THE OCCURRENCE OF MYCOTOXINS IN FEEDSTUFFS IN NATAL AND ASPECTS OF THEIR METABOLISM IN THE RUMEN

by

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DECLARATION

I hereby certify that the results presented are a result of my own investigation.

K Westlake

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CONTENTS

CHAPTER 1

PREFACE

Work by various authors has shown that ruminants are less susceptible to mycotoxin poisoning than monogastric animals. This is especially true of mycotoxicoses caused by trichothecenes.

With this in mind, the object of the present study was to ascertain the extent of mycotoxin contamination of feedstuffs in Natal and to monitor degradation of those toxins found in feedstuffs by ovine rumen fluid *in vitro*.

From subsequent work with pure cultures of rumen anaerobic bacteria it was hoped to identify organisms capable of T–2 toxin degradation and which may therefore play an important role in conferring T–2 toxin resistance to ruminants.

The identification of enzymes capable of T–2 toxin degradation would help to ascertain whether toxin degradation was a result of a specific detoxification process or of fortuitous action of enzymes whose primary function was that of feed degradation.

CHAPTER 2

LITERATURE SURVEY

2.1 HISTORICAL BACKGROUND

The trichothecenes are a chemically related group of biologically active fungal secondary metabolites produced by various members of the Fungi Imperfecti including species of *Fusarium, Myrothecium, Trichoderma, Cephalosporium, Verticimonosporium* and *Stachybotrys* (Ueno, 1977b).

It is only since aflatoxin was determined to be the toxic principle in the death of over 100 000 turkey poults in Britain in 1961 that interest in fungal toxins (mycotoxins) as potential health hazards has arisen. Prior to this, the emphasis in fungal secondary metabolite research was on the screening and production of new antibiotics. Indeed the first trichothecene toxins to be isolated were the Verrucarins, which were found in 1946 during an extensive war-time search for new antibiotics (Brian & McGowan, 1946). At the time of discovery, the single compound isolated was named Glutinosin, but later work by Grove (1968) showed glutinosin to be a mixture of verrucarins A and B. During the search for new antibiotics a large number of Fungi Imperfecti were shown to have anti-fungal activity (Brian & Hemming, 1947). Among these were many species of fungi now known to be capable of trichothecene production. Subsequent efforts to isolate the toxic principle in pure form led to the isolation of trichothecin by Freeman & Morrison in 1948. Later Gotfredson, Grove & Tamm (1967) named the ring system common to all trichothecenes, trichothecane after trichothecin, the first member of the group to be isolated.

In the years following these discoveries much effort was put into the isolation and identification of trichothecenes and fungi responsible for their production and also the identification of toxicoses caused by these toxins. However the first historical report of suspected trichothecene mycotoxicosis was published by Woronin in 1891 (cited by Ueno, 1977a). He described a condition known as 'Taumelgetreide' (Staggering grains) in humans and horses. After consuming these grains, humans exhibited symptoms of headache, vertigo, chills, nausea, vomiting and visual disturbance. Farm animals fed on the same diet exhibited the above symptoms accompanied by feed refusal. From the grains, Woronin was able to isolate the fungis *Fusarium roseum, Gibberella saubinetti, Cladosporium herbarum* and *Helminthosporium sp.*. Since this time more than seventy different trichothecene mycotoxins have been isolated and identified and many of these have been associated with illness and death in both humans and animals.

Ueno (1977a) stated that "Man and farm animals cannot escape from possible exposure to these mycotoxins until all foodstuffs and feedstuffs are free from Fusarium and related fungi".

The importance of these toxins cannot be overemphasised, especially in animal feedstuffs, where a large portion of an animals diet in many parts of the world is comprised of products that are naturally infected with fungal species capable of trichothecene production.

2.2 NATURAL OCCURRENCE

Trichothecenes are produced by various soil fungi including some adapted to growth as saprophytes and plant parasites. These fungi have a worldwide distribution but are most often found in temperate climes where the environmental conditions often affect the amount and type of toxin produced. Various trichothecenes have for example been found in Japan (Yoshizawa, 1983), Thailand (Tsunoda, Toyazaki, Morooka, Nakano, Yoshizawa, Okubo & Isoda, 1968), New Guinea (Gotfredson & Vangedal, 1965) South Africa (Thiel, Meyer & Marasas, 1982); Hungary (Gyimesi, 1965); France (Jemmali, 1983) and the USA (Hsu, Smalley, Strong & Ribelin, 1972). The trichothecene-producing Fusaria are parasitic on a large number of plants including conifers, wheat, maize, carnations, peas, lupines (Seemuller, cited by Smalley & Strong, 1974); oats, forage grasses, sugar beet, stone fruits, citrus fruits, soybean, tomatoes and others (Bilai, cited by Palti, 1978). Species of *Fusarium* occur in all types of soil where farming is practised and in many regions where there is no agriculture. Amongst the Fusaria, some species are found worldwide while others have a much more limited distribution. In the former category, *F. oxysporum* is found in the arctic Tundras, alpine pastures and tropical soils, while in the latter category, *F. sporotrichioides* for example is found almost only in temperate zones (Palti, 1978). The distribution and toxicity of the genus Fusarium has been extensively reviewed by Palti (1978).

The occurrence of other trichothecene – producing fungi including some of the products on which they have been found is summarised in TABLE 1.

TABLE 1 Natural occurrence of some trichothecene-producing fungi (after Smalley & Strong, 1974)

Fungal species	Where found
Myrothecium roridum	Tomato
M. roridum	Cocoa
M. roridum	Coffee
M. roridum	Cotton
M. verrucaria	Dead plant material
M. verrucaria	Pasture plant material
Cephalosporium apii	Celery
C. gregatum	Soybean
C. sacchari	Sugar cane
C. infestans	Human pathogen
C. recifei	Human pathogen
Trichoderma viride	Garlic, lemons
T. roseum	Apples, celery
Trichothecium roseum	Stored fruit and vegetables

Most species of *Myrothecium*, including *M. verrucaria*, are saprophytic. *Myrothecium roridum* however is parasitic on a wide variety of plants (see TABLE 1). These species produce the toxic roridins and verrucarins. According to Tullock (1972), *M. roridum* is synonymous with *Dendrodochium toxicum* and is therefore implicated in dendrodochiotoxicosis of various animals (see Section 2.3.5).

The cephalosporia are responsible for several plant diseases including sugar cane wilt (Edgerton, cited by Smalley & Strong, 1974), and have been implicated as being pathogenic to man (see TABLE 1).

Stachybotrys spp. are saprophytic fungi of worldwide distribution and have been commonly found in hay (Rodricks & Eppley, 1974). Throughout the literature concerning this genus three species are encountered; *S. atra*, *S. alternans*, and *S. chartarum*, however on the strength of identification carried out by the Centraal-bureau voor Schimmelcultures, Baarn, Holland, the three shall be considered synonymous (Hintikka, 1977c) and be referred to as *S. atra*.

The genus *Trichoderma* (TABLE 1) is amongst the most numerous of all soil fungi (Skinner *et al.*, cited by Smalley & Strong, 1974), while *Trichothecia* (TABLE 1) are also common soil fungi. These genera have only limited plant pathogenic capabilities.

2.3 MYCOTOXICOSES AS INDICATORS OF TRICHOTHECENE OCCURRENCE

Mycotoxicoses have been categorised into three groups by Pier, Richland & Cysewski (1980). The groups are (1) acute primary toxicoses, recognised as specific, overt disease when high to moderate amounts of toxin are consumed. (2) Chronic primary mycotoxicoses, seen at moderate to low levels of toxin and taking the form of reduced growth rate, reduced reproductive efficiency and inferior market quality and (3) secondary mycotoxic diseases seen at very low levels of toxin consumption and where the symptoms are those of various infectious diseases. The latter two categories probably occur on a more frequent basis than category (1) but will also be more difficult to detect. Bamburg & Strong (1971), have said 'It has not been established unequivocally that any particular trichothecene is the cause of any individual outbreak of mycotoxicosis under natural conditions. To do so, one must identify the toxic food or feed, establish the presence of the suspected chemical therein by a specific analytical method both qualitatively and quantitively, and demonstrate that the concentration present is able to reproduce the symptoms of the toxicosis when such concentrations are ingested in the same type of diet as the one implicated in the disease."

They go on to say that "Notwithstanding the lack of any such clear-cut proof, circumstantial evidence that various trichothecenes have been responsible for poisoning of both animals and humans beings is impressive." A number of findings that have come to light since the observations of Bamburg & Strong (1971) have lent greater credence to their appraisal of the situation and these will be discussed later. (Section 2.3).

One of the many problems encountered in linking disease symptoms in animals with mycotoxins is that the animals often respond differently under laboratory conditions when compared to the field situation.

However despite these problems a number of animal and human diseases have been designated as trichothecene mycotoxicoses. This has not happened until recent years and the

onset of feed survey programmes where the discovery of trichothecenes has occurred as a result of research into the aetiology of a particular human or animal disease and in some cases the presence of trichothecenes has been assumed rather than proven. At such times the involvement of trichothecenes has been suspected because the disease symptoms have resembled those produced by the trichothecenes (Bamburg & Strong, 1972) and either the presence of trichothecene-producing fungi or the production of disease symptoms by extracts of the fungi, or both, have been taken as indicating the presence of the toxins in the feed. The first positive identification of a trichothecene associated with illness in animals was carried out by Hsu and co-workers in 1972. In this case, they identified T–2 toxin in mouldy corn that was responsible for the death of 20% of a herd of lactating dairy cows in Wisconsin. Prior to this the involvement of trichothecenes had been suspected in several outbreaks of mycotoxicoses in man and animals, without being proven. Such mycotoxicoses included Alimentary Toxic Aleukia (ATA), Stachybotryotoxicosis, Akakabi-Byo (Red mould disease), Dendrodochiotoxicosis and mouldy corn toxicosis which have been considered by Ueno (1977a) to be caused by trichothecenes.

2.3.1 Alimentary Toxic Aleukia (ATA)

The most important trichothecene toxicosis in terms of human health is ATA (Joffe, 1971). During the years 1942–1947 over 10% of the population of Orenburg in Siberia were seriously ill and in many cases died after ingesting products made from overwintered millet, wheat and barley. The clinical symptoms of ATA have been described by a number of authors (Mayer, 1953; Joffe, 1971) and are characterised by vomiting, skin inflammation, diarrhoea, leukaemia, haemorrhage, necrotic angina, sepsis and exhaustion of bone marrow (Forgacs & Carll, 1962). Of the fungi found associated with ATA, *F. sporotrichioides* and *F. poae* were thought to be the major toxin producers. From these species, Olifson (cited by Yagen, Joffe, Horn, Mor & Lutsky, 1977) reported the isolation of three steroids named lipotoxol, sporofusarin and poaefusarin. Lipotoxol was isolated from toxigenic overwintered grain and the latter two from toxigenic cultures of *F. sporotrichioides* and *F. poae*. In separate studies, Olifson (cited by Yagen *et al.*, 1977) and Bukharbayeva & Piotrovski (cited by Leonov, 1977) reproduced the clinical symptoms of ATA in animals using unpurified extracts from cultures of these fungi. However, this could not be taken as conclusive proof of the involvement of these steroids in ATA. This was emphasized in 1973, when Mirocha & Pathre showed the presence of T–2 toxin (2,5%), T–2 tetraol (0,6%), neosolaniol (0,14%) and zearalenone in an unpurified sample of poaefusarin. They could not confirm the presence of steroids in this sample and found the toxicity to be associated with T–2 toxin.

In an attempt to clarify the cause of ATA, Yagen *et al.*, (1977) examined 131 isolates of *F. poae* and *F. sporotrichioides* which were associated with clinical ATA toxicoses in Russia. Selecting the most toxic strain of *F. sporotrichioides*, they were able to isolate six steroids, T–2 toxin, HT–2 toxin and solaniol and in animal studies with T–2 toxin-containing and T–2 toxin-free extracts were able to show that only those extracts containing T–2 toxin elicited on ATA-like syndrome in cats. The steroidal compounds were found to be non-toxic. These results and data showing that the trichothecene diacetoxy scirpenol can induce aleukia in man (Wyatt, Hamilton & Burmeister, 1973) a symptom characteristic of ATA, suggest that T–2 toxin played a significant role in the outbreak of ATA in Russia. Although ATA has not been known to occur since, this serves as an indicator of the potential hazard to human health caused by T–2 toxin and of the importance of fusariotoxicoses.

2.3.2 Mouldly corn toxicosis

Mouldy corn toxicosis is a general term used to describe mycotoxicoses caused by the ingestion of mouldy grain and therefore includes the feed refusal and emesis syndrome of animals thought to be caused primarily by the ingestion of mouldy feed containing deoxynivalenol (vomitoxin) and some of the mycotoxicoses caused by aflatoxin, trichothecenes, zearalenone and others.

The fungal flora and associated mycotoxins found in mouldy feed appear to be linked to the climate of a particular region. Thus, in the USA where mouldy corn toxicosis has occurred frequently, *Aspergillus flavus* is associated with the condition in warmer areas and *Fusarium* species amongst others, in cooler climates. According to Hsu *et al.* (1972) *Fusarium tricinctum* is consistently the most toxic of the fungi isolated from mouldy corn in low-temperature storage and hence the term mouldy corn toxicosis is predominantly associated with contamination of corn by this fungus. Its presence in mouldy corn samples has been correlated with farm outbreaks of mouldy corn poisoning (Smalley, Marasas, Strong, Bamburg, Nichols & Kosuri, 1970) and as previously mentioned, Hsu *et al.* (1972) were the first to identify T–2 toxin in corn contaminated with *Fusarium tricinctum*.

The mycotoxicoses caused by the ingestion of mouldy corn are discussed in later sections depending on the particular mycotoxin involved.

2.3.3 Fusariotoxicoses

Fusariotoxicosis has been described by Kurmanov (1977) as the poisoning of animals resulting from the consumption of roughage and concentrate feed which is contaminated by toxins produced by fungi of the genus *Fusarium*. All species of animals and man are susceptible to these toxins. Among agriculturally important animals, the most susceptible to the *Fusarium* toxins are horses, pigs and chickens. Cattle are quite resistant and this is ascribed to the more complex digestive system of ruminants.

Fusariotoxicoses therefore include ATA, and red-mould poisoning (Sections 2.3.1 & 2.3.6) as well as hyperoestrogenism, bean hull poisoning, leukoencephalomalacia and others.

Fusariotoxicoses of animals have been reported from countries such as Australia, Italy, Russia, USA, Rumania, Poland, Sweden, Finland and others (Kurmanov, 1977).

The toxicosis known as bean hull poisoning occurred in Japan when horses consuming bean hulls contaminated with *F. solani* exhibited symptoms including convulsions and retarded reflexes. Ten to 15% of the affected horses died within 2–3 days (Kanishi & Ichigo, cited by Ueno, 1977a). Analysis of the feed showed the presence of *F. solani* and a number of mycotoxins including T–2 toxin. Toxin was hence implicated in the toxicosis (Ueno, Ishii, Sakai, Kanaeda, Tsunoda, Tanaka & Enomoto, 1972).

Leukoencephalomalacia also affects horses and was reported in the USA as early as 1891 (Mayo, cited by Bridges, 1977). It is caused by the ingestion of maize infected with *F. moniliforme* Sheldon and is characterised by focal liquefaction of the cerebral white matter. This condition has been reviewed recently by Coetzer, Kellerman & Naudé (1985).

Yoshizawa (1983) has reviewed the animal diseases known as fescue foot and hyperoestrogenism caused by the Fusarium metabolites butenolide and zearalenone respectively.

The majority of trichothecene toxicoses can be considered as examples of mouldy corn toxicoses or fusariotoxicoses. Exceptions include Stachybotryotoxicosis and dendrodochiotoxicosis.

2.3.4 Stachybotryotoxicosis

Alimentary toxic aleukia has been described as a trichothecene toxicosis in humans (Section 2.3.1) and is caused by trichothecene-producing fusaria. A second human mycotoxicosis thought to be caused by trichothecene toxins is stachybotryotoxicosis, which also affects animals. It has been reported in Russia (Bilai & Pidoplicko), Hungary (Danko & Tanyi), Yugoslavia (Ozegovĭc, Pavlović & Milosev), Rumania (Drogichi, Moldoveanu & Comsa), Bulgaria (Kunev). (All cited in Pepeljnjak, 1983) and in South Africa (Schneider, Marasas, Dale Kuys, Kriek & Van Schalkwyk, 1979). Clinical symptoms include cough, rhinitis, nasal irritation and cutaneous irritation at the point of contact. Gajdusek (cited by Hintikka, 1977a), noted that cases of human stachybotryotoxicosis have mainly been observed in regions in which the equine disease has also been reported and that the people affected had handled hay or straw contaminated by *S. atra*. Symptoms in these cases were as described above, beginning with isolated rashes and heavy perspiration. Leukopaenia was observed in some patients. According to Drobotko (cited by Hintikka, 1977a) stachybotryotoxicosis in humans can also occur as a general toxicosis as a result of absorption of toxic substances through the skin or by inhalation of the fungal dust.

In animals, the toxicosis is usually acquired by ingestion of toxic material or contact with contaminated bedding and has been reported in many animals including horses (Sarkisov), cattle (Dzhilavyan), swine (Chernov), sheep (Dzhilavyan) and poultry (Sarkisov). (All cited by Hintikka, 1977b). The disease was first recognised in Russia in 1931 when horses, swine, calves and poultry showed signs of shock, somatitis, dermal necrosis, haemorrhage, thrombocytopaenia, leukopaenia, nervous disorders and eventually death from respiratory failure (Forgacs, 1972).

Since then, work by Eppley and co-workers in 1973 and 1976 has shown the presence of macrocyclic dilactone derivatives of trichothecenes, namely stachybotryotoxins A and B and satratoxin C, D, F and G. Satratoxin D was later shown to be synonymous with roridin E (Eppley, 1977) and the former name has therefore been dropped. It has been suggested by Eppley (1977) that the observed toxicity of *S. atra* cultures is due to these toxins, as indicated by similar toxicities in bioassays to those reported for naturally occurring cases.

More recently satratoxins G and H have been detected in seven out of 23 straw samples examined in Hungary. No indication of associated toxicoses was given, nevertheless this information supports the theory that these toxins are responsible for stachybotryotoxicosis. (Sandor, 1984).

2.3.5 Dendrodochiotoxicosis

Dendrodochiotoxicosis is another presumed trichothecene toxicosis initially reported in Russia. Both animals and humans are again affected, but the toxicosis has mainly been reported in horses and is caused by Dendrodochium toxicum (Synonymous with *Myrothecium roridum*) (Tullock, 1972) which grows predominantly upon cellulose-containing substrates such as wheat straw and chaff.

The fungus produces the macrocyclic roridins and verrucarins, members of the trichothecene group of toxins and which have therefore been cited as the toxic factor in contaminated feeds (Mortimer, Campbell, Di Menna & White, 1971). In these cases a number of clinical symptoms appear including anorexia, colic and impaired locomotion, especially in the rear legs.

2.3.6 Red mould disease (Akakabi byo)

In Japan, a number of toxicoses in animals have been attributed to trichothecene poisoning. The toxicosis known as red-mould disease is characterised in man by nausea, vomiting, diarrhoea and headache, feed refusal in horses and haemorrhage of various organs in other animals (Ueno, Ishikawa, Nakajima, Sakai, Ishii, Tsunoda, Saito, Enomoto, Ohtsubo & Umeda, 1971). This toxicosis is caused by the ingestion of cereal crops infected with toxin-producing species of *Fusarium* (Saito & Tatsuno, 1971) and seems to be closely associated with heavy rain and cold weather prevalent in the regions of the Japanese islands facing the Pacific ocean (Ueno *et al.*, 1971).

Several red-mould toxicoses in humans have occurred since 1946 with over 500 people being affected. Also more than 2 500 animals have been affected by similar toxicoses since 1918. However, it wasn't until 1970 that the toxic principle involved was isolated. Two toxins were isolated and identified as deoxynivalenol and nivalenol (Yoshizawa & Morooka, 1977). Since 1970, further studies have shown the isolation of the same toxins from over 100 wheat and barley samples in Japan and although these toxins have yet to be isolated from grain associated with red-mould disease, the implications are that the nivalenol and deoxynivalenol were responsible for red-mould poisoning in animals and man. This toxicosis has not occurred since analytical techniques for the determination of the toxic factors have been developed.

2.3.7 Feed refusal and emesis in swine

The deoxynivalenol isolated in Japan by Morooka in 1971 was found to be identical with deoxynivalenol isolated from a naturally infected corn sample in the USA in 1972 (Forsyth, Yoshizawa, Morooka & Tuite, 1977). This sample of corn had been responsible for feed refusal and emesis in swine. Since then, deoxynivalenol (vomitoxin) has been commonly found in feeds associated with this condition and in further studies, corn infected with *Gibberella zeae* and containing vomitoxin was shown to produce reduced feed intake, poor weight gain, vomiting and diarrhoea (Vesonder, Ciegler & Jensen, 1973). However it has been shown by Forsyth (1974) that feed refusal effects are greater in naturally contaminated corn than with pure vomitoxin fed at the same levels as those found in the corn, suggesting that another factor or factors may also be present.

Other trichothecenes including T–2 toxin (Ellison & Kotsonis, 1973), fusarenone-X and neosolaniol (Ueno *et al.*, 1971; Ueno, Ueno, Litoi, Tsunoda, Enomoto & Ohtsubo, 1971) have also be shown to cause emesis in various test animals, but not in swine.

However in 1977, Vesonder, Ciegler & Jensen showed emesis in swine similar to that caused by feeding corn inoculated with *F. culmorum*, *F. poae* and *F. tricinctum* and containing T–2 toxin, HT–2 toxin, Acetyl T–2 toxin, fusarenone-X and deoxynivalenol.

It would seem therefore that trichothecenes other than deoxynivalenol can cause refusal of feed by swine but that deoxynivalenol is the major factor.

2.4 CHEMISTRY OF TRICHOTHECENE TOXINS

As previously mentioned (Section 2.1) the name trichothecane (FIG. 1) for the ring system common to all trichothecenes was proposed by Gotfredsen *et al.* (1967). Most naturally occurring trichothecenes contain an epoxide ring at C–12, 13 and a double bond at C–9, 10 and are therefore characterised as 12, 13-epoxytrichothec-9-enes (FIG. 2).

The trichothecenes can be divided into two main groups, the macrocyclic and non-macrocyclic trichothecenes. Ueno (1977b) recognised four groups of trichothecenes in which groups A (FIG. 3) and B (FIG. 4) are represented by the non-macrocylic trichothecenes, the macrocyclic compounds represent group D (FIG. 5) and group C is comprised of compounds with a second epoxide function at the C–7,8 position (FIG. 6).

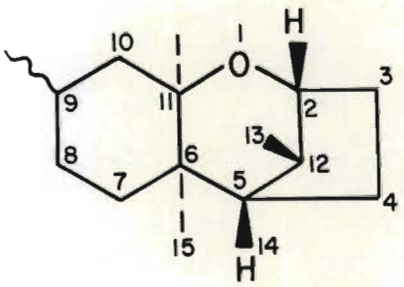


FIG. 1 Structure and numbering system of trichothecane

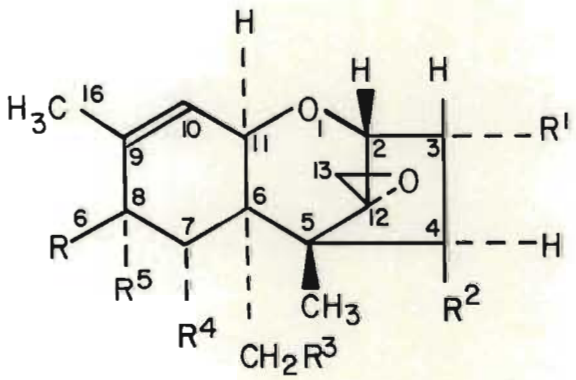
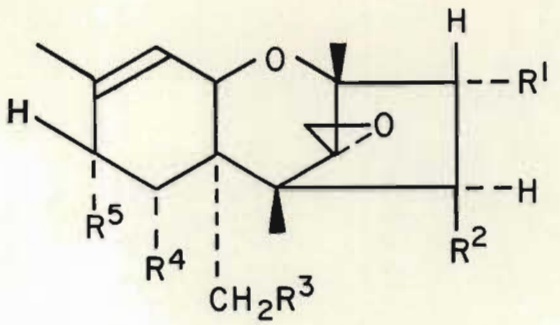
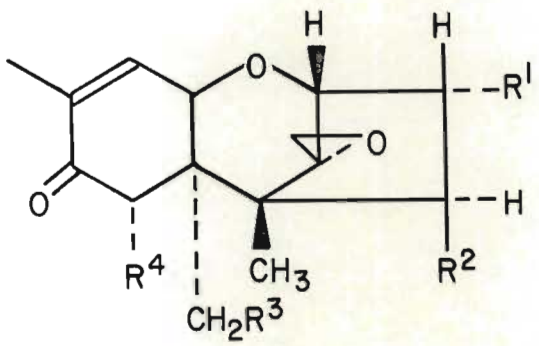


FIG. 2 Structure and numbering system of trichothecene (12,13-epoxytrichothec-9-ene)



Compound	R^1	R^2	R^3	R^4	R^5
Acetyl T-2 toxin	$OCOCH_3$	$OCOCH_3$	$OCOCH_3$	H	$OCOCH_2CH(CH_3)_2$
T-2 toxin	OH	$OCOCH_3$	$OCOCH_3$	H	$OCOCH_2CH(CH_3)_2$
HT-2 toxin	OH	OH	$OCOCH_3$	H	$OCOCH_2CH(CH_3)_2$
T-2 triol	OH	OH	OH	H	$OCOCH_2CH(CH_3)_2$
T-2 tetraol	OH	OH	OH	H	OH
Neosolaniol	OH	$OCOCH_3$	$OCOCH_3$	H	OH
Diacetoxyscirpenol	OH	$OCOCH_3$	$OCOCH_3$	H	H
Monoacetoxyscirpenol	OH	OH	$OCOCH_3$	H	H
Scirpentriol	OH	OH	OH	H	H
Roridin C	H	OH	OH	H	H

FIG. 3. Structures of some group A trichothecenes (after Ueno, 1977b)



COMPOUND	R^1	R^2	R^3	R^4
Nivalenol	OH	OH	OH	OH
Monoacetylnivalenol	OH	$OCOCH_3$	OH	OH
Deoxynivalenol	OH	H	OH	OH
Trichothecin	H	$OCOCH=CHCH_3$	H	H

FIG. 4 Structures of some type B trichothecenes (after Ueno, 1977 b)

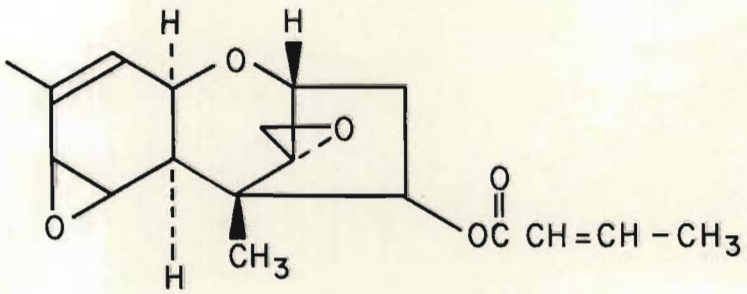
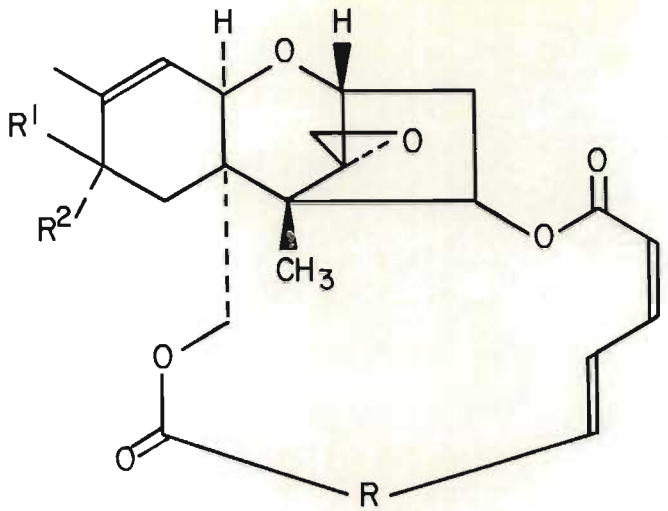


FIG. 5. Structure of crotocin, a group C trichothecene (after Ueno, 1977b)



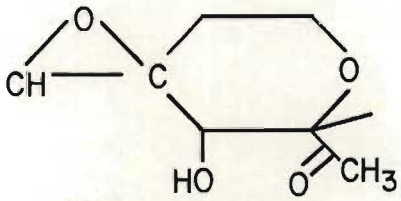
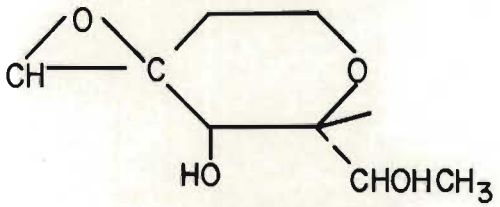
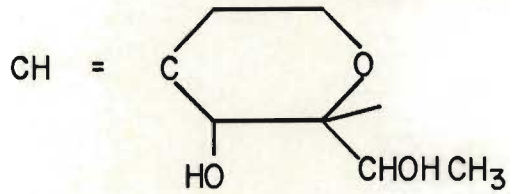
COMPOUND		-R-	R^1	R^2
Verrucarin	A	$-CHOHCHCH_3CH_2CH_2OC(=O)-$	H	H
Verrucarin	J			
Verrucarin	L	$-CH=CCH_3CH_2CH_2OC(=O)-$	H	OH
Roridin	A	$-CHOHCHCH_3CH_2CH_2OCHCHOHCH_3$	H	H
Roridin	E	$-CH=CCH_3CH_2CH_2OCHCHOHCH_3$	H	H
Satratoxin	F		H	H
Satratoxin	G		H	H
Satratoxin	H		H	H

FIG. 6 Structures of some group D trichothecenes (after Ueno, 1977b)

The trichothecenes are usually colourless crystalline compounds and are chemically stable. Pathre & Mirocha (1977) have divided the trichothecenes into two groups based on their solubility. Group A toxins are most soluble in aprotic solvents such as chloroform, ethyl acetate, diethyl ether and acetone and include most of the trichothecenes with ester and hydroxyl functions (e.g. T–2 toxin, neosolaniol and diacetoxyscirpenol). Group B compounds are more soluble in polar solvents such as water, alcohols and acetonitrile. Their chemical reactions have been studied extensively by a number of authors including Dawkins & Grove (1970) and Müller & Tamm (1975).

The feature of greatest interest in the trichothecene structure is the epoxide group at position C12, 13 (FIG. 2) since this group is important not only in the cytotoxicity of trichothecenes but also for the induction of vomiting (Sato & Ueno, 1977).

It has been shown that the epoxide function can be cleaved under highly acidic and alkaline conditions. Prolonged boiling in water produces a new bond between C–10 and C–13 as well as cleaving the epoxide function (Ishii, 1983) (FIG. 7). However the epoxide ring remains intact after treatment with CrO_3, $Pb(OAc)_4$ and MnO_2 (Ishii, 1983). These results therefore indicate that the epoxide ring is fairly stable and can be cleaved by chemical means only under extreme conditions.

The tetracyclic skeleton of the trichothecenes is recognised by signals in ^{1}H–NMR spectra including the AB quartet at about δ3 with J_{ab} = 4 H due to the 12, 13 spiro-epoxy protons. The ^{1}H–NMR spectra of some naturally occurring trichothecenes have been reviewed by Bamburg & Strong (1971) while ^{13}C–NMR spectra have been reviewed by Cox & Cole (1983).

2.5 TRICHOTHECENE PRODUCTION

All of the fungi known to produce trichothecenes are members of the order Hyphomycetales in the Fungi Imperfecti. (Smalley & Strong, 1974). The majority of the toxin producers belong in the family Moniliaceae although the genera *Myrothecium* and *Stachybotrys* are members of the family Dematiaceae. (Smalley & Strong, 1974). The occurrence and distribution of trichothecene-producing fungi has been discussed in Section 2.2.

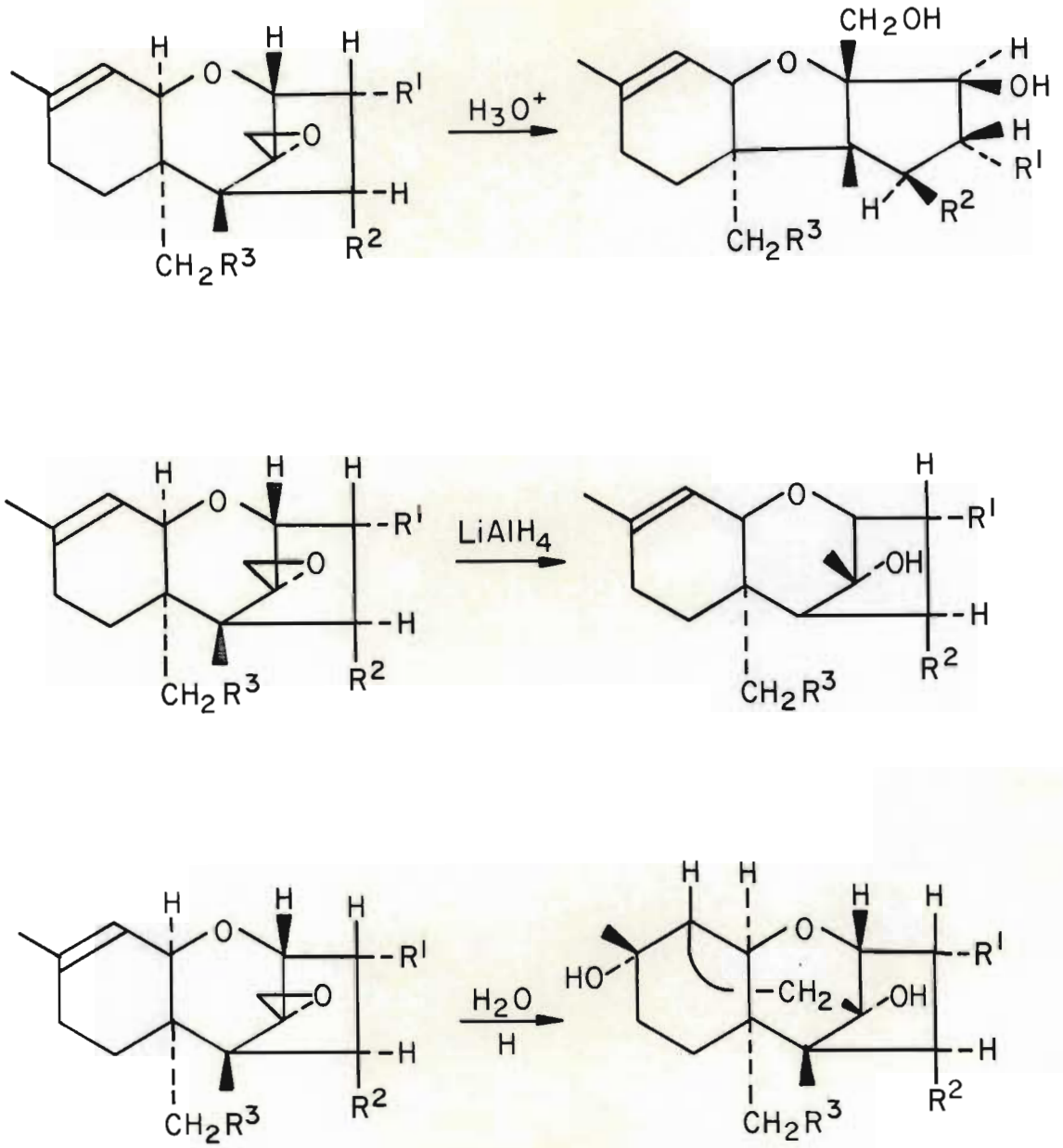


FIG. 7. Removal of the epoxide group of trichothecene under acid, alkali and hydrated conditions (Ishii, 1983)

Trichothecene production in culture

Production of macrocyclic esters

There has been little work done on the optimisation of conditions for the production of macrocyclic trichothecenes. The two major fungi used for the production of these compounds are *M. verrucaria* and *M. roridum* which in culture produce verrucarins A and B and roridin A respectively. (Traxler & Tamm; Tamm; cited by Smalley & Strong, 1974). Roridin E is produced by toxic strains of *S. atra* (Rodricks & Eppley, 1974).

Production of tetracyclic trichothecenes

Much more attention has been given to the strain differences and conditions necessary for the optimisation of production of the tetracyclic trichothecenes which are produced almost exclusively by species of *Fusarium*. The toxins produced by a number of these fungi are shown in TABLE 2.

TABLE 2 **Trichothecene production by some species of *Fusarium***

Toxin	Fungus	Reference
T–2 toxin	*F. tricinctum*) *T. viride*)	Bamburg & Strong (1971)
	F. roseum) *F. lateritium*)	Burmeister, Ellis & Hesseltine (1972)
T–2 toxin, HT–2 toxin, neosolaniol	*F. sporotrichioides*	Scott, Harwig & Blanchfield (1980)
Diacetoxyscirpenol	*F. tricinctum* *F. lateritium*	Bamburg & Strong (1971) Cole & Robinson (1972)
Neosolaniol	*F. roseum*) *F. solani*) *F. tricinctum*) *F. rigidiusculum*)	Ueno, Sato, Ishii, Sakai, Tsunoda & Enomoto (1973)
Deoxynivalenol	*F. graminearum*) *F. roseum*)	Vesonder, Ellis, Kwolek & De Marini (1982)

A number of workers including Bamburg, Riggs & Strong (1968) and Burmeister (1971) have isolated trichothecenes from toxin-producing fungi when grown on autoclaved cereals as substrate. Incubations were conducted at temperatures of 8–12°C and over a 4 to 6 week period.

In liquid media such as Gregory medium (Gregory, Allen, Richer & Peterson, 1952) or peptone-supplemented Czapeks-Dox medium (Ueno, Sawano & Ishii, 1975) yields of toxin are generally low (Smalley & Strong, 1974).

More recently, the use of media absorbed on vermiculite has resulted in high levels of toxin production and has shown toxin purification to be more easily performed than when using media such as autoclaved cereals. Using such a technique, Cullen, Smalley & Caldwell (1982) showed maximum T–2 toxin production after incubation at 19°C for 24 days on modified Gregory medium (2% soya meal, 0,5% corn steep liquor, 10% glucose) absorbed on vermiculite. Using *F. tricinctum* T–340 they were able to obtain T–2 toxin levels of 714 mg/ℓ.

The production of T–2 toxin by*F. acuminatum* grown on maize meal has been shown to be increased by the addition of sorbic acid (Gareis, Bauer, von Montgelas & Gedek, 1984). The use of sorbic acid may therefore be able to further increase production of T–2 toxin on vermiculite media such as that used by Cullen *et al.* (1982).

The effect of temperature on the production of trichothecenes has been reviewed by Smalley & Strong (1974).

2.6 TRICHOTHECENE TOXICITY

The topic of trichothecene toxicity is extensive and a number of reviews on the various aspects have been published (Bamburg & Strong, 1971; Bamburg, 1972; Sato & Ueno, 1977; Ueno, 1983; Mirocha, 1983; Ohtsubo, 1983).

In general all the trichothecenes possess similar biological activity, although the severity of their effects varies from toxin to toxin and is dependant on chemical structure. (Ueno, 1983). They have been shown to be toxic to plants, animals and in some cases bacteria. They can affect the central nervous system, the immune response system and inhibit protein and DNA synthesis. These aspects will be discussed later.

2.6.1 Antibiotic activity

The trichothecenes generally have little effect on bacteria. T–2 toxin for instance, showed no growth inhibition of *Escherichia coli, Staphylococcus aureus* and *Bacillus subtilis* at concentrations below 1 mg/ml (Bamburg & Strong, 1971). Similar results have been obtained for trichothecin, diacetoxyscirpenol and crotocin, amongst others. (Bamburg &

Strong, 1971). However, although inactive against gram-positive bacteria, verrucarin A was shown to cause slight inhibition of growth of the gram-negative *E. coli, Serratia marcescens* and *Protea mirabilis* at concentrations of 50 μg/ml (Horn *et al.*, cited by Bamburg & Strong, 1971).

Bamburg & Strong (1971) have reviewed the toxin effects of trichothecenes on fungi and viruses.

2.6.2 Phytotoxic effects

As described in Section 2.2, many trichothecene-producing fungi are opportunistic plant pathogens, an observation which has prompted investigations into their toxicity to plants. Trichothecin, T–2 toxin and diacetoxyscirpenol have all been shown to be toxic to various plants. This work has been reviewed by Bamburg & Strong (1971).

2.6.3 Animal toxicity

The acute toxicity of trichothecenes is, in general, highest with type D compounds (FIG. 6) with the toxicity of the other groups decreasing in the order type A and type B (Ueno, 1983). However the essential toxic feature of trichothecenes is the 12, 13 epoxide group of the trichothecane nucleus (FIG. 1), for it has been shown that removal of this group from diacetoxyscirpenol almost completely eliminated cytotoxicity to HeLa and hamster kidney cells (Grove & Mortimer, 1969). Similar trends were also shown by Bamburg (cited by Bamburg & Strong, 1971) and Sato & Ueno (1977) presented evidence that the epoxide function is also important in the induction of vomiting. Grove & Mortimer (1969) were also able to show that reduction of the 9, 10 double bond consistently produced lowered cytotoxicity thereby demonstrating that additional molecular features affect the degree of toxicity of a particular compound. Further studies by other workers including Ueno, Nakajima, Sakai, Ishii, Sato & Shimoda (1973), Ueno (1983) as well as Grove and Mortimer (1969) suggest that increasing lipophilic nature afforded by substitution of hydroxyl groups with acetyl groups tends to increase toxicity and that the substituent at C–15 greatly influences the lethal toxicity. However, the observation that tetra-acetylnivalenol is not inhibitory to protein synthesis whereas deacetylated derivatives are inhibitory (Ueno *et al.*, 1973) suggests that the lipophilic nature of the toxin *per se* is not the sole factor controlling toxic response. The situation is further complicated with the observation by Grove & Mortimer (1969) that although scirpentriol and T–2 tetraol (FIG.3) were more water-soluble than their respective diol derivatives they were nonetheless toxic. In these investigations however different assay systems were used and different

types of toxicity monitored and these would probably explain inconsistencies in results. Details of the relative lethal toxicities of some trichothecenes are presented in TABLE 3.

TABLE 3 **Toxicity, Cytotoxicity and Protein Inhibition Effects of Trichothecenes (after Ueno, 1983)**

Type	Trichothecene	Mouse LD_{50} mg/kg Adult I.P.)	Cytotoxicity µg/ml HeLa	Inhibition of protein synthesis µg/ml	Type[a]
A	3-Acetyl T–2 toxin		1.0		
	T–2 toxin	5.2	0.01	0.03	I
	HT–2 toxin	9.2	0.01	0.03	I
	Diacetoxyscirpenol	23.0	0.01	0.03	I
	Neosolaniol		0.1	0.25	
	Verrucarol				ET
B	Nivalenol	4.1	0.3	3.0	I
	Fusarenone–X	3.4	0.1	0.25	I
	Deoxynivalenol	70.0	1.0	2.0	ET
C	Crotocin	810	0.5	1	ET
D	Roridin A	0.5	0.003	0.01	I
	Verrucarin A	0.5	0.005	0.01	I

a) I – Inhibition of initiation step in protein synthesis
ET – Inhibition of elongation – termination step in protein synthesis

The effect of T–2 toxin in cattle is described in Section 5.2 and Mirocha (1983) has described the effect of this toxin in poultry and swine. Diarrhoea is one of the characteristic responses in animals receiving sub-lethal doses (Ueno, 1983) and emesis has been shown in cats, dogs and ducklings (Ueno *et al.*, 1971a). Common symptoms of acute intoxication in experimental animals are diarrhoea, nausea, vomiting and decreases in spontaneous movement and body temperature (Ueno, 1983). Histopathological changes include marked damage to actively dividing cells of the gastrointestinal tract, spleen, thymus and other organs (Saito, Enomoto & Tatsuno, 1969).

2.6.3.1 Dermal toxicity

Brian & McGowan (1946) were the first to report dermal irritation by extracts of trichothecene-producing fungi. Later, the fungi responsible for causing ATA were also shown to possess dermal toxicity (Joffe, 1962). Since then many of the trichothecene toxins have been tested and the dermal highest toxicity has been observed in the macrocyclic trichothecenes (Type D) followed by types A and B in decreasing order.

2.6.3.2 Haematological effects

In T–2 toxin-associated toxicoses of cattle, swine and poultry, leukopeania and thymic involution have been reported in affected animals (Bamburg, Strong & Smalley, 1969). Also, a dose-dependent depression of cell-mediated immune response by T–2 toxin has been reported in mice (Rosenstein, LaFarge-Frayssinet & Lespinats, 1979) and calves (Buening, Mann, Hook & Osweiler, 1982). Depression of total protein, albumin and globin as well as 1 g G and lgM values by T–2 toxin in calves has been shown by Mann, Buening, Hook & Osweiler (1983), while Gentry & Cooper (1983) showed depression, by T–2 toxin, in plasma activity of factors VII, IX, X and XI and in fibrinogen values in calves. Aleukia has been caused in experimental animals by a number of different trichothecenes including T–2 toxin, diacetoxyscirpenol and Verrucarin A. This work has been reviewed by Ueno (1983).

2.6.3.3 Central nervous system disorders

Effects of trichothecenes on the central nervous system of animals are characterised by vomiting. In work with Fusarenone-X (Matsuoka, Kubota & Ueno, cited by Ueno, 1983), results suggested that Fusarenone-X stimulated the chemoreceptor trigger zone in the *medulla oblongata.* In poultry, neural disturbances in trichothecene-treated birds were characterised by abnormal positioning of the wings, hysteroid seizures and impaired righting reflex, presumed to be caused by modification of biogenic amines in the brain (Wyatt, Caldwell, Hamilton & Burmeister; Chi, Mirocha, Kurtz, Weaver, Bates & Shimoda; cited by Ueno, 1983).

2.6.3.4 Genotoxicity

Teratogenic effects in mice have been observed after intraperitoneal administration of T–2 toxin to mice (Stanford, Hood & Hayes, 1975). However, no teratogenicity was observed upon subcutaneous administration of Fusarenone-X (Ito, Ohtsubo & Saito, 1980) nor upon *per os* administration of deoxynivalenol to rats (Morrissey & Vesonder, 1985). Variations in test animals, toxin and administration route prevent thorough evaluation of teratogenic effects of trichothecenes.

There have been only two reports of positive carcinogenic effects of trichothecenes (Schoental, Joffe & Yagen, 1979; Saito, Horiuchi, Ohtsubo, Hatanaka & Ueno, 1980) and in many other investigations, reviewed by Ohtsubo (1983) carcinogenicity has not been shown. Ohtsubo (1983) has emphasised the need for use of pathogen-free environments when conducting such experiments to eliminate the possible effect of secondary infections.

Assays such as the *Rec* assay and the Ames test have also shown Fusarenone-X and T–2 toxin to be non-carcinogenic (Ueno & Kubota, cited by Ueno (1983); Wehner, Marasas & Thiel, 1978). The bulk of evidence therefore suggests that the trichothecenes tested so far are not carcinogenic although LaFarge-Frayssinet, Decloitre, Mousset, Martin & Frayssinet (1981) have emphasised the problems of short-term tests for screening of chemical carcinogens.

2.6.3.5 Cytotoxicity and inhibition of protein and DNA synthesis

The trichothecenes are cytotoxic to eukaryotic cells and their biological activity is closely related to their lethal toxicity to whole animals, dermal toxicity, impairment of immuno-responses and inhibition of macromolecule synthesis (Ueno, 1983). Cytotoxic effects have been shown against HEp2, BHK and HeLa cell lines (Reviewed by Ueno, 1983) and in experiments with HeLa cells, cytotoxicity of the trichothecenes was shown to decrease in the order Type D, Type A and Type B.

In 1968, Ueno, Hosoya, Morita, Ueno & Tatsuno reported that nivalenol inhibited protein synthesis in cultured cells and in cell-free protein-synthesising systems. The site of inhibition was determined to be at the ribosomal level since nivalenol had no effect on the activation reaction for amino acids catalysed by aminoacyl-*t* RNA synthetases. As a result of the different modes of action of individual trichothecenes on polyribosomal behaviour, they were grouped into two types. The first type inhibits the initial step of protein synthesis (I-type) and the second type inhibits the elongation-termination step (ET-type). In experiments with trichodermin, Wei, Campbell, McLaughlin & Vaughan (1974) were able to show binding of this toxin to the 60 S subunit in eukaryotic ribosomes and preferential inhibition of the termination step of protein synthesis. In later work with trichodermin-resistant strains of yeast it was shown that the target for trichodermin was protein L3 of the 60 S ribosomal sub-unit (Fried & Warner, 1981).

Trichothecenes have also been shown to inhibit DNA and RNA synthesis in whole cells in a dose-dependant manner (Ueno & Fukushima, 1968). This work and later developments have been reviewed by Ueno (1983).

The trichothecenes may also exhibit cytotoxic action by binding to thiol enzymes, for work by Ueno & Matsumoto (1975) showed that fusarenone-X caused *in vitro* inactivation of the thiol-containing enzymes creatine phosphokinase, lactate dehydrogenase and alcohol dehydrogenase.

Thus the trichothecenes can interfere with a number of biochemical reactions,especially the inhibition of protein synthesis,within the cell resulting in their overall cytotoxicity. The toxic, cytotoxic and biochemical properties of a number of trichothecenes are shown in TABLE 3.

2.7 TRICHOTHECENE METABOLISM

Metabolic studies on trichothecenes have focused mainly on the metabolism of these toxins in whole animal studies and by liver preparations.

2.7.1 Animal studies

Yoshizawa, Mirocha, Behrens & Swanson (1981) studied the distribution, excretion and metabolism of tritiated T–2 toxin in a lactating dairy cow. After 72 h most of the radioactivity was eliminated in the urine and faeces and approximately 0.2% of the total dose administered was detected in the milk. The added T–2 toxin was almost completely metabolised to other products including neosolaniol, HT–2 toxin and 4-deacetylneosolaniol. Three major unkown metabolites (designated TC–1, TC–3 and TC–6) accounted for most of the products of T–2 metabolism. TC–1 and TC–3 were later identified as 3-hydroxy T–2 toxin and 3'-hydroxy HT–2 toxin (Yoshizawa, Sakamoto, Ayano & Mirocha, 1982). Yoshizawa *et al.* (1982) proposed that these major metabolites were formed by a liver enzyme system, having shown that none of these were found in the rumen content of cows receiving T–2 toxin daily for four consecutive days. They concluded that T–2 toxin was rapidly metabolised by the cow into relatively non-toxic components and that the total residue in edible tissues and milk was negligible.

In studies with mice and rats, Matsumoto, Ito & Ueno (1978) showed that orally administered T–2 toxin was rapidly eliminated in the faeces and urine and was partially metabolised in rats to HT–2 toxin, neosolaniol and three unidentified metabolites. Previous work (Ohta, Matsumoto, Ishii & Ueno, 1978) had shown the selective hydrolysis of the C–4 acetyl residue of trichothecenes including T–2 toxin by a microsomal esterase under *in vitro* conditions,to produce HT–2 toxin. The detection of HT–2 toxin in the later *in vivo* study confirmed the possible role of such an enzyme. Matsumoto *et al.* (1978) also proposed the presence of an esterase in dermal microflora to account for the production of neosolaniol.

The *in vitro* metabolism of T–2 toxin by a rat liver homogenate was shown by Yoshizawa, Swanson & Mirocha (1980) to produce HT–2 toxin, T–2 tetraol, 4-deacetylneosolaniol and an unidentified metabolite. The same metabolites were also produced

when using HT–2 toxin as a substrate. Thus in contrast to the work of Ohta *et al.* (1978) metabolites other than HT–2 were detected. Yoshizawa *et al.* (1980) concluded that in the rat liver *in vitro* system, T–2 toxin is deacetylated preferentially at the C–4 position to give HT–2 toxin which is then converted in a stepwise manner to T–2 tetraol via 4-deacetylneosolaniol.

In *in vitro* studies with liver homogenates of mice and monkeys, Yoshizawa, Sakamoto & Okamoto (1984) showed that metabolism of T–2 toxin to HT–2 toxin, neosolaniol, 4-deacetylneosolaniol, 15-deacetylneosolaniol, T–2 tetraol, 3′-hydroxy T–2 toxin and 3′-hydroxy HT–2 toxin occurred. Because the 3′-hydroxy derivatives of T–2 toxin and HT–2 toxin are more toxic than the parent compound (Yoshizawa *et al.*, 1982) they proposed that the susceptibility of animals to T–2 toxin is dependent on the rates of hydrolysis and hydroxylation in the metabolic interconversion of T–2 toxin metabolites.

2.7.2 Bacterial studies

The metabolism of trichothecenes by rumen micro-organisms is discussed in Section 5.3. Apart from these studies, little work has been done on bacterial modification of this group of toxins. However, Ueno, Nakayama, Ishii, Tashiro, Minoda, Omori & Komagata (1983) demonstrated the conversion of T–2 toxin to HT–2 toxin and T–2 triol by a species of *Curtobacterium* when T–2 toxin was used as a sole carbon and energy source. The T–2 toxin hydrolysing enzymes were found both intracellularly and in the culture filtrate. However activity of the extracellular enzymes was rapidly lost on storage. In the same study it was also shown that diacetoxyscirpenol, neosolaniol and fusarenone-X could also be assimilated by *Curtobacterium sp.* and that these were transformed to scirpentriol, T–2 tetraol and nivalenol respectively.

2.8 OVERVIEW OF TRICHOTHECENE TOXICOSES IN RUMINANTS

One of the major trichothecene toxicoses to affect ruminants is stachybotryotoxicosis which has been demonstrated in sheep and cattle on a number of occasions. The effects of this toxicosis have been previously described (Section 2.3.4).

The trichothecene, T–2 toxin has also been reported to cause a toxicosis in cattle which is usually associated with haemorrhaging. The first report of such a toxicosis was by Hsu *et al.* (1972) who demonstrated the presence of T–2 toxin at levels of 2 mg/kg in

mouldy corn associated with haemorrhaging and death in 20% of a dairy herd in Wisconsin, USA. Later in 1974, Mirocha Schauerhamer & Pathre found 38 μg/kg of T–2 toxin in a commercially prepared cattle feed associated with poor weight gain and in some cases death by haemorrhaging. Also in 1974, Hibbs *et al.* found over 10 μg/kg of an unspecified trichothecene in cattle feed also associated with death due to haemorrhage. In this outbreak the affected animals also showed a decreased platelet count and increased prothrombin time. In another case in which trichothecene analysis was not performed but in which there were high levels of the trichothecene-producing fungus *F. tricinctum* in silage fed to calves, 2% of the animals died of uncontrollable haemorrhage following dehorning or castration (Dahlgren & Williams, 1972). Plasma samples from these animals were deficient in clotting factor VII, a blood clotting factor dependent on vitamin K and it has been postulated by Ribelin (1977) that the known synthesis of vitamin K by rumino-intestinal organisms is inhibited by the trichothecenes.

In experimental studies with cattle, Kosuri, Grove, Yates, Tallent, Ellis, Wolf & Nichols (1970) produced haemorrhages at various sites in the animal after intramuscular injection of T–2 toxin. However, Pier, Cysewski, Richard, Baetz & Mitchell (1976) could not produce extensive haemorrhaging after oral dosage with T–2 toxin. Patterson, Matthews, Shreeve, Roberts, McDonald & Hayes (1979) have also concluded that dietary T–2 toxin does not cause haemorrhaging in calves and pigs.

It is possible that differences in results in the above experiments are due to toxin levels used and route of administration. In this regard Kurmanov (1977) showed that cattle receiving a daily ration of *F. sporotrichiodes* at 200 mg/kg body weight and sheep the same but at a level of 500 mg/kg body weight showed no clinical symptoms of fusariotoxicoses. However, if the rations were increased to 500 mg/kg body weight in cattle and 1 g/kg body weight in sheep, chronic symptoms developed. During this investigation, it was noted that sheep and calves were selective in their feeding of *Fusarium*-contaminated feed while on a voluntary feeding regime and that as a result it was difficult to follow the development of clinical symptoms.

In attempt to elucidate the role of rumen digestion on the development of fusariotoxicosis, experiments were conducted on sheep fistulated in the lower part of the abomasum and through which a culture of *F. sporotrichioides* was introduced at a rate of 500 mg/kg body weight per day. The treatment caused fusariotoxicosis in the animal within 30 min while an animal fed the same diet *per os* for a period of six days remained clinically healthy. After 5 months, the same diet was introduced into the

abomasum of the control animal and clinical symptoms of fusariotoxicosis developed within 30 min. In these investigations, lambs were shown to be more sensitive than sheep when fed *per os*, while both lambs and sheep were highly sensitive to *F. sporotrichioides* when administered directly into the abomasum or by injection under the skin. Kurmanov concluded that "the relative resistance of cattle to toxic compounds of *Fusarium*, in our opinion, is the result of their dependence on the complex rumen digestion which results in a certain degree of detoxification." Because lambs only have a poorly developed rumen, this would also explain the greater sensitivity of lambs when fed *per os*.

Ismailov (cited by Kurmanov, 1977) was unable to cause fusariotoxicosis in cows fed a highly toxic ration of *F. sporotrichioides* on a daily basis. These results also agree with those of Sarkisov (cited by Kurmanov, 1977) and with Hsu *et al.* (1972) who found T–2 toxin to be highly toxic to calves with non-functional rumens while only transitory illness was produced in cattle at levels of 0,37 mg T–2 toxin/kg body weight.

It is also interesting to note the effects of *Stachybotrys* and *Fusarium* toxins increase in feed at low pH values. Pavlov, Dimitrov, Stankoushev and Surtmadjiev (cited by Hintikka, 1977d) showed that sheep fed a toxic culture of *Stachybotrys* in meadow hay and root plants, failed to produce clinical symptoms, but when silage was included in the diet then stachybotryotoxicosis developed. Toxicosis did not occur when alkali was added to the diet. Kurmanov (1977) also showed that cattle and sheep were more susceptible to toxicoses caused by ingestion of *Fusarium* species when fed in diets at low pH values. In these experiments, cattle fed cultures of *F. sporotrichiella* var. *poae* at a dose of 500 mg/kg body weight in silage at pH 3,5 showed symptoms of toxicosis after 20 days, while those fed a combination of forage and beets containing the same level of fungus did not react clinically after a period of 42 days. A similar experiment was conducted on calves and showed that those on a low pH diet developed fusariotoxicosis on the fifth to sixth day while those fed on a normal diet showed fusariotoxicoses on the eleventh to twelfth day. He concluded that "the role of acidified food in the development of fusariotoxicosis in cattle was more obvious than with calves."

CHAPTER 3

OVERVIEW OF TECHNIQUES FOR TRICHOTHECENE ANALYSIS

3.1 CHROMATOGRAPHIC TECHNIQUES

Because they allow the simultaneous detection of a number of toxins, chromatographic techniques are often preferred to bioassay techniques.

3.1.1 Thin-layer chromatography

Thin-layer chromatography (TLC) affords a rapid and simple method of trichothecene analysis and a number of different techniques have been developed. Sample clean-up prior to TLC is often required and after extraction and lipid removal, techniques such as multi-column chromatography (Suzuki, Kurisii, Hoshino, Ichinohe, Nose, Takumaru & Watanabe, cited by Takitani & Asabe, 1983), dialysis (Roberts & Patterson, 1975) and solvent partition (Gorst-Allman & Steyn, 1979) have been employed.

Because trichothecenes do not absorb under UV light, a number of different visualising agents have been used for their detection. These include sulphuric acid (Kotsonis & Ellison, 1975), *p*-anisaldehyde (Scott, Lawrence & Van Walbeek, 1970) nicotinamide 2-acetylpyridine (Sano, Asabe, Takitani & Ueno, 1982) and chromotropic acid (Baxter, Terhune & Qureshi, 1983). Using these reagents trichothecenes can be detected at levels between 25 ng per spot (Sano *et al.*, 1982) and 200 mg per spot (Scott *et al.*, 1970). Densitometric techniques can be used for more quantitative analysis when required (Kotsonis & Ellison, 1975).

3.1.2 High-performance liquid chromatography

Trichothecenes can be analysed using high-performance liquid chromatography (HPLC) combined with a RI detector. Sample derivatisation is not required with this technique and detection limits for T–2 toxin were 2 and 5 μg/g in rye and wheat respectively (Schmidt & Dose, 1984). However, only a small number of trichothecenes have been effectively determined using this technique.

3.1.3 Gas-liquid chromatography

Trichothecene determination by gas-liquid chromatography (GLC) has been shown to be a rapid, sensitive and quantitative technique which allows the simultaneous detection of

a relatively large number of trichothecenes. Scott (1982) has reviewed GLC techniques developed up to 1981, in which derivitisation by *N*-heptafluorobutyrylimidozale (HFBI) and analysis using an electron-capture detector were considered the methods of choice.

More recently, the introduction of capillary columns and bonded liquid phases has greatly enhanced sensitivity and resolution of GLC techniques.

Using a capillary column with SE 52 as liquid phase, Bata, Ványi, Lásztity & Galácz (1984) were able to resolve and quantitate crotocin, scirpentriol, deoxynivalenol, diacetoxyscirpenol, fusarenone-X, T–2 tetraol, HT–2 toxin, T–2 toxin and zearalenone within 26 mins, using Trisil-BT as derivitising agent and a flame-ionisation detector. The detection limits were between 3 and 5 ng per injection. Other liquid phases have been used including OV–1 for the detection of T–2 toxin in plasma (Swanson, Ramaswamy, Beasley, Buck & Burmeister, 1983), DB–5 for the detection of T–2 toxin, HT–2 toxin and diacetoxyscirpenol in cereal grains (Cohen & Lapointe, 1984) and SE–54 for the detection of deoxynivalenol in cereal grains (Cohen & Lapointe, 1982). A packed column with OV–17 as liquid phase has also recently been used for the effective determination of verrucarol, diacetoxyscirpenol, T–2 triol, HT–2 toxin and T–2 toxin as their trimethylsilyl derivatives (Gore, Rougereau & Person, 1984).

HFBI is the most commonly used derivatising agent and while trimenthylsilyl derivatives are also used in GLC analysis, Gilbert, Startin & Crews (1981) have pointed out the necessity of ensuring complete trimethylsilylation when using non-specific detection methods.

3.2 BIOASSAYS AND RADIOIMMUNOASSAYS

Initial bioassays for trichothecene toxins relied on the dermatitic properties of these compounds (Bamburg, Riggs & Strong, 1968; Gilgan, Smalley & Strong, 1966; Wei, Smalley & Strong, 1972). These assays were sensitive to levels of approximately 0.1 μg of toxin but were limited in their toxin specificity.

Other bioassays that have been developed include a protein synthesis inhibition assay (Thompson & Wannemacher Jr, 1984) growth inhibition of protozoal cultures and crustaceans (Bijl, Dive & Van Peteghem, 1981), refusal of toxins in drinking water by mice (Burmeister, Vesonder & Kwolek, 1980) and inhibition of pollen germination (Siriwardana & Lafont, 1978). Although in some cases these assays are sensitive to toxin levels as low as 10 ng/ml, identification of the toxic metabolite is not possible.

As a result of the development of a specific antibody to T–2 toxin it is now possible to use radioimmunoassay (RIA) techniques for T–2 toxin identification and quantitation (Lee & Chu, 1981; Petska, Lee, Lau & Chu, 1981). The sensitivity of such techniques is of the order of 1 ng/ml and with the exception of HT–2 toxin almost no cross-reaction is shown between the antibody and other trichothecene toxins (Chu, Grossman, Wei & Mirocha, 1979). However the titers of antisera to T–2 toxin raised in rabbits, necessary for these assays have been low and there are inherent problems in the standardisation of antisera. With this in mind, Hunter, Brimfield, Miller, Finkelman & Chu (1985) prepared and characterised two monoclonal antibodies to T–2 toxin and developed an assay sensitive to levels of 50 ng per assay. However, HT–2 toxin is also bound by the anti-T–2 toxin antibodies and this limits the use of these antibodies in specific quantitative work.

CHAPTER 4

DEVELOPMENT OF METHODS FOR ANALYSIS OF TRICHOTHECENES USING CHROMATOGRAPHIC TECHNIQUES

4.1 INTRODUCTION

Because GLC techniques facilitate rapid simultaneous analysis of a number of trichothecenes, this technique was adopted in all trichothecene analyses performed, except where otherwise stated.

4.2 METHODS AND MATERIALS

4.2.1 Chemicals

All solvents and reagents were of analytical grade.

Mycotoxin standards were purchased from Sigma Chemical Co., St Louis, MO. USA, except deoxynivalenol which was a generous gift from Dr. P.G. Thiel (MRC, Tygerberg, Cape Town).

N,O-Bistrimethylsilylacetamide was purchased from Merck (Darmstadt, W. Germany).

4.2.2 Gas-liquid chromatographic analysis of trichothecenes

Toxin standards and ethyl acetate extracts of trichothecene – containing samples were derivatised for GLC with N,O-Bistrimethylsilylacetamide (BSA).

Instrumentation

A Hewlett-Packard 5790 gas-liquid chromatograph equipped with flame-ionisation detector was used. The column (SGE, Ringwood, Victoria, Australia) was a capillary wall-coated open tubular (WCOT) column of fused silica with BP 10 as bonded liquid phase. A 3 m length (ID = 0,20 mm) was cut from an original 25 m column.

Operating conditions

Injector and detector temperatures were 280°C and 300°C respectively. Column pressure and the oven temperature programme were as follows:

Column pressure	5,0 p s i
F I D gas	H_2 at 60 ml/min
	Air at 240 ml/min
Septum purge	30 ml/min
Split ratio	10 : 1
Carrier gas (N_2)	60 ml/min

Oven temperature programme:

Initial temperature	210°C
Initial time	4,5 min
Rate	3,0°C/min
Final temperature	240°C
Final time	2,0 min

Preparation of calibration table

Known amounts of each toxin were dissolved in ethyl acetate and added to a screw cap $\frac{1}{2}$ dram vial (Supelco Inc., Bellefonte, PA, USA). Ethyl acetate was evaporated at 60°C under a stream of N_2 in a Pierce 'reactitherm' vessel (Pierce Chemical Co., Rockford, IL, USA). To the dried sample was added 100 μl BSA and the total contents allowed to react in a sealed vial for 1 h at 60°C. After derivatisation, BSA was evaporated under a stream of N_2 and 50 μl ethyl acetate added to the dried sample of which 1 μl amounts were used for injection onto the GLC.

Retention times and peak areas for each toxin were entered into a Hewlett Packard 3390 A integrator and average values obtained for each after injection of 10 samples containing a mixture of T–2 toxin, HT–2 toxin, T–2 triol. T–2 tetraol, verrucarol, deoxynivalenol, neosolaniol, diacetoxyscirpenol, zearalenone and acetyl T–2 toxin.

Trichothecene determination in test samples

Derivatisation of samples was carried out as described in 'Preparation of calibration table'. Samples (1 μl) were then injected onto the column (using a percent retention window of 0,03). This was repeated a further two times. Samples identified as containing trichothecenes were divided into two, to one of which was added 0,25 μg of the suspected toxin or toxins. An increase in peak area in the 'spiked' sample was regarded as positive identification of that particular toxin.

4.3 RESULTS AND DISCUSSION

The gas-liquid chromatographic separation of a standard mixture of trichothecenes and the fusarium toxin zearalenone, as described in 'Methods and Materials' is presented in FIG. 8. Relevant retention times and detection limits are presented in TABLE 4.

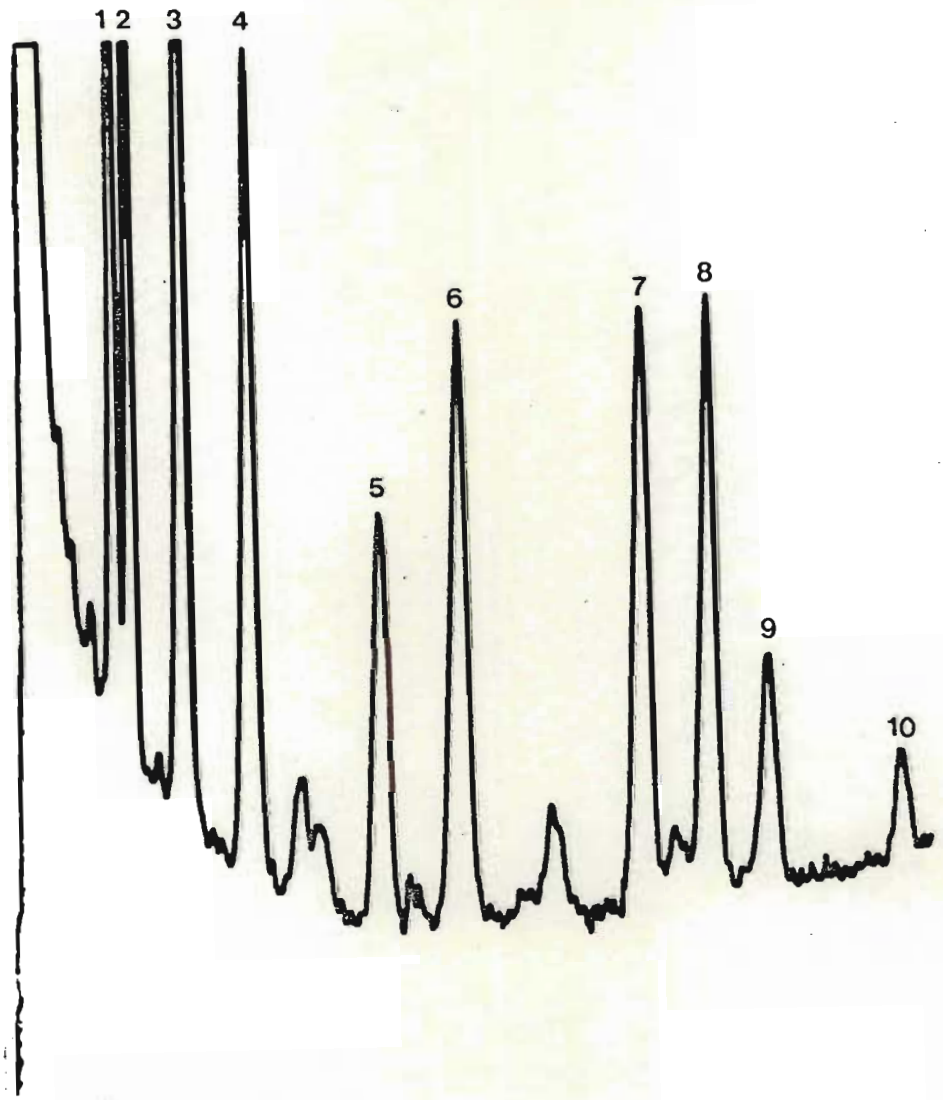
TABLE 4 **Retention times and detection limits of toxins in a standard mixture of trichothecene toxins**

Toxin	Retention Time (Min)	Detection Limit (ng)
Verrucarol	1,41	5
Deoxynivalenol	1,64	20
T–2 tetraol	2,39	10
Diacetoxyscirpenol	3,35	5
Neosolaniol	5,25	5
T–2 triol	6,39	10
HT–2 toxin	9,06	10
T–2 toxin	10,01	10
Zearalenone	10,89	20
Acetyl T–2 toxin	12,77	10

The chromatogram shows that all toxins in the mixture were sufficiently well separated to allow easy identification of the individual toxins and for the majority of toxins analised the detection limits compare favourably with values presented in current literature (Section 3.1.3). Further, because only 3 m of a 25 metre column was used to effect the separation, the effective 'lifetime' of the original column is considerably increased.

In a recent publication, Gilbert *et al.* (1981) have queried the use of BSA as a derivatising reagent for trichothecenes stating that derivatisation is incomplete if this reagent is used alone. However the use of strictly standardised conditions for derivatisation and quantitation by comparison with a standard mixture prepared under identical conditions allows correction for efficiency of derivatisation.

Chromatograms showing the identification of a number of different trichothecenes in ethyl acetate extracts of whole ovine rumen fluid are shown in FIGS.10–12. Because of the use of more than one section of column during the course of those studies, slight differences



1 – Verrucarol
2 – Deoxynivalenol
3 – T–2 tetraol
4 – Diacetoxyscirpenol
5 – Neosolaniol
6 – T–2 triol
7 – HT–2 toxin
8 – T–2 toxin
9 – Zearalenone
10 – Acetyl T–2 toxin

FIG. 8 Gas liquid chromatogram of standard mixture of trichothecene toxins. Operating conditions as described in Section 4.2.2. Signal attenuation = 2^3

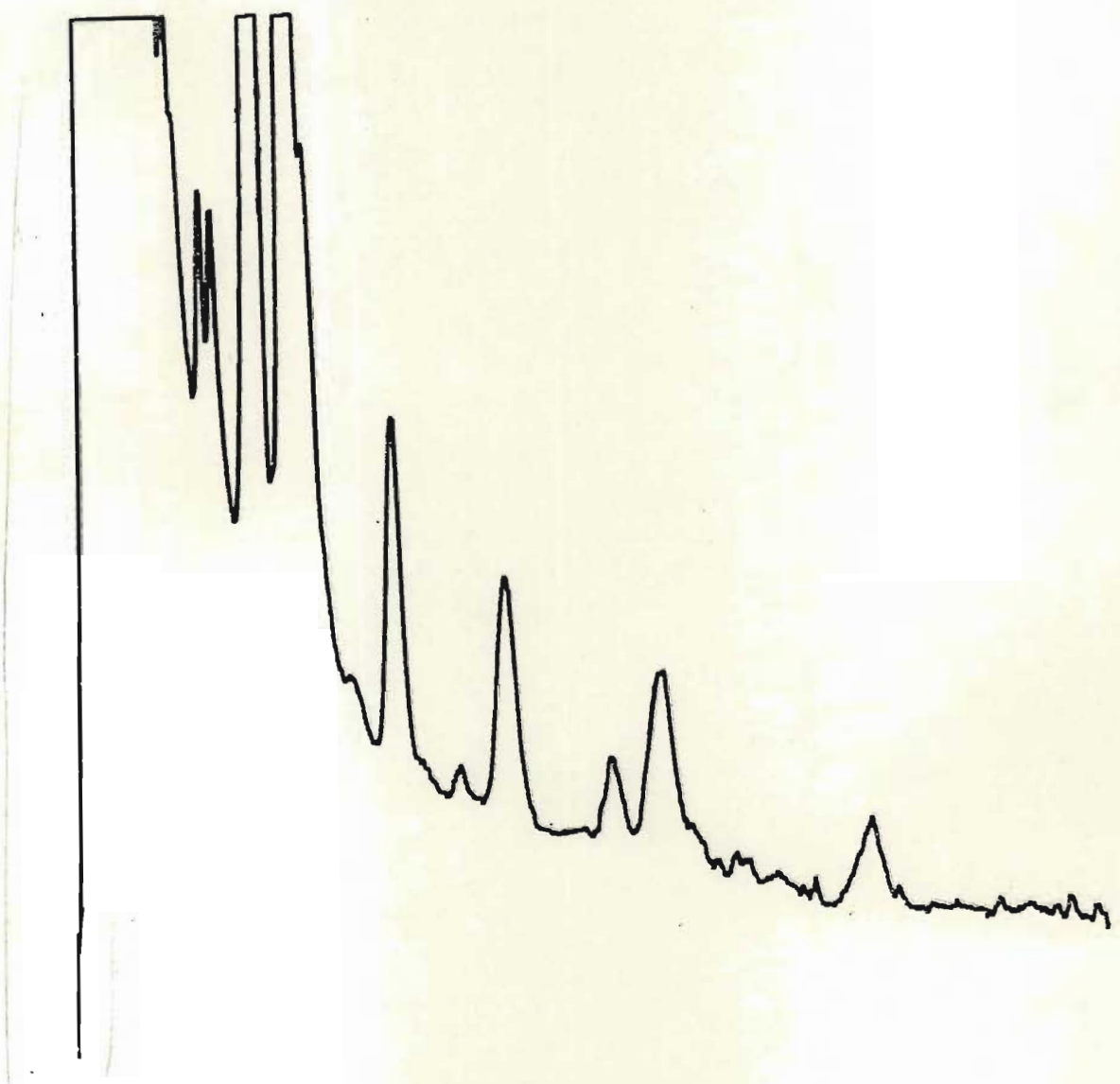
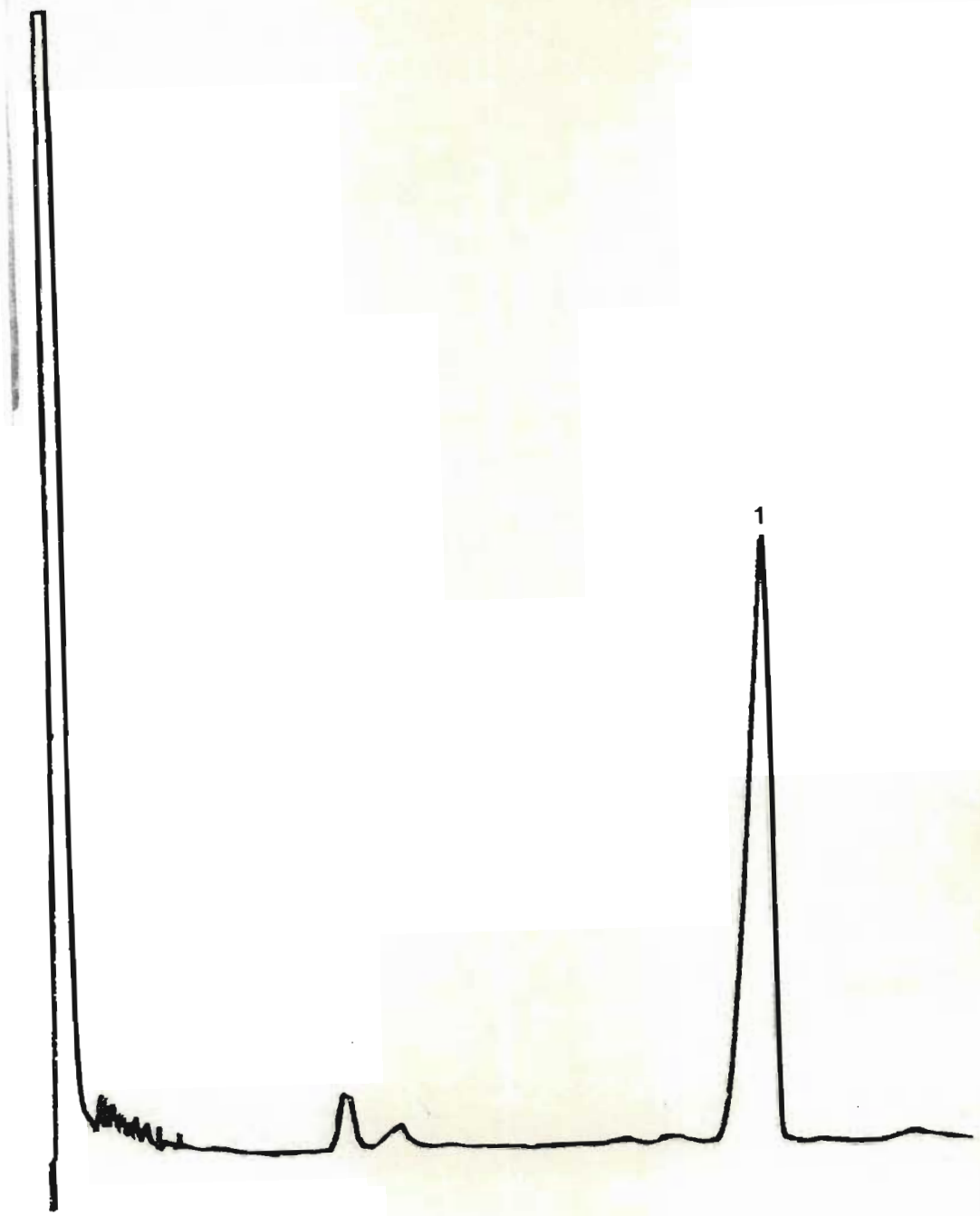
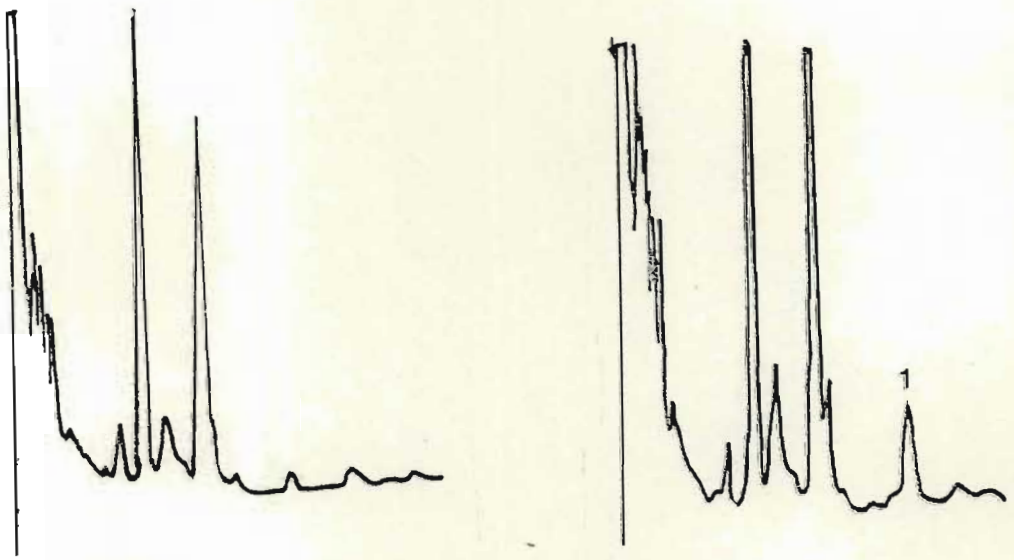


FIG. 9 Gas-liquid chromatogram of toxin-free whole ovine rumen fluid. Operating conditions as described in section 4.2.2 with signal attenuation = 2^3



1 – T–2 toxin

FIG. 10 Identification of T–2 toxin (10 μg/ml) in whole ovine rumen fluid at time O after toxin addition. Operating conditions as described in Section 4.2.2 Signal attenuation = 2^5

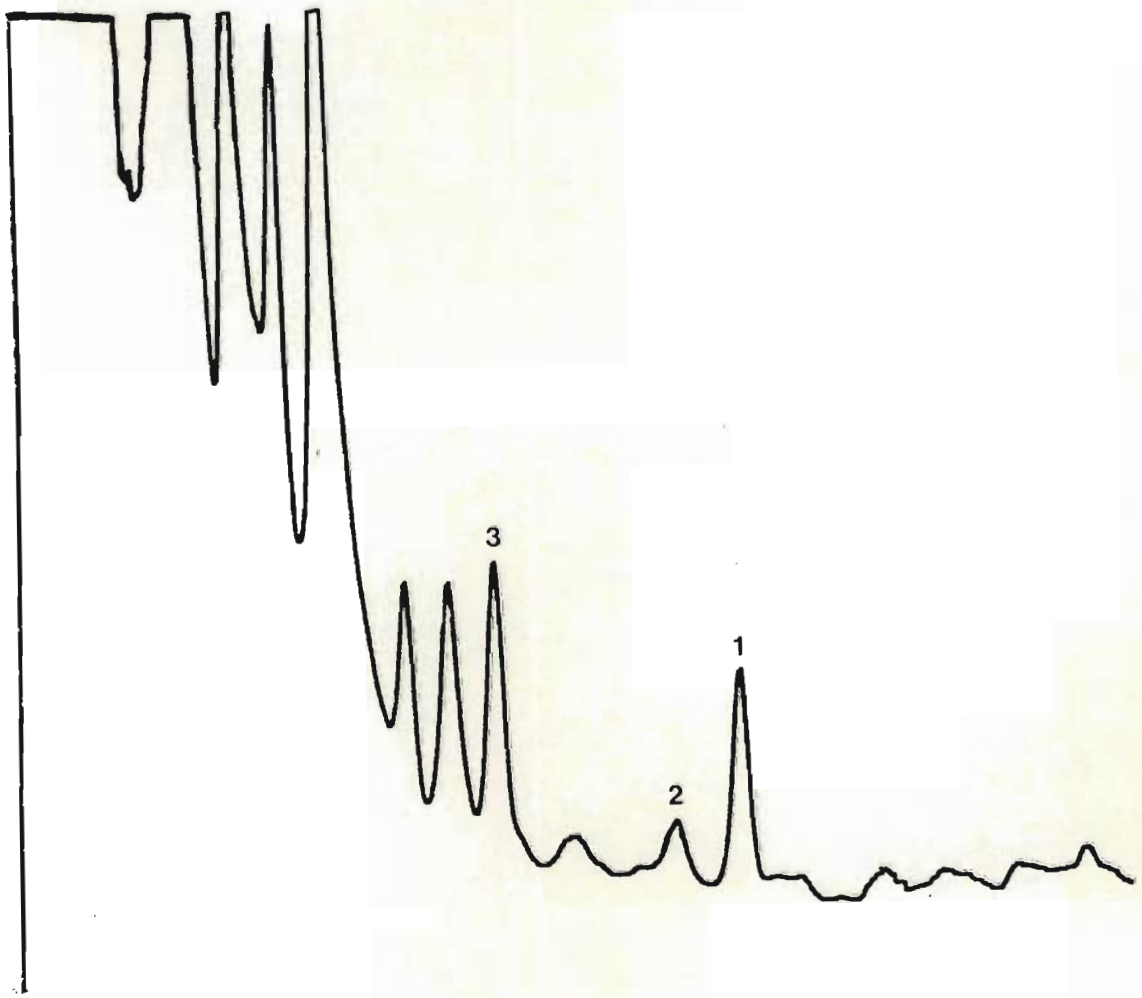


– HT – 2 toxin

+ HT –2 Toxin

1 – HT–2 toxin

FIG. 11 Identification of HT–2 toxin (1 μg/ml) in whole ovine rumen fluid. Operating conditions as described in Section 4.2.2. Signal attenuation = 2^2



1 = T–2 toxin
2 = HT–2 toxin
3 = T–2 triol

FIG. 12 Identification of T–2 toxin (1,0 μg/ml), HT–2 toxin (1,0 μg/ml) and T–2 triol (1,0 μg/ml) in whole ovine rumen fluid. Operating conditions as described in section 4.2.2. Signal attenuation = 2^3

in column length resulted in variation of retention times as compared with FIG. 8. For this reason retention times are not quoted on these chromatograms but peak identification was confirmed in all cases by methods outlined in Section 4.2.2.

FIG. 9 shows the chromatographic separation of an ethyl acetate extract of toxin-free whole ovine rumen fluid. None of the peaks shown were identified but are likely to be fatty acids of varying chain length. These peaks did not interfere with the resolution of toxin peaks. However some interference by the initial broad BSA peak was noted in some samples and in such samples verrucarol and deoxynivalenol could not be quantitated without further evaporation of any excess BSA and rechromatographing. Complete removal of BSA was never achieved but sufficient removal could be obtained to prevent interference with these toxins having short retention times.

The chromatograph presented in FIG. 10 shows that when T–2 toxin was added at 10 μg/ml, signal attenuation could be adjusted so as to facilitate easy identification of toxin peaks against a background of minor peaks from rumen fluid components.

In those situations where toxins were present at low levels and therefore where only low signal attenuation could be used,a comparison of extracts with added toxin with one of rumen fluid alone allowed easy identification of toxin peaks. The chromatograms presented in FIG. 11 show the identification of HT–2 toxin after comparison with a toxin-free control, while that presented in FIG. 12 shows the presence of T–2 toxin, HT–2 toxin and T–2 triol in a spiked sample of rumen fluid. This latter chromatogram also demonstrates that rumen fluid components do not interfere with resolution of toxin peaks.

The identification and quantitation of toxins in bacterial culture studies was also easily performed using methods described in Section 4.2.2 (FIGS. 13, 14).

The chromatogram presented in FIG. 13 shows the peak profile of an ethyl acetate extract of *Butyrivibrio fibrisolvens*, CE 51 while that in FIG. 14 shows a chromatogram of bacterial extract prepared from the same bacterium with added toxins and where toxin concentration has allowed the use of relatively high signal attenuation. The chromatogram presented in FIG. 15 shows FIG. 13 overlayed on FIG. 14 and demonstrates that toxin peaks can be easily distinguished from medium components according to peak retantion time.

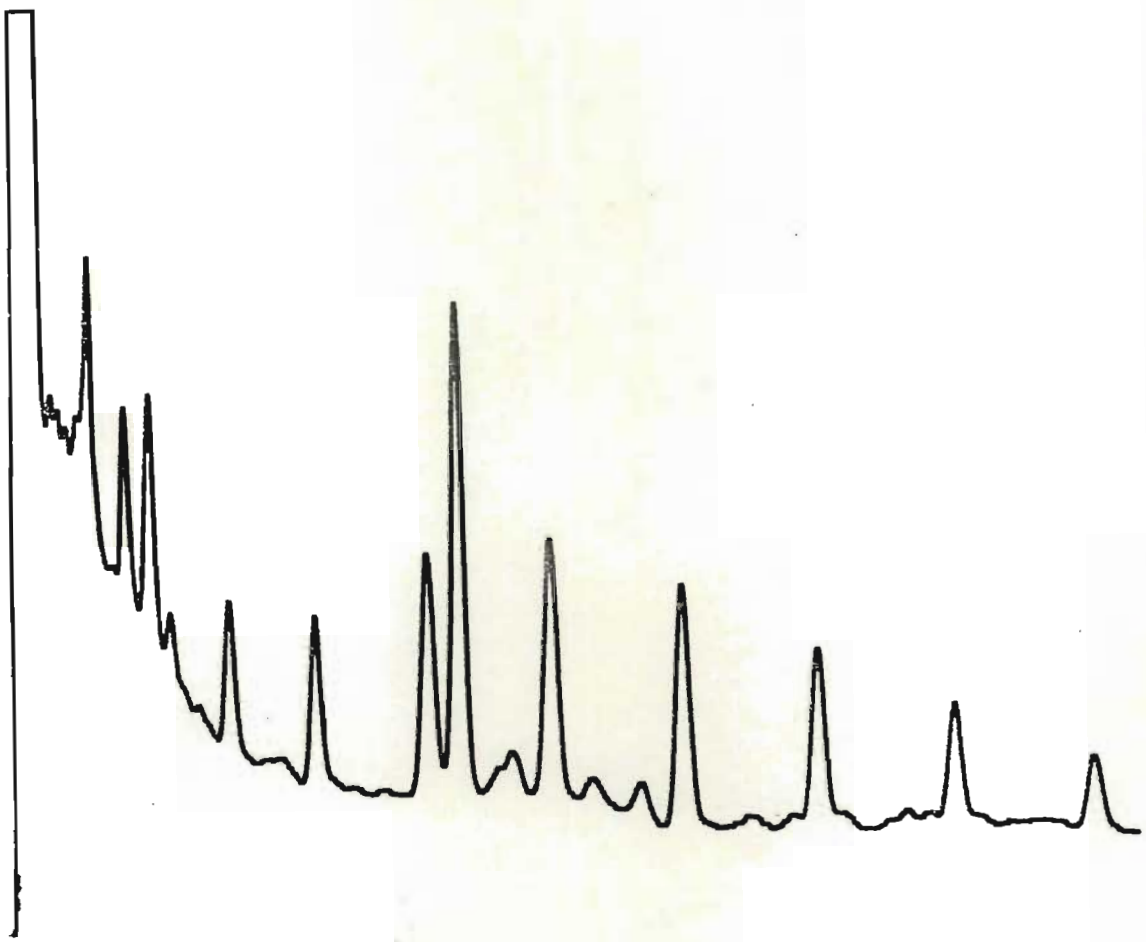
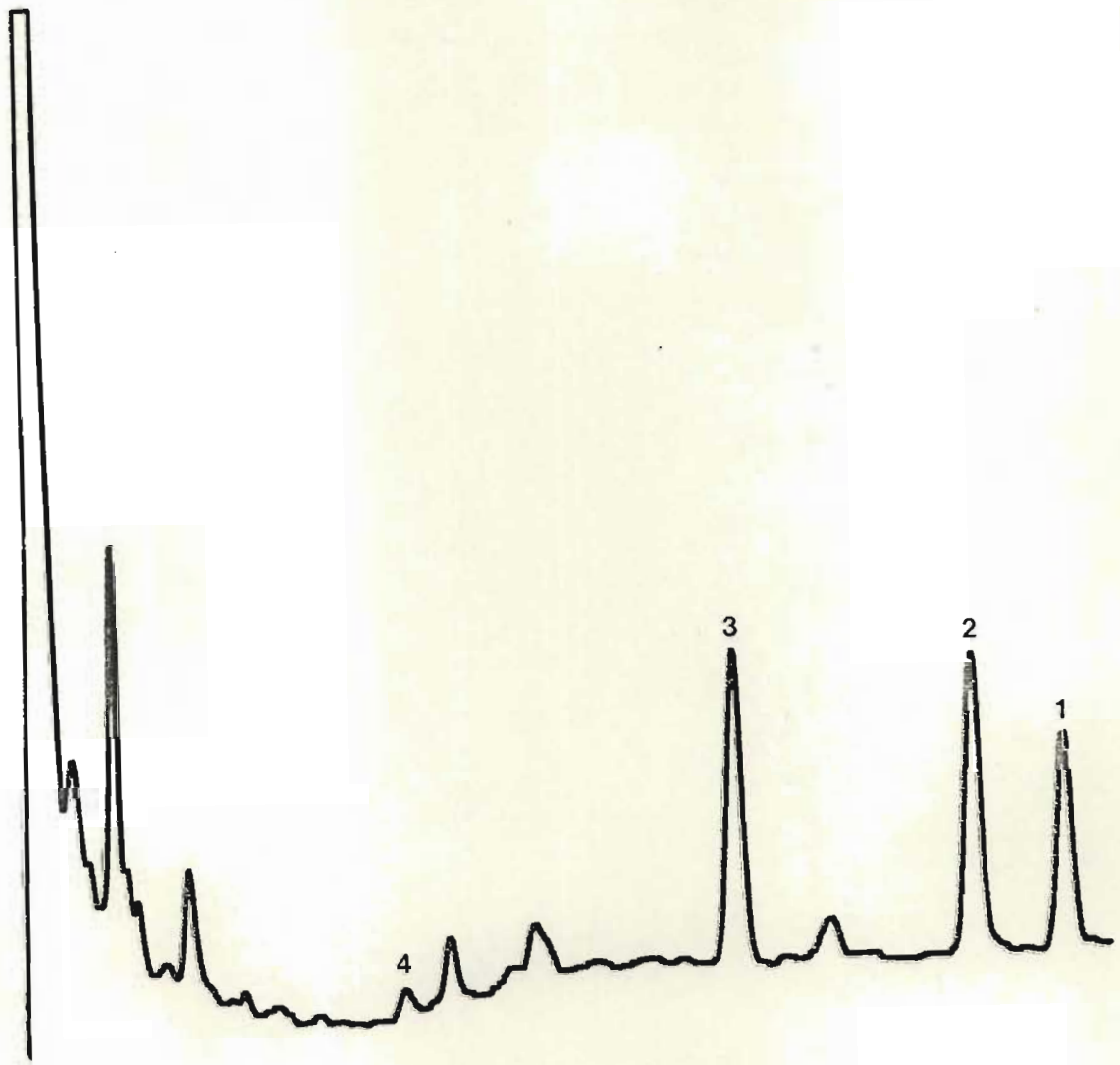
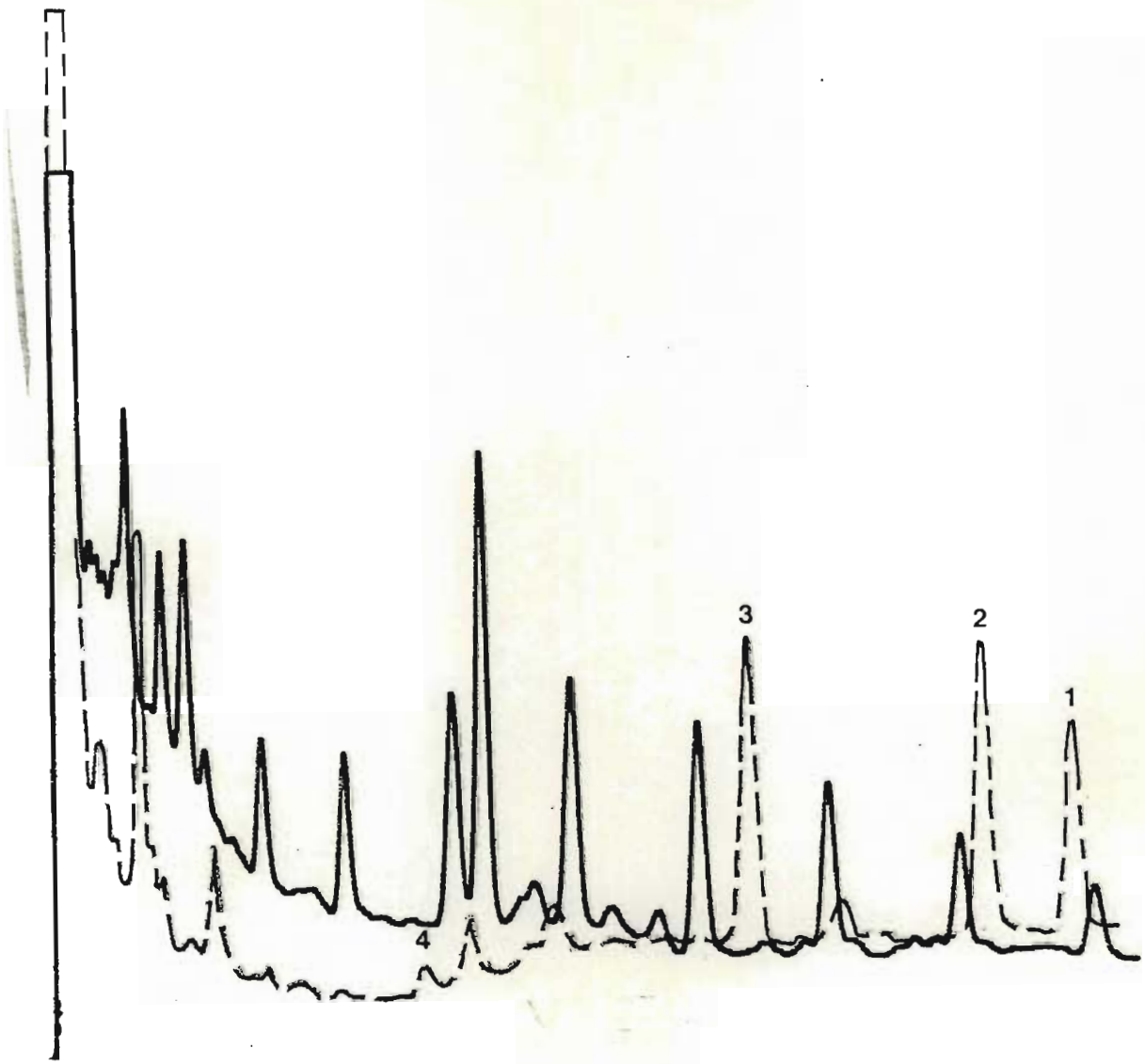


FIG. 13 Gas-liquid chromotogram of a toxin-free ethyl acetate extract of *Butyrivibrio fibrisolvens* strain CE 51. Operating conditions as described in section 4.2.2. Signal attenuation = 2^3



1 = T–2 toxin
2 = HT–2 toxin
3 = T–2 triol
4 = Neosolaniol

FIG. 14 Identification of T–2 toxin (10 μg/ml), HT–2 toxin (10 μg/ml), T–2 triol (10 μg/ml) and neosolaniol (1 μg/ml) in an ethyl acetate extract of *Butyrivibrio fibrisolvens* strain CE 51. Operating conditions as described in section 4.2.2. Signal attentuation = 2^5



1 – T–2 toxin
2 – HT–2 toxin
3 – T–2 triol
4 – Neosolaniol

FIG. 15 Superimposition of toxin containing (--) and toxin free (–) ethyl acetate extracts of *Butyrivibrio fibrisolvens* strain CE 51. Operating conditions as described in Section 4.2.2. Signal attenuation $=2^3$(underlay); 2^5 (overlay)

In some samples and for unknown reasons co-chromatographing of neosolaniol and a rumen fluid component occurred. However these peaks could be resolved with the use of a 4 m length of column and for this reason calibration data relevant to 3 m and 4 m column lengths were stored permanently in integrator memory.

4.4 SUMMARY

A new technique has been developed for trichothecene analysis. This technique facilitates trichothecene identification in rumen fluid and pure cultures of anaerobic bacteria. The use of 3 m column lengths in routine analysis effectively extends the life of the original column and the use of columns of different lengths confers a degree of versatility to the technique which is not available using other gas-liquid procedures.

CHAPTER 5

OCCURRENCE OF MYCOTOXINS IN FEEDSTUFFS IN NATAL

5.1 INTRODUCTION

Only one field outbreak of a trichothecene toxicosis has been reported in the Republic of South Africa. This occurred in 1977 when after unseasonal and heavy summer rain, sheep in the Cape Province died after ingestion of compound feed cubes made from fungal-infested wheat, barley and rye straw (Schneider, Marasas, Dale, Kuys, Kriek & Van Schalkwyk, 1979). The clinical symptoms were typical of stachybotryotoxicosis and were characterised by haemorrhagic septicaemia, anaemia, leukocytopaenia and haemorrhagic tendencies.

Toxigenic strains of *Stachybotrys chartarum* (Synonymous with *S. atra*) were isolated from the wheat and barley straw and after solvent extraction showed the presence of 12, 13-epoxytrichothecenes as determined by thin-layer chromatography and rat skin tests (Schneider *et al.*, 1979). Subsequently the typical clinical signs and pathological lesions of the naturally occurring toxicosis were reproduced in sheep with pure culture material of *S. chartarum* isolated from the wheat.

The only other trichothecene reported to occur naturally in foodstuffs and feeds in Southern Africa is deoxynivalenol which has been found in samples of mouldy maize kernels in South Africa, Transkei and Zambia at levels ranging from 0,07 to 7,40 mg/kg (marasas, Kriek, Van Rensburg, Steyn & Van Schalkwyk, 1977; Thiel, Meyer & Marasas, 1982).
There have been no field outbreaks attributed to deoxynivalenol, but the incidence of deoxynivalenol-contaminated maize was higher in areas of the Transkei with a high rate of human oesophageal cancer than in those areas with a low rate. However, due to the low number of samples involved in this study, statistically valid correlations could not be made. The same trend was also shown with the incidence of *Fusarium verticillioides* but again it is not known whether a causative relationship exists between the incidence of this fungus and oesophageal cancer. (Kriek & Marasas, 1983).

Apart from *F. verticillioides*, two other species of *Fusarium* are commonly associated with animal feeds and human foodstuffs and have been shown to be toxic to experimental animals. These are *F. graminearum* and *F. sacchari var. subglutinans* (Kriek & Marasas, 1983).

More recently, a T–2 toxin-producing strain *F. acuminatum* has been isolated from barley in the Orange Free State. (Rabie, C.J., Pers. Comm.).

This is the first time a T–2 toxin-producing strain of *Fusarium* has been found in the Republic of South Africa.

5.2 METHODS AND MATERIALS

Mycotoxin analysis in feed samples

The samples analysed included animal feedstuffs in general and specific samples of maize and maize products, poultry feed and litter. Many of the general feedstuff samples were obtained from Allerton Veterinary Research Laboratory in Pietermaritzburg and were associated with illness in animals due to unknown causes. These samples were received from throughout Natal. Poultry feed and litter samples were taken from various farms in the Durban-Pietermaritzburg area of Natal, some of these being associated with the poultry disease, ascites. The majority of maize samples were collected from farms in the Durban-Pietermaritzburg area of Natal, while others were of unknown origin.

5.2.1 Chemicals

All solvents and reagents were of analytical grade.

Mycotoxin standards were purchased from Sigma except deoxynivalenol which was a generous gift from Dr P.G. Thiel.

Chromotropic acid was purchased from BDH (Poole, Dorset, England) and N,O-Bistrimethylsilylacetamide was purchased from Merck (Darmstadt, W. Germany).

5.2.2 Preparation of sample extracts

The method employed was a modification of the method of Roberts & Patterson (1975). After milling the sample to pass a 1 mm screen, a 25 g subsample was weighed into a 250 ml virtis homogeniser flask (The Virtis Company, Gardiner NY, USA). Tissue samples were diced prior to homogenisation and 25 g weighed into a similar flask. To the flask was added 100 ml acetonitrile : 4% w/v KCl (9 : 1) and the sample homogenised for 30 min. The aqueous extract was collected by filtering under vacuum, through a

Whatman No. 1 filter paper and the lipids extracted by partitioning twice against 100 ml 2,2,4-trimethylpentane (Iso-octane). To the aqueous extract was added 50 ml 60% ($^{w}/v$) saturated $NaHCO_3$ and this was partitioned three times against 50 ml $CHCl_3$. The $CHCl_3$ extract was collected into a 250 ml round-bottomed evaporating flask by passing through a bed of anhydrous Na_2SO_4 and the aqueous extract retained. After evaporation of the $CHCl_3$ on a rotary vacuum evaporator (Büchi Laboratory-techniques Ltd, Flawil, Switzerland) the extract was reconstituted in a small volume of acetonitrile by washing the flask with two volumes of 2 ml each. Using a pasteur pipette the acetonitrile extract was carefully transferred into $\frac{7}{16}$" diameter dialysis tubing (Previously soaked in distilled water for 1 h) and dialysed overnight against 30% (v/v) aqueous acetone. After dialysis, the aqueous acetone extract was partioned three times against 25 ml $CHCl_3$ and the $CHCl_3$ collected in a 250 ml round bottom flask by passing through a bed of anhydrous Na_2SO_4. After evaporation of the chloroform on a rotary vacuum evaporator the extract was reconstituted in a small volume of chloroform and transferred to a $\frac{1}{2}$ dram vial (Supelco Inc., Bellefonte, PA, USA) for evaporation to dryness at 60°C under a stream of nitrogen. This extract constituted fraction I.

The previously retained aqueous extract was acidified with 1 M H_2SO_4 (diluted 3 : 2 with distilled water) and further extracted with three equal volumes of 50 ml $CHCl_3$. The $CHCl_3$ extract was passed through a bed of anhydrous Na_2SO_4 and collected in a 250 ml round-bottom flask before evaporation of the chloroform on a rotary-vacuum evaporator. The final extract (Fraction II) was prepared under a stream of nitrogen as described for fraction I.

5.2.3 TLC and HPLC analysis

The mycotoxins were separated by 2-dimensional chromatography on silica gel-G aluminium backed chromatoplates, 10 x 10 cm (cut from 20 x 20 cm plates, Merck 5553), using solvent combinations as recommended by Patterson & Roberts (1979). Detection was initially carried out by viewing under long wavelength UV light followed by the use of various spray reagents: 3-methyl-2-benzothiazolinone hydrazone hydrochloride solution (MBTH) for patulin (Scott & Kennedy, 1973), Pauly's reagent (E. Merck pamphlet, 1976) for kojic acid and penicillic acid (with prior treatment for the latter with ammonia vapour (Steyn, 1981)), alcoholic aluminium chloride (Stack & Rodricks, 1971) for sterigmatocystin, and Ehrlich's reagent (Lansden & Davidson, 1983) for cyclopiazonic acid. The trichothecenes were detected by chromotropic acid (Baxter *et al.*, 1983) or nicotinamide-acetyl pyridine reagent (Sano *et al.*, 1982).

Confirmation of positive results was performed by comparison with known standards run on the same chromatoplate and was further verified by 'spiking' the extract with the relevant toxins. Co-chromatography in the solvent systems used and correct colour characteristics when viewed before and after spraying (in both daylight and under UV light) were considered sufficient confirmatory evidence.

The level of mycotoxin contamination was estimated by visual comparison with a series of standards and for aflatoxins, accurate quantitation was performed by HPLC using a Varian Model 5000 liquid chromotograph (Varian instrument group, Palo Alto, CA, USA) with a Beckman Ultrasphere – ODS column, 0,46 x 25 cm (Beckman Instruments Inc., Berkeley, CA, USA). The solvent system used was methanol-acetonitrile-water (1+1+3; v/v/v). The absorbance of aflatoxin was measured at 365 mm.

The quantitation of patulin was performed using the method of Dutton, Westlake & Anderson (1984) but using the liquid chromatograph and column as described above.

5.2.4 Mycological examination

Milled samples weighing 1 g ("as is") were suspended by agitation in 10 ml sterile Ringers solution (Appendix I) and serially diluted to 10^{-6} original concentration with further sterile Ringers solution. Samples of each dilution (1 ml) were then aseptically pipetted into sterile Petri dishes to which was added 20 ml molten Ohio Agricultural Experimental Station Agar (Kaufman, Sumner & Williams, 1963) (Appendix II). The petri dishes were swirled to ensure thorough mixing and allowed to set at room temperature. The petri dishes were then incubated at 25°C for 5 days and fungi present recorded as viable propagules per g original material. *Aspergillus, Penicillium* and *Fusarium* were classified according to their genus using gross morphology and microscopic examination techniques. Other fungi present included species of *Mucor, Rhizopus* and *Trichoderma.*

5.3 RESULTS AND DISCUSSION

A total of 1 129 animal feed samples, poultry litter samples and samples of poultry liver tissue were analysed during the survey period. From these samples, seven different mycotoxins were detected including aflatoxin B_1, T–2 toxin, deoxynivalenol, zearalenone, patulin, kojic acid and sterigmatocystin. Many of the samples containing aflatoxin B_1 also contained aflatoxins B_2, G_1 and G_2 but because aflatoxin B_1 poses the greatest threat

to both human and animal health (Betina, Moulé, Gorst-Allman, Steyn & Heathcote, 1984), only data for this toxin has been presented.

Aflatoxin B_1 was the most commonly occurring mycotoxin detected (TABLES 5 & 6) and was found in an average 27,2% of different animal feeds. The feed samples showing the highest frequency of aflatoxin B_1 occurrence were maize (TABLE 6), oil seeds other than soya bean and mixed feed samples (TABLE 5).

TABLE 5 **Occurrence of aflatoxin B_1 in various animal feeds from Natal, sampled over the period 1982–1983**

Commodity	No. samples examined	No. positive samples	% positive samples	Aflatoxin B_1 concentration (μg/kg)
Cereals other than maize[a]	71	10	14,0	<5–300
Oil seeds[b]	73	31	42,5	<5–2000
Soya Bean	34	5	14,7	<5–20
Animal Feed	42	12	28,6	<5–40
Other Feeds[c]	194	68	35,1	<5–400
Fish Meal	41	1	2,4	20
Other Meals[d]	30	0	0	0
Forage[e]	10	0	0	0
TOTAL	495	127	25,7	<5–20000

a) Includes oats wheat and barley.
b) Includes peanut, cotton seed and sunflower.
c) Includes unspecified material destined for animal consumption.
d) Includes blood, carcass and bone meals.
e) Includes hay, lucerne and silage.

TABLE 6 Incidence of mycotoxins in maize and maize products from Natal, sampled over the period 1982–1983

Commodity	Total No. of samples	Mycotoxin (No. positive samples)		Concentration (μg/kg)
Whole Maize	144	Aflatoxin B_1	(39)	< 5–1500
		T–2 toxin	(4)	< 10
		Deoxynivalenol	(1)	< 10
		Zearalenone	(9)	< 10
		Sterigmatocystin	(4)	< 10
		Kojic acid[a]	(3)	ND
Milled Whole Maize Plant	5	T–2 toxin	(1)	< 10
		Deoxynivalenol	(1)	< 10
Maize Germ Oil Cake	6	Aflatoxin	(2)	50; 200

ND – Not determined.
a) Always found in the presence of aflatoxin.

TABLE 7 Incidence of mycotoxins other than aflatoxin B_1 in agricultural commodities sampled in Natal over the period 1982–1983

Commodity	Total No. samples	Mycotoxin		
		Patulin	Tricho-thecene[a]	Zeara-lenone
Cereals other than maize	71	8	5	ND
Oil Seeds	107	8	1	1
Soya Bean	34	ND	ND	ND
Animal Feed	42	13	3	1
Other Feeds	194	13	8	ND
Fish Meal	41	ND	ND	ND
Other Meals	30	1	1	ND
Forage	10	2	1	ND
TOTAL	529	45 (8,5)[b]	19 (3,59)	2 (0,37)

ND – Not Detected.
a) Includes deoxynivalenol and T–2 toxin.
b) Figure in brackets indicates percentage occurrence of each toxin.

Mycological examination of the same samples showed a relatively high incidence of *A. flavus* and *A. parasiticus* as well as species of *Fusarium* (TABLE 8). Other non-aflatoxin producing species of *Aspergillus* and fungi classified under 'other' were present in more samples but in both cases the presence of such fungi does not present a serious health risk when compared to the toxic potential of Fusaria, *A. flavus* and *A. parasiticus*. Species of *Penicillium* were detected in only 7,8% of samples analysed (TABLE 8). This is in agreement with the low incidence of *Penicillium* toxins of which only patulin was detected (TABLE 7).

TABLE 8 **Incidence of fungi in commodities sampled in Natal over the period 1982–1983**

Commodity	Total No. samples	Fungal incidence (No. of positive samples)				
		Aspergillus spp	*Aspergillus flavus*[a]	*Fusarium*	*Penicillium*	Other[g]
Maize	155	49	57	44	12	42
Other Cereals[b]	71	23	13	18	3	17
Oil Seeds[c]	73	13	23	7	5	25
Soya Bean	34	12	9	3	6	8
Animal Feed	42	16	11	6	6	16
Other Feed[d]	194	63	24	34	13	46
Fish Meal	41	14	4	1	4	5
Other Meals[e]	30	2	5	5	1	15
Forage[f]	10	6	2	4	1	10
TOTAL	650	198 (28,9)[h]	148 (22,7)	122 (25,9)	51 (7,8)	184 (28,3)

a) Also includes *Aspergillus parasiticus.*
b) Includes oats, wheat and barley.
c) Includes peanut cotton seed and sunflower.
d) Includes unspecified materials destined for animal consumption.
e) Includes blood, carcass and bone meals.
f) Includes hay lucerne and silage.
g) Includes species of *Mucor, Rhizopus* and *Trichoderma.*
h) Figure in brackets represents percentage occurrence.

It is evident that the incidence of aflatoxin-producing fungi in feed samples gives little indication of the presence or amount of aflatoxin B_1 present (TABLE 9). The results show the presence of aflatoxin B_1 at various concentrations in samples free of aflatoxin-producing fungi while at the other extreme no aflatoxin was detected in samples where *A. flavus* or *A. parasiticus* were present at levels of greater than 10^6 viable propagules

TABLE 9 **Relationship between aflatoxin B_1 concentration and incidence of aflatoxin-producing *Aspergillus* spp[a]**

No. *Aspergillus* sp/g	No. of positive samples at various concentration ranges of aflatoxin B_1					
	ND	<5	5–9	10-19	20–99	> 100
ND	78	12	0	3	4	1
<10	19	5	0	1	1	1
$10^1 - 10^3$	12	1	1	1	2	0
10^4	3	0	0	1	0	1
10^5	2	0	2	0	0	1
10^6	1	0	0	0	0	0
$> 10^7$	1	0	0	0	0	0

ND – Not detected.

a) Includes *Aspergillus flavus* and *Aspergillus parasiticus.*

per gram of feed material. It is possible that in the former situation, aflatoxin-producing fungi initially present were no longer viable as a result of changing environmental conditions or treatment of the feed with chemical agents such as fungicides. The history of the majority of samples analysed was not known. In the latter case, the most likely explanation is that the fungi isolated were non toxin-producing strains, for if the isolated viable propagules were fungal spores, the high levels detected would suggest previous mycelial growth. Thus the presence in feed samples of potential toxin-producing fungal species cannot be used to indicate the presence of toxin.

Analysis of miscellaneous feed samples showed the presence of four toxins other than aflatoxin B_1. These were patulin, T–2 toxin, deoxynivalenol and zearalenone (TABLE 7). Of these, patulin was the most frequently occurring and as previously mentioned may have been produced by *Penicillium* spp. present in the sample. However patulin is also produced by a number of Aspergilli (Section 6.3) and these may also have been present in those samples where Aspergilli other than *A. flavus* and *A. parasiticus* were detected.

Zearalenone was detected in only 2 samples over the whole survey period and does not therefore pose a significant problem.

Trichothecene occurrence will be discussed later.

Maize is the major crop in South Africa and maize and maize products form a large part of the diet of black Africans as well as being used as a major protein source in mixed animal feeds. Therefore a separate study of mycotoxin occurrence was conducted on maize and maize products. The majority of samples analysed were of whole maize and showed that aflatoxin B_1 was again the most frequently occurring mycotoxin (TABLE 6). Other toxins detected were T–2 toxin, deoxynivalenol, zeralenone, sterigmatocystin and kojic acid. Aflatoxin B_1 concentrations varied between less than 5 μg/kg to as high as 1.5 mg/kg and at the higher level would prove a significant health risk if consumed by animals. For example the LD_{50} for swine has been calculated as 0.6 mg/kg body weight (TABLE 10) and thus although inclusion of such feed in the diet of swine may not prove to be acutely toxic, chronic toxicity and eventual death may occur over a period of time.

TABLE 10 **Median lethal dose (LD_{50}) values for aflatoxin B_1 as a single dose to different animals[a]**

Animal	LD_{50} (mg/kg body weight) p.o.
Duckling	0,3 – 0,6
Pig	0,6
Trout	0,8
Dog	1,0
Guinea Pig	1,4 – 2,0
Sheep	2,0
Monkey	2,2
Rat	5,5 – 17,9
Chicken	6,3
Mouse	9,0

a) After Ciegler, 1975

The incidence of zearalenone was found to be much higher in maize samples than in other animal feed samples. This is in agreement with the fact that zearalenone and deoxynivalenol are the most commonly occurring mycotoxins associated with maize (Mirocha & Christensen, 1974a) and that the producing fungus *Fusarium graminearum* is commonly associated with pathogenic diseases of maize (Sutton, 1982). Deoxynivalenol was found in only two samples and was not associated with zearalenone. Although much less is known of the accumulation of deoxynivalenol in maize, it can also be produced by *Fusarium graminearum* (Caldwell, Tuite, Stob & Baldwin, 1970) as well as *F. tricinctum*,

F. nivale and others (Vesonder, Ellis & Rohwedder, 1981). Work by Scott, Harwig & Blanchfield (1980) has further shown the production of T–2 toxin, HT–2 toxin and neosolaniol by *Fusarium sporotrichioides* isolated from wheat. Thus the occurrence of T–2 toxin, deoxynivalenol and zearalenone in samples analysed may be a result of the growth of a number of different fungi or a result of modification of toxin production in different environmental conditions as shown by Naik; Sherwood & Peberdy and Jarvis (cited by Sutton, 1982).

Optimal conditions for the growth of *F. graminearum* in the field are warm weather with persistent wetness (Sutton, 1982) and thus the low levels of zearalenone and other trichothecenes found in maize samples in South Africa are likely to be a consequence of the relatively dry climate.

It is probable that the presence of T–2 toxin, deoxynivalenol and zearalenone in other animal feeds (TABLE 7) is in most cases due to the use of maize as a protein source in such compound feeds.

Sterigmatocystin and kojic acid were also found in whole maize samples but the fact that the levels were very low and that there have been no other reports of these toxins in animal feeds in South Africa, suggest that they do not pose a significant threat to animal health in this country.

During the survey of poultry feed and litter a total of 142 poultry feed samples were analysed over a two year period and these revealed that samples from broiler houses where poor growth and ascites had been reported, had consistently more aflatoxin than was found in control samples (TABLE 11). Control feeds were random samples mainly obtained from feed millers and hence represent the general occurrence of aflatoxin in feed samples. On the basis of TLC analysis, patulin and trichothecenes were also detected in a small percentage of samples. By comparison with standards, the trichothecenes were identified as T–2 toxin and deoxynivalenol and on the basis of the previous two surveys were probably present in maize used to prepare the compound feed. Patulin has previously been detected in cereals and oil seeds (TABLE 7) which suggests that either or both of these products are responsible for the presence of this toxin in the compound feed.

TABLE 11 **Incidence of mycotoxins in chicken feed, litter and liver in cases of broiler disease**

Sample Type and Disease Condition	Aflatoxin B_1			Other Mycotoxins[b]		
	Total No. of samples examined	Incidence (range)[a]	% positive	Incidence	% positive	Total positive (%)
Feed						
Control	25	3(T–20)	12	1	4	16
Poor growth[c]	75	18(T–80)	24	5	7	31
Ascites	30	9(T–100)	30	2	7	37
Other[d]	12	1(2)	8	ND	–	8
Litter						
Control	72	10(0–40)	14	ND	–	14
Poor growth[c]	63	11(T–1000)	17	3	5	22
Ascites	27	6(T–20)	22	ND	–	22
Other[d]	4	1(T)	25	ND	–	25
Liver						
Healthy	4	ND	–	ND	–	–
Poor growth[c]	9	ND	–	ND	–	–
Ascites	27	5(T–40)	19	ND	–	19
Other[d]	14	ND	–	ND	–	–

a) Concentration (μg/kg) given in parenthesis where T = trace; and ND = not detected.

b) Comprises patulin and trichothecenes.

c) Covers general malaise and nondescript diseases.

d) Covers malabsorption and gizzard erosion.

The occurrence of mycotoxins in litter followed a similar trend to that found in feed samples (TABLE 11). Under normal circumstances one would not expect the presence of toxins in litter to significantly affect the health of the chickens. However, in one series of litter samples analysed, consisting only of groundnut hulls, aflatoxin B_1 was found to be present at levels in excess of 10 mg/kg and was associated with very poor growth rate of broiler chickens.

Examination of liver tissue showed the presence of aflatoxin B_1 in five out of twenty seven birds suffering from ascites (TABLE 11). It is possible that the timing of sampling is a critical factor, since aflatoxin B_1 is rapidly metabolised and excreted by animal tissues (Pier, Heddlestone, Cysewski & Patterson, 1973; Harland & Cardeilhac, 1975). This would result in depletion of toxin levels below detection limits within a short time after ingestion.

TABLE 12 shows the incidence of fungi in the various materials examined. Those capable of mycotoxin production include members of the genera *Aspergillus, Penicillium* and *Fusarium* whilst fungi listed as 'other' include species from the genera *Mucor, Trichoderma Rhizopus* and in the case of litter, a large number of yeasts.

The results show widespread contamination of feed and litter with various fungal genera but with the highest incidence being found in control samples. The reason for this phenomenon is not known. It is also possible that the fungi found in the litter and which are normally regarded as 'storage fungi' (i.e. *Aspergillus, Penicillium*) were present as a result of feed spillage from the feeding troughs onto the litter and subsequent growth in the more moist environment.

It has been shown by Chang & Hamilton (1981) that aflatoxin increases the susceptibility of broilers to infectious bursal disease (IBD) and at the same time alters the symptoms of typical IBD. It has further been shown that aflatoxin can exert a synergistic effect with other conditions such as excess heat, thereby increasing the severity of disease (Wyatt, Thaxton & Hamilton, 1975). Thus the presence of aflatoxin in these samples may increase the susceptibility of the broiler chickens to diseases such as ascites.

TABLE 12 **Incidence of non toxin and toxin-producing fungi found in chicken feed and litter 1982/83 for the Durban–Pietermaritzburg area of Natal**

Sample Type and Disease Condition	Total No. of samples	Number of samples with viable fungal propagules (% total)				
		Aspergillus spp.	*Aspergillus flavus*	*Fusarium* spp.	*Penicillium* spp.	Other[a]
Feed						
Control	25	10(40)	10(40)	5(20)	2(8)	8(32)
Poor growth[b]	75	3(4)	14(19)	15(20)	5(7)	19(25)
Ascites	30	1(3)	3(10)	9(30)	1(3)	3(10)
Other[c]	12	1(8)	3(25)	4(33)	2(17)	4(33)
Litter						
Control	72	29(40)	11(14)	8(11)	ND	71(99)
Poor growth[b]	63	7(11)	8(13)	3(5)	ND	9(14)
Ascites	27	ND	3(11)	1(4)	ND	ND
Other[c]	3	2(50)	2(50)	1(25)	ND	3(75)

a) Other covers non-toxin producers and unidentified fungi; some samples contained more than one genus. Figure in parenthesis is percentage of total samples examined.

b) Covers general malaise and nondescript disease.

c) Covers malabsorption syndrome and gizzard erosion and two cases of aspergillosis.

5.4 SUMMARY

The identification of sterigmatocystin, Kojic acid and T–2 toxin in feedstuffs in South Africa represent the first such reports of the natural occurrence of these toxins in this country. While the occurrence of *Fusarium* species has been reported on a number of occasions in South Africa, South West Africa, Swaziland, Transkei and Zambia (cited by Kriek & Marasas, 1983) only deoxynivalenol of the trichothecenes and the *Fusarium* toxins zearalenone and moniliformin have previously been shown to occur (Marasas *et al.*, 1977; Thiel, Meyer & Marasas, 1982).

The potential toxicity of a number of toxins identified during the survey is presented in TABLE 13. It can be seen that although aflatoxin B_1 represents the greatest hazard, T–2 toxin has been shown to be embryotoxic and teratogenic while the carcinogenic properties of this toxin remain unresolved (Section 2.6.3.4). Further, although the presence of these toxins in feedstuffs largely represents a direct health threat to animals, there is a possibility of carry-over of these toxins into the human food chain.

TABLE 13 **Comparison of biological effects of selected mycotoxins**[a]

Mycotoxin	Biological effect			
	Embryotoxic	Teratogenic	Mutagenic	Carcinogenic
Aflatoxin B_1	+	+	+	+
Aflatoxin M_1	NT	NT	+	+
T–2 toxin	+	+	–	NT
Diacetoxyscirpenol	NT	NT	–	NT
Moniliformin	–	–	NT	NT
Patulin	+	–	+[b]	–(+[c])
Ochratoxin A	+	+	–	–
Zearalenone	+	–	+[b]	NT

a) Adapted from Hayes (1980).

b) *Bacillus subtilis* unactivated system.

c) At site of injection.

d) T–2 toxin carcinogenicity discussed in section 2.6.3.4.

\+ – Positive

– – Negative

NT – Not tested

The incidence of trichothecene and *Fusarium* toxins identified in this study is generally much lower than corresponding results from the USA and Canada where conditions such as feed refusal and emesis are much more prevalent (Section 2.3.7) and may be due to prevailing environmental conditions.

CHAPTER 6

DETAILED BACKGROUND INFORMATION RELEVANT TO STUDIES OF TOXIN DEGRADATION IN THE RUMEN

6.1 RUMEN FUNCTION AND RUMINANT NUTRITION

The principal function of the gastro-intestinal tract of animals is the digestion and absorption of nutrients and the excretion of certain waste products. The majority of animals including man have developed a hindgut fermentation system in which fermentation of ingested food takes place in a region of the gastro-intestinal tract after the stomach. However, in ruminants the fermentation of ingested food occurs in a modified stomach which includes the rumen. Most microbial activity takes place in the rumen and thus fermentation of food in ruminants occurs before reaching the true stomach. (Church, 1979).

Once ingested, the fibrous diet of the ruminant is regurgitated for further mastication before being re-swallowed. This process is known as rumination and has led to the naming and grouping of animals with this characteristic as ruminants. (Church, 1979).

Metabolism in the rumen

The ability of animals to utilise cellulose, hemicellulose and pectin as food depends on the capacity of gastro-intestinal organisms to degrade them and the ability of the host to subsequently utilise these microorganisms and their end products. (Church, 1979).

In ruminants, the host animal and the microbial population share a symbiotic relationship known as mutualism in which the ruminant provides the microorganisms with a means of gathering and selecting food and an environment conducive to the growth of these organisms. In return, the microorganisms provide the host with the enzymes required to hydrolyse cellulose and hemicellulose, a mechanism for detoxification and an ability to utilise non-protein nitrogen for the synthesis of amino acids and thereby enhance the quality of food low in nitrogen. The potential energy available to herbivores also includes the microorganisms themselves and their reduced end products. These reduced products in the form of volatile fatty acids (VFA's) can then undergo oxidative metabolism in the host , thereby releasing the majority of stored energy in the ingested food. (Church, 1979).

Therefore although readily fermentable carbohydrates and proteins are quickly utilised by the microorganisms within the rumen, the production of VFA's from the anaero-

bic catabolism of plant cellulose and hemicellulose renders to the host an energy supply that would not normally be available. (Church, 1979).

6.1.1 Rumen Bacteria

The conditions that prevail within the rumen regulate the numbers and type of micro-organisms present. The rumen bacteria are adapted to live in acidic conditions between pH 5.5 and 7.0 under anaerobic conditions and at a temperature of 39–40°C. The system is essentially isothermal being regulated by the homeothermic metabolism of the host animal. The steady supply of food and continuous removal of fermentation products and food residues maintain relatively constant conditions in which large numbers of bacteria can develop. The removal of acidic fermentation products across the rumen and neutralisation of these products by salivary buffers is an important feature of the rumen, for it allows the maintenance of relatively stable pH conditions. The osmotic pressure does not significantly exceed isotonic levels and ionic concentrations are regulated through dilution, absorption and passage. Thus the rumen can be regarded as a continuous culture system where conditions favourable to the growth of micro-organisms are maintained within relatively narrow limits. (Church, 1979).

Rumen bacteria are predominantly obligate anaerobes but will tolerate some oxygen which can be introduced through feed and water or by diffusion across the rumen wall. The amount of oxygen involved is metabolised quickly and can serve as an electron donor in fermentation reactions. A number of facultative anaerobes are also present but these are not as important for normal rumen function. (Church, 1979).

Classification of rumen bacteria

The most convenient method of classifying rumen bacteria is that according to substrate utilised. Using this classification system, Hungate (1966) identified eight different classes. These are cellulolytic bacteria, hemicellulose digesters, amylolytic bacteria, sugar digesters, acid utilisers, proteolytic bacteria, methanogens and lipolytic bacteria. Church (1979) extended this classification system to include ammonia-producing bacteria and vitamin synthesising bacteria.

6.1.1.1 Lipolytic bacteria and their metabolic activity

Because the degradation of trichothecene toxins in the rumen has been shown to involve ester hydrolysis (Kiessling, Pettersson, Sandholm & Olsen, 1984) it is possible that the lipolytic bacteria are involved in this process.

The first work on the metabolism of lipids in ruminants was conducted by Reiser in 1951 who found a marked decrease in the linolenic acid content of linseed oil when incubated with sheep rumen contents (Reiser, cited by Garton, Lough & Vioque, 1961). This effect was attributed to hydrogenation of double bonds and was substantiated by comparisons of the unsaturation of feed lipids with that of the lipids of rumen contents in sheep (Shorland, Weenink & Johns, 1955). Since then, much work has been conducted on lipid hydrolysis and hydrogenation in the rumen.

The major lipids ingested by ruminants in concentrate-free diets are chloroplast mono- and digalactosylglycerides which have been shown to disappear from the rumen contents within a matter of hours (Dawson & Hemington, 1974a; Dawson, Hemington, Grime, Lander & Kemp, 1974). In animals receiving concentrate supplements, triglycerides present in the diet have also been found to be rapidly hydrolysed (Garton *et al.*, 1961). This hydrolysis occurs as a result of microbial activity (Section 6.1.1.2).

6.1.1.2 Hydrolysis of esterified fatty acids

It has already been mentioned that triglycerides undergo rapid hydrolysis in the rumen. One organism obtained in pure culture and shown to be capable of triglyceride hydrolysis is *Anaerovibrio lipolytica*. This bacterium was found to produce two hydrolytic enzymes, a cell-bound esterase and an extracellular lipase (Henderson, 1973a). The extracellular lipase showed hydrolytic activity towards the triglyceride of olive oil and towards esterified fatty acids of chain length C_{12} and above. However, diglycerides were hydrolysed more rapidly than the corresponding triglycerides. Henderson (1971) suggested that the greater activity towards the diglycerides could be explained by the fact that the normal substrate for the enzyme might be the diglycerides resulting from the hydrolysis of galactosylglycerides by galactosidase. The importance of *A. lipolytica* to lipid hydrolysis in the rumen was shown by Prins, Akkermans-Kruyswijk, Franklin-Klein, Lankhorst & Van Golde (1974) who showed that the rate of hydrolysis of trilinolein was sufficient to account for the rates of hydrolysis of esterified fatty acids in the rumen.

Apart from the galactolipids the major lipids of leaf tissue are in the form of phospholipids and although plant tissue is known to contain phospholipases (Kates, 1970), Dawson & Hemington (1974b) demonstrated the presence of a potent inhibitor of the major phospholipase in mixed pasture grass. Also, Dawson & Kemp (1969) demonstrated rapid hydrolysis of phosphatidylcholine in protozoa-free rumen of sheep. This work therefore suggested that bacteria within the rumen were responsible for phospholipid hydrolysis.

Work by Hazlewood & Dawson (1979) showed phospholipase activity in a non-cellulolytic strain of *Butyrivibrio fibrisolvens*. Maximum activity of the enzyme appeared during the late exponential phase of growth and was associated both with the cells and with the supernatant culture fluid. The phospholipase A produced by the organism deacylated phospholipids to liberate lysophospholipids and unesterified fatty acids. It was not stimulated by calcium ions and showed low activity under aerobic conditions. The same workers subsequently showed the phospholipid deacylating systems had an absolute requirement for either mercaptoethanol, cysteine or dithiothreitol.

In 1979, the same authors isolated a strain of *Butyrivibrio* capable of deacylating plant galactolipids, phospholipids and sulpholipids to obtain sufficient fatty acid for growth.

Thus although very few species of bacteria capable of lipid hydrolysis have been found, those that are capable appear to be able to hydolyse the various plant lipids present in the diet in a rapid manner and must therefore possess the necessary complement of esterases to affect these reactions.

6.1.2 Rumen protozoa

Protozoa in the rumen are primarily ciliated species although a few flagellates are found, particularly in young animals.

Because of their relatively large size, the classification of protozoa is based on morphological characteristics. According to the classification of Hungate (1966) two sub-classes exist within the class ciliata. These are the sub-classes Holotrichia and Spirotrichia of which the former are usually larger and have rows of cilia over the entire body while the latter (commonly known as Oligotricha) are smaller and have 'tufts' of cilia on the anterior parts.

These rumen ciliates have evolved into a highly specialised group adapted to survive in the rumen or closely related habitats. They are anaerobic and can ferment constituents of plant materials for energy (Hungate, 1966).

Due to the difficulty in culturing rumen protozoa, relatively little information is available on their nutrition and metabolism. However various species of holotrich have been shown to be able to utilise glucose, fructose, sucrose, maltose, cellobiose and a number of other carbohydrates (Church, 1979), while species of oligotrichs (entodinio-

morphs) can utilise very little soluble carbohydrate but instead ingest granular starch (Church, 1979).

The uptake and metabolism of linoleic acid by rumen holotrichs has been measured by Girard & Hawke (1978). They found that after 90 min, 84% of the added linoleic acid could be recovered within the cells, mainly as free fatty acid or phospholipid.

6.2 MYCOTOXIN METABOLISM IN THE RUMEN

The first work on mycotoxin metabolism in the rumen was carried out by Hult, Teiling & Gatenbeck (1976), who showed that ochratoxin A could be converted to the non-toxic ochratoxin α and phenylalanine *in vitro* by the contents of the rumen, reticulum and omasum but not the abomasum of the cow. They further calculated that the cow should be able to detoxify ochratoxin A in feed when present at levels of up to 12 mg kg^{-1}.

Later in 1982, Kallela & Vasenius showed in *in vitro* experiments that rumen contents of the cow were also capable of breaking down zearalenone. In this work, they showed that the percentage degradation of zearalenone was lower when added at high levels (1,3 mg per 100 ml). The rumen contents of cattle fed on a concentrate-rich diet was generally more efficient at toxin degradation than the rumen contents of cattle fed on a hay diet. Variation in toxin degrading ability was also shown in samples taken before and after feeding. These latter two effects could be the result of changes in microbial flora of the rumen which have been shown to occur as a result of different diets (Bryant & Burkey, 1953; Michalowski & Muszynski, 1978) and also the time of sampling (Nakamura & Kanegasaki, 1969; Leedle, Bryant & Hespell, 1982).

Also in 1982, Pettersson, Kiessling & Ciszuk confirmed the earlier work of Hult *et al* in studies with ochratoxin A and further showed that rumen protozoa were more efficient at degrading ochratoxin A than rumen bacteria. Work by Kiessling, Pettersson, Olsen & Tideman (1982) showed that rumen microorganisms had no effect on aflatoxin B_1 and that zearalenone was degraded to zearalenol α and β by rumen protozoa only. Studies with diacetoxyscirpenol showed that this trichothecene could be deacetylated to monoacetoxyscirpenol by rumen bacteria but to a slightly greater extent by rumen protozoa. They concluded that because the breakdown products of zearalenone were more polar than zearalenone itself these would be more toxic to the host because of the increased rate of absorption into the bloodstream, while the breakdown of diacetoxy-

scirpenol represented a detoxification mechanism because the latter is a weaker inhibitor of protein synthesis. Thus no single rule or process could be applied to the metabolism of mycotoxins in the rumen from the point of view of the host.

In 1984, Kiessling, Pettersson, Sandholm & Olsen extended investigations to include the effect of intact rumen fluid, rumen protozoa and rumen bacteria on the metabolism of aflatoxin B_1, ochratoxin A, zearalenone, T–2 toxin, diacetoxyscirpenol and deoxynivalenol. They found that aflatoxin B_1 and deoxynivalenol were unaffected by any of the preparations while the other four toxins were degraded to a greater extent by protozoa than by bacteria. Breakdown products of zearalenone, ochratoxin and diacetoxyscirpenol were as previously reported while T–2 toxin was deacetylated to HT–2 toxin. The breakdown of ochratoxin A, zearalenone and diacetoxyscirpenol was inhibited to varying degrees by the addition of milled feed to the diet and this effect was thought to be due to either direct substrate inhibition or as a result of the inherent change in the protozoal population. With the exception of zearalenone, all breakdown processes were considered to be detoxification processes.

Although Kiessling *et al.* (1984) did not detect any breakdown products of deoxynivalenol, King, McQueen, Levesque & Greenhalgh (1984) using similar *in vitro* studies showed that nearly all added deoxynivalenol was transformed to a single breakdown product within 24 h when added at levels of up to 10 μg/g. Above this level toxin breakdown was significantly reduced. The breakdown product was identified as 3α, 7α 15-trihydroxytrichothec – 19, 12 – dien – 8 – one in which the epoxide group of deoxynivalenol had been reduced. Studies with 3-acetoxydeoxynivalenol showed that this compound was first deacetylated to deoxynivalenol before epoxide reduction as above. Evidence has been presented by Sato & Ueno (1977) that the epoxide group on the trichothecene nucleus is important in the cytotoxicity and induction of vomiting by these compounds and thus the epoxide reduction could be considered as a detoxification mechanism and may therefore explain the tolerance of ruminants to this group of compounds. The reduction of epoxide to olefin in the rumen has been previously demonstrated by Ivie (1976) in studies with insect juvenile hormone mimic. This work supports the above data and suggests the presence of an epoxide reductase in the rumen.

6.3 AFLATOXIN, PATULIN, OCHRATOXIN AND ZEARALENONE

Aflatoxin

The aflatoxins are produced by the fungus *Aspergillus flavus (A. flavus)* and the closely related *A. parasiticus* and comprise a group of related metabolites of which the most common are aflatoxins B_1, B_2, G_1 and G_2. Of these, aflatoxin B_1 is the most toxic to living organisms, with the toxicity of the remainder decreasing in the order aflatoxin G_1, B_2 and G_2 (Betina, *et al.*, 1984). The structure of aflatoxin B_1 is shown in FIG. 16.

Aflatoxin B_1 is embryotoxic, teratogenic, mutagenic and carcinogenic (Hayes, 1980) and has been associated with primary liver cancer in humans. (Patterson & Jones, 1978). Also, when aflatoxin B_1 is ingested by mammals, it is metabolised in the liver by the hepatic microsomal mixed-function oxidase (Lynch, Smith, Covey & Gordon, 1973) and converted to aflatoxin M_1 and other metabolites. These metabolites are excreted in the milk (Kiermeier, 1977) and although the carcinogenicity of aflatoxin M_1 is considerably less than aflatoxin B_1 (Wong & Hsieh, 1976) the presence of this toxin in milk represents a further risk to human health.

The toxicity of aflatoxin B_1 to various animals is given in TABLE 10.

The effects of aflatoxin on ruminants

There have been a number of reported cases of aflatoxin poisoning in cattle which indicate that young animals (up to 6 months of age) are more severely affected than adult cattle (Loosemore & Markson, 1961; Clegg & Bryson, 1962).

Sheep appear to be relatively resistant to aflatoxins, for in a study performed by Lewis, Markson & Allcroft (1967), lesions were only observed after 5 years exposure to a toxic feed containing between 1 and 1,75 mg/kg. However Armbrecht & Shalkop (1972) found that aflatoxin at levels of 2 mg/kg of body weight was lethal to rams.

It has been suggested by Mathur, Smith & Hawkins (1976a) that the greater resistance to aflatoxin poisoning of mature ruminants compared to young animals may be a result of some detoxifying ability of the rumen microflora. Mathur *et al.* (1976b) could not detect fluorescent compounds other than aflatoxin B_1 after incubation of aflatoxin

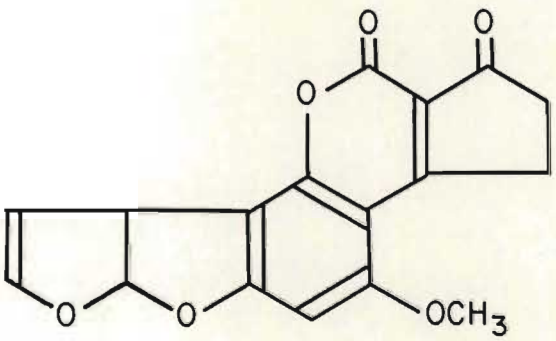


FIG. 16 Structure of aflatoxin B_1

B_1 with rumen microorganisms. Kiessling *et al.* (1984) reported similar results. However, Engel & Hagemeister (1978) were able to demonstrate degradation of aflatoxin B_1 in the rumen and omasum of dairy cows. In *in vitro* studies, they were able to show that 40% of the added aflatoxin was degraded. Thus the potential of rumen microflora to degrade aflatoxin B_1 remains unclear.

In studies on the effect of aflatoxin B_1 on the rumen microflora. Mathur *et al.* (1976b) showed that the morphology and physiology of *Streptococcus bovis* was affected by the toxin, while Beuchat & Lechowich (1971) obtained similar results using *Bacillus megaterium*. Fehr & Delage (1970) also showed that aflatoxin B_1 caused a decrease in volatile fatty acid production (VFA) in *in vitro* studies with rumen microorganisms.

Patulin

Patulin is a mycotoxin produced by a number of fungal genera and species including *Penicillium urticae, P. expansum, A. clavatus, A. terreus* and *Byssochlamys nivea.* The chemical structure of patulin is shown in FIG. 17.

Patulin has been found in a number of commodities including wheat, flour, various fruits and fruit juices, meat and poultry feed (Ciegler, 1977) and in toxicity studies has been shown to be carcinogenic at the site of injection in rats (Dickens & Jones, 1961). However, it has not been shown to be carcinogenic by *per os* administration. Topical application led to dermal irritation in humans (Dalton, 1952) and animals (Hofmann, Mintzlaff, Alperden & Leistner, 1971). Intravenous perfusion of 0,1 g into humans had no ill effects. (De Rosnay, Martin-Dupont & Jensen, 1952) and this has led to doubts about the seriousness of patulin as a mycotoxin.

Data implicating patulin in mycotoxicoses are primarily circumstantial but involve cattle in all cases. *P. urticae* was isolated from malt feed believed to be responsible for the deaths of over 100 dairy cattle in Japan (Hori & Yamamoto, 1953). Subsequent tests with the pure culture grown on malt grain produced nervous symptoms, brain haemorrhage and death in a bull and mice (Hori, Yamamoto, Azewa, Matsuki, Hamaguichi & Soraoka, 1954). In France, pulmonary oedema and congestion were found in dead cows fed on wheat which had been contaminated with *A. clavatus* (Moreau & Moreau, 1960). The same fungus was found in malt that had caused toxic poisoning of cows in Germany (Schultz, 1968). There have been very few other reports of naturally occurring toxicoses thought to be due to patulin.

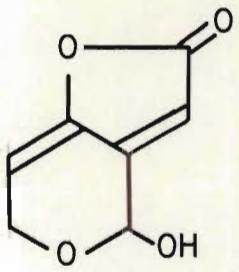


FIG. 17. Structure of patulin

Ochratoxin

The ochratoxins comprise a group of closely related metabolites produced by species of *Penicillium* and *Aspergillus* and which were first discovered in 1965 (Van der Merwe, Steyn, Fourie, Scott & Theron, 1965). Ochratoxin A (FIG. 18) is the only member of the group to have been found as a widespread natural contaminant of feed. (Krogh, 1977) and has been shown to cause renal damage in mice, rats, dogs, pigs, ducklings, hens and rainbow trout (Krogh, 1977). However, no ill effects have been observed in ruminants and they are therefore thought to be less sensitive, due to degradation of the toxin in the rumen (Hult *et al.*, 1976).

The metabolism of ochratoxin A by rumen microorganisms is discussed in Section 6.2.

Zearalenone

Zearalenone (FIG. 19) is a mycotoxin produced by a number of species of *Fusarium* including *Fusarium graminearum* and can cause a hyperoestrogenic syndrome described in section 2.3.3.

Zearalenone has been found in a number of food and feed commodities including corn, wheat, barley, oats, hay, commercial animal rations and corn silage and has been found in most parts of the world where maize and other cereals are grown (Mirocha, Pathre & Christensen, 1977).

Zearalenone has a profound effect on the fertility of pigs but only a moderate effect on bovine fertility. Kallela & Vasenius (1982) investigated the effect of rumen fluid on zearalenone degradation. This work is discussed in Section 6.2. (Mirocha, Pathre & Christensen, 1977).

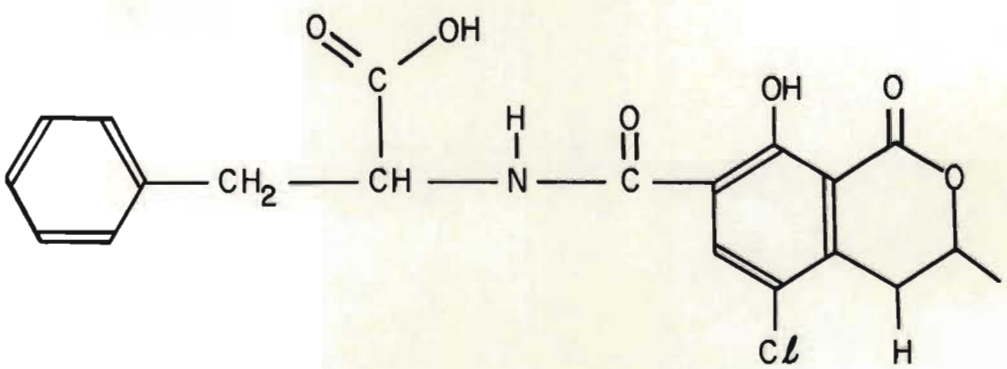


FIG. 18 Structure of ochratoxin A

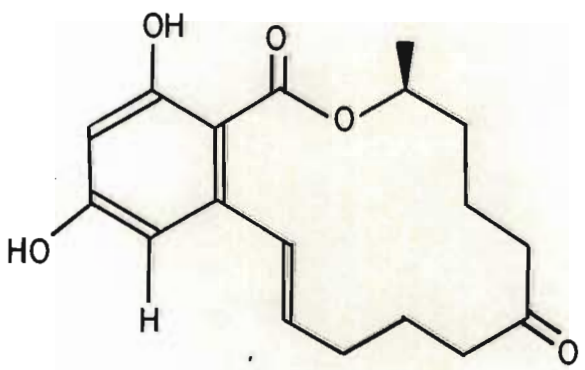


FIG. 19 Structure of zearalenone

CHAPTER 7

MYCOTOXIN DEGRADATION BY OVINE RUMEN FLUID AND OVINE RUMEN FLUID PREPARATIONS

7.1 INTRODUCTION

Studies described in Chapter 5 have shown the presence, to varying degrees, of aflatoxins, patulin, zearalenone, kojic acid, sterigmatocystin, T–2 toxin and deoxynivalenol in various feedstuffs sampled from Natal. Further, because it has been shown that ruminants and especially sheep are more resistant to mycotoxins than monogastric animals (Section 2.8) a number of experiments were conducted to determine the effect of rumen fluid and rumen fluid preparations on mycotoxin metabolism.

7.2 METHODS AND MATERIALS

7.2.1 Chemicals

All solvents and reagents were of analytical grade and sources, where required, have been noted in the text.

7.2.2 Toxin degradation by whole ovine rumen contents

Intact donor sheep were maintained on a daily ration of lucerne hay. Rumen fluid was withdrawn from these sheep directly, without exposure to air, into a sealed flask maintained at 39°C, using an adaptation of the suction-strainer technique of Raun & Burroughs (1962) as used by Dennison & Marais (1980). McDougall's artificial saliva buffer was made up according to Baumgardt, Taylor & Cason (1962) (Appendix III), warmed to 39°C in a temperature-controlled water bath, and flushed with CO_2 to pH 7.0. After dilution of whole rumen contents with this buffer (20 ml rumen fluid : 80 ml buffer), the toxins to be tested, made up as concentrated solutions in dimethyl formamide (DMF) were added to the final desired concentration. To each flask (100 ml screw-cap polyethylene bottles), prepared in triplicate, was added 0.5 g oven-dried lucerne, milled to pass a 1 mm screen. The solution in each bottle was swirled to suspend the feed particles and after flushing with CO_2 the bottle was sealed and incubated in a horizontal position at 39°C. An additional set of triplicate flasks were prepared as controls and to which no toxin was added. After thorough mixing, 20 ml samples were withdrawn for toxin analysis at various time intervals and extracted with three equal volumes of solvent ($CHCl_3$ for aflatoxin and zearalenone, ethyl acetate for all other toxins). The aqueous

layer was retained and the organic layer was collected in a 100 ml round bottom flask after passing through a bed of anhydrous Na_2SO_4. The solvent was evaporated by rotary vacuum evaporation and reconstituted in a small volume of extraction solvent before transferring to a half dram vial and further evaporation of the solvent under a stream of N_2. Toxins and any breakdown products were first analysed using the TLC procedure previously described (Section 5.2.3) and later quantitated using HPLC and GLC techniques described in Section 5.2.3 and 4.2.2 respectively.

The retained aqueous layer containing undigested lucerne was filtered through a previously dried and weighed 9 cm Whatman filter paper (grade 01) and the retained lucerne substrate was dried to constant weight at 70°C. The decrease in sample weight with time was used as an index of microbial activity.

7.2.3 Toxin degradation by artificial salivary buffer

Mycotoxin degradation in artificial salivary buffer was performed as in Section 7.2.2 but without added rumen fluid.

7.2.4 Toxin degradation by autoclaved whole rumen fluid

Mycotoxin degradation in autoclaved whole rumen fluid was performed as in Section 7.2.2 after cooling to 39°C.

7.2.5 Determination of percentage toxin recovery

In all toxin degradation investigations the percentage recovery of toxin was determined after analysing the amount of toxin recovered at time 0. Analytical techniques have been previously described (Sections 4.2 and 5.2.3). Subsequent toxin levels were adjusted to 100% recovery according to the determined percentage recovery value.

7.2.6 Isolation of bacterial and protozoal preparations from whole rumen fluid

Rumen fluid was collected as described in Section 7.2.2. Bacteria and protozoa were separated using a modification of the method of Pettersson *et al.* (1982). After centrifugation (200 x g, 10 min) of the whole rumen fluid, the protozoal pellet was resuspended in saliva buffer to the original volume. A bacterial preparation was obtained by adding dioctylsodium sulphosuccinate at 1 mg/ml to the supernatant obtained after centrifugation. Incubation of toxins and toxin analyses were performed as previously described (Sections 4.2, 5.2.3, 7.2.2) using buffered sterile-filtered rumen fluid as

a control. Cell-free rumen fluid was obtained after filtering whole rumen ingesta through a 90 mm membrane filter (0,22 μm) with a 75 mm AP-type prefilter (Millipore Corporation, Bedford, MA. (USA).

7.2.7 Bacterial and protozoal growth in the presence of T–2 toxin

Approximately 2 ℓ of whole rumen ingesta was obtained from four sheep fed lucerne hay. The ingesta was withdrawn from rumen cannulae using gentle suction on a sampling tube (i.d. = 20 mm). The rumen ingesta was collected in a 2 ℓ conical flask after filtering through two layers of cheesecloth. The flask was allowed to stand at 39°C in an anaerobic cabinet (65% N_2 – 30% CO_2 – 5% H_2 gas phase) for approximately 1 h during which time protozoa settled to the bottom of the flask. After removal of partially digested forage from the top of the flask, the contents were carefully decanted into a second flask until only the protozoal layer remained. This layer was then centrifuged (200 x g, 15 min) and after returning to an anaerobic cabinet and decanting the supernatant, the combined protozoal pellets were resuspended in 25 ml anaerobic diluent (protozoal inoculum). The decanted supernatant represented the bacterial inoculum to which was added dioctyl sodium sulphosuccinate (1 mg/ml).

Eight previously prepared sterile 4 oz McCartney flat bottles (300 ml) contained 200 ml sterile modified Medium 10 (Appendix IV) with 1% glucose as sole carbon and energy source. Streptomycin (2 mg/ml) and penicillin (2 mg/ml) were added to four of the bottles. To the antibiotic containing bottles was added 5 ml protozoal inoculum and 5 ml bacterial inoculum was added to the remaining four bottles. After pre-incubation at 39°C for 15 min, T–2 toxin (100 μg/ml in DMF) was added to two each of the media containing bacteria and protozoa and an equivalent volume of DMF was added to the remaining media. After thorough mixing, samples were taken for protein analysis (2 ml) using the method of Lowry, Rosebrough, Farr & Randall (1951) and estimation of protozoal numbers by diluting 1 ml in 9 ml formol saline (Appendix V). The numbers of entodiniomorph and holotrich protozoa were then determined by counting in a Nageotte chamber (0,50 mm, Walter Schrenck Erben, D–6238, Hofheim W. Germany) and observation under phase – contrast microscopy (Nikon Optiphot, 100 x magnification). Samples for all analyses were taken t_0 and after 6 h (t_6).

7.3 RESULTS AND DISCUSSION

All toxin concentration values have been expressed as a percentage of that originally present at t_0. The toxin concentration at t_0 is a measure of the efficiency of recovery

of toxin using methods described previously (Section 7.2.2). Average percentage recovery values determined in this way are presented in TABLE 14.

TABLE 14 Percentage recovery values of mycotoxins from whole rumen fluid

Mycotoxin	Concentration (μg/ml)	% recovery[a] efficiency
Aflatoxin B_1	10.0	85
Aflatoxin G_1	10.0	85
Patulin	10.0	87
Ochratoxin A	10.0	b
Zearalenone	10.0	b
T–2 Toxin	10.0	78
HT–2 Toxin	10.0	75
Deoxynivalenol	10.0	53
Diacetoxyscirpenol	10.0	80
Neosolaniol	10.0	63

a) Average values of all relevant experiments

b) Due to the qualitative nature of these results, recovery efficiencies of these toxins could not be determined.

7.3.1 Aflatoxin degradation studies

Using methods described previously (Section 7.2.2) the effect of whole rumen contents on the degradation of aflatoxins B_1 and G_1 when added at various concentrations was investigated. The results indicate (TABLE 15) that aflatoxin B_1 was not degraded by whole rumen contents. In comparison, data presented in TABLES 16 and 17 show a similar small reduction in recovery of aflatoxin B_1 in controls with buffer alone or with autoclaved rumen fluid. These data are in agreement with the work of Kiessling *et al.* (1982, 1984) who showed that ovine rumen fluid had no effect on the degradation of aflatoxin B_1.

Any increase in aflatoxin concentration with time is probably due to slight variation in recovery efficiency.

The use of percentage digestion of lucerne hay as an index of microbial activity showed many inconsistencies in results probably due to sampling problems. Despite these inconsistencies, the data presented in TABLE 15 suggest that some digestion did occur but that in all cases (except where aflatoxin B_1 was added at a concentration of 0,05 μg/ml)

TABLE 15 Aflatoxin B_1 degradation by whole ovine rumen contents

Time (h)	Repeated experiments	Concentration aflatoxin B_1 (μg/ml)[a]											
		10,0		Average %[b] digestion lucerne hay	1,0		Average % digestion lucerne hay	0,1		Average % digestion lucerne hay	0,05		Average % digestion lucerne hay
0	1	10,0	(10,0)	5,1	1,0	(1,0)	1,2	0,1	(0,1)	7,5	0,05	(0,05)	5,3
	2	10,0			1,0			0,1			0,05		
	3	10,0			1,0			0,1			0,05		
3	1	9,7			0,89			0,09			0,05		
	2	9,1	(9,5)	6,7	0,41*	(0,91)	9,5	0,09	(0,08	7,5	0,04	(0,04)	19,7
	3	9,7			0,93			0,08			0,04		
12	1	9,7			0,97			0,08			0,04		
	2	9,5	(9,6)	13,9	0,91	(0,93)	16,3	0,08	(0,08)	9,2	0,04	(0,04)	12,1
	3	9,6			0,91			0,08			0,04		
24	1	9,6			0,98			0,08			0,04		
	2	9,1	(9,2)	10,1	0,90	(0,92)	12,9	0,03*	(0,075)	8,5	0,04	(0,04)	18,9
	3	9,0			0,89			0,07			0,04		

a) Values presented are average values and have been presented to nearest significant figure
b) Average of three experiments to nearest significant figure. Time 0 valve represents amount recoverable before digestion
*) Results not included in average

TABLE 16 Mycotoxin breakdown after incubation in artificial salivary buffer

Time (h)	Toxin concentration (μg/ml)[a,b]									
	B_1	G_1	PAT	OCH[c]	Z[c]	T–2	HT–2	DON	DAS	NEO
0	10,0	10,0	10,0	10,0	10,0	10,0	10,0	10,0	10,0	10,0
1	9,6	9,8	9,8	10	10	9,5	9,4	9,8	9,6	9,6
3	9,2	9,6	9,7	10	10	9,7	9,4	9,7	9,6	9,2
12	9,2	9,1	9,7	10	10	9,4	9,5	9,2	9,4	9,3

a) Average values of experiment conducted in triplicate

b) t_o values adjusted to amount toxin added and subsequent values adjusted in accordance with percentage recovery

c) Approximate values determined after TLC and by comparison with standards of known concentration

KEY:

B_1	–	Aflatoxin B_1	HT–2	–	HT–2 toxin
G_1	–	Aflatoxin G_1	DON	–	Deoxynivalenol
PAT	–	Patulin	DAS	–	Diacetoxyscirpenol
OCH	–	Ochratoxin A	NEO	–	Neosolaniol
Z	–	Zearalenone			
T–2	–	T–2 toxin			

TABLE 17 Mycotoxin breakdown by autoclaved whole ovine rumen contents

Time (h)	Toxin concentration (μg/ml)[a,b]									
	B_1	G_1	PAT	OCH[c]	Z[c]	T–2	HT–2	DON	DAS	NEO
0	10,0	10,0	10,0	10,0	10,0	10,0	10,0	10,0	10,0	10,0
1	9,6	9,6	9,3	10	10	8,2	9,2	9,1	9,6	9,6
3	9,1	9,1	9,2	10	10	9,2	9,4	9,7	9,8	9,2
12	9,6	9,8	9,2	10	10	9,2	9,4	9,6	9,2	9,2

a) Average values of experiment conducted in triplicate.

b) t_o values adjusted to the amount toxin added and subsequent values adjusted in accordance with percentage recovery.

c) Approximate values determined after TLC and by comparison with standards of known concentration.

KEY:

B_1 – Aflatoxin B_1
G_1 – Aflatoxin G_1
PAT – Patulin
OCH – Ochratoxin A
Ż – Zearalenone
T–2 – T–2 Toxin
HT–2 – HT–2 toxin
DON – Deoxynivalenol
DAS – Diacetoxyscriponol
NEO – Neosolaniol

no noticeable digestion occurred after 12 h incubation, suggesting little microbial activity after this time.

It is generally accepted that under *in vitro* conditions protozoa remain viable for no longer than 6 h of incubation due to an accumulation of fermentation end products and a consequent reduction in pH.

Furthermore, since the experimental bottles used in these studies were opened to the atmosphere for sampling at timed intervals, before being regassed, and sealed, viability would be reduced further. Thus *in vitro* incubations of longer than 4–6 h are unlikely to represent the ruminal situation where conditions are favourable for the balanced growth of all groups of organisms. In their work in 1982 and 1984, Kiessling *et al.* monitored toxin degradation for periods of up to 3 h and thus avoided long term incubations and their inherent problems. Thus in further studies incubations were carried out over a maximum period of 12 h but particular attention was paid to data obtained within the first three hours.

Studies on the degradation of aflatoxin G_1 were performed at concentrations of 1,0 and 10,0 μg/ml because of limitations imposed by analytical techniques on the sensitivity of detection. The results show (TABLE 18) that no significant degradation of this toxin occurred in rumen fluid when compared to that in buffer alone (TABLE 16) or in autoclaved rumen fluid (TABLE 17). There were again variations in lucerne digestion data although consistently higher values were obtained at the lower toxin concentration which would indicate lower microbial intoxication. As in results obtained with aflatoxin B_1 there was little or no microbial activity after 12 h as measured by percentage digestion of lucerne.

The increase in concentration of T–2 toxin and deoxynivalenol (TABLE 17) probably occurred as a result of minor changes in the efficacy of solvent extraction.

These results, therefore, indicate that after ingestion by ruminants, aflatoxins B_1 and G_1 they are absorbed into the blood stream essentially unaltered. These results are in agreement with those of Kiessling *et al.* (1982, 1984) and Mathur *et al.* (1976b). However the observations that cattle up to 6 months of age with an incompletely developed rumen are more resistant than adult cattle (Loosemore & Markson, 1961) would appear to be independent of rumen function. Data concerning the resistance of sheep to aflatoxin poisoning is contradictory (Section 6.3) but from the results obtained, any resistance shown by sheep is also likely to be independent of rumen function unless variables such as diet composition promote the growth of a specific microorganism or group of microorganisms capable of aflatoxin degradation and which would not be present at such levels on other feed regimes. Factors affecting the breakdown of aflatoxin in the rumen were beyond the scope of the present

TABLE 18 **Aflatoxin G_1 breakdown by whole ovine rumen contents**

Time (h)	Repeated experiments	Aflatoxin G_1 concentration (μg/ml)[a]			
		10,0[b]	Average % digestion lucerne hay	1,0[b]	Average % digestion lucerne hay
0	1	10,0	7,2	1,0	10,1
	2	10,0		1,0	
3	1	9,1	12,2	0,91	16,2
	2	9,5		0,93	
12	1	8,7	12,7	0,89	19,2
	2	9,1		0,86	
24	1	8,8	13,3	0,86	21,4
	2	8,9		0,88	

a) Values presented are average values and have been presented to the nearest significant figure

b) Values expressed relative to corrected values at t_o

studies and therefore further investigation was not continued. It is however important to note that because dietary composition can affect the relative numbers of the rumen microbial population (Mackie, Gilchrist, Robberts, Hannah & Schwartz, 1978) relevant comparisons of data are difficult.

Because of the proven ability of the liver and specifically the hepatic microsomal mixed – function oxidase (Lynch *et al.*, 1973) to detoxify aflatoxin B_1 it is likely that this is the major organ of aflatoxin detoxification in ruminants and other animals.

As a result of variability in data of percentage digestion of lucerne in these studies, the addition of dried lucerne (0,5 g/100 ml) as substrate was continued in subsequent studies but determination of percentage digestion was not performed.

7.3.2 Degradation of ochratoxin, zearalenone and patulin

Previous work has shown that ochratoxin A can be degraded to ochratoxin α and phenylalanine (Hult *et al.*, 1976) and recent work showed that zearalenone can be degraded in *in vitro* studies with rumen fluid (Kallela & Vasenius, 1982).

Because previous work (Section 5.3) showed that patulin and zearalenone were present in feed commodities in Natal, similar investigations were conducted on these two toxins. The method used is described in Section 7.2.2.

Further discussion is based on average values obtained from three experiments performed in triplicate (TABLE 19). These results show that all three toxins were broken down to a large extent after 12 h incubation and that when added at 1,0 μg/ml (100 μg total) the average percentage degradation was was approximately 75%.

Although the concentration of ochratoxin could not be accurately determined, comparison with standards of known concentration showed that the degradation rates (as measured at 3 h) were approximately 194 μg/h per ℓ and 555 μg/h per ℓ when added at 1.0 and 10.0 μg/ml respectively. (Kiessling *et al.* (1984) found that the maximum rate of degradation of ochratoxin A was 155 μg/h per ℓ and 517 μg/h per ℓ when added at 0.2 and 4.7 μg/ml respectively. Because of the semi-quantitative nature of the present work, the difference in toxin concentrations used and lack of knowledge concerning the diets used in the work of Kiessling *et al.*, (1984) a direct comparison of results is not possible. However despite this, the degradation rates are of the same order when added at both

TABLE 19 **Degradation of patulin, ochratoxin A and zearalenone by whole ovine rumen contents**

Time (h)	Repeated experiments	Toxin concentration (μg/ml)[a,b]					
		Patulin		Ochratoxin A[c]		Zearalenone[c]	
		10,0	1,0	10,0	1,0	10,0	1,0
0	1	10,0	1,0	10,0	1,0	10,0	1,0
	2	10,0	1,0	10,0	1,0	10,0	1,0
	3	10,0	1,0	10,0	1,0	10,0	1,0
1	1	9,1	0,81	7,5	0,75	10,0	0,75
	2	9,9	0,70	10,0	0,5	10,0	0,75
	3	9,2	0,51	7,5	0,5	7,5	0,75
3	1	8,4	0,61	10,0	0,5	7,5	0,5
	2	8,1	0,41	7,5	0,5	5,0	0,5
	3	8,6	0,29	7,5	0,25	7,5	0,25
12	1	6,0	0,23	2,5	0,25	5,0	0,25
	2	6,5	0	5,0	0	5,0	0,25
	3	6,8	0	5,0	0	5,0	0,25

a) Values presented are average values and have been presented to the nearest significant figure

b) All values expressed relative to corrected values at t_o

c) Toxin concentration determined by comparison with standards of known concentration after TLC

high and low concentrations and both show higher degradation rates when toxin is added at higher concentrations. Under normal circumstances one would assume that the toxin degrading activity of the rumen was constant regardless of amount of toxin added. However the results obtained both in the current study and the work of Kiessling *et al.* (1984), do not support this.

It is possible that the addition of toxins at high concentration causes a change in the relative numbers of rumen microorganisms or in the enzyme make up of individual organisms. However further studies would have to be conducted before a satisfactory explanation of this phenomenon could be obtained.

The breakdown products of ochratoxin A were shown by Hult *et al.* (1976), to be ochratoxin α and phenylalanine. Kiessling *et al.* (1984) considered this as a detoxification mechanism which will therefore confer to ruminants increased resistance to ochratoxin A toxicoses.

As in ochratoxin A studies the use of semi-quantitative techniques in the analysis of zearalenone result in only very approximate values for the degradation rates of this toxin. When added at 1,0 μg/ml the degradation rate of zearalenone was the same as that for ochratoxin (194 μg/h per ℓ), as determined after incubation for 3 h. However this is more likely to be a reflection of analytical system employed. This result does show however that the rate of degradation is of the same order. When added at 10 μg/ml the approximate degradation rate of zearalenone was found to be 1 111 μg/h per ℓ and thus significantly higher than the rate when added at the lower concentration. It must be pointed out that very little degradation of zearalenone occurred after 3 h and that although the result presented is an average value, the rate of degradation would change by greater than 10% were visual estimations to be in error by selecting the second of two standards, the intensity of which varied slightly from that of the first.

In their studies in 1984, Kiessling *et al.* determined the degradation rate of zearalenone when added at 2,8 μg/ml to be 367 ± 83 μg/h per ℓ and 281 ± 80 μg/h per ℓ in the absence and presence of feed respectively. The latter result more closely approximates the value of 194 μg/h per ℓ obtained in the present studies. Again the rate of degradation in this study was higher at the higher toxin concentration and this phenomenon has been previously discussed in relation to ochratoxin breakdown.

In their studies, Kiessling *et al.* (1984) showed that the rumen protozoa were largely responsible for degradation of zearalenone. No such differentiation was made in the present studies.

Kallela & Vasenuis (1982) showed that when added at 3 μg/ml, zearalenone was degraded by approximately 17% after incubation for 4 h with bovine rumen fluid. This rate is significantly lower than the values determined in the present study and by Kiessling *et al.* (1984). However the use of bovine rumen fluid precludes direct comparison of results.

Kiessling *et al.* (1984) showed that zearalenone was degraded to α - zearalenol and β - zearalenol and concluded that such degradation in combination with the action of liver metabolism would lead to a product which would be less toxic to the animal.

No previous studies have been performed on the degradation of patulin by rumen fluid *in vitro*. The results obtained (TABLE 19) show that when added at 1,0 μg/ml no patulin remained in two out of three studies after 12 h incubation. The actual rates of degradation (as measured at 3 h) were 187 μg/h per ℓ and 544 μg/h per ℓ when added at 1,0 and 10,0 μg/h per ℓ respectively and are therefore very similar to the rates obtained for the degradation of ochratoxin A. Breakdown products were not identified and as with ochratoxin A and zearalenone higher rates of degradation were observed at higher toxin concentrations.

7.3.3 Trichothecene degradation by whole ovine rumen contents

Because ruminants and especially sheep have been shown to be particularly resistant to trichothecene toxicoses (Section 2.8) the majority of toxin degradation studies by whole ovine rumen fluid were performed on this group of toxins. The methods employed have been described previously (Section 7.2.2).

Initial studies (TABLE 20) show that after 3 h incubation an average 3,35 μg/ml of T–2 toxin remained (as determined from eight separate studies conducted in triplicate). This represents a T–2 toxin degradation rate of approximately 2 220 μg/ℓ per h. In similar studies, Kiessling *et al.* (1984) calculated a T–2 toxin degradation rate of 1 730 μg/h per ℓ using ovine rumen fluid. Although there is an approximate 20% difference in these rates, the values are of the same order when compared to the degradation rates of ochratoxin and patulin. Therefore, despite differences in experimental design similar trends in T–2 toxin degradation are evident.

TABLE 20 **Degradation of T–2 toxin by whole rumen contents**

Time (h)	T–2 toxin concentration (μg/ml)[a,b]							
	10,0	10,0	10,0	10,0	10,0	10,0	10,0	10,0
0	10,0	10,0	10,0	10,0	10,0	10,0	10,0	10,0
1	5,3	6,2	5,3	5,5	6,8	5,1	4,2	4,8
3	3,9	4,4	3,4	3,4	3,9	4,9	1,7	1,2
12	0,8	1,4	0,7	1,7	1,9	4,6	0,3	0

a) Average values of experiment conducted in triplicate

b) Values expressed relative to corrected values at t_o

It is apparent from these results that T–2 toxin is much more susceptible to breakdown by rumen fluid than other mycotoxins previously tested in this study and is therefore an important factor in conferring T–2 toxin resistance to sheep.

Because of results obtained in these studies, similar studies were conducted on the degradation of a number of different trichothecenes by whole ovine rumen fluid.

The following discussion is based on the average degree of toxin degradation after 3 h incubation and as determined from two experiments performed in triplicate (TABLE 21).

The rate of T–2 toxin degradation was calculated as 2 000 μg/h per ℓ, indicating that the rumen fluid activity was very similar to that calculated in previous experiments (2 200 μg/h per ℓ).

The rate of HT–2 toxin degradation was approximately 1 830 μg/h per ℓ indicating that the activity of rumen fluid against HT–2 toxin is approximately 83% of the activity against T–2 toxin.

Neosolaniol was not degraded and the degradation rates for deoxynivalenol and diacetoxyscirpenol were 2 220 and 2 067 μg/h per ℓ respectively.

These latter two results contrast markedly with those of Kiessling *et al.* (1984) who were unable to show any degradation of deoxynivalenol by ovine rumen fluid and determined the rate of degradation of diacetoxyscirpenol to be approximately 845 μg/h per ℓ. However in similar studies with ovine rumen fluid King *et al.* (1984) showed that nearly all deoxynivalenol was transformed to a single breakdown product within 24 h when added at levels of up to 10 μg/g. No degradation rates were calculated in this investigation.

The discrepancy in the above results with those of Kiessling *et al.*, (1984) may be due to the low recovery rate of deoxynivalenol as determined in this study (TABLE 14).

Comparison of data in Tables 16, 17 and 21 shows that the factor responsible for trichothecene degradation is likely to be the rumen microflora as the extent of degradation of all but neosolaniol is much greater upon incubation with whole ovine rumen contents than with either artificial salivary buffer or autoclaved ovine rumen contents.

Thus it has been shown that T–2 toxin, HT–2 toxin, deoxynivalenol and diacetoxyscirpenol can all be degraded to varying extents by whole ovine rumen contents. In these investigations, however, no attempt was made to identify breakdown products and therefore further studies were conducted to identify breakdown products of T–2 toxin after incubation in ovine rumen fluid.

TABLE 21 Degradation of a number of different trichothecene toxins by whole rumen contents

Time (h)	Toxin concentration (μg/ml)[a,b]									
	T–2 Toxin		HT–2 Toxin		Deoxynivalenol		Diacetoxyscirpenol		Neosolaniol	
	10,0	10,0	10,0	10,0	10,0	10,0	10,0	10,0	10,0	10,0
0	10,0	10,0	10,0	10,0	10,0	10,0	10,0	10,0	10,0	10,0
1	6,7	5,2	9,2	8,6	6,3	3,2	5,5	5,5	9,7	9,7
3	3,8	4,2	4,6	4,4	3,9	2,8	3,9	3,7	9,5	9,7
12	2,0	1,8	0,9	2,9	1,3	0,9	1,2	0,9	9,3	9,2

a) Average values of two experiments conducted in triplicate

b) Values expressed relative to corrected values at t_o

7.3.4 Identification of T–2 toxin breakdown products after incubation in whole ovine rumen fluid

The methods used in this investigation have been described previously (Section 7.2.2). Three separate experiments were performed in triplicate and further discussion of results is based on average values obtained from these investigations.

The rate of T–2 toxin degradation was calculated as approximately 2 055 μg/h per ℓ (TABLE 22) thus showing close agreement with previous estimations (2 220 and 2 000) and indicating that provided feeding regimes and sampling times are identical then repeatable results can be obtained using separate batches of rumen fluid. In these studies the same four sheep fed a constant diet of lucerne hay over a prolonged period were used for the supply of all rumen fluid and this may also be an important factor contributing to the reproducibility of results.

Two breakdown products were formed and identified as HT–2 toxin and T–2 triol using previously described techniques. The mechanism of T–2 toxin breakdown therefore involves deacetylation at the R_2 (C–4) and R_3 (C–15). (See FIG. 3 for structures).

The average total amount of toxin present after 3 h (T–2 toxin, HT–2 toxin and T–2 triol) represents approximately 92% of the T–2 toxin added at time 0 (after adjusting for recovery efficiency). It is possible that the remaining 8% is present in the form of another degradation product which was not detected using the methods employed but is more likely due to experimental error and variation in recovery efficiency.

From the results it would appear that T–2 toxin is first deacetylated to HT–2 toxin which is then further deacetylated to T–2 triol. Assuming no further degradation of T–2 triol, the rate of formation of this breakdown product was calculated as 266,7 μg/h per ℓ which is significantly lower than the rate of degradation of T–2 toxin. This would imply that the enzyme or enzymes involved in the degradation of T–2 toxin have variable substrate specifities and that the Km of a particular reaction is dependent on the position of the acetyl group on the trichothecene nucleus. Because of the order of formation of breakdown products it would seem that the acetyl group at C–4 is more susceptible to attack than that at C–15.

Kiessling *et al.* (1984) found that T–2 toxin was deacetylated to HT–2 toxin only and concluded that this represented a detoxification process due to the decreased lipophilic nature of HT–2 toxin and therefore the extent of absorption into the host which would therefore be absorbed to a lesser extent into the host animal's bloodstream.

TABLE 22 **Breakdown produced after incubation of T–2 toxin with whole rumen contents at 39°C**

Time (h)	Toxin concentration (μg/ml)[a,b]								
	Experiment 1			Experiment 2			Experiment 3		
	T–2	HT–2	Triol	T–2	HT–2	Triol	T–2	HT–2	Triol
0	10,0	0	0	10,0	0	0	10,0	0	0
1	5,8	2,2	0,5	5,1	3,9	0,2	5,1	2,6	0,4
3	4,2	5,0	0,8	3,6	4,5	0,8	3,7	4,1	0,8
12	2,9	5,9	1,2	1,4	6,1	1,6	1,1	6,1	1,7

a) Average values of experiment conducted in triplicate

b) Values expressed relative to corrected values at t_o

KEY:

T–2 – T–2 toxin
HT–2 – HT–2 toxin
Triol – T–2 triol

The further degradation of the HT–2 toxin to T–2 triol as determined in these studies would thus represent a more effective detoxification process if this protective mechanism is based on the lipophilic nature of the breakdown product.

However, in studies conducted by Yoshizawa *et al.* (1981) HT–2 toxin and neosolaniol, amongst other metabolites, were found in the blood of a cow fed a diet containing T–2 toxin. Because of the relatively rapid rate of breakdown of T–2 toxin in the rumen it is unlikely that much unmetabolised T–2 toxin will be absorbed into the blood (although this will be dependent on the initial level of toxin). This suggests that the reduced lipophilic nature of the breakdown product does not preclude absorption by the host animal.

No neosolaniol was detected in the present investigations.

7.3.5 T–2 toxin degradation by protozoal, bacterial and cell-free preparations of ovine rumen fluid

The previous investigation has shown that T–2 toxin can be degraded to HT–2 toxin and T–2 triol by whole ovine rumen fluid. A subsequent investigation was therefore performed in order to determine the active component of rumen fluid responsible for this degradation. The methods employed have been previously described (Section 7.2.2).

Results show (TABLE 23) that there was no toxin-degrading activity in the cell-free preparation of rumen fluid but that both bacterial and protozoal preparations were capable of T–2 toxin degradation. This further indicates that the enzyme or enzymes responsible for T–2 toxin degradation are cell bound.

Further discussion is based on average values obtained from two experiments performed in triplicate and show that toxin degrading activity is highest in protozoal preparations (TABLE 23). Degradation rates were 2 083 µg/h per ℓ and 1 780 µg/h per ℓ for the protozoal and bacterial preparations respectively. (Because of the inter action between bacteria and protozoa in the rumen the sum of these rates does not necessarily give the overall degradation rate). In similar studies, Kiessling *et al.* (1984) showed that bacterial activity against T–2 toxin was approximately 45% of the protozoal activity when T–2 toxin was added at 20 mg/l. In the present study, bacterial activity was shown to be approximately 85% of the protozoal activity when T–2 toxin was added at 10 mg/l. As already mentioned no indication is given of the diet or time of sampling in the work of Kiessling *et al.* (1984) and as these factors will affect the microbial

TABLE 23 Degradation of T–2 toxin by protozoal, bacterial and cell-free preparations from whole rumen fluid

Time (h)	T–2 Toxin concentration (μg/ml)[a,b]					
	Experiment 1			Experiment 2		
	Bacterial preparation	Protozoal preparation	Cell-Free preparation	Bacterial preparation	Protozoal preparation	Cell-Free preparation
0	10,0	10,0	10,0	10,0	10,0	10,0
1	6,7	6,9	10,0	6,4	5,8	9,7
3	4,8	4,1	9,6	4,5	3,4	9,9
12	2,8	1,5	9,6	2,8	1,9	9,7

a) Average values of experiment conducted in duplicate

b) Values expressed relative to corrected values at t_o

TABLE 24 **Susceptibility of bacterial and protozoal preparations from ovine rumen fluid preparations to T–2 toxin as determined by total protein measurement**

Time (h)	Protein concentration (μg/ml)							
	Bacteria				Protozoa			
	– Toxin		+ Toxin		– Toxin		+ Toxin	
	1[a]	2[a]	1	2	1	2	1	2
0	1 303	723	1 156	799	1 189	1 120	175	1 435
6	1 832	1 381	1 788	1 516	1 462	2 194	210	1 623
	(+529)[b]	(+658)	(+632)	(+717)	(+273)	(+1074)	(+35)	(+188)
	Av^{GE}	= 593.5	Av^{GE}	= 674.5	Av^{GE}	= 673.5	Av^{GE}	= 111.5

a) Experiment conducted in duplicate

b) Bracketed figures show change in protein concentration

composition of rumen fluid, direct comparisons cannot be made between the two values. However it is clear that the enzymes responsible for T–2 toxin degradation are cell bound and that the highest activity appears to reside in the protozoa.

As previously described (Sections 2.6.1 and 2.6.3) the trichothecenes show greater toxicity to eukaryotic than prokaryotic cells. In order to investigate the toxicity of T–2 toxin to bacterial and protozoal preparations of ovine rumen fluid a further investigation was performed using methods described in Section 7.2.7. The results (TABLE 24) show that after averaging changes in protein concentration there was a significant decrease in total protozoal protein in the presence of T–2 toxin (111,5 μg/ml) compared with that in the absence of T–2 toxin (673,5 μg/ml). This represents an approximate 83% decrease in total protozoal protein due to the presence of T–2 toxin. Whereas the addition of toxin to the bacterial preparation was associated with an increase in the total amount of protein present at 6 h by approximately 14%. Thus T–2 toxin is significantly more toxic to the protozoa than the bacteria and in the latter case may even be assimilated by the rumen bacteria and serve as an energy source. This has been previously demonstrated in *Curtobacterium sp.* (Ueno *et al.*, 1983). Certainly, no inhibition of bacterial growth is shown by T–2 toxin.

Direct enumeration of the protozoa in the presence and absence of T–2 toxin showed that the average decrease in numbers in the presence of toxin was approximately 22% greater (TABLE 25) than the decrease in the absence of toxin. Although there was a decrease in numbers even in the absence of toxin this is probably a consequence of the extreme difficulty encountered in maintaining viable rumen protozoa over prolonged periods. The ratios of holotrich to entodiniomorph protozoa showed little change in the presence of toxin suggesting that these two major groups of rumen protozoa are equally susceptible to the effects of T–2 toxin.

7.4 SUMMARY

Studies on toxin degradation by whole ovine rumen fluid have shown that all of the toxins examined except aflatoxins B_1 and G_1 and neosolaniol were degraded to varying extents. Despite variations in experimental design, many of the toxin-degradation rates were similar to values reported in the literature and showed that the degradation of T–2 toxin occurred much more rapidly than the degradation of other toxins. T–2 toxin was shown to be broken down to HT–2 toxin and T–2 triol by successive deacetylations at C–4 and C–15 on the trichothecene nucleus and that the activity was enzyme

TABLE 25 Toxicity of T–2 toxin (10 μg/ml) to a protozoal preparation from ovine rumen fluid as determined by direct counting

Time (h)	Protozoal concentration (count/ml)							
	– Toxin				+ Toxin			
	1[a]		2[a]		1		2	
0	38,0	(28,9 : 71,1)[b]	34,0	(32,3 : 67,7)	39,8	(28,6 : 71,4)	39,6	(29,8 : 70,2)
6	21,8	(29,3 : 70,7)	20,4	(30,6 : 69,4)	20,8	(26,3 : 73,7)	20,2	(26,4 : 73,6)
	[16,2][c]		[13,6][c]		[19,0][c]		[19,4][c]	
	[AV^{GE} = 14,9][c]				[AV^{GE} = 19,2][c]			

a) Experiment conducted in duplicate

b) Bracketed figures ( ) show ratio holotrich protozoa : Entodiniomorph protozoa

c) Bracketed figures [ ] show decrease in protozoal numbers between 0 and 6 h and derived average values

associated and cell bound. The rumen protozoa were shown to have a slightly greater T–2 toxin degrading ability (17%) than the bacteria but were also shown to be more susceptible to the toxic effects of T–2 toxin than the rumen bacteria.

Because it is impossible to prepare a protozoal preparation from rumen fluid that is free from contaminating bacteria and because of the contribution of these bacteria to the metabolic process being monitored, further investigations were performed in order to determine the ability of pure cultures of rumen bacteria to degrade trichothecene degradation.

CHAPTER 8

TOXIN DEGRADATION BY PURE CULTURES OF RUMEN BACTERIA

8.1 INTRODUCTION

The degradation of a number of mycotoxins by whole ovine rumen contents as well as by protozoal and bacterial preparations from ovine rumen fluid has been demonstrated (Chapter 7). However, sterile-filtered rumen fluid preparations did not possess toxin-degrading properties, suggesting that the toxin degrading factors are cell-bound. Furthermore the lack of toxin-degrading activity in autoclaved rumen fluid suggests that these factors are enzymes.

In view of these results and because studies with whole rumen fluid do not facilitate investigations on toxicity to individual species within the microbial populations, a number of further experiments were performed in order to measure the toxicity of mycotoxins to various pure cultures of anaerobic rumen bacteria and to determine any toxin-degrading action of the same bacteria. Especial attention was paid to studies with trichothecenes.

8.2 METHODS AND MATERIALS

8.2.1 Chemicals

All solvents and reagents were of analytical grade and have been noted in the text, where necessary. Bacterial cultures were obtained from the Anaerobic Microbiology Division, LMCB, CSIR and Rumen Biochemistry section, ADSRI at Onderstepoort, South Africa and were maintained on agar slopes above liquid N_2 at approximately -80°C.

8.2.2 Bacterial strains

Butyrivibrio fibrisolvens strains have been previously characterised (Shane, Gouws & Kistner, 1969). The other bacteria utilised were *Anaerovibrio lipolytica* strain 5 S (Henderson & Hodgkins, 1973) and *Selenomonas ruminantium* (ATCC 1920 S).

8.2.3 Preparation of anaerobic media for growth and toxicity studies

Narrow neck 1 oz McCartney bottles equipped with tight-fitting rubber bungs (Sarmcol 254, Howick, Natal), were gassed for 3 mins with anaerobic gas mixture (30% CO_2 : 65% N_2 : 5% H_2) by inserting a narrow needle attached to the gas line into the McCartney bottle. Gas flow rate was metered via flow meters. Once gassed, the rubber bungs were firmly secured using a metal screw cap with holes for media injection.

These bottles were then autoclaved at 121°C for 30 mins. Separately prepared medium was sterilised at 121°C for 30 mins in a 500 ml Schott bottle equipped with a gas inlet line and medium delivery line, to the end of which was attached a 25 ml stainless steel repeating syringe (Socorex, France). After sterilisation, the medium was allowed to cool while gassing with anaerobic gas mixture. Once cool, the medium was dispensed in 10 ml volumes via the syringe into the previously sterilised and gassed 1 oz McCartney bottles.

Maintenance slopes were prepared using the same medium with inclusion of 2,0% (w/v) agar. After sterilisation the bottles were racked at a slight angle to the horizontal.

8.2.4 Preparation of bacterial inoculum for use in small-scale batch experiments

Agar slopes and liquid media were prepared as in Section 8.2.2. Stock cultures of bacteria maintained on maintenance agar slopes (Appendix VI) and stored above liquid nitrogen at approximately -80°C were allowed to thaw for approximately 3 h. To these slopes was added 2 ml anaerobic diluent (Appendix VII) via a sterile 2 ml syringe Becton, Dickinson & Co., Parsippany, N.J. USA) and the bottle was gently shaken to suspend the bacteria. One ml of bacterial suspension was withdrawn into the syringe and 0,2 ml used to inoculate each of three previously prepared maintenance agar slopes. After overnight incubation at 39°C the surface of the slopes and water of syneresis were checked for visible growth and after replacing one slope in liquid nitrogen, a further 2 ml anaerobic diluent was added to a second slope showing bacterial growth. After gently shaking the slope to suspend the growing bacteria, 1 ml was withdrawn into the syringe and 0,2 ml used to inoculate each of three 1 oz McCartney bottles containing 10 ml sterile modified medium 10 (Appendix IV). Three serial transfers were completed and resulting inoculum used for further studies.

8.2.5 Preparation of medium for large-scale batch culture of bacteria

Ten litres of modified medium 10 minus $NaHCO_3$ and reducing agent were dispensed into each of two 20 ℓ aspirator bottles. These were tightly sealed with rubber bungs equipped with ports for gassing, medium transfer, medium dispensing and gas venting. The aspirator bottles were then sterilised in an automatic autoclave (Drayton Castle, Middlesex, England) equipped with a temperature probe which was inserted into a third 20 ℓ aspirator bottle containing 10 ℓ water. This acted as a temperature monitor in the centre of the fluid body and controlled the sterilisation programme. After sterilisation the aspirator bottles were allowed to cool to approximately 50°C in the autoclave and were then removed. The medium was then transfered from one bottle to

the other using anaerobic gas mixture to provide a positive pressure. Reducing agent (Appendix IX) and $NaHCO_3$ (Appendix X) were then added by injection through a septum in the medium transfer line. The complete medium was then gassed with anaerobic gas mixture at a pressure of approximately 3,4 kPa for a minimum of 12 h.

8.2.6 Preparation of inoculum for large-scale batch culture of bacteria

Modified medium 10 (200 ml) was prepared and sterilised in a Schott bottle with screw cap and rubber seal equipped with lines for gassing, medium inoculation, medium transfer and venting. After gassing the medium for approximately 1 h, bacterial inoculum (10 ml) (prepared as in Section 3.8) was added to the medium by means of 20 ml sterile syringe (Summit, interchangeable) and the medium incubated at 39°C in a temperature-controlled water bath until sufficient growth had occurred (OD ≈ 0,7 – 0,8) for transfer to 20 ℓ medium.

8.2.7 Harvesting of bacterial cells grown in large-scale batch culture

Bacterial inoculum prepared as in Section 8.2.3 was transferred via the medium transfer line, to 20 ℓ medium prepared as in Section 8.2.4 using anaerobic gas mixture to create a positive pressure. Samples were then taken at regular time intervals via the medium dispensing line until an OD of 0,8 – 1,0 had been attained (equivalent to late log phase). The medium dispensing line was then connected to the inlet line of a zonal rotor with eight tube continuous flow system (Szent-Gyorgy & Blum) and the medium was centrifuged at 4°C and 27 500 x g at a flow rate of approximately 1,5 ℓ/h in a Servall refrigerated/automatic centrifuge, model RC–2 (Ivan Sorvall Inc, Connecticut, USA). After centrifugation, the rotor tubes were transferred to an anaerobic cabinet, model 1 024 (Forma Scientific, Ohion, USA) and the pelleted bacteria washed into a 100 ml screw-cap bottle with anaerobic diluent (Appendix VII). The bottle was tightly sealed and the bacteria stored at -10°C for further use.

8.2.8 Isolation of tri-butyrin hydrolysing bacteria

Approximately 1 ℓ of whole rumen contents were withdrawn from the rumen of a fistulated sheep using a sampling tube (ID 20 mm). Samples were obtained by applying gentle suction to the tube by mouth. After filtering the rumen contents through two layers of cheesecloth into a 1 ℓ conical flask, the flask was transferred to an anaerobic cabinet and a 1 ml sample serially diluted to 10^{-8} in anaerobic diluent. Using a repeating micropipette (Eppendorf Multipette 4780) ten volumes of 20 µl each of the 10^{-6} – 10^{-8} dilutions were pipetted through a template onto previously prepared

dishes containing modified medium 10 plus tributyrin (1%) as sole carbon and energy source. After incubation at 39°C for four days in an anaerobic environment in screw cap jars, individual colonies were transferred to slopes of maintenance medium and after overnight incubation were stored at approximately -80°C over liquid nitrogen.

8.2.9 Growth and toxin degradation by pure cultures of rumen bacteria

Modified medium 10 was prepared as described previously (Section 8.2.2). After sterilisation, mycotoxins prepared as concentrated solutions in DMF were injected into the medium-containing McCartney bottles to the required concentration. Medium inoculated with DMF only, served as a control. All tests and controls were prepared in triplicate. After pre-equilibration to 39°C in a temperature-controlled water bath, each bottle was inoculated with 0,2 ml bacterial inoculum (prepared as in Section 8.2.3) and the OD immediately determined. According to the total number of bottles used, subsequent OD readings were taken at 15, 20 or 30 min intervals until stationary phase was reached. Upon reaching stationary phase, each bottle was opened and the bacterial suspension containing toxin was extracted three times with an equal volume of solvent ($CHCl_3$ for aflatoxin and ethyl acetate for trichothecenes). After evaporation of the solvent at 60°C under a stream of N_2 and reconstitution in 200 μl ethyl acetate the extract was analysed by GLC methods described in Section 4.2.2.

Optical density data was entered onto a computer (Olivetti P6060) and a linear regression programme used to calculate growth rate and its correlation coefficient.

8.3 RESULTS AND DISCUSSION

To the authors knowledge there has been only one report of mycotoxin toxicity studies with pure culture of anaerobic bacteria from the rumen. (Mathur *et al.*, 1976b).

Data presented in TABLE 26 show average percentage recovery values of various mycotoxins, calculated from results obtained in relevant experiments.

Studies on the susceptibility of *Butyrivibrio fibrisolvens* strain CE 51 to aflatoxin B_1 toxicity (TABLE 27) showed that aflatoxin B_1 had no effect on the growth rate of this bacterium. Because of the possible effect of a number of variables, including the growth status of inoculum, on the lag time, variation in lag time (TABLE 27) is not considered to be significant. Previous work (Mathur *et al.*, 1976b) has shown that aflatoxin B_1 altered the morphology and physiology of *Streptococcus bovis* but did not report on the effects of aflatoxin B_1 on the growth rate of this bacterium. Engel & Hagemeister (1978) showed that 40% of added aflatoxin B_1 was degraded by bovine rumen contents *in vitro*.

TABLE 26 Percentage recovery of mycotoxins in pure culture investigations with *B. fibrisolvens* strain CE 51 grown in medium 10 with added glucose (1% w/v)

Mycotoxin	Concentration (μg/ml)	% Recovery[a]
Aflatoxin B_1	10,0	86
T–2 Toxin	10,0	76
HT–2 Toxin	10,0	78
Acetyl T–2 toxin	10,0	76
Deoxynivalenol	10,0	59
Diacetoxyscirpenol	10,0	76
Verrucarin A	10,0	61
Zearalenone	10,0	b
Ochratoxin A	10,0	b

a) Average % recovery calculated from all experiments conducted with each particular toxin

b) Due to semi-quantitative nature of analysis, recovery efficiencies were not calculated

TABLE 27 The effect of aflatoxin B_1 addition (10 μg/ml) on the growth rate (μ) of different strains of Butyrivibrio fibrisolvens grown on medium 10 containing 1% w/v glucose

Strain	+ Aflatoxin B_1					– Aflatoxin B_1				
	μ	$\bar{\mu}$	r	$\bar{r}$	AV^{GE} LAG TIME (h)	μ	$\bar{\mu}$	r	$\bar{r}$	AV^{GE} LAG TIME (h)
CE 46	0,65		0,996			0,64		0,998		
	0,70	0,67	0,996	0,997	4,2	0,64	0,64	0,999	0,998	3,8
	0,65		0,998			0,65		0,998		
CE 51	0,62		0,87			0,59		0,998		
	0,50	0,54	0,993	0,992	4,6	0,45	0,53	0,996	0,996	4,6
	0,49		0,996			0,55		0,995		
CE 52	0,51		0,996			0,59		0,997		
	0,57	0,53	0,998	0,997	1,3	0,45*	0,53	0,950*	0,988	2,0
	0,50		0,996			0,55	(0,57)[a]	0,995	(0,996)[a]	
CE 56	0,45		0,997			0,48		0,990		
	0,52	0,48	0,995	0,996	3,2	0,47	0,49	0,978	0,985	3,7
	0,47		0,995			0,51		0,988		

a) Bracketed values are average values when results marked with * have not been included in mean

KEY : μ – Specific growth rate (h^{-1})
$\bar{\mu}$ – Average specific growth rate (h^{-1}–
r – Correlation coefficient
$\bar{r}$ – Average correlation coefficient

However in previous work in the present study (Section 7.3.1) and in work by Kiessling *et al.* (1984) no degradation of aflatoxin B_1 was shown with ovine rumen contents *in vitro*. Because of the use of rumen contents from different test animals, a direct comparison of results cannot be made, however assuming that loss of aflatoxin B_1 in studies with *B. fibrisolvens* CE 51 (TABLE 28) is due to experimental procedure it is clear that this bacterium cannot breakdown aflatoxin B_1 during growth.

TABLE 28 **The susceptibility of aflatoxin B_1 to degradation by four strains of *Butyrivibrio fibrisolvens* grown on 1% (w/v) glucose**

Strain	% toxin remaining after incubation to stationary phase[a]
CE 46	95
CE 51	91
CE 52	98
CE 56	95

a) Average value of experiment conducted in triplicate

Studies on the toxicity of T–2 toxin to four strains of *B. fibrisolvens* (TABLE 29) showed that this toxin had no significant effect on the growth rate of two of the bacteria strains (CE 52, CE 56) but that in strains CE 46 and CE 51 there was a marked increase in growth rate. Previous studies with *Curtobacterium sp.* (Ueno *et al.*, 1983) showed that this bacterium could assimilate T–2 toxin when present as a sole carbon source and identified HT–2 toxin and T–2 triol as breakdown products. Studies on the assimilation of T–2 toxin by *B. fibrisolvens* were not conducted in the present investigation and it is unlikely that in the presence of 1% glucose, T–2 toxin would be utilised as a source of carbon. However if rumen bacteria have the ability to assimilate T–2 toxin this would be an important factor conferring resistance to trichothecene toxins in ruminants.

Data presented in TABLE 30 shows that in T–2 toxin degradation studies with *B. fibrisolvens*, HT–2 toxin, T–2 triol and neosolaniol were produced to varying degrees by the four strains tested. In similar studies on the effect of T–2 toxin on *B. fibrisolvens* strains grown on cellobiose, the same breakdown products were produced but for unknown reasons the relative proportions were very different (TABLE 31). In the latter studies there was no significant effect of T–2 toxin on the growth rate of the same four bacterial strains (TABLE 32) although the growth rate on 1% (w/v) cellobiose (TABLE 32) was consistently lower than that on 1% (w/v) glucose (TABLE 29). This is to be expected since glucose

TABLE 29 The effect of T–2 toxin on the growth rate (μ) of different strains of *Butyrivibrio fibrisolvens* grown on 1% (w/v) glucose

Strain	+ T–2 Toxin (10 μg/ml)					– T–2 Toxin				
	μ	$\bar{\mu}$	r	$\bar{r}$	AV^{GE} lag time (h)	μ	$\bar{\mu}$	r	$\bar{r}$	AV^{GE} lag time (h)
CE 46	0,73		0,999			0,68		0,999		
	1.07	0,90	0,963	0,981	6,3	0,63	0,69	0,996	0,994	5,5
	1,50*		0,707			0,77		0,989		
CE 51	0,75		0,999			0,05*		0,429		
	0,76	0,76	0,992	0,995	6,2	0,64	0,64	0,998	0,997	4,4
	1,06*		0,892			0,64		0,996		
CE 52	0,84*		0,997			0,58		0,981		
	0,57	0,61	0,990	0,992	1,6	0,58	0,60	0,993	0,989	0,6
	0,64		0,994			0,64		0,993		
CE 56	0,35*		0,815			0,53		0,982		
	0,43	0,51	0,993	0,992	0,8	0,51	0,54	0,999	0,992	0,3
	0,59		0,991			0,59		0,995		

* Results not included in average

KEY: μ – Specific growth rate (h^{-1})
$\bar{\mu}$ – Average specific growth rate (h^{-1})
r – Correlation coefficient
$\bar{r}$ – Average correlation coefficient

TABLE 30 **Degradation of T–2 toxin by four strains of *Butyrivibrio fibrisolvens* when grown on 1% (w/v) glucose**

Strain	Toxin concentration (% of added T–2 toxin)[a]				
	T–2 toxin	HT–2 toxin	T–2 triol	Neosolaniol	Total
CE 46	69	16	3	9	97
CE 51	57	22	3	10	92
CE 52	48	33	12	0	93
CE 56	28	38	9	14	89

a) Average value of experiment conducted in triplicate

TABLE 31 **Degradation of T–2 toxin by four strains of *Butyrivibrio fibrisolvens* when grown on 1% (w/v) cellobiose**

Strain	Toxin concentration (% of added T–2 toxin)[a]				
	T–2 toxin	HT–2 toxin	T–2 triol	Neosolaniol	Total
CE 46	61	12	14	0	87
CE 51	60	19	5	7	91
CE 52	75	13	5	0	93
CE 56	67	8	6	9	90

a) Average value of experiment conducted in triplicate

TABLE 32 The effect of T–2 toxin on the growth rate (μ) of different strains of *Butyrivibrio fibrisolvens* grown on 1% (w/v) cellobiose

Strain	+ T–2 Toxin (10 μg/ml)					– T-2 Toxin				
	μ	$\bar{\mu}$	r	$\bar{r}$	AV^{GE} lag time (h)	μ	$\bar{\mu}$	r	$\bar{r}$	AV^{GE} lag time (h)
CE 46	0,64		0,995			0,64		0,998		
	0,62	0,64	0,993	0,994	8,6	0,65	0,64	0,999	0,998	7,9
	0,65		0,993			0,64		0,998		
CE 51	0,53		0,998			0,59		0,998		
	0,52*	0,54	0,967	0,994	8,9	0,49	0,54	0,996	0,996	6,7
	0,56		0,990			0,55		0,995		
CE 52	0,56		0,997			0,59		0,997		
	0,58	0,57	0,990	0,994	3,3	0,45*	0,57	0,950	0,996	2,1
	0,56		0,994			0,55		0,995		
CE 56	0,51		0,997			0,48		0,990		
	0,46	0,47	0,998	0,997	2,8	0,47*	0,50	0,978	0,989	2,0
	0,44		0,996			0,51		0,988		

* Results not included in average

KEY: μ – Specific growth rate (h^{-1})
$\bar{\mu}$ – Average specific growth rate (h^{-1})
r – Correlation coefficient
$\bar{r}$ – Average correlation coefficient

can be more rapidly fermented than cellobiose. In both studies the lag time was greater when cells were grown in the presence of T–2 toxin. When grown on 1% (w/v) glucose, the lag time in the presence of T–2 toxin was between 13 and 62% longer than in the absence of toxin and when grown on 1% (w/v) cellobiose the lag time in the presence of toxin increased by between 8 and 36% depending on the strain. Thus it is possible that the enzymes responsible for T–2 toxin degradation are inducible rather than constitutive.

It is interesting to note that in both investigations neosolaniol was produced as well as HT–2 toxin and T–2 triol. This suggests that T–2 toxin can be degraded in two ways. The first by a deacetylation at C–4 to produce HT–2 toxin and subsequent deacetylation at C–15 to produce T–2 triol while the production of neosolaniol would involve a single step deacetylation at C–8 (FIG. 20). This implicates the presence of two different enzymes with differing specificities according to the position of the ester linkage on the trichothecane nucleus.

Previous *in vitro* studies with ovine rumen fluid (Section 7.3; Kiessling *et al.*, 1984) have failed to show the presence of neosolaniol as a breakdown product of T–2 toxin. This observation may be a result of the relatively low levels of neosolaniol present (below detection limits) due to the production of this trichothecene by only a limited number of rumen microorganisms. That this may be so is emphasised by the fact that in studies with *Selenomonas ruminantium* and *Anaerovibrio lipolytica*, HT–2 toxin and T–2 triol were the only breakdown products detected (TABLE 33). In these studies the presence of T–2 toxin (10 μg/ml) had no effect on the growth rate of the bacteria tested and as with *B. fibrisolvens* strains CE 46 and CE 51 the growth rate of *S. ruminantium* was enhanced (TABLE 34). In these studies, the average lag time for *A. lipolytica* increased by 21% in the presence of T–2 toxin but there was little change in the lag time of *S. ruminantium*.

Because previous studies were conducted using only one concentration of T–2 toxin, the effect of various concentrations of T–2 toxin on the growth of *B. fibrisolvens* was investigated. Results show (TABLE 35) that at concentrations as high as 500 μg/ml growth rate was not significantly affected and that although no growth occurred in one experimental incubation with T–2 toxin at 1 mg/ml, the average growth rate, although lower than that without added toxin, was within the limits of variation due to experimental procedure when compared to variation in growth rates at T–2 toxin concentrations as low as 2,0 and 10 μg/ml.

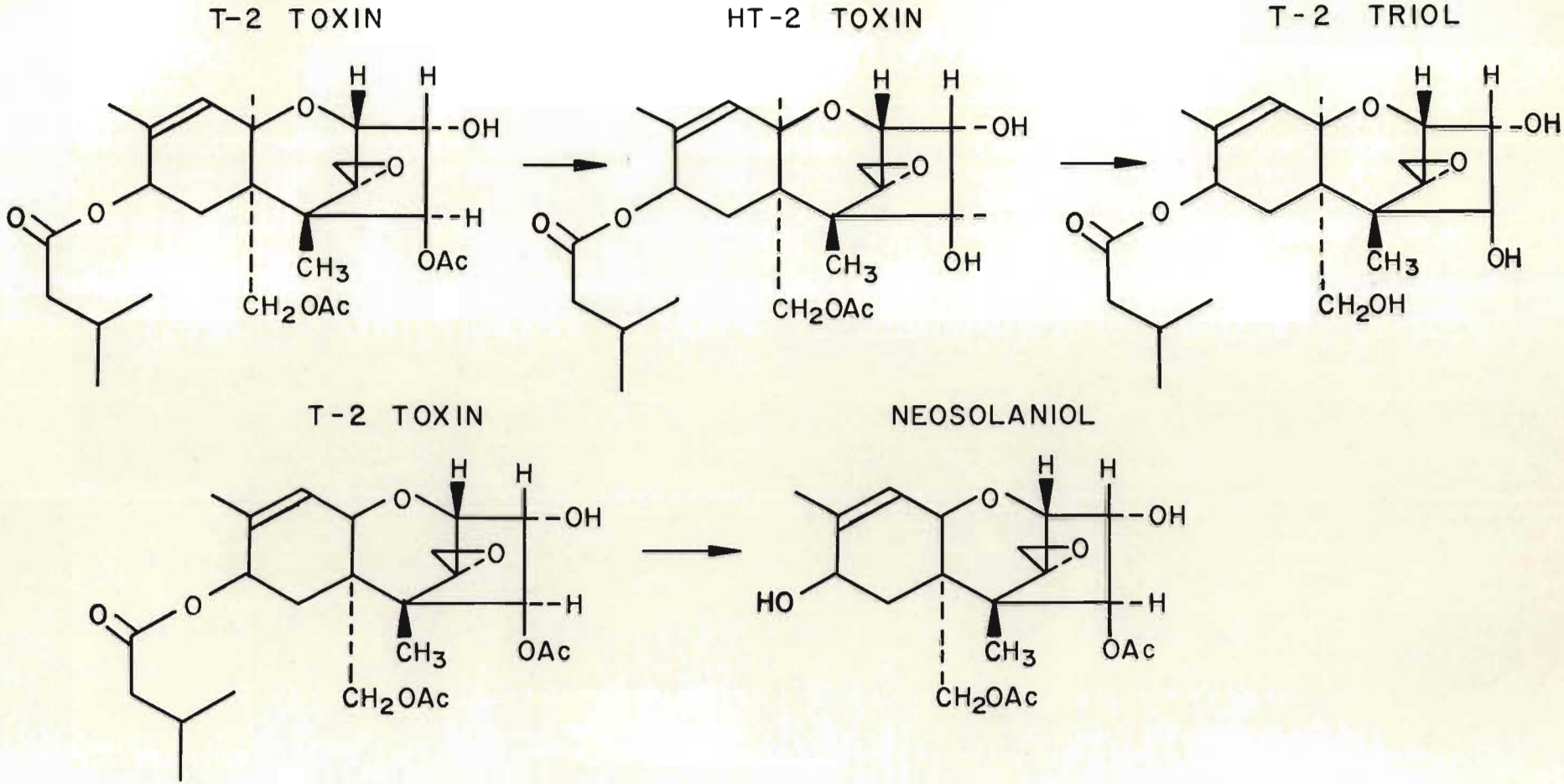


FIG. 20 Different pathways of T-2 toxin degradation in Butyrivibrio fibrisolvens strain CE 51

TABLE 33 Degradation of T–2 toxin (10 μg/ml) by *Selenomonas ruminantium* and *Anaerovibrio lipolytica* grown on 1% (w/v) glucose

Bacterium	Toxin concentration (% of added T–2 toxin)[a]			
	T–2 Toxin	HT–2 Toxin	T–2 Triol	Total
Selenomonas ruminantium	62	18	10	90
Anaerovibrio lipolytica	59	22	7	88

a) Average value of experiment conducted in triplicate

TABLE 34 **Effect of T–2 toxin on growth rate (μ) of *Selenomonas ruminantium* and *Anaerovibrio lipalytica* grown on 1% (w/v) glucose**

Bacterium	+ T–2 Toxin (10 μg/ml)					– T–2 Toxin				
	μ	$\bar{\mu}$	r	$\bar{r}$	AV^{GE} lag time (h)	μ	$\bar{\mu}$	r	$\bar{r}$	AV^{GE} lag time (h)
Selenomonas	0,32*		0,998			0,84		0,998		
ruminantium	0,95	0,90	0,998	0,998	1,3	0,76	0,80	0,994	0,997	1,4
	0,85		0,998			0,79		0,998		
Anaerovibrio										
lipolytica	0,49	0,52	0,991	0,991		0,45		0,991		
	0,55		0,990		4,2	0,51	0,52	0,993	0,994	3,3
		No Growth				0,59		0,99		

* Result not included in average

KEY:
- μ – Specific growth rate (h^{-1})
- $\bar{\mu}$ – Average specific growth rate (h^{-1})
- r – Correlation coefficient
- $\bar{r}$ – Average correlation coefficient

TABLE 35 The effect of different concentrations of T–2 toxin on the growth rate of *Butyrivibrio fibrisolvens* strain CE 51 grown on 1% (w/v) glucose

Concentration (μg/ml)	μ	$\bar{\mu}$	r	$\bar{r}$	AV^{GE} lag time (h)	Concentration (μg/ml)	μ	$\bar{\mu}$	r	$\bar{r}$	AV^{GE} lag time (h)
0	0,51		0,999			10,0	0,5		0,998		
	0,64	0,55	0,994	0,997	1,2		0,5	0,51	0,998	0,998	0,5
	0,51		0,997				0,4		0,998		
0,1	0,53		0,999			50,0	0,59		0,999		
	0,51	0,52	0,999	0,999	0,7		0,48	0,51	0,998	0,999	0,9
	0,51		0,999				0,45		0,999		
0,5	0,56		0,999			100,0	0,59		0,999		
	0,59	0,55	0,998	0,998	0,5		0,48	0,53	0,999	0,999	0,4
	0,49		0,997				0,53		0,999		
1,0	0,45		0,997			500,0	0,40		0,998		
	0,49	0,55	0,998	0,998	0,4		0,62	0,51	0,998	0,998	0,6
	0,48		0,998				0,50		0,999		
2,0	0,43		0,999			1000,0	0,50	0,49	0,996	0,997	0,4
	0,45	0,48	0,997	0,998	0,4		0,48		0,998		
	0,58		0,997				No Growth				

KEY:
- μ – Specific growth rate (h^{-1})
- $\bar{\mu}$ – Average specific growth rate (h^{-1})
- r – Correlation coefficient
- $\bar{r}$ – Average correlation coefficient

Because results in previous studies are contradictory (c.f. TABLE 29) the variations in lag times (TABLE 35) are to be caused by factors other than toxin concentration. Such factors would include the growth status of the inoculum. Thus contrary to previous indications TABLE 29 this would suggest that the enzymes responsible for T–2 toxin degradation are constitutive. This matter is discussed further in Chapter 9.

The production of breakdown products at high T–2 toxin concentrations (TABLE 36) was similar to results described previously (TABLE 29).

TABLE 36 **Degradation of T–2 toxin by *Butyrivibrio fibrisolvens* strain CE 51 grown on 1% (w/v) glucose**

Concentration (μg/ml)	Toxin concentration (% added T–2 toxin)[a]				
	T–2 toxin	HT–2 toxin	T–2 triol	Neosolaniol	Total
1 000	42	27	12	10	91
500	44	20	12	11	87
100	61	20	9	0	90

a) Average value of experiment conducted in triplicate

Results presented in TABLE 37 show that growth of *B. fibrisolvens* was not significantly affected by the mycotoxins acetyl T–2 toxin, HT–2 toxin, deoxynivalenol, diacetoxyscirpenol, verrucarin A, zearalenone or ochratoxin A. Lag times were similar to those previously described (TABLE 35) and were not significantly affected by the toxin added. Identification of possible breakdown products of toxins other than acetyl T–2 toxin and HT–2 toxin could not be performed due to lack of relevant standards and equipment.

Acetyl T–2 toxin was broken down to T–2 toxin, HT–2 toxin and T–2 triol indicating and initial deacetylation at C–3 and HT–2 toxin was degraded to T–2 triol (TABLE 38).

TABLE 37 The effect of different mycotoxins (10 μg/ml) on the growth rate of *Butyrivibrio fibrisolvens* strain CE 51 grown on 1% (w/v) glucose

Toxin (10 μg/ml)	μ	$\bar{\mu}$	r	$\bar{r}$	AV^{GE} lag time (h)	Toxin (10 μg/ml)	μ	$\bar{\mu}$	r	$\bar{r}$	AV^{GE} lag time (h)
Control (Toxin-free)	0,51 0,55 0,53	0,53	0,999 0,998 0,998	0,998	0,5	DAS	0,46 0,56 0,51	0,51	0,999 0,996 0,997	0,997	0,4
T–2 Toxin	0,50 0,49 0,58	0,52	0,996 0,994 0,996	0,995	0,5	VERR A	0,63 0,51 0,51	0,55	0,998 0,997 0,998	0,998	0,4
HT–2 Toxin	0,49 0,33* 0,49	0,49	0,999 0,996 0,998	0,998	0,8	Z	0,54 0,57 0,56	0,56	0,998 0,998 0,999	0,998	0,5
AT–2 Toxin	0,48 0,53 0,49	0,50	0,998 0,996 0,998	0,997	0,4	OCH	0,29* 0,52 0,52	0,52	0,998 0,996 0,999	0,997	0,6
DON	0,53 0,50 0,50	0,51	0,998 0,998 0,998	0,998	0,1						

* Result not included in average

KEY:
AT–2 – Acetyl T–2 toxin
DON – Deoxynivalenol
DAS – Diacetoxyscirpenol
VERR A – Verrucarin A
Z – Zearalenone
OCH – Ochratoxin A

TABLE 38 The degradation of various trichothecene toxins by *Butyrivibrio fibrisolvens* strain CE 51 grown on 1% (w/v) glucose

Toxin added (10 μg/ml)	Toxin concentration (% of added T–2 toxin)			
	T–2 toxin	HT–2 toxin	T–2 triol	Neosolaniol
Acetyl T–2 toxin	29	10	0	0
T–2 toxin		23	8	4
HT–2			7	0

TABLE 39 The effect of T–2 toxin on four isolates of tri-butyrin hydrolysing bacteria grown on 1% (w/v) glucose

Bacterium	+ T–2 toxin (10 μg/ml)			– T–2 toxin				
	μ	r	AV^{GE} lag time (h)	μ	$\bar{\mu}$	r	$\bar{r}$	AV^{GE} lag time (h)
KTB 9		No growth		0,45		0,991		
				0,51	0,52	0,989	0,993	0,2
				0,59		0,999		
KTB 10		No growth		0,53	0,56	0,993	0,995	
				0,58		0,997		0,8
						No growth		
KTB 13		No growth		0,54	0,55	0,994	0,994	
				0,55		0,994		0,2
						No growth		
KTB 16	0,56	0,991	0,2	0,45		0,996		
				0,57	0,46	0,993	0,995	0,3
				0,37		0,996		

KEY: μ – Specific growth rate (h^{-1})
$\bar{\mu}$ – Average specific growth rate (h^{-1})
r – Correlation coefficient
$\bar{r}$ – Average correlation coefficient

Of all the bacteria tested, the four tri-butyrin-hydrolysing isolates, obtained as described in Section 8.2.6 were the only organisms whose growth rate was significantly affected by T–2 toxin to the extent where three out of four isolates did not grow at all (TABLE 39). This indicates that the esterase activity of rumen bacteria does not confer resistance to T–2 toxin and that different esterase-type enzymes with varying substrate specificities are present amongst anaerobic rumen bacteria.

8.4 SUMMARY

Studies with aflatoxin B_1 showed that this mycotoxin was not toxic to several strains of *Butyrivibrio fibrisolvens* nor was it broken down by this organism.

T–2 toxin (10 μg/ml) was not toxic to the same strains of *B. fibrisolvens* when grown on either 1% (w/v) glucose or 1% (w/v) cellobiose nor to *B. fibrisolvens* strain CE 51 at concentrations as high as 1 mg/ml. However all strains of *B. fibrisolvens* tested were capable of T–2 toxin degradation and two different breakdown routes were shown, both involving de-esterification type reactions. These results imply the presence of more than one esterase-type enzyme with different specificities according to the position of the ester group on the trichothecane nucleus.

T–2 toxin was also found to be non-toxic to *Selenomonas ruminantium* and *Anaerovibrio lipolytica* and both organisms were capable of breaking down T–2 toxin to HT–2 toxin and T–2 triol.

Studies with a number of different trichothecenes also showed that these were non-toxic to *B. fibrisolvens* strain CE 51.

Of the bacteria tested all but those isolated on a tributyrin-containing medium were resistant to T–2 toxicity as judged by growth rate studies.

CHAPTER 9

ESTERASE STUDIES WITH RUMEN ANAEROBIC BACTERIA

9.1 INTRODUCTION

Previous work (Chapter 8) showed that *Butyrivibrio fibrisolvens, Anaerovibrio lipolytica* and *Selenomonas ruminantium* were capable of T–2 toxin degradation.

Two different T–2 toxin breakdown pathways were demonstrated each involving deacetylation of the parent molecule and suggesting the presence of esterase-like enzyme activity.

Since *B. fibrisolvens* had both types of enzyme activity during degradation of T–2 toxin it was chosen for further enzyme studies.

9.2 METHODS AND MATERIALS

9.2.1 Chemicals

All solvents and reagents were of analytical grade and have been noted in the text where necessary.

9.2.2 Lysis of bacterial cells and preparation of cell-free homogenates and bacterial membrane fractions

Although *Butyrivibrio fibrisolvens* is classified as a gram-negative bacterium the cell wall ultrastructure is more typical of gram positive bacteria. (Bryant, 1984). Preparation of membrane and cytosol fractions of this bacterium was performed as detailed in FIG. 21.

9.2.3 Protein determination

Measurement of protein was performed using the Biorad assay procedure (Biorad Protein assay kit II. Cat. No. 500 – 0002, Biorad Laboratories, D – 8000, Munich, W. Germany).

A calibration curve prepared using bovine serum albumin (1,2 mg/ml) was linear between 0 and 120 μg protein (FIG. 22).

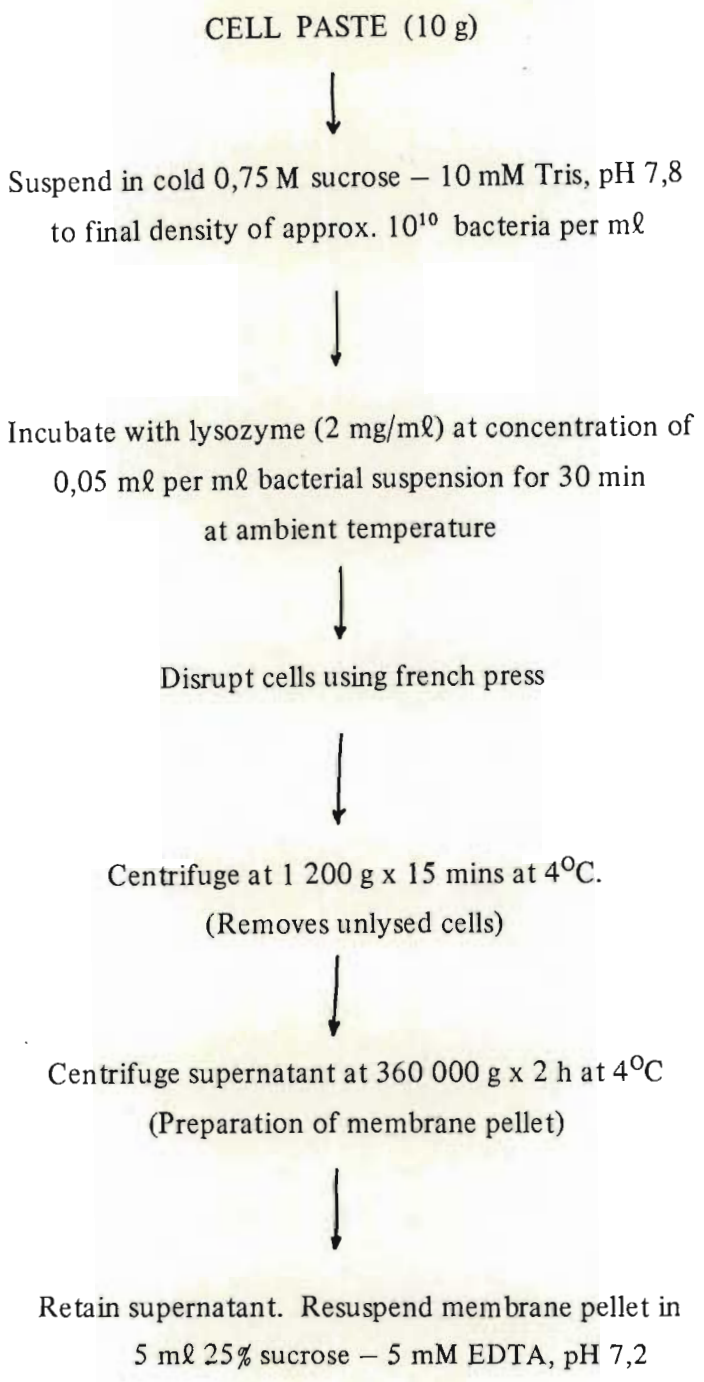


FIG. 21 **Flow chart for preparation of membrane and cytosol fractions from *Butyrivibrio fibrisolvens***

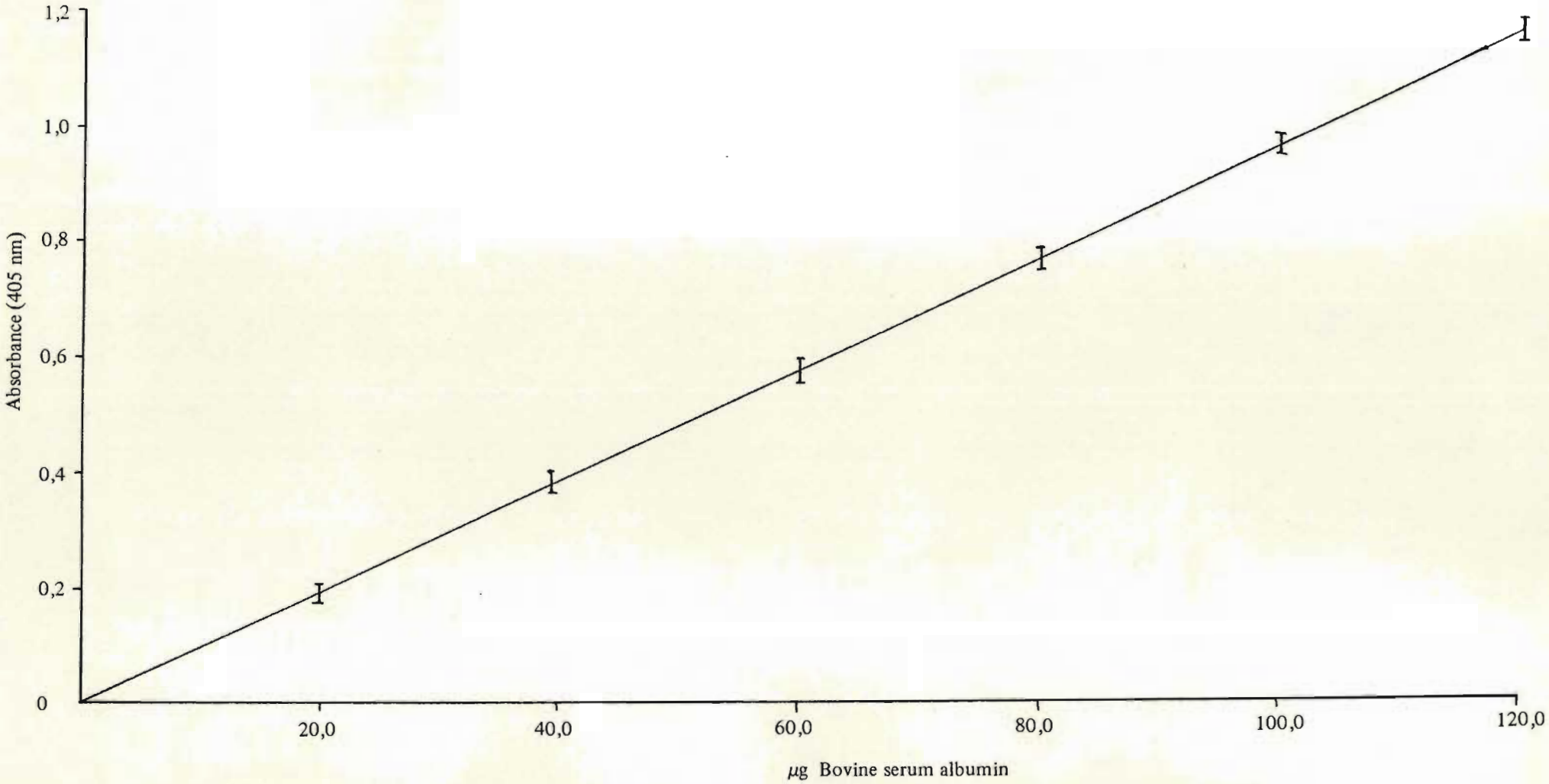


FIG. 22 **Standard protein curve obtained using BioRad protein assay procedure**

9.2.4 Measurement of esterase activity

Esterase activity was standardised using porcine liver esterase type II EC 3.1.1.1 (Sigma). The enzyme was diluted 1 : 500 with 0,1M phosphate buffer pH 6,0 , 1 mM dithiothreitol before use. Release of *p*-nitrophenol from *p*-nitrophenyl-acetate was monitored with time at 405 nm on a scanning Beckman 25 spectrophotometer at 39°C. The reaction mixture contained :

2,0 ml phosphate buffer (0,1 M) pH 6,0, 1 mM Dithiothreitol
0,5 ml enzyme
0,01 ml *p*-nitrophenylacetate (0,251M) in DMF

Formation of -nitrophenol was measured against a 'blank' containing:

2,5 ml 0,1 m phosphate buffer pH 6,0, 1 mM dithiothreitol
0,01 ml *p*-nitrophenylacetate (0,251M) in DMF

Esterase activity in cell preparations was measured at 39°C after pre-equilibration of reaction components in a temperature-controlled water bath.

9.2.5 Fractionation of cell extracts by ammonium sulphate precipitation

Cytosol and membrane fractions prepared as in Section 9.2.2 were brought to 30% (w/v) saturation with ammonium sulphate and allowed to stand at 4°C for 1 h before centrifuging (12 000 x g, 15 mins, 4°C). The precipitate was collected (0–30% fraction) and the supernatant brought to 40% (w/v) saturation and after standing for 1 h at 4°C was recentrifuged as above. The precipitate was collected (30–40% fraction). Other fractions (40–50%; 50–60%) were prepared in a similar manner. Protein measurement and esterase activity of each fraction and the final supernatant were measured by methods described previously (Sections 9.2.3 and 9.2.4).

9.2.6 Gel filtration on Sephadex G–150

Sephadex G–150 (40–120 mesh, Pharmacia, Sweden) was allowed to swell for 24 h in 0,05 M phosphate buffer pH 7,0 before use. The column (70 x 2,5 cm) was packed at ambient temperature with the preswollen gel. Once packed two bed-volumes of buffer were passed through the column (flow rate 30 ml/h) before sample application. Samples were eluted with 0,05 M phosphate buffer pH 7,0 and effluent collected in 6 ml

fractions using an Isco Golden retriever model 328 (Instrumentation Specialities Co. Inc. Lincoln, Nebraska, USA). Protein concentration was measured at 280 nm using an Isco Model UA–2 UV analyser. Protein peak samples were pooled for enzyme analysis.

9.2.7 Toxin degradation by fractions obtained after gel filtration on Sephadex G–150

Pooled fractions obtained as described previously (Section 9.2.6) were pre-incubated at 39°C for 10 min after which time T–2 toxin was added at 100 μg/ml in DMF. Samples (10 ml) were withdrawn at various time intervals and extracted three times with an equal volume of ethyl acetate. The pooled ethyl acetate extracts were then dried at 60°C under a stream of nitrogen and analysed by GLC as described previously (4.2.2).

9.3 RESULTS AND DISCUSSION

Initial studies showed that esterase activity was higher in whole homogenates of *Butyrivibrio fibrisolvens* than in *Anaerovibrio lipolytica* and *Selenomonas ruminantium* (TABLE 40) as determined by the release of *p*-nitrophenol from *p*-nitrophenyl acetate. However none of these bacteria showed any activity against -nitrophenyl laurate, stearate or palmitate. Therefore in subsequent esterase assays esterase activity was monitored using *p*-nitrophenyl acetate as sole substrate.

TABLE 40 Esterase activity in whole homogenates of *Butyrivibrio fibrisolvens*, *Selenomonas ruminantium* and *Anaerovibrio lipolytica*

Bacterium	Esterase activity (μ moles np formed/min/mg protein)
Butyrivibrio fibrisolvens	473,8
Anaerovibrio lipolytica	239,6
Selenomonas ruminatium	135,7

Key np – *p*-nitrophenol

When using the assay technique described in Section 9.2.4 it was found necessary to make up fresh substrate in DMF on a daily basis. Failure to do so resulted in appreciable release of *p*-nitrophenol in the control.

Since previous work (Hazlewood & Dawson, 1979) showed that the phospholipid deacylating system of *B. fibriosolvens* had an absolute requirement for either mercapto-ethanol, cysteine or dithiothreitol, dithiothreitol (1 mM) was included in all buffers used.

Because whole cells of *B. fibrisolvens* had the highest esterase activity, further studies were conducted on this organism only.

Cytosol and membrane fractions of *B. fibrisolvens* were prepared and esterase activity measured as described previously (Section 9.2.2). The results show (TABLE 41) that there was roughly equivalent activity in the cytosol fraction and in the membrane fraction in the absence of Triton X–100. However the addition of 0,25% (w/v) Triton X–100 to the membrane fraction caused a significant increase in esterase activity. The esterase activity in the presence of Triton X–100 was measured over the initial 30 secs of reaction since in the later stages the production of a precipitate (presumably due to reaction of the buffer with Triton X–100) masked any *p*-nitrophenol production.

TABLE 41 **Esterase activity in sub-cellular fractions of *Butyrivibrio fibrisolvens***

Fraction	Esterase activity (μ moles np formed/min/mg protein)
Cytosol	96,7
Membrane fraction + Triton X–100 (0,25% w/v)	169,6
Membrane fraction – Triton X–100	95,9

The increase in membrane esterase activity in the presence of Triton X–100 suggests that some but not all esterase activity is tightly bound to the cell membrane.

Because the highest activity was found in this membrane fraction it was used in further work in enzyme purification. This was initially performed using ammonium sulphate precipitation and results show that the highest activity was found in the 50–60% saturation fraction. (TABLE 42). Further purification of this fraction by gel-filtration on Sephadex G–150 showed the presence a number of peaks of which peak 1 (FIG. 23) showed esterase activity of 588,8 μmoles np formed/min/mg protein. No attempt was made to purify this fraction but the molecular weight of the protein was determined as being approximately 65 000.

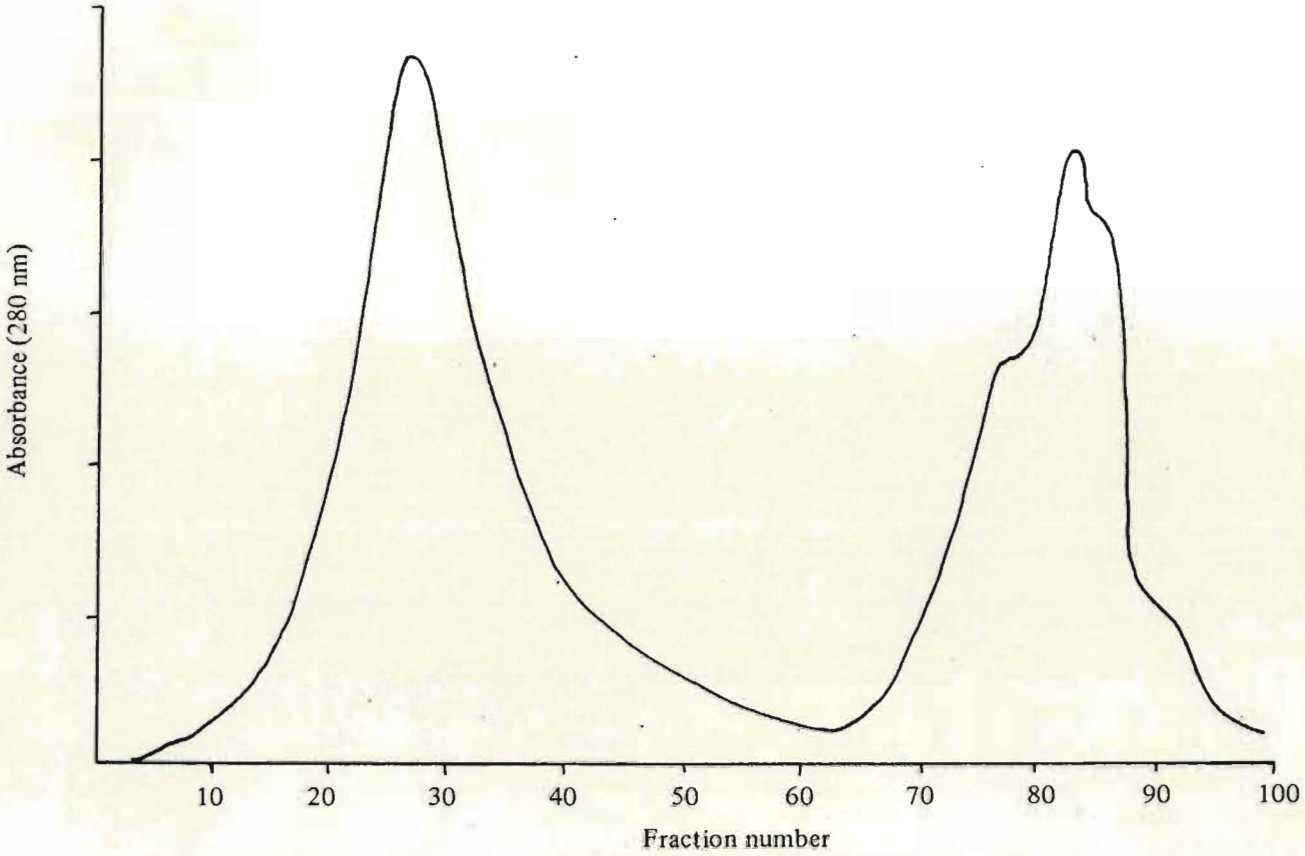


FIG. 23 **Elution profile on Sephadex G–150 of membrane fraction from *Butyrivibrio fibrisolvens***

TABLE 42 Esterase activity in membrane fractions of *Butyrivibrio fibrisolvens* after precipitation by $(NH_4)_2\ SO_4$

Fraction ($\%(NH_4)_2\ SO_4$)	Esterase activity (μ moles np formed/min/mg protein)
0 – 30 %	129,4
30 – 40 %	12,2
40 – 50 %	3,2
50 – 60 %	395,6
Final supernatant	5,1

Subsequent toxin degradation studies showed that this fraction was capable of degrading T–2 toxin to HT–2 toxin and T–2 triol (TABLE 43). This result suggests that at least one esterase-like enzyme capable of degrading T–2 toxin is present within the cell membrane of *B. fibrisolvens*. There was no production of neosolaniol and therefore further studies are required to locate an enzyme that can degrade T–2 toxin to neosolaniol as shown in Chapter 8.

Because the bacteria used in these studies were grown in the absence of T–2 toxin, these results suggests that at least one enzyme capable of T–2 toxin degradation is a constitutive enzyme. Previous work (Hazlewood & Dawson, 1979) has shown the presence of a cell-bound esterase in *B. fibrisolvens* that is capable of deacylating phospholipids and it is possible that such an enzyme is also responsible for the T–2 toxin degradation shown in the present studies.

TABLE 43 Degradation of T–2 toxin by protein peak 1 from *Butyrivibrio fibrisolvens*

Time (h)	Toxin concentration (% of added T–2 toxin)			
	T–2 toxin	HT–2 toxin	T–2 triol	Total
0	92	0	0	92
0,25	74	14	2	90
0,5	64	18	8	90
1,0	50	26	13	89

9.4 SUMMARY

Preliminary studies on the esterase of *B. fibrisolvens* have shown the presence of a membrane-bound enzyme capable of T–2 toxin degradation to HT–2 toxin and T–2 triol. The esterase activity in whole cells of *B. fibrisolvens* was higher than in either *S. ruminantium* or *A. lipolytica.*

SUMMARY

Initial studies showed the presence of a number of mycotoxins including aflatoxin B_1, patulin, zearalenone, kojic acid and T–2 toxin in animal feed commodities sampled from Natal. Many of these samples had been associated with illness in domestic animals.

A new gas-liquid chromatographic method was developed for the quantitation of trichothecenes. This method facilitated the detection of ten trichothecenes in less than thirteen minutes.

In order to elucidate possible detoxification pathways in ruminants, studies were conducted to determine the effect of whole ovine rumen fluid and ovine rumen fluid preparations on the breakdown of various mycotoxins including the major toxins identified in animal feedstuffs.

These results showed that ovine rumen fluid was capable of breaking down patulin, ochratoxin, zearalenone and a number of trichothecenes including T–2 toxin. The toxin degradation rates when added at 10 μg/ml were as follows ochratoxin (555 μg/h per ℓ), zearalenone (111 μg/h per ℓ), patulin (544 μg/h per ℓ) and T–2 toxin (2 220 μg/h per ℓ). Thus T–2 toxin was broken down at much quicker rate than the other mycotoxins tested. HT–2 toxin, deoxynivalenol and diacetoxyscirpenol were also broken down.

Studies with T–2 toxin showed that this compound was broken down to HT–2 toxin and T–2 triol and therefore that degradation occurs via successive deacetylations at C–4 and C–15. The rate of HT–2 toxin degradation was much slower than that of T–2 degradation.

Cell-free preparations from ovine rumen fluid could not degrade T–2 toxin but both bacteria and protozoa obtained from such fluid were able to do so, the rates of degradation being 1 780 μg/h per ℓ and 2 083 μg/h per ℓ respectively. However although the rumen protozoa were more efficient at T–2 toxin degradation they were also susceptible to the toxic effects of this toxin. T–2 toxin was not toxic to the bacterial preparation and in fact total bacterial protein increased in the presence of T–2 toxin compared to a toxin-free control.

Subsequent studies with pure cultures of rumen anaerobic bacteria showed that aflatoxin B_1 was not toxic to several strains of *Butyrivibrio fibrisolvens* nor was it broken down by this bacterium.

Similar work with T–2 toxin showed that it was not toxic to four different strains of *B. fibrisolvens* at 10 μg/ml nor to strain CE 51 when added at concentrations as high as 1 mg/ml. Furthermore, all four strains were capable of T–2 toxin degradation and in the case of strain CE 51, only 57% of T–2 toxin remained after incubation to stationary phase of bacterial growth. Breakdown products were HT–2 toxin (22%), T–2 triol (3%) and neosolaniol (10%). These results implied the presence of more than one esterase-type enzyme with different specificities according to the position of the ester group on the trichothecane nucleus.

T–2 toxin was also found to be non-toxic to *Selenomonas ruminantium* and *Anaerovibrio lipolytica* and both organisms were capable of breaking down T–2 toxin to HT–2 toxin and T–2 triol but not neosolaniol. *A. lipolytica* was slightly more active than *S. ruminantium* with only 59% of added T–2 toxin remaining at the end of the growth phase compared to 62% for *S. ruminantium.* Generally, however, the amount of T–2 toxin left at the end of the growth phase was approximately the same for all bacteria.

The mycotoxins, acetyl T–2 toxin, HT–2 toxin, deoxynivalenol, diacetoxyscirpenol, verrucarin A, zearalenone and ochratoxin A had no effect on the growth rate of *B. fibrisolvens* strain CE 51. [However because of lack of facilities only the breakdown of acetyl T–2 toxin and HT–2 toxin could be monitored.] In these cases HT–2 toxin was broken down to T–2 triol and acetyl T–2 toxin was broken down in a similar manner to T–2 toxin but with a prior deacetylation occurring at C–3.

Of the bacteria tested all but those isolated on a tributyrin – containing medium were resistant to T–2 toxicity as judged by growth rate studies.

Enzyme studies with *B. fibrisolvens* strain CE 51 showed the presence of a membrane-bound esterase capable of breaking down T–2 toxin to HT–2 toxin and T–2 triol. The esterase activity in whole cells of *B. fibrisolvens* was higher than in either *S. ruminantium* or *A. lipolytica.*

APPENDIX I

Ringers solution

NaCl	2,25 g
KCl	0,105 g
$Ca\ Cl_2\ \ 2\ H_2O$	0,06 g
$Na\ HCO_3$	0,05 g
Sodium lauryl sulphate	0,10 g
Distilled H_2O	1 000 ml

Dispense 9,0 ml into narrow neck McCartney bottles and autoclave at 121°C for 15 min.

APPENDIX II

Ohio Agricultural Experimental Station agar.[a]

Glucose (Dextrose)	5,0 g
Yeast extract	2,0 g
Sodium nitrate	1,0 g
Magnesium sulphate	0,5 g
Potassium dihydrogen phosphate	1,0 g
Desiccated Oxbile (Oxoid L50)	1,0 g
Sodium propionate	1,0 g
Agar (Oxoid L13)	20,0 g
Distilled H_2O	1 000 ml

Procedure: Dissolve the above with heating and adjust to pH 6,0. Dispense into media bottles and autoclave at 115 ^{o}C for 10 mins. Once cool, add 1 ml each of sterile streptomycin (0,5%) and sterile chloramphenicol (0,5%).

a) Kaufmann, Sumner & Williams (1963).

APPENDIX III

McDougalls artificial saliva buffer. [a]

$Na.H\,CO_3$	9,8 g
$Na_2\ H\,PO_4$	3,7 g
KCl	0,570 g
NaCl	0,47 g
$MgSO_4\ .\ 7H_2O$	0,12 g
$CaCl_2\ .\ 2H_2O$	0,053 g
Distilled water	1 000 ml

Dispense in 1 ℓ amounts and store at 4°C.

a) McDougall (1948).

APPENDIX IV

Weakly-buffered cellulose[a] medium based on medium 10 of Caldwell and Bryant[b]

Component	Concentration in medium	Stock solution/suspension		
		Concentration (g/l)	Designation	Vol added in 500 ml batch of medium (ml)
Distilled water				402,5
Cellulose, ball-milled	1,0 g/l	20,0	Cellulose	25,0
Trypticase (Merck 7213)	2,0 g/l	100,0	Trypticase sol^{n} [c]	10,0
Yeast extract (Merck 3753)	0,5 g/l	50,00	Yeast extract sol^{n} [c]	5,0
K_2HPO_4	1,7 mM	11,84	Mineral sol^{n} 1	12,5
KH_2PO_4	1,3 mM	7,09		
$(NH_4)_2SO_4$	3,4 mM	17,97		
NaCl	0,76 mM	1,78	Mineral sol^{n} 2	12,5
$MgSO_4 . 7H_2O$	0,38 mM	3,75		
$CaCl_2 . 2H_2O$	0,41 mM	2,41		
Haemin	0,0015 mM	0,1	Haemin sol^{n} [d]	5,0
Na-acetate	29,7	178,33		
Na-propionate	8,05	59,60		
Na-butyrate	4,35	38,36	VFA-sol^{n} [e]	5,0
Na-isobutyrate	1,08	8,50		
Na-valerate	0,92 mM	9,39		
Na-isovalerate	0,91 mM	9,31		
Na-DL–α–methyl butyrate	0,92 mM	9,41		
Indigocarmine	0,0044 mM	5,0	Indigocarmine	0,5
$Na_2S . 9H_2O$	1,04 mM	125,0	Sulphide[f]	1,0
Cysteine $HCl.H_2O$	1,42 mM	125,0	Cysteine[f]	1,0
Na_2CO_3	12,26 mM	32,5	Na_2CO_3 [g]	20,0

Continued /.....

Appendix IV – Continued

a) Substituted with 1% cellobiose or glucose as required.

b) Caldwell & Bryant (1966).

c) Required volume of stock solution prepared with warm distilled water shortly before use.

d) Solvent: 500 ml ethanol + 500 ml 0,05 M NaOH. Stock solution stored at 4°C.

e) Approximate volumes of acids : acetic 170 ml, propionic 60 ml, butyric 40 ml, all others 10 ml each; acids mixed, chilled in ice bath; pH adjusted to 7,5 with approximately 460 ml 10 M NaOH. Vol made up to 1 ℓ and solution stored at room temperature.

f) Solutions prepared with exclusion of O_2, heat-sterilised separately under N_2 and added aseptically to bulk of previously sterilised (121°C x 30 mins) medium.

g) Combined with the appropriate volume of VFA-salts solution, heat sterilised, cooled, equilibrated with gas mixture of 65% N_2 : 30% CO_2 : 5% H_2 and added aseptically to bulk of sterile medium. pH of medium equilibrated with above gas mixture at 39°C. pH =7,0 ± 0,05.

APPENDIX V

FORMOL SALINE

0,8% NaCl in formaldehyde (37%)

APPENDIX VI

Maintenance Medium[e]

Component	Amount added/500 ml
Bacto agar (Difco)	10,0 g
Cellobiose	0,25 g
Xylan	0,25 g
Starch	0,25 g
Bacto-casitone (Difco B259)	2,5 g
Mineral sol[n]	37,5 ml
Mineral sol[n]	37,5 ml
Clarified rumen fluid[b]	200,0 ml
Deionised water	174 ml

After steaming above components for 30 min add Cysteine. HCl . H_2O, Na_2 S . $9H_2O$[c] (10 ml) and $NaHCO_3$[d] (35 ml) to bulk of medium using aseptic techniques. Equilibrate at 39°C with gassing (30% CO_2 : 65% N_2 : 5% H_2) for 1 h.

a) See Appendix IV.
b) See Appendix VIII.
c) See Appendix IX.
d) See Appendix X
e) Bryant & Robinson (1962).

APPENDIX VII

Anaerobic diluent[b]

Component	vol added/l
Mineral sol[n]	37,5 ml
Mineral sol[n]	37,5 ml
Indigo carmine[a]	10,0 ml
$NaHCO_3$	6,37 g
Deionised water	925,0 ml

a) See Appendix IV

Boil deionised water, measure and place in 1 ℓ McCartney bottle. Gas with 100% CO_2. Add Mineral sol[ns] 1 and 2, indigo carmine and $NaHCO_3$. Gas for 30 min with 100% CO_2. Add 0,5 g solid cysteine HCl and dispense into pre-gassed (100% CO_2) McCartney bottles and sterilise.

b) Bryant & Burkey (1953).

APPENDIX VIII

Preparation of clarified rumen fluid

Donor sheep maintained on lucerne hay diet. Collect rumen fluid 2 h after feeding and strain through two layers of cheesecloth. Centrifuge at 2 500 x g for 40 min at 4°C. Collect supernatant and centrifuge at 15 000 x g for 20 min at 4°C. Saturate with anaerobic gas mixture. Freeze supernatant until required for media preparation.

APPENDIX IX

Preparation of Cysteine . HCl . H_2O : Na_2S . $9H_2O$ solution

Weigh 1,25 g Cysteine . HCl . H_2O[a] into 100 ml flask and dissolve with 20 ml 1,0 M NaOH. Add 1,25 g Na_2S . $9H_2O$[b] and dissolve in cysteine solution while gassing with 100% N_2. Make up to 100 ml with deionised water and dispense in required amounts into N_2 purged 1 oz McCartney bottles. (Final pH 11,08). Sterilise at 121°C for 25 mins.

a) cysteine . HCl . H_2O (Koch Light 1424 h)

b) Na_2S . $9H_2O$ (Koch Light 5121 c)

APPENDIX X

Preparation of sodium bicarbonate solution

Prepare solution of $NaHCO_3$ (9,1% w/v) in volumetric flask with deionised water. Dispense as required.

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