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Protection Against Trichothecene Mycotoxins

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Environmental Health Hazards

Commission on Life Sciences

National Research Council

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PREFACE

In response to a request from the Department of the Army to the National Academy of Sciences, a committee was formed in the National Research Council's Commission on Life Sciences to study means by which human health can be protected against exposure to trichothecene mycotoxins. The appointments were completed in October 1982, and the committee finished its work in September 1983.

The impetus for this study was concern for the welfare of civilians and U.S. military personnel who may be exposed to high levels of mycotoxins used offensively during military actions. No judgments are made in this report regarding evidence for the extent and occurrence of such actions in the past. Although the report focuses on the trichothecene class of mycotoxins, the committee recognizes that there may be other classes of biologically produced chemical agents whose toxicities differ from those of the trichothecenes and which may deserve further study. Because trichothecenes are part of the natural environment and can cause widespread injury to plants, animals, and humans, the committee considered their biological behavior in the biosphere as a whole. Accordingly, the recommendations in this report may also be applied to protection of human populations exposed to trichothecenes from natural sources.

The Committee on Protection Against Mycotoxins addressed many scientific questions, which fall into six broad topics: natural occurrence, methods of detection and quantitation, decontamination and detoxification, long-term environmental effects, responses of biological systems, and prevention and treatment. To examine these issues, the committee formed from among its members two closely interacting subgroups. One subgroup dealt with the first four topics, focusing on the chemical and environmental aspects of trichothecenes. The other subgroup addressed the latter two topics, studying various aspects of animal and human health. Each subgroup contributed to the work of its counterpart through discussions and shared writing efforts. The committee as a whole reviewed this report, and resulting comments have been incorporated into the text.

A broad literature search was conducted to gather the information needed by the committee during its study. The effort extended beyond a review of the vast scientific literature on trichothecene mycotoxins to include unpublished material from scientists involved in research, officials from federal agencies and the military, and scientific representatives from foreign governments. Only information available to the public was considered.

The committee is indebted to Dr. Benjamin J. Wilson, who contributed substantially to the description of the biological effects in humans contained in Chapter 6, despite an illness that necessitated his resignation from the committee.

David W. Talmage
Chairman
Committee on Protection
Against Mycotoxins

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TABLE OF CONTENTS

		Page
	EXECUTIVE SUMMARY	1
1	INTRODUCTION	12
2	THE TRICHOTHECENE MYCOTOXINS: THEIR STRUCTURE, NATURAL PRODUCTION, AND LEVELS OF OCCURRENCE	17
	Chemical Structures of Trichothecenes	17
	Trichothecene-Producing Fungi	22
	Factors Affecting Natural Production of Fusarium Toxin	26
	Natural Levels of Trichothecenes	33
	Estimated Levels of Trichothecenes from Possible Military Use	35
	Conclusions and Recommendations	36
3	DETECTION, IDENTIFICATION, AND QUANTITATION	44
	Sampling	44
	Considerations in Analysis	46
	Rapid Field Screening	47
	Quantitative Analysis of Trichothecenes	58
	Confirmatory Tests	64
	Conclusions and Recommendations	65
4	DECONTAMINATION AND DETOXIFICATION	70
	Chemical Methods of Detoxification	70
	Enzymatic Decomposition	76
	Decontamination of Water Supplies	76
	Processing Mycotoxin-Contaminated Commodities	77
	Disposal	79
	Conclusions and Recommendations	79
5	LONG-TERM ENVIRONMENTAL EFFECTS	83
	Environmental Chemistry	83
	Water Contamination, Leaching, and Mobility	84
	Uptake by Plants	84
	Degradation in Natural Environments	84
	Degradation in Microbial Cultures	85
	Needed Research	86
	Conclusions and Recommendations	90

EXECUTIVE SUMMARY

Trichothecenes contained in cereal grains have been recognized as a recurring public health problem for many years. Thus, much of the literature on the human health effects of these natural toxins consists of anecdotal information grounded in a long history of exposures in civilian settings. More recently, trichothecenes and their effects on humans have attracted national and international attention because of their reported use in warfare. This study arose out of a concern about such uses and the potential harm these substances could cause among civilian and military populations that might be exposed to them. Accordingly, this report is intended to assist in the protection not only of U.S. Armed Forces against the adverse effects of trichothecenes but also of civilian populations that may come into contact with these toxins during peace or war.

The committee neither supports nor refutes the evidence for the military use of mycotoxins in embattled areas. This consideration was not contained in its charge. Rather, it concentrated on the following two major objectives:

- examination of the environmental and biological behavior of the trichothecene class of mycotoxins to determine what protective measures can be taken and

- development of the means for optimizing the protection of human beings against the effects of trichothecenes through appropriate prophylaxes and treatments.

The committee approached its task by characterizing the occurrence of trichothecenes, their metabolism, their adverse effects in the environment, and their adverse effects in humans. These characterizations included potential methods for the specific, sensitive, and rapid detection of trichothecenes; the practical means for their destruction, disposal, and environmental decontamination; possible prophylaxes to prevent their toxic action in humans; and treatments for trichothecene-induced health effects.

THE TRICHOTHECENE MYCOTOXINS: THEIR STRUCTURES, NATURAL PRODUCTION, AND LEVELS OF OCCURRENCE

Trichothecenes are a chemically related group of biologically active secondary metabolites produced by certain species of fungi belonging to the genera *Fusarium*, *Acremonium*, *Trichoderma*, *Trichothecium*, *Myrothecium*, *Stachybotrys*, and *Cylindrocarpon*. Although *Fusarium* species, perhaps the most important source of

trichothecenes, occur worldwide in habitats as diverse as deserts, tidal salt flats, alpine mountain regions, and the tropics, not all of them produce trichothecenes. In many regions of the world, the extent to which trichothecene-producing species are present is not yet known. Furthermore, confusion regarding the correct taxonomic identification of these fungi has resulted in erroneous identification of species responsible for producing trichothecenes. Forthcoming publications on the taxonomic classification of Fusarium species should provide a promising format for rectifying this situation.

The trichothecenes are biosynthesized by fungi during the depletion of one or more nutrients. This phase of metabolism is controlled by four factors: age of the hyphae; the type and degree of nutrient exhaustion; physical conditions, e.g., oxygen tension, light, pH, and temperature; and the effects exerted by physical and chemical factors on the enzymes of secondary metabolism, in terms of which pathways are selected and, hence, which metabolites will be produced. Small changes in physical and chemical factors can result in dramatic changes in the quantity and composition of various dominant secondary metabolites as well as variations in the many minor metabolites.

Few studies have been conducted, either in the field or during post-harvest storage, to determine the exact conditions under which trichothecenes are formed. In some laboratory studies, trichothecenes have been biosynthesized from Fusarium in liquid cultures. Since the conditions of trichothecene production can be carefully controlled in such studies and minor metabolites can be more readily detected, this technique holds promise of providing insight into the pathways of biosynthesis.

Levels of trichothecenes such as T-2 toxin, 4-deoxynivalenol (DON), diacetoxyscirpenol (DAS, or anguidine), and nivalenol have been measured primarily in agricultural commodities. Most measurements indicate that the concentrations range from 0 to 10 µg/g. Reported values may generally be biased toward higher mean levels than actually exist, because most measurements have been made in response to outbreaks of mycotoxicosis rather than on the basis of random sampling. A recent study has shown that 89% of randomly collected samples contained levels less than 4 µg/g in regions of Nebraska and Kansas with a history of trichothecene occurrence. In the event of deliberate release of trichothecenes, the average concentration could be more than two orders of magnitude greater than typical levels produced from natural sources.

DETECTION, IDENTIFICATION, AND QUANTITATION

Collection of field samples of trichothecenes is one of the first and most important steps in the chemical analysis of these toxins.

Because there may be several different purposes for collecting samples, each requiring a different type of analysis and different levels of purity, no one protocol or set of recommendations can be established. However, for a given type of chemical analysis, careful attention should be given to sampling procedures. After collection, techniques to reduce or eliminate chemical breakdown or biodegradation should be applied to maintain sample integrity.

The procedures for detecting and determining trichothecenes may require sophisticated equipment and may be time-consuming and complicated when high sensitivity is required. The physical and chemical properties of these substances preclude the use of simple analytical methods at the present time.

Criteria used to evaluate methods for determining trichothecenes in cereal grains, foods, and feeds have been published. Recovery of trichothecenes added to uncontaminated samples should be at least 70%, with a coefficient of variation no greater than 30% and a detection limit lower than 0.1 μg/g of sample. For samples in which trichothecenes are deliberate contaminants, the detection limit could be higher than that for naturally contaminated cereals.

Several methods are suitable for rapid field screening. In one, rapid thin-layer chromatography (TLC) could be used to detect deliberately released samples. Such a method has already been developed and modified for analysis of DON on a variety of agricultural commodities. Assays of trichothecenes by the enzyme-linked immunosorbent assay (ELISA) technique can be performed with relatively simple portable laboratory equipment and completed within several hours. The feasibility of immunological assays has been demonstrated in published experiments with T-2 toxin as an antigenic determinant. It seems probable that similar assays can be developed for other members of the trichothecene family.

Remote sensing, either active or passive, is another possible method of rapid detection, although the feasibility of this technique has yet to be demonstrated. The LANDSAT program of the National Aeronautics and Space Administration is an example of orbital, passive remote sensing in which narrow bands of visible and infrared radiation provide imagery of the earth's surface. Similar techniques might be used to detect the clouds of mycotoxin called "yellow rain." Laser remote sensing is an active system that has been used to measure aerosol and gaseous constituents of the atmosphere. Although this technique offers a number of potential advantages over chemical methods for rapid detection, some general difficulties are associated with their application in studies of trichothecene aerosols. Other methods of detection involve the use of susceptible biological systems such as seeds of higher plants; the skins of rabbits, guinea pigs, or rats; brine shrimp; and certain lower organisms. Such tests are

sensitive, but often lack specificity and are time-consuming. Because of their simplicity, however, some of these tests may be well suited for corroborative testing.

A number of good methods exist for the quantitation and confirmation of trichothecenes in the laboratory. Gas-liquid chromatography is the most highly developed and widely used method for quantitation. Gas chromatography-mass spectrometry has been used for a number of years to confirm the identity of Fusarium toxins as well as to quantify them. Tandem mass spectrometry is another method for confirmation and can be used for screening.

DECONTAMINATION AND DETOXIFICATION

In the event of deliberate release, levels of exposure to trichothecenes are likely to be several orders of magnitude higher than levels from natural sources. Military personnel and civilians would be contaminated as would exposed equipment, foods, and water supplies. Thus, methods of removal or chemical detoxification of trichothecenes are needed to protect not only those directly exposed but also those exposed through contaminated food and water, which could pose a hazard for weeks or months. In the agricultural research community, there has been considerable experience with the decontamination and detoxification of aflatoxins--a group of mycotoxins that do not fall into the trichothecene class. Since there has been little research to develop such procedures for trichothecenes, the methods used for aflatoxins should be studied to determine their applicability to trichothecenes.

The most effective agent for decontaminating T-2 toxin is sodium hypochlorite. However, the products of the reaction with this chemical have not been studied to determine whether they are toxic. This concern about potential toxicity stems from findings that the reaction of aflatoxin with sodium hypochlorite results in the formation of products that are carcinogenic and mutagenic.

One promising approach for the detoxification of trichothecenes is to devise chemical procedures for selectively attacking the epoxide ring, a functional group whose presence is necessary for the toxicity of these compounds. Epoxides are normally very reactive toward nucleophiles, acids, and bases. In trichothecenes, however, they are shielded sterically to the extent that the usual reagents do not react with the epoxide ring under mild conditions. Suitable reagents may be developed to enable the epoxide ring to break under mild conditions. Potential candidates include certain catalysts, "super nucleophiles," "super acids," and triphenylphosphine.

Another strategy is to use enzymes to destroy trichothecenes. The best sources of such enzymes are probably microorganisms; however, little effort has been directed toward the extraction of large quantities of enzymes from this source. An advantage of using enzymes is that many of them are not harmful to humans, but they lack the broad scope of action that characterizes many chemical reagents.

Little work has been done on the removal of mycotoxins from water supplies. Several promising methods should be investigated to determine their effectiveness. Methods also need to be developed for decontaminating foods or transforming them into other useful products. Ammoniation is the most effective and convenient means of detoxifying aflatoxins in agricultural commodities. The use of this process to decontaminate trichothecene-infected wheat samples is now being examined.

Studies have been conducted to determine the distribution of trichothecene mycotoxins among product fractions during the processing of agricultural commodities. Investigators studying the wet milling of corn contaminated with T-2 toxin found that two-thirds of the toxin was removed in the steepwater, 4% was present in the starch, and the remainder was distributed between gluten, fiber, and germ. Grains infected with mycotoxins have very little economic value; however, they can be used as substrates for alcohol production by fermentation without transferring the toxin to the distilled alcohol.

Disposal methods have not been applied to trichothecenes. Aflatoxin-contaminated agricultural commodities or laboratory wastes have been incinerated and buried. The applicability of these or similar procedures for disposing of trichothecenes should be tested to ensure their effectiveness.

LONG-TERM ENVIRONMENTAL EFFECTS

To minimize long-term effects on human health and on crops and animals, the persistence and degradation of trichothecenes in the biosphere should be characterized. There have been no studies showing whether such substances will leach through soil to contaminate groundwater or whether there is appreciable lateral translocation through waters or in eroding soil particles to contaminate adjacent surface waters. Furthermore, there have been no studies to determine whether plants used for human food and by domestic animals may assimilate trichothecenes from contaminated soils.

Except for one study of aflatoxin, there have been no efforts to evaluate the possibility that any mycotoxin is degraded in soils by biotic or abiotic mechanisms. The concentrations of T-2 toxin, aflatoxin, and some other mycotoxins are known to decrease over time

in microbial cultures, indicating that degradation by enzymes has occurred, but the enzymes involved in these microbial transformations have not been characterized or purified. In a few instances, the products of the microbial process have been identified, but each of those products represented only a modest change in the parent mycotoxin. Extensive microbial degradation has yet to be shown.

To characterize long-term effects caused by trichothecene residues, studies should be conducted to assess their stability in sterile systems at defined pH; their photochemical degradation in water and aerosols; their mobility in soils; their metabolism and persistence in soils, water (including irrigation facilities), and activated sludge and other waste-treatment systems; their breakdown by edible plants; their persistence on buildings and equipment; and their uptake, accumulation, and deposition by crop plants, forage plants, aquatic plants, invertebrates, fish, domestic animals, and game animals. In parallel with these studies, other investigations should be conducted to determine the effects on processes that are important in the maintenance of soil fertility, effects on the ability of soils to degrade plant materials, and effects of persistent residues on crop plants and critical species supporting agricultural production, including pollinating insects. Several analytical alternatives can be used to achieve the levels of accuracy, precision, and sensitivity required for environmental studies.

RESPONSES OF BIOLOGICAL SYSTEMS

Biological Effects in Humans

There is little definitive information on the toxic effects produced in humans by specific trichothecene mycotoxins, with the exception of DAS. Extensive descriptions of human toxicoses, presumably caused by unidentified trichothecenes, emanated from studies of persons who had ingested *Fusarium*-contaminated grain products in the USSR and in Japan. The investigators in the Soviet Union provided excellent descriptions of alimentary toxic aleukia (ATA), first identified in that country. They described this disease as "septic angina," because it included necrotizing tonsillitis, fever, hemorrhagic diathesis, and severe intoxication.

The clinical course of ATA is divided into four stages. The first stage involves local irritation of the oral mucous membranes, developing into hyperemia of the oral mucosa accompanied by weakness, fever, nausea, emesis, and sleep disturbances. In the second stage (the leukopenic stage), alterations include leukopenia, granulopenia, and progressive lymphocytosis. Electrocardiographic changes occur in some individuals. In the third stage (the hemorrhagic stage), petechial rash develops on the skin and spreads over the body. In

addition, the affected persons experience severe pharyngitis and ulcerative changes of the larynx, which in the most severe cases result in death by strangulation. During this stage, severe hemorrhagic diathesis of the nasal, oral, gastric, and intestinal mucosa may also occur.

In the fourth stage (the recovery stage), the subjects are susceptible to various secondary infections. Convalescence for some is prolonged, lasting several weeks. Although the cases of ATA reported in the Soviet Union are believed by many to have been associated with exposure to T-2 toxin and possibly other trichothecene mycotoxins, the etiology of this disease cannot be established.

DAS (anguidine) has been administered by intravenous infusion in both phase I and phase II clinical evaluations for the treatment of human malignancies. In these studies, the antitumor activity was minimal or absent, but a variety of toxic effects were recorded in patients given intravenous daily doses ranging from 0.2 mg/m^2 (0.005 mg/kg body weight [bw]) to 6.0 mg/m^2 (0.154 mg/kg bw). Nausea and vomiting accompanied by myelosuppression, hypotension, diarrhea, central nervous system dysfunction, fever, chills, stomatitis, erythema, and hair loss were frequently reported. In some patients, the hypotension was severe and required removal from chemotherapeutic treatment. A few patients died as a result of the treatment. Many of the signs and symptoms of DAS toxicity observed in human cancer patients have also been observed in persons reportedly exposed to "yellow rain" attacks; however, some of the alterations described for these persons have not been observed in patients treated with DAS. These differences may be related to the differences in dose, duration, and route of exposure between the two groups.

Because of the unknown exposure doses in the "yellow rain" reports and evidence that other agents may have coexisted in mixtures with the trichothecenes, the data are considered unreliable for the determination of the biological effects of toxic trichothecenes in humans. For this reason, data derived from controlled experiments in animals must be used to predict the biological effects of these toxins in humans.

Biological Effects in Animals

There is a substantial amount of toxicological data from animal studies that can be used to evaluate the biological effects of trichothecene mycotoxins in humans. Because certain trichothecenes such as T-2 toxin, DAS, and DON are natural contaminants of agricultural feedstuffs, considerable research effort has been expended in defining their toxic effects in both laboratory and domestic animals. The routes of exposure used in these studies

include parenteral administration or oral dosing, either with the toxin alone or mixed with diets. Unfortunately, there are virtually no reports of studies in which animals have been subjected to topical or aerosol exposures.

The most toxic trichothecenes, i.e., those with lower LD_{50}s, include T-2 toxin, 4-acetylnivalenol (fusarenon-x), nivalenol, and DAS; one of the least toxic trichothecenes is DON. Toxicities vary among the various species and routes of exposure; however, the differences are not great, indicating similar modes of action and rates of uptake among species for all exposure routes.

Metabolic studies indicate that trichothecene mycotoxins are rapidly absorbed from the gastrointestinal tract and rapidly distributed throughout the body. Peak concentrations appear in the blood within the first few hours after administration. Later, relatively high concentrations of trichothecenes are present in the bile, gallbladder, liver, kidneys, and intestines. Some studies indicate that T-2 toxin is excreted into the intestine via the bile. In others, however, significant amounts were found to be eliminated in the urine. T-2 toxin seems to be rapidly metabolized to HT-2 toxin. Several other metabolic degradation products have been identified in urine and feces, but the degradation pathways leading to their formation are unknown.

Trichothecene mycotoxins are some of the most potent inhibitors of protein synthesis. Moreover, they have an unusually broad spectrum of inhibition, being effective inhibitors of protein synthesis in fungi, plants, and animals. It appears likely that their toxicity is directly related to their role as inhibitors of protein synthesis, which would explain the necrotic changes observed in rapidly dividing cells.

These toxins damage a variety of organs and tissues. The threshold dose for certain of these effects varies with species exposed and the trichothecene administered. Nonetheless, the alterations in clinical signs, tissue morphology, or function are similar among the various species studied and reflect damage to target tissues that have a component of rapidly dividing cells--gonads, gut, bone marrow, and lymphoid tissues. The most susceptible organs and tissues are the mucous membranes of the digestive system, the skin, and the hematopoietic tissues, including bone marrow, erythrocytes, leukocytes, and thrombocytes. In several animal species, the lymphoid and immunologic tissues have been found to be altered, leading to significant immunosuppressive changes. In addition to these effects, lesions and hemorrhages have been produced in the digestive system, liver, kidney, and heart of various species. Testicular lesions have also been noted, and DON has been shown to be embryotoxic; however, no significant teratologic abnormalities have been observed in mice

treated with the same compound. In another study, however, mice treated with T-2 toxin produced offspring with significantly more malformations than did controls. Few long-term feeding studies have been completed, but the evidence accumulated thus far indicates that these toxins are not tumorigenic, and essentially all the trichothecene mycotoxins evaluated in tests for mutagenicity have been negative.

Biological Effects in Plants, Insects, and Microorganisms

Most of the trichothecene mycotoxins are cytotoxic to plants grown in tissue culture. They are potent inhibitors of cell growth, and germinating seeds are highly sensitive to their action. Many Fusarium species that produce trichothecenes are plant pathogens; however, their pathogenic potency has generally not been related to the production of phytotoxic trichothecenes, whose role in plant pathogenesis has not yet been determined.

Verrucarin A and roridin A exert insecticidal action against Mexican bean beetles (Epilachna varivestis), and several trichothecene mycotoxins have larvicidal activity against greenbottle flies (Lucilia). However, T-2 toxin and DAS are inactive as contact insecticides against fruit flies (Drosophila).

A few trichothecenes have been studied for their antimicrobial activity. No appreciable antibacterial activity has been detected, but antifungal activity has been observed. Trichothecin has systemic fungicidal activity that has been used in controlling plant diseases.

PREVENTION AND TREATMENT

No specific therapy for trichothecene toxicity is known. The guiding principle followed when dealing with any toxic substance is to avoid or limit exposure. One effective way to limit exposure is to provide protective clothing and masks to people at risk. Such simple materials as a waterproof cloth or plastic sheeting, or any type of basic shelter, could significantly reduce cutaneous exposure. Skin absorption could also be minimized by such measures as a change of clothing and possibly bathing in uncontaminated water with soap or detergent. Specific decontamination procedures might entail the use of readily accessible materials such as chlorine bleaches, soap, and large amounts of uncontaminated water to wash the contaminated area.

Since trichothecenes may persist in the environment, they may gain access to the body by translocation through rain or dew, by the ingestion of contaminated water or foodstuffs, or by inhalation of dusts. Exposure by these routes could be minimized by appropriate

decontamination procedures or by removing individuals from contaminated areas.

The uptake of ingested toxic substances can sometimes be reduced by ingesting either nonspecific binding agents such as charcoal or specific binding agents; however, no specific binding agents are known for trichothecenes. Lotions or creams may also be applied to the skin to reduce absorption. Two types of prophylaxis--immunologic and enzymatic--have been suggested for protection against trichothecene mycotoxins, but there are likely to be strict limits on the effectiveness of both. The beneficial effects of immunization are limited by the amount of antibody that can be produced and the relatively large amount required to neutralize a toxin. The beneficial effects of enzymatic inducers are limited by the necessary delays between administration and induction and the general absence of significant reductions in toxicity, even after liver enzymes are induced.

RECOMMENDATIONS

Of the many potentially productive areas of research described in this report, the committee has listed below the eight that are most promising. As stipulated in its charge, the committee restricted its study to the trichothecene mycotoxins. However, it was well aware that there are other potent biologically produced toxins that may present similar risks to human health as a result of their natural occurrence or deliberate release. Therefore, when developing its research program, the Army should view the following recommendations in this broader perspective.

1. Taxonomic groups of fungi known to produce trichothecenes are globally distributed; however, naturally occurring trichothecenes have been reported in relatively few countries. Thus, a program should be undertaken to obtain a better characterization of the global distribution of these toxins.

2. Protocols for field sampling should be developed. Further research is needed on the application of thin-layer chromatography (TLC) and the enzyme-linked immunosorbent assay (ELISA) for the identification and quantitation of various trichothecenes present in a variety of sample materials. Other methods that are less promising but nonetheless warrant attention include remote sensing techniques and assays based on the susceptibility of living organisms to trichothecenes. Gas-liquid chromatography (GLC) and mass spectrometry (MS) should be used to validate new methodologies.

3. Chemical and enzymatic reactions that degrade trichothecenes to form nontoxic products should be investigated. The chemistry of the sterically protected epoxide ring should also be studied.

4. Methods of reclaiming trichothecene-contaminated commodities by transforming them into useful foods, feeds, and products should be developed.

5. The persistence and leaching of trichothecenes and the products of their nonbiological and microbial degradation in different types of soils and waters need to be evaluated. Information is also required on the uptake by plants growing in contaminated soils and on the behavior of trichothecenes in terrestrial and aquatic food chains. These studies involve different levels of complexity and expense, but many of them should produce results within a relatively short period.

6. In the broad field of research on the biological effects of T-2 toxin and certain other trichothecene mycotoxins, studies in the following three areas have potential for providing the most useful information in developing protective and therapeutic measures for exposed populations: (1) toxicokinetics following topical and inhalation exposures under a variety of environmental conditions; (2) the effects of continuous or intermittent long-term exposures by single or multiple routes; and (3) the effects produced by combined exposure to trichothecenes and other mycotoxins. These studies should include clinical, clinicopathological, immunological, morphological, and functional evaluations, with particular attention to cardiovascular, central nervous, and immune systems, as well as organs and tissues that are established targets. Detailed research recommendations are described at the end of Chapter 6.

7. There is a need to identify the best methods for preventing exposure through the use of protective barriers and decontamination procedures.

8. Methods for reducing the toxic effects of trichothecenes should be developed. Such efforts should be focussed not only on protective measures (as stipulated in Recommendation 7) but also on ways to reduce absorption of the toxin and its binding to ribosomes, and to increase its rate of degradation and elimination. The methods adopted should be based on sound quantitative studies of the cutaneous pharmacokinetics of these coumpounds.

The military is encouraged to continue seeking the advice of nonmilitary scientists in its research programs. As a result of this review of new techniques and new data, the committee recommends that the military draw from the diverse expertise in the scientific community in order to take full advantage of discoveries as soon as possible.

Chapter 1

INTRODUCTION

Mycotoxin-induced diseases have affected human populations since ancient times. The first recorded reference has been attributed to Galen, who observed in the first century, A.D.:

> Thus, of the many people who are forced to eat such food [stored barley and wheat] in time of famine, some die from a putrid or pestilential fever, others are seized by a scabby (itching) and leprosy-like skin condition (Lieber, 1970).

Over the last four centuries, numerous documented illnesses and epidemics in England, France, and the American colonies, attributed at the time of their occurrences to other causes, may in fact have been caused by mycotoxicoses. It was not until this century that episodes of mycotoxicoses in humans and animals were scientifically documented. Some of the earliest work was done by scientists in the USSR, who recorded the incidence of alimentary toxic aleukia in that country between 1932 and 1947 (Gajdusek, 1953). In 1944, the peak year, more than 10% of the population was affected in some districts and many fatalities occurred. The cause of the disease was found to be associated with the ingestion of grain that had overwintered in the field. Food had been very scarce that year in many parts of the Soviet Union, and grain left in the field throughout the winter was eaten by many people.

Investigators tracing the loss of 100,000 turkeys and ducklings in the United Kingdom during 1960 discovered that a mold toxin had been produced on feeds contaminated with Aspergillus flavus (Kraybill and Shimkin, 1964; Spensley, 1963). The fungus was subsequently found to produce compounds capable of causing cancer of the liver in laboratory animals (International Agency for Research on Cancer, 1971, 1972). Since then, many studies have been focussed on the effects of mycotoxins in foodstuffs and farm animals.

More recently, mycotoxins and their effects on humans have attracted national and international attention because of their reported use as warfare agents. Inhabitants of embattled areas in

Afghanistan, Laos, and Kampuchea described the aerial release of a yellow substance as granules or as mists that "fell like rain." This later became known as "yellow rain" (Haig, 1982). As the issue of the possible use of these toxins as chemical warfare agents gained prominence and public awareness, "yellow rain" became popularized as a generic name for mycotoxin mixtures purportedly used in this manner.

Although the composition of yellow rain per se has not yet been determined and may vary, field samples have been reported to contain T-2 toxin (a trichothecene mycotoxin) and other mycotoxins (Haig, 1982; National Defence Headquarters, 1982; Schiefer, 1982; Schultz, 1982).

The study reported herein was undertaken at the request of the U.S. Department of the Army to analyze the biological behavior of T-2 toxin and related trichothecenes so that protective measures against their actions can be appropriately and efficiently designed. The request arose from concern about the potential harm these substances could pose to the civilian and military populations that might be exposed to them.

The trichothecene-producing fungi simultaneously biosynthesize many similar, but chemically distinct, toxins in this class. Some of these substances have yet to be isolated and characterized. Despite the possible differences in their structure, the specific modes of toxin action in both humans and animals appear to be identical and the inherent toxicities of the trichothecenes are relatively similar. The Committee on Protection Against Mycotoxins concluded that a thorough analysis of trichothecene-induced health effects could not be complete unless the entire class of trichothecenes were considered.

Various fungal species produce a host of other classes of mycotoxins such as aflatoxins, rubratoxins, and ochratoxins. Although this report focusses specifically on the trichothecene class of mycotoxins, some studies of aflatoxins and certain other mycotoxin classes are discussed when the results may be applicable to the trichothecenes.

During the course of the study, the committee reviewed the literature on T-2 toxin and related trichothecene mycotoxins. It found that most reported information and ongoing research efforts pertained to T-2 toxin, HT-2 toxin, diacetoxyscirpenol (DAS), nivalenol, and deoxynivalenol (DON).

The committee had two major objectives:

- an examination of the chemical, environmental, and biological behavior of the trichothecene class of mycotoxins so that appropriate protective measures can be taken and

- development of the means for optimizing the protection of human beings against the effects of trichothecenes through appropriate prophylaxes and treatments.

The committee approached its task by characterizing the occurrence and natural degradation of trichothecenes; their metabolism; their adverse effects in the environment; and their toxicity in both humans and animals. These characterizations included potential methods for the specific, sensitive, and rapid detection of trichothecene toxins under battlefield conditions; the practical means for their destruction, disposal, and environmental decontamination; possible prophylaxes to prevent their toxic action in humans; and treatments for trichothecene-induced adverse health effects.

When appropriate, the committee considered the published reports of adverse human health effects in embattled areas where these agents have purportedly been used. However, its members neither support nor refute the evidence for their military use in such areas. This consideration was not contained in the committee's charge.

Much of the literature on the human health effects of trichothecenes consists of anecdotal information that is grounded in a long history of incidence in civilian settings. For example, the natural occurrence of trichothecenes in cereal grains has been recognized as a recurring health problem for many years. Accordingly, this report is intended to assist in the protection against the adverse effects of trichothecenes not only for U.S. Armed Forces but also for civilian populations that may also come into contact with these toxins during peace or war.

In addition to studying the published literature, the committee examined research programs currently under way. As an extension of this effort, it has suggested avenues for future research that is likely to lead to the development of measures to protect large populations within civilian and military contexts.

The committee's findings are presented in seven chapters. In Chapter 2, it discusses the occurrence of mycotoxins, including their natural production, natural levels of occurrence, and levels that might be expected under conditions of deliberate release. The detection, identification, and quantitation of mycotoxins, including methods of sampling, methods for rapid field screening, and methods for laboratory analysis, are described in Chapter 3. Chapter 4 examines the decontamination of mycotoxins, including the treatment of affected foodstuffs and methods of disposal. In Chapter 5, the committee describes environmental effects, including the degradation and fate of mycotoxins. The responses of biological systems to mycotoxins, including systemic effects in humans and animals, toxicokinetics, and mechanisms of action are described in Chapter 6.

These five chapters form a basis for possible approaches to prophylaxes for and treatments of humans affected by mycotoxicosis, as discussed in Chapter 7. The committee's major recommendations are contained in Chapter 8.

This report does not contain references to the entire known body of literature or to all the information that was made available to the committee. Rather, the committee cited only those references that it found to be the most pertinent to its charge. It was also aware of the possibility that there may be additional, but inaccessible information in the classified files of the Department of State and the Department of Defense. The reader is referred to the Bibliography at the end of this document for a listing of the literature reviewed by the committee.

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Chapter 2

THE TRICHOTHECENE MYCOTOXINS: THEIR STRUCTURE, NATURAL PRODUCTION, AND LEVELS OF OCCURRENCE

Trichothecene-producing fungi and their toxins are distributed throughout various regions of the world. Information about the trichothecene toxins that might be found in a specific area can be provided by knowledge of their chemistry and biosynthesis, the taxonomic characterization of fungi present in that region, the specific toxins that such fungi produce, and the environmental factors affecting the ability of fungi to produce toxins. This information will assist in selecting the best techniques for the detection, identification, and quantitation of trichothecenes.

CHEMICAL STRUCTURES OF TRICHOTHECENES

The trichothecenes are a chemically related group of biologically active fungal metabolites produced in culture by various species of Fusarium, Trichothecium, Trichoderma, Acremonium (Cephalosporium), Cylindrocarpon, Myrothecium, Stachybotrys, and possibly Dendrodochium. They all have a basic tetracyclic sesquiterpene structure, as shown in Figure 2-1. The skeletal structure includes a six-membered oxygen-containing ring, an epoxide group in the 12,13 position, and an olefinic bond in the 9,10 position. The trichothecenes bear oxygen-containing substituents located at one or more of positions 3, 4, 7, 8, and 15. These substituents may be hydroxyl, esterified hydroxyl, keto (position 8 only), or epoxide (position 7,8 only) groups or combinations thereof. The verrucarins and roridins constitute a special subgroup characterized, respectively, by a macrocyclic ester or an ester-ether bridge between positions 4 and 15.

The trichothecenes can be classified into groups according to their structural characteristics. Ueno (1980) has proposed a classification scheme consisting of four such groups, as shown in Figure 2-1 and Table 2-1. The trichothecene molecule, the simplest member of group A, has no substituted functional groups at positions 3(R_1), 4(R_2), 15(R_3), 7(T_4), and 8(R_5). Trichodermol has a hydroxyl functional group at position 4. The remaining members of group A possess hydroxyl or acyloxyl functional groups at position 3, 7, 8, or 15. Group B consists of trichothecenes possessing a carbonyl

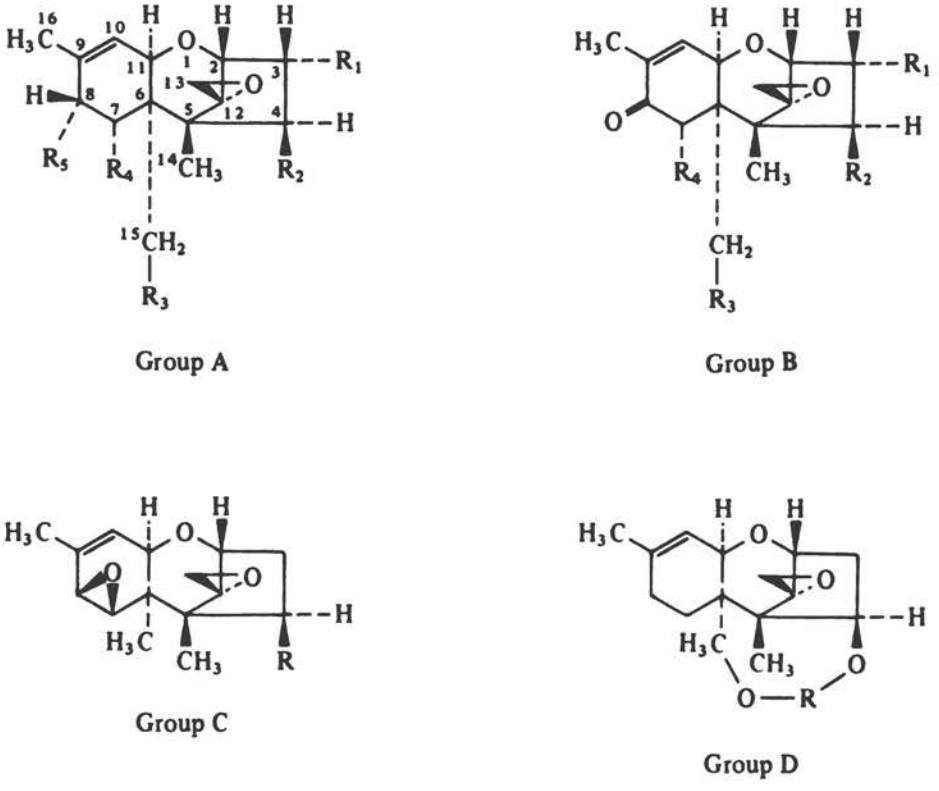


Figure 2-1. Basic Ring Structure of Trichothecenes.

functional group at position 8. Stepwise acetylation of nivalenol, the tetrahydroxylated derivative, yields mono-, di-, tri-, and tetracetylnivalenol. Group C posesses a second epoxide function at the 7,8 position. Group D comprises the macrocyclic trichothecenes.

Table 2-2 lists melting points, molecular weights, molecular extinction coefficients, and other selected physical properties of trichothecenes.

TABLE 2-1

Trichothecene Mycotoxins Listed by Structural Group[a]

Group A

Trichothecenes	R_1	R_2	R_3	R_4	R_5
Trichothecene (scirpene)	H	H	H	H	H
Trichodermol (roridin C)	H	OH	H	H	H
Dihydrotrichothecene	H	H	OH	H	OH
Scirpen-4,8-diol	H	OH	H	H	OH
Verrucarol	H	OH	OH	H	H
Scirpentriol	OH	OH	OH	H	H
T-2 tetraol	OH	OH	OH	H	OH
Pentahydroxyscirpene	OH	OH	OH	OH	OH
4-Deacetylneosolaniol	OH	OH	OH	H	OH
Trichodermin	H	OAc	H	H	H
Deacetylcalonectrin	OAc	H	OH	H	H
Calonectrin	OAc	H	OAc	H	H
Diacetylverrucarol	H	OAc	OAc	H	H
4-Monoacetoxyscirpenol	OH	OAc	OH	H	H
4,15-Diacetoxyscirpenol (DAS)	OH	OAc	OAc	H	H
7-Hydroxydiacetoxyscirpenol	OH	OAc	OAc	OH	H
8-Hydroxydiacetoxyscirpenol (neosolaniol)	OH	OAc	OAc	H	OH
7,8-Dihydroxydiacetoxyscirpenol	OH	OAc	OAc	OH	OH
7-Hydroxy-8-acetyldiacetoxyscirpenol	OH	OAc	OAc	OH	OAc
8-Acetylneosolaniol (8-Acetyl-DAS)	OH	OAc	OAc	H	OAc
NT-1	OH	OAc	OH	H	OAc
NT-2	OH	OAc	OH	H	OH
HT-2 toxin	OH	OH	OAc	H	$OCOCH_2CH(CH_3)_2$
T-2 toxin	OH	OAc	OAc	H	$OCOCH_2CH(CH_3)_2$
Acetyl T-2 toxin	OAc	OAc	OAc	H	$OCOCH_2CH(CH_3)_2$

Group B

Trichothecenes	R_1	R_2	R_3	R_4
Trichothecolone	H	OH	H	H
Trichothecin	H	$OCOCH{=}CHCH_3$	H	H
Deoxynivalenol (DON)	OH	H	OH	OH
3-Acetyldeoxynivalenol	OAc	H	OH	OH
5-Acetyldeoxynivalenol	OH	H	OH	OAc
3,15-Diacetyldeoxynivalenol	OAc	H	OAc	OH
Nivalenol	OH	OH	OH	OH
4-Acetylnivalenol (fusarenon-X)	OH	OAc	OH	OH
4,15-Diacetylnivalenol	OH	OAc	OAc	OH
4,7,15-Triacetylnivalenol	OH	OAc	OAc	OAc
Tetracetylnivalenol	OAc	OAc	OAc	OAc

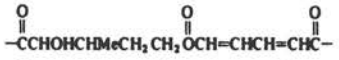
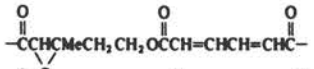
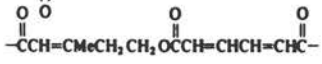
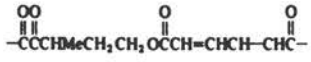
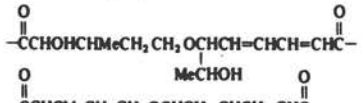
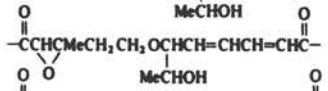
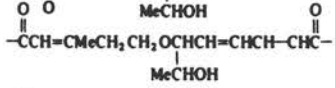
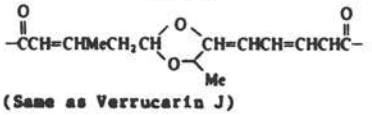
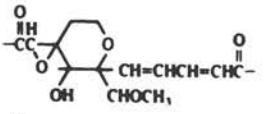
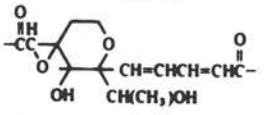
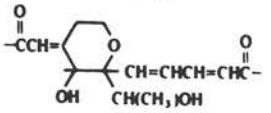
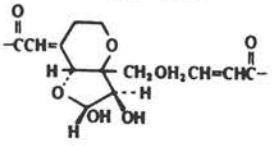
[a]Adapted from Ueno, 1980.

TABLE 2-1 (Continued)

Group C

Trichothecenes	R
Crotocol	OH
Crotocin	$OCOCH{=}CHCH_3$

Group D

Trichothecenes	R
Verrucarin A	
Verrucarin B	
Verrucarin J	
2'-Dehydroverrucarin A	
Roridin A	
Roridin D	
Roridin E	
Roridin H	
Satratoxin C	(Same as Verrucarin J)
Satratoxin D	(Same as Roridin E)
Satratoxin F	
Satratoxin G	
Satratoxin H	
Vertisporin	

Physical Properties of Selected Trichothecenes

Toxin	Description	Molecular Weight	Melting Point (°C)	$[\alpha]_D^{25}$[a] solvent	ξ Solvent, max[b]	R_f value[c]	References
T-2 toxin[d]	White needles	466	151-152	$+15_{EtOH}$[e]	[f]	0.40[g]	Bamburg and Strong, 1971
HT-2 toxin	Pale yellow oil	424	-	NR[h]	[f]	0.09[g]	Cole and Cox, 1981
Neosolaniol	Crystals	382	171-172	NR	[f]	0.22[g]	Cole and Cox, 1981
T-2 tetraol	NR	298	NR	NR	[f]	0.32[i]	Cole and Cox, 1981 Wyllie and Morehouse, 1977
Diacetoxyscirpenol (DAS)	Crystals	366	162-164	NR	[f]	0.36[g]	Bamburg and Strong, 1971 Cole and Cox, 1981 Wyllie and Morehouse, 1977
Monoacetoxyscirpenol	Crystals	324	172-173	NR	[f]	0.21[g]	Cole and Cox, 1981 Wyllie and Morehouse, 1977
Scirpentriol	Prisms	282	189-191	$0_{Acetone}$	[f]	0.35[i]	Cole and Cox, 1981 Wyllie and Morehouse, 1977
Nivalenol	Crystals	312	222-223	$+21.5_{EtOH}$	$7,500_{MeOH,\ 218}$	0.42[i]	Bamburg and Strong, 1971 Cole and Cox, 1981
Fusarenon-X	Hexagonal bipyramid crystals	354	91-92	$+64.3_{EtOH}$	$6,500_{MeOH,\ 220}$	0.77[i]	Cole and Cox, 1981 Wyllie and Morehouse, 1977
Nivalenol diacetate	Crystals	396	135-136	NR	$6,200_{MeOH,\ 220}$	0.90[i]	Cole and Cox, 1981 Wyllie and Morehouse, 1977
Deoxynivalenol (DON)	Needles	296	151-153	$+6.35_{EtOH}$	$4,500_{EtOH,\ 218}$	0.62[i]	Cole and Cox, 1981 Wyllie and Morehouse, 1977
Monoacetoxydeoxynivalenol	NR	338	185-186	$+430_{MeOH}$[j]	$5,900_{EtOH,\ 219}$	0.80[i]	Cole and Cox, 1981
Verrucarol	Crystals	266	158-159	-39_{CHCl_3}[k]	$7,900_{EtOH,\ 195}$	0.06[g]	Bamburg and Strong, 1971 Cole and Cox, 1981
Roridin A	Crystals	532	198-204	$+168.5_{CHCl_3}$	$18,600_{EtOH,\ 263}$	0.70[g]	Cole and Cox, 1981
Trichodermol (roridin C)	Needle crystals	250	116-119	-33.5_{CHCl_3}[j]	[f]	0.86[g]	Bamburg and Strong, 1971 Cole and Cox, 1981
Trichodermin	Crystals	292	45-46	-10.2_{CHCl_3}[j]	$2,400_{205}$	0.67[g]	Bamburg and Strong, 1971 Cole and Cox, 1981
Crotocin (antibiotic T)	Colorless prisms	332	126-128	$+7.17_{CHCl_3}$[j]	$21,800_{211}$	0.67[l]	Cole and Cox, 1981
Crotocol	Crystals	264	154	-6.4_{CHCl_3}[l]	$4,830_{EtOH,\ 210}$	0.50[l]	Cole and Cox, 1981

[a] The specific optical rotation at 25°C using the sodium D line.
[b] Molar extinction coefficient at specified wavelengths. More extensive IR, ^{13}C-NMR, ^{1}H-NMR, and mass spectral data can be found in Cole and Cox, 1981.
[c] A relatively polar solvent is required for thin-layer chromatography separation of more polar trichothecenes such as DON and T-2 tetraol.
[d] Solubility for all toxins similar. Soluble in acetone, ethyl acetate, chloroform, acetonitrile, ethanol, and methanol. Relatively insoluble in water and petroleum ether. Solubility varies with different toxins in benzene, toluene, and diethyl ether. (Wyllie and Morehouse, 1977).
[e] At 26°C.
[f] End absorption is less than or equal to 230 nm.
[g] Chloroform-methanol (98:2 v/v).
[h] Not reported.
[i] Chloroform-methanol (5:1 v/v).
[j] At 20°C.
[k] At 22°C.
[l] Ethanol-ethyl acetate-acetone (1:4:4).

TRICHOTHECENE-PRODUCING FUNGI

The fungi that produce trichothecenes are all members of the anamorph (i.e., asexual state) class Hyphomycetes of the subdivision Deuteromycotina (Fungi Imperfecti) (Carmichael et al., 1980). Where their teleomorphs (sexual states) are known, they are in the subdivision Ascomycotina, class Pyrenomycetes, mainly in the order Hypocreales. One or two perfect states[1] are included in the related order Sphaeriales. Of the seven imperfect[1] genera with perfect stages in the Hypocreales order, five are species of Nectria and another belongs to the closely related genus Hypomyces. The production of trichothecenes by fungi may have important taxonomic implications, suggesting close natural relationships among these otherwise diverse species of imperfect fungi.

Most of the species of imperfect fungi that produce trichothecenes without the macrocyclic ring are members of the family Moniliaceae, which includes the genera Fusarium, Acremonium, Trichoderma, and Trichothecium. This family is characterized by their hyaline or brightly colored conidia, which are usually produced in phialidic conidiogenous cells. Two important trichothecene-producing genera (Myrothecium and Stachybotrys) are morphologically different, having either dark conidia or conidiophores. Both of these genera, which are assigned to the family Dematiaceae (Ellis, 1971), produce trichothecenes with the macrocyclic "bridge." A genus belonging taxonomically in the Hypocreales order Cylindrocarpon is exceptional in that it produces macrocyclic trichothecenes (isororidin E, epoxyisororidin E, and epoxy- and diepoxyroridin H) (Matsumoto et al., 1977).

Fusarium

Seventeen of the 30 species of Fusarium listed in the manual written by Nelson et al. (in press) have been reported to produce toxins. These include F. merismoides (= F. episphaeria, in part), F. decemcellulare (= F. rigidiusculum), F. nivale, F. poae (= F. tricinctum), F. sporotrichioides (= F. tricinctum), F. chlamydosporum (= F. tricinctum), F. avenaceum (= F. roseum), F. semitectum (= F. roseum), F. equiseti (= F. roseum), F. scirpi (= F. roseum), F. acuminatum (= F. roseum), F. heterosporum (= F. roseum), F. culmorum (= F. roseum), F. graminearum (= F. roseum), F. lateritium, F. oxysporum, and F. solani. The species names in parentheses are those of the Snyder and Hansen system (Snyder and Hansen, 1940, 1941, 1945,

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[1]The perfect state is synonomous with the teleomorph (sexual) state. The imperfect state is synonomous with the anamorph (asexual) state.

1954). In that system an attempt was made to simplify the more detailed system of Wollenweber and Reinking (1935). In some cases several species from the latter system were bunched together into one species; F tricinctum and F. roseum are two notable examples of such aggregation. F. tricinctum includes F. poae, F. tricinctum sensu stricto, F. sporotrichioides, and F. chlamydosporum. Of these species F. sporotrichioides is the most potent producer of toxins and F. tricinctum sensu stricto does not produce toxins. The use of the terms F. tricinctum and F. roseum without further specification has probably been a source of confusion in attempts to identify correctly the fusaria discussed in the literature.

Another problem has been the incorrect identification of species. The F. solani isolates originally reported to produce neosolaniol have now been correctly identified as F. sporotrichioides (Ishii and Ueno, 1981). The F. nivale isolate originally used in the characterization of nivalenol, 4-acetylnivalenol, and 4,15-diacetylnivalenol has similarly been correctly identified as F. sporotrichioides (Ueno, 1980). The problems of identifying Fusarium and various proposed taxonomic systems have been discussed by Toussoun and Nelson (1968), Raillo (1950), Gordon (1952), Messiaen and Cassini (1968), Bilai (1970), Booth (1971), and most recently by Marasas et al. (in press) and by Nelson et al. (in press).

The conidia in species of Fusarium are endoconidia produced on either monophialides or polyphialides. They may occur as 0 to 3 septate, pyriform, fusoid-to-oval microconidia and as straight or curved 3 to 10 or more septate macroconidia. The presence or absence of microconidia and chlamydospores together with the shape of both the macroconidia and microconidia are used to delineate sections, groups, or species (Booth, 1971; Snyder and Hansen, 1941, 1945; Toussoun and Nelson, 1968). Of the perfect stages recognized in the 30 Fusarium species (Nelson et al., in press), nine are species of Gibberella, two of Calonectria, two of Nectria, and one of Micronectriella (Booth, 1971).

The fusaria occur widely in nature on many hosts and substrates and are among the most common of all the fungi. Many of the species are parasitic on higher plants and cause serious economic losses. These aspects have been discussed by Booth (1971) and by others.

The associations of fusaria with mycotoxicoses are well known. Detailed reviews of these relationships have been written by Bamburg and Strong (1971), Bilai (1970), Joffe (1965), Mirocha et al. (1971), Saito and Tatsuno (1971), Smalley et al. (1970), and Ueno (1980), among others.

Trichothecium

In the genus Trichothecium, only T. roseum--one of approximately four species--is known to produce toxic trichothecenes. A monograph describing this small genus was published by Rifai (1966). T. roseum, the major species in this genus, is distributed worldwide. It usually occurs as a saprophyte on decaying vegetable matter. Occasionally, it becomes a weak or secondary parasite on stored fruits and vegetables (U.S. Department of Agriculture, 1960).

Trichoderma

Of approximately 20 species recognized in the genus Trichoderma, only T. viride and T. lignorum are known to produce trichothecenes (von Arx, 1974). In a monograph on this genus, however, Rifai (1969) has reduced the number of acceptable species to nine, thereby leaving only T. viride as a producer of trichothecenes in this genus.

Species of Trichoderma are commonly isolated from soil, decaying wood, and other moist vegetable debris (Rifai, 1969). T. viride can frequently be isolated from moldy corn or other moldy feeds and is often associated with mycotoxicoses (Smalley, personal observations, 1973). Members of this genus are mainly saprophytic, but T. viride apparently has limited parasitic capabilities (U.S. Department of Agriculture, 1960).

Taxonomically, the conidiogenous cells in Trichoderma are monophialidic with hyaline to green nonseptate conidia that gather in moist balls at the mouth of the phialide (Rifai, 1969). Most of the species accepted as belonging to the Trichoderma genus have perfect stage counterparts, which all belong to the genus Hypocrea of the Hypocreales order (Rifai, 1969).

Acremonium

In the genus Acremonium (= Cephalosporium), only A. crotocinigenum --one of approximately 20 to 70 species variously ascribed to this genus--has been shown to produce toxic trichothecenes (Achilladelis and Hanson, 1969; Schol-Schwarz, 1965). It is unique in that it produces a trichothecene with a second epoxide in the basic structure (see Table 2-1). Since many other species of Acremonium have been shown to produce antibiotics (Roberts, 1952), and since trichothecenes have antibiotic properties, other toxic trichothecene producers may ultimately be found in nature.

Nomenclatural problems also exist in this genus. Perfect stages of Acremonium species that have been described are members of the

Ascomycotina (Ascomycetes) class, either in the order Eurotiales genus Emericellopsis (Backus and Orpurt, 1961; Durrell, 1963), in the order Hypocreales genus Nectria (von Arx, 1974), or elsewhere (Wallrothiella, Mycocitrus, Pelaronectriella, Hyprocrea, Trichosphaerella, and others) (Carmichael et al., 1980).

Conidiogenous cells in Acremonium are monophialidic; the phialides arise directly and singly from the vegetative hyphae and produce nonseptate hyaline conidia borne in balls (Barron, 1968). Members of this genus occur widely in nature, both as saprophytes and, occasionally, as parasites on plants and animals.

Cylindrocarpon

The genus Cylindrocarpon is closely related to Fusarium, separated from it only by the absence of a foot cell bearing some kind of heel (Booth, 1971, p. 11). The genus is characterized by large, hyaline, multiseptate macrospores with ends borne in phialides. Cylindrocarpon species are common in soil and on twigs, leaves, or fruits of many plants. Perfect stages are recognized in Nectria, Calonectria, and others (Carmichael et al., 1980). The genus has been described in a monograph by Booth (1966); several new species have been reported more recently by Carmichael et al. (1980).

The single unspecified Cylindrocarpon sp. reported to produce macrocyclic trichothecenes is apparently rare. None of several Cylindrocarpon isolates derived from moldy feeds studied in Wisconsin proved to be toxic (Smalley, 1983).

Myrothecium and Stachybotrys

In the genus Myrothecium, two of the 13 recognized species, M. roridum and M. verrucaria, have been intensively studied for their toxic trichothecene-producing capabilities (Bamburg and Strong, 1971; Böhner et al., 1965; Domsch and Gams, 1972; Härri et al., 1962; Traxler and Tamm, 1970; Traxler et al., 1970). In the other dematiaceous genus, Stachybotrys, only one of the eight recognized species (S. atra = S. alteranans) (Ellis, 1971) has been shown to produce toxic trichothecenes (Rodricks and Eppley, 1973). These genera also differ from the other known trichothecene producers in that their toxins, called verrucarins and roridins, possess a unique "bridge structure" as a part of the basic trichothecene moiety (Bamburg and Strong, 1971). (See Table 2-1.)

The conidiogenous cells in Myrothecium are phialides, borne in sessile sporodochia, and the conidia they produce are hyaline to pale olive in color and are nonseptate (Tulloch, 1972). In Stachybotrys,

the conidiogenous cells are also phialidic, but cluster in groups at the apex of each hyphal stipe or branch. The conidia are nonseptate, hyaline to pale olive, and aggregate in large, slimy heads that are often black and glistening (Bisby, 1945; Ellis, 1971; Verona and Mazzucchetti, 1968). The perfect stage, and the only one known for Myrothecium, is in the genus Nectria in the order Hypocreales (N. bactridioides) (Tulloch, 1972). In the genus Stachybotrys, the only described perfect stage was identified as Melanopsammina in the order Sphaeriales (M. pomifromis) (Barron, 1968; Booth, 1957).

Members of the genus Myrothecium are commonly isolated from soils, especially from those high in organic matter (Ellis, 1971). One species (M. inundatum) is found only on dead mushrooms (Russula ajusta), whereas most of the common species have been found on dead and dying leaves of various plants (Tulloch, 1972). Most of the species carry on a saprophytic existence, but one species (M. roridum) is a plant pathogen causing serious leaf spots and diebacks on a number of hosts in temperate and tropical regions.

Studies of verrucarins and roridins--the toxic trichothecenes produced by Myrothecium--have been focussed mainly on antibiotic production and cytotoxicity. Toxic fungi used in these studies have generally not been obtained from foods or feeds involved in animal intoxication, but, rather, fungus isolates from type culture collections have been used in industrial screening trials .

Bilai and Pidoplichko (1970) have described two species of Dendrodochium (D. toxicum and D. caucasicum) in their book on mycotoxins and mycotoxicoses. One of these (D. toxicum) has been studied extensively as the cause of a trichothecene-like intoxication in farm animals in the Soviet Union. Tulloch (1972) suggests that D. toxicum is synomymous with Myrothecium roridum. Both Carmichael et al. (1980) and Barron (1968) consider Dendrodochium as a distinct genus closely related to the genera Myrothecium and Volutella. As with Myrothecium, its perfect state is Nectria. Dendrodochium is occasionally observed in soil, but is more commonly found growing saprophytically on bark.

Some of the known species of Stachybotrys occur naturally on moist, decaying tree branches in the tropics. These species include S. theobromae, S. nephrospora, S. cylindrospora, S. kampalensis, and S. dichroa) (Ellis, 1971). S. parvispora has been observed on decaying leaves of various tropical plants. S. atra is found widely on paper, seeds, soil, textiles, dead grass, straw, and forage matter (Barron, 1968; Ellis, 1971). There have been no reports that they are parasitic on plants.

FACTORS AFFECTING NATURAL PRODUCTION OF FUSARIUM TOXIN

The natural production of trichothecenes is affected by the physiology of the fungi and also by certain environmental and genetic

factors. To explore these factors, the committee focused on the following four issues with emphasis on Fusarium:

- principles of secondary metabolite biosynthesis of mycotoxins;
- in-vitro biosynthesis of trichothecenes by Fusarium;
- production of Fusarium toxins (trichothecenes and zearalenone) in nature; and
- implications of the in-vitro data for large-scale production of Fusarium toxins.

Principles of Mycotoxin Biosynthesis

The literature relating to secondary metabolism of fungi is too vast to summarize in this report. Thus, this discussion is intended to present only basic ideas relating to mycotoxins. Fungi, especially Deuteromycetes, are prolific in their secondary metabolites (> 1,500 compounds are known). Determination of the exact mechanism by which each one is synthesized is a problem of immense proportions. Useful reviews of this subject have been written by Bu'Lock (1975, 1980), Demain (1972), Martin and Demain (1980), and Malik (1982).

Primarily metabolism of fungi comprises the basic biochemical activities of the cell (e.g., glycolysis and the tricarboxylic acid cycle). If all nutrients are supplied in the appropriate quantities for optimum biomass production, the fungal cells will grow rapidly and virtually no secondary metabolites will be biosynthesized. In liquid cultures, the depletion of one or more nutrients induces the stationary phase, during which growth is "unbalanced" and the production of secondary metabolites is possible. The type and degree of nutrient exhaustion influence the secondary metabolites produced. The age of hyphae also influences this process. The ability of hyphae to produce secondary metabolites in the stationary phase varies (Minaeva and Maksimova, 1982).

Increasing the complexity further, physical factors such as oxygen tension, light, pH, and temperature also play a critical role in secondary metabolism. For example, an initial temperature of < 22°C is required for the induction of zearalenone synthesis by Fusarium graminearum (Hidy et al., 1977). Moreover, interactions between the factors may be important determinants of biosynthetic pathways. For example, temperature affects oxygen tension, and small changes in oxygen tension are known to have potent biological effects (Shih and Marth, 1974).

The final and most daunting aspect is the enzymology of secondary metabolism. Little is known about it. Many secondary metabolites are rather large molecules, and their biosynthesis can involve the sequential action of a number of enzymes, some of which are relatively

nonspecific. This means that a particular enyzme can react with several precursors and may even re-react with the products. The net effect is that biosynthetic pathways are often complicated by a complex matrix of possible reactions.

In summary, secondary metabolism in fungi is controlled by four overlapping factors: the age of the hyphae, the type and degree of nutrient exhaustion, the physical conditions, and the effects the physical and chemical factors have exerted on the enzymes of secondary metabolism with regard to which pathways are selected and, hence, which metabolites will be produced. Small differences in the chemical and physical conditions can result in dramatic changes in the quantity and composition of various dominant secondary metabolites as well as variations in the many minor metabolites.

In-Vitro Production of Trichothecenes by Fusarium

In one of the first reports of a trichothecene being produced in large quantities, the formation of T-2 toxin by solid substrate fermentation was described (Burmeister, 1971). In this study, white corn grits were inoculated with Fusarium tricinctum and incubated for 3 weeks at 15°C. Gram quantities were produced for animal feeding studies. The amount of toxin formed in the grits decreased as the incubation temperature was raised to 20°C, 25°C, and 32°C. More recently, Cullen et al. (1982) reported that strains of Fusarium produced high levels of T-2 toxin when cultured on certain media absorbed on vermiculite. Modified Gregory medium (2% soya meal, 0.5% corn steep liquor, 10% glucose) inoculated with F. tricinctum T340 strains yielded maximum (714 mg/liter) T-2 toxin production within 24 days at 19°C. Vogel synthetic medium N (Vogel, 1956) supplemented with 5% glucose yielded 353 mg/liter after 12 to 14 days of incubation at 15°C.

The usual method for producing deoxynivalenol (DON) is to grow F. graminearum on sterilized corn or rice. Various studies have been conducted to determine the effects of the isolate, moisture content, the type of grain used, temperature, and other factors on yield (Vesonder et al., 1982). Although this method is simple and results in the production of reasonable quantities of DON, it is slow (incubation times are long), tedious, not amenable to scaling up to large quantities, and produces culture extracts that require extensive cleanup. Another simple way to obtain material containing reasonable quantities of DON is to inoculate field corn with a toxigenic isolate of F. graminearum, allow it to grow for 2 months, and then harvest it. Other trichothecenes, such as T-2 toxin, can be produced on nutrient-saturated vermiculite (Cullen et al., 1982). However, all these methods give little information that furthers our understanding of the process of biosynthesis. Knowledge of the correct conditions

for the production of a specific trichothecene in liquid culture is necessary for studies of biosynthesis and for large-scale production (e.g., in fermentators).

Fusarium trichothecenes produced in liquid cultures include DON (Miller et al., in press a; Wu et al., 1981; Yoshizawa and Morooka, 1975), fusarenon X (Ueno et al., 1970), nivalenol, T-2 toxin, neosolaniol (Ueno et al., 1975), 3-acetyldeoxynivalenol (Blight and Grove, 1974; Miller et al., in press a), 15-acetyldeoxynivalenol (Miller et al., in press a), 4,15-diacetoxyscirpenol (DAS) (Brian et al., 1961), and numerous minor metabolites. Japanese investigators (Ueno et al., 1970, 1975; Yoshizawa and Morooka, 1975) have been the most successful users of this strategy. In most cases, however, they have accepted very low yields and produced large volumes of culture for extraction.

Most of the knowledge about the large-scale production of DAS is considered proprietary by industry and is therefore inaccessible. The best available information on medium-yield liquid culture production of a trichothecene is the work on 3- and 15-acetyldeoxynivalenol and DON (Miller et al., in press a).

The physical-chemical environmental factors needed for the induction of specific secondary metabolites must be considered when inducing Fusarium to produce trichothecenes such as DON. There are two other aspects that must also be considered. One is the necessity of using a strain of Fusarium that produces at least some DON. Once a system showing good yields is developed, isolates can be tested and mutated to improve yields. The second is to derive the appropriate medium, which at present is an empirical procedure. However, it is possible to identify types of useful media for the production of metabolites produced via similar pathways (Demain, 1972). It is also useful to consider the composition of the substrates on which the fungus produces the desired toxin in nature.

Ueno et al. (1970, 1975) have been fairly successful in producing various trichothecenes by using a protein nitrogen source. These investigators have demonstrated that aeration and carbohydrate concentration are also important in the biosynthesis of trichothecenes by Fusarium. Miller et al. (in press a) have shown that the biosynthesis of DON is induced by carbohydrate exhaustion, a narrow pH range, relatively low oxygen concentrations, and a requirement for amino acid nitrogen. Figure 2-2 shows some of these phenomena in the synthesis of 15-acetyldeoxynivalenol (15-A-DON) and DON by F. graminearum M69. Carbohydrate exhaustion precedes significant mycotoxin production. The production of 15-A-DON occurs when the cells are in the stationary phase and are going into the death phase.

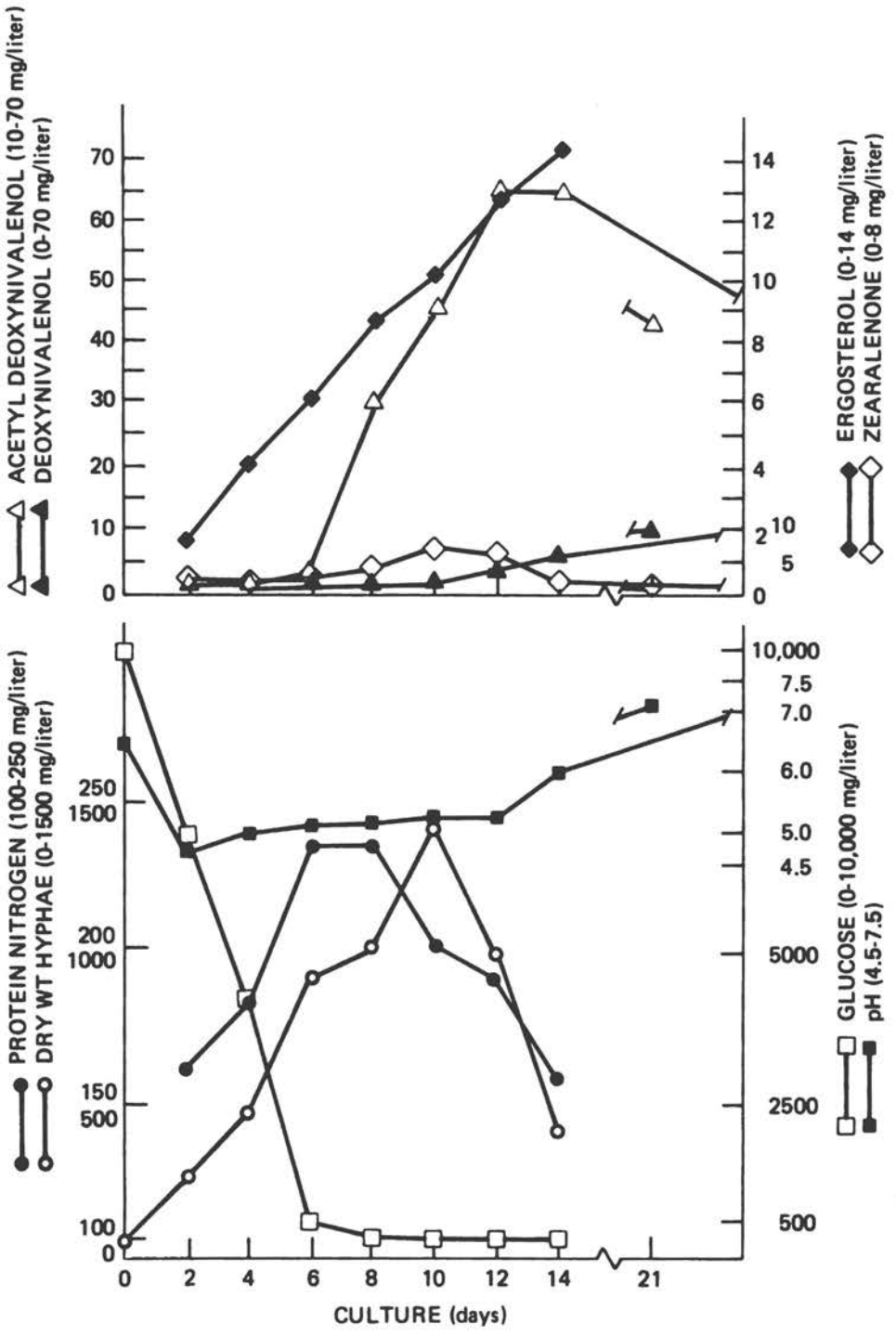

Figure 2-2. Formation of 15-A-DON, DON, and zearalenone with time relative to pH, dry weight, protein, glucose, and ergosterol concentrations in the medium. (Miller, et al., in press a).

Many tests have been performed with fully defined media in this fermentation, and the specific changes in the semidefined medium used during the course of the fermentation have been analyzed. These studies have shown that the synthesis of DON, at least for appreciable yields, requires protein or amino acid nitrogen. Studies on the effects of different plant carbohydrates on toxin production and, more importantly, toxin ratios have shown that major and minor mycotoxin yields are extremely variable, depending on the carbohydrate supplied. (Compare Ueno et al., 1970 and Ueno et al., 1975.) This would be expected because the metabolism of different sugars results in the accumulation of different primary metabolites.

For various reasons, liquid culture methods are more useful for the detection of minor metabolities. Studies using these methods with F. graminearum and F. roseum ATCC 28114 at Agriculture Canada in Ottawa and at the National Research Council in Halifax have led to the identification of DAS, T-2 toxin, a then new metabolite 15-A-DON, 3-acetyldeoxynivalenol (3-A-DON), DON, another as yet unnamed but well-characterized trichothecene produced in appreciable quantities, a number of undescribed trichothecenes produced in lesser quantities, and precursor metabolities of DAS and T-2 (Miller, et al., in press a). F. graminearum had not previously been thought to produce some of the aforementioned compounds (Ichinoe et al., in press). The North American isolates of F. graminearum tested did not produce 3-A-DON--the known intermediate of DON formation (Tamm and Breitenstein, 1980; Yoshizawa and Morooka, 1975). All 25 isolates tested by the Canadian investigators to date also produce appreciable quantities of butenolide lactone.[2]

As noted above, the isolates of F. graminearum tested in North America produce 15-A-DON, in contrast to Japanese isolates of F. graminearum, thus demonstrating the variability of the pathways to DON in that species. Studies using carbon-13 compounds have shown that the labels are not always found on the same part of the DON molecule, further illustrating the profound variability of intermediate metabolites.

The Production of Fusarium Toxins (Trichothecenes and Zearalenone[3]) in Nature

Little has been done to determine the mechanism and conditions required for the formation of Fusarium toxins in the field and in

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[2]Miller, J. D. 1983. Personal communication. Agriculture Canada, Ottawa, Canada.

[3]Zearalenone formation and occurrence are also considered in this discussion because this toxin has often been reported along with trichothecenes in field outbreaks. The presence of zearalenone in a feed or cereal grain is regarded as indicative not only of Fusarium invasion but also of the possible presence of trichothecenes.

storage. Some reports of mycotoxicosis outbreaks caused by Fusarium species have related toxin formation in the temperate zones to cold, wet weather. Corn produced in the United States during the unusally cool and wet summer of 1972 was heavily infected with Fusarium graminearum, and a relationship existed between the fungal infection and outbreaks of feed refusal and emesis in swine (Tuite et al., 1974). Low temperatures (below 21°C) and 9 days of rain during the silking period were suggested as ideal conditions for the fungal outbreak. DON, believed to be responsible for feed refusal and emesis, was isolated from upper midwestern corn grown in 1972 (Vesonder et al., 1973, 1976).

In a study of wheat grown in Virginia in 1975, investigators found zearalenone in 19 of 42 samples. Later, DON was detected in several of the zearalenone-positive samples (Shotwell et al., 1977). The weather was unusually cold and rainy during the 1975 growing season for wheat in Virginia. During the next 5 years, 1976 to 1980, such weather did not occur, and when the wheat was examined for zearalenone, it was found to be negative (Shotwell and Hesseltine, in press).

Some Fusarium species severely infect maturing grain sorghum during warm, very humid weather (Schroeder and Hein, 1975). An isolate of Fusarium from head-blighted sorghum produced 10 times as much zearalenone when grown at a constant 25°C for 19 days than when grown for 6 days at 25°C followed by 13 days at 10°C -- conditions commonly used for zearalenone production.

Most studies of trichothecene and zearalenone formation have been designed to produce maximum yields under laboratory conditions for feed trials. Too little effort has been made to simulate field conditions for toxin formation. Bennett et al. (1981) produced 30 μg/g zearalenone and approximately 20 μg/g DON by injecting corn ears in the field with Fusarium isolates. The corn was harvested and stored unprotected for one winter. A new trichothecene isolated from this corn had one less hydroxyl group than DON. No effort was made in this study to relate weather conditions to toxin production. However, data from 1 year's production of a mycotoxin in the field have little meaning because of the many uncontrollable variables.

In the detailed field study conducted by Miller et al. (in press b), corn ears were infected with an F. graminearum isolate that only produced 15-A-DON in vitro. They found that fungal biomass peaked several weeks before harvest. The dominant toxin in the infected ears was DON, indicating that the plant had hydrolyzed the A-DON to DON. In-vitro studies have shown that leaf tissue of the hybrid of corn used in this field experiment has the ability to hydrolyze A-DON to DON. The field study also indicated that DON concentrations declined with the decrease in biomass, suggesting that plant enzymes further hydrolyzed DON. This also seems to occur in vitro. The possibility

that plant enzymes may play a role in the formation and concentration of trichothecene toxins in crops was suggested in 1975 (Yoshizawa and Morooka, 1975). This phenomenon has also been observed with macrocyclic trichothecenes (Jarvis et al., 1981) and less complex trichothecenes (Ghosal et al., 1982).

Implications of In-Vitro Data for Large-Scale Production of Toxins by F. graminearum

As suggested earlier, liquid culture methods open up the possibility for large-scale production of Fusarium trichothecenes. Agriculture Canada and the Atlantic Regional Laboratory of the National Research Council of Canada operate a batch production system for the production of A-DON, which is then chemically converted to DON (an approximately 90% overall yield) for chemical and toxicological studies. Batch cultures can yield more than 100 mg/liter broth (approximately 100,000 μg/g dry weight) of A-DON in 8 days, as well as some DON and many other trichothecenes. This route was chosen because A-DON is easier to crystallize and purify than DON. Somewhat higher yields could be obtained with the isolates used. In principle, it is possible to derive isolates that could increase the yield of any trichothecene. The production of various trichothecenes in fermentors is also entirely feasible.

NATURAL LEVELS OF TRICHOTHECENES

Fusarium species occur worldwide in habitats as diverse as deserts, tidal salt flats, and alpine mountain regions. They are widely distributed in the tropics (Bugnicourt, 1939; Wollenweber et al., 1925); however, the extent to which trichothecene-producing species of Fusarium or other genera occur in the tropics has not been well defined. There have been reports of naturally occurring T-2 toxin in India (Bhat et al., 1978; Rukmini and Bhat, 1978), zearalenone and DON in Zambia and the Transvaal province of South Africa (Marasas et al., 1977), and macrocyclic trichothecenes in Brazil (Jarvis et al., 1981; Kupchan et al., 1977). There are few other data on trichothecene levels in the tropics.

In general, studies on the occurrence of trichothecenes have been limited and, for the most part, performed in developed countries. Although perhaps close to 100 derivatives of trichothecenes have been characterized in the laboratory, general information regarding natural occurrence exists for only four of these -- T-2 toxin, DON, DAS, and nivalenol. Table 2-3 summarizes the results of these studies. They provide a reasonable estimate of trichothecene levels that can be expected to occur naturally on agricultural commodities under favorable conditions of mold growth. One can observe from the table that, with few exceptions, the concentrations range from near zero to 10 μg/g.

TABLE 2-3

Reported Natural Occurrences of Trichothecenes: Levels and Location

Trichothecene[a]	Concentration (μg/g)	Feedstuff	Country	References
DON	1.8	Corn	USA	Mirocha et al., 1976
DON	3	Corn	USA	Vesonder et al., 1973
DON	0.5-10.7	Corn	USA	Vesonder et al., 1978
DON	8	Corn	USA	Ishii et al., 1975
DON	15-40	Corn	USA	Vesonder et al., 1976, 1979
DON	8	Corn	USA	Yoshizawa and Morooka, 1977
DON	12	Corn	USA	Forsyth et al., 1977
DON	1.0	Mixed feed	USA	Mirocha et al., 1976
DON	7.9	Corn	Canada	Vesonder and Ciegler, 1979
DON	1.4	Feed	Canada	Vesonder and Ciegler, 1979
DON	0.3-8.5	Winter wheat	Canada	Trenholm et al., 1983
DON	0.6	Corn	France	Jemmali et al., 1978
DON	1.3-7.9	Corn	Austria	Vesonder and Ciegler, 1979
DON	1-20	Corn	Austria	Lew et al., 1979
DON	7.4	Corn	Zambia	Marasas et al., 1977
DON	0.25-4.0	Corn	Transkei	Marasas et al., 1979
DON	2.5	Corn	South Africa	Marasas et al., 1977
DON	5	Barley	Japan	Yoshizawa and Morooka, 1977
T-2	2.0	Corn	USA	Hsu et al., 1972
T-2	0.08	Mixed feed	USA	Mirocha et al., 1976
T-2	25	Barley	Canada	Puls and Greenway, 1976
T-2	0.02	Corn	France	Jemmali et al., 1978
T-2	4.0	Corn	India	Ghosal et al., 1978
T-2	Not reported	Safflower	India	Ghosal et al., 1977
T-2	Not reported	Sorghum	India	Rukmini and Bhat, 1978
DAS	0.5	Mixed feed	USA	Mirocha et al., 1976
DAS	31.0	Corn	Germany	Siegfried, 1977
DAS	1.0-1.5	Corn	Hungary	Ueno, 1980
DAS	14	Corn	India	Ghosal et al., 1978
DAS	Not reported	Safflower	India	Ghosal et al., 1977
NIV	4.8	Corn	France	Jemmali et al., 1978
NIV	1.0-4.0	Barley	Japan	Yoshizawa and Morooka, 1977

[a]DON = Deoxynivalenol; DAS = Diacetoxyscirpenol; NIV = Nivalenol.

Reported levels of occurrence may be generally biased toward higher mean levels than may actually exist. This is so because most measurements have been made in response to reports of animal illnesses resulting from the ingestion of contaminated foodstuffs or the appearance of moldy discoloration in affected agricultural products. In a recent study of DON levels in hard red winter wheat, investigators from the Northern Regional Research Center of the U.S. Department of Agriculture collected 161 samples from regions of Nebraska and Kansas known to have scabby wheat in 1982 (O. L. Shotwell, 1983, personal communication). These samples were not collected in response to reported effects from trichothecenes. Rather, they were selected randomly so that they would be representative of the entire wheat crop grown in the region. Of the samples analyzed, 89% contained $\leq$ 4 µg/g, 67% contained $\leq$ 2 µg/g, and 42% contained $\leq$ 1 µg/g. The average DON level in Grade U.S. No. 1 wheat was 0.77 µg/g.

As Hesseltine (1976) and Jarvis (1971) have indicated, the levels at which mycotoxins occur is a complex function of physical, chemical, and biological factors. The concentration of trichothecenes in a given area can change rapidly when any of these factors change.

ESTIMATED LEVELS OF TRICHOTHECENES FROM POSSIBLE MILITARY USE

In order to proceed with the selection of the most appropriate techniques for detecting, identifying, and quantitating trichothecenes and the evaluation of measures to protect exposed populations from their deleterious effects, the committee estimated the possible levels of exposure that might result from their deliberate release. Using the estimated exposure levels of other chemical agents and the relative LD_{50} values for those agents,[4] the committee estimated that the offensive use of trichothecenes could produce concentrations of approximately 1 g/m^3 in the exposure cloud, and 1 g/m^2 on the ground.[4] To compare these levels with the natural level of occurrence, one can estimate the concentration of trichothecene per square meter of an infected cornfield. A productive crop yields about 800 g of husked corn per square meter.[5] Assuming a concentration of 10 µg/g, there would be an average of 0.008 g (8,000 µg) of trichothecene per square meter of cornfield -- more than two orders of magnitude less than might be expected from deliberate release.

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[4]The source material was taken from Chemical and Bacteriological (Biological) Weapons and the Effects of Their Possible Use, a report published by the United Nations (1970).

[5]A yield of 100 bushels of corn per acre equals 0.024 bushels per square meter. Each bushel contains about 70 lbs. of husked corn; 0.024 bushels contains 1.7 lbs. There are 454 g in a pound; 1.7 lbs. equals 772 g (U.S. Department of Agriculture, 1981).

CONCLUSIONS AND RECOMMENDATIONS

More than 50 trichothecenes have been isolated and characterized either from laboratory cultures or from natural sources. It is likely that other trichothecenes remain to be discovered. Therefore, studies should be conducted to isolate and identify such presently uncharacterized trichothecenes.

Trichothecenes occur naturally as mixtures rather than as individual substances. In most reports of natural occurrence, T-2 toxin, DAS, nivalenol, and DON have been identified. The quantity of trichothecenes produced, and possibly the composition of such mixtures, is influenced by the genetic potential of the producing fungi, temperature, humidity, and the growth medium. Studies should be conducted to determine the composition of the naturally occurring mixtures and to measure the quantity of T-2, DAS, nivalenol, DON, and other known toxic trichothecenes in those mixtures.

Taxonomic groups of fungi known to produce trichothecenes are globally distributed; however, naturally occurring trichothecenes have been reported in relatively few countries. Thus, a program should be undertaken to obtain a better characterization of the global distribution of these toxins.

More studies are also needed to determine the mechanism by which trichothecenes form in nature.

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Chapter 3

DETECTION, IDENTIFICATION, AND QUANTITATION

A variety of techniques are available for use in the detection, identification, and quantitation of trichothecene mycotoxins in different environments. Selection of the most effective techniques is dependent on knowledge of the types and estimated concentrations of the trichothecenes that are present, as discussed in Chapter 2.

SAMPLING

There are several reasons for collecting samples for the analysis of mycotoxins, especially trichothecenes, in war zones. The most important reason is to protect civilians and military personnel from adverse effects resulting from exposure to chemical warfare attacks. To initiate remedial actions immediately, it is necessary to collect samples as soon as possible after an attack to determine the toxins used. Such samples would include wipe samples taken from surfaces of equipment, houses, clothes, and food supplies--from anything in direct line of the chemical attack that would be a measure of the exposure. Part of these samples would be assayed by rapid screening tests that could be performed in the field or at stations set up near combat areas. Results of the sample analyses must be obtained before appropriate detoxification procedures can be selected to eliminate further hazards to military personnel and civilian populations. Another important reason for sample collection is to provide direct evidence to the countries involved and to world organizations that such an attack did indeed occur.

Urine, blood, and biopsy samples must be taken to determine the extent of mycotoxin exposure. The results would be used in choosing the course of treatment to alleviate adverse effects. Autopsy samples should also be collected to study the ultimate effect of trichothecenes and zearalenone and to provide further evidence of a chemical warfare attack. Finally, environmental samples should be taken to evaluate contamination of vegetation, water, soils, and food sources, such as crops, after an attack. Residual trichothecenes that persist in the environment must be eliminated by appropriate detoxification procedures. Control samples corresponding with the

vegetation, soil, water supplies, crops, and food samples should be collected in nearby areas where chemical attacks have not taken place to assess possible occurrence of natural trichothecenes.

Because there is such a diversity of samples to be collected in a war zone, it is not possible to design one protocol or to make one set of recommendations such as those used in collecting samples of agricultural commodities for mycotoxin analysis. For example, to obtain a 50-g subsample for the analysis of aflatoxin in corn, Davis et al. (1980) recommended that a 4.5 kg primary sample be taken by probing a lot (e.g., from a truck load, a loaded barge, or a silo) of corn at at least five representative points or by using a continuous or stream sampler at a spout, elevator leg, or conveyor belt as the lot is being moved. After chemical attack, bulk samples of grains and food supplies should be collected in this manner, then ground sufficiently fine to pass through a No. 20 sieve and thoroughly blended, thereby providing a subsample for analysis. Some small samples should be selected if it appears likely that they are very contaminated. Others, e.g., environmental samples, should be collected in such a manner that they are representative of large lots, including water supplies, foods, crops, and vegetation. The number of samples collected will be determined by the situation or opportunity, but as many as possible should be obtained without compromising proper handling. Sample size will be determined by the type of material or circumstances. A small "wipe" sample from surfaces in the center of a chemical attack is sufficient for analysis.

After collection, sample integrity must be maintained until analysis. Although trichothecenes are relatively stable, there can be chemical changes such as oxidation, acid hydrolysis, and instability in a specific matrix. Biological degradation would take place in the presence of microorganisms, bacteria, yeasts, fungi, and a little moisture. There will be no additional trichothecene production in undried samples unless trichothecene-producing fungi are present.

Porous bags made of burlap, paper, or cloth should be used to collect samples, and care should be taken to ensure that they do not become wet. Plastic water-tight containers provide perfect fermentation vessels when they contain a little moisture, microorganisms, and organic material. Although these containers can be used for liquids, water-tight, air-tight plastic containers should be used for solids only when they contain drying agents such as Drierite (calcium sulfate with an indicator) or highly active silica gel. Some grocery products are currently protected in this manner. Low concentrations (0.1-0.5%) of propionic acid have been used to suppress microbial growth during grain storage. Animal feeds have been protected from microbial contamination and spoilage by the use of a solid product called Monoprop (a 1:1 mixture of propionic acid and an inert support, which is a horticultural vermiculite). The stability of trichothecenes in the presence of low concentrations of

propionic acid should be tested before use because aflatoxin levels in corn have decreased during storage with Monoprop (Shotwell et al., 1983).

In general, solid samples obtained for the analysis of trichothecenes should be shipped dry and protected from moisture. Most samples, especially body tissues, are best preserved by shipping them in dry ice. Since blood cannot be frozen if cells are to be analyzed separately from the serum, samples should be refrigerated and heparin or ethylenediaminetetraacetate (EDTA) added to prevent clotting. Sodium azide added to milk samples stabilizes them for mycotoxin analysis; the milk samples must be refrigerated rather than frozen. Lyophilization (freeze-drying) has been used to remove water to obtain stable solids; however, the process affects the analysis of aflatoxin in dairy products and tissues, possibly because aflatoxin complexes with proteins in the samples. Lyophilization may not interfere with trichothecene analysis. Time in transit must be minimized to ensure that the time between sample collection and sample analysis is as short as possible.

CONSIDERATIONS IN ANALYSIS

The detection and determination of trichothecenes in samples implicated in chemical warfare are difficult because those samples are not, or cannot be, as easily defined as samples routinely collected from grains, foods, and mixed feeds. Because mycotoxin levels are likely to be much higher in chemical warfare samples than in naturally contaminated agricultural commodities, however, it may be possible to use analytical procedures with higher detection limits. The limiting factor in the analysis of chemical warfare samples will be the sample size available in the field. In Chapter 2, the committee estimated that levels of 1 g/m^2 (100 $\mu g/cm^2$) are likely to be found in such situations. Since there are standard detection methods with sensitivity to submicrogram levels, the size of the sample would probably not have to be large.

Much of the methodology developed for detecting and determining trichothecenes in agricultural commodities is applicable to all types of samples. However, the procedures require sophisticated equipment and have always been time-consuming and complicated primarily because the physical and chemical properties of these substances (e.g., their inability to fluoresce) preclude the use of simple analytical methods at the present time.

The criteria used to evaluate methods for determining trichothecenes in cereal grains, foods, and feeds were outlined by Scott (1982). The percent recovery of trichothecenes added to uncontaminated samples should be $\geq$ 70%, with a coefficient of

variation of ≤ 30% and a detection limit below 0.1 μg/g sample. The detection limit for samples to which trichothecenes have been deliberately added could be higher than the limit for naturally contaminated cereals, foods, or feeds. All screening and quantitative analytical procedures for mycotoxins involve three steps: sampling and extraction, purification of the extract if necessary, and detection or quantification of the mycotoxin (trichothecenes) in the partially purified extracts.

RAPID FIELD SCREENING

The requirements for a rapid field screening method are minimum equipment, disposable items such as plastic test tubes, relatively nontoxic reagents or chemicals that do not require special handling, and minimal assay time. Rapid screening tests are used widely to detect aflatoxins, which are carcinogenic mycotoxins, in corn, peanuts, and pistachio nuts at the earliest buying stages. In suspect corn lots, for example, aflatoxins can be identified on farms or at country elevators and terminals by inspection with a 365-nm ultraviolet light ("black light") in less than 5 minutes (Shotwell and Hesseltine, 1981; Shotwell et al., 1972). The presence of these substances can also be established by a "minicolumn" screening method in 10 minutes (Association of Official Analytical Chemists, 1980). The minicolumns are prepacked, and all items for the test are supplied in kits. Another method, thin-layer chromatography (TLC), can be simplified for use under field conditions. The enzyme-linked immunosorbent assay (ELISA) could also be modified so that it could be supplied in a kit form (Pestka et al., 1981).

Thin-Layer Chromatography

A method that may be particularly useful in detecting warfare samples might be one based on a rapid TLC screening method developed for analysis of deoxynivalenol (DON) on a variety of agricultural commodities (Trucksess et al., in press). This method has been applied to corn, wheat, bran, milo, barley, soybean meal, rye, and most mixed feeds. Its detection limit is 0.3 μg/g.[1] It has also been used to detect and determine T-2 toxin and diacetoxyscirpenol (DAS). The trichothecenes are extracted with an 84:16 acetonitrile-water solution in a blender or Erlenmeyer flask on a wrist action shaker. The extract is passed through a chromatography column containing neutral alumina and activated charcoal (Darco G-60) to remove impurities before TLC. These columns are commercially available.[2] A vacuum source is used to speed up column

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[1]Romer, T. 1983. Personal communication. Romer Labs., Inc., Washington, Mo. 63090.
[2]Myco-Lab Company, P.O. Box 321, Chesterfield, Mo. 63107.

development. The solvent for TLC silica gel plates is toluene-acetone (1:1), and the blue color in the DON zone is developed by spraying with a 20% solution of hydrated aluminum chloride ($AlCl_3 \cdot 6H_2O$) and heating at 130°C. The same type of procedure may be used for other trichothecenes. The method requires a minimum of equipment and could be modified for use in the field. The 20% hydrated aluminum chloride can be incorporated into the silica gel used in preparing the TLC plates. In the field, the heat source could be provided by a hot jeep engine or a cigarette lighter after the plates are dried.

A Myco-Chek[3] kit can be used to identify DON, fusarenon-X (FUS), and other trichothecenes in cereal grains and mixed feeds in a "go, no-go" situation. The names of the solvents and reagents in the kit are not given in the instructions, but the extractant is probably ethyl acetate. The extract is purified for TLC on coated microscope slides. Anisaldehyde is probably the agent used to develop the color of trichothecenes on the developed slides with heat. The very first kit was not satisfactory, but the manufacturer claims that the method has been improved. The equipment in the original kit, however, would be applicable in the field and might be used in a modification of the rapid TLC method described above.

The reaction of 4-(p-nitrobenzyl)pyridine (NBP) with the 12,13-epoxy group of trichothecenes has been suggested as the basis of a rapid detection method (Takitani et al., 1979) and is being developed as a field test. A 1% solution of NBP in a 2:3 chloroform-carbon tetrachloride solvent is sprayed on a developed TLC plate to react with trichothecenes. The plates must be heated to 135°-150°C for 10 minutes for the reaction to take place. The blue color becomes visible after spraying with a solution of tetraethylene pentamine or other base; the background is white. The reaction of NBP with trichothecenes can be used to quantitate the mycotoxins as well as to detect them. The reflectance spectra of the blue zones are scanned with a spectrophotometer. Once again, cigarette lighters or heated engines could be used as field heat sources for these tests.

The use of chromotropic acid (the disodium salt of 4,5-dihydroxy-naphthalene-2,7-disulfonic acid dihydrate) has been proposed to improve the visualization of trichothecenes on developed TLC plates (Baxter et al., 1983). It reacts with both group A and B trichothecenes and has been tested with T-2 toxin, DAS, DON, HT-2 toxin, T-2 triol, and T-2 tetraol. Depending on the trichothecenes used, colors of the spots on developed TLC plates are purple, brown, or grey under visible light and blue-white, bright blue, or intense black under ultraviolet light (365 nm). The detection limits under UV

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[3]LSB Products, Manhattan, Kan.

light are 0.05 to 0.1 μg per spot, compared with 0.05 μg/ng DON per spot obtained with the 20% hydrate aluminum chloride.

Immunological Methods

Immunological assays for trichothecenes have the potential for providing high sensitivity and good reliability. Moreover, they can be performed relatively quickly. For example, assays of T-2 toxin by the ELISA technique can be performed in a modestly equipped laboratory and completed within several hours.

The feasibility of immunological assays has been demonstrated in published experiments with T-2 toxin as antigen (Chu et al., 1979; Fontelo et al., 1983; Lee and Chu, 1981a,b; Pestka et al., 1981); however, the titers of antibodies elicited so far are quite low. It seems probable that similar assays can be developed for other members of the trichothecene family. As indicated below, preliminary results suggest an approach to obtaining higher antibody titers. There is also a possibility that the time required for assays can be reduced.

Immunological assays do have certain limitations. For example, they are probably not as definitive as mass spectrometry because of the possibility that immunological cross-reactions occur among related compounds, such as the cross-reaction between T-2 toxin and HT-2 toxin observed by Chu et al. (1979). Such cross-reactions would not, however, necessarily nullify the conclusion that a trichothecene is present. Also, unexpected contaminants might conceivably interfere with assays. Another significant limitation is the requirement for a different set of reagents (for example, toxin conjugates and antibody) for each different toxin that is to be identified. As immunological assays are further developed and tested, the importance of these posssibly negative factors should become more evident.

Since trichothecenes are relatively small molecules, it is necessary to conjugate the trichothecene to a protein carrier in order to immunize animals. Bovine serum albumin has been used as a carrier for this purpose in most published experiments. The chemical modifier is generally attached to the hydroxyl group at the 3 position of T-2 toxin. This group is first modified with succinic anhydride. The protein carrier is then attached with a carbodiimide reagent.

Both radioimmunoassays (RIAs) (Chu et al., 1979; Lee and Chu, 1981a,b) and ELISAs (Pestka et al., 1981) have been applied in the detection of T-2 toxin. More recently, Fontelo et al. (1983) have developed an improved RIA. Antibodies against DAS and DON have been prepared, and quantitative assays for these two compounds are under

development.[4] Programs with the goal of producing monoclonal mouse antibodies are under way at several laboratories.

When laboratory facilities are limited, ELISA tests are much more practical than RIA because of the bulkiness and expense of the equipment needed to measure radioactivity. The end point of the ELISA assay is the development of a color in solution that can be seen or estimated quantitatively with an inexpensive portable spectrophotometer or colorimeter. In the assay for T-2 toxin, the stability of reagents over a period of at least several weeks should not present a problem, espeically if reagents can be refrigerated.[4] Further research will be needed to establish the stability of each reagent under a variety of conditions.

The limit of sensitivity of RIA or ELISA is determined by the binding affinity of the antibody for its antigen. Roughly speaking, the concentration at which an antigen is detectable is of the same order of magnitude as the reciprocal of the association constant (K_a) of the antigen-antibody reaction. K_a is expressed as reciprocal molarity (M^{-1}). This assumes that the antibody does not undergo bivalent or multivalent attachment to an individual antigen molecule. When such attachment occurs, the effective binding affinity or "avidity" is markedly increased. Studies by Lee and Chu (1981a,b), Pestka et al. (1981), Fontelo et al. (1983), and F. S. Chu[4] indicate that RIA or ELISA are each capable of detecting as little as 0.001 µg/ml (1 ppb) of T-2 toxin.

Although the ELISA is much more practical than RIA when laboratory facilities are limited, research on RIA should not be neglected. If samples are sent back to established laboratories for evaluation, RIA would be useful for checking results obtained by ELISA.

To illustrate the requirements for equipment and time, a typical procedure will be described. A sample to be assayed for T-2 toxin is homogenized and extracted with an 80:20 methanol-water solution. After centrifuging or filtering, the supernatant is diluted with water to a methanol concentration of 10%. This solution is passed through a Sep-Pak™C_{18} cartridge;[5] T-2 toxin will adhere to the matrix. The toxin is eluted with 80% methanol and either diluted to 10% or dried to remove methanol and then redissolved in a 10:90 methanol-water solution.

In one variation of the ELISA, the wells of polystyrene microculture plates (capacity per well is about 0.4 ml) are coated

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[4]Chu, F. S. 1983. Personal communication. University of Wisconsin, Madison.

[5]Waters Associates, Milford, Mass.

with a T-2 polylysine conjugate (formed in the well by coupling T-2 hemisuccinate to polylysine) and saturated with another protein such as bovine serum albumin (BSA) that is irrelevant to the assay. (Such precoated plates may be stored for long periods.) For a control sample, rabbit anti-T-2 antiserum is then added, allowed to stand for 45 minutes, and washed out. The next reagent added is a solution of goat anti-rabbit IgG that has been conjugated with horseradish peroxidase (HRP). After standing for 30 minutes, the solution is washed out and a mixture of hydrogen peroxide and 2,2'-azino-di-3-ethyl-benzthiazoline-6-sulfonate (ABTS) is added to each well. Hydrolysis of ABTS by HRP yields a blue-green product whose concentration can be measured with a spectrophotometer or colorimeter. The amount of color developed in a fixed period (often 20 minutes) is a measure of the amount of HRP in that well.

To assay for a sample containing an unknown concentration of T-2 toxin, the sample is mixed with the anti-T-2 antiserum before the latter is added to the well. The presence of T-2 toxin in the unknown sample will cause a reduction in the amount of anti-T-2 bound to the well and, therefore, in the amount of goat-IgG-HRP bound in the next stage. This in turn will reduce the amount of colored product produced. To quantify T-2 in unknown samples, the method is first standardized by using samples with a known content of T-2 as inhibitors. If T-2 is absent from the unknown sample, no effect on color development will be noted.

Possibility of a Rapid Assay Suitable for Use Under Field Conditions. The ELISA would be simplified if the sample were simply dissolved in a suitable solvent, such as methanol or methyl cellosolve, diluted with water, and tested. Because the toxin concentrations in samples obtained from a war zone are likely to be higher than those resulting from natural contamination, purification on Sep-Pak™ can probably be omitted. Since the nature of the other components in the mixture might vary greatly, depending on the way in which the toxin was released and the sample from which it was retrieved (such as a leaf or stone), considerable experimentation will be needed to ascertain whether unpurified material is likely to give reliable results. Assays (such as RIA) have been carried out successfully on serum and urine samples containing higher levels of T-2 toxin without the use of the purification procedure (Lee and Chu, 1981a). The presence of 10% methanol or methyl cellosolve does not appear to interfere with the ELISA.

The principle of a very rapid assay, which has been applied to other antigens (unrelated to trichothecenes), is as follows. The antigen (antigen X) is conjugated to a hydrolytic enzyme, which is capable of releasing a colored product within minutes from a colorless substrate. Conjugates are prepared in such a manner that the activity of the enzyme is inhibited when antibody to X is added. Presumably, the mechanism of inhibition is steric hindrance. The presence of X in

an unknown sample is ascertained through its ability to combine with the antibody and to prevent the inhibition of enzyme activity that would otherwise occur. Quantitation is achieved by the use of standards containing known amounts of X.

The use of trichothecenes as antigens has not been developed to the extent that they can be used in this type of assay. However, this may be a possible in the future. The method is so simple that it would not require laboratory facilities. Again, the possible need for preliminary isolation of the toxin must be studied.

Low Antibody Titers. T-2 toxin conjugated to proteins for the purpose of immunization has been found to be strongly immunosuppressive in mice.[6] This has greatly hampered efforts to produce monoclonal antibodies in mice and may well account for the low titers achieved elsewhere in rabbits.[7] In an attempt to circumvent this problem, nontoxic synthetic analogs of T-2 toxin have been prepared.[7,8] The structure of one of these analogs -- deoxyverrucarol -- is shown in Figure 3-1.

In preliminary experiments, it was found that relatively high titers of anti-T-2 toxin antibodies are obtained in BALB/c mice when conjugates of deoxyverrucarol are used for immunization.[6] High antibody titers of antideoxyverrucarol have also been obtained in rabbits.[7] Since deoxyverrucarol lacks many of the side chains of T-2 toxin, the antibodies elicited may well be cross-reactive with other trichothecenes. Preliminary results show that the antibody has some cross reactivity with DAS.[7] Other techniques for obtaining higher titers of antibodies are also being pursued.[6]

Another approach to immunization is to use T-2-BSA conjugates with a small number of T-2 groups per molecule of BSA. The rationale for this is that such conjugates should be less toxic. Significantly higher antibody titers in mice and rabbits[7] have been obtained with this type of antigen.

Monoclonal Antibodies to Trichothecenes. The use of monoclonal antibodies, necessarily of mouse or rat origin, would provide great advantages. Virtually unlimited supplies of antibody could be prepared, and the properties of different batches of antibodies should be invariant. The antibodies would of course have to exhibit specificity and high affinity for the antigen. However, the efforts to prepare monoclonal antibodies to trichothecenes undertaken in 1982[6] have been beset with problems, as described above. Although

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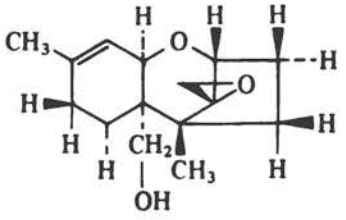
[6]Hunter, K. W., Jr. 1983. Personal communication. Uniformed Services University School of Medicine, Bethesda, Md.
[7]Chu, F. S. 1983. Personal communication. University of Wisconsin, Madison.
[8]Jarvis, B. B. 1980. Personal communication. University of Maryland, College Park.



Deoxyverrucarol

Figure 3-1. Deoxyverrucarol -- a synthetic T-2 toxin analog.

improved titers of antibodies were recently obtained in BALB/c mice, preparation of monoclonal antibodies has not been reported as of September 1983.

Scope of Current Research. Studies are continuing on the development of assay systems, the preparation of monoclonal antibodies,[9,10] and the preparation of synthetic analogs of trichothecenes for use as immunogens.[9,11,12] Liposomes containing trichothecene antigens will be prepared and tested for immunogenicity.[10,13]

Comments

In general, it is not difficult to produce antibodies and to develop quantitative immunological assays for hydrophobic compounds of

[9]Jarvis, B. B. 1983. Personal communication. University of Maryland, College Park, Md.
[10]Fontelo, P., and J. Hewetson. 1983. Personal communication. Ft. Detrick, Md.
[11] Chu, F.S. 1983. Personal communication. University of Wisconsin, Madison, Wisc.
[12]Falklam, T. 1983. Personal communication. Battelle Memorial Institute, Columbus, Ohio.
[13]Callahan, R. 1983. Personal communication. Vestar Research, Inc., Pasadena, Calif.

low molecular weight, such as most of the trichothecenes. The difficulties encountered so far appear to be largely attributable to the cytotoxicity of these molecules, even when introduced as a conjugate to a protein molecule. The approaches mentioned above -- use of modified trichothecenes or of conjugates containing smaller numbers of toxin molecules -- are logical, and preliminary data suggest that both methods are promising. These results have not yet been published. The production of higher titers of antibodies will of course facilitate the development of assays and may also permit the production of monoclonal antibodies in mice or rats. (When titers are very low, it is difficult to obtain monoclonal antibodies because of the low frequency of clones secreting the desired product.) It remains to be seen whether these modified approaches will yield antibodies of sufficiently high affinity ($>1 \times 10^9$ M^{-1}) to be useful in RIA and ELISA.

There are several parameters that can be varied and tested for their effects on antibody titer and affinity. These include the structure of the modified toxin; the position in the toxin to which the linking groups are attached; the side chain in the protein to which the attachment is made; the number of toxin groups per molecule of protein; and the nature of the protein carrier. In addition, various protocols for immunization can be tested. The nature of the protein carriers may be of particularly great importance, but little emphasis has been given to this factor in the investigations published so far.

Those doing research in this area have continued to make substantial progress. In view of the many factors involved, it will be necessary to bring additional personnel into these projects if there is to be an expeditious development of assays for an array of trichothecenes. The amount of effort to be expended will depend on decisions regarding the urgency with which the assays are needed. It might be desirable, however, to delay any rapid expansion of effort until the newer data on antibody production are reviewed.

Ribosome Concept

An alternative quantitative approach might utilize eukaryotic ribosomes that possess a single trichothecene binding site associated with ribosomal protein L3. Binding of trichothecenes to this site inhibits protein synthesis and appears to be responsible for the toxicity of this family of compounds. For T-2 toxin, the affinity is on the order of 1×10^{-6} M^{-1} for free ribosomes. A competitive binding assay using this receptor would have a workable sensitivity of approximately 1×10^{-6} M, or approximately 0.4 ppm. The sensitivity could be extended somewhat by preconcentrating the extracts.

Such an assay would have some potential advantages, including universal detection of the trichothecenes of interest, sensitivity to individual trichothecenes more or less proportional to their toxicity, homogeneity of binding characteristics, and ready availability of essentially any quantity of ribosomes from sources such as yeast.

The principal disadvantage of this approach is its limited sensitivity, as compared to ELISA. Technical difficulties could arise in the extraction, purification, and stabilization of the ribosome trichothecene receptor.

Remote Sensing of Airborne Trichothecenes

Remote sensing involves the use of photographic or other optical methods to sense and identify objects at a distance. The systems designed to achieve this objective may be classified as active, i.e., those that involve the use of lasers or other light sources, and passive, i.e., those based on the radiation naturally present during daylight or emanating from heated objects. Remote sensing systems have been used on the ground and aboard aircraft for many years. More recently, they have been placed on board satellites for earth reconnaissance. The LANDSAT program of the National Aeronautics and Space Administration (National Oceanic and Atmospheric Administration, in press) is an example of orbital, passive remote sensing that uses narrow bands of visible and infrared radiation to provide imagery of the earth's surface. Similar techniques might be used to detect clouds of mycotoxin yellow rain.

Over the past 15 years, laser remote sensing has been developed for the remote measurement of aerosol and gaseous constituents of the atmosphere (Carswell, 1983). Some methods depend on single wavelength techniques based on Raman scattering, fluorescence, and elastic scatter of photons. Another method involves the pulsing of a laser at two different wavelengths, and determinations are based on the ratio of the two signals received at these wavelengths. This method takes advantage of the dependence of absorption intensities or volume backscatter coefficients (needed for the detection of aerosols) on wavelength. The multiwavelength method has received the most attention and has perhaps the greatest potential as a remote sensing technique for warfare agents.

Remote sensing offers a number of potential advantages over chemical methods of rapid detection. The instruments used in this method can detect warfare agents before they strike an intended target. For such current systems, the advanced warning time is estimated to be 1 to 4 minutes. Since the system is computer automated, no personnel need to be exposed during measurement. Moreover, detection is rapid: signals can be interpreted instantaneously. Detection is also possible at night, and under

optimal conditions, it is chemical-specific. Remote sensors can be multifunctional: they can be programmed to detect a wide range of chemicals and different classes of chemicals.

There are some general disadvantages to remote sensing. Systems are more expensive, by several orders of magnitude, than simple chemical methods. Furthermore, gases, smokes, impurities, and various other foreign chemicals can easily mask the signals from warfare agents. The enemy can sabotage detection efforts by adding interfering chemicals, by arbitrarily changing the physical characteristics of aerosol agents, or by releasing chemicals that could trigger false alarms.

There is very little information available for assessing the feasibility of laser remote sensing of trichothecenes. Research efforts in this area have been minimal. Some potential difficulties are associated with the use of remote sensing techniques for these toxins. For example, trichothecenes used as a warfare agent could well be delivered in a complicated chemical mixture, which could vary from episode to episode of use. It may be difficult to obtain representative spectral information needed for remote detection.

Trichothecenes have no appreciable vapor pressure and, hence, exist only as airborne aerosols. The spectral resolution from backscattered aerosol signals is typically less sharp than signals obtained from absorbing gases and vapors. Thus, it would probably be more difficult to obtain chemical-specific information for trichothecenes than for gases and vapors. Moreover, the presence of impurities and natural aerosols will interfere with the signal and make detection and specification more difficult.

Some problems in aerosol detection are not applicable to gases and vapors. The volume backscatter coefficient is a function not only of wavelength but also, in some cases, of particle size distribution. When the laser wavelengths are comparable to the mean particle radius, the dependence of backscatter on wavelength curves is a function of the size distribution of the particles. When there is no prior knowledge of the size distribution, and when the size distribution can be arbitrarily changed from episode to episode, the dependence of the backscatter signature on the size distribution greatly complicates the signal processing requirements.

Trichothecenes characteristically exhibit strong infrared absorptions in the range from 2.5 μm to 25 μm. Lasers operating in this range have been well developed, but particles with a mean radius less than 50 μm (0.05 mm) may be difficult to identify because of the constraints described above. If the mean particle size is larger, some chemical-specific information may be extracted. Release of trichothecenes as large particles (0.1 mm to 1 mm) may in fact be the

desired mode of delivery. Such particles would drop out of the atmosphere quckly and in high concentrations, whereas smaller particles would tend to remain in the air longer and be dispersed over wide areas. A limiting factor in the use of remote sensing is the short residence time of the large particles in the atmosphere due to rapid gravitational settling. A cloud formed from an explosion 30 meters above the ground with particles ranging from 0.1 mm to 1 mm could fall to the ground in less than 1 minute. Thus, the 1- to 4-minute advance warning of this system would be to no avail.

Since trichothecene aerosols have been reported to appear as a yellow cloud, they might be detectable with lasers in the visible or ultraviolet region. However, no trichothecenes absorb in the visible region, and only groups B and D (Figure 2-1) absorb in the ultraviolet region. The yellow coloration may be due to the presence of impurities or other inert substances. A method based on detection of impurities or inert substances is probably not advisable because of the variability in the composition that can occur.

Trichothecene aerosol can be produced by dissolving the toxins in a volatile organic solvent and spraying the solution into the atmosphere. One indirect approach for detecting trichothecenes is based on detection of the solvent vapors that would be released during this process; however, this method could lead to a false alarm if the atmosphere were sprayed with solvent vapors alone.

It is much easier experimentally to detect the presence of a cloud than it is to determine the chemical nature of the cloud by remote sensing. The information obtained is limited, but in conjunction with other information, it may prove useful in helping to protect civilian and military personnel.

A number of formidable experimental difficulties can be expected from the application of remote sensing to trichothecenes if the goal is unambiguous identification of the chemical composition of a suspect cloud or ground deposit. Given the current level of information, it is not clear that these difficulties can be easily overcome. Preliminary information on the spectral and scattering characteristics of trichothecene aerosols should be carefully assessed in order to determine the feasibility of this system.

Biological Methods of Detection

Pathre and Mirocha (1976) have reviewed and evaluated the biological methods for detecting trichothecenes. These toxins exhibit characteristic antibiotic, phytotoxic, cytotoxic, and dermatitic activity. Certain assays are based on the ability of the toxins to produce these detectable effects in susceptible biological systems.

Trichothecenes have been also detected by their fungistatic and antiprotozoal activity and by their ability to inhibit the growth and germination of seeds of higher plants. Burmeister and Hesseltine (1970) reported that 0.5 μg of T-2 inhibited the germination of pea seeds by 50% when the seeds were soaked overnight in a solution of the toxin.

Dermal toxicity tests for rabbits, guinea pigs, and rats have been used extensively. These tests are capable of detecting trichothecenes at submicrogram levels. However, Pathre and Mirocha (1976) have noted that the sensitivity of skin tests varies from animal to animal and that partially purified extracts containing T-2 and DAS at levels as high as 10 to 20 μg per application failed to produce any observable reaction in some tests in rats.

Brine shrimp are also sensitive to trichothecenes, as indicated by reports of the LC_{50} (lethal concentration in liquid medium required to kill 50% of the test animals) for several toxins. The dried eggs of brine shrimp hatch within 24 to 28 hours when placed in a suitable medium at ambient temperature. Because the larvae are phototrophic, they can be easily separated from the eggs. In an application of the bioassay technique, Eppley (1974) found that the effects of T-2 and DAS can be detected at concentrations of approximately 0.125 μg/0.5 ml.

Recently, Hannan[14] reported that trichothecenes inhibit the luminescence of certain dinoflagellates. T-2 concentrations of 1 μg/ml have been found to reduce light intensity by 40% in a solution containing 300 organisms per milliliter. Intensity is measured by a photometer attached to an amplifier. To maximize the effect, the solution containing the toxin is kept in the dark for 0.5 to 1 hour.

Biological tests are sensitive, but they often lack specificity and are time consuming. Because of their simplicity, however, some of them may be well suited for corroborative field testing.

QUANTITATIVE ANALYSIS OF TRICHOTHECENES

Table 3-1 summarizes conditions and reagents used in analytical methods for trichothecenes that have been tested for percent recoveries and detection limits. Regardless of the kind or size of sample being analyzed, the detection limit of the procedures must be considered.

Solvents used to extract trichothecenes from agricultural commodities have been methanol-water (1:1) (Bennett *et al.*, in press;

[14]Hannan, P. 1983. Personal communication. Naval Research Laboratory, Washington, D. C.

TABLE 3-1

Quantitative determination of trichothecenes

Reference for Method	Sample Material	Extraction Solvent	Purification of Extracts	Detection/ Quantitation	Trichothecene Recovery and Dectection[a] Toxin	% Recovery	Detection Limit (ng/g)
Scott et al. 1981	Wheat Barley Corn Soybeans	Methanol-water (1:1)	a. 30% Aqueous ammonium sulfate precipitation b. Ethyl acetate extraction (two evaporations) c. Silica gel column wash: toluene-acetone (95:5); elute methylene chloride-methanol (95:5)	GLC-EC or GLC-MS (SIM)[b] of HFB[c] derivatives	DON DAS T-2 HT-2	72-80 74-79	10 60 500 20
Romer et al., 1978	Corn Mixed feeds	Methanol-water (1:1)	a. 30% Ammonium sulfate precipitation b. Chloroform extraction and potassium hydroxide partition c. Silica gel column washes: benzene and acetone-benzene (5:95); elute: ethyl ether	GLC-EC of HFB derivatives	T-2 DAS	105 97	-- --
Kamimura, 1981	Corn Barley Wheat Rice	Methanol-water (95:5)	a. Amberlite XAD-4; elute methanol b. Florisil column elute chloroform-methanol (9:1)	TLC silica gel 60 and spray 20% $AlCl_3 \cdot 6H_2O$ (365 nm UV), or 0.32% dinitrophenylhydrazine or 20% H_2SO_4; GLC-FID[d] or EC of TMS[e] derivatives	NIV DON FUS-X DAS NS T-2 HT-2 ZE	71-82 89-93 78-90 78-102 98-108 72-82 87-90 82-90	20-50 20-50 20-50 100-500 100-500 100-500 100-500 10-50
Yamamoto, 1975	Grains Beans	a. Aqueous methanol/hexane b. Hexane c. Three times with chloroform	a. Silica gel chromatography b. Preparative TLC chromatography of TMS ethers	GLC-FID of TMS derivatives	T-2 DAS FUS-X	80-90 71-86 63-82	30-70 10-20 10-20
Kuroda et al., 1979	Grain Foods	a. Methanol/water b. Hexane	a. XAD-2 column chromatography b. Florisil column chromatography	GLC-FID TMS derivaties	DON NIV FUS-X T-2 DAS DON NIV FUS-X	102-111 56- 73 89- 96 --- --- 94- 96 62- 69 95- 99	 50 1,000 250 3-15 3-15 6-30

Table 3-1 - continued.

Reference for Method	Sample Material	Extraction Solvent	Purification of Extracts	Detection/ Quantitation	Trichothecene Recovery and Dectection[a] Toxin	% Recovery	Detection Limit (ng/g)
Ilus et al., 1981	Barley Wheat Oats	a. Ethyl acetate b. Aqueous methanol	Preparative TLC	Capillary GLC-FID	T-2 HT-2 NS	80	50
Collins and Rosen, 1981	Corn and its wet-milled products	Aqueous methanol	a. Aqueous $FeCl_3$, chloroform extraction b. Aqueous KOH c. Preparative TLC	GLC-FID GLC-MS	T-2	84-100	
Collins and Rosen, 1979	Milk	Sodium sulfate (400 g) in ethyl acetate	Preparative TLC	GLC-MS, EI*f*, CI*f*, TMS derivatives	T-2 T-2	72-96 60-104	3(CI) 6(EI)
Bijl et al., in press	Corn	Methanol-water (1:1)	None	Capillary GLC with FID, EC, or MS of free trichothecenes or TMS derivatives	DAS T-2 T		10
Cohen and La Pointe, 1982, and in press	Wheat Oats Berley Corn	Chloroform-ethanol (80:20)	One or two of following: Sep-pak silica cartridge, Sephadex LH20, Lipedex 5000, Cyano column	Capillary GLC with EC with HFB esters; also GLS-MS-SIM	DON T-2 DAS HT-2	90	50 10 10 50
Pathre and Mirocha, 1978	Rice Corn Mixed feeds	Water-methanol (60:40)	a. Saturated sodium chloride plus ethyl acetate b. Acetonitrite and petroleum ether c. Preparative TLC	GC-MS-SIM of TMS derivatives or TLC	DON	46	200
Sana et al., 1982	Applied to DON Stds. only Corn	Methanol-water (95:5)	a. Amberlite XAD-4, elute methanol b. Florisil column, elute chloroform-methanol (9:1)	TLC, nicotinamide, plus 2-acetylpyridine in alkali, acid treatment, fluorescence	DON FUS T-2 NIV		
Schmidt et al., 1982	Rice Corn Oats Wheat Rye Peas	Acetonitrile-4% potassium chloride (9:1)	Sep-pak C-18 cartridge	Two-dimensional TLC	HT-2	88	

Table 3-1 - continued.

Reference for Method	Sample Material	Extraction Solvent	Purification of Extracts	Detection/ Quantitation	Trichothecene Recovery and Dectection[a] Toxin	% Recovery	Detection Limit (ng/g)
Schmidt et al., 1981	Rice	Acetonitrile-4% potassium chloride (9:1)	Sep-pak C-18 cartridge	Reverse phase HPLC[&], infrared detector	T-2 HT-2		
Szathmary et al., 1980	Feeds Corn	a. Water b. Ethyl acetate c. Methanol	a. Petroleum ether b. Methanol-water (1:2)	Capillary GC-MS or capillary GC-FID of TMS derivatives	T-2 DON DAS NS ZE		50-100
Takitani et al., 1979	T-2 or FUS-X in rice and stds. of tricho-thecenes	Methanol	Silica gel and Florisil columns	TLC, 4-(p-nitrobenzyl)-pyridine	T-2 FUS HT-2 NS DAS FUS-X NIV DON		
Trucksess, in press	Wheat Corn	Acetonitrile-water (84:16)	Activated charcoal-neutral alumina column	Silica gel 60 TLC plates dipped in 15% $AlCl_3 \cdot 6H_2O$	DON	77-93	40
Schweighardt et al., 1980	Mixed feeds	a. Ethyl ether b. Methanol-water	a. Lead acetate percipitation b. Chloroform extraction c. Sep-pak C_{18} cartridge	Reverse phase HPLC[&]	ZE DON	70-80 25-35	2 25
Palmisano et al., 1981	Corn	Methanol-1% sodium chloride (55:45) and hexane	a. Chloroform extraction b. Sep-pak C-18 cartridge	GC-FID differential pulse pology raphy	T-2 DON		50

[a]DON = deoxynivalenol, NIV = nivalenol, FUS-X = fusarenon-X, DAS = diacetoxyscirpenol, T-2 = T-2 toxin, HT-2 = HT-2 toxin, NS = neosolaniol, ZE = zearalenone.
[b]GLC-EC = gas liquid chromatography-electron capture, GLC-MS (SIM) = GLC-mass spectrometry (selected ion monitoring).
[c]HFB = heptafluorobutyrates.
[d]FID = flame ionization detection.
[e]TMS = trimethysilyl.
[f]EI = electron impact ionization, CI = chemical ionization.
[&]HPLC = high performance liquid chromatography.

Kamimura et al., 1981; Romer et al., 1978), methanol-water (95:5) (Yamamoto, 1975), and ethyl acetate (Ilus et al., 1981; Kuroda et al., 1979; Scott, 1982). The advantage of using a solvent less polar than methanol-water (1:1) is that zearalenone, an estrogenic mycotoxin produced by fusaria, can also be determined quantitatively. Zearalenone is biosynthesized along with trichothecenes such as DON by the same fusaria species (Collins and Rosen, 1981). Solvents used for extracting trichothecenes from samples such as agricultural commodities, forages, tissues, and fluids should be chosen not only because they remove practically all of a given mycotoxin but also because they remove a minimum amount of substances that would interfere with quantitation.

Some substances that will interfere with quantitation usually are present in extracts and have to be removed by "cleaning up" or partially purifying the extracts. Methods used to purify crude extracts containing trichothecenes have been liquid-liquid partition (Bennett et al., in press; Collins and Rosen, 1979; Kamimura et al., 1981; Romer et al., 1978), precipitation of impurities with ammonium sulfate (Bennett et al., in press; Kamimura et al., 1981; Romer et al., 1978;) or ferric chloride (Collins and Rosen, 1979), column chromatography with silica gel (Bennett et al., in press; Kamimura et al., 1981; Mirocha et al., 1976; Romer et al., 1978) or Florisil (Yamamoto, 1975); ion-exchange chromatography (Yamamoto, 1975), or preparative TLC (Collins and Rosen, 1979; Ilus et al., 1981; Kuroda, 1979; Mirocha et al., 1976).

Preparative TLC is a tedious process and may result in low recoveries of trichothecenes. However, this is the least complicated method of quantitating trichothecenes in partially purified extracts (Yamamoto, 1975). Colors have been developed in TLC zones containing trichothecenes by spraying them with 20% aluminum chloride, 20% sulfuric acid, 0.32% phenylhydrazine, anisaldehyde, or 10% sulfuric acid and 10% glacial acetic acid in methanol and heating at 110°C. Quantities of a given mycotoxin in a TLC zone are determined by visual comparisons of these zones with zones containing standards on the sample plate or with a densitometer. The development of specific colors can be used to confirm identity and to detect and determine trichothecenes. The reaction products of the epoxy ring of trichothecenes with 4-(p-nitrobenzyl)-pyridine (Takitani et al., 1979) or with nicotinamide and 2-acetylpyridine (Sano et al., 1982) can be quantitated on TLC plates either visually or densitometrically. The fluorescence of the naphthylidine derivative of trichothecenes formed by reactions with nicotinamide and 2-acetylpyridine can be enhanced by adding paraffin-hexane (Uchiyama and Uchiyama, 1978).

Gas liquid chromatography (GLC) has been used most effectively for measuring trichothecene derivatives (trimethylsilyl ethers or heptafluorobutyrates) in solutions. The derivatives are detected by flame ionization detectors (FID) (Collins and Rosen, 1979; Kuroda et al., 1979; Mirocha et al., 1976; Yamamoto, 1975), electron capture (EC)

detectors (Bennett et al., in press; Kamimura et al., 1981; Romer et al., 1978; Yamamoto, 1975), or mass spectrometry (MS) with or without selected ion monitoring (SIM) (Bennett et al., in press; Collins and Rosen, 1979; Ilus et al., 1981). Detection by electron capture is more sensitive than by FID; GC-EC is used commonly to measure pesticides. The distinct advantage of gas chromatagraphy-mass spectrometry (GC-MS) SIM is that it not only serves as a method of quantitating trichothecenes but also confirms the identity of the individual trichothecenes. High performance liquid chromatography (HPLC) has been suggested as a method of quantitation (Bennett et al., in press).

The method developed by Scott et al. (1981) for wheat, corn, barley, and soybeans has been used to determine DON in hundreds of Canadian wheat samples since 1980. It has also been applied to DAS, T-2 toxin, and HT-2 toxin. Recoveries and detection limits have been established for DON, DAS, T-2 toxin, and HT-2 toxin (Table 3-1). In the method of Scott et al. (1981), the extraction solvent is methanol-water (1:1); the extract is purified by ammonium sulfate precipitation and silica gel chromatography; quantitation is accomplished by GLC-EC or GLC-MS (SIM) of the heptafluorobutyrate derivatives. It is a very time-consuming method, but it is widely used. In 1982, this method was modified at the Northern Regional Research Center of the U.S., Department of Agriculture for application to several hundred samples of hard red winter wheat and soft red winter wheat (Bennett et al., in press). The modifications did not lower the recoveries of DON or increase the detection limits in wheat, but they increased the number of samples that can be analyzed in a day by a factor of two or three. This modified method, now being used by industries and commercial analytical laboratories, still needs to be evaluated for DAS, T-2 toxin, HT-2 toxin, and other trichothecenes. In fact, a number of reported analytical methods for trichothecenes have not been tested for recoveries or detection limits.

The macrocyclic trichothecenes, satratoxins G and H produced by Stachyobotrys atra, were detected and determined in cereal grains by an HPLC method (Stack and Eppley, 1980). The mycotoxins were extracted by methanol-water (55:45) in the presence of hexane followed by partitioning into chloroform. Before HPLC, the extract was purified on a silica gel column. Recoveries of added satratoxins G and H from wheat samples averaged 65% for G and 71% for H (200-1,000 μg/g). The lower limit of detection for wheat was 0.2 μg/g. There were comparable recoveries in analyses of corn, oats, and barley samples. Quantitation of toxin levels by HPLC has also been applied to other trichothecenes, such as DON.

The trichothecenes are unique in that they all have the 12, 13-epoxy function, which might be used as a selective screening method. In an enzymatic assay procedure reported by Foster et al. (1975), glutathione-S-epoxide-transferase was used and an analysis for the unreacted glutathione in the reaction mixture provided an indirect measure of epoxide concentration. Submicrogram amounts could be measured

in this procedure, but unfortunately it was not applied to trichothecene detection in naturally contaminated samples. This procedures promises to be useful since it would be desirable to detect the epoxide ring to determine the possible presence of trichothecenes. However, the authors and other scientists have had difficulty in preparing the enzyme necessary for the method.

Romer[15] suggested that all trichothecenes could be hydrolyzed to five alcohols. Type A trichothecenes would yield T-2 tetraol and scirpenetriol; Type B would give DON and nivalenol (NIV); macrocyclics yield verrucarol (see Table 2-1 for a listing of the types of trichothecenes). The heptafluorobutyrates of the alcohols could be prepared for TLC on GLC-EC or MS. Romer has not pursued this approach. If necessary, the hydrolytic procedure could be used to determine the presence of trichothecenes (type A or B or macrolides)in a sample.

Of the methods reported for the quantitation of trichothecenes, two are to be tested collaboratively under the auspices of the Association of Official Analytical Chemists, because a number of laboratories are using them. The methods are considered to be reliable and to have high recoveries (80-95%) and low detection limits (0.01 to 0.5 μg/g). The first method is an improvement of the Romer method.[16] It involves the use of columns of neutral alumina and charcoal and TLC with an aluminum chloride spray to develop color (Trucksess et al., in press). The color on TLC plates is measured either visually or with a densitometer. The second method is a modification (Bennett et al., in press) of the method reported by Scott et al. (1981). Quantitation is done by GC-EC or by GC-MS.

CONFIRMATORY TESTS

In confirmatory tests for the previously mentioned trichothecenes, color spray agents are used for developed TLC plates and GC-MS (SIM). GC-MS has also been used for rapid screening of milk for T-2 toxin and HT-2 toxin; the lower limit of detectability is 0.3 g/g and recovery is approximately 70% at 1.25 μg/g (Pareles et al., 1976). GS-MS has also been applied as a rapid screening technique for zearalenone and DON in cereal grains[17] and to confirm the identity of trichothecenes and zearalenone in Southeast Asia (Mirocha et al., in press; Rosen and Rosen, 1982).

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[15]Romer, T. 1981. Trichothecenes Symposium, Washington, D. C., October 26-30, 1981. Association of Official Analytical Chemists.
[16]Romer, T. 1982. Personal communication. Romer Labs., Inc., Washington, Mo. 63090.
[17]Payne-Wahl, K. 1983. Personal communication. Agricultural Research Service, U.S. Department of Agriculture, Peoria, Ill.

The new technique of tandem MS, also called mass spectrometry-mass spectrometry (MS-MS), has been applied to crude extracts of wheat, corn, oats, and rice to detect DON and zearalenone, two mycotoxins produced by the same Fusarium species, in levels below 1 ppm (Bennett et al., in press; Plattner, in press). With MS-MS, one stage of mass separation is used to select the compound of interest (DON or zearalenone) from the crude extract, and a second stage is used for analysis after dissociation by collision with a target gas. MS-MS is an elegant means of rapid screening for mycotoxins as well as a method for confirming the identity of mycotoxins, such as DON, in agricultural commodities and other substrates.

CONCLUSIONS AND RECOMMENDATIONS

Sampling procedures should be developed for the rapid identification and quantitation of trichothecenes under field conditions; for monitoring dose levels in individuals after an exposure; and for evaluating persistence of the toxins. Suitable protocols for the preservation of samples are also needed.

For rapid analysis of trichothecenes in the field, TLC appears to be the simplest and best developed method presently available and its efficacy has been well established in the testing of agricultural products. Moreover, the cost of TLC analyses is modest. Thus, the application of this method to trichothecenes in a variety of samples should be studied.

Immunological assays for trichothecenes, now under active development, appear promising for future use. Such assays are more time-consuming than TLC, but they are potentially more sensitive and specific and may provide better quantitation. Considerable effort is needed to improve the production of antibodies and to develop immunological assays for a variety of trichothecenes. Particular attention should be given to ELISA, since this is the most promising assay for use under field conditions.

Remote sensing techniques would be of great value, but their feasibility has not been established. These methods should be explored, initially at the theoretical level.

Methods based on toxicity to microorganisms, plants, and animals are nonspecific and generally time-consuming. However, some of them are simple to use and may provide corroborative evidence. Their lack of specificity may permit the identification of groups of compounds.

A number of reliable methods for the quantitation and confirmation of trichothecenes are more suitable for the laboratory than for the field. GLC is the most highly developed and widely used method of

quantitation. GC-MS has been used for a number of years to confirm the identity of Fusarium toxins as well as to quantify them. Tandem MS is another method for confirmation and can be used for screening.

The presence on ribosomes of receptors with specificity for trichothecenes suggests an alternative method for quantitative analysis of these toxins. Preliminary experiments on the feasibility of this method should be considered.

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Chapter 4

DECONTAMINATION AND DETOXIFICATION

In the event of deliberate release, levels of exposure to trichothecenes are likely to be several orders of magnitude higher than levels from natural sources. Military personnel, civilians, equipment and hardware, foods, and water supplies may become contaminated, and the contaminated food and water could affect the health of populations dependent on them. The effects could last for weeks or months. Thus, methods of removal or chemical detoxification of trichothecenes from food and water should be developed not only to protect persons directly exposed but also to prevent others from becoming exposed through contact with a contaminated surface or the consumption of contaminated food or water.

There is a long history of experience in the agricultural research community relating to the decontamination and detoxification of aflatoxins. Since there has been little research in this area on trichothecenes, it is hoped, and is quite possible, that some of the methods developed for aflatoxins may be applicable to trichothecenes.

CHEMICAL METHODS OF DETOXIFICATION

Aflatoxins

After a number of tests on laboratory animals revealed that aflatoxins were potent carcinogens, a study was conducted to identify chemical reactions that might be used for detoxification (Trager and Stoloff, 1966). Loss of fluorescence and/or change in R_f[1] on thin-layer chromotography (TLC) plates were used as indicators of reactions. Evidence of detoxification was obtained through bioassays with chick embryos or tissue cultures. The reactions were primarily additions and oxidations involving the olefinic double bond of the terminal furan ring and oxidation of the phenol formed upon opening of

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[1] R_f is the ratio of the movement of the zone of interest to the movement of the solvent front on a TLC plate.

the lactone ring (Figure 4-1). Contact of aflatoxins B_1, B_2, G_1, and G_2 with gaseous chlorine, chlorine dioxide, and nitrogen dioxide, and treatment with 5% sodium hypochlorite, resulted in detoxification.

The use of sodium hypochlorite has been recommended by the Association of Official Analytical Chemists (1980) for the detoxification of aflatoxins and other mycotoxins. A 3% to 5% solution of sodium hypochlorite has been used to destroy aflatoxin on laboratory glassware, TLC plates, and protective clothing, as well as in spills, biological media and substrates, and solutions of aflatoxins. More recently, the reaction of aflatoxins with sodium hypochlorite solutions has been studied in depth by the International Agency for Research on Cancer (IARC) (Castegnaro et al., 1981). Approximately 5% of the original aflatoxin B_1 was found to be converted to 2,3-dichloroaflatoxin B_1, a potent carcinogen and mutagen. Treatment of the mutagenic 2,3-dichloro derivative with 5% acetone inactivated it. IARC now recommends that a 1% to 3% sodium hypochlorite solution be used, followed by the addition of 5% acetone (Castegnaro et al., 1980).

Natarajan et al. (1975) reported that sodium hypochlorite was effective in destroying aflatoxins during the preparation of peanut protein isolates from raw peanuts and defatted peanut meals. The important factors in reducing aflatoxin levels in protein isolates to nondetectable levels were pH (which ranged from 8 to 10) and sodium hypochlorite concentrations (which ranged from 0.4% to 0.15%). Sodium hypochlorite was added to the aqueous suspension of ground raw peanuts.

Ammoniation is the most effective and convenient means of detoxifying aflatoxin in agricultural commodities. Both aflatoxin-contaminated cottonseed meals and peanut meals can be detoxified with ammoniation at 93°C under a pressure of 45 psig (Gardner et al., 1971). Application for approval of this method to detoxify cottonseed meal under pressure at high temperatures has been made to the Food and Drug Administration (FDA) by the Southern Regional Research Center of the U.S. Department of Agriculture.

When it became known that aflatoxin contamination of corn could be a problem, especially in the southern United States, a detoxification method using gaseous ammonia at ambient temperatures and atmospheric pressures was developed at the Northern Regional Research Center (NRRC) for on-farm application (Bagley, 1979). The process can be implemented in standard metal storage bins with epoxy-coated walls or polyethylene-lined walls or in any other air-tight system. The process takes takes 1 to 3 weeks, depending on temperature. The treatment of aflatoxin-containing corn with this method has been approved by the North Carolina Department of Agriculture for all animal feed except dairy feed intended for use and/or sale in North

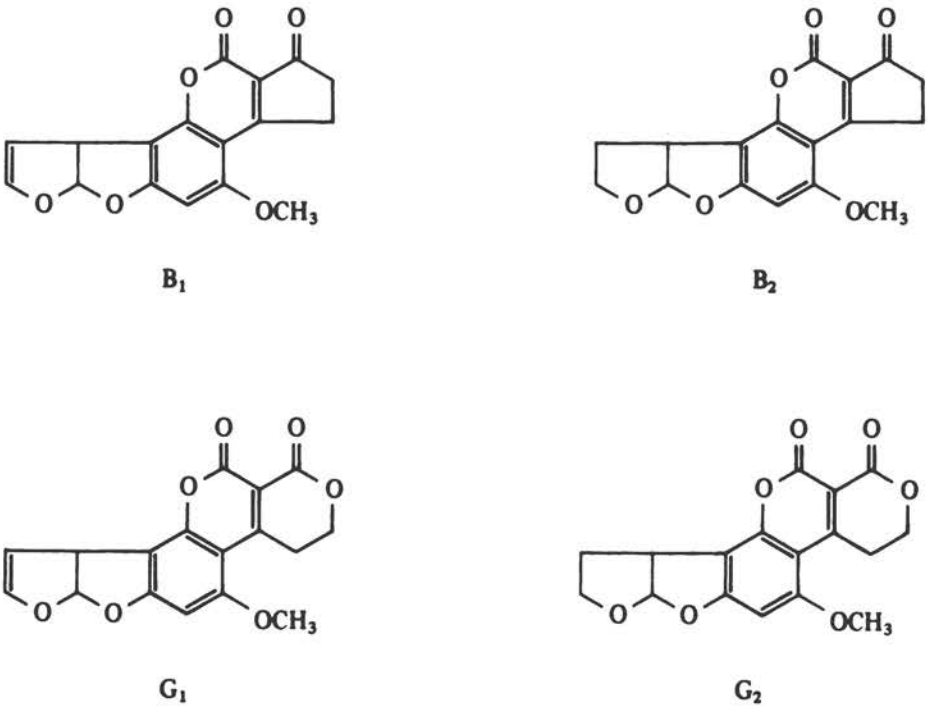

Figure 4-1. Structure of aflatoxins B_1, B_2, G_1, and G_2.

Carolina. The NRRC has applied to the FDA for approval of the ammoniation process to detoxify aflatoxin-contaminated corn for use as animal feed.

Contaminated cottonseed is being ammoniated in Arizona in large polyethylene bags designed for ensiling. Cottonseed is placed in the bags with an end loader, the bags are closed, and ammonia is passed through the bags.

Codifer (1976) has reported that formaldehyde and calcium hydroxide have been used to inactivate aflatoxin in peanut meal. The treatment of peanut meal containing 0.57 µg/g aflatoxin under reflux at atmospheric pressure in the presence of 2.0% calcium hydroxide and 1.0% formaldehyde lowered the aflatoxin level to 0.005 µg/g, which is lower than the action level of 0.02 µg/g proposed by the FDA (1979).

Bennett et al. (1981) found that formaldehyde alone was effective in decontaminating three lots of corn containing zearalenone in concentrations of 3.5, 8.0, and 33.5 µg/g. p-Formaldehyde vapors were circulated through beds of corn at ambient temperatures and atmospheric pressure for 10 days. Zearalenone levels were decreased

in all three lots; the lot containing the highest concentration (33.5 μg/g) contained 2.1 μg/g after the 10th day. However, recent findings indicate that formaldehyde vapor may be carcinogenic to humans (International Agency for Research on Cancer, 1982). Zearalenone is a Fusarium mycotoxin with estrogenic properties. It often occurs naturally with deoxynivalenol (DON).

Sodium bisulfite has also been studied as a reagent that could be used to detoxify aflatoxin-contaminated corn because it is effective in reducing aflatoxin levels (Moerck et al., 1980). The treated corn has not been tested in laboratory animals to demonstrate that it is biologically safe.

Trichothecenes

The most effective decontaminating agent found for T-2 toxin is sodium hypochlorite. The efficiency of this agent can be increased by the addition of small amounts of alkali. The products of the reaction should be studied to ensure they are not as toxic as, or more toxic than, the original trichothecene--T-2 toxin. As mentioned before, aflatoxin B_1 forms 2,3-dichloroaflatoxin B_1 in the presence of sodium hypochlorite, necessitating treatment with 5% acetone to destroy the mutagenic and carcinogenic 2,3-dichloro compound (Castegnaro et al., 1980). Methanol, which is used in some procedures as an organic solvent, is itself attacked by hypochlorite reagents to form a complex mixture of products, including formaldehyde. Tert-butyl alcohol would be a better choice of solvent.

Other methods of detoxification and decontamination are being pursued. Preliminary results indicate that soaking contaminated corn in 10% sodium bisulfite detoxified DON completely (Hamilton, 1983). In a simpler procedure, soaking the corn in pure water reportedly removed 30% of the DON (Hamilton, 1983). Studies have also been initiated to determine the effectiveness of ammoniation in the decontamination of wheat samples infected with DON (Schneider, 1983).

One strategy for detoxifying trichothecenes is to devise chemical procedures for attacking the toxin molecule selectively, taking advantage of specific structural features or reactive functions. All trichothecenes contain a 12,13-epoxy group, a substituent necessary for toxicity. Thus, transformations of diacetoxyscirpenol (DAS) that open the spiro epoxide ring result in compounds that lack the cytotoxicity of DAS (Grove and Mortimer, 1969) (Figure 4-2). Epoxide rings of triacetylscirpenol will open at elevated temperatures in the presence of acid to form a rearrangement product. M. D. Grove,[2] who

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[2]Grove, M. D. 1983. Personal communication. Agricultural Research Service, U.S. Department of Agriculture, Peoria, Ill.

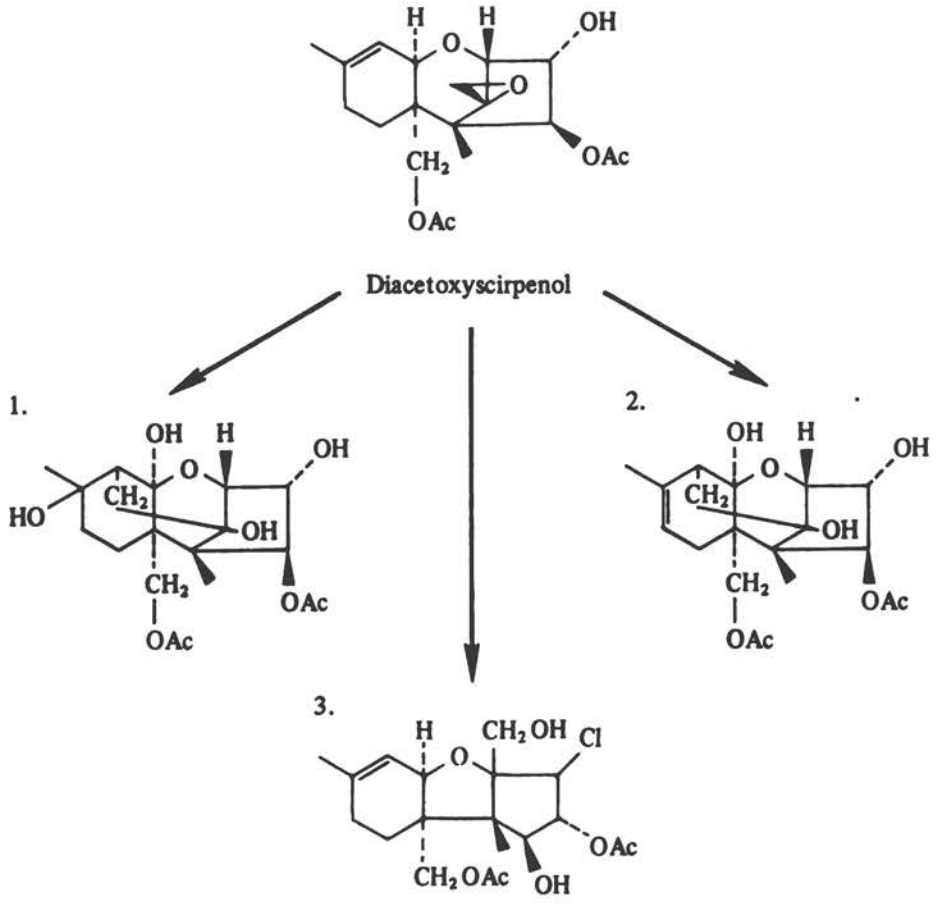

Figure 4-2. Transformation and rearrangements to form three different noncytotoxic products from diacetoxyscirpenol.

studied similar reactions of T-2 toxin, found that products similar to those obtained from DAS are formed. A bioassay in mice demonstrated that these products were not active.

Epoxides, or substituted ethylene oxides, are normally very reactive toward nucleophiles, acids, and bases. However, the epoxide in these compounds is shielded sterically to the extent that the usual reagents do not attack under mild conditions. The chemical literature documents extensive studies on the reactions of epoxides with acidic and basic reagents. For example, Sigg et al. (1965) reported the reaction of DAS with sodium ethoxide under vigorous conditions. They also noted the inactivity of the epoxide against chromium trioxide and lead tetraacetate. Other investigators have found that the sterically hindered epoxide is slowly attacked by various acids at elevated temperatures.

A promising approach is to develop suitable catalysts that would allow reactions to proceed under less vigorous conditions. One theory, which has never been tested, is to utilize "supernucleophiles," perhaps in nonsolvating solvents, to attack the relatively inert epoxide. An example might be sodium thiophenoxide in dimethylformamide (DMF) or dimethylsulfoxide (DMSO). A precedent is the cleavage of phenacylesters from penicillins at room temperature, which occurs within seconds (Sheehan et al., 1964).

Other good nucleophiles that can be used in aqueous media are sodium thiocyanate and hydroxylamine. The latter attacks esters and lactones and, thus, could destroy other toxins such as zearalenone. Sodium borohydride opens epoxides and might be a reductive method of inactivation.

Another untested idea is to use "super acids" such as trifluoromethane-sulfonic acid (CF_3SO_3H), which is powers of 10 stronger than ordinary, laboratory-strength acids such as sulfuric and hydrochloric acids, in solvents that cannot be readily protonated. Another "super acid" is "magic acid"--hydrofluoroboric acid plus antimony pentafluoride ($HBF_4 + SbF_5$). The plan would be to bring about acid-catalyzed ring openings and rearrangements under mild, ambient conditions.

Yet another approach might be to treat the epoxide-containing mycotoxin with triphenylphosphine, which is reported to extract oxygen to yield the corresponding olefin. Since this reagent presumably attacks the epoxide from the oxygen on the less hindered side, the reaction might proceed under relatively mild conditions.

All the foregoing discussions regarding supernucleophiles, super acids, and triphenylphosphine need experimental verification and development.

ENZYMATIC DECOMPOSITION

Enzyme technology offers a mild means of destroying organic compounds. It can be used to convert toxins to nontoxic derivatives. Most enzymes are not harmful or have little effect on humans; hence, they may be exceptionally effective in protecting personnel who have been exposed to mycotoxins.

Enzymes are highly specific for individual chemicals, classes of chemicals, or certain chemical groupings. Therefore, an enzyme preparation that is an effective detoxifying agent for one chemical or class of chemicals is useless on a different chemical or class. For this reason, enzymes do not have the broad scope of action that characterizes many chemical reagents.

Enzymes that act on mycotoxins exist in many organisms. This has been amply demonstrated in the studies discussed in Chapter 5. Microorganisms are probably the best source of enzymes that can be used to destroy mycotoxins because they can easily be grown in large quantities and because optimal conditions for enzyme production can be established readily. These enzymes should not be difficult to obtain and purify, although little attention has yet been given to microbial enzymes that metabolize mycotoxins. The studies that have been conducted have dealt largely with intact microbial cells or microbial cultures. If the enzymes are intracellular, methods will have to be developed to extract them. They will also need to be purified in order to avoid possible deleterious reactions among people who are treated with crude preparations. Means will also have to be found to prevent loss of enzyme activity during storage, if the enzymes obtained do not store well. In addition, a single enzymatic reaction may modify the molecule but not bring about detoxication; hence, it is necessary to determine whether one or several enzymes are needed to destroy the harmfulness of the toxin.

DECONTAMINATION OF WATER SUPPLIES

Very little work has been done to remove mycotoxins, much less trichothecenes, from water supplies. Several possible methods should be investigated to determine their effectiveness. For example, filtration through activated charcoal or a bleaching filter is one possible removal process. Reverse osmosis is another. Good, portable reverse osmosis machines have already been developed for separating whey in cheese-making. Furthermore, more research is required to determine the solubilities and stabilities of trichothecenes in water.

PROCESSING MYCOTOXIN-CONTAMINATED COMMODITIES

If an agricultural commodity such as a cereal grain or oilseed is contaminated with a mycotoxin, the distribution of the toxin among product fractions during processing must be determined. In 1971, the FDA ordered a recall of corn meal allegedly contaminated with aflatoxin (Anonymous, 1971). This action led to a pilot-plant study of the fate of aflatoxin during dry-milling of contaminated corn (Brekke et al., 1975). Three different 90- to 135-kg lots of corn containing aflatoxin B_1 in concentrations of 0.013, 0.160, and 0.510 μg/g were subjected to the dry-milling process. The prime products, which are used in foods, accounted for 49% to 60% of the product yield but only 6% to 10% by weight of the aflatoxin in the original. Highest levels of aflatoxin occurred in the germ, hulls, and degermer fines--by-products used in animal feeds. Therefore, the addition of these by-products to animal feeds would be less acceptable than the use of the original contaminated corn. In another study, Bennett et al. (1976) examined three lots of zearalenone-containing corn that had been dry milled. They found that the grit fractions used for foods contained the lowest levels of zearalenone and that the germ and feed fractions contained the highest levels.

Wet-milling of corn containing aflatoxin, zearalenone, or T-2 toxin has also been investigated. Commercial corn of acceptable grade for use by the wet-milling industry is almost entirely free of aflatoxin, but the finding of several commercial aflatoxin-containing samples in the late 1960's led to a study of the fate of the toxin during this process (Yahl et al., 1971). The aflatoxin was found primarily in steepwater (39% to 42%) and fiber (30% to 38%); the remainder was observed in gluten (14% to 17%) and germ (6% to 10%). Increases in aflatoxin concentrations in the fractions compared with the concentration in the original corn were as follows: steepwater, 4- to 5-fold; fiber, 2.5- to 3-fold; gluten, 1- to 1.5-fold; and germ, no increase. These fractions, which are used in feed, accounted for 96.7% to 98.3% of the aflatoxin contained in the original corn. The starch fraction, which would be used in food, accounted for 1.0% to 1.2% of the aflatoxin in the original corn. Although it seems advantageous to have the starch fractions relatively free of aflatoxin, the wet-milling industry depends on the feed by-products for profit.

Three lots of corn naturally contaminated with zearalenone in concentrations of 0.9, 4.1, and 9.4 μg/g were used to study the distribution of the toxin in fractions resulting from the wet-milling process (Bennett et al., 1978). The starch fractions used for food were devoid of zearalenone; in the fractions used for animal feed, however, amounts of zearalenone were present in the following order: gluten > solubles > fiber > germ. An investigation of bench-scale wet-milling of corn contaminated with T-2 toxin revealed that the

pattern of T-2 distribution in the products was similar to that observed in the wet-milling of aflatoxin- or zearalenone-containing corn (Collins and Rosen, 1981). Two-thirds of the T-2 toxin was removed in the steepwater, 4% was found in the starch, and the remainder was distributed between gluten, fiber, and germ. More T-2 toxin than zearalenone was concentrated in the germ fraction during wet-milling.

"Distressed" grains, i.e., grains infected with fungi and/or contaminated with mycotoxins, have very little economic value. They cannot be used either in feeds or in foods. Therefore, there have been a number of investigations on the use of mycotoxin-contaminated grains as substrates for ethanol production by fermentation. The studies have shown: (1) little degradation of the toxins during fermentation; (2) no toxin in the distilled alcohol; and (3) toxin accumulation in the spent grains used in feeds.

Bothast et al. (1982) reported an integrated process for ammonia inactivation of aflatoxin-contaminated corn and ethanol fermentation. The aflatoxin is degraded by the addition of 1% ammonia (based on the weight of the grain) during the liquefaction step of the fermentation process. The integrated process produces more (aflatoxin-free) ethanol than fermentations on the same corn with no ammonia treatment. In this process, aflatoxin levels were decreased 80% to 85%.

The fate of ochratoxin during the brewing process to produce ethanol has also been studied (Nip et al., 1975). About 38% of the added ochratoxin (1 μg/g) was degraded during brewing. Most of the remainder was recovered from spent grain, and a small amount was recovered in the yeast and the beer.

The use of zearalenone-contaminated corn in ethanol production was investigated by Bennett et al. (1981). Two lots of yellow corn, severely damaged by Fusarium and contaminated with 8.0 and 33.5 μg/g concentrations of zearalenone, were used for the fermentation. In the original corn, zearalenone was concentrated in the residual solids, which are generally used for animal feed; in the ethanol fraction, no zearalenone was found. Treatment with formaldehyde significantly reduced the level of zearalenone in the fermentation solids used for animal feeds. Treatment with ammonia also reduced levels of zearalenone, but not as effectively as formaldehyde.

In 1980, when a portion of the Canadian wheat crop became contaminated by DON, experiments were undertaken to determine what portion of the DON in the original wheat would appear in the milled flour. Since DON was found in Canadian wheat in 1981 and 1982 and in wheat grown in eastern Nebraska and Kansas in 1982, the studies were extended. Three research groups have reported that the level of DON

in flour is one-third to three-fourths the level in the wheat from which it is milled (Schneider, 1983; Scott, 1983; Seitz, 1983).

DISPOSAL

Incineration or burial has been used to dispose of aflatoxin-contaminated agricultural commodities or laboratory wastes. Tests should be conducted on the trichothecenes to determine the temperature at which they are completely destroyed and rendered suitable for disposal. These tests should include not only pure trichothecenes but also compounds likely to be formed when materials surrounding or containing the trichothecenes exert protective effects against destruction by heat.

Although aflatoxin-contaminated agricultural commodities have been buried as a disposal procedure for years, there have not been adequate tests of the stability of aflatoxins in soil. In fact, aflatoxin-contaminated peanut meals have been used as fertilizers. If burial of trichothecene-contaminated material is considered as a potential method of disposal, the stability of these mycotoxins in the soils should be investigated -- even though it is known that, in comparison to aflatoxins, the trichothecenes are much more thermally stable and resistant to acids, bases, and other chemicals.

CONCLUSIONS AND RECOMMENDATIONS

The toxicity of trichothecenes is associated with the presence of a sterically shielded epoxide group. Research on reactions that break the epoxide ring under mild conditions of pH and temperature should be encouraged. Some promising approaches include hydration reactions and attack by highly reactive reagents such as supernucleophiles, super acids, and triphenylphosphine.

Sodium hypochlorite treatment under alkaline conditions has been proposed as a method to inactivate T-2 toxin. However, structures and toxicities of the resulting products are unknown. Products of the reaction of T-2 toxin with sodium hypochlorite in the presence of alkali and other chemicals used for detoxification should be investigated.

The thermal decomposition of T-2 toxin is affected by the organic and/or inorganic materials in which the toxin occurs. Thus, destruction of T-2 toxin by heat and other physical means should be studied in different materials.

Methods for reclaiming trichothecene-contaminated agricultural commodities by transforming them into useful foods, feeds, and products should be developed.

The use of enzymes may be an effective and essentially nonhazardous technique for detoxifying mycotoxins and protecting personnel from their deleterious actions. Studies should be initiated to determine whether enzymes that bring about mycotoxin detoxification can be obtained and used for protecting personnel.

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Chapter 5

LONG-TERM ENVIRONMENTAL EFFECTS

If trichothecenes or other biologically produced toxins are used as weapons against an agricultural society, those civilian populations would be denied free access to land and to the uncontaminated water and food supplies needed to sustain their lives. Major disruption might also be anticipated if such toxic agents were used against undefended populations in heavily technological nations. Thus, one facet of defense against such toxins involves minimizing not only the immediate effects but also the long-term hazards to civilian populations.

In this chapter, the committee has identified the information that is needed to aid in coping with the long-term effects on public health and agriculture that are associated with the deliberate release of trichothecenes. Of primary importance in minimizing such effects and protecting populations is the characterization of the fate and persistence of trichothecenes in the biosphere. This information is essential if chronic hazards are to be avoided after the immediate hazards have been reduced.

ENVIRONMENTAL CHEMISTRY

Studies of the organic chemistry of soils, waters, and sediments have been hindered by the poor recovery of chemicals, which often occurs even when pure chemicals are added to samples of these environments. Most analytical studies have been focussed on pesticides. The recoveries in these investigations have ranged from 0 to 50%. Further confounding of quantifications and identifications can be attributed to the sorption, conjugation, possibly oligomerization, and as yet uncharacterized reactions that occur in these environments. It is well known that a high percentage of some pesticides added to soils are present in a fraction known as "bound residues," which have not yet been characterized.

There is very little published information on the problems that may be encountered when analyzing, quantifying, and identifying mycotoxins and/or products formed from them in soils, waters, and sediments.

Moreover, there are few data on mycotoxin concentrations in soils, sediments, or waters, either spatially or with time. Plans for such studies are not known to exist.

WATER CONTAMINATION, LEACHING, AND MOBILITY

Many toxic chemicals move downward through soil and contaminate groundwaters, which are often used as sources for drinking water. Other toxins are translocated with eroding soil particles into adjacent surface waters, where some of the chemicals move laterally for considerable distances. Contamination of surface waters is of great importance not only because they are used for drinking and bathing but also because they provide fish for human consumption. With one exception, however, there have been no studies to determine whether mycotoxins will leach through soil to contaminate groundwater or whether there is appreciable lateral translocation through groundwaters or with eroding soil particles to contaminate adjacent surface waters. In that one study, Henrickson and Grant (1971) reported that aflatoxin B_1 and G_1 did not move through soil. No substantive conclusions can be drawn from that study because the soils were not characterized and the volumes of liquid, concentration of chemical, height of soil column, and period of leaching were not specified.

UPTAKE BY PLANTS

Plants used as food for humans and domestic animals may assimilate toxic chemicals in the soils in which they grow. Nevertheless, with one exception, research apparently has not been conducted to examine the possibility that plants will take up mycotoxins from contaminated soils. In that one study, lettuce seedlings were found to take up aflatoxin B_1 from soil and transfer it to above-ground portions of the plant (Mertz *et al.*, 1981). In addition, trichothecenes may be taken up from solution by the roots of certain species of plants (Jarvis *et al.*, 1981), and this may indicate the potential for uptake in soils.

DEGRADATION IN NATURAL ENVIRONMENTS

Many organic compounds are subject to biodegradation or other types of chemical changes in soils and waters. These changes may involve photochemical reactions, nonbiological reactions not involving light, or biodegradation. In soils and waters, microbial processes are the dominant and often the sole means of biodegradation. In the absence of some degradative mechanism, toxic chemicals will persist for very long periods, since the only significant process for decreasing their concentrations are volatilization and leaching. Because of the need

to establish the persistence of mycotoxins, investigations designed to monitor their longevity and to determine whether the processes of loss are biological or nonbiological are quite important.

Products formed during the degradative sequences are often as toxic as the parent chemical, sometimes less toxic, and occasionally, far more toxic. Because of the likelihood that products of different toxicity will be generated in natural waters and soils, information is needed on the products of degradation or other transformations in these environments. These products include those that are generated by photochemical reactions, by nonbiological reactions proceeding in the absence of sunlight, and by microbial processes.

Microbial degradation and transformations are especially important because they are usually the only processes by which organic compounds are converted to inorganic products in the absence of intense sunlight. Since only inorganic products are formed when there is complete degradation, microbial processes result in a total detoxification of the contaminated area. In some circumstances, photochemical reactions may bring about an extensive breakdown of the material, but in only a few instances does such degradation proceed to yield inorganic products. The microbial degradative reactions may involve not only mineralization (the complete conversion of organic molecules to inorganic products) but also cometabolism. In cometabolism, the responsible microorganisms do not grow at the expense of the chemical. The breakdown is therefore slow, and organic products similar to the parent molecule are generated (Alexander, 1981).

The possibility that any mycotoxin is degraded by biotic or abiotic mechanisms in natural waters or in soil has not been evaluated, except for one study of aflatoxin in soils. In that study, only a small amount (6.3% to 14%) of the added aflatoxin was mineralized (i.e., completely degraded biologically and converted to carbon dioxide) in 112 days. The quantity of extractable aflatoxin B_1 declined rapidly, and aflatoxin B_2 and G_2 were formed quickly. However, the aflatoxins B_2 and G_2 that were generated were more persistent and only disappeared after 77 and 49 days, respectively (Angle and Wagner, 1980).

Except for the study of aflatoxins cited above, products formed from any mycotoxin in soils or water have yet to be identified.

DEGRADATION IN MICROBIAL CULTURES

In microbial cultures, the concentrations of several toxins decrease with time, indicating that degradative enzymes exist and are functioning. Such reports exist for T-2 toxin (Cullen *et al.*, 1982),

aflatoxins (Ciegler et al., 1966; Detroy et al., 1971; Schroeder, 1966), gliotoxin (Brewer and Taylor, 1966), sporidesmin (Dingley et al., 1962), patulin (Burroughs, 1977), rubratoxin (Emeh and Marth, 1978), and epipolythiadioxopiperazines (Taylor, 1971). In a very few instances, products of the microbial process have been characterized, but each of the products represented only a modest change of the parent mycotoxin. Extensive microbial degradation has yet to be shown.

Nakayama et al. (1980) isolated 12 bacterial strains from soil. Each of the strains was capable of utilizing T-2 toxin as a sole carbon source. Two strains were Nocardia-like, six were strains of Bacillus, and four were unidentified. T-2 toxin was transformed to HT-2 toxin, T-2 triol, and T-2 tetraol by one of the unidentified strains (Nakayama et al., 1980).

Other transformations are the conversion of T-2 toxin to HT-2 toxin by Fusarium (Yoshizawa and Marooka, 1975); the acetylation and deacetylation of 12,13-epoxytrichothec-9,10-enes (Claridge and Schmitz, 1978); the deacetylation of tetracetylnivalenol to 4,7,15-triacetylnivalenol and of 3-acetyldeoxynivalenol to deoxynivalenol by Fusarium (Yoshizawa and Marooka, 1975, 1974); the conversion of aflatoxin B_1 to a product with the chromatographic characteristics of aflatoxin B_2 by Penicillium raistrickii (Ciegler et al., 1966); the conversion of aflatoxin B_1 to aflatoxicol (Doyle et al., 1982); the transformation of verrucarins A and B to the corresponding 16-hydroxy compounds and to 3'-hydroxyverrucarin A by Rhizopus arrhizus (Pavanasasivam, 1980); the metabolism of zearalenone to 6',8'-dihydroxyzearalenone by Fusarium roseum (Steele et al., 1977); the bacterial metabolism of ochratoxin A (Wegst and Lingens, 1983); and the cleavage of the isocrotonic ester and consequent detoxication of trichothecin and crotocin by Penicillium chrysogenum (Horvath and Varga, 1961). The enzymes involved in these microbial transformations have not been characterized or purified.

NEEDED RESEARCH

To characterize chronic threats posed by trichothecene residues, studies to assess the following components of environmental persistence and degradation should be conducted: stability in sterile aqueous systems at defined pH; photochemical degradation of compounds in water and as aerosols; mobility in soils; metabolism and persistence in soils, water (including irrigation facilities), activated sludge and other waste-treatment systems, and crop plants; persistence on buildings and equipment; and uptake, accumulation, and disposition by crop plants, forage plants, aquatic plants, invertebrates, fish, domestic animals, and game animals.

In parallel with these studies, consideration should be given to possible detrimental effects on the long-term capacity of the environment to support the population after a chemical attack. These studies should examine effects on processes that maintain soil fertility (e.g., nitrogen fixation), effects on the ability of soils to degrade plant materials, and effects of persistent residues on crop plants and on species of animals that support agricultural production, including pollinating insects.

Studies such as these are conducted routinely by commercial laboratories to obtain safety data for the registration of pesticides and veterinary drugs. Experimental designs and protocols for studies of trichothecenes should be similar to those proposed in the guidelines for the registration of pesticides (U.S. Environmental Protection Agency, 1982); however, emphasis should be given to increasing the numbers of soil types and geographic areas included.

The committee anticipates that the environmental behavior of T-2 toxin, DON, and verrucarins will be representative of the behavior of other similar trichothecenes and that less extensive data will be required to estimate the long-term hazards that other trichothecenes might present following an attack.

Standard practice in pesticide studies is to test 5 times the projected use level. Similarly, studies of environmental effects must take into account the anticipated ambient concentrations. Samples of trichothecenes from Southeast Asia indicate that peak environmental concentrations were on the order of 100 μg/g (Haig, 1982), but actual use levels may have been higher because of dilution and degradation effects. Most tests would likely be conducted at concentrations of at least 100 μg/g. Others may be performed at 500 μg/g. Experimental protocols will have to account for the fact that investigators will be at risk from such exposures. At a minimum, hematology studies before, during, and after the investigations should be mandatory for all laboratory personnel. Disposal of experimental materials is an additional consideration.

Requisites for Conducting Persistence Studies

Certain materials must be available before this set of studies can be conducted. There must be an adequate supply of pure T-2 toxin or other trichothecenes as well as carbon-14-labeled T-2 toxin of adequate purity and specific activity. Carbon labels are preferred to tritium labels for such studies, since they enable investigators to follow the fate of the carbon skeleton. Ideally, the label should be uniform. Failing this, one or more of carbons 6 through 11 should be labelled, since this portion of the molecule is likely to exist longest in the environment.

Adequate analytical methods are also essential in the conduct of such studies. They must be carefully designed and validated before use. Current methods for the analysis of trichothecenes are discussed in a separate section of this report. However, since the studies described above are dependent on the application of robust analytical methods, they are discussed further in this chapter.

There are several analytical alternatives for attaining the levels of accuracy, precision, and sensitivity required for environmental studies. The selection of the method to be used will be dependent on other properties of the various techniques. Included among the factors to be considered are: (1) requirements for relatively high rates of sample analysis using semiautomated instrumental methods; (2) use of readily available equipment, reagents, analytical software, and skills; (3) selectivity of detection, which will be reflected in minimal sample preparation requirements; (4) simplicity of sample preparation to minimize variability due to manipulation; (5) modest labor costs per sample; and (6) robust analytical methods yielding data that will not vary appreciably with changes in personnel or techniques.

In studies of persistence, many thousands of assays are necessary to achieve sensitivity limits of between 0.01 and 0.05 ppm. Resources expended on an innovative program of method development and careful method validation may result in an overall reduction in experimental costs. Following an initial exploratory phase, in which parallel radiochemical and chemical analyses are used to determine the suitability of various portions of a method, a validation study must be conducted.

In practice, the development stage is less structured than the validation phase, and generally requires considerable innovation and experimentation if the resulting method is to be optimum. Simplicity should be sought in preference to lengthy complex procedures. The radioisotope recoveries measured at the various intermediate stages of the analysis should be used to assess the reliability of each step of the method. The radiolabel data often expedite optimization of the method by indicating the source of losses during sample preparation.

In a typical, well-designed validation procedure, six or more levels of radio-labeled toxin are added to the sample matrix. The concentrations normally include one-half the concentration of the projected quantification limit and twice the projected upper limit of the method. One sample should consist of the unamended sample matrix. Each matrix containing the radiochemical should be analyzed at least three, and preferably six, times in such a manner that the analyses are true replicates rather than multiple instrumental analyses of the same extract. The instrumental analyses are often done in triplicate so that any variability due to instrumental sources

can be assessed. The analyst should be experienced in the use of the method and, preferably, should not be aware of the content of the samples. The radio-labeled substance is often used during validation both as a secondary check on recoveries and as a control for sample-dependent interferences.

The analytical methods of choice for hazard assessment studies often include the use of gas-liquid chromatography (GLC) and/or high-performance liquid chromatography (HPLC). Gas chromotography-mass spectrometry (GC-MS) is usually used for confirmation rather than as a primary method. In metabolism studies involving a radio-labeled substance, HPLC is the method of choice, since the radioactive metabolites may be easily recovered and then quantified by scintillation counting. Since real time scintillation detectors do not generally have the sensitivity required, counting is best done after separation. Thin-layer chromatography (TLC) can serve a similar function, but there is less sensitivity and more difficult, labor-intensive recovery with this method. Both instrumental methods are usually automated quite easily and can be used to process at least 30 samples within 24 hours.

For the trichothecenes, routine analysis using GC to determine the heptafluorobutyryl derivatives (Scott et al., 1981) is probably preferable to HPLC, unless chemical derivatives suitable for pre- or postcolumn detection are developed. Enhanced selectivity might be attained with this approach. Some possible derivatization reactions that may prove useful include reaction of the epoxide with a chromophore such as a nitrophenol to yield a colored product in a basic environment (as the phenolic group is blocked), reaction of the ester groups with hydroxylamine and subsequent detection via formation of a ferric complex, and postcolumn electrochemical detection using electrochemical production of active bromine and its consequent loss in the presence of a double bond.

If biological samples are to be analyzed, the radioactive test substance should be introduced into an appropriate organism. Since the trichothecenes are hydroxylated compounds, conjugates will be produced. Methods should be developed to recover as much of the biologically incorporated label as possible. For example, a mixture of proteases, glucuronidases, and sulfatases must be used to maximize recovery of zearalanol[1] from the rat liver when the compound is introduced into a live animal. The more simplistic approach of adding different levels of zearalanol to the liver orginally led to a method

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[1] Zearalanol is a derivative of zearalanone. It contains a saturated bond at the 10,11 position and a hydroxyl group at the 6 position.

that was apparently valid but did not reflect actual tissue levels of zearalanol.

An additional requirement for conducting persistence studies is toxicological data on the test substance. This requirement is met by the available data on the trichothecenes.

Any protocols developed in this area should be reviewed by nonmilitary scientists in the field of environmental chemistry and analysis, and an independent contractor should monitor the conduct of the studies to ensure the quality of the experimental work. The methods to be used should be evaluated in at least two other laboratories before initiating extensive studies. If the studies are conducted under contract, the competing laboratories should be required to demonstrate their ability to perform accurate analyses of samples with a known toxin content.

CONCLUSIONS AND RECOMMENDATIONS

Current research programs do not address the persistence of trichothecenes in nature and the hazards of long-term exposure to these toxins. In addition, there is little information on the chemical behavior of mycotoxins in soils and waters, their uptake by plants, or their transfer through food chains. Studies are not known to be under way to determine the periods of resistance of mycotoxins in waters and soils. No studies have been conducted on the leaching, degradation in nature, or products formed in waters and soils following treatment with trichothecenes, and there are essentially no data on any other mycotoxin.

Studies should be conducted to establish the persistence, leaching, and susceptibility to degradation of mycotoxins in various soil types and natural waters. Such studies might be modeled after those commonly used to assess the hazards presented by pesticides.

The products of nonbiological and microbial degradation in these environments should be established, and the persistence of these products should be determined.

Information is required on the uptake of mycotoxins by plants growing in contaminated soils and on the behavior and reactions of mycotoxins in terrestrial and aquatic food chains.

Analytical methods for soils, water, physiological fluids, and tissues of plants and animals should be developed and validated before any of the studies outlined are undertaken. Protocols should be reviewed by nonmilitary scientists, and independent contractors should monitor the conduct of studies.

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Chapter 6

RESPONSES OF BIOLOGICAL SYSTEMS

BIOLOGICAL EFFECTS IN HUMANS

Historical Studies

Alimentary Toxic Aleukia. There is little definitive information on the toxic effects in humans produced by specific trichothecene mycotoxins, with the exception of diacetoxyscirpenol (DAS). Extensive descriptions of toxicoses in humans, presumably caused by unidentified trichothecenes, were provided in reports of illness caused by ingestion of Fusarium-contaminated grain products in the Soviet Union and Japan. These foodborne illnesses were first reported at the beginning of the 19th century, but it was not until the 1950's and 1960's that the trichothecene mycotoxins were first isolated and identified (Yagen et al., 1977). Evidence linking Fusarium metabolites to the outbreaks was based on experiments in fungi isolated from naturally contaminated food and preserved as laboratory cultures before analyses for trichothecene production (Ueno et al., 1972; Yagen et al., 1977).

Uraguchi et al. (1961) described an outbreak of foodborne disease that occurred in the suburbs of Tokyo in 1954. He reported that 25 persons who had consumed Fusarium-infected rice subsequently suffered from nausea, vomiting, and drowsiness. Human mycotoxicoses resembling alimentary toxic aleukia (ATA) have been recognized in the Soviet Union for many decades. Ueno (1977b) cited the review by Woronin (1891) of the so-called "Taumelgetreide" (staggering grains) found in the Ussuri district of eastern Siberia. People consuming the grain had developed headache, vertigo, chills, nausea, vomiting, and visual disturbance.

Gajdusek (1953) has provided an excellent description of ATA. This disease was first identified in the Soviet Union as "septic angina" because it included necrotizing tonsillitis, fever, hemorrhagic diathesis, and severe intoxication. Sarkisov (1944) reported that Fusarium sporotrichioides or toxins produced by this fungus were responsible for the disease.

The clinical course of ATA was divided into four stages (Gajdusek, 1953; Joffe, 1971; Mayer 1953). Within the first few hours after ingestion of contaminated food, local irritation of the oral mucous membranes occurred. As intoxication developed, there was hypermia of the oral mucosa accompanied by weakness, fever, nausea, emesis, and sleep disturbances. In mild cases, these signs and symptoms disappeared within a few days after the contaminated food was removed. However, in the more severe cases, the high fever continued and was accompanied by acute esophagitis, gastritis, and gastroenteritis. Cyanosis and tachycardia were observed in a few individuals and, in rare cases, circulatory failure and convulsions occurred.

When contamination was great or the exposure duration was extended, the disease progressed to the second stage--the leukopenic stage. The alterations observed included leukopenia, granulopenia, and progresseive lymphocytosis. Electrocardiographic changes were recorded in some individuals. This stage lasted between 2 and 4 weeks, but Gajdusek (1953) noted that it may extend to 8 weeks. When the contamination was discontinued and concentrations consumed were low, complete recovery followed.

When the consumption of contaminated food was greater and larger amounts of toxins were ingested, the disease progressed to the third stage--the hemorrhagic stage, which was characterized by the presence of petechial rash on the skin of the chest and other areas of the body. Initially, the petechiae were localized in small areas, but they became more numerous and widespread as time progressed. The exposed persons also developed severe pharyngitis. This was described by Gajdusek (1953) as "catarrhal, diphtheritic, necrotic, or gangrenous." The tissue in the throat was necrotic with the production of a brown exudate that contained microflora usually found in agranulocytic angina. The most severe cases had intensive ulcerative and gangrenous changes of the larynx, which resulted in aphonia and death by strangulation.

In this third stage, exposed individuals also had severe hemorrhagic diathesis of the nasal, oral, gastric, and intestinal mucosa and the peripheral blood had reduced numbers of formed elements. Leukopenia was marked and leukocytes were as low as 100 to 200/mm^3. Erythrocyte counts usually ranged from 2 to 2.5 million/mm^3; however, some persons had counts less than 1 million/mm^3. The hemoglobin values were usually below 40% and in some individuals, below 10%. Blood loss from hemorrhages and impaired erythropoiesis resulted in severe anoxia. Gajdusek (1953) reported that the platelet counts ranged from 20,000 to 80,000/mm^3, compared to normal counts of 200,000 to 300,000/mm^3, and that hemorrhages normally occurred when platelet counts were less than 50,000/mm^3. Sedimentation rates were increased to more than 50 mm/hr,

and the clotting and clot retraction times were prolonged. Rumple-Leeds phenomenon (capillary fragility) was observed in approximately one-half of the patients examined.

At this stage of illness, patients remained conscious; however, they had elevated temperature (38.9 to 40°C), hypotension, and a weak pulse. Deaths occurred during this stage, which lasted for 2 weeks. In the more severe outbreaks of poisoning, fatality rates were as high as 50%.

The fourth stage of ATA was the recovery stage. During this period, exposed individuals were susceptible to various secondary infections, including pneumonia. Convalescence was prolonged, lasting several weeks for some patients. Most of the patients were 10 to 40 years of age. No illness was detected in nursing infants whose mothers had ATA.

Stachybotryotoxicosis. Gajdusek (1953) also reviewed the Soviet literature on stachybotryotoxicosis in horses and humans. He noted that signs and lesions of the disease were suggestive of mycotoxicosis, and that many of the toxic effects reported in these studies were the same as those now known to be induced by trichothecenes produced by other fungal genera (i.e., Fusarium, Trichothecium, Trichoderma, Acremonium, Cylindrocarpon, and Myrothecium).

In equine animals, the disease has been subdivided into the following three stages: (1) a cutaneous stage, in which the skin and mucous membranes are affected; (2) a generalized toxicosis involving the blood-forming organs; and (3) a neurologic stage, involving a loss of appetite, digestive disturbances, and the initiation of secondary infections (Hintikka, 1977). Drobotko (1945) implicated Stachybotrys atra (= alternans) as the biological agent responsible for human cases of "septic angina" resulting from the ingestion of overwintered grain. The symptoms were similar to those seen in the equine disease and were indistinguishable from the symptoms of fusaria-produced ATA.

In early experimental work, humans were found to be sensitive to the mold and to the extracts, both of which produced dermatitis when applied to the skin. Signs and symptoms in individuals exposed to contaminated hay or hay dust included dermatitis, conjunctivitis, cough, rhinitis, laryngitis, pharyngitis, irritation and bleeding from nasal passages, fever, and, rarely, leukopenia (Drobotko, 1945).

Eppley (1977) reported that cultures of S. atra contained at least five trichothecenes, three of which belonged to the roridin-verrucarin group. All these trichothecenes caused skin lesions in rabbits and were lethal for chick embryos.

Dendrodochiotoxicosis. Bilai (1960) and other Soviet investigators have mentioned Dendrodochium toxicum as a fungus capable of producing a highly potent, but unidentified toxin. However, Tulloch (1972) has classified this organism as Myrothecium roridum, a recognized producer of the macrocyclic trichothecenes--roridins and verrucarins. Ueno (1983) alluded to Dendrodochium-produced toxicoses in humans, but did not elaborate upon this subject.

Clinical Effects of Known Trichothecene Toxins

Although ATA is believed by many to be a disease involving T-2 toxin and possibly other trichothecenes, its etiology cannot be established. The toxic effects produced in humans by trichothecene mycotoxins have been established only for DAS (anguidine), which has been used as a chemotherapeutic agent in both phase I and II evaluations for treatment of human malignancies (Table 6-1). DAS was selected for clinical trials based on its activity in mice with P388 and L1210 leukemia (Murphy et al., 1978). In addition, activity was observed against Line 38 colon adenocarcinomas in mice (Helman et al., 1976).

Phase I clinical trials are conducted to establish the maximum tolerated dose (MTD) in humans and to determine the toxic effects. In the Phase II trials, the drug is screened against a range of tumors for potential activity. In these clinical trials, the antitumor activity of DAS was minimal or absent, but a variety of toxic effects were recorded.

In a phase I clinical evaulation of DAS, the compound was given to 39 patients with malignant neoplasms (Murphy et al., 1978). It was administered by rapid intravenous infusion daily for 5 days at 2-week intervals. At doses greater than 3.0 mg/m^2 body surface area or 0.077 mg/kg body weight (bw),[1] signs of toxicity including nausea and vomiting were observed. Myelosuppression, which occurred in 11 of 25 (79%) of patients, was mild to moderate at doses of 3 mg/m^2 (0.077 mg/kg bw) or above, reaching a peak 12 days after initiation of the treatment. Less commonly observed signs and symptoms of toxicity included hypotension, diarrhea, central nervous system (CNS) disturbances, fever and chills, a generalized burning sensation, erythema, and stomatitis. No antitumor activity was observed.

Goodwin et al. (1978) recorded the responses of cancer patients treated with DAS. They observed toxicity in patients given doses ranging from 0.2 mg/m^2 (0.005 mg/kg bw) to 6.0 mg/m^2 (0.154 mg/kg bw) for 5 days by intravenous injection (IV push). Total leukocytes

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[1]The mg/m^2 values were converted to mg/kg body weight by assuming a 70-kg patient with a body surface area of 1.8 m^2.

TABLE 6-1. Toxic Effects in Human Cancer Patients Given Diacetoxyscirpenol (Anguidine)

Clinical Observation	Patients Experiencing Toxic Effect (%), by Study				
	Murphy et al., 1978	Diggs et al., 1978	Yap et al., 1979	Bukowski et al., 1982	Thigpen et al., 1981
Nausea and vomiting	100	74	87	49	64
Myelosuppression	79	[a]	[a]	64	19
Hypotension	20	37	31	50	24
Diarrhea	20	10	NR[b]	NR	NR
Central nervous system dysfunction	20	16	38	12	4
Fever and chills	12	84	33	NR	NR
Stomatitis	8	NR	4	4	NR
Erythema	12	26	22	NR	4
Hair loss	NR	NR	4	NR	4

[a]Myelodepression given as moderate; number of patients not specified.
[b]Not reported.

and platelet numbers were reduced in some of the subjects. After three doses of 3.0 mg/m^2 (0.077 mg/kg bw), nausea was accompanied by signs of CNS dysfunction, including drowsiness, nervousness, confusion, anxiety, depression, convulsions, hallucinations, and coma. Gastrointestinal tract disturbances and diarrhea were present in patients given the higher doses. When the method of administration was changed to 48-hour infusion, the CNS symptoms were absent, but thrombocytopenia was evident by day 4 of treatment. Fever, chills, and vomiting were minimal during the 8-hour infusion period, but signs were more severe in patients given 4-hour infusions. No significant long-term alterations were observed in renal, heptic, or pancreatic functions or in serum protein concentrations, total protein, albumin, ceruloplasmin, immunoglobulins, or lipoproteins.

DAS was used in a phase II treatment of adenocarcinoma of the colon and rectum (Diggs et al., 1978). It was administered intravenously in doses of 5.0 mg/m^2 (0.129 mg/kg bw) daily for 5 days and repeated every 3 weeks. Patients with hepatic disease were given 3.5 mg/m^2 doses. The compound was added to 150 ml of dextrose and given to the patient by intravenous infusion for 2 to 3 hours. No patient had a measureable regression of neoplastic disease. Signs and symptoms of toxicity included fever (16/19 patients), nausea and vomiting (14/19 patients), chills (8/19 patients), hypotension (7/19 patients), CNS disturbances (3/19 patients), and diarrhea and headache (2/19 patients). Acute hypotension was severe enough in some patients to require large amounts of intravenously administered fluids, but most episodes of hypotension were transient. Myelosuppression was moderate.

A similar set of clinical signs and symptoms of toxicity was observed in patients with advanced breast cancer, who were treated with DAS at a dose of 5.0 mg/m^2 (0.129 mg/kg bw) daily for 5 days repeated every 3 weeks (Yap et al., 1979). Patients with hepatic disease were given 3.0 mg/m^2 doses. Nausea and vomiting were most frequently observed (87% of cases). Other evidences of toxicity were fever and chills (33%), hypotension (31%), skin erythema (22%), CNS disturbances (38%), burning sensation (4%), and hair loss (4%). In most patients, mild myelosuppression occurred between the second and third weeks of treatment.

DAS was used in a phase II treatment of gastrointestinal malignancies (Bukowski et al., 1982). The compound was administered as a 4-hour intravenous infusion at doses of 3.0 or 4.5 mg/m^2 (0.077 or 0.116 mg/kg bw) daily for 5 days. Partial remission was observed in eight of 81 patients with colon cancer and in a single patient with carcinoma of the common bile duct. The toxicity of DAS was varied. Myelosuppression was mild to moderate; thrombocytopenia and leukopenia occurred in 19% of the patients. Nausea and vomiting was common (49% of the patients). Mild hypotension was documented in 37 patients, and severe hypotension was observed in 10 patients. CNS toxicity manifested by hallucinations and/or confusion was observed in 12% of the patients.

DAS was used in a phase II trial in patients with sarcomas and metastatic disease (Thigpen et al., 1981). The compound was given in daily doses of 4.5 mg/m^2 (0.116 mg/kg bw) as a 4-hour intravenous infusion for 5 consecutive days and repeated every 21 days. None of 25 treated patients showed any measureable antineoplastic response to the drug treatment. The most significant adverse effect, myelosuppression, was found in 16 of the 25 patients as leukopenia and/or thrombocytopenia. Nausea and/or vomiting occurred in 16 of the 25 patients, hypotension in 6, and hallucination, erythema, stomatitis, and alopecia in one patient each.

In the United States, three accidental cases of trichothecene poisoning are known. A laboratory research assistant working with T-2 toxin developed a severe facial skin irritation that required prolonged nonspecific dermatologic treatment before recovery.[2] In the other two cases (Bamburg, 1969), a crude extract of Fusarium tricinctum accidentally got inside the plastic gloves of two laboratory investigators, who washed their hands with mild detergent within 2 minutes. Nevertheless, an increasing burning sensation developed in the fingers of both hands 4 to 8 hours afterward, followed 1 day later by numbness in the fingers, which within 3 days lost all sensitivity. Skin on the tips of the fingers that had the most contact with the toxic extract became white and hard and began to peel in large, thick pieces. New skin regained normal sensitivity by the 18th day after contact.

Recent reports (Department of External Affairs, 1982; Haig, 1982; National Defence Headquarters, 1982; Schiefer, 1982; Shultz, 1982) implicate the presence of trichothecene mycotoxins, notably T-2 toxin, in "yellow rain," which allegedly was used for military purposes in Southeast Asia and in Afghanistan. In these reports, four major effects of "yellow rain" on human health were emphasized, namely, epithelionecrosis on exposed areas of skin, acute gastroenteritis, convulsion seizures, and death in approximately 25% of the persons reported to have been exposed. Many of the signs and symptoms of toxicity observed in DAS-treated human cancer patients are the same as those reported to have been observed in persons exposed to "yellow rain" attacks. Observations common to both sets of human patients include nausea and vomiting, cutaneous irritation, CNS dysfunction, hypotension, chills, fever, and myelosuppression.

However, other alterations observed in persons reportedly exposed to "yellow rain" have not been found in patients treated with DAS. These differences may be related to the differences in dose and in the duration and route of exposure between the two groups. All five DAS-treated patients who died from treatment had received the compound by intravenous infusion under hospital conditions.

Because of unknown exposure doses in the yellow rain reports and evidence that other agents may have coexisted in mixtures with the trichothecenes, the data were considered unreliable in determining biological effects of toxic trichothecenes in humans. For this reason, data derived from controlled experiments in animals must be used to predict the biological effects of these toxins.

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[2] Wilson, B. J. 1983. Personal communication. Vanderbilt University, Nashville, Tenn.

BIOLOGICAL EFFECTS IN ANIMALS

Because certain trichothecene mycotoxins, such as T-2 toxin, DAS, and deoxynivalenol (DON), are found as natural contaminants of grains and processed feedstuffs, considerable research effort has been expended in defining their toxic effects in various species of domestic and laboratory animals. In these studies, various routes of exposure have been used, including parenteral injection, oral dosing, and dietary administration. In many of the studies with rodents, however, injection (e.g., intravenous, intraperitoneal, and subcutaneous) has been the major route of administration. Test substances have included grains contaminated with defined fungal species as well as purified mycotoxins.

In contrast to the few data on responses of humans to the trichothecenes, especially to T-2 toxin, substantial toxicological data on animals are available for evaluations of the biological effects of several specific trichothecene mycotoxins. However, virtually none of these data have been derived from topical or aerosol exposure of experimental animals.

Comparative Toxicity

The LD_{50} values of the trichothecenes vary somewhat with the particular toxin and animal species studied (Tables 6-2 and 6-3). The more toxic members of the group, i.e., those with lower LD_{50}s, include T-2 toxin, 4-acetylnivalenol (fusarenon-x), nivalenol, and DAS. One of the least toxic compounds in this group is DON. There are differences among the various species in their susceptibility to the trichothecenes, but the differences are not great, considering the LD_{50} values obtained by oral and other routes of administration. For most of the animals examined in the LD_{50} studies, the dose-response curves for lethality were relatively steep, indicating limited individual animal variation. Similar LD_{50} values for T-2 toxin have been obtained in studies in the chicken, rat, and guinea pig. In mice, slightly greater LD_{50} values were obtained for T-2 toxin and DAS, but very similar values were obtained for 4-acetylnivalenol.

These toxins produce damage in a variety of organs and tissue. The threshold dose for certain of the tissue effects varies both in respect to the species exposed and the trichothecene mycotoxin used (Table 6-4). The alterations, either in clinical signs or tissue morphology or function, are similar among the various species studied and reflect damage to the target tissues that have a component of rapidly dividing cells such as the gonads, gut, bone marrow, and lymphoid tissues.

Table 6-2. LD_{50}s of Several Trichothecene Mycotoxins in Various Animals

Trichothecene	Animal	Route of Administration	LD_{50}(mg/kg)	Reference
T-2 Toxin	Chicken	Oral	4.0	Hoerr et al., 1981
	Chicken	Oral	3.6	Sato and Ueno, 1977
	Rat (Holtzman)	Oral	4.0	Marasas et al., 1969
	Rat	Oral	5.2	Sato and Ueno, 1977
	Trout fingerlings	Oral	6.1	Marasas et al., 1969
	Swine	Intravenous	1.21	Weaver et al., 1978a
	Guinea pig	Oral	3.1	DeNicola et al., 1978
	Mouse (ddYS strain)	Intraperitoneal	5.2	Sato and Ueno, 1977
	Mouse	Oral	10.5	Sato and Ueno, 1977
DAS	Chicken	Oral	5.0	Hoerr et al., 1981
	Swine	Intravenous	0.376	Weaver et al., 1978b
	Mouse	Intraperitoneal	23.0	Sato and Ueno, 1977
	Mouse	Intravenous	12.0	Sato and Ueno, 1977
	Rat	Intravenous	1.3	Sato and Ueno, 1977
	Rat	Intraperitoneal	0.75	Sato and Ueno, 1977
	Rat	Oral	7.3	Sato and Ueno, 1977
	Rabbit	Intravenous	1.0	Sato and Ueno, 1977
Nivalenol	Mouse (DDD or DDN strains)	Oral	4.1	Sato and Ueno, 1977
4-Acetylnivalenol				
	Mouse (DDS strain)	Intraperitoneal	3.3	Ueno et al., 1969
	Mouse	Subcutaneous	4.2	Sato and Ueno, 1977
	Mouse (DDD strain)	Intravenous	3.4	Ueno et al., 1971b
	Mouse (DDD strain)	Per os	4.5	Sato and Ueno, 1977
	Mouse (DDD strain)	Subcutaneous	4.2 - 4.6	Sato and Ueno, 1977
	Rat (Wistar stock)	Oral	4.0	Sato and Ueno, 1977
	Rat	Not reported	4.4	Sato and Ueno, 1977
Solaniol	Mouse (ddYS)	Intraperitoneal	14.5	Ishii et al., 1971
DON	Chick	Oral	140.0	Huff et al., 1981
Neosolaniol	Mouse (ddYS)	Intraperitoneal	14.5	Ueno et al., 1972
HT-2 toxin	Mouse	Intraperitoneal	9.0	Sato and Ueno, 1977
Diacetylnivalenol	Mouse	Intraperitoneal	9.6	Sato and Ueno, 1977

Table 6-3. LD_{50}s of Trichothecene Mycotoxins in the Mouse and Broiler Chicken

Animal	Trichothecene	LD_{50} (mg/kg)
Mouse (ddYS strain)[a]	T-2 toxin	5.2
	DAS	23.0
	Neosolaniol	14.5
	HT-2 toxin	9.0
	Nivalenol	4.1
	4-Acetylnivalenol	3.3
	Diacetylnivalenol	9.6
Chicken[b]	8-Acetylneosolaniol	3.2
	DAS	3.8
	T-2 toxin	4.9
	HT-2 toxin	7.2
	Neosolaniol	24.9
	T-2 tetraol	33.8

[a]Data from Ueno et al., 1973.
[b]Data from Chi et al., 1978.

Comparative Effects by Route of Exposure

For many toxic materials, the route of exposure alters the pathologic effects and clinical signs. However, a number of the toxic responses to the trichothecene mycotoxins are similar, regardless of the exposure route. The acute LD_{50}s of several trichothecene mycotoxins are similar for both oral and parenteral exposures (Tables 6-2 and 6-3). Once they enter the systemic circulation, trichothecenes affect rapidly proliferating tissues independently of exposure route. Therefore, oral, parenteral, and cutaneous exposures produce gastric and intestinal lesions; hematopoietic and immunosuppressive effects described as radiomimetic in nature; CNS toxicity resulting in anorexia, lassitude, and nausea; suppression of reproductive organ function; and acute vascular effects leading to hypotension and shock.

Although a number of toxic effects are common to multiple routes of exposure, route-specific effects have also been observed. For example, topical exposure leads to local cutaneous necrosis and inflammation (Hayes and Schiefer, 1979; Marasas et al., 1969; Ueno et al., 1970). Oral exposure results in upper gastrointestinal tract lesions as well as systemic effects (Chi et al., 1977b,c; Chi and Mirocha, 1978; Hoerr et al., 1982a; Wyatt et al., 1972).

Table 6-4. Toxic Effects of Trichothecenes in Several Animals at Different Dose Levels

Trichothecene	Animal	Toxin Intake[a]	Effect	Reference
T-2 toxin	Cattle	0.16 mg/kg[b]	Enteritis; abomasal ulcers	Pier et al., 1976
		0.64 mg/kg	Death (calf); bloody feces, enteritis; abomasal and rumenal ulcers; coagulopathy	Pier et al., 1976
		0.6 mg/kg	Suppressed lymphoblastogenesis	Buening et al., 1982
	Swine	12 ppm	Reduced pig and litter size	Weaver et al., 1977
		8 ppm	No effects detected	Weaver et al., 1977
	Chickens			
	Broiler	4 ppm	Oral necrosis	Wyatt et al., 1972
	Broiler	4 ppm	Neural effects: seizures, loss of righting reflex	Wyatt et al., 1973
	Broiler	4 ppm	Hepatic hematoma	Wyatt et al., 1973
	Broiler	8 ppm	Coagulopathy	Doerr et al., 1974
	Broiler	4 ppm	Reduced gain	Wyatt et al., 1973
	Broiler	1.5-3 mg/kg	Decreased rate of gain	Hoerr, 1982b
	Broiler	2.5 mg/kg (single)	Thymic and bursal necrosis	Hoerr, 1981
	Broiler	3 mg/kg	Malformed feathers	Hoerr, 1982b
	Laying hen	20 ppm	Decreased egg production and shell quality	Wyatt et al., 1975
	Cats	0.6-1.0 mg/kg	Hematopoietic depression, hemorrhage, gastroenteritis	Palti, 1978
	Trout	6.1 mg/kg	LD_{50} (fingerlings)	Marasas et al., 1969
		8.0 mg/kg	Sloughing of intestinal mucosa	Marasas et al., 1969
	Rats	3.8-4.0 mg/kg	LD_{50} (21-day female)	Marasas et al., 1969
DAS	Swine	0.38 ppm	Hemorrhagic enteritis	Pathre and Mirocha, 1977
	Chickens			
	Broiler	2.5-3.5 mg/kg	Decreased rate of gain	Hoerr, 1982b
	Broiler	2.5-3.5 mg/kg	Decreased hematocrit	Hoerr, 1982b
	Broiler	2.7 mg/kg[c]	Thymic and bursal necrosis	Hoerr, 1981
	Rats	7.3 mg/kg[c]	LD_{50}	Ueno, 1980

[a]Toxin intake in mg/kg/day or feed content in ppm (μg/g) taken ad libitum.
[b]Daily dose.
[c]Single dose.

Studies of inhalation exposure to trichothecene toxins have not been reported. This route of exposure could result in lesions in both the respiratory and gastrointestinal tracts as well as local tissue necrosis, immunosuppression, and the previously discussed route-independent effects. Several factors could alter the response produced by a given dose.

The entire respiratory tract would be exposed to the inhaled material. After absorption from the nasal mucosa, from the upper respiratory tract mucosa, and from the lungs, the toxin will appear in the bloodstream and will be distributed to other organs and tissues. For approximately 24 hours after an inhalation exposure, the toxin deposited in the upper respiratory tract will be cleared by ciliary activity and will be swallowed, resulting in gastrointestinal exposure to the toxin. Therefore, inhalation exposures will be followed by exposure of other tissues and organs at varying dose rates.

The major subdivisions of the respiratory tract differ in their structure, size, function, and response to inhaled particles. They also have different methods and rates of particle elimination. Therefore, the effective tissue dose of inhaled of trichothecene particles is a function of regional deposition within the respiratory tract, retention times at the deposition sites and along the clearance pathway, the biological effects of the toxin, and the physicochemical properties of the inhaled particles.

Airborne particle concentration and size distribution are major factors in determining the relative amount of toxin deposited in the major subdivisions of the respiratory tract. Total and regional deposition of 0.1- to 10- μm particles has been determined (Davies et al., 1972; Hatch and Gross, 1964; Heyder et al., 1973; Lippmann and Altshuler, 1975; Stuart, 1973; Task Group on Lung Dynamics, 1966).

The aerosal size measurement most commonly used in health-related literature is the aerodynamic diameter, which includes both the particle density and its airborne behavior due to various forces. The numerical value of the aerodynamic diameter is equal to the diameter of a unit density sphere having the same terminal settling velocity as the particle under consideration.

To consider the deposition, retention, and removal of inhaled particles, the respiratory system can be divided into three major subdivisions based on clearance mechanisms. (1) The extrathoracic airways include the nasal passages, oropharynx and larynx, and the upper trachea. Particles in these regions are removed through the nostrils or by the movement of mucus into the gastrointestinal tract within minutes to hours after deposition. (2) The tracheobronchial tree, or conducting airways, are lined with ciliated and secreting

cells that distribute inspired air to the gas-exchange spaces within the lung. Inert, insoluble particles deposited in this region are carried within 1 day toward the larynx, and the material is swallowed (Albert et al., 1969). Soluble particles clear more rapidly, presumably into the bronchial blood flow (Yeates et al., 1973). (3) Beyond the terminal bronchioles is the region of gas exchange in the lungs. Aveolar clearance of insoluble particles may be due to penetration of the aveolar wall and transport via the lymphatic system, slow dissolution in physiologic fluids, or phagocytosis by lung aveolar macrophages and transport to the lymphatic circulation. Soluble particles are cleared much more rapidly from the aveolar region via the pulmonary blood flow; their half-lives are as short as minutes.

The extent to which soluble forms of the trichothecenes will produce toxic effects in the respiratory system cannot be deduced from available data. Nonetheless, many of the trichothecenes are sufficiently soluble to be cleared rapidly from the respiratory tract (Table 2-2) and transported into the general circulation where they add to the overall body burden of the toxin.

Because trichothecene toxins are persistent in the environment, contact with a dried residue may occur after an area has been sprayed and may result in toxic effects. If the residue material coats dust particles or can be aerosolized by mechanical action, both skin and inhalation exposure would result from contact with such a residue. Under most situations, resuspended soil particles are larger than the 3 to 4 μm maximum size required for significant deep lung deposition. Thus, respiratory tract exposure would involve toxin deposition in the upper respiratory and tracheobronchial region, followed by gastrointestinal tract exposure after clearance.

Skin Exposures

Skin contact with either contaminated feed, extracts of fungal cultures, or purified trichothecenes results in cutaneous reactions consisting of severe local irritation, inflammation, desquamation, and necrosis (Busby and Wogan, 1979; Marasas et al., 1969; Pier, 1981; Sato and Ueno, 1977; Smalley and Strong, 1974; Ueno, 1970). These reactions have been produced by exposing several animal species to various trichothecenes. Typical are the minimum effective dose data reported by Ueno et al. (1970) for guinea pigs (0.2 μg/2 x 2-cm spot) and mice (1 μg/2 x 2-cm spot).

In addition to the local effects, the trichothecene toxins penetrate the skin and enter the systemic circulation. Extensive cutaneous exposure to T-2 toxin would presumably induce acute toxic reactions; however, this route of exposure has not been extensively

studied. There is also no information on trichothecene application to other than the intact skin, or on the application of trichothecene mixtures or mixtures of trichothecenes and compounds that enhance cutaneous penetration. Once cutaneous necrosis occurs, however, the kinetics of absorption through the exposed skin may be expected to change.

Massive cutaneous contact is difficult to prevent when the sources of exposure are sprays, coarse mists, and, to a lesser extent, fine mists that have been deliberately used to contaminate the environment. Given the stability of T-2 and similar toxins, residues from the intentional dispersal of these toxins may be expected to be present on plants and other surfaces as well as attached to dust particles and dissolved in the waxy coating of fruits, vegetables, and edible foliage.

Multiple routes. Most exposures to deliberately released trichothecenes will probably involve several routes. Although no multiple-route exposures have been studied, the data allow some extrapolation. For example, since LD_{50}s do not vary greatly between the oral and injection exposure routes and toxicokinetic data suggest that distribution within the body is rapid, a combined oral and cutaneous exposure in mice would probably have an LD_{50} of 3 to 10 mg/kg bw. The oral dose component would be rapidly absorbed from the gastrointestinal tract, and the blood concentration from this source would be augmented by the toxin entering the boodstream after penetrating the skin. Toxic manifestations would be those associated with oral ingestion combined with the local inflammatory and necrotic effects of dermal exposure. The addition of inhalation exposure to the other two routes of exposure would increase both the extent and duration of gastrointestinal tract exposure because of the mucociliary clearance of the toxin from the upper airways, unless the toxin were soluble and rapidly cleared from these airways via the lymphatic and vascular circulation. In any case, the inhaled dose would add to the overall body exposure to the toxin. The material deposited by inhalation in the upper respiratory tract would probably result in necrosis of the mucosa of the nasal passages, larynx, and pharynx.

Clinical Signs

Clinical signs of toxicity in animals given various trichothecenes vary somewhat with species, specific trichothecene used, and dose. In avian species given T-2 toxin or DAS, signs of toxicity have included feed refusal, weight loss or reduced weight gain, and reduced spontaneous activity (Hoerr et al., 1981; Palyusik and Koplik-Kovaks, 1975; Richard et al., 1978; Wyatt et al., 1972, 1973). Other abnormalities described in chickens given T-2 toxin in doses up to 16 µg/g diet for 13 to 21 days included abnormal positioning of

wings, hysteroid seizures, and impaired righting reflexes (Wyatt et al., 1973). In chicks given either T-2 toxin or DAS in multiple oral doses up to 3.5 mg/kg bw, there was a delayed maturation of feathers. The barbs of the remiges were dishevelled and oriented to the rachis at acute angles (Hoerr et al., 1982c).

Emesis, posterior paresis, staggering gait, lethargy, and feed refusal were observed in swine given T-2 toxin (Weaver et al., 1978a) or DAS (Weaver et al., 1978b). Cutaneous necrosis was found on the legs and snouts of pigs that had been in contact with feed contaminated with T-2 toxin (Pier, 1981). Emesis has also been observed in dogs (Matsuoka et al., 1979) and in cats (Lutsky et al., 1978; Sato et al., 1975) given T-2 toxin. Additional signs of toxicity in the cats included anorexia, posterior paresis, lassitude, and weakness. Reduced weight gain was observed in rats given T-2 toxin (Marasas et al., 1969) and in mice given T-2 toxin or 4-acetylnivalenol (Hayes et al., 1980; Ueno et al., 1971b). These animals also experienced reduced feed consumption, reduced weight gain, lethargy, ruffled fur, diarrhea, and perioral dermatitis. Decreased activity, lethargy, and hypothermia occurred in mice given nivalenol (Saito et al., 1969). Calves given feed containing T-2 toxin or capsules containing the toxin became dehydrated, lost their appetites, developed necrotic lesions of the lips, had loose feces containing small amounts of blood, and had reduced rates of body weight gain (Pier, 1981; Pier et al., 1976).

Mucous Membrane and Cutaneous Effects

T-2 toxin and other trichothecene mycotoxins alter various tissues as a result of their irritant and/or cytotoxic activities. The mucosa of the digestive system and the epidermis of the skin are susceptible to the irritant activity when either are topically contaminated.

Organs and tissues such as lymphoid and myeloid tissues, intestinal epithelium, and gonads, which have a component of rapidly dividing cells, are most susceptible to the effects of orally or parenterally administered trichothecenes.

Alterations observed at the necropsy of test animals given oral doses of T-2 toxin and other trichothecenes have been prominent in the mucosa of the digestive system. These changes have included raised yellow caseous plaques of the oral mucosa and tongue (i.e., necrotizing stomatitis and glossitis) in chickens (Chi and Mirocha, 1978; Chi et al., 1977b,c; Moran et al., 1982) and turkey poults (Richard et al., 1978). There have also been reports of mucosal ulcerations of chicken crops (Hoerr et al., 1982), white caseous plaques of turkey poult crops (Richard et al., 1978), and multiple, raised proliferative or ulcerated lesions of the tongue and buccal

mucosa of pigs given DAS (Weaver et al., 1977). Such oral lesions have been produced in chicks and hens by dietary T-2 toxin (Chi et al., 1977b,c; Hoerr et al., 1982a,b; Wyatt et al., 1972) and by DAS (Chi and Mirocha, 1978; Hoerr et al., 1982b).

Oral lesions also occurred in a high percentage of chicks fed T-2 toxin at 4 μg/g feed during a 3-week period. The lesions were circumscribed, proliferative yellow caseous plaques located at the margin of the beak and on the mucosa of the hard palate and tongue (Chi et al., 1977b,c). The lesions also occurred in some chicks fed T-2 toxin at 2 μg/g feed. Lesions have appeared as early as 7 days after feeding DAS or T-2 toxin at a concentration of 5 mg/kg bw (Hoerr et al., 1982c). The focal fibrinopurulent glossitis in turkey poults fed a diet containing T-2 toxin concentrations of 10 μg/g consisted of a crust composed of necrotic epithelium and inflammatory cells (Richard et al., 1978). Erosion and ulceration of the squamous epithelium occurred beneath the crust. The surviving epithelium and adjacent submucosa were markedly infiltrated by heterophils.

Mucosal ulceration was observed in the gizzards of chickens fed T-2 toxin at 4 μg/g (Chi et al., 1977c) and in White Leghorn hens fed T-2 toxin at 4 and 8 μg/g (Chi et al., 1977b). Hayes et al. (1980) observed hypertrophy and hyperkeratosis in the squamous epithelium of the esophageal portion of the stomach of mice fed T-2 toxin at 20 μg/g.

Integument Effects

T-2 toxin and other trichothecenes produce cutaneous damage when applied topically in sufficiently high concentrations (Hayes and Schiefer, 1979; Marasas et al., 1969; Ueno et al., 1970). Observations of laboratory animals and humans indicated that skin contact with either contaminated feed or extracts of trichothecene toxins results in cutaneous reactions consisting of severe focal irritation, inflammation, and necrosis.

The alterations produced in the skin by the trichothecenes are semiquantitative within a range of toxin concentrations. A skin test has been proposed as a means of detecting the presence and estimating the potency of the skin-irritating toxins in contaminated feedstuffs (Chung et al., 1974; Hayes and Schiefer, 1979). There are, however, differences in the potency between individual trichothecenes in producing skin irritation. In one study, DAS was more irritating than 4-acetylinivalenol (fusarenon-X), which in turn was more irritating than nivalenol (Ueno et al., 1970).

There are also differences among animal species in their response to the cutaneous irritancy of the trichothecenes. For example, studies of the cutaneous responses of the rabbit, guinea pig, and

mouse to several trichothecens indicated that the guinea pig was the most sensitive and the mouse the least sensitive (Ueno et al., 1970). Chung et al. (1974) reported that the rabbit was more sensitive to trichothecenes than were weanling rats or young guinea pigs and that the rabbit skin test was reliable to at least 0.01 µg T-2 toxin per spot.

Several trichothecenes have been tested for their cutaneous irritation in several animal species. A few days after 1 µg of 4-acetylnivalenol was applied to the skin of the guinea pig, hyperemia and hemorrhages developed followed by crust formation (Ueno et al., 1971a,b). When applied to the dehaired skin of the back, DAS (0.2 µg) and 4-acetylnivalenol caused reddening of the test area within 12 hours after administration; doses of 10 or 100 µg of these toxins caused petechial hemorrhages and crust formation after a few days. Nivalenol was less irritating: a dose of 100 µg did not produce edema at the site of application (Ueno et al., 1970, 1973). However, severe cutaneous lesions were produced by the topical application of larger doses of nivalenol. These included atrophy of hair follicles, cellular infiltrates into the dermis, and necrosis of the epidermis (Saito et al., 1969). The minimum dose of solaniol for skin irritation in the rabbit was about 1.0 µg (Ishii et al., 1971). Both crotocin (Glaz et al., 1960) and trichothecin (Freeman, 1955) have produced cutaneous irritation in guinea pigs.

Cutaneous responses to T-2 toxin and DAS were studied in rats and rabbits (Hayes and Schiefer, 1979). Clinically, reactions to T-2 toxin applied topically (2 or 3 µl of an 80 µg/ml ethyl acetate solution) were visible by 12 hours after administration as flat hyperemic plaques, which increased in redness to a maximum at 48 hours and were covered by a moist exudate by 24 to 30 hours. The redness subsided after 48 hours and treatment sites were covered by a dry friable exudate. The application sites appeared as small, smooth, pink hairless spots by the 6th day after administration. Microscopically, vascular sequestration of neutrophils was evident 3 hours after dosing. Necrosis of dermal fibroblasts was detected between the 6th and 12th hours, and neutrophilic cell infiltration of the dermis was present by the 12th hour. Neutrophils had penetrated the epidermis 24 hours after dosing. Within the first 24 hours, epidermal changes included necrosis and spongiosis of basal and squamous cells, subepidermal edema, and coagulative necrosis with desquamation of the epidermis in the more severe reactions. Hyperplasia of the epidermis was evident after 24 hours; it was prominent at 48 hours and persisted over the 14-day observation period. Subepidermal fibroplasia appeared by day 6.

In dose-response studies, Hayes and Schiefer (1979) found that rabbits were more sensitive than rats to the skin irritancy of T-2 toxin and DAS, that there was marked variation in sensitivity among

individual rats, and that most rats responded to concentrations above 15 μg/ml. Reaction intensities were modified by the solvents used in these studies. Both reaction frequencies and intensities were similar for ethyl acetate and methanol solvents, but were much lower for corn oil and dimethylsulfoxide.

Lesions in feathers were observed in chickens 12 through 24 hours after they were given T-2 toxin by crop gavage (Hoerr et al., 1981). The changes consisted of necrosis of several layers of cells: the regenerative layer of cells at the base of the feathers, the basilar layer of the ramus and barb ridges, and the intermediate layer of the axial and barbula plates. Necrosis of the stratum germination cells at the neck of the feather follicle was observed in chickens given DAS.

Systemic Effects

T-2 toxin and other trichothecenes have produced alterations in various organs and tissues. These systemic effects may be observed when the toxins are administered by several routes of exposure including oral (by feed, capsule, or intubation), intravenous, or intraperitoneal administration. As mentioned above, the tissues with the most severe alterations are those with a component of rapidly dividing cells. Thus, the gut, lymphoid (including immunologic) tissues, hematopoietic tissues, and the gonads are most affected when these toxins are given to various animal species. However, other tissues may be altered as well.

Hematopoietic Tissues. Trichothecenes produce alterations in hematopoietic tissues and hemograms in animals such as the mouse, rat, guinea pig, cat, and chicken. Among the trichothecenes that have been studied for these effects are T-2 toxin, DAS, nivalenol, 4-acetylnivalenol, and DON. Gross examination reveals that the bone marrow is pale, edematous, and may contain focal hemorrhages.

DeNicola et al. (1978) reported necrosis of the myeloid tissue of the bone marrow of guinea pigs given T-2 toxin by gavage at doses of 2.5 or 5.0 mg/kg bw. Necrosis of bone marrow cells was observed in mice given nivalenol intraperitoneally (Saito et al., 1969) and in rats and guinea pigs given 4-acetylnivalenol by the same route (Ueno et al., 1971b).

In mice fed a diet containing T-2 toxin at 20 μg/kg, the bone marrow became hypocellular during the first 3 weeks of feeding (Hayes et al., 1980); however, regeneration with development of a cellular marrow was evident by day 41. Developing erythrocytes had disappeared from the the splenic red pulp and bone marrow by day 14, but regeneration was observed in some mice by day 28.

In chickens given a single oral dose of T-2 toxin or DAS, necrosis of the erythroid and granulocytic regions of the bone marrow was observed at 6 hours and was most severe at 24 hours. Necrosis was accompanied by marked cellular depletion (Hoerr et al., 1981).

In guinea pigs, the subchronic administration of 0.5-0.75 mg/kg bw doses of T-2 toxin produced hematologic abnormalities (DeNicola et al., 1978). The number of erythrocytes was reduced, and erythrocytic morphology was altered. The effects included basophilic stippling, fragmentation, increased polychromasia and anisocytosis, and an increase in nucleated cells. Both total leukocytic and total lymphocytic cell counts were greatly decreased in the bone marrow, and the myeloid:erythroid ratio (M:E ratio) was markedly reduced in comparison to the controls.

Hematologic alterations were also observed in domestic cats given T-2 toxin. These included leukopenia, anemia with reduced packed cell volume (PCV) and hemoglobin values, thrombocytopenia, and morphologic alterations of neutrophils and erythrocytes (Lutsky et al., 1978).

Patterns of abnormal erythropoiesis were evident in anemic mice fed 20 µg/g doses of T-2 toxin (Hayes et al., 1980). Abnormalities in erythroid cells included cytoplasmic stippling in rubricytes and metarubricytes, nuclear fragmentation in metarubricytes, and an increased ratio of cytoplasm to nucleus in rubricytes.

T-2 toxin was reported by Doerr et al. (1976) to produce aberrations in the coagulation of blood from young broiler chickens. Both factors VII and X were reduced, the greatest reduction occurring in factor VII. T-2 toxin was fed to the animals in dietary concentrations of 1.0, 2.0, 4.0, 8.0, and 16 µg/g for 3 weeks in order to study coagulation of blood (Doerr et al., 1974). At 8 and 16 µg/g, there were significant increases in prothrombin times, but not in decalcification times in plasma.

Lymphoid and Immunologic Tissues

Degenerative and necrotic alterations have been produced in the lymphoid tissues of several animal species by several trichothecene mycotoxins. The species affected include the mouse, rat, guinea pig, cat, chicken, turkey, pig, and cattle. The trichothecenes studied include T-2 toxin, DAS, nivalenol, and 4-acetylnivalenol. Gross examination revealed that lymhoid organs such as the thymus, spleen, and lymph nodes were decreased in size after exposure.

- Lymphoid Tissues. Necrosis of lymphoid cells was observed in the lymph nodes, Peyer's patches, and peribronchiolar and palpebral

lymphoid follicles in guinea pigs given by gavage 2.5 or 5.0 mg/kg bw doses of T-2 toxin (DeNicola et al., 1978). Cats given multiple oral doses of T-2 toxin developed leukopenia, enlarged hemorrhagic mesenteric lymph nodes (Lutsky et al., 1978), and necrosis of lymphoid cells in the spleen and lymph nodes (Sato et al., 1975).

In pigs given T-2 toxin intravenously in doses as high as 3.2 mg/kg bw, Weaver et al. (1978a) observed necrosis of lymphoid cells in the spleen, mesenteric lymph nodes, and Peyer's patches of the ileum and lymphoid nodules of the cecum. It was also found in the germinal centers of the spleen and mesenteric lymph nodes of pigs given intravenous DAS doses up to 5.0 mg/kg bw (Weaver et al., 1978b). Cortical atrophy of the thymus occurred in calves given T-2 toxin at a dose of 0.3 or 0.6 mg/kg bw (Osweiler et al., 1981).

In the chicken, some lymphoid tissue lesions appeared in the thymus, bursa of Fabricius, cecal tonsil, and spleen less than 1 hour after a single gavage dose of T-2 toxin (2.0-2.5 mg/kg bw) or DAS (2.7 mg/kg bw) (Hoerr et al., 1981). Necrosis and depletion of lymphocytes were observed during the first 24 hours; partial or complete restoration of normal histologic features occurred by the 72nd or 168th hour after dosing. T-2 toxin caused more severe lesions than did DAS. In the thymus, necrotic lymphocytes were first present at 6 hours. Initially, necrosis was more severe in the thymic medulla of T-2 toxin-treated chickens, but lymphoid depletion became severe in both the cortex and medulla. Necrosis occurred early in the bursa of Fabricius after 1 hour, involving both the cortex and medulla, and was followed by severe atrophy at 18 hours with a return to normal histologic features in most chicks by 168 hours. Lesions in the cecal tonsil and spleen were similar to those in the thymus and bursa; they consisted of lymphoid cell necrosis and depletion. Atrophy of the thymic cortex was observed in turkey poults given 10 µg/g oral doses of T-2 toxin (Richard et al., 1978).

Hayes et al. (1980) described depletion of lymphocytes in lymphoid tissues of mice fed a diet containing 20 µg/g T-2 toxin. They reported that lymphocytes had disappeared from the thymic cortex by the 7th day of feeding. The spleen and lymph nodes had little follicular activity. Thymic-dependent lymphoid populations of the periarteriolar sheaths in the spleen, the paracortical regions of lymph nodes, and intraepithelial lymphocytes of the small intestines were decreased. There was also a decrease in the B-cell-dependent lymphoid populations in the intestinal lamina propria, the medullary cords of lymph nodes, and in the splenic cords.

Mice given 8-hydroxydiacetoxyscirpenol (neosolaniol) intraperitoneally in doses of 11 mg/kg bw developed necrosis of lymphoid cells in the lymph nodes at 3 to 6 hours, in the follicles of the spleen at 6 to 12 hours, and in the thymic cortex at 6 to 24 hours

after dosing (Sato et al., 1978). In mice given nivalenol by oral, subcutaneous, or intraperitoneal routes, necrosis of lymphoid cells was observed in the lymph nodes, thymus, spleen, and lymph follicles in other tissues (Saito et al., 1969). Such lesions were also found in rats given 4-acetylnivalenol (Ueno et al., 1971b).

• Immunologic Tissues. T-2 toxin and other trichothecenes produce widespread degenerative and necrotic changes in the lymphoid cells of the spleen, thymus, lymph nodes, and bone marrow. Because of this damage, functional abnormalities of the immune system would be expected and, in fact, have been described.

The immunosuppressive effects of Fusarium extracts, T-2 toxin, and DAS have been investigated by in vivo and in vitro methods (LaFarge-Frayssinet et al., 1979). In these studies, mice were given Fusarium extracts by intraperitoneal injection of doses that were one-half the LD_{50}, followed in 24 hours by a dose that was one-quarter the LD_{50}; other mice received a 15-day treatment of one-twelfth the LD_{50} daily. In the high dose mice, the spleen and thymus weights were decreased; the weight loss was greater for the thymus. The proliferative response of their splenic cells to phytohemagglutinin (PHA) was depressed as early as day 3 and reached its maximum on day 10, but the response had returned to normal by day 19 after dosing. The proliferative response of the splenic cells to lipopolysaccharides (LPS) was elevated on day 3 but was depressed on day 10 after dosing. Antibody response to sheep erythrocytes was lowered on days 3, 7, and 10. In mice given the 15-day treatment, antibody response to sheep erythrocytes was low, but the proliferative response of splenic cells to PHA and LPS were elevated. When Fusarium extracts and purified T-2 toxin or DAS were added to splenic cells in vitro, the proliferative responses to PHA and LPS were inhibited and the degree of inhibition was greater for T-2 toxin (LaFarge-Frayssinet et al., 1979). For thymic cells in vitro, inhibition of PHA response was produced by Fusarium extract at 2 to 10 ng/ml, by T-2 toxin at 2 to 10 ng/ml, and by DAS at 0.05 to 20 ng/ml.

The inhibitory effect of crude Fusarium poae extracts, T-2 toxin, and DAS on the synthesis of antibody to sheep erythrocytes and the effect of T-2 toxin on skin graft rejection were studied in mice by Rosenstein et al. (1979). Both the extracts and toxin were injected intraperitoneally. After the extract was administered daily for 7 days at one-fifth the LD_{50}, there was a decrease in thymus and spleen weights accompanied by a fall in antibody titer. Both T-2 toxin and DAS inhibited antibody formation against sheep erythrocytes at doses that did not significantly reduce the thymus weight. Administration of T-2 toxin significantly increased the period required for skin graft rejection.

The effects of T-2 toxin on the immune system were studied in male rhesus monkeys (Jagadeesan et al., 1982). The T-2 toxin was given orally in doses of 100 μg/kg bw daily for 4 to 5 weeks. At the end of the treatment, total leukocyte counts were reduced, but hemoglobin and PCV values and erythrocyte counts were not changed. There were also reductions in the bactericidal activity of neutrophils, in the number of T- and B-cells, in lymphocyte transformation by phytohemagglutinin, and in immunoglobin concentrations. All the affected values had returned to nearly normal 5 months after the toxin administration was discontinued.

Disturbances in bovine immune system response were produced by T-2 toxin given orally to calves at doses of 0.6 mg/kg bw daily for 43 days (Buening et al., 1982). A significant decrease in lymphocytic response of the T-2 toxin group to phytohemagglutinin was observed 1, 8, and 29 days after toxin administration. There were also significant decreases in the responsiveness of lymphocytes to concanavalin A and pokeweed mitogen on the 29th day after toxin administration. Neutrophil chemotaxis under agarose was significantly decreased by toxin administration, but no significant differences in neutrophil random migration under agarose, NBT reduction, or glucose uptake were observed.

Humoral components of the bovine immune system were altered by the oral administration of T-2 toxin at doses of 0.6 mg/kg bw daily for 43 days (Mann et al., 1982). A large, sharp decrease in total serum protein was recorded at the 7th day after toxin administration. Protein fractions, including gamma, alpha, $beta_1$, and $beta_2$, were significantly lower in the treated calves. The albumin/globulin ratio of the toxin-treated group was elevated throughout the period of treatment, but there were no significant changes in albumin concentrations in the serum. The serum concentrations of IgM and IgA were much lower in treated calves, but the serum concentrations of IgG were not changed. The quantities of complement C3 were reduced by toxin treatment.

The effects of T-2 toxin on the immune responses of growing swine have also been studied (Rafai and Tuboly, 1982). Cross-bred, 4-week-old pigs were fed T-2 toxin at dietary concentrations of 5 μg/g for 25 days, resulting in an average daily T-2 toxin intake of 1.25 mg. This treatment resulted in feed refusal (49% to 77%) and decreased weight gain. PVC values were not altered by the dietary T-2 toxin, but leukocyte counts were decreased. Adrenocortical activity was increased, as indicated by elevated plasma cortisol concentrations. Blast transformation was decreased, as measured by 3H-thymidine incorporation after an antigenic stimulus by Clostridium perfringens (u.c. antigen). IgG-positive cell counts were also depressed by toxin treatment as was immune-rosette formation. Levels of toxin-neutralizing antibodies to necrotic enteritis B vaccine were

significantly lower in the sera of treated pigs. These data indicate that there were disturbances in the function of both T- and B-lymphocytes in T-2 toxin-treated swine.

• Digestive System. Trichothecene mycotoxins, including T-2 toxin, DAS, nivalenol, 4-acetylnivalenol, and DON, produce alterations in various segments of the digestive system of several animal species such as the rat, cat, mouse, guinea pig, chicken, turkey, trout, and pig. In studies of effects on the digestive system, the oral route of exposure is the route most frequently used. Oral doses of T-2 toxin and other trichothecenes are rapidly absorbed and produce lesions not only in the digestive system organs but in other tissues as well.

Clinically, damage to the gut is manifested by diarrhea and, sometimes, blood-tinged feces. Gross changes in the digestive system have included fluid gut contents, red or white hepatic foci, gastrointestinal tract hyperemia, and hyperemia of the gallbladder in chickens given T-2 toxin or DAS (Hoerr et al., 1981).

• Alimentary Canal. Gastrointestinal hyperemia and hemorrhages have been observed in cats given T-2 toxin (Lutsky et al., 1978), in calves given verrucarin A or roridin A (Mortimer et al., 1971), in pigs given DAS (Weaver et al., 1978b), in mice given 4-acetylnivalenol (Ueno et al., 1971b), and in guinea pigs given T-2 toxin (DeNicola et al., 1978). Denudation of rumenal papillae, erosion of the rumenal wall, and abomasal ulceration were observed in calves given T-2 toxin in contaminated feed or in capsules (Pier, 1981; Pier et al., 1976).

Single oral doses of T-2 toxin (2.0-2.5 mg/kg) and DAS (2.7 mg/kg) produced alterations in portions of the intestinal tract of chickens (Hoerr et al., 1981). The alterations included necrosis of the superficial mucosa of the proventriculus, the mucosal glands of the ventriculus (by DAS), the epithelium of the tips of the duodenal villi after 6 hours, and crypt epithelium in both the small and large intestine 12 hours after dosing. Minimal digestive tract lesions were observed in broiler chickens fed diets containing DON in concentrations up to 210 μg/g (Moran et al., 1982).

Sloughing of the intestinal mucosa was observed in rainbow trout fed diets containing T-2 toxin (Marasas et al., 1967). The loss of intestinal mucosa was great. In the fish given doses of 13.3 or 33.3 mg/kg bw, the shed mucosa literally covered the bottoms of the tanks.

Signs of enteritis (i.e., loose feces) were observed in 1-month-old Jersey calves given T-2 toxin daily in capsules in doses of 0.08, 0.16, 0.30, and 0.60 mg/kg bw. Bloody feces were present at the two higher doses (Pier et al., 1976). At necropsy, the investigators observed that the calves given the two higher doses had ulcers in the abomasum and rumen and abomasitis with edema.

Gastrointestinal lesions were observed in guinea pigs given T-2 toxin by gavage at doses of 2.5 or 5 mg/kg bw. Mucosal alterations were present in several segments, but were most severe in the cecum and stomach. In the stomach, there were hemorrhages of the fundic mucosa, necrosis, and multifocal ulcerations. Focal villi necrosis with pseudomembrane formation and casts within crypts were present in the small intestine. The cecal alterations were mucosal hemorrhages and necrosis (DeNicola et al., 1978).

In domestic cats given T-2 toxin orally, Lutsky et al. (1978) observed mucosal necrosis in the stomach and intestines and necrotic debris in the intestinal crypts. In another study of T-2 toxin in domestic cats, Sato et al. (1975) found extensive necrosis of the mucosa of the small and large intestine.

In a time course study of the changes produced in mice by 2.5 mg/kg bw doses of 4-acetylnivalenol administered intraperitoneally, Sato et al. (1978) found intestinal crypt cell necrosis by the third hour after dosing. The necrosis reached a maximum after the sixth hour. Crypt debris was cleared by 24 hours, and the villi were short and edematous.

Necrosis of intestinal mucosal crypts and villi, especially in the duodenum and jejunum, was observed in mice and rats given nivalenol orally (Saito et al., 1969). Necrosis of the small intestinal villi and crypt epithelia accompanied by mucosal erosion and ulceration was found in mice given 4-acetylnivalenol (Ueno et al., 1971b).

Weaver et al. (1978b) reported the production of gastrointestinal lesions in pigs given DAS by intravenous injection at doses ranging from 0.30 to 0.50 mg/kg bw. The lesions found in the small intestine, colon, and cecum were characterized by mucosal congestion and hemorrhage, edema of the submucosa, and necrosis of the villi and crypt epithelia. Hemorrhagic lesions were not produced in swine by intravenously administered T-2 toxin (Weaver et al., 1978a), but necrosis of villi and crypt epithelia of the small and large intestines was observed. Pier et al. (1976) did not find extensive hemorrhages in calves fed T-2 toxin nor did Patterson et al. (1979) find hemorrhagic syndrome in calves and pigs given T-2 toxin or cultures of Fusarium tricinctum.

• Liver. Hepatic lesions were produced in chickens given a single oral dose of T-2 toxin or DAS (Hoerr et al., 1981). Disseminated hemorrhagic foci of coagulative necrosis found 1 to 24 hours after dosing was accompanied by biliary duct hyperplasia, which was most prominent in the portal triads adjacent to the foci of necrosis. Necrosis with mucosal ulceration of the gallbladder at 24 hours was followed at 72 hours by cholecystitis with congestion, edema, and inflammatory cell infiltration. In later studies, necrosis of biliary

duct epithelium, cholestasis, and cytoplasmic vacuolation of hepatocytes were observed in chickens given multiple doses of T-2 toxin or DAS (Hoerr et al., 1982b). Weaver et al. (1978b) found congestion and hemorrhages in the gallbladder of pigs given DAS by intravenous injection.

Other Organs and Tissues. Histopathologic alterations have been observed in other organs and tissues, but these findings have been uncommon and poorly documented, except for the alterations in the reproductive system. The studies are described below.

- Kidney. Mild necrotic lesions in the tubular epithelium of the kidney were observed in chickens given either single or multiple oral doses of T-2 toxin or DAS (Hoerr et al., 1981, 1982b).

- Cardiovascular System. Foci of hemorrhages were observed in the heart of a pig given an intravenous DAS dose of 0.43 mg/kg bw (Weaver et al., 1978b), and myocardial hemorrhages were noted in mice given lethal doses (subcutaneous LD_{50}, 4.6 and 4.2 mg/kg bw male and female mice) of 4-acetylnivalenol (Ueno et al., 1971b). No lesions were found in the hearts of chickens given oral doses of T-2 toxin (up to 3.0 mg/kg bw) or DAS (up to 3.5 mg/kg bw) (Hoerr et al., 1982b) or fed diets containing T-2 toxin provided as a corn culture of Fusarium sporotrichioides (Hoerr et al., 1982a).

Lesions were found in the heart and arteries of aged (12- to 27-month-old) Wister-Porton rats given three to eight oral doses of T-2 toxin (Schoental et al., 1979). Since these cardiovascular alterations have been found in several rat strains and have been associated with aging (Anver and Cohen, 1979), it is unlikely that the cardiovascular lesions in the treated rats were produced by the T-2 toxin.

- Central Nervous System . Few reports contain descriptions of morphologic alterations in the brain and spinal cord of animals given trichothecenes, although there have been some reports of CNS dysfunction (Wyatt et al., 1973). Meningeal hemorrhage was observed in the brain of domestic cats given T-2 toxin both orally and subcutaneously (Sato et al., 1975). Subarachnoidal hemorrhages were described in rabbits given 5 mg/kg bw doses of 4-acetylnivalenol twice weekly for 4 weeks (Ueno et al., 1969). Ueno et al. (1974) described petechial hemorrhages in the brain of a cat given corn contaminated with DON produced by F. graminearum (Gibberella zeae).

- Endocrine Organs. Alterations were found in the thyroid glands of chickens given multiple oral doses of T-2 toxin or DAS (Hoerr et al., 1982b). The follicles were small, contained pale-staining colloid, and had tall follicular epithelia.

• Reproductive Organs. Mice given nivalenol developed testicular lesions: the spermatogenic cells were reduced in number, some of the blastic cells were necrotic, and multinucleated spermatic giant cells were present in the seminiferous tubules (Saito et al., 1969). In guinea pigs given T-2 toxin by gavage at doses of 2.5 and 5 mg/kg bw, there was degeneration and necrosis of the spermatogenic cells of the seminiferous tubules of the testis. Only primitive spermatogenic and Sertoli cells remained in the seminiferous tubules, and many of these were necrotic as evidenced by nuclear pyknosis and karyorrhexis (DeNicola et al., 1978). Suppression of spermatogenesis was observed in ganders and male turkeys fed F. graminearum cultures containing trichothecene mycotoxins (Palyusik et al., 1971). Necrosis of cellular components of the ovaries was observed in rabbits given 5 mg/kg bw doses of 4-acetylnivalenol twice weekly for 4 weeks (Ueno et al., 1969).

In comparison to control hens, White Leghorn hens fed a diet containing 8 µg/g T-2 toxin consumed less feed and produced fewer eggs with thinner shells and lower hatchability (Chi et al., 1977b). In another study, 50-week-old White Leghorn hens were fed 0.5 µg/g DAS for 4 weeks (Allen et al., 1982). A gradual decrease in hatchability was observed, and most of the embryo mortality occurred during the first week of incubation. Hatchability rapidly returned to normal upon withdrawal of the DAS-contaminated feed.

• Respiratory System. The effects of trichothecenes on the respiratory system have not been defined for any route of toxin administration. As discussed in the beginning of this chapter, stachybotryotoxicosis in humans involves the upper respiratory tract; however, no inhalation toxicity data for trichothecenes have been reported. Nonetheless, there is good reason to believe that both local and systemic toxic effects will result from inhalation exposure. Since data from animal experiments indicate that the mycotoxins are rapidly absorbed and distributed after oral administration, rapid absorption from the lung could be anticipated.

Recent in vitro studies suggest that both DAS and T-2 toxin affect the function of pulmonary macrophages (Gerberick et al., in press a,b). The investigators found that alveolar macrophages obtained from rats by tracheal lavage were less viable, smaller, and less numerous than in untreated cultures. The observed macrophage cytotoxicity and reduced phagocytic activity indicate that inhalation of T-2 toxin and similar trichothecenes could result in increased secondary infections as well as suppression of pulmonary immune function.

• Eye. Ocular lesions were produced in rabbits by cultured filtrates of Myrothecium verrucaria containing roridin A and verrucarin A. The filtrates were instilled into the conjunctival sac (Mortimer et al., 1971). The investigators observed reddening and

edema of the conjunctival membranes within 1 to 2 days after instillation. Later, the cornea became opaque and vascularized. In some eyes, the corneas developed scars that persisted (as did the opacity) for as long as 5 months (Mortimer et al., 1971). In another study, four drops of a 1% solution of trichothecin instilled into the conjunctival sac of a rabbit caused slight inflammation of the conjunctiva of the nictitating membrane and eyelids (Freeman, 1955).

Mutagenicity

Essentially all the trichothecenes evaluated in mutagenicity tests have been negative. For example, Ueno and Kubota (1976) found that T-2 toxin and 4-acetylnivalenol were negative in a bacterial system using a recombination-deficient mutant strain of Bacillus subtilis. When tested for mutagentic activity with Salmonella typhimurium TA98 and TA100, both mycotoxins were negative with and without preincubation with S9 microsomal fraction. They were also found to be inhibitory at concentrations of 100 to 1,000 µg/plate (Ueno et al., 1978). 4-Acetylnivalenol did not induce 8-azaguanine-resistant mutants in cultures of FM3A cells from the C3H mouse mammary carcinoma cell line (Umeda et al., 1977).

Wehner et al. (1978) reported the results of mutagenicity tests with S. typhimurium strains TA98, TA100, TA1535, and TA1537 for six trichothecenes: monoacetoxyscirpenol, DAS, triacetoxyscirpenol, T-2 toxin, DON, and 3-acetyldeoxynivalenol. All the trichothecenes were negative for mutagenicity both with and without metabolic activation.

Kuczuk et al. (1978) reported that T-2 toxin and DAS were negative for mutagenicity when tested with and without metabolic activation using S. typhimurium strains TA1535, TA1537, and TA1538 and Saccharomyces cerevisiae strain D3.

Carcinogenicity

Present evidence indicates that trichothecene toxins are not tumorigenic. However, few long-term feeding studies with these compounds have been completed. Schoental et al. (1979) reported the occurrence of neoplasms in Wistar-Porton rats given three to eight doses of T-2 toxin ranging from 0.2 to 0.4 mg/kg bw. Too few animals were used in this study to determine the tumorigenicity of T-2 toxin, since the observed tumors could well have been the "background" rate for the strain of rat used.

No tumorigenic activity was found when 4-acetylnivalenol was fed to male Donryu rats for 1 or 2 years at concentrations of 3.5 ppm (which equalled 50 µg/rat/day) or 7 ppm (which equalled 105 µg/rat/day) in

ground pelleted feed (Saito et al., 1980). At the end of the test, there were 35 rats in the 3.5 ppm, 2-year group, 19 rats in the 7 ppm, 1-year group, and 14 rats in the 7 ppm, 2-year group.

Teratogenicity

Trichothecenes studied for their effects on embryonic and fetal development in mice include T-2 toxin (Hood et al., 1978; Stanford et al., 1975), acetylnivalenol (Ito et al., 1980), and 4-deoxynivalenol (Khera et al., 1982).

T-2 toxin dissolved in propylene glycol was administered by intraperitoneal injection into mated, sexually mature female mice on one of days 7 through 11 of gestation at doses of 1.0 or 1.5 mg/kg bw. A number of deaths resulted among the treated females, primarily among those given 1.5 mg/kg, and there was reduced survival of the offspring of dams at both dose levels (Stanford et al., 1975). In this study, approximately 37% of the fetuses from females given 1.0 or 1.5 mg/kg T-2 toxin on day 10 were grossly malformed. The most frequent anomalies were bent, shortened, or missing tails and limb malformations, including oligodactyly and syndactyly. Exencephaly, open eye, and retarded jaws were also found.

Hood et al. (1978) administered T-2 toxin dissolved in propylene glycol to mated female CD-1 mice in intraperitoneal doses of 0.5 mg/kg bw on day 10 of gestation. The toxin produced grossly malformed fetuses, principally with tail and limb anomalies. A higher incidence of malformations was observed when T-2 toxin doses of 0.5 mg/kg were combined with ochratoxin A doses of 4 mg/kg.

Acetylnivalenol was found to be embryotoxic, but not teratogenic, in DDD-strain mice (Ito et al., 1980). The mycotoxin was dissolved in saline and given by subcutaneous injection at doses of 0.63 or 2.6 mg/kg bw or mixed in the diet at 5, 10, or 20 μg/g. Embryo lethality was 100% when the 20 μg/g diet was fed for 7 days during the middle period of pregnancy, and it was 40% in dams fed the 10 μg/g diet for the same period.

4-Deoxynivalenol was embryotoxic when dissolved in distilled water and given for four consecutive days (days 8 through 11 of pregnancy) by esophageal intubation to mated female Swiss-Webster mice (Khera et al., 1982). The incidence of resorptions was 100% at doses of 10 or 15 mg/kg bw, and 80% at 5 mg/kg bw. In the latter group, the number of live fetuses and average fetal weight were lower than those of the controls. Skeletal malformations were present in the fetuses of the 1.0, 2.5, and 5 mg/kg groups, and the incidence was related to dose. The skeletal anomalies included lumbar vertebrae with fused arches or

partly absent centra, absent or fused ribs, and missing, fused, or scrambled sternebrae.

Subchronic and Chronic Effects

Lesions were found in the nonglandular portion of the stomach of DDD mice fed T-2 toxin at 10 or 15 μg/g for 12 months (Ohtsubo and Saito, 1977). The alterations included hyperplasia, hyperkeratosis, and acanthosis of the squamous epithelium. Such changes were found within 13 weeks after feeding was started. They were diffuse in mice fed the high dose and focal in those fed the low dose. The lesions were consistently observed during the 12-month feeding period, but most of them subsided 3 months after cessation of feeding. Mice fed 4-acetylnivalenol had mild lesions in the nonglandular stomach (Ohtsubo and Saito, 1977).

Wistar rats fed T-2 toxin at concentrations of 5, 10, or 15 μg/g for 4 weeks also had lesions in the nonglandular stomach, including hyperplasia, hyperkeratosis, and acanthosis of the squamous epithelium. The lesions were diffuse and severe in the rats fed 15 μg/g, focal but definite in those fed 10 μg/g, and minimal in rats fed 5 μg/g (Ohtsabo and Saito, 1977).

BIOLOGICAL EFFECTS IN PLANTS, INSECTS, AND MICROORGANISMS

In the few studies conducted on various plants in tissue culture, most of the trichothecenes tested have been found to be cytotoxic. When added to agar cultures of tobacco callus, T-2 toxin produced a 50% growth inhibition at concentrations of 6×10^{-3} μM (Helgeson et al., 1973; Stahl et al., 1973). In the same test, the standard growth inhibitors actinomycin D or cycloheximides induced a 50% inhibition at approximately 10^{-1} μM. Helgeson et al. (1973) concluded that T-2 toxin was the most active agent yet tested for inhibition of cell growth in tissue cultures of tobacco callus.

There appears to be a good correlation between dermal toxicity, cytotoxicity, and phytotoxicity to higher plants among the various trichothecenes. In fact, the original purification of DAS was accomplished as a part of a phytotoxicity assay (Brian et al., 1961). Phytotoxicity has also been reported for T-2 toxin by Marasas et al. (1971), Burmeister and Hesseltine (1970), Burmeister et al. (1971), and others (Ueno et al., 1971b), who have suggested the use of these plant systems to detect low-order trichothecene contamination in feeds and foods. As might be expected from the cytotoxicity information, the germinating seeds of higher plants are also extremely sensitive to the action of trichothecenes (Burmeister and Hesseltine, 1970; Marasas, 1969; Marasas et al., 1971; Ueno et al., 1971b).

Trichothecin inhibited the development of lesions from tobacco necrosis virus on beans inoculated with doses ranging from 20 to 100 mg/liter, but the investigators suggested that the effects were exerted mainly on host plant susceptibility to the virus rather than directly on the viral particles (Bawden and Freeman, 1952).

Many Fusarium species that produce trichothecenes are known to be pathogenic in plants. In general, however, their pathogenic abilities have not been related directly to the production of phytotoxic trichothecenes (Smalley et al., 1970). Bilai (1970a,b) and Seemüller (1968) have discussed the phytopathological and taxonomic significance of the Fusaria in the section Sporotrichiella, which contains the major trichothecene-producing fungi. Bilai (1970b) observed that F. sporotrichioides is widely distributed throughout the world and that its varieties are found on a number of cultivated plants such as wheat, oats, forage grasses, sugar beets, stone fruits, citrus fruits, maize, soybean, peas, lupine, tomato, and carnation. The phytotoxic trichothecenes may play a role in the pathogenesis of various plant diseases but no definitive studies have been reported.

Plant pathogenicity by authentic strains of F. sporotrichioides has been not been observed in most studies; however, the parasitic capabilities of F. poae have been documented (Cullen, 1981; Fisher et al., 1983; Seemüller, 1968). A T-2 toxin-producing isolate of F. sporotrichioides (Andrews et al. 1981; Cullen, 1981) was observed to be pathogenic for the Eurasian water milfoil (Myriophyllum specatum). Myrothecium roridum produces toxic roridins and verrucarins and is parasitic on a variety of host plants. It infects gardenias (Fergus, 1957), tomatoes (Stevenson and McCollock, 1947), cotton (Cognee and Bird, 1964), pansies (Preston, 1943), gloxinias (Littrell, 1965), snapdragons (Preston, 1943), soybeans (Schiller et al., 1978), cantaloupes (McLean and Sleeth 1961), cocoa (Deighton, 1955), coffee (Nag Raj and George, 1958; Scheiber and Zentmyer, 1968), and red clover (Cunfer and Lukezic, 1970). In New Zealand, Myrothecium spp. is normally part of the mycoflora of pasture plant leaves (Mortimer et al., 1971). The parasitic activities of M. roridum on coffee berries are of interest as a possible means by which trichothecenes contaminate food intended for consumption by humans (Nag Raj and George, 1958; Scheiber and Zentmyer, 1968). The potential for the intoxication of animals by trichothecenes produced by Myrothecium spp. seems great because of the many host plants involved.

Several species of Acremonium are known to produce plant diseases, but many have not been tested for trichothecene production. For example, A. apii causes brown spot of celery (Smith and Ramsey, 1951); A. album, balsam fir canker (Hepting, 1971); A. diospyri, persimmon wilt (Smith and Ramsey, 1951); A. gregatum, brown stem rot of soybean (Dickson, 1956); A. sacchari, sugar cane wilt (Edgerton, 1959); and A. deformans, leaf wilt of barbasco plants (Crandall, 1950). A few

species are pathogenic to humans, e.g., Acremonium (=Cephalosporium) infestans, A. kileense, A. modurae, A. recifei, and A. spinosum (American Type Culture Collection, 1982). Apparently, none of the parasitic Acremonium species have been examined for the production of trichothecenes.

Trichoderma viride and Trichothecium roseum have limited capabilities as plant pathogens. T. viride is the agent that causes rot in stored garlic and lemons, but apparently does not commonly infect other stored fruits and vegetables (U.S. Department of Agriculture, 1960). T. roseum is occasionally parasitic on apple fruits, and it also causes pink rot in stored celery (U.S. Department of Agriculture, 1960). Although this list of fungi that are both plant pathogens and producers of trichothecenes is impressive, the role of trichothecene production in true pathogenic processes is unclear.

T-2 toxin and DAS are inactive as contact insecticides against Drosophila (Cole and Rolinson, 1972; Smalley, unpublished data, 1973). However, Kishaba et al. (1962) found that culture filtrates of Myrothecium roridum inhibit the feeding and cause the death of Mexican bean beetles (Epilachna varivestis). Using this as a bioassay, investigators obtained the pure form of two compounds that were chemically identical with verrucarin A and roridin A (Bamburg and Strong, 1971; Cole and Rolinson, 1972). Cole and Rolinson (1972) described the larvicidal activities of DAS, purified from Fusarium lateritium, against 48-hour, first-instar larvae of the blowfly Lucilia sericata. They reported an LC_{50} of 7.5 mg/ml. In work with culture filtrates, these investigators found larvicidal activities in culture extracts obtained from trichothecene-producing strains of M. verrucaria, M. roridum, A. crotocinigenum, and Trichothecium roseum. They attributed this observation to the larvicidal activity of the respective mycotoxins, i.e., verrucarins, roridins, crotocin, and trichothecin. In these studies, pure trichothecin and trichothecolone were found to have an LC_{50} of approximately 100 mg/liter against L. sericata larvae.

A few trichothecenes have been studied for antibiotic or cytotoxic properties. None of them have been found to have appreciable antibacterial properties (Bamburg and Strong, 1971), but some of them have antifungal activity (Glaz et al., 1960). Burmeister and Hesseltine (1970) reported that T-2 toxin in a 50 µg/assay disc was not bacteriostatic to a number of bacterial species, but that it was slightly toxic to 6 of 11 fungi tested. Cytotoxic activity was greatest against yeasts, although Penicillium digitatum was also inhibited. Filter paper discs containing 4 µg or more of T-2 toxin had detectable fungistatic activity against Rhodotorula rubra, the most sensitive organism studied. The other trichothecenes with antifungal activity (verrucarin A and crotocin) also were active

against a number of fungi, especially yeasts (Glaz et al., 1959; Härri et al., 1962).

Trichothecin has been studied intensively in the Soviet Union and elsewhere for its practical usefulness in controlling plant diseases (Akhmedov, 1967; Askarova and Joffe, 1962; Babaeo, 1964; Kiryukhina, 1970; Kuz'mina, 1966; Kublanovskaya and Sukhacheva, 1961; Nechaeva, 1969; Sil'yanova, 1970). To a lesser extent, trichodermin has also been tested for activity against plant diseases (Tillaev, 1964). The fungicidal activity of trichothecin in plants is apparently systemic. Thus, it has been used experimentally to control fusarial and verticillium wilts of cotton (Babaeo, 1964; Kublanovskaya and Sukhacheva, 1961), fusarial wilt of pea and chick-pea (Kuz'mina, 1966), fusarial wilt of flax (Gurinovich and Kobyyrea, 1964), fusariosis of maize (Nechaeva, 1969), wilt smut of wheat (Sil'yanova, 1970), and downy mildew of tobacco (Kiryukhina, 1970). Data obtained from these studies suggest that the toxic trichothecenes prevent cell growth of the pathogen.

TOXICOKINETICS

Of the few papers published on the toxicokinetics of the trichothecenes, most are concerned only with orally administered toxins. Nevertheless, a review of the current literature can provide a useful introduction to the toxicokinetics of the trichothecenes and indicate those areas in which additional studies may be required.

Absorption. In cell-free systems, the inhibition of in vitro protein synthesis by trichothecenes is very rapid. At high doses, the inhibition is generally complete within a few minutes after administration of the toxin (Cundliffe et al., 1974; Wei et al., 1974a). Inhibition of protein synthesis is also very rapid in single cells in culture, typically being completed within 10 to 15 minutes (Wei et al., 1974a).

The cellular transport mechanism has not been studied in detail. Results from absorption studies in animals indicate, however, that the mechanism must be capable of quickly transporting quantities of trichothecenes through the cell membrane to the cytoplasmic and membrane-bound ribosomes of the cell. Such studies in animals have been conducted with three routes of exposure: dermal, injection, and ingestion. All of them have shown that the biological responses to the trichothecenes are rapid. Within a few minutes, a dermal response to high levels of a trichothecene can be observed, and in many animals, the ingestion of food containing trichothecenes results in severe emesis (Forsyth et al., 1977).

Feeding studies with trichothecene concentrations too low to produce an emetic response have been conducted in several domestic animals to determine if a health hazard exists when trichothecenes are contaminants of feedstuffs. In one such study, 6-week-old chicks were given a single dose of 3H-labeled T-2 toxin (Chi et al., 1978b). These animals excreted 60% of the labeled compound within 24 hours and 81% after 48 hours. Concentrations of T-2 toxin or its metabolites reached a maximum in most tissues 4 hours after administration. However, maximum concentrations were not observed in the muscle, skin, and bile until 12 hours after dosing. At this time, 42% of the radioactivity had been excreted and 38% had been recovered in the gastrointestinal tract and feces. In another study, maximum concentrations were reached in the plasma of a lactating cow within 8 hours and in milk within 16 hours after dosing (Yoshizawa et al., 1981). Seventy-two hours after administration, almost all the radioactivity had been eliminated in the urine and feces in a ratio of 3:7. Only 0.2% of the dose was recovered from the milk. The kinetics of absorption appear to be similar in swine (Robison et al., 1979a,b).

In mice, the absorption of a single oral dose of 3H-labeled T-2 toxin was rapid (Matsumoto et al., 1978). After the concentration in the blood peaked within 1 hour, there was a slower phase in which the trichothecenes and their metabolites were distributed via the blood to different tissues.

Metabolism

Our knowledge of the metabolism of administered trichothecenes in mammals is largely restricted to T-2 toxin and its metabolites. The first step in the metabolic inactivation of the trichothecenes is deacetylation. Studies have shown that the C-4 acetyl residue of T-2 toxin is removed by a nonspecific carboxyesterase localized in the microsomal fraction (Ellison and Kotsonis, 1974; Ohta and Ueno, 1976; Ohta et al., 1977). The same enzyme or others in the same fraction can also remove C-3 acetyl groups. Considerable variations among organs and test animals have been observed in the activities of these microsomal enzymes (Ohta and Ueno, 1976; Ohta et al., 1977, 1978).

Several metabolic degradation products have been identified in the feces and urine of mammals treated with various trichothecenes. 4-Acetylnivalenol is partially converted to nivalenol by the removal of the acetyl group from the C-4 position. HT-2 toxin results when the acetyl group on the number 4 carbon of T-2 toxin is removed. T-2 tetraol is produced by the hydrolysis of all the ester groups present in T-2 toxin (Matsumoto et al., 1978; Ueno et al., 1971b).

In chicks, T-2 toxin was converted to HT-2 toxin, T-2 tetraol, and neosolaniol. The neosolaniol was produced when the isovaleric acid

residue was removed from the ester group at the C-8 position of the T-2 toxin (Chi et al., 1978a). A number of other metabolic intermediates were detected in these studies but were not identified.

Similar metabolic transformations involving deacetylations have been observed in fungi. For example, Penicillium chrysogenum can enzymatically hydrolyze the isocrotonic esters at the C-4 position of crotocin and trichothecin (Horvath and Varga, 1961). In addition, Yoshizawa and Marooka (1975a,b) have demonstrated that the mycelia of Fusarium spp. converted 3-acetyldeoxynivalenol to DON. F. solani and F. nivale converted 4-acetylnivalenol to nivalenol and tetracetylnivalenol to 4,7,15-triacetylnivalenol. Acetyl T-2 toxin is converted to T-2 toxin and then to HT-2 toxin by the same fungi.

The metabolic fate of 3-H labeled T-2 toxin was examined in a lactating cow (Yoshizawa et al., 1981). The major metabolites of this trichothecene were unidentified compounds designated TC-1, TC-3, and TC-6. Unmetabolized T-2 toxin represented less than 20% of the total radioactivity 4 hours after administration of the toxin. This indicated that the metabolism of the toxin proceeds relatively rapidly. TC-1 and TC-3 were also found in the urine and feces. These investigators found the same metabolites in chickens given T-2 toxin (Yoshizawa et al., 1981). They reported that the unknown metabolites were not HT-2 toxin or T-2 tetraol, which were detected along with neosolaniol at much lower concentrations (Yoshizawa et al., 1981). Subsequently, Yoshizawa et al. (1982) identified TC-1 as the 3'-hydroxy derivative of T-2 toxin and TC-3 as the 3'-hydroxy derivative of HT-2 toxin. Thus, an important step in the metabolism of T-2 toxin was the introduction of the 3'-hydroxy group into the isovaleric acid residue that forms the ester at the C-8 position in T-2 toxin (Yoshizawa et al., 1982). In vitro, the major pathway to T-2 tetraol formation was the conversion of HT-2 toxin and 4-deacetylneosolaniol (Yoshizawa et al., 1980). The position of these new intermediates in the degradation pathway and their relative toxicities are unknown.

The metabolism of T-2 toxin was followed in rats and mice given a single oral dose. T-2 toxin, HT-2 toxin, and two unidentified compounds were isolated from rat feces. HT-2 toxin, neosolaniol, and three unidentified compounds were the major metabolites found in the urine. In both rats and mice, the unidentified metabolites represented the bulk of the administered T-2 toxin (Matsumoto et al., 1978). Those metabolites were probably the same as the 3'-hydroxy derivatives of T-2 toxin and HT-2 toxin identified by Yoshizawa et al. (1982). They have also been produced by F. heterosporium as natural products (Cole et al., 1981).

Generally, there is a gradual reduction in toxicity as a result of the deacetylations of T-2 toxin to HT-2 toxin and to the ultimate

deacetylated product, T-2 tetraol. T-2 toxin is 1.5 times as toxic as HT-2 toxin. HT-2 toxin is 3.4 times as toxic as neosolaniol, which in turn is 1.4 times as toxic as T-2 tetraol (Chi et al., 1978a). Because many of the metabolic derivatives have been found in feces and urine, these metabolic transformations may be important components of the elimination process, which apparently is the most important detoxification mechanism.

In enterohepatic circulation, material circulates between the liver and the intestines in a loop from the bile to the intestines into the blood, and then to the liver, where it is redistributed to the bile. Since high concentrations of T-2 toxin have been found in the bile, it is likely that blocking the enterohepatic circulation should result in a more rapid elimination of the toxin through the feces. If elimination is the main mechanism of metabolic detoxification, steps can be taken to enhance this process.

Distribution

There have been a few studies to determine the distribution of trichothecenes in different tissues over time. Ueno et al. (1971b) reported that 4-acetylnivalenol was distributed throughout the bodies of mice within 30 minutes after subcutaneous administration. The liver contained the largest amount of the trichothecene, and the kidneys contained the second largest amount. Matsumoto et al. (1978) studied the tissue distribution of a single oral dose of radio-labeled T-2 toxin in mice. In these laboratory animals, the peak concentration of the radioactivity in blood occurred 1 hour after administration of the toxin. Three and one-half hours after administration, most of the toxin and metabolites were found in the liver, kidney, and stomach. In the bile, there was a high specific activity of the toxin and its metabolites.

The tissue distribution of orally administered T-2 toxin has been examined in chicks (Chi et al., 1978b). The organs and the tissues, excluding the gastrointestinal tract, rapidly acquired T-2 toxin or its metabolites. In most tissues, the maximum concentrations were reached after 4 hours. In the muscle, skin, and bile, however, maximum concentrations were found 12 hours after dosing. Of all the tissues sampled, the highest specific activity was observed in the bile.

The distribution of tritium-labeled T-2 toxin was followed in swine for 18 hours after administration of a single oral dose (Robison et al., 1979a). The largest amounts of the toxin or its metabolites were found in the muscles and the liver, and the bile had the highest specific activity. The kidney also contained relatively high concentrations, as indicated by the radioactivity.

In a study by Yoshizawa et al. (1981), a lactating cow was given a single oral dose of T-2 toxin. In the plasma, the peak concentration was reached within 10 hours after dosing; in the milk, it occurred within 16 hours. At 72 hours, the liver contained twice the concentration of the other tissues, excluding the gastrointestinal tract.

These results indicate that the toxin is distributed throughout the body within 12 hours. The high concentrations found in the bile, gallbladder, liver, kidneys, and intestines probably reflect the role of these tissues in excretion of the toxin.

Retention

Animal experiments designed to determine tissue retention of the trichothecenes have not been conducted. Typically, experiments have been terminated after 24 to 72 hours when significant amounts of radioactivity from the toxin or its metabolites remained in the animals. The chemical structure of these retained metabolites has not been determined. Thus, there are no data on the possible retention of these toxins in various organs and tissues.

Excretion

The excretion of trichothecenes has been examined in several species of animals. Studies on the elimination of 4-acetylnivalenol in mice have provided data suggesting that the kidney is the main route of elimination for subcutaneous doses, since most of the administered dose was eliminated in the urine within 12 hours (Ueno et al., 1971b). When chicks were given a single oral dose of T-2 toxin, 50% of the toxin was eliminated within 12 hours (Chi et al., 1978b). Most of the trichothecene was eliminated through the gastrointestinal tract, possibly without equilibrating with the rest of the organs and tissues. Nevertheless, the presence of more than one-half the maximum radioactivity in these organs 48 hours after administration indicates a slow release of absorbed T-2 toxin and metabolites (Chi et al., 1978b). The highest specific activities were reached in the bile. The data suggest that T-2 toxin was excreted into the intestine via the bile and that the liver was a major organ for excretion (Chi et al., 1978b).

Robison et al. (1979a) have studied the excretion of T-2 toxin in swine during the 18 hours following administration of a single oral dose. At a dose of 0.1 mg/kg bw, 22% of the compound was excreted in the urine and 25% in the feces. At a dose of 0.4 mg/kg, which

approaches the emetic dose, 17% was excreted in the urine but only 0.8% in the feces. These data suggest that the higher dose may have interfered with elimination via the gastrointestinal tract.

The rate of elimination of a single oral 0.4 mg/kg bw dose of T-2 toxin in a lactating cow was followed for 72 hours after dosing (Yoshizawa et al., 1981). Thirty percent of the dose was eliminated within the first 24 hours, mostly through the kidneys. After 24 hours, the feces were the principal route of elimination, and by 72 hours almost all the toxin or its metabolites were eliminated. In a study of mice by Matsumoto (1978), 57% of a single oral dose of T-2 toxin was eliminated in the feces and 12% in the urine. Observations of these animals for 96 hours after dosing indicated that most of the dose was eliminated during the first 24 hours. These data indicate that most of the toxin administered in doses substantially less than the LD_{50} is eliminated relatively quickly through the feces and urine. Most of the eliminated toxin is metabolically modified, but the importance of this modification in the excretion process is not known.

MECHANISMS OF ACTION

The trichothecenes are not only the most potent small molecule inhibitors of protein synthesis in eucaryotic cells but they also have an unusually broad spectrum of inhibition, being effective inhibitors of protein synthesis in fungi, plants, and animals. It is likely that the toxic effects observed in whole animals, including necrosis of rapidly dividing cells, are directly related to their role as inhibitors of protein synthesis on 80S eucaryotic ribosomes. This inhibitory characteristic explains most, if not all, of the observed cellular metabolic effects of these toxins.

Ueno et al. (1968) first demonstrated that two of the trichothecenes, nivalenol and 4-acetylnivalenol, inhibited protein synthesis in rabbit reticulocytes and ascites cells. Ohtsubo et al. (1972) reported that 4-acetylnivalenol inhibited protein synthesis. McLaughlin and colleagues found that trichodermin inhibited protein synthesis by interfering with the activity of peptidyl transferase, which is required for elongation and termination (Hansen and Vaughan, 1973; Stafford, 1972; Stafford and McLaughlin, 1973; Wei and McLaughlin, 1974; Wei et al., 1974b). They reported that trichodermin preferentially inhibited the termination step of protein synthesis. The discovery of trichodermin's unique site of action served as a stimulus for the examination of a number of other trichothecenes in several laboratories primarily concerned with the mechanism of protein synthesis (Barbacid and Vazquez, 1974; Carrasco et al., 1973; Carter et al., 1976; Cundliffe et al., 1974; Mizuno, 1975; Schindler, 1974; Tate and Caskey, 1973; Wei and McLaughlin, 1974; Wei et al., 1974a).

Although a number of interesting questions remain to be answered, the mechanism by which protein synthesis is inhibited by trichothecenes is well understood.

Cytotoxicity

The inhibition of protein synthesis by trichothecenes occurs in a variety of eucaryotic cells (Barbacid and Vazquez, 1974; Carter et al., 1976; Inderlied et al., 1980; Notario et al., 1982; Ohtsubo et al., 1972; Smith and David, 1981; Stafford and McLaughlin, 1973; Ueno et al., 1968; Wei et al., 1974a). In yeast and mammalian cells, it has been proven to be the primary mechanism of toxicity (McLaughlin et al., 1977; Stafford and McLaughlin, 1973; Wei et al., 1974b).

Human (HeLa) cells in tissue culture were highly sensitive to trichothecenes; growth was inhibited at trichodermin concentrations of less than 1 µg/ml (McLaughlin et al., 1977; Wei et al., 1974a). In yeast, trichodermin at 34 µM (40 µg/ml) inhibited protein synthesis by 97%, polysaccharide synthesis by 60%, and RNA synthesis by 86% (McLaughlin et al., 1977). This pattern of macromolecular synthesis is typical of inhibitors that act on protein synthesis.

The substantial inhibition of RNA synthesis is a secondary effect of the inhibition of protein synthesis, and it may be important in the cumulative toxicity. The systems that control the rate of stable RNA synthesis in eucaryotic cells respond to the lack of protein synthesis by inhibiting the synthesis of rRNA. Since rRNA accounts for 80% of the total cellular RNA, all inhibitions of protein synthesis in eucaryotic cells act strongly to inhibit RNA synthesis through the control mechanisms that balance and regulate macromolecular metabolism (McLaughlin et al., 1977).

The generation time for the yeast strain used in the above experiment is 180 minutes (i.e., it takes each yeast cell approximately 180 minutes to pass through the eucaryotic cell cycle, which consists of the following four phases: G_1, S, G_2, M). Cells require newly synthesized protein to exit the G_1 phase and enter the S phase (Mitchison, 1971), during which DNA is synthesized. Inhibitors of protein synthesis prevent cells from entering the S phase, thereby blocking most DNA synthesis (Mitchison, 1971). A small amount of DNA synthesis continues to occur, however, because some cells in the S phase when the inhibitor is added finish their DNA synthesis. Also, unscheduled or repair-related DNA synthesis is not strongly blocked by inhibitors of protein synthesis (Mitchison, 1971). The pattern with which DNA synthesis is inhibited by trichothecenes is consistent with a primary effect on protein synthesis.

The effect of different concentrations of trichodermin and verrucarin A on protein synthesis in yeast is shown in Figure 6-1. No biphasic inhibition curve has been observed with any of the trichothecenes in either yeast or mammalian cells (McLaughlin et al., 1977). This suggests that there is only one site of action for the trichothecenes. The trichothecene-resistant mutants that have been isolated in Saccharomyces cerevisiae, Schizosaccharomyces pombe, and mammalian cells support this point of view (Berry et al., 1978; Carter et al., 1980; Gupta and Siminovitch, 1978; Rivera et al., 1980; Schindler et al., 1974; Wei, 1974a). In each case, resistance to the trichothecenes was associated with a genetic alteration of ribosome structures that made the ribosomes resistant to the mechanism of action of the trichothecenes. Thus, evidence from cytotoxicity studies and genetic studies indicates that the primary toxic effect of the trichothecenes is caused by their properties as potent inhibitors of protein synthesis.

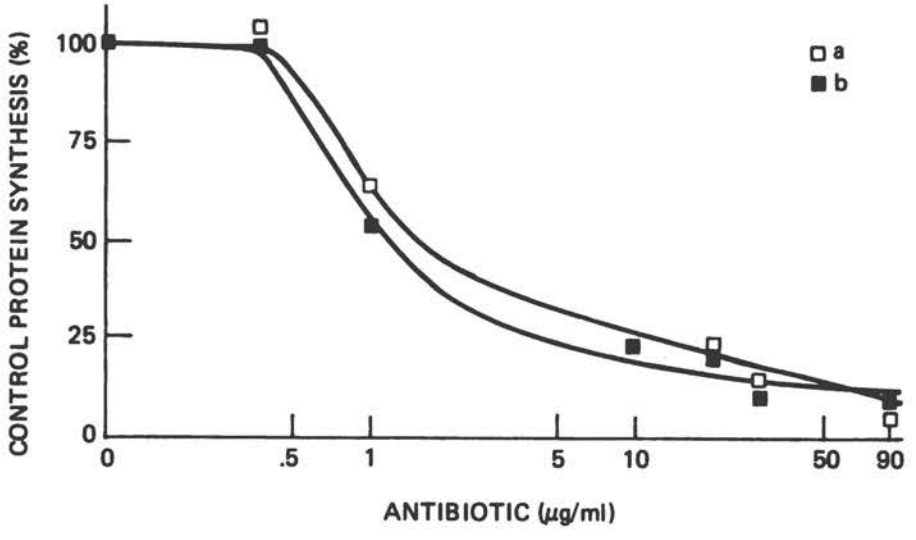

Figure 6-1: Inhibition of protein synthesis with increasing concentrations of (a) trichodermin or (b) verrucarin A in an S62 x S185 diploid strain of Saccharomyces cerevisiae. The rate of protein synthesis was determined over 1 hour. From McLaughlin et al., 1977, with permission.

Inhibition of Protein Synthesis

In eucaryotic cells, protein synthesis takes place on polysomes. The ribosomes of these polysomes translate an mRNA molecule that codes for one polypeptide chain. The general pattern of eucaryotic protein synthesis and the role of polysomes in the ribosome cycle is presented schematically in Figure 6-2. During initiation (I), ribosomal subunits from the ribosomal subunit pool join the mRNA at the initiation region (region 1). Initiation is a complex process involving several initiation factors and peptidyl transferase, which is an integral part of the 60S ribosomal subunit, to form the first peptide bond. After initiation, the ribosomes then undergo approximately 100 to 200 elongation (E) events as the amino acids are added one at a time to the growing polypeptide chain to complete the protein (region 2). Each elongation event requires elongation factors I and II peptidyl transferase. Finally, in response to a termination (T) codon on the mRNA, the complete polypeptide chain is released from the ribosome, which is then released from the mRNA (region 3). This termination event requires a release factor and peptidyl transferase. When protein synthesis is blocked by an initiation inhibitor (region 1), the entry of new ribosomal subunits in polysomes is prevented and the ribosomes on polysomes complete their elongation and termination events and enter the subunit pool. Thus, initiation inhibitors rapidly convert polysomes into monosomes and ribosomal subunits. Inhibitors of either elongation or termination prevent protein synthesis without causing the conversion of polysomes to monosomes.

Figure 6-2: Model of protein synthesis and the ribosome cycle in eucaryotic cells. I = inhibition, E = elongation, and T = termination. Numbers 1, 2, and 3 indicate the regions of action. From McLaughlin et al., 1977, with permission.

Most inhibitors of protein synthesis act on one specific part of the process. For example, cycloheximide and anisomycin, and their families of derivatives, inhibit the elongation process. The trichothecenes are atypical in that they may be divided into two broad functional groups, depending on whether or not they affect initiation or elongation-termination (Table 6-5) (Cundliffe et al., 1974; McLaughlin et al., 1977; Ueno et al., 1968).

Table 6-5. Inhibition of Protein Synthesis by Trichothecenes

Trichothecenes Affecting Initiation	Trichothecenes Affecting Elongation or Termination
Scirpentriol	Trichodermin
15-Acetoxyscirpendiol	Trichodermol
Diacetoxyscirpenol	Crotocol
Verrucarin A	Trichothecolone
T-2 toxin	Crotocin
Nivalenol	Trichothecin
4-Acetylnivalenol	Verrucarol

The group of trichothecenes that inhibit elongation-termination can be further divided into those that primarily affect elongation and those that primarily affect termination. This division can be based on an in vivo test determining whether polysomes reform on preexisting mRNA after an initiation inhibitor is removed. Under these conditions, an elongation inhibitor will block polysome reformation because it acts immediately after the completion of the initiation process, whereas a termination inhibitor will allow rapid polysome reformation. Results obtained with this test indicate that trichodermin acts as a termination inhibitor both in yeast and in mammalian cells. In yeast, it inhibited termination 5 to 10 times as effectively as it inhibited elongation, despite the fact that on the average more than 100 elongation events occur for every termination event in protein synthesis (Stafford and McLaughlin, 1973; Wei and McLaughlin, 1974; Wei et al., 1974b).

One component that all three processes have in common is peptidyl transferase, which is an integral part of the large ribosomal subunit, and all the toxic trichothecenes are potent inhibitors of its activity (Barbacid and Vazquez, 1974; Carrasco et al., 1973; Carter et al., 1976; Cundliffe et al., 1974; Mizuno, 1975; Schindler, 1974; Tate and Caskey, 1973; Wei and McLaughlin, 1974; Wei et al., 1974a,b). This suggests that the site of action of the trichothecenes is the peptidyl

transferase itself or a nearby region of the ribosome. The data indicate that inhibitors of protein synthesis are also inhibitors of peptidyl transferase (McLaughlin et al., 1977). Some trichothecenes and their inhibitory effects on both protein synthesis and peptidyl transferase are shown in Table 6-6.

The effects of peptidyl transferase activity on initiation deserve comment. Experimental data have established that the ribosomes inhibited during initiation actually formed several peptide bonds. Both peptidyl transferase and ribosome movement were blocked before the ribosome moved beyond the initiation site on the mRNA (Mizuno, 1975; Smith et al., 1975). Thus, some of the trichothecenes inhibit initiation by a special type of steric process because of the sheer bulk of the ribosome.

All the trichothecenes appear to produce toxicity through a basic mechanism involving the inhibition of peptidyl transferase as the key step. However, the diverse effects of the different trichothecenes either on initiation or on elongation-termination require additional consideration. Two models have the potential for explaining the effects of trichothecenes on protein synthesis (McLaughlin et al., 1977). In both of them the trichothecenes inhibit peptidyl transferase, but only under certain conditions that normally exclude a first effect on elongation. In one model, the trichothecene would only bind to the ribosome at certain stages in the ribosome cycle. For example, an initiation inhibitor would bind to the ribosomes only in the first stage of the cycle. Once bound, it would be effective in inhibiting peptidyl transferase and blocking initiation. The different conformations of the ribosome during the cycle would alter the ribosomal binding constants of the individual trichothecenes.

In the other model, the toxin would bind to the ribosome with equal affinity through the ribosome cycle, but it would only interfere with peptidyl transferase when the ribosome assumes certain unique configurations during the cycle. All the trichothecenes inhibit peptidyl transferase in the in vitro assay. In vivo, however, they would be much more inhibitory against peptidyl transferase activity in certain ribosomal configurations (Wei and McLaughlin, 1974).

Data are not yet sufficient to indicate which of the two proposed models is preferable. In principle, studies with radioactive toxin should enable one to distinguish between the two models, but the results to date have not been conclusive (Cannon et al., 1976; Wei et al., 1974a).

Data obtained in studies using radio-labeled trichodermin indicate that each ribosome has only one binding site for trichodermin. The binding site is located on the 60S ribosome, consistent with the action of peptidyl transferase (Barbacid and Vazquez, 1974; Wei et

TABLE 6-6 Comparative in Vitro and In Vivo Protein Synthesis of the Trichothecenes[a]

	Synthetic Activity Remaining for Different Tricothecene Concentrations (%)				
	In Vitro Peptidyl Transferase Assay		In Vivo Protein Synthesis Assay		In Vivo Site of Action
	5 μg/ml	20 μg/ml	1 μg/ml	10 μg/ml	
Control	100	100	100	100	–
Trichodermol (R_1 = H, R_2 = OH, R_3 = H)	73	40	40	13	E[b]
Verrucarol (R_1 = H, R_2 = OH, R_3 = OH)	91	67	68	63	E
Scirpentriol (R_1 = OH, R_2 = OH, R_3 = OH)	9	5	11	2	I[c]
15-Acetoxyscirpendiol (R_1 = OH, R_2 = OH, R_3 = OAc)	19	11	9	1	I
Diacetoxyscirpenol (R_1 = OH, R_2 = OAc, R_3 = OAc)	26	15	4	0	I
Verrucarin A (R_1 = H, R_2, R_3 = Dicarboxylic acid ester: $-CCHOHCHCH_3CH_2CH_2-$ (O, O) $OCCH = CHCH = CHC-$)	42	30	23	0	I
Crotocol (C-7, C-8 = β-epoxy)	98	94	79	49	E
Trichothecolone (C-8 = O, C-7 = H)	88	68	74	23	E
Crotocin (R_2 = $OOCCH = CHCH_3$)	34	22	11	2	E
Trichothecin (C-8 = O, R_2 = $OOCCH = CHCH_3$)	8	3	6	2	E

[a] From McLaughlin et al., 1977, with permission.
[b] E = point of elongation.
[c] I = point of initiation.

al., 1974a). Wei et al. (1974a) reported that the binding constant for ribosomes from rabbit reticulocytes was 9.2×10^5. A report by Barbacid and Vasquez (1974) indicated that yeast ribosomes have a binding constant of 5.5×10^{-5} and human ribosomes, a binding constant of 1.5×10^{-6}.

Several studies have provided data suggesting that all the trichothecenes that are effective inhibitors of protein synthesis compete for the same binding site on the ribosome. Table 6-7 demonstrates a general correlation between the ability of a compound to compete for the trichodermin binding site and its effectiveness as an inhibitor of peptidyl transferase activity (McLaughlin et al., 1977). Also, anisomycin and narciclasine, two structurally unrelated antibiotics that inhibit peptidyl transferase activity, compete for the same binding site as trichodermin and probably occupy binding sites that overlap those that bind trichodermin (Jimenez et al., 1975a,b; Schindler et al., 1974).

Table 6-7. Effectiveness of Competition for the Trichodermin Binding Site[a]

Compounds Tested	[^{3}H]-Trichodermin Binding (%)	Peptidyl Transferase Inhibition (%)
Trichodermin	100	93
Trichothecin	100	97
Scirpentriol	104	95
15-Acetoxyscirpendiol	97	89
Crotocin	79	78
Diacetoxyscirpendiol	78	85
Verrucarin A	66	70
Trichodermol	49	60
Trichothecolone	28	32
9,10-Dihydrotrichodermin	14	10
Verrucarol	11	33
9,10-Dihydrotrichodermol	6	13
4-Epitrichodermol	3	7
Trichodermone	2	1
Crotocol	7	6

[a]From McLaughlin et al., 1977, with permission.

Natural Mechanisms of Resistance to Trichothecenes

It has been possible to isolate yeast and mammalian cell mutants that are resistant to the toxic effects of the trichothecenes. One group of resistant mutants possesses altered 60S ribosomal subunits, which have a lowered affinity for the trichothecenes (Berry et al., 1978; Carter et al., 1980; Gupta and Siminovitch, 1978; Rivera et al., 1980; Schindler et al., 1974; Wei, 1974a).

Although the mutants were identified as being resistant to trichodermin, they were found to be resistant to all the trichothecenes and to the structurally unrelated compounds anisomycin and narciclasine. This pattern of cross resistance is consistent with the data indicating that there is one overlapping binding site for all these compounds.

Resistance is due to a single recessive gene in yeast and mammalian cells. In Saccharomyces cerevisiae, the mutation has been mapped to a locus on chromosome XV (Schindler et al., 1974). It has been possible to isolate the gene for resistance using recombinant DNA technology and to establish that it encodes for protein L-3, the largest protein of the 60S ribosomal subunit (Fried and Warner, 1981). Protein L-3 is presumed to be the major structural component of peptidyl transferase. The mutation conferring resistance renders the mutants 20 to 100 times less sensitive to the toxic effect of the trichothecenes (McLaughlin et al., 1977), indicating that the trichothecenes exert their toxic effect by inhibition of protein synthesis. This primary effect must be at least 20 to 100 times more important for toxicity than any secondary effect. Myrothecium verrucaria produces trichothecenes and possesses a natural resistance to their toxic effects. The 60S ribosomal subunits from this organism have a very low affinity for T-2 toxin and are resistant to its toxic effects (Hobden and Cundliffe, 1980).

In addition to resistant mutants with altered ribosomes, it has been possible in S. cerevisiae and S. pombe to isolate mutants defective in other genes with resistance to the toxic effects of trichothecenes. In S. cerevisiae, Stafford and McLaughlin (1973) have shown that this second type of resistant mutant does not alter the ribosomes and involves an altered cellular permeability or affinity for the toxin. This mutation also confers substantial resistance.

Structure-Function-Toxicity Relationships Among the Trichothecenes

Because all active trichothecenes bind to the same ribosomal binding site but produce widely different effects on protein synthesis, their structure-function relationships require close scrutiny. Ueno (1977) has observed that the epoxide function is

essential for toxic action. (See Figure 2-1 and Table 2-1.) Wei and McLaughlin (1974) have reported that reduction of the 9,10 double bond leads to a reduction of this action.

The following structural correlations can be made from the information contained in Tables 6-5 and 6-6. Substitution at the R_2 position (Figure 2-1) enhances the ability of the compound to act as an inhibitor of peptidyl transferase. All the compounds that have substitutions only at R_2 (with H at R_1 and R_3) are inhibitors of elongation or termination. Although verrucarol (with H at R_1 and OH at R_2) is an inhibitor of elongation, all of its esterified derivatives are inhibitors of initiation. Scirpentriol (with OH at R_1, R_2, and R_3) and its derivatives are inhibitors of initiation. Substitution at R_1 and R_3, which are located on the same side of the molecule, produces an inhibitor of initiation. Substitution at R_2, which projects away from the other side of the molecule, produces an inhibitor of elongation or termination. These structure-function relationships may be of considerable value to toxicologists since most of the very toxic compounds are inhibitors of initiation.

Synergistic Effects

The possibility of synergistic relationships among the various trichothecene toxins has not been investigated. It is perhaps more likely that such relationships might exist between trichothecene toxins and other mycotoxins with different mechanisms of action. In acute toxicity trials conducted by Lindenfelser *et al.* (1974), combinations of aflatoxin B_1 and T-2 toxin appeared to produce a synergistic lethal response in mice. In view of the small number of animals involved in that study, however, their findings must be regarded as tentative. Nonetheless, their results suggest that trichothecenes might form synergistic lethal combinations with other chemical toxins that cause general cellular damage. This possibility deserves exploration.

CONCLUSIONS AND RECOMMENDATIONS

Exposure of various test animals to sufficiently large doses of T-2 toxin, DAS, and other trichothecene mycotoxins can result in an immediate systemic illness with the development of lesions in various organs and tissues. Secondary or long-term effects of such exposure-related toxicoses resulting in incapacitating diseases and death are equally important; however, the effects produced by chronic exposure to these toxins have not been adequately studied.

The systemic effects reviewed above were produced by doses of toxin administered orally and parenterally. There are no data on systemic effects produced by topical or respiratory tract exposures.

The effects described below have been observed in nearly all animal models given effective doses of toxic trichothecenes and can be ascribed to the irritant and cytotoxic effects on rapidly dividing cells.

- Epithelionecrosis involving cutaneous surfaces, mucocutaneous junctions, and the mucosa of the upper alimentary canal results from direct contact between the trichothecene toxins (e.g., T-2 and DAS) and the epithelial surface. It is not found following parenteral or oral administration, except at points of direct contact with contaminated feedstuffs (e.g., lips, tongue, esophagus, and stomach). The irritant action results in immediate discomfort, changes in ability to mediate physiologic homeostasis through cutaneous heat loss or conservation, and disruption of an effective defensive barrier to secondary infection by opportunistic microbial pathogens. Cutaneous necrosis is observed within 24 hours after application of minimal quantities of T-2 toxin.

- Gastroenteritis with epithelial cell (crypt) necrosis follows both oral and parenteral administration of T-2 toxin, DAS, or other trichothecenes. Such lesions result when the toxin is brought to the intestinal tissues via the bloodstream and causes localized irritation of the intestinal mucosa. The immediate reactions include diarrhea and, in certain monogastric mammals such as the pig and cat, vomiting. Mucosal necrosis with disruption of the gut epithelium by erosions and ulcerations produces foci at which opportunistic microbial pathogens can invade the intestine and enter the bloodstream.

- Necrosis of hematopoietic tissues and hematologic changes are produced by T-2 toxin, DAS, and other trichothecenes affecting both the formed elements and humoral portions of the blood. Coagulopathy involving multiple factors (e.g., factors VII and X) has been observed as have changes in differential serum protein contents, including lowered levels of gamma, alpha, $beta_1$, and $beta_2$ protein fractions. Evidence of depressed hematopoiesis includes lowered PCV levels, leukopenia, hypoactive marrow, and thrombocytopenia in some animal species, but not in all. The development of a hemorrhagic disease syndrome is an uncommon manifestation of toxicosis resulting from T-2 toxin.

- Necrosis of lymphoid tissues (i.e., spleen, thymus, and lymph nodes) and suppression of immunologic mechanisms have been observed in several test animals. The effects include evidence of both quantitative and qualitative suppression of the cell-mediated immune

system and reduced concentrations of some immunoglobulins, including I_gA and I_gM, and some components of complement C3. These reactions, coupled with depressed phagocytic function of both macrophages and neutrophils and the disruption of natural barriers to microbial invasion because of epithelial necrosis, are extremely important secondary effects of trichothecene toxicosis.

- CNS effects are uncommonly observed in experimental studies in animals, but have been found in chickens given T-2 toxin and in human patients given DAS. Morphologic changes to account for the clinical observation of CNS dysfunction have not been described.

- Skin penetration is a very likely route of exposure to trichothecenes deliberately released in the air. Toxin penetrating intact or broken skin will contribute to the systemic concentrations of toxin resulting from uptake from other exposure routes.

- Inhalation appears to be an important route of exposure that has not been investigated. This route is unique in that the effective dose delivered into the body and the distribution of this dose are a function of the toxin's physical state and physicochemical form at the time of the exposure. The toxin's solubility in biological fluids is especially important in determining the degree to which inhaled toxin represents a local dose to the respiratory system or provides an additional contribution to the overall body concentrations of the toxin.

- There have been no direct inhalation toxicity studies to elucidate the toxicology of inhaled T-2 toxin.

- Current biological data have been derived from oral administration and parenteral injection to the virtual exclusion of all other routes of exposure. Thus, there is a need to study both local and systemic effects resulting from topical and inhalation exposure. Topical exposure studies should include effects of environmental temperature and relative humidity. Whole animal studies, including extensive clinicopathological and pathological examinations, are needed to investigate effects from these routes of exposure.

- There are no definitive data relating to long-term effects of trichothecene-induced toxicosis. Some studies have demonstrated that persons recovering from ATA have experienced reduced resistance to secondary infections. Part of this reduced resistance has been attributed to hematopoietic suppression, leukopenia, and disruption of epithelial barriers. Recent experiments in animals imply that immunosuppressive activity follows sublethal exposure to T-2 toxin. Thus, effects on the immune response appear to be important sequelae that need additional emphasis in future research.

- Interaction of trichothecene toxins with other substances, particularly other mycotoxins such as aflatoxin, may well promote augmented responses to the point of synergism because of their different mechanisms for inhibiting protein synthesis. This is an important area for which there are virtually no experimental data.

- Cardiovascular lesions have been observed following experimental dosing of rats with T-2 toxin. These results are problematic because of the natural occurrence of similar lesions in the strain of rats studied. This response, if verified, may be relatable to the myocardial lesions observed in persons reportedly exposed to yellow rain. Long-term toxicity studies are recommended to resolve this issue.

- Studies in which clinical signs of CNS disturbances have resulted indicate that trichothecenes cross the blood-brain barrier. Therefore, morphological, histochemical, and biochemical studies should be undertaken to determine the effects of trichothecenes on the CNS.

- Data indicate that T-2 toxin is metabolized relatively rapidly and that this process results in the formation of toxic metabolites. It is important to examine this degradative pathway for several toxins in several species exposed by topical, oral, and inhalation routes of exposure. It may be possible to enhance the degradative metabolism by inducing the enzymes involved or by administering degradative enzymes that may be developed through genetic engineering.

- Excretion appears to be a very important component of the natural process by which subjects recover from toxicosis. The role of toxin metabolism in that process needs to be defined. Since toxins are excreted largely through the urine and feces, it may be possible to enhance the process through simple means. Data also indicate that the trichothecenes or their metabolites are reabsorbed from the intestinal tract. It may be possible to identify a compound that, when administered orally, will inhibit this process and break the enterohepatic circulation loop. If metabolism is important to the excretion process, then enhancing metabolism may enhance excretion.

- It is important to determine the mechanism by which the toxin is transported into the cell. There are no data on this subject. It may be possible to design inhibitors of cellular transport that will serve as antidotes against the toxin.

- Because all the trichothecenes bind to the same site on the ribosome, it may be possible to prepare an effective antidote by developing chemicals that will bind to this site with a high affinity without seriously inhibiting protein synthesis. It is likely that

direct information on the mechanism of binding would also be produced from this effort.

- Studies should be undertaken to define the effect that trichothecene inhibition of protein synthesis exerts on macromolecular metabolism. At the cellular level, investigations should be conducted to determine if part of the observed toxicity is due to unbalanced macromolecular synthesis and growth and if in vitro studies can be devised to further understanding of the stages of recovery from trichothecene toxicosis. The biochemical elements of recovery need to be carefully defined. These studies should focus on several types of trichothecenes since their metabolism may well be different.

- The mechanism of genetic resistance to the trichothecene toxins should be studied in several organisms to determine the range of natural resistance mechanisms. It is possible that several of these mechanisms can be adopted for treatment of exposed victims. For example, a common mechanism of antibiotic resistance is the production of enzymes that inactivate the antibiotic. If such enzymes exist for the trichothecenes, they can be produced in large quantities by recombinant DNA or conventional technology and used to treat exposed persons.

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Chapter 7

PREVENTION AND TREATMENT

No specific therapy for trichothecene-induced toxicosis is known. However, a number of possible approaches to the development of specific therapeutic agents are discussed in Chapter 6. At present, the only effective prophylaxis is avoidance or reduction of exposure and the only therapy is symptomatic and supportive.

PREVENTION

Prevention By Reducing Exposure

Since it is presumed that the toxic trichothecenes will be delivered as an aerosolized liquid or powder, procedures such as providing the individuals in the exposure area with protective clothing and masks could be effective. Materials such as a waterproof cloth and plastic sheeting or any type of simple shelter would significantly reduce cutaneous exposure following an attack. Rapid departure from the area would also limit exposure.

Prevention By Avoiding Exposure

Respirators would provide considerable protection against inhalation exposure to the aerosolized trichothecenes (American National Standards Institute, 1980; Pritchard, 1977). Since these mycotoxins do not exist in a vapor phase, respirators using filtration should be effective. Those that would prevent droplets from gaining access to the respiratory tract should also afford protection. In addition, charcoal filters of the type commonly used to limit exposure to toxic substances in a vapor phase could be used effectively, since they should provide a barrier to droplet exposure as well as to absorption. A study should be conducted to identify appropriate protective respiratory masks and procedures.

Prevention by Decontamination

At present, there is only fragmentary qualitative and quantitative information on the factors involved in percutaneous penetration. After this information has been obtained, the validity of the following suggestions should be reevaluated.

After initial exposures, further absorption in trichothecene-exposed persons could be effectively reduced by such simple measures as a change of clothing and, possibly, bathing in uncontaminated water with soap or detergent. However, the number of minutes or hours after exposure in which washing would be effective has not been determined for humans.

Specific decontamination procedures should be developed. For example, further absorption may be minimized by decontaminating exposed surfaces with readily accessible agents such as chlorine bleaches or with soap and large amounts of uncontaminated water.

Contamination of persons from residual toxin concentrations could result if they remain in or enter areas where spraying had occurred. Since the trichothecenes may persist in the environment, they could gain access to the body either by translocation through rain or dew, by the ingestion of foodstuffs from the contaminated area, by inhalation of dusts, or by the ingestion of contaminated water. Exposure by these routes could be prevented by instituting appropriate decontamination procedures, by removing people to areas with safe water and food supplies, or by replacing essential supplies in the contaminated area.

Uptake of orally ingested toxic substances can sometimes be considerably reduced by ingesting either specific binding agents such as desferrioxamine (Catseh and Harmuth-Hoene, 1975) or nonspecific binding agents such as charcoal (Corby and Decker, 1970). In addition to decreasing absorption of oral doses, these agents may also facilitate the elimination of substances already absorbed.

It might be possible to develop protective lotions or creams that could be applied to the skin to reduce absorption. An effective barrier cream would reduce the need for protective clothing, which has considerable disadvantages for persons working in certain contaminated environments.

PROPHYLAXIS

Two types of prophylaxis--immunological and enzymatic--were specifically considered as means for providing protection against trichothecenes, but there are likely to be strict limitations on the effectiveness of both.

Immunological Prophylaxis

The injection of antibodies that bind absorbed trichothecenes could have practical, but limited, benefit. That is, the amount of antibody that can be produced is relatively small in comparison to the large amount (200 mg) required to neutralize 1 mg of toxin.

The total amount of immunoglobin in the human body is approximately 1 g/kg bw. In the best antiserum produced so far in rabbits, less than 0.01% of the immunoglobin served as an antibody to trichothecenes.[1] Even if this percentage could be increased 100-fold by better immunogens (see Chapter 3), the antibody level would be only 10 mg/kg, or enough to neutralize 0.05 mg of toxin. This is well below the lethal range (approximately 3 to 10 mg/kg bw) of known trichothecenes, but might be enough to protect against lower concentrations acting on the skin or mucous membranes.

Paradoxically, antibodies may be more effective against agents that are more toxic than trichothecenes. The trichothecenes have relatively low binding constants (10^6 M/liter or less) (Barbacid and Vasquez, 1974) for a receptor on ribosomes of which there are 10^5 per cell. Therefore, the lethal dose is approximately 10^6 molecules per cell. It is conceivable, therefore, that a more toxic agent with the same mechanism of action might be either formed naturally or synthesized. Such an agent would have a higher binding constant for ribosomes ranging from 10^7 to 10^8 M/liter and would require less than 10^5 molecules per cell to be effective. Therefore, the use of active immunization for prophylaxis might be effective against localized exposure of known trichothecenes with a lethal dose of approximately 1 mg/kg bw, but it could also conceivably be effective against the systemic effects of more toxic derivatives if their lethal concentrations were less than 0.05 mg/kg bw.

One of the possible disadvantages of immunological prophylaxis is its specificity. In general, there is a relationship between specificity and binding affinity. Thus, antibodies that bind to a toxin tightly enough to compete with cellular receptors are likely to be highly specific. It is conceivable, however, that an antibody could be formed against a common determinant on all trichothecenes.

Enzymatic Inducers

One possible prophylactic measure for reducing exposure to trichothecenes would be to induce enzymes that increase their rate of metabolism to nontoxic substances. Some trichothecenes are known to be metabolized fairly rapidly, but the exact molecular pathway for this degradation is not known (see Chapter 6). The metabolic rate may

[1] Chu, F.S. 1983. Personal communication. University of Wisconsin, Madison.

be enhanced by agents that induce liver enzymes, e.g., barbiturates (Neal, 1980). However, these drugs are not likely to be very effective in either prophylaxis or treatment. For prophylaxis, these agents would need to be administered within a day or two of expected exposure, and the maximum reduction in toxicity is likely to be less than twofold. The effect of enzyme inducers administered after exposure to trichothecenes will be even less than that achieved before exposure. By the time the enzymes are induced (24 hours or longer), most of the toxin will already have been metabolized. Since the trichothecenes act by interfering with protein synthesis, enzyme inducers may be totally ineffective when given after exposure to the toxin.

TREATMENT

No specific treatment of trichothecene-induced toxicosis is known. In Chapter 6, the committee identified a number of possible areas of research that could lead to the development of such agents. The following paragraphs describe some of these areas:

- The passive administration of passively or actively induced antibodies that could bind absorbed trichothecenes. The effectiveness of this measure will depend on the production of hybridomas making monoclonal antibodies specific for various trichothecenes and the ability of this technique to produce sufficient quantities of antibodies to provide active protection. (See above section entitled Immunological Prophylaxis.)

- Agents that prevent the binding of the trichothecenes to their site of action on ribosomes. Such substances may prevent binding of absorbed trichothecenes to the 80S ribosome, thereby interfering with their mechanism of action. If sufficiently active, they could displace substances already bound.

- Enzymes that selectively degrade or detoxify trichothecenes. In theory, these enzymes could be produced and administered passively to exposed individuals. This procedure is a departure from established therapy, and its initial use would probably be accompanied by many and perhaps very difficult problems.

- Agents that enhance the excretion of trichothecenes into the bile or prevent their reabsorption by the intestines. This use of such agents is a well-established therapeutic principle with demonstrated clinical value in a number of intoxications.

- Potential clinical manifestations known or believed to occur after exposure to trichothecenes. These manifestations should be studied, and general clinical support measures currently in use should be evaluated with a view toward integrating them for large-scale application.

- Supportive care in the treatment of trichothecene-induced toxicosis. Diarrhea, fluid and blood loss, and resulting hypovolenic shock are an important result of the known toxicity of trichothecene substances. Thus, appropriate replacement with fluids, either orally or intravenously, and replacement of blood loss when necessary should significantly reduce mortality and morbidity. The use of adequate fluid management should have significant benefit. The cutaneous effects caused by trichothecenes are analogous, at least in some stages, to clinical situations involving therapy for third-degree burns.

- Inhalation exposures to aerosolized mycotoxins. In humans, inhalation of large amounts of trichothecenes may produce effects similar to those in so-called adult respiratory distress syndrome (ARDS), with its associated reduction in oxygenation and ventilation. Monitoring of arterial blood gases and supplemental oxygen therapy may be useful in the treatment of individuals exposed to aerosolized mycotoxins. Other effects, such as central nervous system difficulties and renal failure, can be treated by appropriate symptomatic means.

- Cardiovascular support therapy. Since cardiovascular complications may result from trichothecene exposure, it may be of value to use nonspecific measures involving cardiovascular support, for example, drug therapy, cardiac pacing, and cardiac monitoring. As better definitions of exact physicological defects in the cardiovascular system become known, a more systematic approach to supportive therapy can be made.

- Antibiotic therapy for secondary bacterial infection. When secondary bacterial infections occur, especially in the lung and skin, antibiotic therapy may be effective. Therapy for hypovolemia may be effective in restoring the fluid lost from the gastrointestinal tract and the skin and in reestablishing intravascular fluid balance.

- Replacement of formed elements of the blood. Consideration should be given to replacement of the formed elements of the blood, such as platelets and white blood cells, in a manner analogous to the chemotherapy given to patients who have developed marked suppression of the bone marrow as well as infection and bleeding. Therapy with white blood cell and platelet transfusion is cumbersome and requires support services of the type found only in tertiary care hospitals. It would not be suitable for use in remote areas of developing countries or for situations involving mass casualties.

- Detoxification agents. When more information becomes available on trichothecene persistence and the mechanism of skin damage, it may be possible to recommend specific agents to detoxify the toxins or their damaging metabolites. In the interim, common practice dictates that severe irritant dermatitis may be more rapidly improved by the

application of uncontaminated water compresses. Topical corticosteroids might then be added, but the efficacy of these agents should be validated in an appropriate model. When the areas affected by dermatitis are large, fluid and electrolyte balance may require supervision. When secondary infection develops, systemic or topical antibiotics should be effective. Thus, the use of prophylactic antibiotics to treat effects of trichothecenes should be considered in further research.

CONCLUSIONS AND RECOMMENDATIONS

No specific therapy for trichothecene toxicity is known. At present, the only effective prophylaxis is avoidance and the only therapy is symptomatic and supportive. In nonspecific supportive therapy, many frequently used drugs such as corticosteroids may be harmful. Thus, they should be administered to humans only with great care pending the completion of more definitive information from studies in animals.

Of all the areas of research discussed above, the committee believes that the following subjects deserve greatest emphasis:

1. investigation of the best methods of preventing exposure with protective barriers and decontaminatin procedures;

2. development of specific therapeutic agents for the treatment of acute trichothecene toxicity, the rationale for which is described in greater detail in Chapter 6;

3. determination of the efficacy of immunological prophylaxis, but only after better immunological agents are developed, as described in Chapter 3; and

4. provision of general supportive measures to sustain life using current technology.

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Chapter 8

RECOMMENDATIONS

Of the many potentially productive areas of research described in this report, the committee has listed below the eight that are most promising. An elaboration of these recommendations and the rationale behind them are provided in the preceding chapters.

The suggested research is aimed at protecting populations against exposure to trichothecene mycotoxins and their deleterious effects. The committee understands that its recommendations represent a very broad and ambitious undertaking. Thus, before any research program is adopted, it will be necessary to identify priorities for specific studies based on available expertise, anticipated needs, and available funds.

As stipulated in its charge, the committee restricted its study to the trichothecene mycotoxins. However, it was well aware that there are other potent biologically produced toxins that may present similar risks to human health as a result of their natural occurrence or deliberate release. Therefore, when developing its research program, the Army should view the following recommendations in this broader perspective.

1. Taxonomic groups of fungi known to produce trichothecenes are globally distributed; however, naturally occurring trichothecenes have been reported in relatively few countries. Thus, a program should be undertaken to obtain a better characterization of the global distribution of these toxins.

2. Protocols for field sampling should be developed. Further research is needed on the application of thin-layer chromatography (TLC) and the enzyme-linked immunosorbent assay (ELISA) for the identification and quantitation of various trichothecenes present in a variety of sample materials. Other methods that are less promising but nonetheless warrant attention include remote sensing techniques and assays based on the susceptibility of living organisms to trichothecenes. Gas-liquid chromatography (GLC) and mass spectrometry (MS) should be used to validate new methodologies.

3. Chemical and enzymatic reactions that degrade trichothecenes to form nontoxic products should be investigated. The chemistry of the sterically protected epoxide should also be studied.

4. Methods of reclaiming trichothecene-contaminated commodities by transforming them into useful foods, feeds, and products should be developed.

5. The persistence and leaching of trichothecenes and the products of their nonbiological and microbial degradation in different types of soils and waters need to be evaluated. Information is also required on the uptake by plants growing in contaminated soils and on the behavior of trichothecenes in terrestrial and aquatic food chains. These studies involve different levels of complexity and expense, but many of them should produce results within a relatively short period.

6. In the broad field of research on the biological effects of T-2 toxin and certain other trichothecene mycotoxins, the following three areas have potential for providing the most useful information in developing protective and therapeutic measures for exposed populations: (1) toxicokinetics following topical and inhalation exposures under a variety of environmental conditions; (2) the effects of continuous or intermittent long-term exposures by single or multiple routes; and (3) the effects produced by combined exposure to trichothecenes and other mycotoxins. These studies should include clinical, clinicopathological, immunological, morphological, and functional evaluations, with particular attention to cardiovascular, central nervous, and immune systems, as well as organs and tissues that are established targets. Detailed research recommendations are described at the end of Chapter 6.

7. There is a need to identify the best methods for preventing exposure through the use of protective barriers and decontamination procedures.

8. Methods for reducing the toxic effects of trichothecenes should be developed. Such efforts should be focused not only on protective measures (as stipulated in Recommendation 7), but also on ways to reduce absorption of the toxin and its binding to ribosomes, and to increase its rate of degradation and elimination. The methods adopted should be based on sound quantitative studies of the cutaneous pharmacokinetics of these compounds.

The military is encouraged to continue seeking the advice of nonmilitary scientists in its research programs. As a result of this review of new techniques and new data, the committee recommends that the military draw from the diverse expertise in the scientific community in order to take full advantage of discoveries as soon as possible.

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