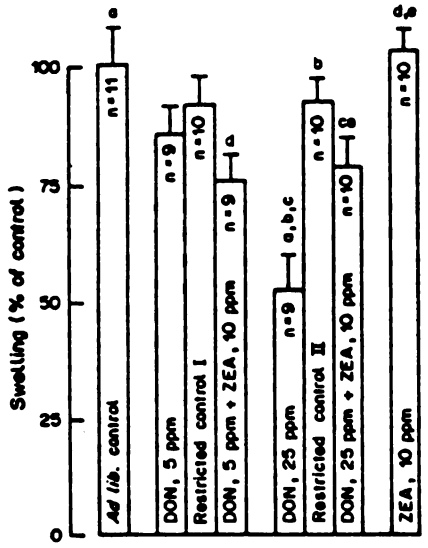


Fig. 2. Delayed hypersensitivity response in mice exposed to dietary deoxynivalenol (DON) and/or zearalenone (ZEA) for 3 wk. Results are presented as a percentage of the *ad lib.* control response. Means (± SEM) marked with the same letter (a–e) differ significantly from each other—a by Dunnetts *t* test ($P < 0.01$), b, d and e by the Bonferroni *t* test ($P < 0.01$) and c by the Bonferroni *t* test ($P < 0.05$).

the *ad lib.* and restricted controls ($P < 0.01$) and was significantly ($P < 0.05$) lower than that found for the group given 25 ppm DON with 10 ppm ZEA (Fig. 2). In contrast, mice subjected to the identical dietary mycotoxin treatments for 8 wk did not show delayed hypersensitivity responses significantly different from those of the *ad lib.* or restricted controls (data not shown).

## DISCUSSION

Consumption of agricultural staples infected by Fusarium has been associated with impaired health and increased mortality in both humans and animals (Côté *et al.* 1984; Joffe, 1978; Ueno, 1983a). While the direct toxicity of Fusarium toxins is undoubtedly a factor in such outbreaks, immunosuppression resulting from mycotoxin ingestion is likely to contribute to the aetiology of these mycotoxicoses. The data presented here and summarized in Table 3 demonstrate that dietary exposure to naturally occurring levels of DON and ZEA caused statistically significant effects on host resistance, humoral immunity and cell-mediated immunity in the mouse. The observation that DON, ZEA and food restriction independently modified the function of various immune parameters (Table 3) illustrates the complexity encountered in specifically identifying the mechanisms by which Fusarium toxins cause immunosuppression.

Infection with *L. monocytogenes* has been used extensively as a model for assessing the effects of chemicals on host resistance (Tripathy & Mackaness, 1969). Listeria challenge elicits an early immune response, allowing rapid quantitative assessment of the course of the infection. T cell-independent macrophages play a role in the early stages of the defence against Listeria (Newborg & North, 1980), whereas subsequent resistance and survival of the host is T cell-mediated (Mackaness, 1962 & 1969), with the generation of specifically sensitized T cells and activated macrophages. Recently, Tryphonas *et al.* (1986) reported that dietary DON caused a reduced, dose-related, time-to-death interval following challenge with *L. monocytogenes*. The potential of DON and ZEA to alter Listeria resistance in the mouse was assessed here by monitoring effects on bacterial counts in the spleen and liver on days 1 and 4 after infection, the time points when macrophage and cell-mediated immunity, respectively, are likely to be expressed (Dean *et al.* 1980; Tripathy & Mackaness, 1969). Initial experiments using a pair-feeding design indicated that 2-wk exposure to DON with ZEA significantly increased splenic Listeria counts on both day 1 and day 4 (Table 1). A subsequent experiment using a restricted dietary design showed the statistically significant effects of ZEA alone, DON alone, and DON with ZEA on splenic Listeria counts after 4 days (Fig. 1). Thus, macrophage and cell-mediated immune function might both have been altered by the mycotoxin treatments. Differences among liver bacterial counts were not detectable in any of the Listeria experiments. The inability to detect subtle immune alterations in this organ may have been due to the greater capacity of the liver to detoxify DON and ZEA or to the more enhanced anti-Listeria resistance in the liver than in the spleen (Pietrangeli *et al.* 1983).

Although increased mortality and diminished cell-mediated immunity have been observed previously in mice exposed to the synthetic oestrogen, diethylstilboestrol (Dean *et al.* 1980; Luster *et al.* 1980, 1982 & 1984), this, to our knowledge, is the first report of immunotoxicity by ZEA. The ability of oestrogens to compromise host resistance to Listeria correlates with oestrogenic potency and may be thymus-mediated (Luster *et al.* 1984) or related to their capacity to

Table 3. Summary of significant immunological alterations observed in the B6C3F$_1$ mouse after dietary exposure to deoxynivalenol (DON) and/or zearalenone (ZEA) or after dietary restriction*

| Treatment | Duration of feeding... | Resistance to Listeria | | PFC response to SRBC | | Delayed hypersensitivity response | |
|---|---|---|---|---|---|---|---|
| | | 2 wk | 8 wk | 2 wk | 8 wk | 3 wk | 8 wk |
| DON | | Decreased† | No effect | Decreased† | No effect‡ | Decreased‡ | No effect |
| ZEA | | Decreased | No effect | No effect | No effect | No effect | No effect |
| DON + ZEA | | Decreased | No effect | No effect | No effect | No effect | No effect |
| Reduced feed intake | | No effect | No effect | No effect | Increased | No effect | No effect |

PFC = Plaque forming cell SRBC = Sheep red blood cells

*Summary based on comparison with the *ad lib.* control.

†No effect when based on comparison with the restricted control.

‡Decreased when compared to the restricted control.

inhibit interleukin-2 production in lymphocytes and the subsequent proliferation of antigen-sensitized T lymphocytes required for recovery (Pung *et al.* 1984 & 1985). Repeated sc injection of zearalenol, an *in vivo* metabolite of zearalenone, does not increase susceptibility to Listeria in the B6C3F$_1$ mouse (Pung *et al.* 1984). Administered in this way, zearalenol causes macrophage activation, as do other oestrogens, but zearalenol fails to suppress splenic lymphoproliferative responses in a manner similar to that found with more potent oestrogens (Luster *et al.* 1984). However the two latter studies employed a 3–5-day rest period between oestrogen injection and the final immune function assay. Since zearalenone and its metabolites are metabolized and cleared rapidly *in vivo* (Mirocha *et al.* 1981), our ability to detect a significant effect on Listeria resistance is probably related to the continuous dosing of zearalenone in the diet before and after Listeria challenge.

Dietary DON significantly increased splenic Listeria counts over those in *ad lib.* controls (Fig. 1), but although the splenic counts of DON-treated animals were consistently higher than those of the restricted controls, these differences were not significant. Thus, diminished Listeria resistance in DON-treated mice may, in large part, be a result of a nutritional effect associated with DON-induced food refusal rather than a reflection of direct immunotoxicity of this mycotoxin. It should also be noted that the combined effects of ZEA and DON on Listeria resistance were slightly higher than the effects of either toxin alone, with a statistically significant difference being detectable in one experiment between a DON-fed group and a combined treatment group (Table 1).

Of particular interest was the fact that even though we observed significantly decreased resistance to Listeria after 2 wk dietary exposure to DON and ZEA, similar immunosuppression by those toxins was not evident after the 8-wk exposure (Tables 1 & 3). This observation may be the result of one or more factors. First, the immune response to Listeria in the 8-wk mouse may have matured to a point where it was resistant to the possible effects of dietary mycotoxins (Patel, 1981). Secondly, age-related differences in toxin-metabolizing enzymes may exist between the 2-wk and 8-wk experimental mice (Doull, 1975). Thirdly, the more prolonged exposure to DON and ZEA may have resulted in the induction of appropriate detoxification enzymes, making the animals more tolerant to these toxins and thus decreasing observable immune suppression. Nevertheless our results lead to the conclusion that exposure to DON and ZEA over a prolonged period did not result in cumulative and increased damage to immune function compared to the short-term exposure.

Depression of humoral immune function by trichothecenes, including DON, has been reported previously (Rosenstein *et al.* 1979; Tryphonas *et al.* 1984), but differences in toxin, dose, animals and route of exposure hinder comparisons between the studies. Humoral immune function was assessed here by measuring the splenic plaque-forming response to the T-dependent antigen SRBC. This response was lower in mice ingesting 25 ppm DON for 2 wk than in mice in the *ad lib.* and restricted control groups. Our inability to detect an effect by DON at the 5 ppm level is consistent with a similar observation made by Tryphonas *et al.* (1986). The response of either restricted control group after 2 wk was not significantly different from that of the *ad lib.* control group. After exposure for 8-wk, however, both restricted control groups showed a significant increase in PFC/$10^6$ cells compared to *ad lib.* controls and the percentage increase was proportional to the degree of feed restriction (Table 2). Values for PFC/spleen were not significantly different, because of decreased total spleen counts in the restricted groups. Moderate dietary restriction has previously been reported to enhance some immune functions, including the PFC response to SRBC (Keusch *et al.* 1983). Both groups of mice ingesting DON alone for 8 wk showed some decrease in response compared to their respective restricted control group (and the effect with 25 ppm DON was statistically significant) but not compared to the *ad lib.* control group. Thus depression by DON actually restored humoral responses to the level of the *ad lib.* controls, illustrating the importance of dietary controls in trichothecene immunotoxicity studies.

Cell-mediated immunity is essential for resistance to various infections, tumour immunity and allograft rejection. Delayed hypersensitivity is a slowly evolving inflammatory response that can serve as a model for cell-mediated immune function. Significant effects of 25 ppm DON on delayed hypersensitivity were observable after 3 wk when compared to values for either *ad lib.* or restricted controls (Fig. 2) indicating that, in contrast to the Listeria results, depressed cell-mediated immunity in this case was likely to be the direct result of immunotoxicity of DON rather than an indirect effect of reduced food intake. It is noteworthy that co-administration of 10 ppm ZEA significantly decreased the inhibitory effects of 25 ppm DON. After 8 wk, food restriction apparently diminished the delayed hypersensitivity response, but this observation was not significant. As was found with the 8-wk Listeria study, exposure to DON did not decrease the delayed hypersensitivity response. Again, the age-related factors mentioned above for Listeria resistance may have contributed to this observation.

On a mechanistic level, trichothecenes inhibit protein synthesis in eukaryotic cells by interfering with peptide initiation or elongation/termination steps (McLaughlin *et al.* 1977). DON potentially exerts the immunotoxic effects described here via direct action on lymphocytes (Gyongyossy-Issa & Khachatourians, 1985) and macrophages (Gerberick & Sorenson, 1983). *In vitro* studies indicate that the levels of the trichothecene T-2 toxin required to inhibit lymphocyte function (Gyongyossy-Issa & Khachatourians, 1985) are 10–100 times lower than those required to inhibit macrophage function (Gerberick & Sorenson, 1983), suggesting that lymphocytes are more likely to be a prime target for immunosuppression. Individual subsets of lymphocytes appear to be equally susceptible to trichothecenes (Forsell *et al.* 1985; Forsell & Pestka, 1985). Preferential toxicity towards lymphocytes may be related to differences in detoxification capacity, membrane permeability or ribosomal affinity for trichothecenes. Recently, it has been demonstrated that DNA, RNA and protein synthesis in murine lymphocytes is inhibited by a

threshold dose of T-2 toxin of approximately $1 \times 10^5$ molecules per lymphocyte (Gyongyossy-Issa & Khachatourians, 1985). This level corresponds to an earlier estimate for specific T-2 toxin receptors on murine lymphocytes by Gyongyossy-Issa & Khachatourians (1984). The intriguing possibility that trichothecene action on lymphocytes is mediated by a receptor merits further investigation.

In conclusion, the results presented here confirm and extend previous work implicating Fusarium toxins in immunosuppression. The implications of this report are particularly significant because DON and ZEA are two of the most frequently occurring Fusarium toxins, and because the dietary levels of DON and ZEA used here realistically represent the concentrations of these toxins encountered in wheat and corn after natural infection by *F. graminearum*. Furthermore, exposure of the B6C3F$_1$ mouse to these mycotoxin levels does not result in detectable histopathological damage (Forsell *et al.* 1985) but, as shown here, it can cause subtle immunosuppressive effects. Interpretation of these data requires two key considerations. First, immune effects by these toxins are undoubtedly modulated by decreased nutrient intake caused by mycotoxin-induced food refusal. Secondly, although we have specifically described effects on systemic immune parameters, it is very likely that Fusarium toxins have their greatest effect on gut-associated lymphoid tissue, before they are absorbed and subsequently metabolized by detoxification enzymes. Acute doses of DON cause severe lesions in the gastro-intestinal tract of the B6C3F$_1$ mouse (Forsell *et al.* 1987), while chronic effects may be much more subtle. The potential for effects of dietary DON on mucosal immunity have been verified by our recent observation that dietary exposure to 10 ppm of this mycotoxin increases serum IgA levels (Forsell *et al.* 1985). Thus, while it is clear that dietary Fusarium toxins can significantly affect immune function, future investigations should be directed towards the clarification of the nutritional effects on immune function that can be brought about by mycotoxin ingestion, a thorough understanding of the interactions between dietary mycotoxins and specific cell sub-populations of the systemic and mucosal immune systems, and the assessment of the potential of mycotoxins to modify the host defence against infectious agents acting at the gastro-intestinal level.

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