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THE BIOLOGICAL PROPERTIES OF THREE TRICHOTHECENE MYCOTOXINS PRODUCED BY FUSARIA

by

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University of Cape Town

Dedicated to my parents

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ABSTRACT

The highly toxic fungal metabolite, neosolaniol monoacetate, was isolated and purified from cultures of *Fusarium sambucinum*. Since little is known about its toxic properties, the biological effects of this trichothecene were compared to those caused by diacetoxy=scirpenol in male Wistar rats. The lesions caused by the two toxins were very similar. Chronic exposure to either toxin led to a significant decrease ($P<0.05$) in red blood cell counts and a significant increase ($P<0.05$) in platelet size. The major pathological lesions observed were atrophy of the actively dividing cells of the bone marrow, thymus, spleen and lymph nodes.

The reported species difference in T-2 toxin toxicity was investi=gated by determining the deacylation rate of T-2 toxin to HT-2 toxin, one of the first steps in the detoxification of this trichothecene. The high deacylation rate catalysed by rat microsomes correlated with the low sensitivity of this species to T-2 toxin, whereas the low deacylation rates with cat and monkey microsomes agreed with their high sensitivity. In contrast to this, the apparently high toxicity of T-2 toxin to humans does not correlate with the high deacylation rate observed in human hepatic microsomes.

Involvement of the UDP-glucuronyltransferases in the detoxification of T-2 toxin was studied with rat and pig hepatic microsomes. T-2 toxin and two of its metabolites, HT-2 toxin and T-2 tetraol, did not appear to act as substrates for these enzymes under the *in vitro* conditions used.

LIST OF ABBREVIATIONS

ATA	Alimentary toxic aleukia
CSIR	Council for Scientific and Industrial Research
DAS	Diacetoxyscirpenol
DMSO	Dimethylsulfoxide
ECD	Electron capture detector
GC	Gas chromatography
HFBI	Heptafluorobutyrylimidazole
i.d.	Internal diameter
i.m.	Intramuscular
i.p.	Intraperitoneal
i.v.	Intravenous
MeOH	Methanol
MRC	South African Medical Research Council
NMA	Neosolaniol monoacetate
p.o.	per os
RIND	Research Institute for Nutritional Diseases
TCA	Trichloroacetic acid
TLC	Thin layer chromatography

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CHAPTER 1

INTRODUCTION

1.1 HISTORICAL BACKGROUND

Mould induced food poisoning, mycotoxicosis, has posed a threat to human health for more than 2000 years. It has been suggested that food-borne fungi could have been involved in some of the 10 plagues of Egypt during biblical times (1).

During the Middle Ages St. Anthony's fire, later known as ergotism, claimed the lives of many thousands of people in Europe (2). The victims contracted the disease after ingesting bread made from rye infected with the fungus *Claviceps purpurea*. The main syndrome of this disease was gangrene of the extremities caused by peripheral thrombosis of the arteries. Outbreaks of ergotism were recorded until recently. In the seventeenth century it was realized that the fungi, *Claviceps purpurea* and *C. paspali* produced ergot alkaloids that were responsible for ergotism.

During the 1940's a disease known as alimentary toxic aleukia (ATA) was recorded in Russia. It has been estimated that hundreds of thousands of people died after the consumption of overwintered grain infected with the fungi *Fusarium sporotrichioides* and *F. poae* (3).

A major animal mycotoxicosis was reported in 1960 in Britain when more than 100000 turkeys died from Turkey X disease caused by ingestion of a batch of Brazilian groundnut meal. A blue fluorescent

substance, isolated from this meal, was found to be produced by Aspergillus flavus and A. niger and this led to the subsequent discovery of the aflatoxins (4). The effect of this episode of avian disease was to stimulate research into the toxins, myco= toxins, produced by food-borne fungi and the possible threats these mycotoxins pose to humans and animals.

Over the last two decades many different mycotoxins have been identi= fied and studied with the emphasis largely on the aflatoxins and their effects on human and animal health. Over the past five years a definite shift in emphasis has taken place towards investigations on the Fusarium toxins and especially the group of sesquiterpene toxins known as the trichothecenes. This shift in emphasis can to some extent be attributed to the allegations that these toxic compounds were used as chemical warfare agents in "Yellow Rain" (5).

1.2 MYCOTOXICOSES ASSOCIATED WITH TRICHOTHECENES

The trichothecenes are a group of secondary metabolites produced by various fungal genera such as Fusarium, Stachybotrys, Myrothecium, Trichoderma and Cephalosporium (6, 7) (Section 1.3). The trichothe= cenes produced by Fusaria have received more attention than the macro= cyclic trichothecenes produced by the other genera because of their involvement in human and animal diseases.

Certain Fusarium species have been implicated in human diseases such as alimentary toxic aleukia (ATA), Urov or Kashin-Beck disease,

Akakabi-byo or scabby grain intoxication, and oesophageal cancer (8). To date the trichothecenes have been shown to be involved in the etiology of only one of these diseases, ATA.

ATA is a disease that claimed the lives of hundreds of thousands of people in the USSR during the final years of the Second World War. The victims consumed overwintered grain infected with _F. sporotrichioides_ and _F. poae_ (9). ATA is characterized by stomatitis, dermatitis, haemorrhage, suppression of the haematopoeitic system, and depletion of the bone marrow with resultant leukopaenia, thrombo=cytopaenia and immunosuppression (3, 8).

Cultures of _F. sporotrichioides_ and _F. poae_ isolated from the over=wintered grain, when administered to various animal species, successfully reproduced the symptoms of ATA in cats and monkeys. The trichothecene, T-2 toxin, isolated from these cultures produced the characteristic ATA symptoms in cats (10), substantia=ting the role of T-2 toxin in the etiology of ATA.

Outbreaks of Akakabi-byo or scabby grain intoxication of humans have been reported in the USSR and Japan. In the USSR the outbreak resulted from the consumption of bread made from scabby rye infected by _F. graminearum_. The symptoms observed included headache, vertigo, shivering chills, nausea, vomiting and visual disturbances. In Japan sporadic occurrences of scabby wheat, barley, oats, rye and rice caused by _F. graminearum_ were frequently associated with out=breaks of human mycotoxicoses characterized by anorexia, nausea,

vomiting, headache, abdominal pain, diarrhea, chills, giddiness and convulsions. There were also reports that F. sporotrichioides may be involved in Akakabi-byo (8). F. graminearum and F. sporo=trichioides have been shown to produce various trichothecenes such as deoxynivalenol, nivalenol, fusarenon-X, diacetoxyscirpenol (DAS), neosolaniol and T-2 toxin (11). Although deoxynivalenol and nivalenol are known to occur naturally in scabby grains in Japan, these two trichothecenes have not been directly implicated in an actual case of human mycotoxicosis. It is thus not known whether nivalenol and/or deoxynivalenol are responsible for the clinical symptoms or whether other toxins or factors are involved (8).

The Fusaria have also been associated with various animal myco=toxicoses with haemorrhagic, estrogenic, emetic and feed refusal effects as well as with fescue foot, bean hulls poisoning and equine leukoencephalomalacia. The haemorrhagic, feed refusal and emetic syndromes are the only ones where the trichothecenes have been shown to play a role (8).

The haemorrhagic syndrome is associated with the ingestion of moldy cereals contaminated with F. sporotrichioides This disease is characterized by bloody diarrhea, necrotic oral lesions, haemorrhagic gastro-enteritis and extensive haemorrhages in many organs and has affected cattle, pigs and poultry. Many of the pathological lesions observed in field outbreaks have been reproduced experimentally with cultures of toxic strains of F. sporo=trichioides, crude extracts of cultures and crystalline trichothe=

cenes such as T-2 toxin and DAS (8).

Sporadic field outbreaks of feed refusal and emetic syndrome in pigs are linked with the ingestion of F. graminearum infected cereals, especially corn and barley. It has been shown that the trichothe= cene, deoxynivalenol, occurs in cereals infected by F. graminearum at levels capable of inducing characteristic clinical signs of these syndromes under experimental conditions. It is possible that deoxynivalenol alone is not responsible for the feed refusal syndrome and that other factors may be involved (8, 12).

1.3 THE PRODUCTION OF THE TRICHOTHECENES

The trichothecene mycotoxins are produced by various fungal genera from two families of imperfect fungi, Dematiaceae and Moniliaceae (7).

1.3.1 Trichothecene Producers from the Family Dematiaceae

The family Dematiaceae have two trichothecene producing genera, Myrothecium and Stachybotrys. Two Myrothecium species are known to produce trichothecenes. M. verrucaria produces verrucarin A, B, J (6, 7, 13) and the trichoiverroids (13). (Section 1.4). The trichoiverroids include trichodermadiene, trichoverrins A and B, roridin L-2 and trichoverritone. M. roridum produces the roridins (A, D, E and H) (6, 7, 13) and also roridin L-2, trichoverritone, 16-hydroxytrichodermadienediols A and B, and 16-hydroxyroridin L-2 (13).

Only one species from the genus *Stachybotrys*, *S. atra*, is known to produce trichothecenes (6, 7). The macrocyclic trichothecenes produced by this species are known as satratoxins C, D, F, G and H (7).

1.3.2 Trichothecene Producers from the Family Moniliaceae

The trichothecene producing genera in this family are *Fusarium*, *Cephalosporium*, *Trichoderma* and *Trichothecium* (7). The genus *Fusarium* has received the most attention because of its involvement in human and animal mycotoxicoses (7, 8, 13). The Fusaria occur widely in nature on many hosts and substrates. They are of the most commonly occurring fungi and many of the species are para= sitic to higher plants, causing severe economic losses (6, 7).

The taxonomy of the genus *Fusarium* is complex as different classi= fication systems are used in different countries. Wollenweber and Reinking (14) were the first to publish a *Fusarium* classifi= cation system. Snyder and Hansen reduced the number of *Fusarium* species from 65, according to Wollenweber and Reinking, to 9. Nelson *et al* (15) recently published a classification system that includes the advantages of the different systems, as well as their own suggestions, to aid in the practical identification of the Fusaria. This classification system comprises 15 *Fusarium* species reported to produce trichothecenes (8). The Fusaria produce both type A and type B trichothecenes (Section 1.4.2). These species are *F. nivale*, *F. sporotrichioides*, *F. poae*, *F. tri=*

cinctum, F. avenaceum, F. semitectum, F. equiseti, F. acuminatum, F. sambucinum, F. graminearum, F. lateritum, F. moniliforme, F. oxysporum, F. solani and F. culmorum.

In the genus Cephalosporum there is only one species known to produce trichothecenes (7). Crotocin and crotocol are produced by C. crotocinigenum (6, 7). The genus Cephalosporum occurs widely in nature, both as saprophites and occassionally as parasites on plants and animals (7).

In the genus Trichoderma, T. viride and T. polysporum are known to produce trichodermin and trichodermol (6,7). Trichothecium roseum, the only species in the genus Trichothecium to produce trichothe= cenes, produced trichothecin and trichothecolone (6, 11).

1.4 CHEMICAL NATURE OF THE TRICHOTHECENES

1.4.1 Chemical Properties

There are more than 40 naturally occurring trichothecenes and they all have a basic tetracyclic sesquiterpene structure (6, 11). The structure and numbering system is shown in Figure 1.1. Most of the trichothecenes have an epoxide ring at C-12,13 and a double bond at C-9,10 and are therefore called 12,13-epoxytrichothec-9-enes (6, 7). The naturally occurring mycotoxins have a minimum of six chiral centres at 2, 4, 5, 6, 11 and 12. For the elucidation of the molecular configuration, the C-14 methyl was used as an arbitrary

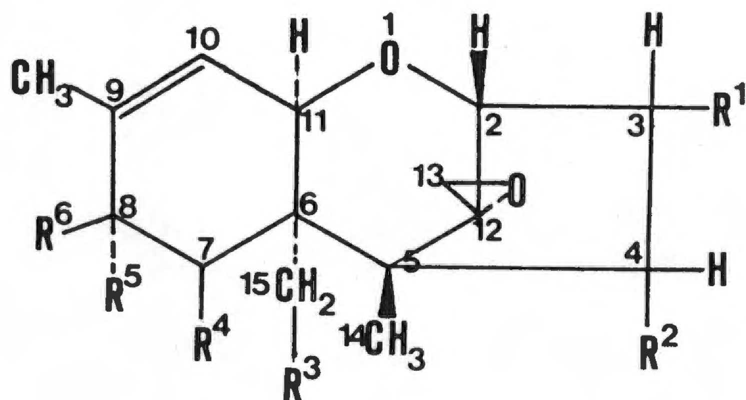


Fig. 1.1 Structure and numbering system of the trichothecenes

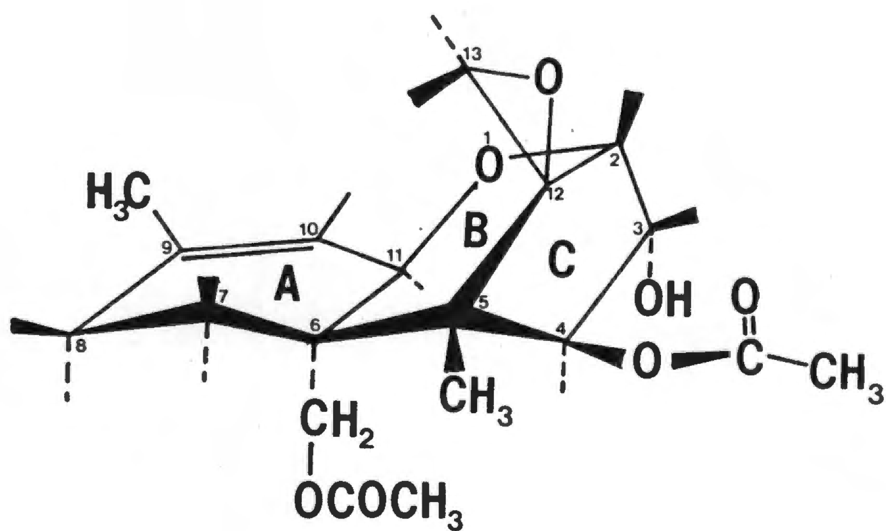


Fig. 1.2 Stereochemical configuration of DAS.

reference point and was assigned the β-configuration. Figure 1.2 represents the stereochemical structure of DAS. The presence of the 9,10 double bond leads to a 1,2 diplanar conformation for the six membered A ring, which is typical of substituted cyclohexanes. six-membered oxa-ring B adopts a chair configuration, and the five membered ring C an envelope form (11).

The trichothecenes are usually colourless, crystalline compounds. They are generally soluble in organic solvents such as alcohol, acetone, ethyl acetate and chloroform, but are only slightly soluble in water (11).

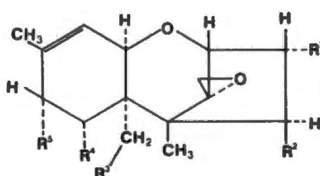
The group A and C trichothecenes (Section 1.4.2) do not show any absorbance or fluorescence in the ultraviolet or visible regions of the spectrum. This is due to the absence of any conjugated double bonds (16). The group B trichothecenes show absorbance at 215-226 nm due to the presence of a carbonyl group and group D at 195-263 nm due to conjugated double bonds in their macrocyclic structure.

1.4.2 Structural Classification of the Trichothecenes

The trichothecenes have been classified into four groups according to their chemical characteristics. Groups A and B differ mainly by the presence (B) or absence (A) of a carbonyl function at C-8. Examples of trichothecenes belonging to group A are T-2 toxin, diacetoxyscirpenol, scirpentriol and trichodermin (Table 1.1) while nivalenol, fusarenon-X, deoxynivalenol and trichothecin belong

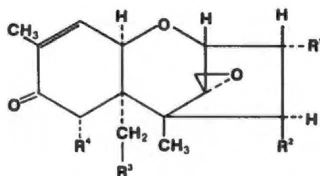
to group B (Table 1.2). Group C has only one member, namely crotocin (Table 1.3). Group D consists of compounds that possess a macrocyclic ester bridge between carbons 4 and 15. Examples of this group are the verrucarins and roridins (Table 1.4) (17).

TABLE 1.1 STRUCTURES OF GROUP A TRICHOTHECENES



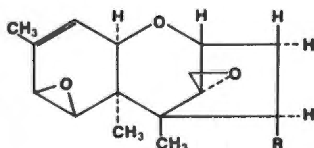
COMPOUND	R^1	R^2	R^3	R^4	R^5
T-2 toxin	OH	$OCOCH_3$	$OCOCH_3$	H	$OCOCH_2CH(CH_3)_2$
HT-2 toxin	OH	OH	$OCOCH_3$	H	$OCOCH_2CH(CH_3)_2$
T-2 triol	OH	OH	OH	H	$OCOCH_2CH(CH_3)_2$
T-2 tetraol	OH	OH	OH	H	OH
Diacetoxyscir=penol	OH	$OCOCH_3$	$OCOCH_3$	H	H
Neosolaniol	OH	$OCOCH_3$	$OCOCH_3$	H	OH
Neosolaniol monoacetate	OH	$OCOCH_3$	$OCOCH_3$	H	$OCOCH_3$
Trichodermin	H	$OCOCH_3$	H	H	H

TABLE 1.2 STRUCTURES OF GROUP B TRICHOTHECENES



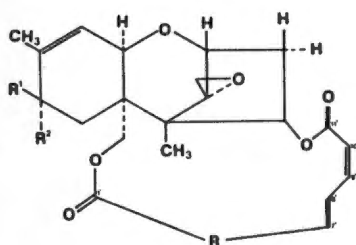
COMPOUND	R^1	R^2	R^3	R^4
Trichothecin	H	$OCOCH{=}CHCH_2$	H	H
Nivalenol	OH	OH	OH	OH
Fusarenon-X	OH	$OCOCH_3$	OH	OH
Deoxynivalenol	OH	H	OH	OH

TABLE 1.3 THE STRUCTURE OF THE GROUP C TRICHOTHECENES



COMPOUND	R
Crotocin	$OCOCH{=}CHCH_3$

TABLE 1.4 STRUCTURES OF GROUP D TRICHOTHECENES



COMPOUND	R	R^1	R^2
Verrucarin A	$-CHOHCHCH_3\ CH_2\ CH_2\ \overset{O}{\overset{\parallel}{OC}}\ -$	H	H
Verrucarin B	$-\overset{O}{CH-CH_3}\ CH_2\ CH_2\ \overset{O}{\overset{\parallel}{OC}}\ -$	H	H
Roridin D	$-\overset{O}{CH-CCH_3}\ CH_2\ \overset{\vert}{O}CHCHOHCH_3$	H	H
Roridin J	$-CH{=}CCH_3\ CHOHCH$ (ring: O, O, CH_3)	H	H
Satratoxin F	$-\overset{O}{CH-C}$ (ring: O, OH, $C(=O)CH_3$)	H	H
Satratoxin H	$-CH{=}C$ (ring: O, OH, $CHOHCH_3$)	H	H

1.5 THE BIOLOGICAL ACTIVITY OF THE TRICHOTHECENES

1.5.1 Toxicity

The trichothecenes have been shown to be toxic to a number of different animal species. The LD_{50} values depend on the route of administration and the animal species (6, 18). For example, the LD_{50} for T-2 toxin is 5.2 mg/kg bodyweight in mice when administered i.p. and 10.5 mg/kg when administered p.o. (18, 19). Of the A type trichothecenes T-2 toxin is the most toxic to animals while nivalenol is the most toxic type B trichothecene (18). Verrucarin A and J are the most toxic of the type D trichothecenes and are even more toxic than T-2 toxin (18). More studies have been performed on the biological action of the type A and B trichothecenes than on the other types because of their more frequent natural occurrence.

The toxicity of the 12,13-epoxytrichothec-9-enes depends largely on the nature of the substituents on the cyclic skeleton of the molecules. The removal of the C-8 isovaleryl residue in T-2 toxin (LD_{50} = 4.97 mg/kg), by hydrolysis to form a hydroxyl group (neosolaniol; LD_{50} = 24.87 mg/kg) decreases the toxicity, whereas its replacement by an acetate group (neosolaniol monoacetate (NMA); LD_{50} = 3.22 mg/kg) increases the toxicity to broiler chicks. The toxicity of T-2 toxin to broiler chicks was also reduced by deacylation to HT-2 toxin (LD_{50} = 7.22 mg/kg) and T-2 tetraol (LD_{50} = 33.79 mg/kg) (20).

A wide variety of symptoms are observed in experimental animals when

treated with trichothecenes including ataxia, diarrhea, haemorrha= ging, emesis, anorexia, hypothermia and disturbances of the central nervous system (10, 21-26).

1.5.2 The Effect of the Trichothecenes on the Immune System

1.5.2.1 The effect on lymphoid tissues

Mice treated with T-2 toxin, fusarenon-X, neosolaniol, HT-2 toxin or DAS suffered from atrophy of the thymus (27-30) together with a reduction in weight of the thymus (30, 31). Atrophy was also reported in cats, rats and guinea pigs treated with fusarenon-X (28) while T-2 toxin produces similar results in avians (32).

The cell numbers in mouse thymus are decreased by T-2 toxin treat= ment (33) while major changes occur in the cortical region (34). This region of the thymus contains many lymphocytes. At a cellular level it has been shown that fusarenon-X treatment results in karyorrhexis of the actively dividing cells (28).

As in the thymus, the major lesion seen in the spleens of various species is atrophy of the lymphoid tissue after treatment with T-2 toxin (10, 21, 27, 31, 35, 36). Fusarenon-X and DAS have similar effects on the spleen (21, 28, 37).

Administration of T-2 toxin to cats results in the formation of amyloid material in the spleen. This could indicate that T-2 toxin,

like other cytotoxic drugs, may cause an increase in amyloid inducing factors from decaying lymphoid cells (10). In mice, treatment with fusarenon-X results in an increase in non-lymphocytic cells in the spleen (37). It would thus appear that trichothecenes affect the lymphoid tissue in spleen in two ways, namely by reducing lymphocytic cells and by increasing non-lymphocytic cells. Karyorrhexis is observed in the spleens of animals treated with trichothecenes (28, 35) and pyknotic nuclei are noticed in some animals (35).

The treatment of animals with trichothecenes results in atrophy of the lymph nodes (27, 36, 38-40) and regular haemorrhaging is noticed (10, 22), primarily affecting the mesenteric lymph nodes (10). T-2 toxin seems mostly to affect actively dividing cells (39) and in swine pyknotic nuclei and karyorrhexis of cells in the lymph nodes are observed (35).

1.5.2.2 <u>The effect on antibody synthesis</u>

Treatment of mice with T-2 toxin and DAS inhibits antibody synthesis after immunization with sheep red blood cells (30, 34, 41). Fusarenon-X decreases the anti-DNP, IgE and IgG formation in mice (37). Similar effects are observed in mice treated with deoxynivalenol (42).

Inhibition of antibody synthesis appears to be due to a direct effect of the trichothecenes on the antibody producing cells.

In monkeys, T-2 toxin causes the reduction of the B-cell population (43). T-2 toxin also causes suppression of splenic B-cell proli= ferative responses and reversible inhibition of B-cell stimulation by mitogens in mice, lambs and pigs (44-47) while it is also cytotoxic to lymphocyte cell cultures (46).

1.5.2.3 The effect on cell-mediated responses

Evidence for the effect of the trichothecenes on the cell-mediated response was provided when it was shown that T-2 toxin and DAS prolong the period required for skin graft rejection in mice (30, 48).

Another immune response mediated by sensitized T-lymphocytes is termed delayed hypersensitivity. This term merely describes the slow appearance of secondary responses in the skin after immuni= zation (49). In mice T-2 toxin enhances the delayed hypersensitivity response (48, 50) and this can be explained by the preferential inhibition of the proliferation of a subset of T-lymphocytes which otherwise reveal suppressor functions (38, 48).

As with humoral immunity, the lymphocytes responsible for cell-mediated immunity are directly affected by the trichothecenes. Fusarenon-X causes suppression of mouse lymphocyte functions *in vivo* and *in vitro*. This inhibition is much more pronounced with T-cells than with B-cells (37).

1.5.3 The Effect of the Trichothecenes on the Haematopoeitic System

1.5.3.1 The effect on red blood cells

The treatment of rats and cats with DAS and T-2 toxin results in a decrease in red blood cell levels (10, 21, 39). Red blood cell levels in laying hens and chickens are however not affected by T-2 toxin treatment (51, 52). In swine, neither i.v. administered T-2 toxin nor dietary DAS has an effect on red blood cell levels (51, 52). T-2 toxin causes haemolysis in *in vitro* experiments by a mechanism, similar to that induced by H_2O_2 and polyoxyethylene surfactants, involving a free radical reaction mechanism (53). Other studies support the hypothesis that T-2 toxin induces haemolysis by interacting with the cell membrane (54).

1.5.3.2 The effect on white blood cells

The white blood cell population is more susceptible to the effect of the trichothecenes than red blood cells (55). Treatment of several animal species with T-2 toxin results in a decrease in their white blood cell counts (31, 38, 39, 47). Some reports however claim that T-2 toxin has no effect on white cell numbers in swine (35). DAS causes a reduction of white blood cell numbers in rats and young chickens (21, 40), while those of swine are unaffected (56). Administration of fusarenon-X and neosolaniol to mice results in a temporary leukocytosis (31).

The different subpopulations of white blood cells are affected differently by the trichothecenes. Monkeys develop leukocyto= paenia after T-2 toxin treatment due to a reduction in the number of polymorphonuclear white blood cells, while the lymphocyte numbers remain unchanged (36). T-2 toxin has a similar effect on calves as on monkeys (55). In young chickens treatment with T-2 toxin and DAS results in rapid necrosis of lymphocytes (40).

1.5.3.3 The effect on blood platelets

The administration of T-2 toxin to mice, monkeys, rabbits and guinea pigs results in a reduction in platelet number (31, 36, 57, 58), while in calves T-2 toxin has no effect on the platelet number (55). Terminally ill cancer patients treated with DAS as an anti-neoplastic agent developed thrombocytopaenia (59). Two other trichothecenes, fusarenon-X and neosolaniol, also cause a reduction in platelet number in mice (31).

1.5.3.4 The effect on blood coagulation

T-2 toxin increases the blood coagulation times in cows, rats, rabbits, lambs and guinea pigs (22, 44, 58, 60, 61). The coagulation patterns of plasma from T-2 toxin-treated guinea pigs diluted with plasma from untreated animals point to a deficiency of coagulation factors as the principle cause of prolonged clotting times (58).

T-2 toxin decreases different coagulation factors in various animal species. In rabbits, treatment with T-2 toxin results in a 40% decrease in the activities of factors VII, VIII, IX, X and XI (57). Factors VII, IX, X and XI and fibrinogen values are decreased in calves (62). In chickens, treatment with T-2 toxin results in a decrease in the concentrations of factors VII, X, prothrombin and fibrinogen (63).

The trichothecenes have a direct inhibitory effect on the aggregation of platelets. When either collagen or ADP is used as aggregating agent _in vitro_, T-2 toxin, HT-2 toxin, DAS and deoxy= nivalenol, in decreasing order of potency, inhibit the aggregation of bovine platelets (64, 65). A dose related inhibition of human platelet aggregation by T-2 toxin has also been reported (66).

Thromboxane A_2, which is involved in aggregation of platelets, is spontaneously and rapidly converted to thromboxane B_2 which is completely inactive. Thromboxane B_2 release from collagen-stimu= lated platelets is decreased in the presence of trichothecenes (64-66). It therefore seems that the trichothecenes somehow inhibit the synthesis of thromboxane A_2 or the release of thromboxane B_2. T-2 toxin changes platelet membrane permeability (66) and could change the secretion of thromboxane B_2.

1.5.3.5 _The effect on the bone marrow_

Bone marrow is severely affected in animals treated with trichothe=

cenes. T-2 toxin, HT-2 toxin, DAS, neosolaniol and fusarenon-X affect the actively dividing cells in bone marrow of mice, cats, guinea pigs, young chickens and rats (38-40, 52, 67, 68) while those of swine are not affected by T-2 toxin treatment (35).

The pathological lesions in the bone marrow are cellular degene= ration (28, 68), hypoplasia (69, 70) and karyorrhexis (28). All cells in the bone marrow are not affected to the same extent by the trichothecenes. In mice erythropoeisis is more susceptible to T-2 toxin than myelopoeisis and thrombopoeisis (69, 70). In rats, myeloid cells are more susceptible to T-2 toxin than lymphoid cell lines (67).

1.5.3.6 <u>The effect on serum cholesterol and hematocrit levels</u>

Cholesterol levels increase in the serum of cows, rats, laying hens and broiler chicks after T-2 toxin treatment (22, 51, 61, 71, 72). Swine and terminally ill cancer patients treated with T-2 toxin and DAS respectively, show no changes in their serum cholesterol (35, 59).

The hematocrit of monkeys, broiler chicks, rabbits, calves and guinea pigs decreases after T-2 toxin administration (36, 55, 58, 60, 71) while DAS treatment has no effect on the hematocrit level in swine (56). This decrease in hematocrit correlates with the decrease in red blood cells observed in animals treated with the trichothecenes (Section 1.4.3.1).

1.5.4 The Effect of the Trichothecenes on the Hepatobiliary System

The livers of animals appear to be prime targets for the action of trichothecenes. Chickens administered T-2 toxin i.m. present with enlarged and haemorrhagic livers (73) while monkeys and rats treated with DAS show a decrease in liver weight (21).

The lipid composition of liver is influenced by trichothecenes. Albino rats receiving dietary T-2 toxin have small areas of focal fatty change in their livers (53) and in calves fatty degeneration is observed after T-2 toxin treatment (74). Laying hens and rats treated with deoxynivalenol and T-2 toxin, respectively, have increased lipid levels in the liver (75, 76). The increased lipid content consists mainly of triglycerides, free cholesterol and phosphatidyl choline. Studies with [1-^{14}C] acetate suggest that the synthesis of these lipid components is reduced in the rat livers. Despite this decreased synthesis of lipids, the total lipid content increases, suggesting impaired secretion of lipids from the liver to blood (75).

Microscopical examination of affected livers reveals cytoplasmic degeneration of cells (77) as well as irregular cell nucleus size in the liver (21). Liver cell damage is also indicated by changes in the levels of serum enzymes. The increase in the levels of isocitrate dehydrogenase, alanine transaminase, aspartate transaminase and glutamate pyruvate transaminase in animals treated with T-2 toxin and DAS indicates damage to the hepatobiliary system

(56, 73). In animals where the liver is not affected by the trichothecenes, these enzyme levels are unchanged (35, 52, 56, 73). Animals treated with T-2 toxin and DAS however show a decrease in alkaline phosphatase activity (22, 36, 73, 78), probably due to impaired synthesis.

An enlarged gall-bladder is observed in chickens and hens receiving T-2 toxin (72). In young chickens T-2 toxin and DAS cause rapid necrosis of the extrahepatic biliary tract mucosa and of the gall bladder mucosa (40) as well as bile duct proliferation in rats and chickens (21, 40).

1.5.5 The Effect of Trichothecenes on the Gastrointestinal Tract

Treatment of rats, cows, mice and guinea pigs with T-2 toxin and DAS results in haemorrhaging of the intestine (21, 22, 68, 69). Very high doses of T-2 toxin result in the shedding of the intestinal mucosa in mature trout (79). Excessive damage to the actively dividing cells of the intestinal mucosa is observed in various animal species treated with fusarenon-X, neosolaniol, T-2 toxin and DAS (27-29, 38-40). Karyorrhexis, mitotic injury and cellular degeneration occurs in some animals (28, 29, 35) while others show hyperplasia of the mucosa (56, 70).

Trichothecenes also affect the intestinal crypts and villi. Swine and young chickens treated with T-2 toxin develop pyknotic nuclei and karyorrhexis of the crypt cells in the jejenum and ileum (35, 40).

In mice fusarenon-X causes a shortening of the intestinal villi (80).

One of the symptoms of trichothecene mycotoxicosis, diarrhea, was studied in fusarenon-X treated mice (80). As fusarenon-X inhibits peristalsis of the intestine, increased fluidity of the intestinal contents is thought to be responsible for the watery diarrhea. Fusarenon-X increases the permeability of the intestinal lumen.

1.5.6 The Effect of Trichothecenes on the Cardiovascular System

Oral and i.v. administration of T-2 toxin to rats results in an increase in blood pressure (25, 61, 81) followed by a decrease in blood pressure to below normal values as the rats near death (61, 81). As T-2 toxin causes no change in the blood pressure of pithed rats, animals with their central nervous system destroyed, it seems that the effect on the blood pressure occurs via the effect of T-2 toxin on the central nervous system (81). Fusarenon-X increases the blood pressure in mice, but in rats the blood pressure decreases (24).

In rats and guinea pigs, T-2 toxin causes an increase in heart rate followed by a decrease shortly before death (61, 81). Similar results were obtained with cats and guinea pigs treated with fusarenon-X (28). Fusarenon-X however has no effect on the heart rate of mice (24). T-2 toxin has no effect on the heart rate of pithed rats (81)

implying that the heart rate is affected via the central nervous system. T-2 toxin however has a mild but irreversible effect on the heart rate of isolated perfused rat hearts (82). In guinea pigs T-2 toxin causes myocardial conduction abnormalities (83) and thus the effect of the trichothecenes on the heart rate seems to be mediated by disturbances in the conduction of impulses.

Rats treated with T-2 toxin develop severe cardiovascular lesions (84, 85). The lesions in the heart consisted of interstitial oedema, focal cellularity and damage to single or groups of myocytes. After 10 daily administrations of T-2 toxin cardiomyo=phathy-like changes are seen with hypertrophy, focal fibrosis and abundant cellularity, especially in the subendothelial regions of the left ventricle (85).

The vasculature of animals is also affected by the trichothecenes. In rats T-2 toxin damages the vascular epithelium (24) and in a bovine ear perfusion system it results in a dose dependent vasocon=strictor response (86). The results from the ear perfusion system indicate that the natural defense mechanisms of the animal to stress or the response to stressor substances, such as histamine and norepinephrine, is impaired by T-2 toxin.

1.5.7 The Dermal Toxicity of Trichothecenes

The application of DAS, T-2 toxin and neosolaniol to the skin of various animal species results in crust formation that heals gradually (21, 22, 27, 79, 87). Haemorrhages are also observed

after the application of T-2 toxin and neosolaniol to the skin of mice (87). Low doses of T-2 toxin and DAS on mouse and rabbit skin result in a non-specific acute dermal inflammatory reaction that is characterized by hyperaemia, oedema and neutrophil exudation, and variable degrees of necrosis of the epidermis (87). The subcutaneous application of nivalenol and fusarenon-X results in oedamatous change with degeneration and necrosis of the subcutaneous tissue (88). The reaction of the trichothecenes on the skin of animals has been used in the detection of these toxic compounds.

1.5.8 The Carcinogenicity of the Trichothecenes

Marasas *et al* (79) observed that T-2 toxin fails to induce hepatomas in trout and rats. There are, however, reports that T-2 toxin causes hepatomas and neoplastic lesions in rats and mice (25, 89. 90). The trichothecenes are not mutagenic in the *Salmonella typhimurium* mutagenicity test but cause chromosomal aberrations in guinea pigs (90). The overall impression is that the trichothecenes are not carcinogenic *per se* but can influence carcinogenesis by reducing the immunological capability (90; section 1.4.2).

1.5.9 The Effect of Trichothecenes on Macromolecular Synthesis

The trichothecenes are very potent and specific inhibitors of protein synthesis (91) in a broad spectrum of organisms including fungi, plants and animals (92). The mode of action of inhibition has been studied extensively and it is now accepted that they act

mainly by interfering with the active centre of the ribosomal peptidyl transferase thereby preventing the completion of the ribosomal cycle (91, 93-97). Two mechanisms have been proposed - in the one instance, the trichothecenes only bind to the ribosome at certain stages of the ribosomal cycle, while in the other they bind throughout the ribosomal cycle, only interfering with peptidyl transferase when the ribosome assumes certain unique configurations during the cycle (92).

The ability of the trichothecenes to inhibit various stages of protein synthesis has been used to classify them into three categories, namely inhibitors of chain initiation (I), inhibitors of chain elongation (E) and/or inhibitors of chain termination (T). (90, 95, 96, 98, 99).

The chain initiation inhibitors are further divided into two groups: those that only inhibit the function of intact ribosomes (I_2) and those that can also prevent formation of the initiation complex (I_1)(90, 96).

The E or I type behaviour is dependent upon the concentration of some trichothecenes. Thus they are divided into classes of I type showing partial E type behaviour at high concentrations (I_2-E) and E-type showing I type behaviour at low concentrations (E-I_2) (90, 96). Table 7 gives examples of the different classes of inhibitors.

The termination inhibitors are either unsubstituted or carry only

a small substituent at the C-4 position. Trichothecenes with an ester group at C-4 or other positions are normally elongation inhibitors. Introduction of an ester function on the C-15 position will make a compound that shows pure E-type behaviour become an E-I_2 type compound. Compounds show type I behaviour if, in addition to an ester at C-15, either the C-3 or C-4 alcohol groups are acylated (88, 98-100).

The trichothecenes have also been shown to inhibit DNA synthesis in intact cells (90, 101), although no effect on DNA polymerases and thymidine kinase *in vitro* is observed. It seems that DNA synthesis inhibition is a secondary effect to protein synthesis inhibition (90).

TABLE 1.5 Classes of protein synthesis inhibitors

Trichothecenes	Class
Trichodermol	T
Trichothecin	E
Crotocin	E
T-2 toxin	I_2
HT-2 toxin	I_2
Diacetoxyscirpenol	I_2-E
Nivalenol	I_2
Scirpentriol	E-I_2
Verrucarin A,B,J.	I_2

1.6 METABOLISM OF THE TRICHOTHECENES

1.6.1 Xenobiotic Metabolism

Humans and animals are frequently exposed to foreign compounds, xenobiotics, that can enter their bodies and exert harmful effects. To reduce the harmful affects, various defense mechanisms exist, including the so-called drug metabolising enzyme systems, whereby the excretion of the compounds is fascilitated (102).

The drug metabolising enzymes occur in tissues such as kidneys, skin and intestine but the liver, because of its size and the relatively high concentrations of these enzymes, is the major site of drug metabolism. Some compounds may however not be metabolised primarily by the liver, depending on factors such as structure of compounds, enzyme specificity and site of entry into the body.

Drug metabolism is arbitrarily divided into two phases. The reactions occurring in phase 1 usually involve the conversion of a hydrophobic xenobiotic to a more polar, hydroxylated molecule (102). Phase 2 reactions are conjugation reactions whereby polar molecules are attached to a compound or to a hydroxylated product from a phase 1 reaction. These highly polar water soluble conjugates can then readily be excreted in the bile or urine (102).

The major phase 1 reactions are oxidation, reduction and hydrolysis. Enzymes involved in oxidation include cytochrome P_{450}, azo-reduc=

tases and nitroreductases. The major enzymes catalysing conjugation reactions in phase 2 of drug metabolism are glutathione S-trans= ferases, sulfotransferases and UDP-glucuronyltransferases.

A wide spectrum of compounds are capable of inducing the synthesis of drug metabolising enzymes, e.g. medical drugs, carcinogens, pesticides, cigarette smoke and various environmental pollutants. In drug metabolism, induction is defined as an increase in the total activity of the enzymes. Phenobarbital and 3-methylcholanthrene are two of the best known and most effective inducing agents. Pheno= barbital can induce a wide variety of enzymes such as cytochrome P_{450}, monoamine oxidase, epoxide hydratase, glutathione S-trans= ferases and UDP-glucuronyltransferases. 3-Methylcholanthrene induces cytochrome P_{448} (a form of cytochrome P_{450} with different specificity), epoxide hydratase, UDP-glucuronyltransferases and glutathione S-transferases. Although some substrates of the drug metabolising enzymes can act as inducers it is not necessary for a substance to be a substrate to have inducing activity (102).

1.6.2 Metabolism of T-2 toxin

Hayes and Schiefer (69) observed that mice receiving dietary T-2 toxin initially showed signs of inhibition of haematopoeisis which they later overcame while still consuming the food containing T-2 toxin. When rats were treated with phenobarbital before receiving T-2 toxin the mortality rate decreased (61). These observations pointed to an inducible enzyme system that is

involved in the detoxification of T-2 toxin.

Tissue distribution studies were performed in mice (28, 77), chickens (103) and swine (104) using [^{3}H] T-2 toxin or fusarenon-X. Radioactivity determinations in various tissues, excreta and blood of mice treated with [^{3}H] T2-toxin (p.o.) (77) indicate that there is no accumulation of radioactivity in any of the organs. The bile of these animals registers the highest specific radioactivity, indicating that T-2 toxin and/or its degradation products are mainly excreted via the liver and the biliary excretory system. Of the total dose of radioactivity, 57% is recovered in the faeces and 12% in the urine.

The radioactivity level in the blood after p.o. administration shows the occurrence of two peaks after 1 hour and 24 hours, respectively. The first peak is thought to be due to the rapid uptake of the toxin and the second peak is considered to originate from the reabsorption of T-2 toxin and/or its degradation products excreted via the bile into the intestine (77). Tissue distribution studies in chickens (103) and swine (104) after p.o. dosing of [^{3}H] T-2 toxin give results similar to those observed in mice (77).

The maximum levels of radioactivity in tissues of mice occur 30 minutes after s.c. injection of [^{3}H] fusarenon-X (28). As with T-2 toxin no specific accumulation in any tissue is observed, while the major route of excretion seems to be via the kidneys in the urine.

Ellison and Kotsonis (105) studied the *in vitro* metabolism of T-2 toxin and observed that when it is incubated with the post-mitochon= drial supernatant of bovine or human liver, HT-2 toxin is formed by the action of liver esterases which remove an acetyl group from C-4 on the T-2 toxin molecule. The conversion of T-2 toxin to HT-2 toxin using post-mitochondrial supernatant prepared from rat liver was demonstrated by Ohta *et al* (106). They followed the conversion gas chromatographically and showed that the amount of HT-2 toxin formed is equal to the decrease in the amount of T-2 toxin. When HT-2 toxin is incubated with hepatic post-mitochondrial supernatant, no decrease in the amount of HT-2 toxin is observed. The subcellular fraction with the highest deacylating activity is the microsomal fraction (106, 107). Kinetic analysis of the formation of HT-2 toxin reveals that the K_m value for the rat enzyme is 2.7×10^{-4} M (106).

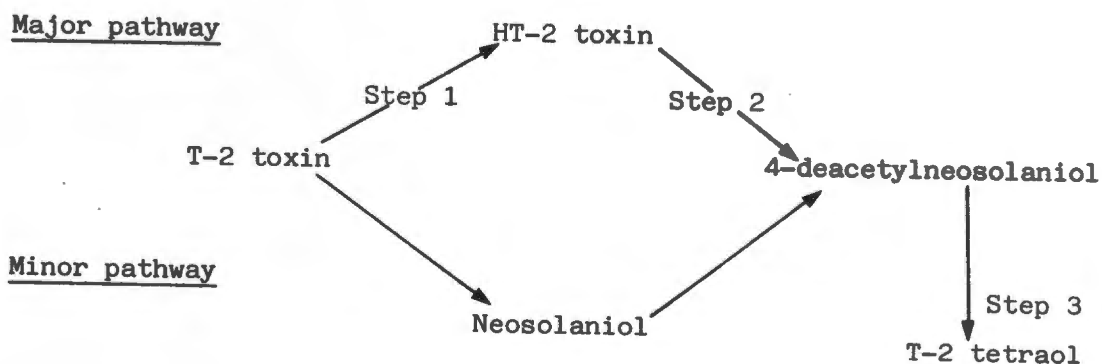
Ohta *et al* (106) also investigated the possible role of cytochrome P_{450} in the formation of HT-2 toxin. Eserine and diisopropylphos= phofluoridate (DFP), known inhibitors of esterases, completely inhibit the formation of HT-2 toxin, confirming that a microsomal esterase catalyzes this reaction and that cytochrome P_{450} is not involved. In the presence of microsomes and NADPH a decrease in the formation of HT-2 toxin is observed (106) which can be explained by the formation of 3'hydroxy T-2 toxin and 3'hydroxy HT-2 toxin (108).

The specificity of the microsomal esterases was examined by Ohta

_et al_ (106) by incubating various trichothecenes with microsomes prepared from rat and rabbit liver. They concluded that the enzyme attacks the C-4 acetyl residue of the trichothecenes and that the nature of the substituents at C-3 and C-8 influences the enzymatic hydrolysis of the C-4 acetyl residue. A hydrophobic group in the C-8 position appears to be essential for deacylation at C-4.

Yoshizawa (110) found that by incubating T-2 toxin with post-mitochondrial supernatant from rat liver for 60 minutes, T-2 toxin is completely metabolised to four metabolites, HT-2 toxin, T-2 tetraol and two unknown metabolites. When HT-2 toxin is incubated with the post mitochondrial supernatant, T-2 tetraol and the two unknown metabolites are formed, suggesting that the two unknown metabolites and T-2 tetraol are formed from T-2 toxin via HT-2 toxin.

Yoshizawa (110) proposed the following pathways for T-2 toxin metabolism:

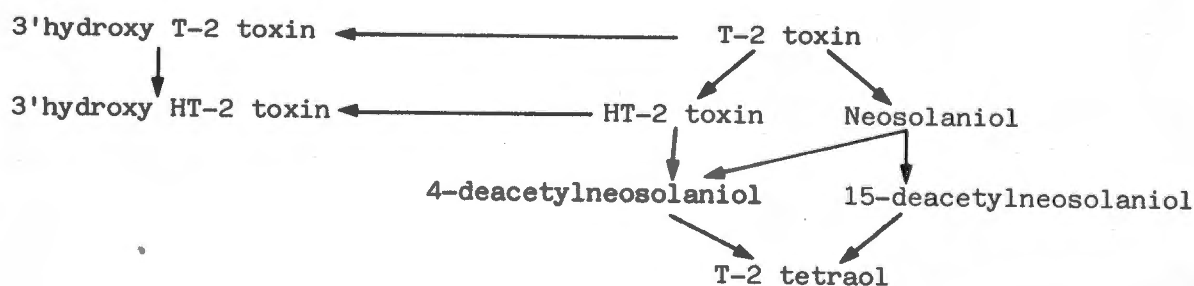


The reactions rates of the different steps were investigated by Yoshizawa and Sakamoto (107) using different subcellular fractions of the liver. The deacylation rates of steps 1 to 3 differ conside= rably in the different species, as shown below, when using post-mitochondrial supernatant.

SPECIES	(nmol product/mg/10 min)		
	STEP 1	STEP 2	STEP 3
Rabbit	128.5	0.7	3.6
Rat	31.4	0.8	0.4
Swine	19.3	0.1	1.7
Cow	4.9	0.2	0.3
Chicken	3.3	0.0	0.0

They also indicated that step 1 occurs in microsomes, step 2 in cyto= sol and step 3 in both microsomes and cytosol. Different enzymes appear to catalyze the different steps as the esterase inhibitor, eserine, affects the deacylation steps differently (107).

The proposed pathway shown in the diagram above can be elaborated by including the role of cytochrome P_{450}, as demonstrated by Yoshizawa et al (108).



Cell culture studies indicated that Chinese hamster ovary (CHO) cells metabolise T-2 toxin primarily to HT-2 toxin while African green monkey (VERO) cells form an unknown metabolite, more polar than T-2 toxin (111). O'Brien et al (112) observed that T-2 toxin is rapidly metabolised to HT-2 toxin by rat hepatocytes, and then more slowly to a multiple of unidentified metabolites. In contrast to Trusal and Watiwat (111), O'Brien et al showed that VERO cells were not able to metabolise T-2 toxin.

After the administration of T-2 toxin to swine glucuronides of T-2 toxin, HT-2 toxin, 3'hydroxy T-2 toxin and 3'hydroxy HT-2 toxin appear in their urine and bile (113). This would imply that the UDP-glucuronyltransferases are also involved in the detoxification of T-2 toxin. There are no reports of any in vitro studies undertaken to elucidate the role of the UDP glucuronyltransferases in the detoxification of T-2 toxin.

A variety of metabolites are found in the excreta of mice (77), chickens (114, 115) and cow (116) after the administration of T-2 toxin. The majority of these metabolites found are the same as those observed in in vitro experiments, but new ones such as 3'-acetoxy-3-hydroxy-HT-2 toxin and 3'-hydroxy-2-hydroxy HT-2 toxin are also present. A schematic representation of the metabolic pathway, as elucidated by in vivo and in vitro studies, is shown in Figure 1.3.

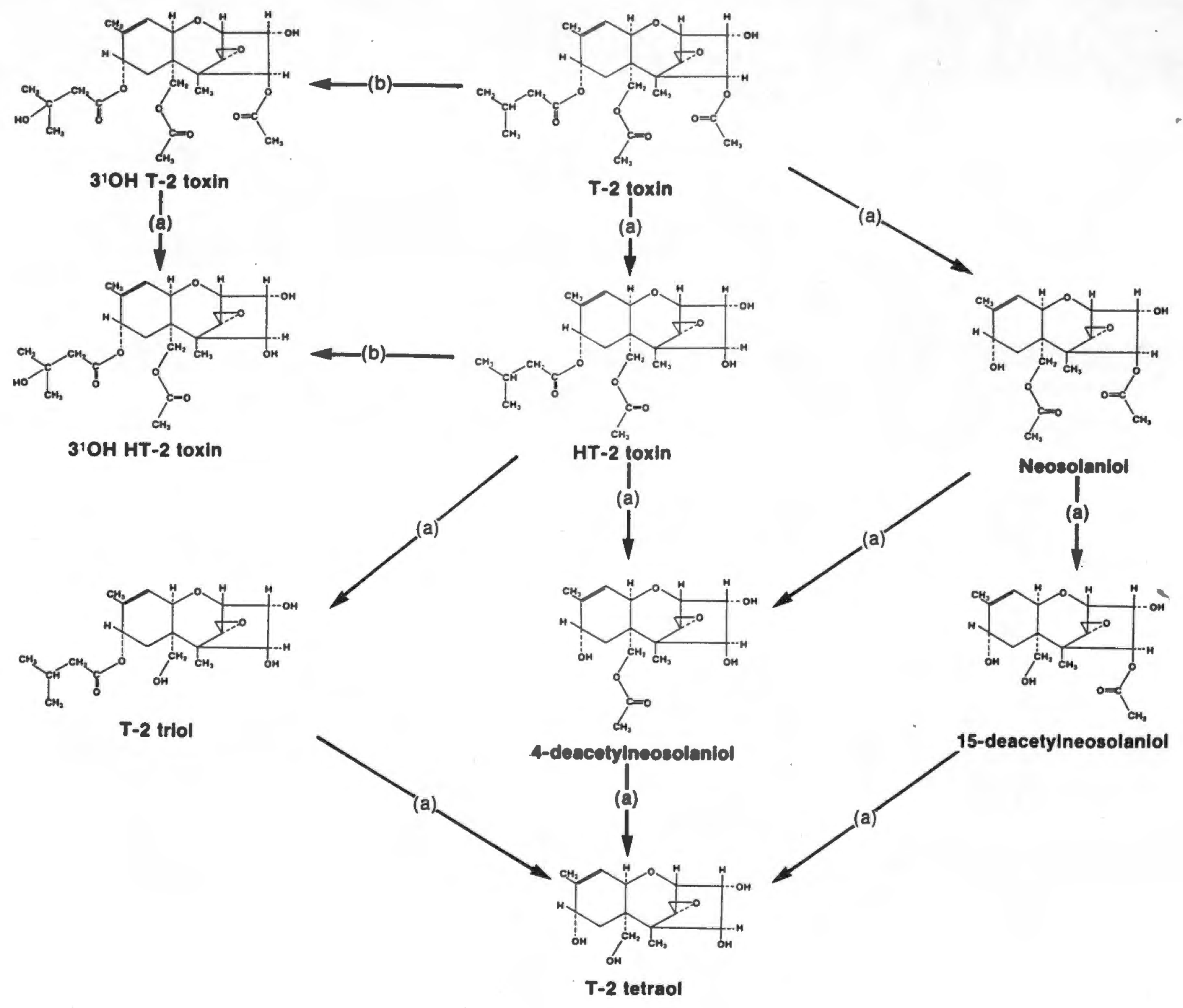


Fig. 1.3 The detoxification of T-2 toxin. Reactions denoted (a) are catalysed by esterases and (b) by cytochrome P_{450}.

1.7 OBJECTIVES

The trichothecenes have a wide spectrum of toxic effects on animals, some of which are haemorrhaging, ataxia, diarrhea, reduction in numbers of red blood cells, white blood cells and platelets, damage to actively dividing cells and suppression of the immuno= logical system. They have also been implicated in a number of human and animal diseases.

T-2 toxin is one of the most frequently occurring trichothecenes in foodstuffs (117). The acute and chronic toxic effects have been well studied in various animal species, indicating considerable differences in the toxic effects between species (Section 1.5). In order to elucidate the relationship between the detoxification of T-2 toxin and its toxicity, the deacylation rate of T-2 toxin was compared using rat, cat, monkey and human hepatic microsomes. Glucuronides of T-2 toxin and its metabolites have been detected in swine bile and urine (113). Since there have been no reports on the glucuronidation of T-2 toxin under _in vitro_ conditions, the glucuronidation of T-2 toxin was studied using rat and pig hepatic microsomes.

Although neosolaniol monoacetate (NMA) appears to be at least as toxic as the well known T-2 toxin (118), little is known about its mode of action or toxic properties. It was necessary to isolate sufficient amounts of the mycotoxin in order to study the biological effects of NMA.

Isolation was done from cultures of a South African isolate of *Fusarium compactum* strain MRC 1293 known to produce NMA in culture (119). The biological effects of NMA were studied in the male Wistar rats and the effects were compared to those of another highly toxic trichothecene, diacetoxyscirpenol.

CHAPTER 2

EXPERIMENTAL

2.1 THE ISOLATION OF NEOSOLANIOL MONOACETATE

2.1.1 Culture of Fungi

Yellow maize kernels (400 g) suspended in water (400 mℓ) were auto= claved on 2 consecutive days in 2 liter fruit jars for 1 hour at 120 kPa. An inoculum of Fusarium compactum strain MRC 1293 was pre= prepared by adding distilled water to a lyophilized culture obtained from the toxic fungal collection of the South African Medical Research Council. The sterilized maize was inoculated and incubated for three weeks at 25°C in the dark. Cultures were dried at 45°C, ground and stored in airtight containers at 4°C.

Rice (1 kg) was soaked overnight in distilled water (425 mℓ) and 100 g quantities were placed in 250 mℓ Erlenmeyer flasks and autoclaved on 2 consecutive days for 20 minutes at 120 kPa. Inoculums of F. compactum strain MRC 1293 and F. sambucinum strain MRC 1903 were prepared, as described above. After inoculation, the rice was incubated for 3 weeks at 27°C in the dark and stored at 4°C.

2.1.2 Extraction of Maize Cultures

Three different procedures were used for the extraction of maize cultures:

(a) Ethyl acetate extraction [Mirocha (120)]:

The maize culture (50 g) was moistened with water (30% w/v), blended with 50 mℓ ethyl acetate for 2 minutes at full speed in a Sorvall Omni=

mixer and filtered through Whatman no. 1 filter paper in a Buchner funnel. The ethyl acetate extraction was repeated 3 times, the filtrate combined, dried over anhydrous Na_2SO_4 for 3 hours and the solvent removed *in vacuo* at 40°C. The dried extract was partitioned between acetonitrile and petroleum ether in a separating funnel and the 2 layers were stored separately in Erlenmeyer flasks. The maize residue, remaining after ethyl acetate extraction, was extracted 3 times with 50 mℓ $MeOH:H_2O$ (2:3) by blending and filtration, as described. Extracts were combined and stored at 4°C.

(b) Aqueous methanol extraction:

The maize culture (50 g) was extracted 3 times with 50 mℓ $MeOH:H_2O$ (1:1) by blending and filtration, as described above. Extracts were combined and stored at 4°C.

(c) Methanol/chloroform extraction:

The maize culture (50 g) was extracted 3 times with 50 mℓ $MeOH:CHCl_3$ (2:8) by blending and filtration, as described in (a). Extracts were combined and stored at 4°C.

The extracts prepared by the different extraction procedures (a, b and c) were diluted to 200 mℓ with the respective solvents and 1:3; 1:15; 1:63; and 1:255 dilutions were made. The toxicity of 500 μℓ aliquots from each dilution was determined by the brine shrimp assay (Section 2.2).

2.1.3 Extraction of Rice Cultures

Rice cultures were extracted according to the method of Mirocha (120). The cultures (81 g) were extracted three times with 80 mℓ ethyl acetate by blending and filtration, as described in section 2.1.2. Extracts were dried over anhydrous Na_2SO_4, the ethyl acetate evapo= rated *in vacuo* and the residue partitioned between acetonitrile and petroleum ether, as described in section 2.1.2.

2.1.4 Fractionation of Extracts from Maize Cultures

The acetonitrile fraction (Section 2.1.2) was evaporated *in vacuo* and the residue (1.1 g), dissolved in a small amount of $MeOH:CH_2Cl_2$, was fractionated on a silica gel (Kieselgel 60:40 g) column (2 x 50 cm) using $MeOH:CH_2Cl_2$ (1:19) as eluent. Aliquots (250 μℓ) of 10 mℓ fractions of the eluate were tested for brine shrimp toxicity (Section 2.2). The toxic fractions were combined, the solvent evaporated *in vacuo* at 40°C and the residue (0.65 g), redissolved in a small amount of $MeOH:CHCl_3$ and further purified on a Sephadex LH-20 (30 g) column (2 x 35 cm) using $MeOH:CHCl_3$ (1:39) as eluent. Aliquots (100 μℓ) of 5 mℓ fractions of the eluate were tested for toxicity with the brine shrimp toxicity assay (Section 2.2). Toxic fractions were combined and analysed for the presence of NMA by gas chromatography (GC) (Section 2.3).

The fractions containing NMA were combined, the solvent removed *in vacuo* at 40°C and the residue (60 mg), redissolved in a small amount of $MeOH:CH_2Cl_2$, applied to a silica gel (Kieselgel 60:40 g) column (2 x 50 cm) using $MeOH:CH_2Cl_2$ (1:19) as eluent. Fractions of the eluate (5 mℓ) were analysed by thin layer chromatography (TLC) using

ethyl acetate:CH_2Cl_2 (3:7) as developing solvent (Section 2.4).

2.1.5 Fractionation of Extracts from Rice Cultures

The acetonitrile fractions obtained from rice cultures of *F. sambucinum* strain MRC 1903 and *F. compactum* strain MRC 1293 (Section 2.1.3) were individually fractionated by silica gel chromatography. The solvent was removed *in vacuo* at 40°C and the residues (0.34 g from *F. compactum*, and 0.72 g from *F. sambucinum*), redissolved in small amounts of MeOH:CH_2Cl_2, were applied to silica gel (Kieselgel 60:40 g) columns (2 x 50 cm) using MeOH:CH_2Cl_2 (1:19) as eluent. Fractions (10 mℓ) were collected and analysed by TLC using ethyl acetate: CH_2Cl_2 (3:7) as developing solvent (Section 2.4). Fractions contai= ning NMA were combined and analysed by GC to confirm the presence of NMA (Section 2.3).

The solvent was removed *in vacuo* at 40°C from the combined fractions obtained from the silica gel column fractionation of the *F. sambucinum* extract and the residue (0.32 g), redissolved in a small amount of ethyl acetate:CH_2Cl_2, was applied to a silica gel (Kieselgel 60:20 g) column (1.5 x 39 cm) using ethyl acetate:CH_2Cl_2 (3:7) as eluent. Fractions (10 mℓ) were collected and analysed by TLC using ethyl acetate:CH_2Cl_2 (3:7) as developing solvent (Section 2.4). The fractions containing NMA were combined and the solvent removed *in vacuo* at 40°C (residue weight 0.13 g). Final purification of NMA was obtained by crystallization using benzene/hexane as solvent. Crystals were dried in a dessicator and weighed.

2.1.6 Bulk Extraction of Rice Culture

A bulk extraction of a rice culture of *F. sambucinum* strain MRC 1903 (1 kg) was performed. Two batches of 500 g were each extracted 3 times with 500 mℓ ethyl acetate as described in Section 2.1.2. The ethyl acetate extracts were combined, dried over anhydrous Na_2SO_4 and the solvent removed *in vacuo* at 40°C. The residue was partitioned between petroleum ether and acetonitrile, as described in section 2.1.2, and the solvent was removed from the acetonitrile fraction *in vacuo*. This residue (4.2 g), redissolved in a small amount of $MeOH:CH_2Cl_2$, was fractionated on a silica gel (Kieselgel 60:300 g) column (5 x 500 cm) using $MeOH:CH_2Cl_2$ (1:19) as eluent. Fractions (50 mℓ) were collected and analysed by TLC (Section 2.4). The fractions containing NMA were combined, dried under vacuum and the residue (1.64 g), redissolved in ethyl acetate:CH_2Cl_2, was chromato=graphed on a silica gel (Kieselgel 60:100 g) column (3 x 45 cm) using ethyl acetate:CH_2Cl_2 (3:7) as eluent. Fractions (10 mℓ) were collected and analysed by TLC (Section 2.4). Fractions containing NMA were combined and the solvent evaporated *in vacuo* at 40°C. The residue was dissolved in benzene and NMA was crystallized from benzene/hexane.

2.1.7 Purity of NMA

The purity of the NMA crystals was assessed using GC (Section 2.3), melting point and mass spectroscopy. A Reichert melting point apparatus was used to determine the melting point and the mass spectro=scopy was performed at the National Chemical Research Laboratory of the CSIR.

2.2 BRINE SHRIMP TOXICITY ASSAY

The brine shrimp toxicity assay was performed by a modification of the method of Eppley (121). Brine shrimp (Artemia salina) eggs (0.2 g), obtained from a local pet shop, were incubated in 200 mℓ artificial sea water (122; Table 2.1 for 24 hours at 28°C).

TABLE 2.1 Ingredients for artificial sea water

Amount	Ingredient
46.954 g	NaCl
9.962 g	$MgCl_2$
7.834 g	Na_2SO_4
2.204 g	$CaCl_2$
1.328 g	KCl
0.384 g	$NaHCO_3$
0.192 g	KBr
0.052 g	H_3BO_3
0.048 g	$SrCl_2$
0.006 g	NaF

The pH of the artificial sea water was adjusted to 6.7 with 1 M HCl and the solution was made up to 2 liters with distilled water.

Polytop glass vials were washed with soap water, rinsed thoroughly with methanol and distilled water and dried at 100°C. Aliquots of the fractions to be tested were placed in clean polytop glass vials and stored at room temperature (covered with tissue paper) until the solvent evaporated completely (ca. 1 day). The brine shrimp larvae (ca.30) were drawn up in a plastic disposable syringe in a

volume of 1 mℓ and added to the vials containing the samples to be tested. Vials in which pure solvent was evaporated served as controls. The vials containing the brine shrimp larvae were incubated at 28°C for 48 hours.

The number of dead brine shrimp larvae were counted under a light microscope after 24 and 48 hours. Brine shrimp larvae were considered to be dead when no positive forward movement or movement of the legs were observed. After the 48 hour count all the shrimps were killed by adding acetonitrile and the total number of larvae per vial counted. The percentage mortality was then calculated for each vial.

$$\% \text{ mortality} = \frac{\text{number of dead larvae}}{\text{total number of larvae}} \times \frac{100}{1}$$

2.3 GAS CHROMATOGRAPHIC DETERMINATIONS

GC determinations were performed on a Varian model 3700 gas chroma= tograph fitted with a capillary column and electron capture detector (ECD). The trichothecenes were derivatized by acylation of the hydroxyl groups with heptafluorobutyrylimidazole (HFBI) (Figure 2.1) to increase the volatility of the compounds as well as to provide electron-absorbing halogen atoms enabling detection by ECD.

A modification of the method of Romer _et al_ (117) was used for the derivatization of samples and trichothecene standards. The samples were derivatized in 4 mℓ screw-top vials after the solvent was removed under a stream of nitrogen. Toluene:acetonitrile (95:5; 1 mℓ) was added to the vials and mixed with 0.1 mℓ HFBI (Pierce

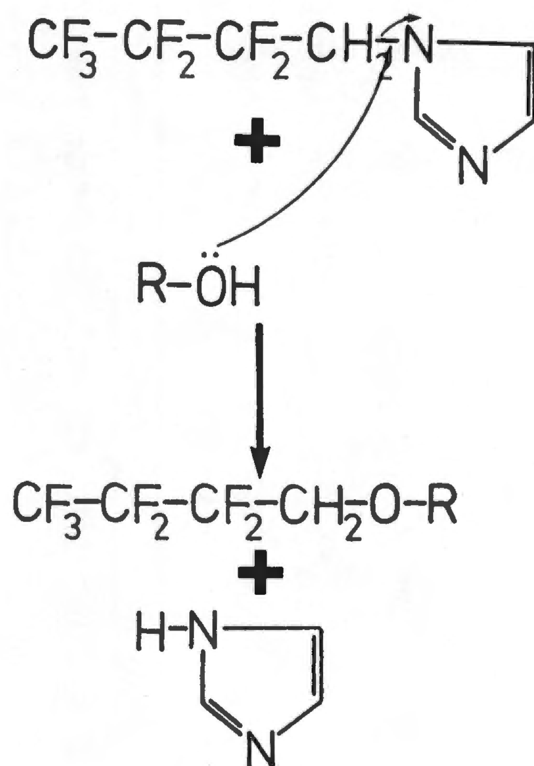


Fig. 2.1 The derivatization of hydroxyl groups by HFBI.

Chemical Company). After 1 hour incubation at 60°C, 1 mℓ 0.01 M phosphate buffer (pH 6.0) was added, the contents mixed and allowed to separate. An aliquot of the organic layer was evapo=rated to dryness, dissolved and appropriately diluted in benzene.

Aliquots of derivatized standards and sample extracts (1 µℓ) were injected using a split injection system onto a bonded SE-30 fused silica column (25 m x 0.32 mm i.d.). Helium was used as carrier gas and an ECD fitted with a ^{63}Ni radioactive source was used to detect the trichothecenes. Table 2.2 summarizes the exact chromato=graphic conditions.

TABLE 2.2 GC conditions for trichothecene analyses

Injector temperature	200°C
Detector temperature	300°C
Column temperature	180°C-250°C at 5°C/min
Carrier gas	Helium; 1.6 mℓ/min
Make-up gas	Nitrogen; 30 mℓ/min
Split ratio	10:1
Attenuation	64

2.4 THIN LAYER CHROMATOGRAPHIC ANALYSES

TLC analyses were performed on pre-coated silica TLC plates (Kieselgel 60, 0.25 mm thickness; Merck (SA)). Samples and standards (12.5 µg T-2 toxin, HT-2 toxin, T-2 tetraol and 6.3 µg NMA) were applied in spots 2 cm from the bottom of the TLC plate and 0.8 cm apart. TLC

plates were developed until the solvent front was 2 cm from the top of the plate. The plates were dried at room temperature, sprayed with a p-anisaldehyde mixture and heated at 120°C for 10 minutes. The p-anisaldehyde mixture consisted of 0.5 g p-anisaldehyde, 85 mℓ methanol, 10 mℓ glacial acetic acid and 5 mℓ H_2SO_4. The R_f values of the reddish-brown spots in the sample chromatograms were compared to those of the trichothecene standards.

2.5 THE BIOLOGICAL EFFECTS OF DAS AND NMA ON WISTAR RATS

2.5.1 Experimental Animals

Male and female Wistar rats (ca. 200 g bodyweight) bred at the Research Institute for Nutritional Diseases, MRC, Tygerberg, were used. The animals were housed in individual cages and allowed free access to rat cubes (Epol Ltd, Cape Town) and tap water.

2.5.2 Treatment of Animals

Male and female Wistar rats were used in a preliminary experiment. Male rats were divided into four groups with two animals per group. Three groups received, respectively, p.o. 3, 1 and 0.2 mg DAS/kg bodyweight, while a control group received the solvent dimethyl=sulfoxide (DMSO), 3 times a week, i.e. on Mondays, Wednesdays and Fridays, over a period of 4 weeks. The female rats received the same treatment as the male rats.

In the main experiment male Wistar rats were divided into two treat=ment groups. The rats in the experimental group (30 animals)

received p.o. 1 mg DAS/kg bodyweight (0.3 mℓ/200 g bodyweight), and the rats in the control group (30 animals) received DMSO (0.3 mℓ/200 g bodyweight) three times a week, for a period of 5 weeks. The experiment conducted with NMA was carried out in an analogous way.

2.5.3 Collection of Blood and Tissue Samples

During the preliminary experiment with DAS, rats were anaesthetized weekly by an i.p. injection of Sagatal (Pentobarbitone: Maybaker (S.A.) (Pty) Ltd, Port Elizabeth; 0.85 mℓ/kg bodyweight). Blood samples were collected via cardiac puncture, without opening the chest cavity, in Na^{+}EDTA washed plastic disposable syringes and transferred to Na^{+}EDTA vacuum tubes (Lab and Scientific, Cape Town).

In the main experiments with DAS and NMA, five rats from each group were sacrificed weekly. The rats were anaesthetized with Sagatal and blood samples collected by cardiac puncture. All organs and one femur were placed in buffered formalin for pathological exami= nation. A bone marrow impression was performed on the remaining femur.

2.5.4 Haematological Parameters

2.5.4.1 Haematological determinations

Blood platelets were counted with a Coulter counter Model Z_F accor= ding to the method supplied with the Coulter Platelet Kit (Coulter Diagnostics, Hialea, Florida). Blood was drawn up into a plastic

tube (2 mm i.d. x 4 cm) supplied with the platelet kit and allowed to stand for approximately 1 hour for the red blood cells to sediment, whereafter the platelet rich plasma (33 $\mu\ell$) was diluted to 25 mℓ with Isoton II (Coulter Electronics) and counted. The platelet numbers in the dilution was converted to the count in blood using the conversion table supplied. The hematocrit was determined with a Microhematocrit bench top centrifuge (Hawksley, England).

Red blood and white blood cell counts and other haematological para= meters were determined by the department of Haematology, Tygerberg Hospital, Tygerberg. The differential white blood cell counts were performed by the Pathology Section, RIND, MRC, Tygerberg.

2.5.4.2 Operation of Coulter Counter

The Coulter counter operates on a principle whereby particles or cells suspended in an electrolyte can be sized and counted by passing them through an aperture through which an electrical current is flowing. The particles or cells displace an equal volume of electrolyte, creating a change in the resistance in the path of the electrical current. The resulting current change is directly proportional to the size of the particles or cells. By drawing a specific volume of sample through the aperture and counting the number of voltage changes the number of particles or cells in the suspension can be determined.

Platelet rich plasma does not only contain platelets, but also red blood cells and other cellular debris which are likely to interfere with platelet counts. The cellular debris will result in voltage changes smaller than those resulting from platelets passing through the aperture and red blood cells will lead to voltage changes greater than those caused by platelets. The Coulter counter can be set to count only voltage impulses larger than a certain thres= hold level.

Two threshold levels can be determined experimentally to allow for the counting of platelets. The lower threshold setting will allow the counting of voltage changes caused by blood platelets and red blood cells, while discarding voltage changes caused by cellular debris. The upper threshold level will only count voltage changes caused by red blood cells. By subtracting the counts at the upper threshold setting from those at the lower threshold setting the counts due to platelets can be obtained. Lower and upper threshold levels suitable for the counting of rat platelets were determined to be 5 and 50, respectively.

To assess the effects of the toxins on blood platelet sizes, counts were also obtained at threshold levels of 15 and 25.

2.5.5 Pathological Examination of Tissues

The pathological examination of the tissues was performed by the pathology section, RIND, MRC, Tygerberg.

2.6 IN VITRO STUDIES ON THE METABOLISM OF T-2 TOXIN

2.6.1 Preparation of Microsomes

Microsomes were prepared from rat, human, cat, pig and monkey livers. In the study on the glucuronidation of T-2 toxin (Section 2.6.4) hepatic microsomes were prepared from a pig and phenobarbital induced rats. Male Wistar rats received 0.1% phenobarbital in their drinking water for five days prior to sacrifice. Induced and un= induced rats were starved for 16 hours, sacrificed by decapitation and the livers removed. A pig liver was obtained from the Maitland abbattoir. A male cat was obtained from a farm near Durbanville and the liver was removed under halothane anaesthesia. Delft animal Centre, Kuilsriver, supplied a liver biopsy from a female Vervet monkey. The monkey was anaesthetized with ketamine hydrochloride and exsanguinated. A human liver biopsy was obtained from a kidney donor that died from head injuries (Tygerberg Hospital, Tygerberg).

The livers were homogenized in 3 volumes (v/w) ice-cold 0.25 M sucrose in a Sorvall omnimixer. After filtration through 4 layers of cheesecloth, the homogenate was further treated in a Dounce homoge= nizer using 5 strokes with a "loose" plunger and 8 strokes with a "tight" plunger. All procedures were carried out on ice. The homogenate was centrifuged at 9000 x g for 20 minutes in a Sorvall centrifuge (4°C; SS-34 rotor) and the resulting supernatant was centrifuged for 1 hour at 100000 x g in a Beckman L8-70 M ultracentri= fuge (4°C; Type 65 rotor). The microsomal pellet was suspended in 50 mM Tris buffer (pH 7.4) containing 150 mM KCl and stored in 3 mℓ

quantities at -80°C. Before use the microsomal preparation was centri= fuged at 100000 x g for 1 hour and the pellet resuspended in the buffer mentioned in the text. The protein concentration was determined by the method of Lowry et al. (123).

2.6.2 Preparation of T-2 tetraol from T-2 toxin

T-2 tetraol was prepared from T-2 toxin by the method of Wei (124). T-2 toxin (500 mg) was incubated in 25 mℓ 1 N ammonia in methanol:water (4:1 v/v) for 72 hours at room temperature. The ammonia solution was removed in vacuo at 40°C and T-2 tetraol was isolated by chromatography on a silica gel (Kieselgel 60: 50g) column (2 x 50 cm) using ethyl acetate:acetone:methanol (50:50:1 v/v) as eluent. Fractions containing T-2 tetraol, as detected by TLC (Section 2.4), were combined and filtered through a Millex-SR 0.5 μm Filter unit (Millipore). On evaporation of the solvent in vacuo at room temperature, white crystals formed. 210 mg T-2 tetraol was recovered. The purity of T-2 tetraol was assessed by GC (Section 2.3).

2.6.3 Microsomal UDP-glucuronyltransferase Activity

UDP-glucuronyltransferase activity using phenolpthalein as substrate was determined according to the method of Halac and Bonevardi (125) Microsomes from phenobarbital induced rats were resuspended in 50 mM Tris-HCl buffer (pH 8.0) (Section 2.6.2). The incubation system were prepared as follows: (Total volume = 1 mℓ)

16 mM $MgCl_2$

1.6 mM UDP-glucuronic acid (Sigma Chemicals)

0.671 mM phenolphthalein (Merck)

50 mM Tris-HCl buffer (pH 8.0)

1 mg microsomal protein/mℓ

The incubation mixture, without UDP-glucuronic acid, was preincubated at 37°C for 3 minutes after which UDP-glucuronic acid was added to initiate the reaction. After 10 minutes 5 mℓ of 0.4 M glycine buffer (pH 10.5) was added. The mixture was centrifuged at 2000 x g for 10 minutes and the concentration of phenolphthalein was determined spectrophotometrically on a Beckman spectrophotometer ($\varepsilon = 12.6\ cm^2/\mu mole$ at 555 nm). UDP-glucuronyltransferase activity was expressed as nmoles phenolphthalein conjugated/min/mg protein.

2.6.4 The Glucuronidation Assay for T-2 toxin, HT-2 toxin and T-2 tetraol

Hepatic microsomes from a pig and phenobarbital induced rats were resuspended in 100 mM Tris-HCl buffer (pH 8.0) (Section 2.6.1). The microsomal UDP-glucuronyltransferases were activated by the addition of Triton X-100 according to the method of Boutin (126). The microsomal suspension was homogenized in a Dounce homogenizer in the presence of Triton X-100 (BDH Chemicals) (3.8 μℓ/10 mg microsomal protein) using 4 strokes of a "tight" plunger. The incubation mixture was prepared as follows: (Total volume = 1 mℓ)

0.6 mM T-2 tetraol or 0.429 mM T-2 toxin

16 mM $MgCl_2$

4 mM UDP-glucuronic acid

100 mM Tris-HCl buffer (pH 8.0)

5 mg microsomal protein/mℓ

Incubations were carried out in triplicate. The incubation mixture without the toxin was preincubated for 3 minutes at 37°C whereafter the specific toxin was added to initiate the reaction. Controls lacked UDP-glucuronic acid. After an incubation period of 30 minutes the reaction was stopped by the addition of 0.2 mℓ 10% trichloroacetic acid (TCA), mixed well and placed on ice. The ice-cold reaction mixtures were centrifuged in a Sorvall centrifuge (4°C) at 2000 x g for 15 minutes and an aliquot (0.6 mℓ) of the supernatant was passed through a Sep-pak C_{18} cartridge (Millipore). The cartridge was washed 3 times with 2 mℓ water to remove salts present in the supernatant while the toxins were eluted with 2 washes of 2 mℓ methanol and collected in 4 mℓ screw-top vials.

The eluates were dried under a stream of nitrogen and the residues were derivatized and analysed by GC (Section 2.3). The concentrations of the toxins present in the eluates were determined from standard curves compiled on each day, using 1 μℓ injections of the following concentrations of derivatized toxin:

T-2 toxin: 250, 500, 750, 1000 ng/mℓ

HT-2 toxin: 75, 150, 225, 300 ng/mℓ

T-2 tetraol: 50, 100, 150, 200 ng/mℓ.

Illustrations of the standard curves are presented in figures 2.2, 2.3 and 2.4.

2.6.5 Deacylation Assay for T-2 toxin

The microsomes prepared from rat, human, cat and monkey livers were resuspended in 50 mM Tris-HCl buffer containing 150 mM KCl (pH mentioned in the text) (Section 2.6.2).

The standard incubation mixture consisted of the following in a total volume of 1 mℓ:

T-2 toxin
50 mM Tris-HCl buffer containing 150 mM KCl
microsomes.

After preincubation of the microsomes for 3 minutes at the required temperature T-2 toxin was added to initiate the reaction. The reaction was stopped by the addition of 0.2 mℓ 10% TCA and the substrate and products were isolated using a Sep-pak C_{18} cartridge, as described in Section 2.6.4. Gas chromatography was used to monitor the conversion of T-2 toxin to HT-2 toxin (Section 2.3 and 2.4).

2.6.5.1 Effect of temperature

The effect of temperature on the deacylation reaction with rat microsomes (1 mg microsomal protein/mℓ) and T-2 toxin (0.5 mM) was determined by duplicate determinations at pH 7.4 and at 25, 30, 37 and 42°C (Incubation time 10 minutes).

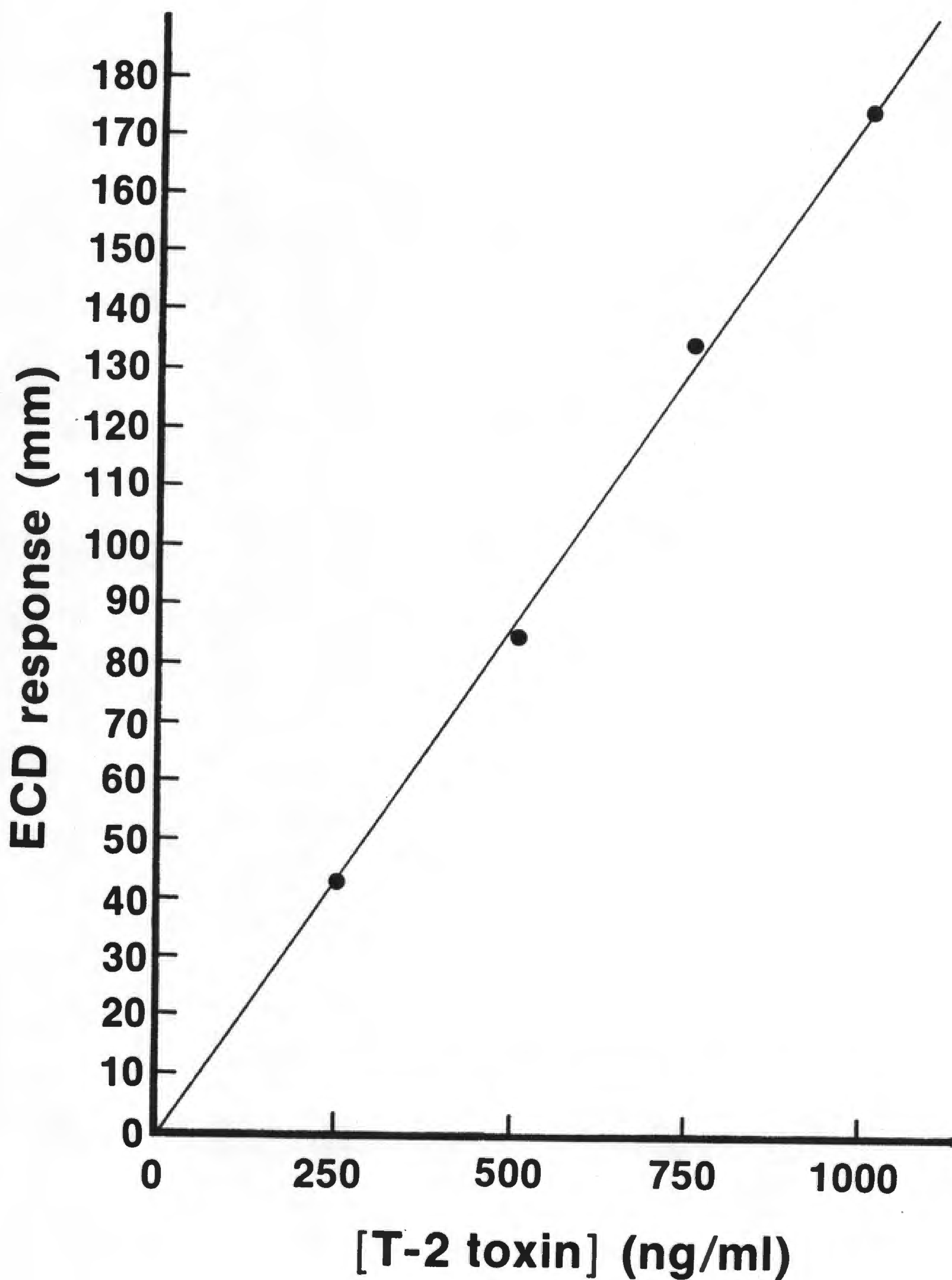


Fig. 2.2 Standard curve for the quantification of T-2 toxin by GC.

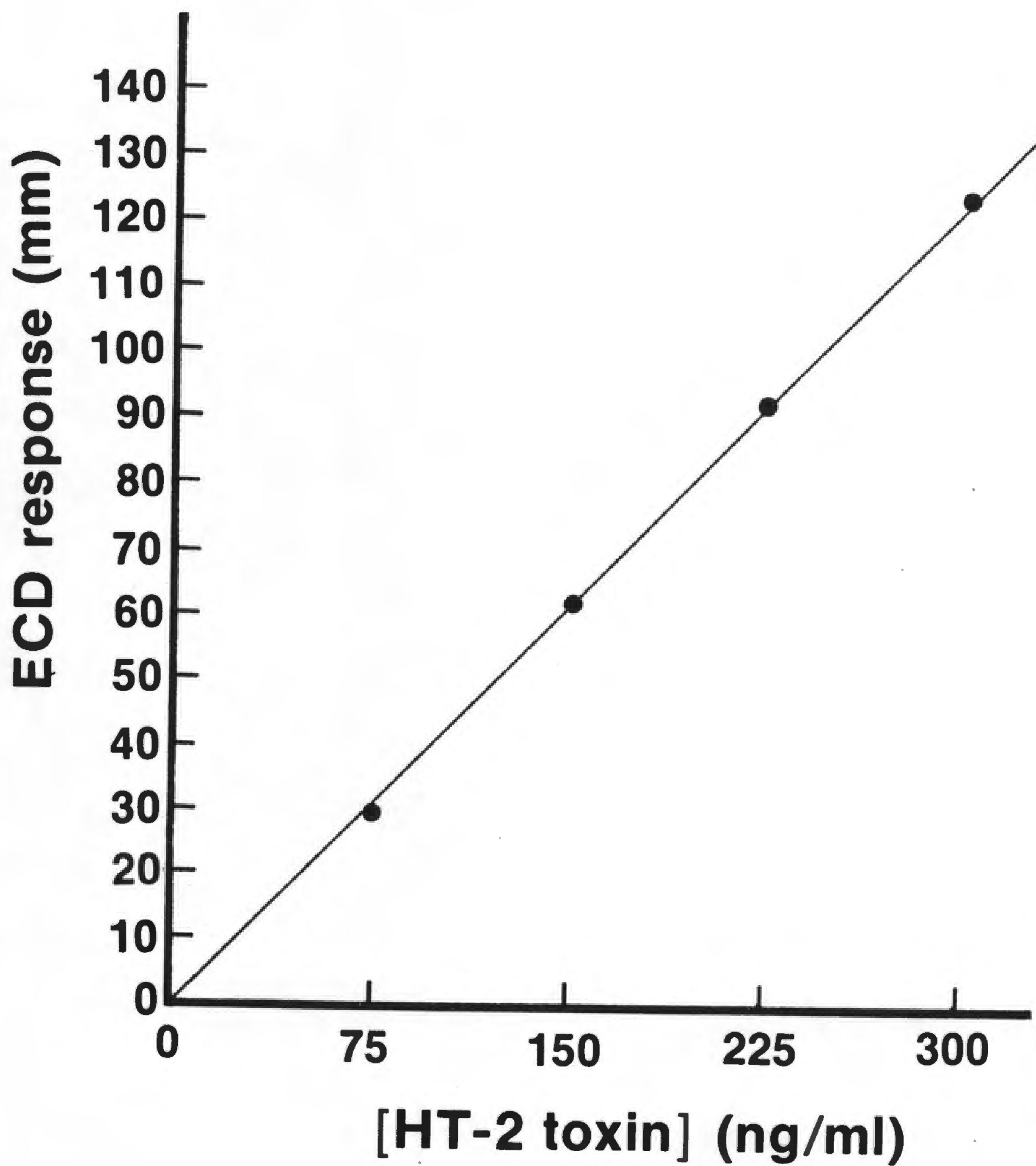


Fig. 2.3 Standard curve for the quantification of HT-2 toxin by GC.

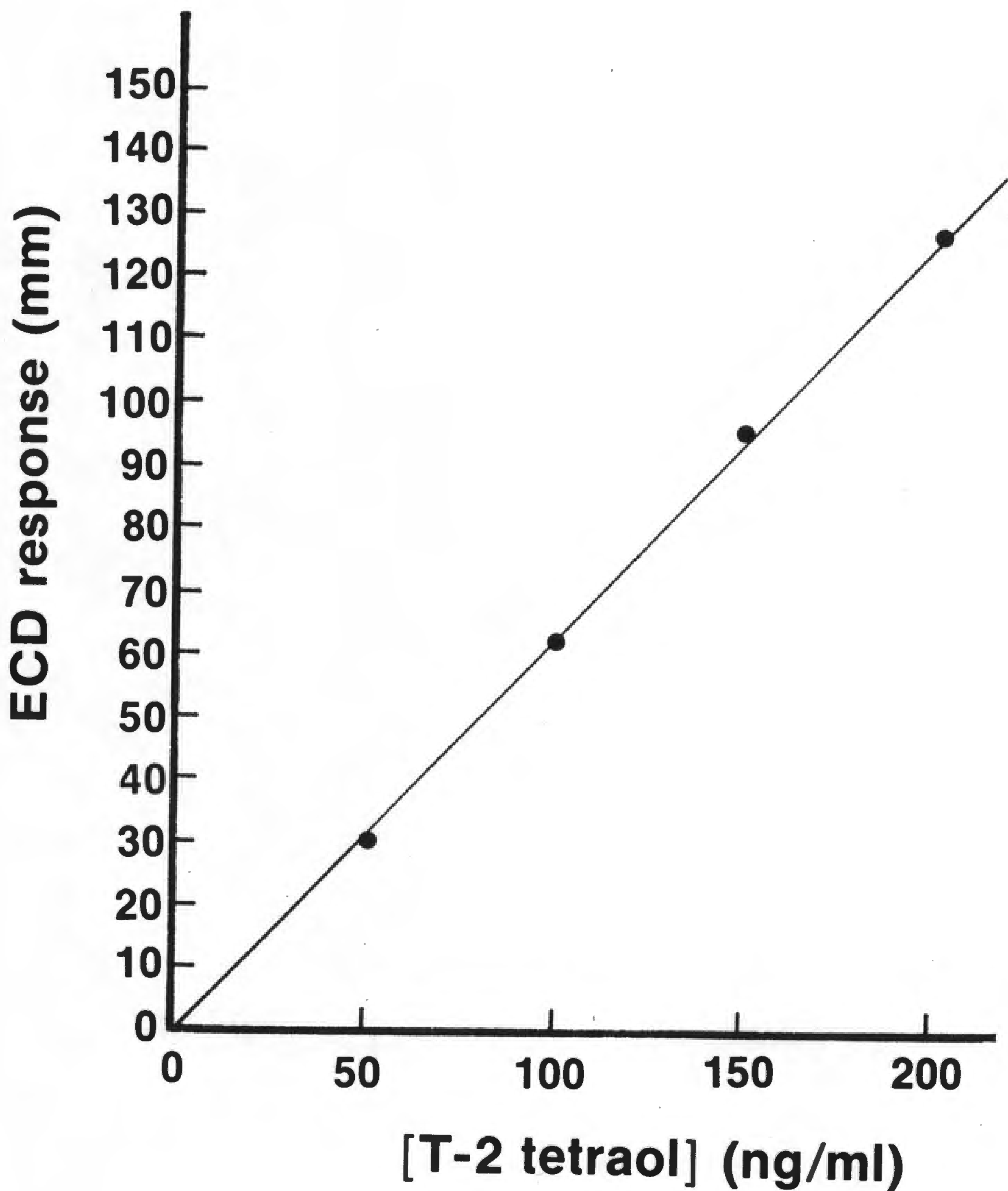


Fig. 2.4 Standard curve for the quantification of T-2 tetraol by GC.

2.6.5.2 Effect of pH

The effect of pH on the deacylation reaction was determined by performing assays at pH 7.0, 7.4, 8.0 and 8.5 for rat microsomes and at pH 7.0, 7.4, 8.0, 8.5 and 9.0 for microsomes from cat, monkey and human liver at 37°C as in table 2.3.

TABLE 2.3 ASSAY CONDITIONS FOR DETERMINING THE pH OPTIMUM

Source	Microsomal protein concentration (mg/mℓ)	T-2 toxin concentration(mM)	Incubation time (min)
rat	1	0.50	10
human	1	0.56	5
cat	2	0.56	30
monkey	1	0.56	15

As a control, T-2 toxin (0.56 mM) was incubated without microsomes at 37°C for 30 minutes with buffer at pH 7.4, 8.0, 8.5 and 9.0.

2.6.5.3 Time course study

Microsomes from the different sources were incubated (in duplicate) at 37°C and pH 7.4 for different time intervals as shown in table 2.4.

TABLE 2.4 ASSAY CONDITIONS FOR THE TIME COURSE STUDY

Source	Microsomal protein concentration (mg/mℓ)	T-2 toxin concentration (mM)	Incubation times (min)
rat	1.0	0.50	5, 10, 15
human	1.0	0.56	5, 10, 20
cat	2.0	0.56	15, 30, 45, 60
monkey	1.0	0.56	10, 20, 40

2.6.5.4 Kinetic studies

In order to determine the kinetic constants of the deacylation reaction the initial velocities of the deacylation reaction were determined at pH 7.4 and 37°C, using the conditions in table 2.5.

TABLE 2.5 ASSAY CONDITIONS FOR THE KINETIC STUDIES

Source	Microsomal protein concentration (mg/mℓ)	Incubation time (min)	T-2 toxin concentra= tions (mM)
rat	1	10	0.14, 0.28, 0.42, 0.56, 0.70
human	1	5	0.27, 0.54, 0.8, 1.07, 1.34
cat	2	30	0.27, 0.54, 0.8, 1.07, 1.34
monkey	1	15	0.27, 0.54, 0.8, 1.07, 1.34

2.7 STATISTICAL ANALYSIS

All data are represented as means ± standard deviations. Multiple comparisons were performed on the haematological para= meters obtained from the *in vivo* experiments (Section 4.5) using Tukey's (140) and Gabriel's (141) tests. $P < 0{,}05$ was considered to be significant. The statistical analyses were performed by the Insti= tute of Biostatistics, MRC, Tygerberg. The Student's T-test was used to determine statistically significant differences in *in vitro* results.

2.8 MATERIALS

All reagents used were of analytical grade and obtained from Merck (SA).

HT-2 toxin standard were obtained from Makor Chemicals, Israel. DAS and T-2 toxin were previously isolated in our laboratory.

NMA standards were obtained as gifts from Dr K Ishii, Japan, and Dr R Cole, U.S.A.

CHAPTER 3

RESULTS

3.1 ISOLATION OF NMA FROM MAIZE CULTURES OF FUSARIUM COMPACTUM

Cultures of F. compactum strain MRC 1293 grown on maize were extracted with different solvent mixtures (Fig. 3.1) (Section 2.1.2) and dilutions of the extracts were subjected to the brine shrimp toxicity assay (Section 2.2), as it has previously been used to isolate tricho= thecenes (121). The toxicities of the extracts at comparable concentrations are represented in Figures 3.2 and 3.3.

The ethyl acetate, MeOH:H_2O (1:1) and MeOH:$CHCl_3$ (2:8) extracts were toxic to brine shrimp larvae, and both the undiluted and 1:3 dilutions resulted in a 100% mortality. The 1:15 dilution of the ethyl acetate extract was slightly more toxic than the corresponding dilutions of MeOH:H_2O (1:1) and MeOH:$CHCl_3$ (2:8) extracts, while no toxicity was observed with higher dilutions. The residue remaining after ethyl acetate extraction was re-extracted with MeOH:H_2O (2:3) to determine whether ethyl acetate extraction removed all toxic material. Toxicity could however still be recovered from the maize residue.

After partitioning of the dried ethyl acetate extract between petro= leum ether and acetonitrile (Section 2.1.2), the bulk of the toxicity was recovered in the acetonitrile phase, while only a minute amount of toxicity was present in the petroleum ether phase (Fig. 3.3). Twenty-one percent (w/w) of the ethyl acetate residue was recovered in the acetonitrile phase and 79% (w/w) in the petroleum ether phase.

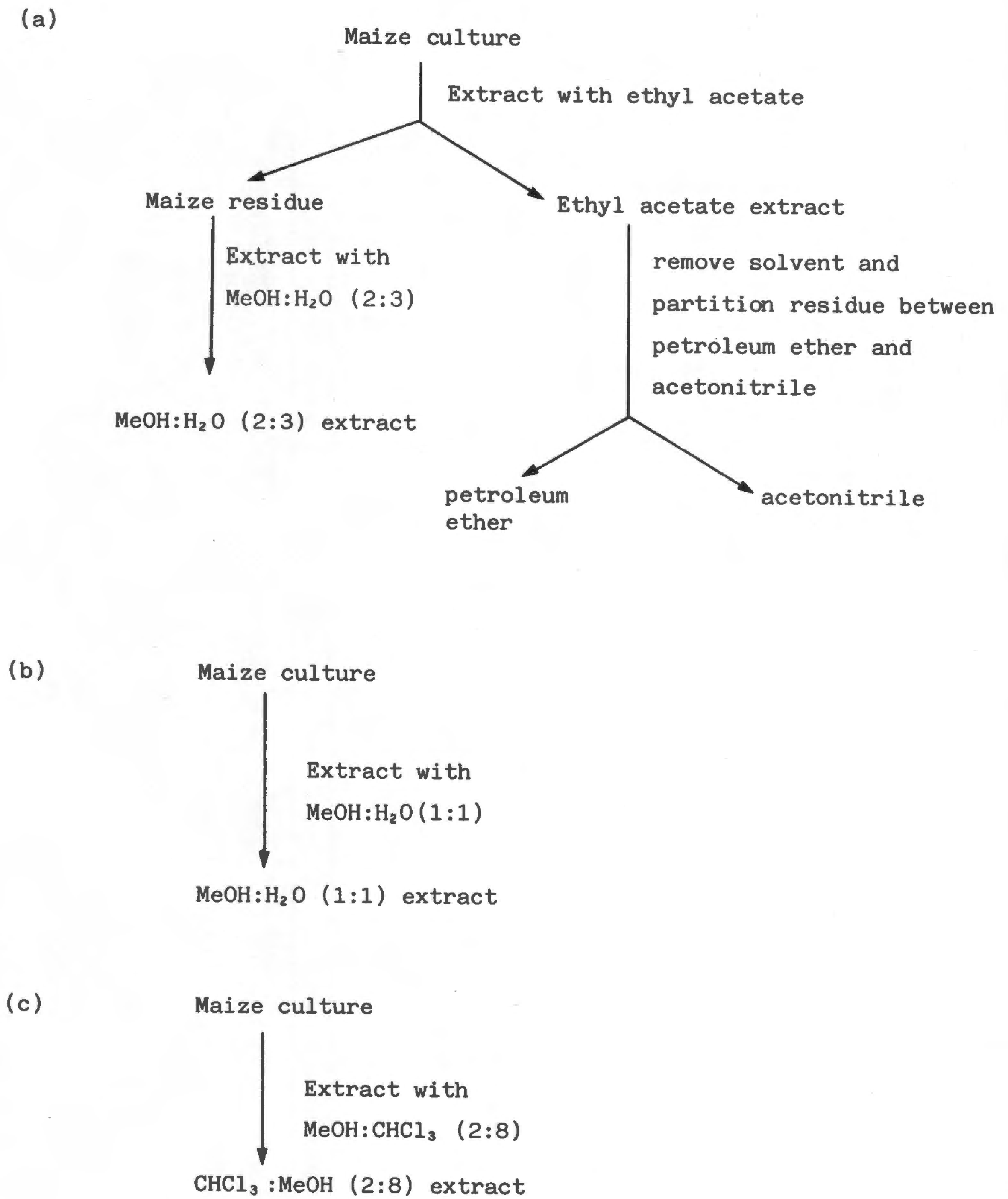


Fig. 3.1 The extraction of maize cultures with different solvent mixtures (Section 2.1.2).

Fig. 3.2 Toxicities of the ethyl acetate, MeOH:H_2O (1:1) and MeOH:$CHCl_3$ (2:8) extracts towards brine shrimp at 24 hours. (Section 2.1.2). ——·——·—— represents the mortalities of controls and where not shown the mortality is 0.

Fig. 3.3 Toxicities of the MeOH:H_2O (2:3), petroleum ether and acetonitrile extracts towards brine shrimps at 24 hours (Section 2.1.2). ——·——·——·—— represents the mortalities of the controls and where not shown the mortality is 0.

Silica gel and Sephadex LH-20 column chromatography were used for the further purification of the acetonitrile extract (Section 2.1.4). Fractions from the silica column were tested for brine shrimp toxicity (Section 2.2). Toxic fractions (15-28, Fig. 3.4) were combined and subjected to further fractionation by Sephadex LH-20 column chromatography (Section 2.1.4). Two major toxic areas were observed (Fig. 3.5). Fractions 2 to 7 (A), 8 to 14 (B) and 20 to 30 (C) were combined, and an aliquot of each of A, B and C was subjected to GC analysis (Section 2.3). The results indicated the presence of NMA in fraction A.

Fraction A was further fractionated by silica gel column chromatography (Section 2.1.4) and the eluate subjected to TLC analysis (Section 2.4). Fractions 4 to 8 contained a compound(s) that had the same R_f value (0.17) as the NMA standard. Fractions 4 and 5 (I) and 6, 7 and 8 (II) were combined, respectively, and analysed by GC (Section 2.3). NMA was present in fraction I (Fig. 3.6), but the chromatogram indicated the presence of many other contaminating compounds.

3.2 ISOLATION OF NMA FROM RICE CULTURES OF F. COMPACTUM AND F. SAMBUCINUM

Rice cultures of *F. compactum* strain MRC 1293 and *F. sambucinum* strain MRC 1903 were extracted with ethyl acetate and the extract was partitioned between acetonitrile and petroleum ether (Section 2.1.3). The acetonitrile extracts were fractionated by silica gel column chromatography (Section 2.1.5). Fractions were subjected to TLC

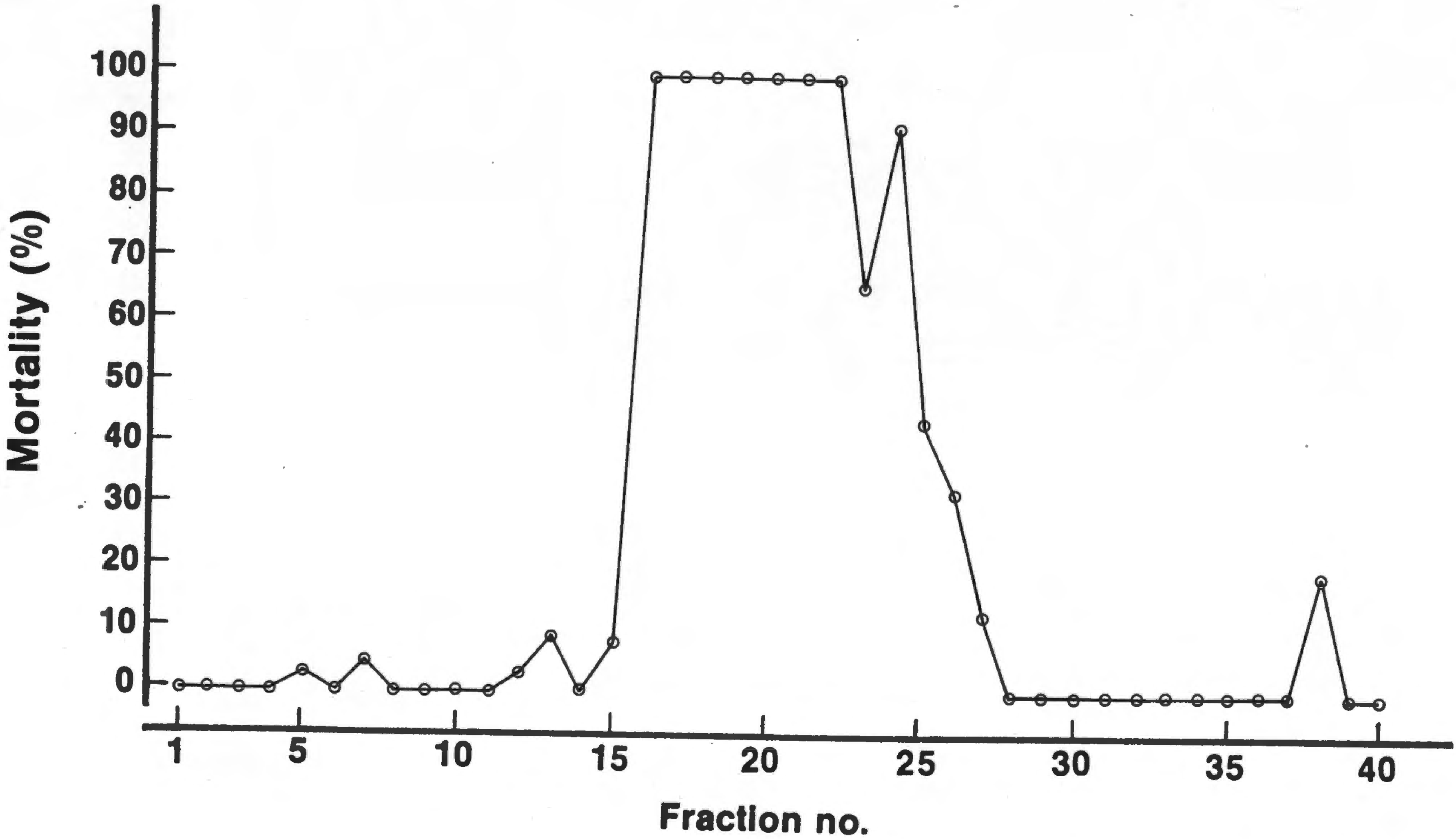


Fig. 3.4 Brine shrimp toxicity of fractions collected from the silica column used for the purification of the acetonitrile extract (Section 2.1.4).

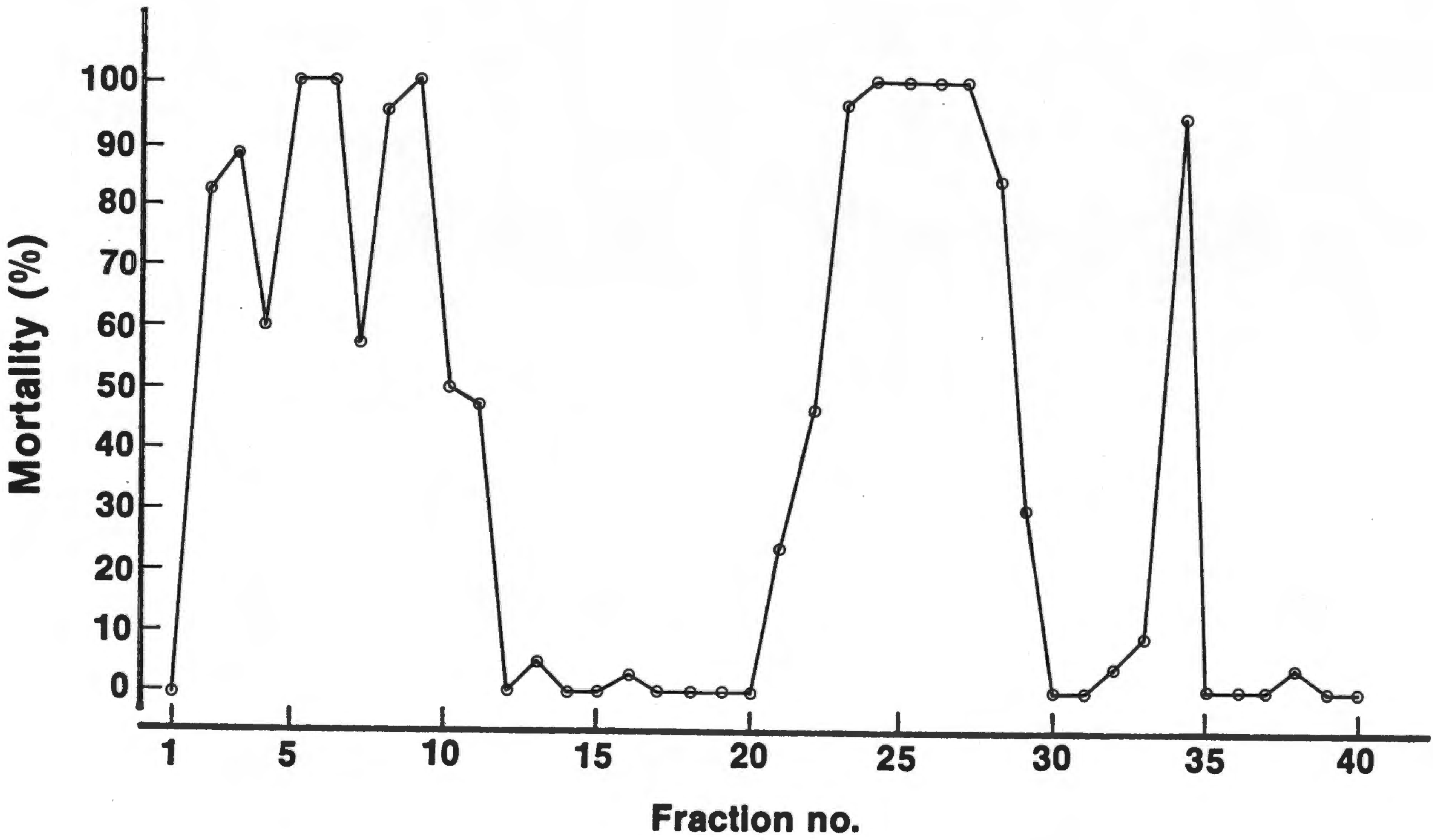


Fig. 3.5 Brine shrimp toxicity of fractions collected from the Sephadex LH-20 column (Section 2.14).

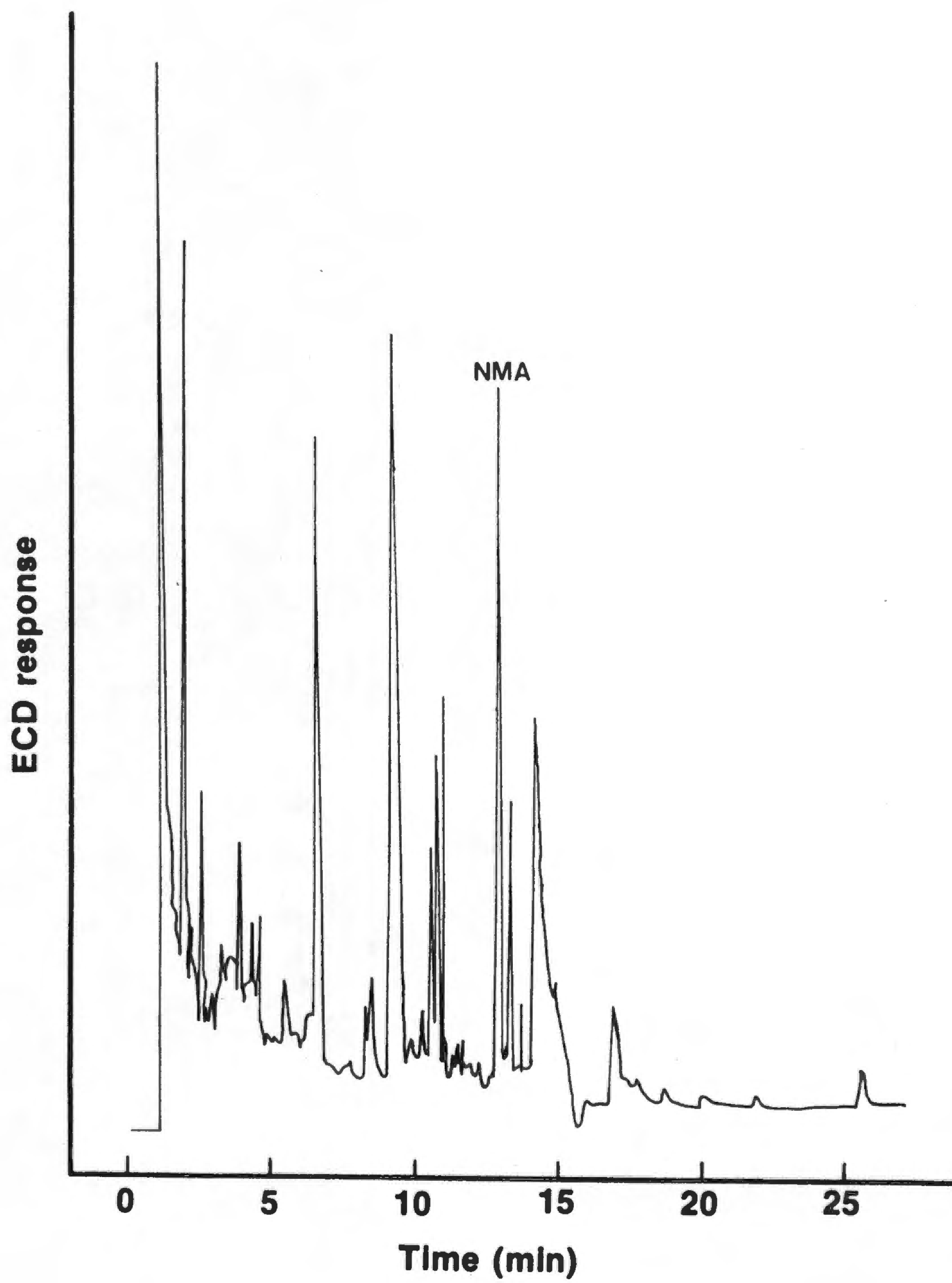


Fig. 3.6 GC analysis of fraction I (Section 3.1).

analysis (Section 2.4) and those fractions containing NMA (R_f = 0.17) were combined and subjected to GC analysis (Fig. 3.7). The amount of NMA produced by F. sambucinum was more than that produced by F. compactum. The F. sambucinum extract was further purified on another silica column (Section 2.1.5). TLC analysis was performed on the fractions and those containing NMA were combined.

Pure NMA (29 mg) was recovered from this material by crystallization from benzene:hexane (Section 2.1.5). This represents a yield of 358 mg/kg rice culture. A large scale extraction of the rice culture (Section 2.1.6) yielded only 50 mg/kg. The melting point of the crystalline NMA was found to be 190-192°C and GC analysis revealed only one peak at the same retention time as the NMA standard. The mass spectrum of the crystalline NMA is given in Figure 3.8.

3.3 BIOLOGICAL EFFECT OF DAS AND NMA IN RATS

3.3.1 Preliminary Experiments

Male and female Wistar rats received p.o. 3.0, 1.0 and 0.2 mg DAS/kg bodyweight three times a week over a 4 week period (Section 2.5.2) and blood samples were collected as described in section 2.5.3. The results indicated that the dosage level of 3.0 mg DAS/kg could not be used for chronic studies as the rats died within two weeks after the commencement of the experiment. The male and female rats receiving 1.0 and 0.2 mg DAS/kg survived the 4 week experimental period. Blood platelet levels were not influenced at any of the dosage levels. The only change observed in haematological parameters

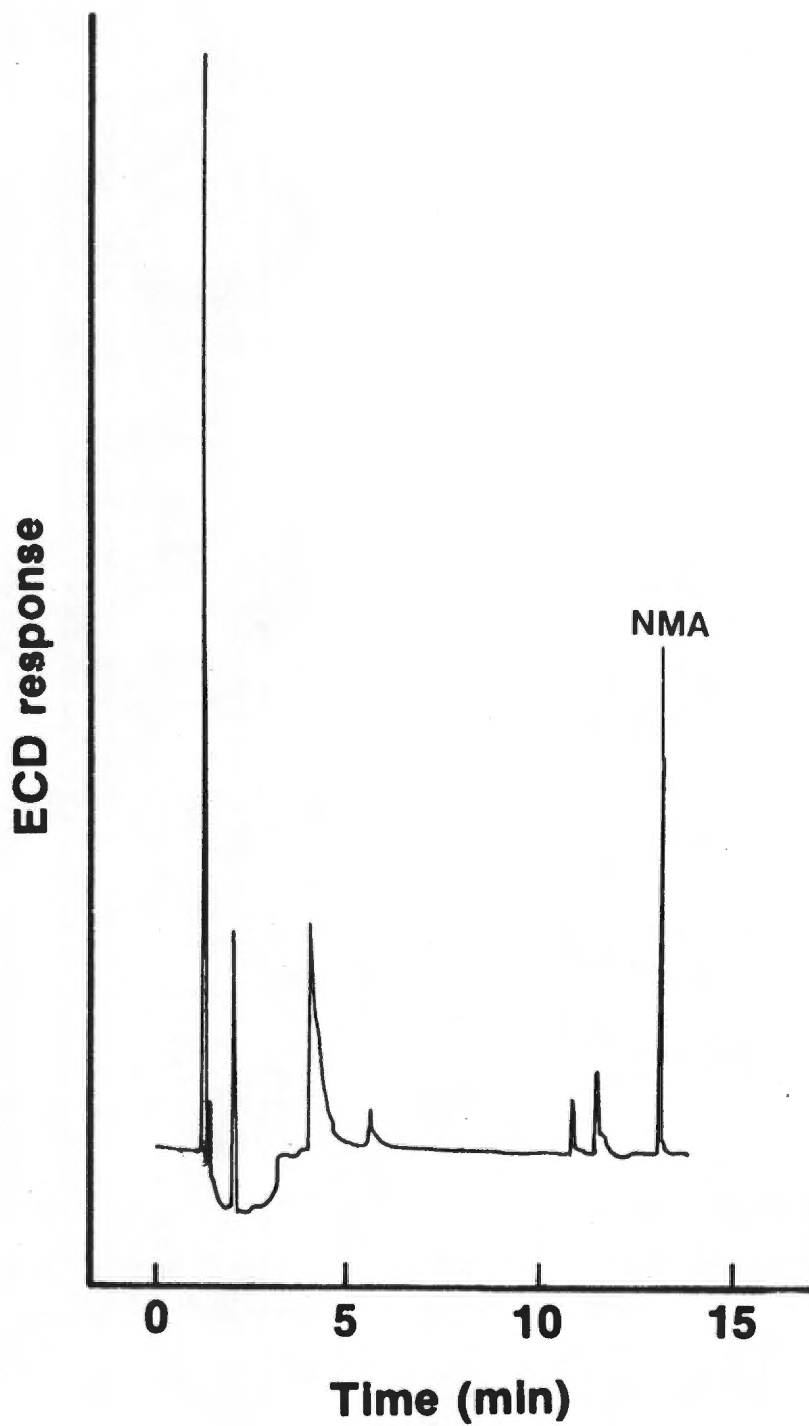


Fig. 3.7 GC analysis of the combined column fractions containing NMA, as determined by TLC, resulting from the fractionation of the acetonitrile extract of rice on a silica column (Section 2.1.5).

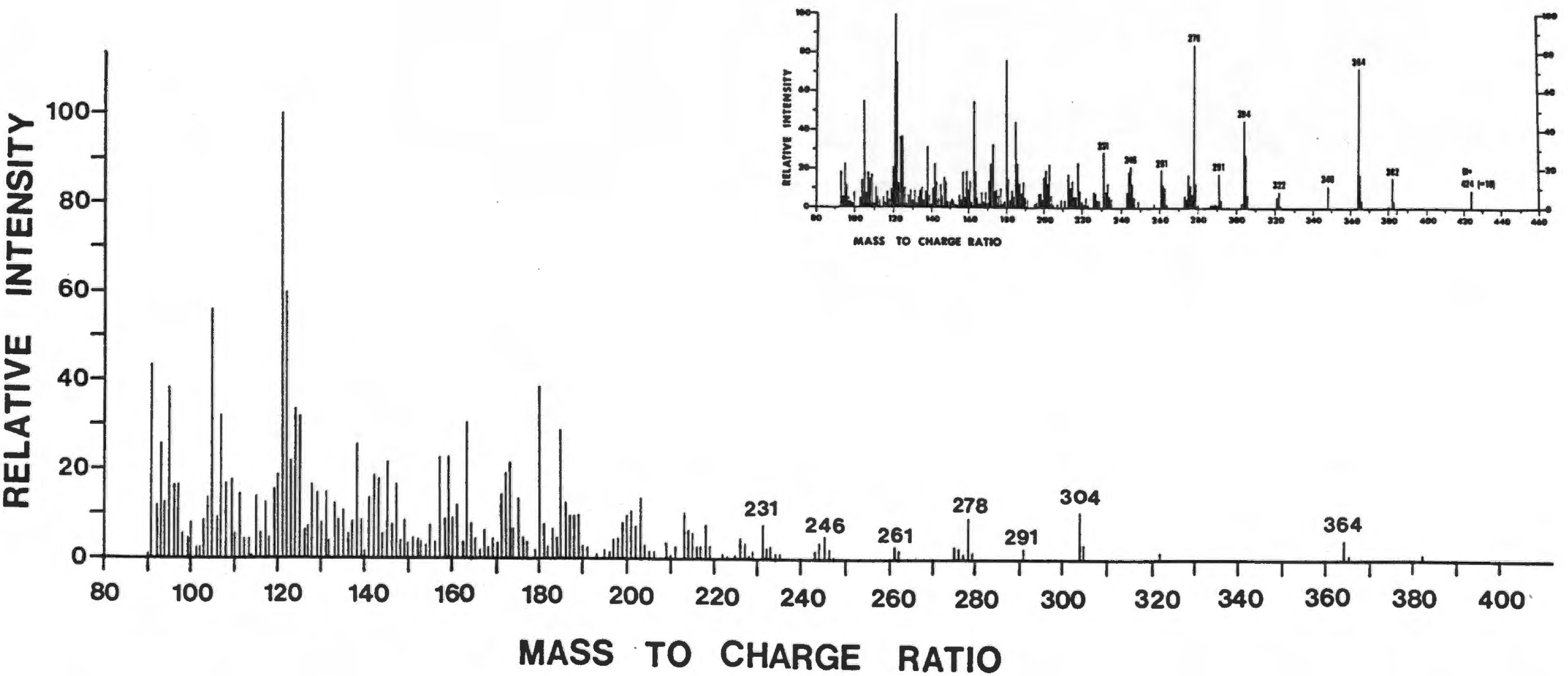


Fig. 3.8 The mass spectrum of isolated NMA. Insert - published mass spectrum (17).

was a decrease in the hematocrit (% red blood cells in blood) of rats treated at both levels of DAS. Treatment with DAS resulted in a reduced growth rate, when compared with the controls. The changes observed in the hematocrit and growth rate were more pronounced in the rats receiving 1.0 mg/kg than in those receiving 0.2 mg/kg. Male rats were more susceptible to DAS than females at a dosage level of 1.0 mg/kg, while at a level of 0.2 mg/kg there was no difference between males and females. Further studies were performed with male rats at a dose of 1.0 mg DAS/kg.

3.3.2 The Effect of DAS and NMA on Male Wistar Rats

Male Wistar rats received 1 mg DAS/kg bodyweight and 1 mg NMA/kg bodyweight, respectively, p.o. three times a week over a six week period (Section 2.5.2). The changes in haematological parameters and pathological changes in the organs were examined.

Treatment of rats with DAS and NMA slightly reduced the increase in bodyweight in rats receiving the toxins, when compared with that in the control animals (Fig. 3.9). The coats of rats treated with the toxins were not as shiny and smooth as those of the controls. The administration of DAS resulted in two cases of severe diarrhea, while no case of diarrhea occurred in any of the rats treated with NMA or in the controls.

3.3.2.1 The effect of DAS and NMA on the haematological parameters of rats

The blood platelet levels of control rats varied considerably over

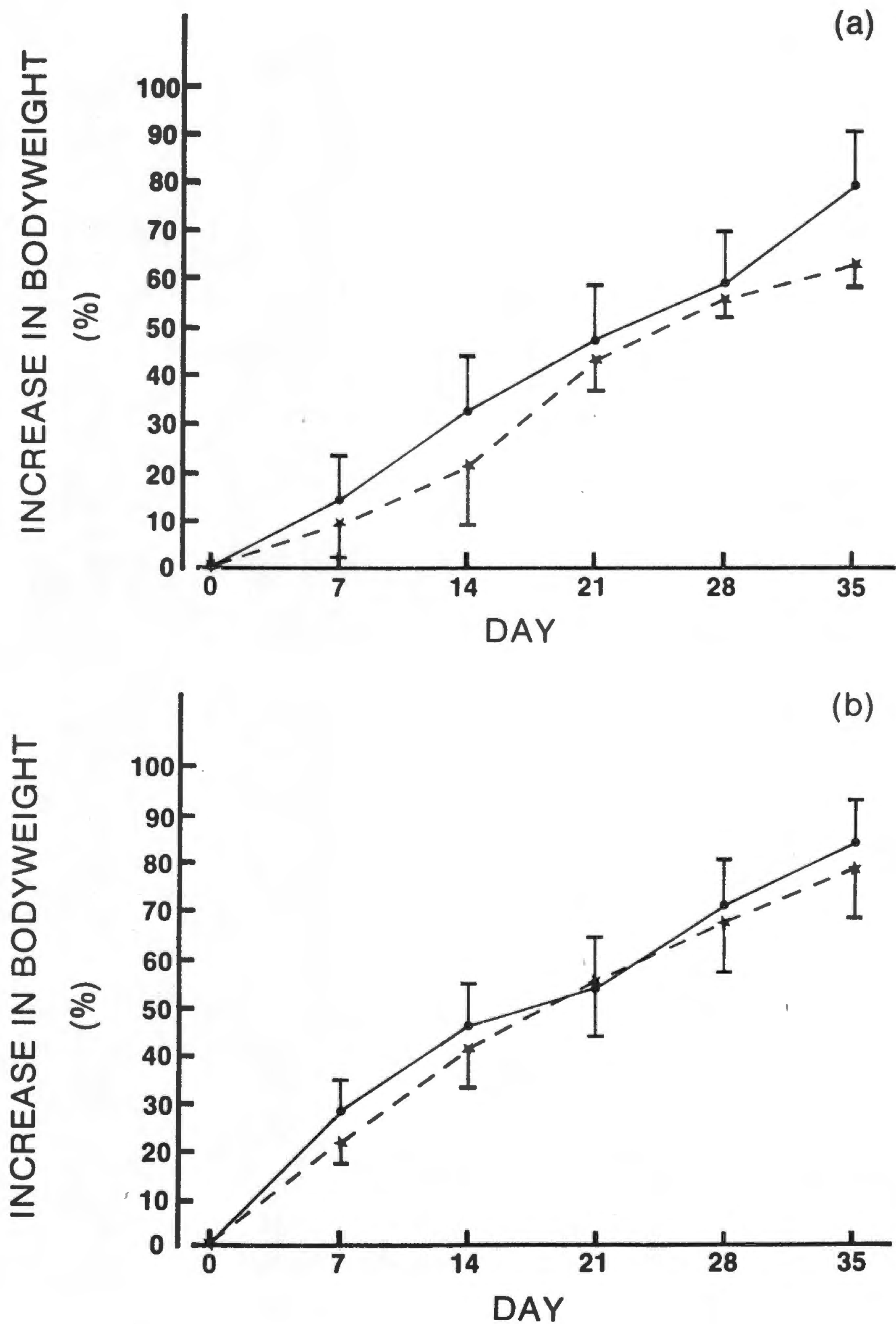


Fig. 3.9 The effect of DAS (a) and NMA (b) on the bodyweight of rats. Rats were treated as described in Section 2.5.2 (—— controls; ----- treated).

the experimental period (Fig. 3.10). DAS clearly had no significant effect on the platelet levels when compared with that in control animals. Although it appears as if treatment with NMA resulted in a decrease in platelet levels, statistical analysis (Section 2.7) indicated that this decrease was not significant.

The only significant effect on blood platelets was a change in size after treatment with either of the toxins. (Fig. 3.11 and 3.12). Absolute sizes of platelets were not measured but rather the relative numbers of platelets counted in the Coulter counter at different threshold settings (Section 2.5.4.2). Treatment with both DAS and NMA resulted in a significant increase in the number of larger platelets. This change was already observed one week after the commencement of the experiment and lasted the whole experimental period.

DAS and NMA had no significant effect on the total white blood cell levels in rats (Fig. 3.13) or on the lymphocyte, neutrophil or monocyte counts (Fig. 3.14, 3.15, 3.16). While it appeared as if there was a reduction in the eosinophil count from day 21 onwards this decrease was not statistically significant (Fig. 3.17).

Treatment of rats with DAS and NMA resulted in a significant reduc= tion in the red blood cell counts when compared with controls (Fig. 3.18). The red blood cell counts of the control rats showed a tendency to increase as the rats aged. The same tendency was observed with rats receiving the toxins but the counts were lower

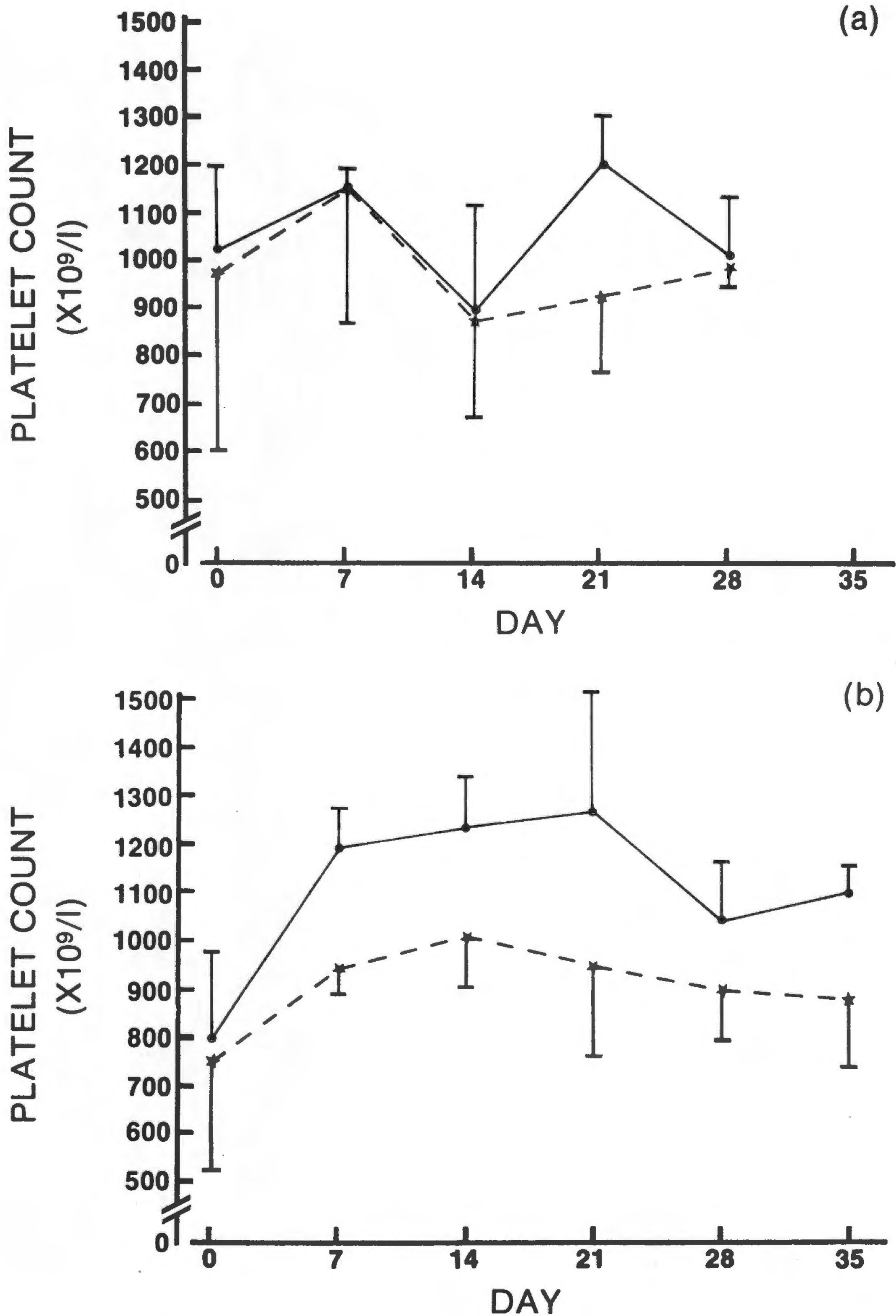


Fig. 3.10 The effect of DAS (a) and NMA (b) on the platelet levels of rats. Rats were treated as described in Section 2.5.2 (—— controls; ----- treated).

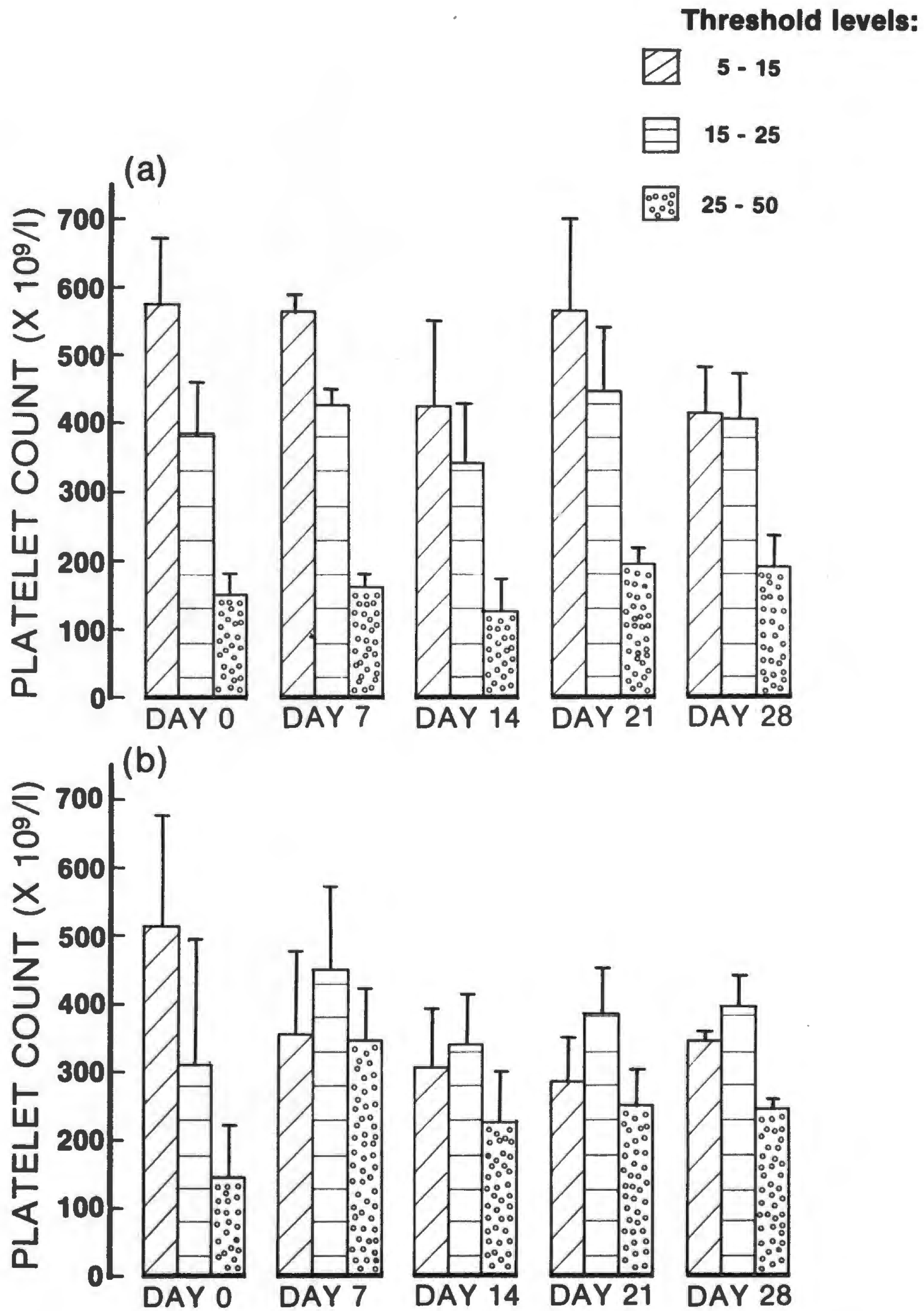


Fig. 3.11 The effect of DAS on the size distribution of platelets. Rats were treated as described in Section 2.5.2 ((a) control; (b) treated).

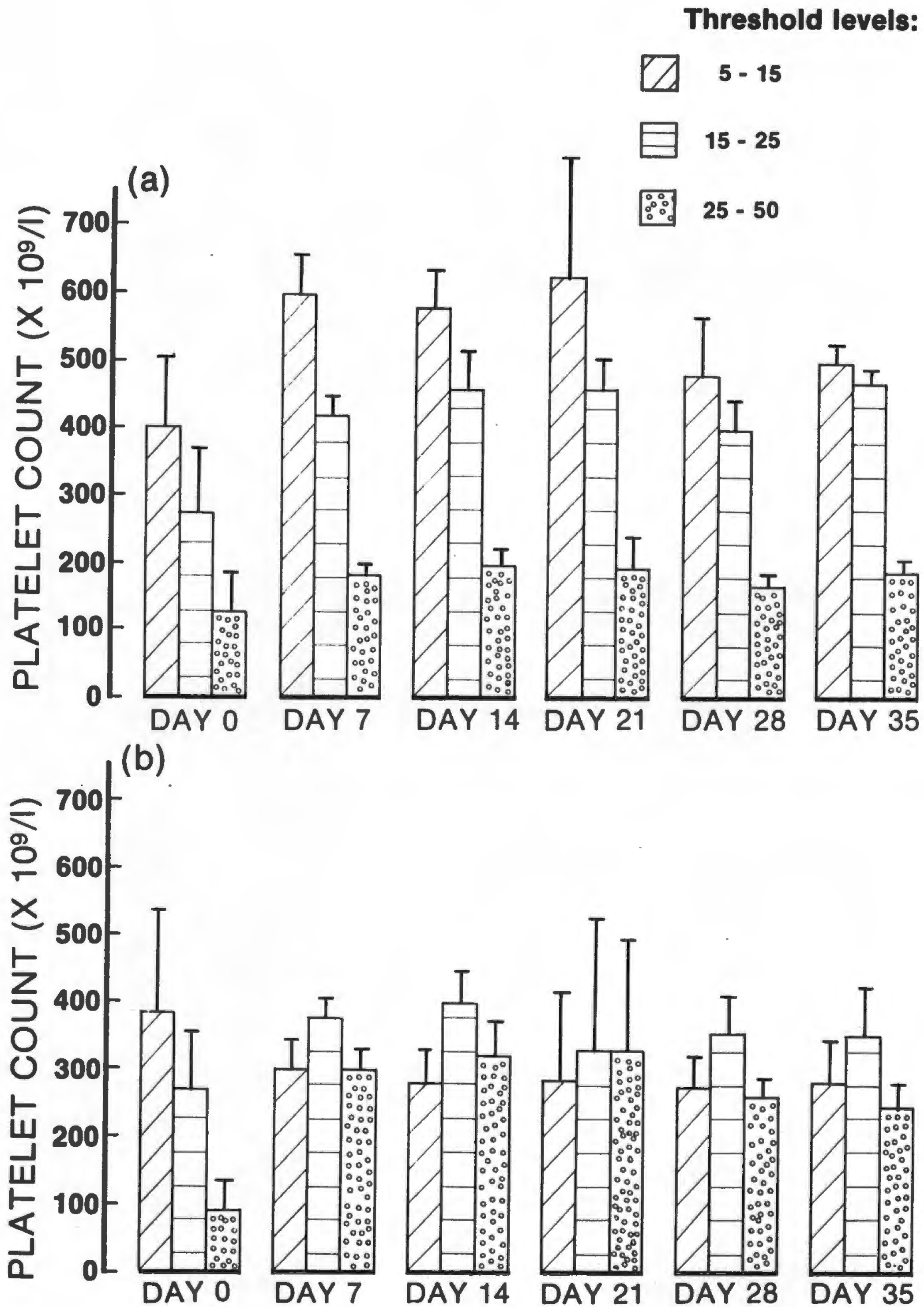


Fig. 3.12 The effect of NMA on the size distribution of platelets. Rats were treated as described in Section 2.5.2 ((a) control; (b) treated)

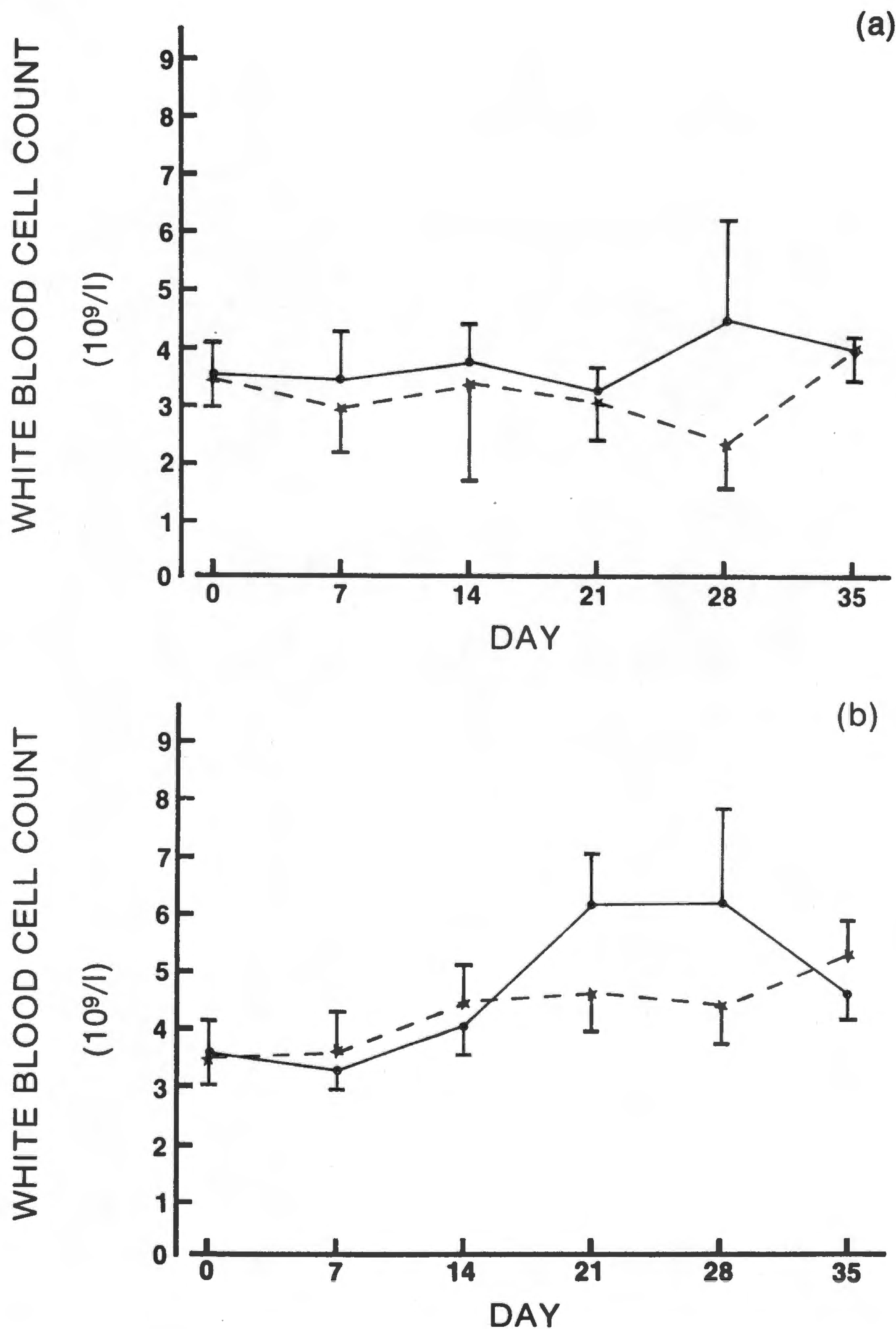
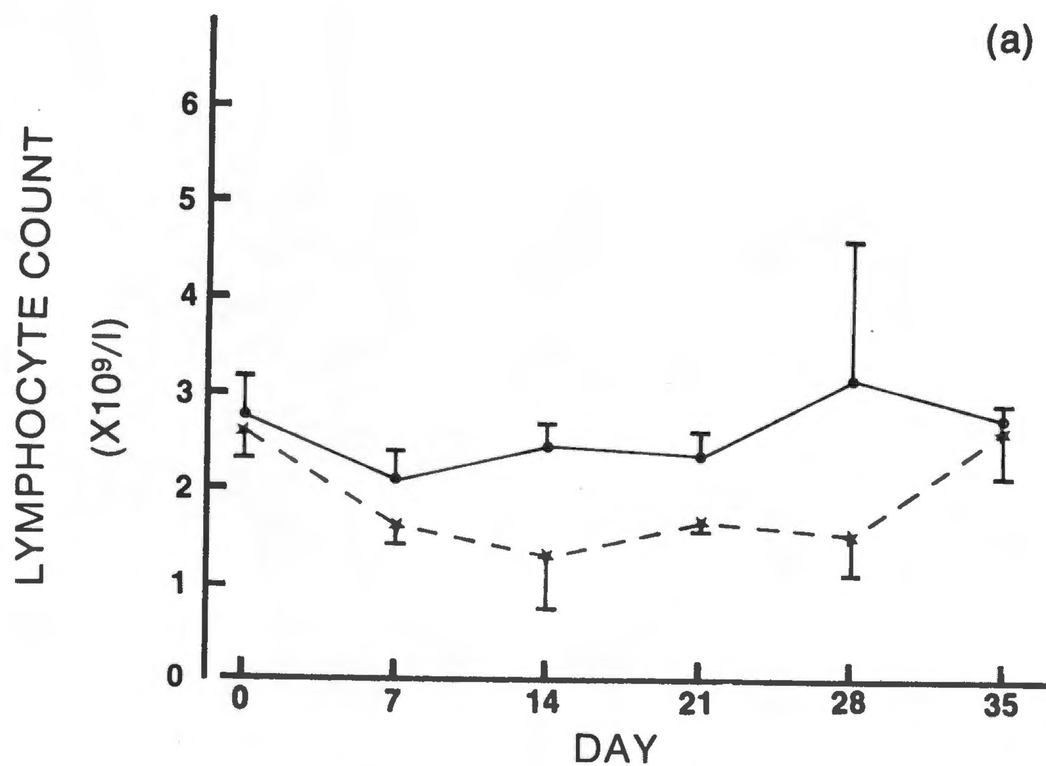


Fig. 3.13 The effect of DAS (a) and NMA (b) on the total white blood cell counts. Rats were treated as described in Section 2.5.2 (—— control; ----- treated).



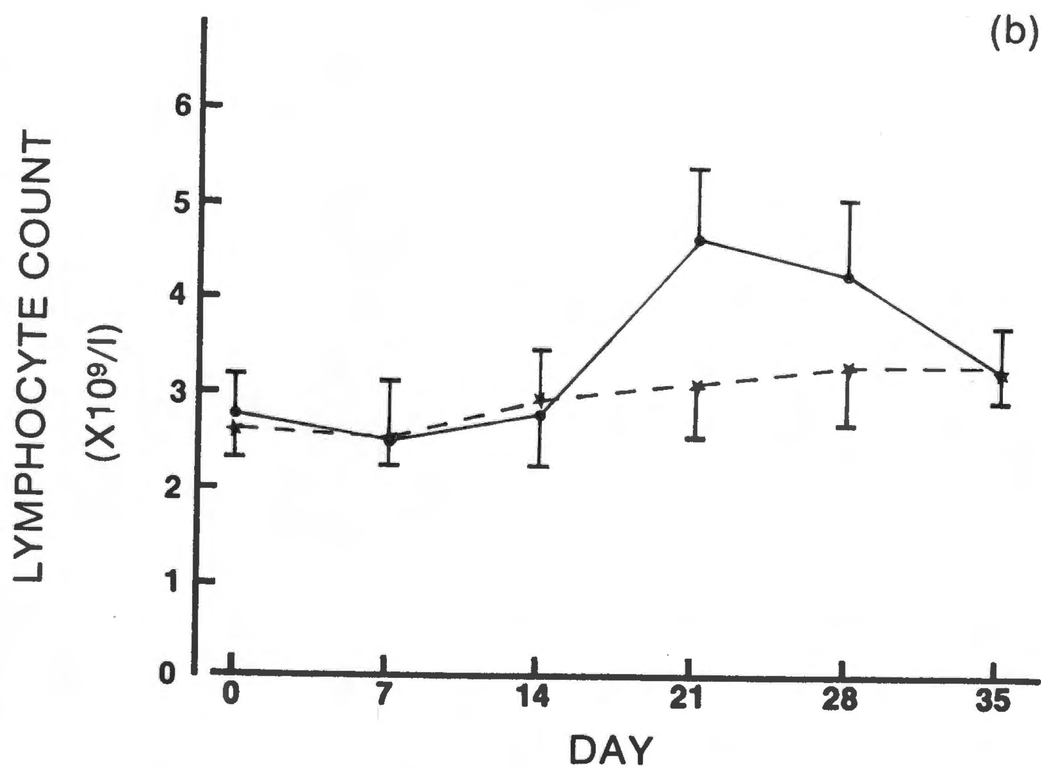


Fig. 3.14 The effect of DAS (a) and NMA (b) on the lymphocyte counts. Rats were treated as described in Section 2.5.2 (—— control; ----- treated).

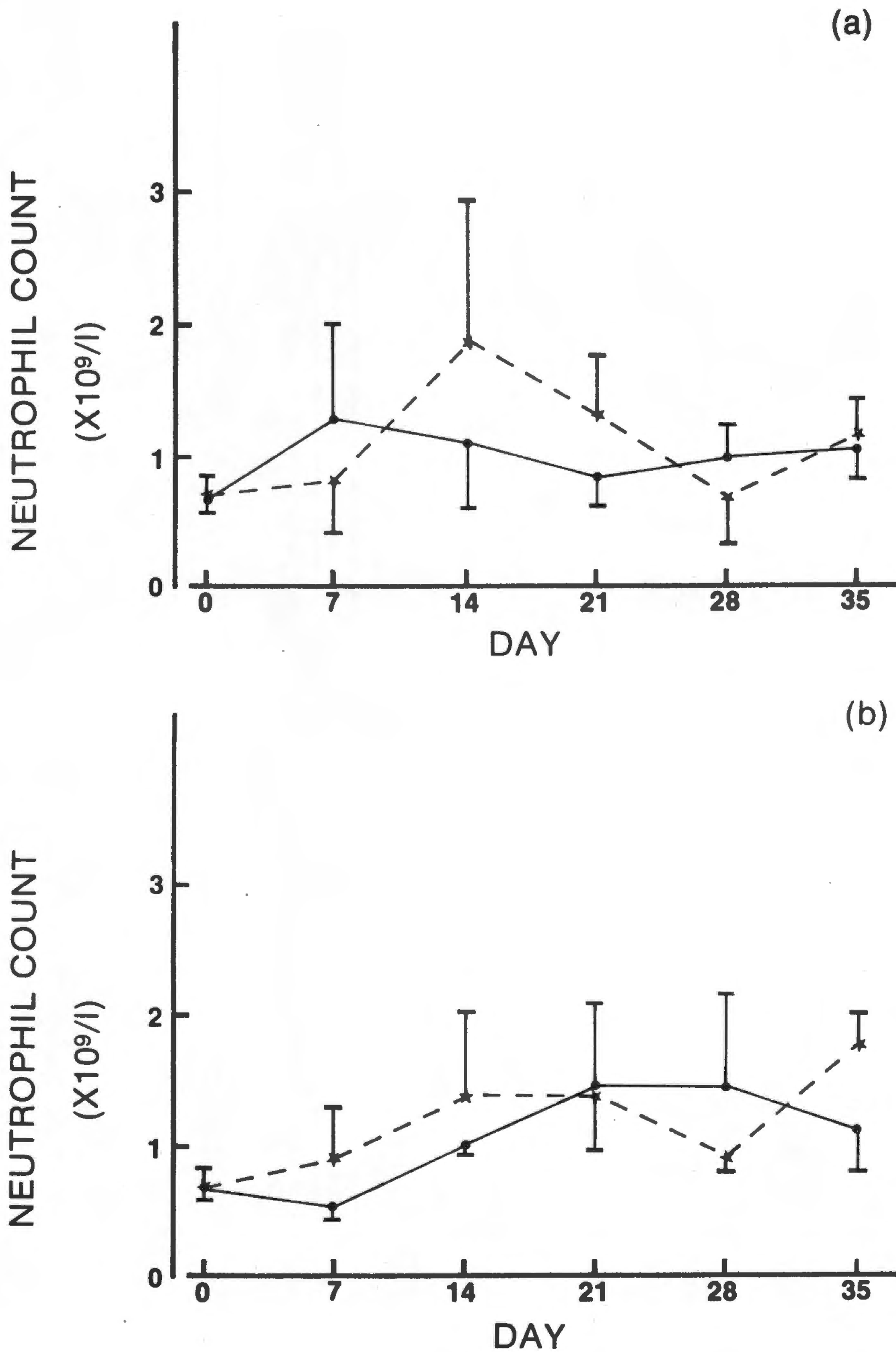


Fig. 3.15 The effect of DAS (a) and NMA (b) on the neutrophil counts. Rats were treated as described in Section 2.5.2 (—— control; ----- treated).

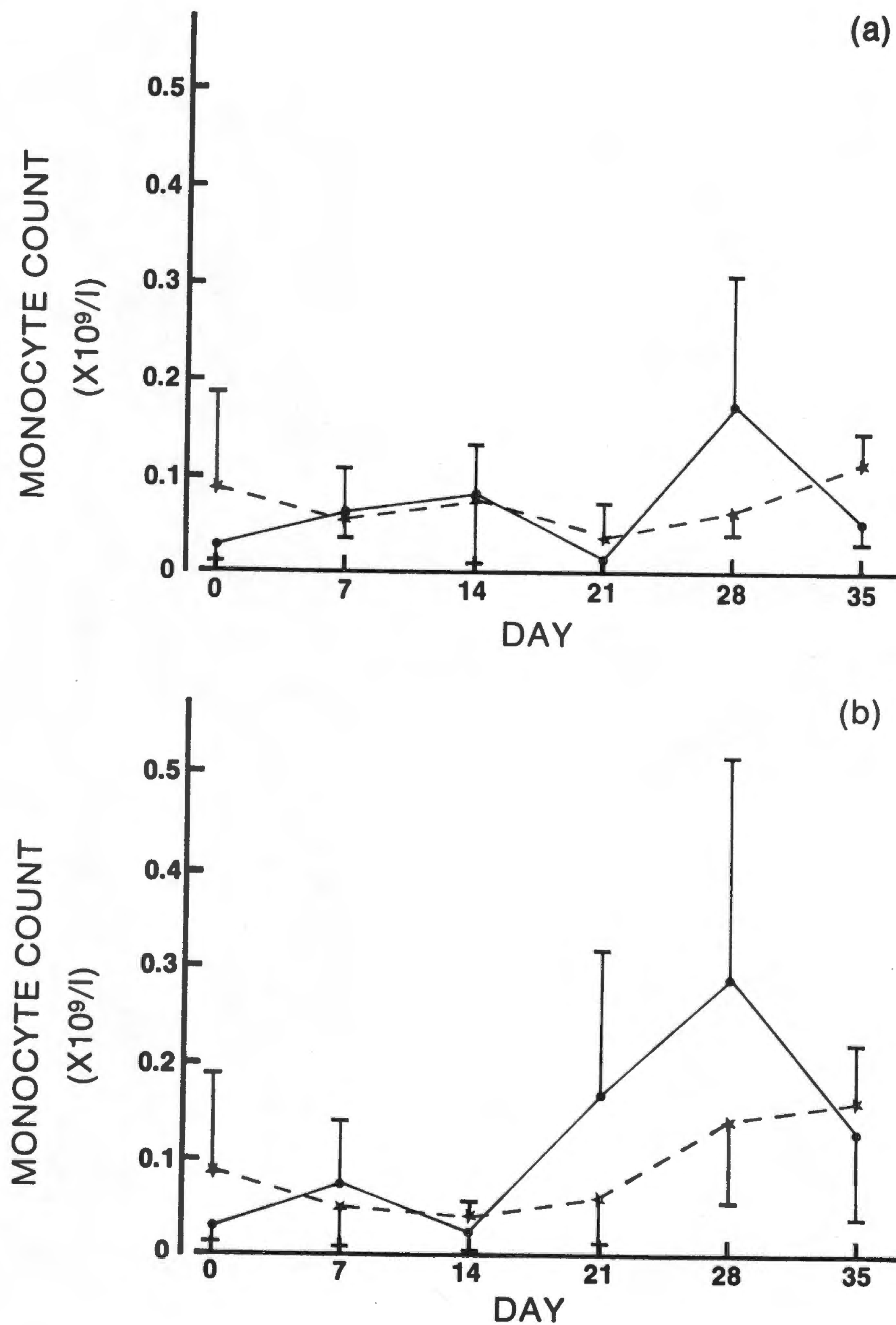


Fig. 3.16 The effect of DAS (a) and NMA (b) on the monocyte counts. Rats were treated as described in Section 2.5.2 (——control; ----- treated).

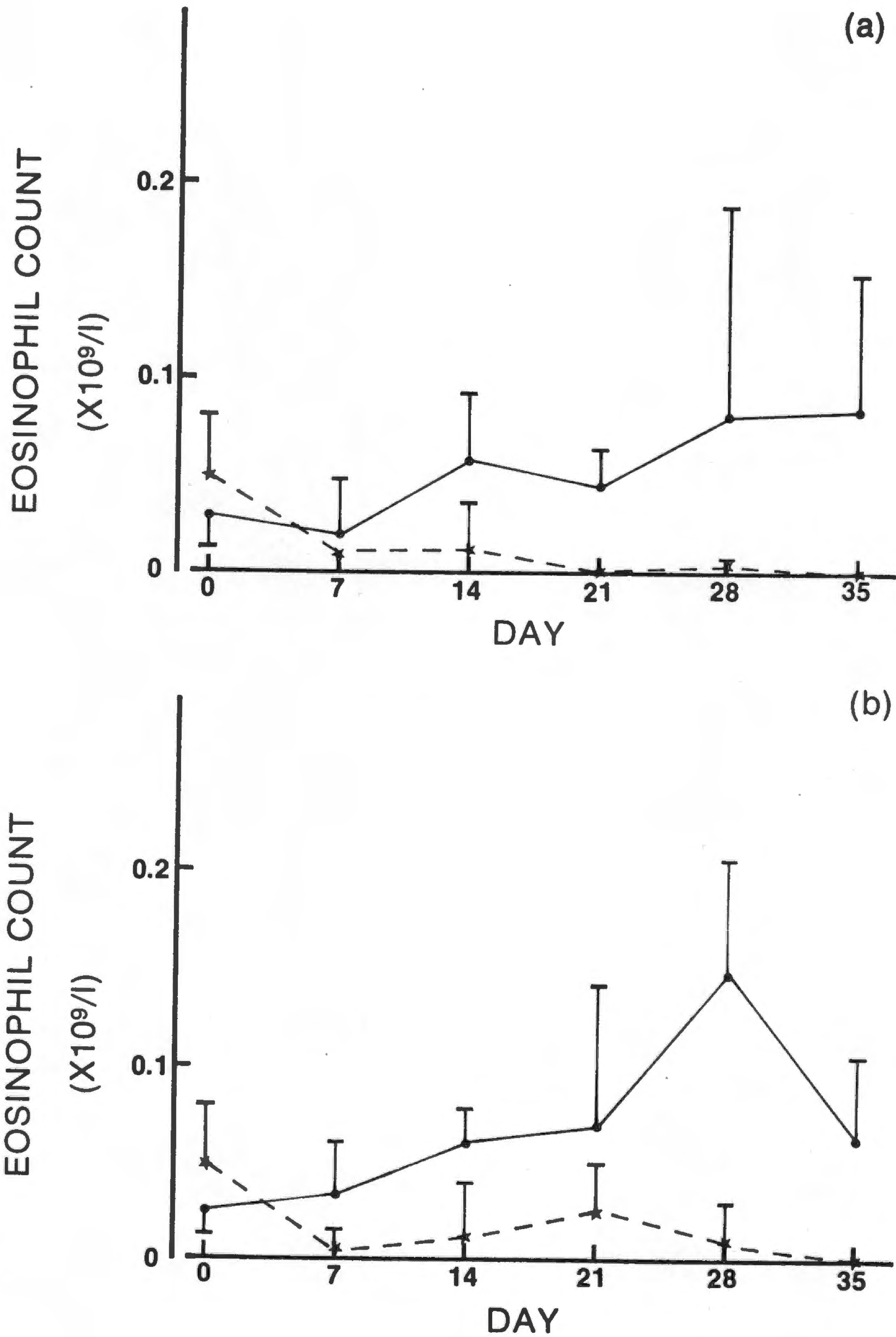
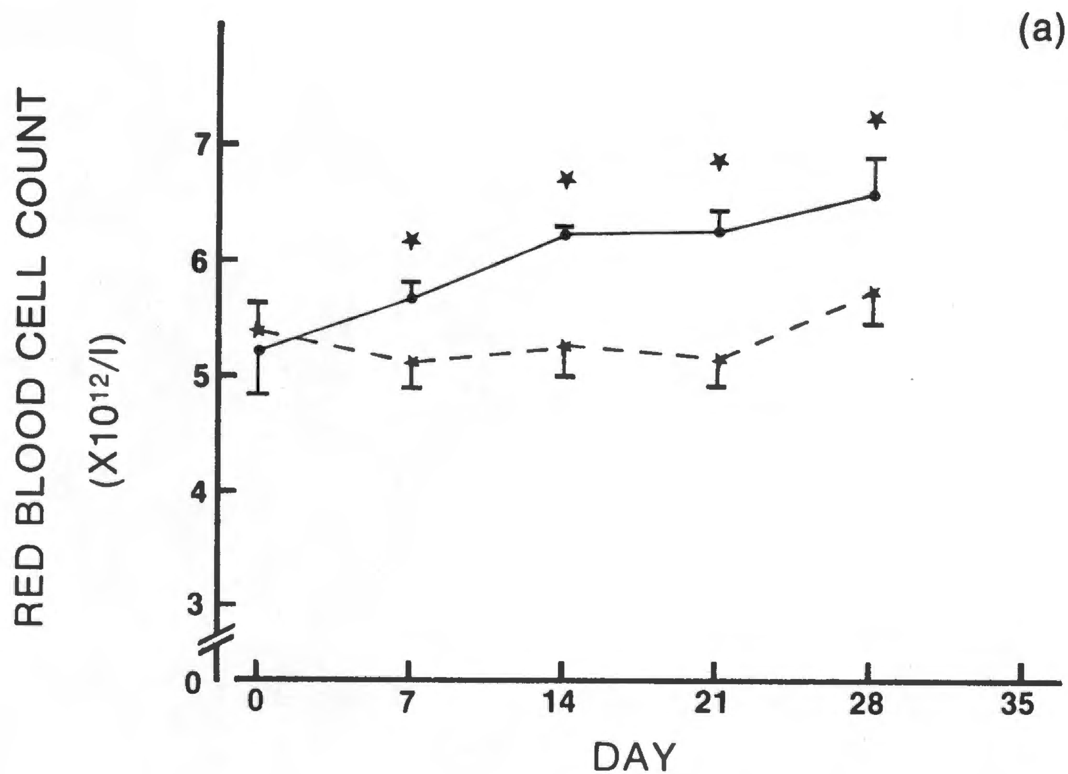


Fig. 3.17 The effect of DAS (a) and NMA (b) on the eosinophil counts. Rats were treated as described in Section 2.5.2 (—— control; ----- treated).



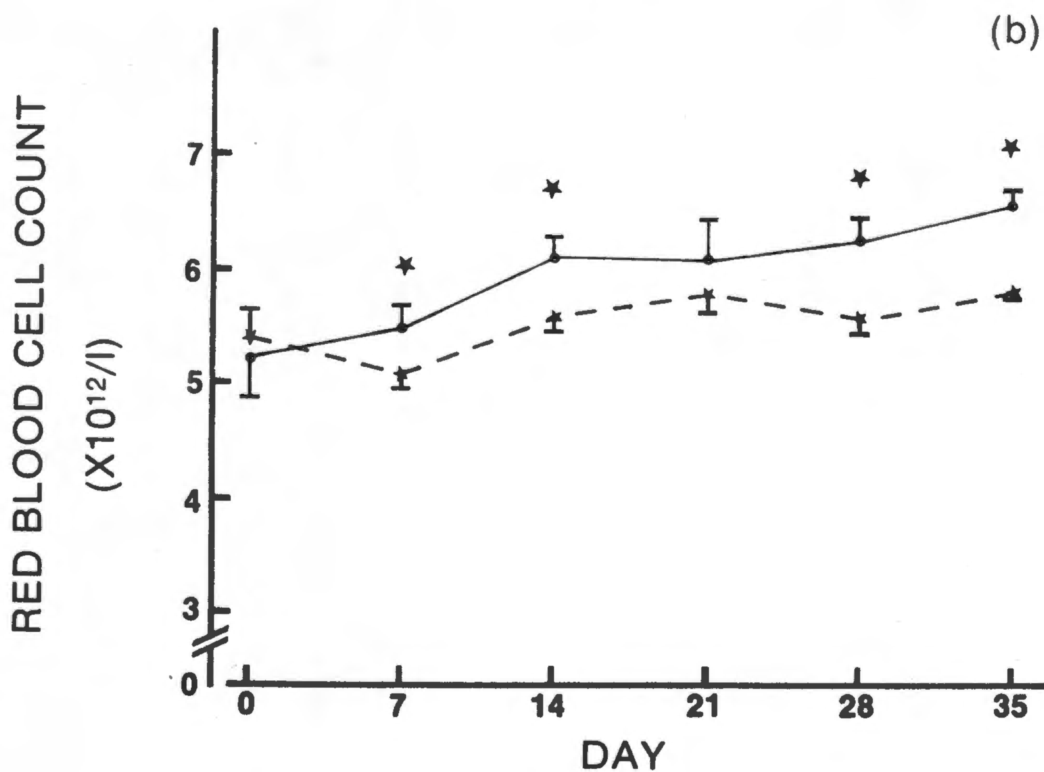


Fig. 3.18 The effect of DAS (a) and NMA (b) on the red blood cell counts. ✶ indicates statistically significant differences ($P < 0.05$). Rats were treated as described in Section 2.5.2. (—— control; ----- treated).

than that of the control groups.

The amount of haemoglobin in the red blood cells and their mean cell volumes (Fig. 2.19 and Fig. 3.20) were not affected by treatment with DAS and NMA. In contrast, treatment with DAS and NMA resulted in a decrease in the amount of haemoglobin present in blood (Fig. 3.21). The haemoglobin content of rat blood increased as the rats aged, but the rats treated with NMA and DAS had significantly lower haemoglobin contents than controls. The hematocrit of the rats increased with age in the controls as well as in those rats treated with the toxins (Fig. 3.22). The hematocrit was however significantly lower in those animals treated with the toxins.

3.3.2.2 Pathological changes

The animals treated with DAS and NMA generally presented with mild pathological changes. Affected spleen tissue in rats treated with DAS showed moderate or mild atrophy. Most significant were the depletion of the lymphoid population of periarteriolar sheaths and follicles, lymphocyte depletion and reticular cell predominance in the red pulp.

Treatment with DAS resulted in lymphocyte disappearance and an increasing number of granulocytes in the thymic cortex. No changes were observed in the thymus of rats treated with NMA.

Bone marrow and lymphatic node atrophy was observed in rats treated with DAS and a bone marrow impression smear showed a predominance

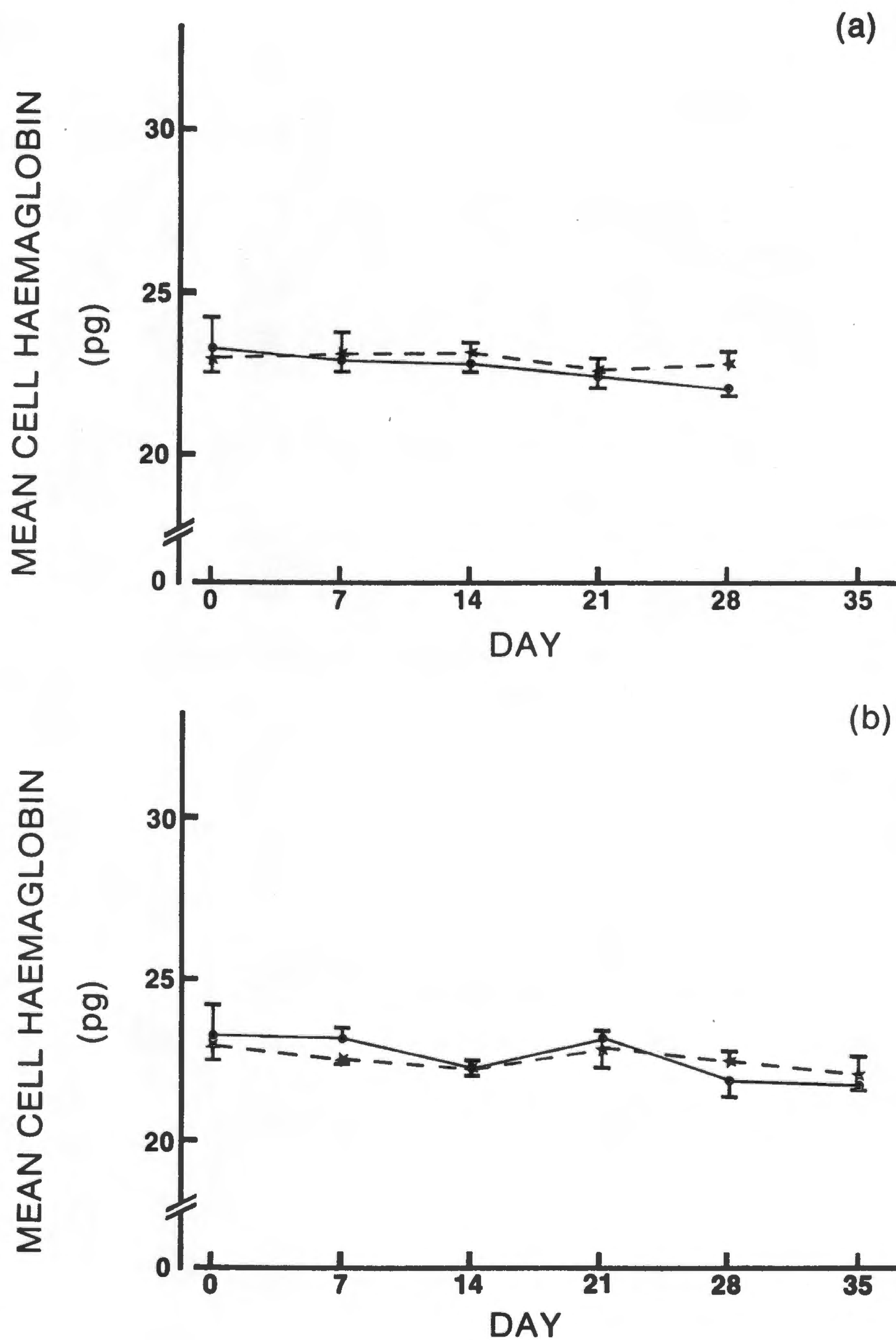
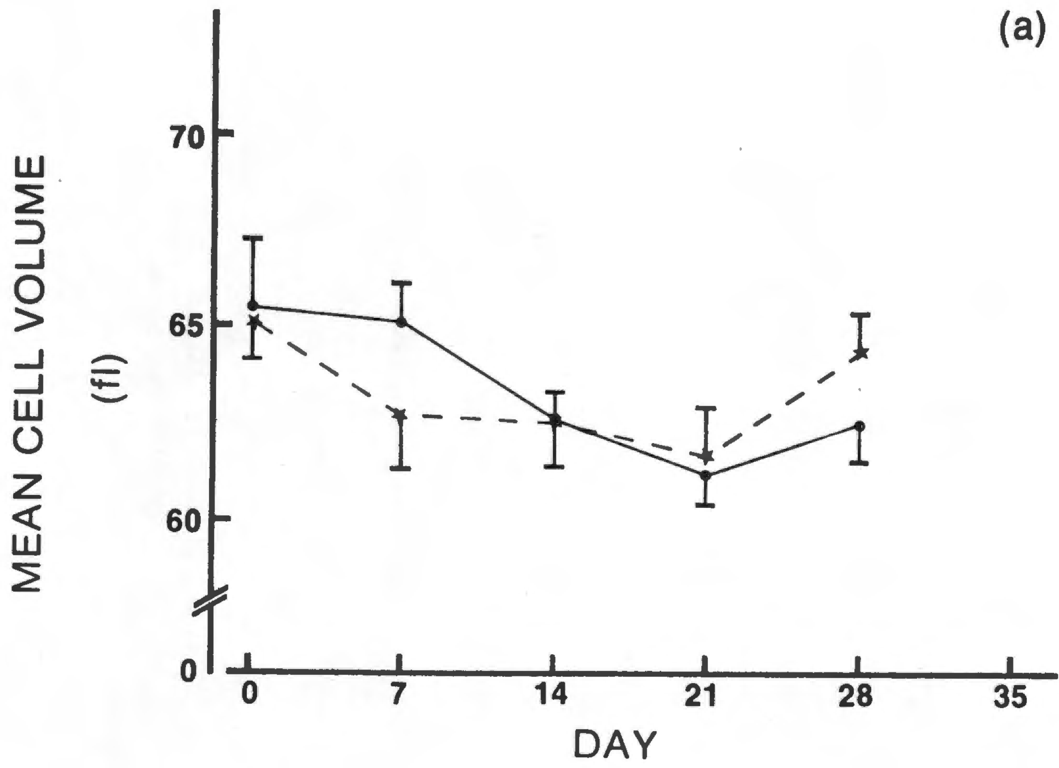


Fig. 3.19 The effect of DAS (a) and NMA (b) on the mean cell haemoglobin of red blood cells. Rats were treated as described in Section 2.5.2 (—— control; ----- treated).



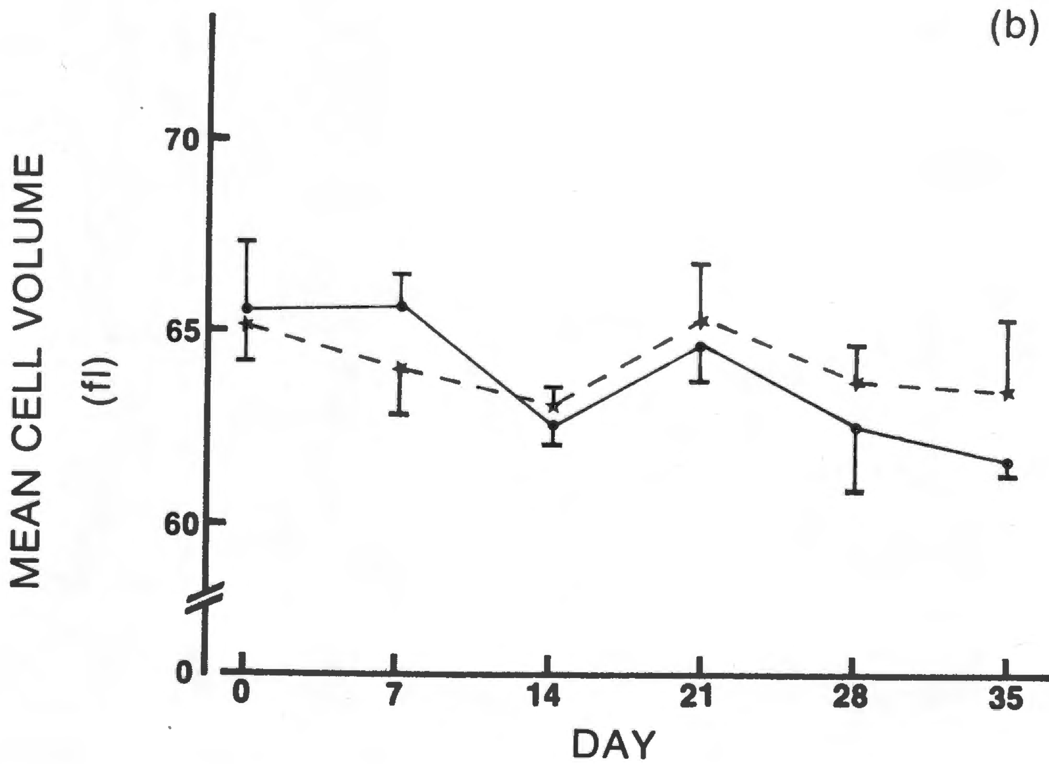


Fig. 3.20 The effect of DAS (a) and NMA (b) on the mean cell volume. Rats were treated as described in Section 2.5.2. (—— control; ----- treated).

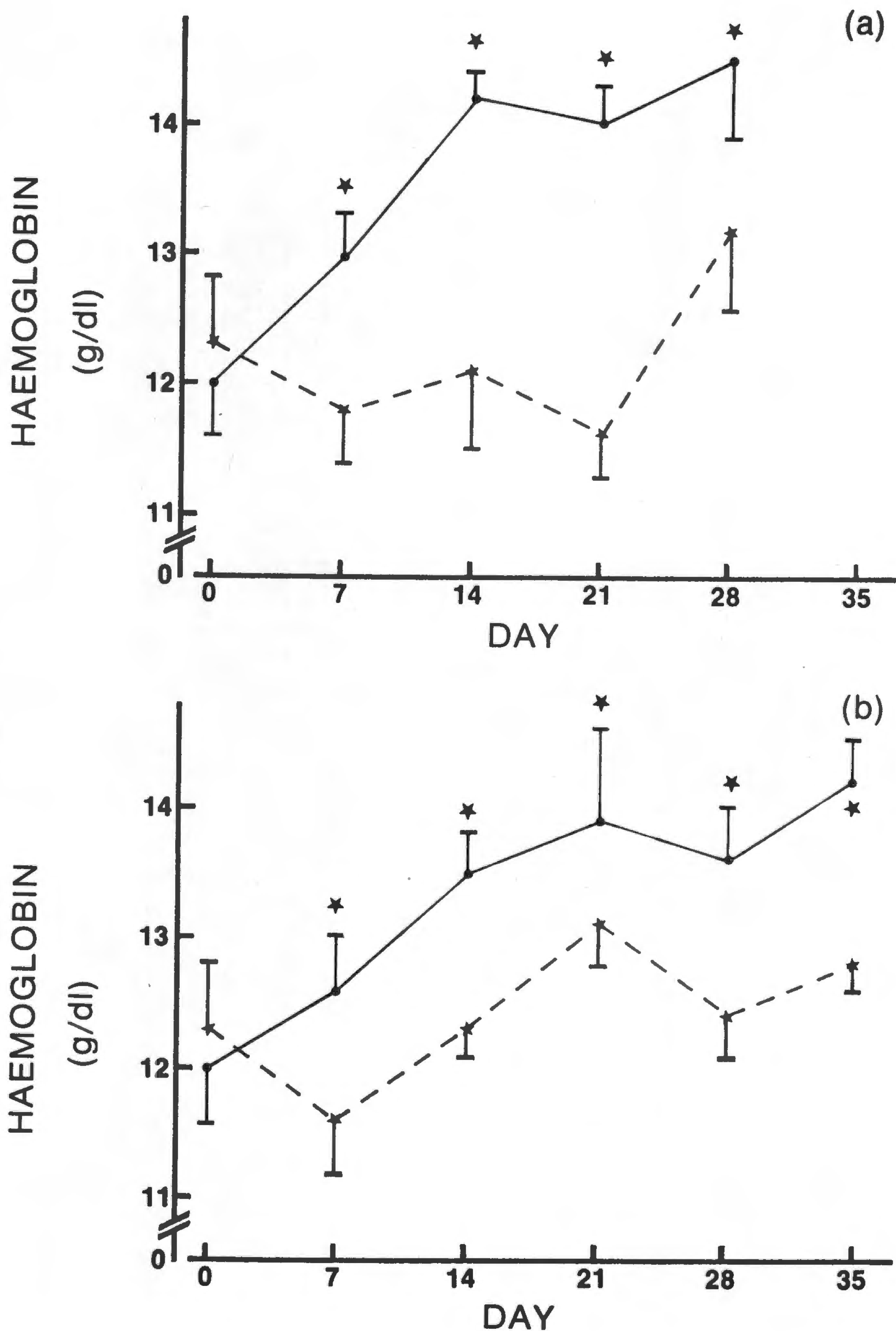


Fig. 3.21 The effect of DAS (a) and NMA (b) on the total haemoglobin content of blood. * indicates statistically significant differences (P <0.05). Rats were treated as described in Section 2.5.2 (—— control; ----- treated).

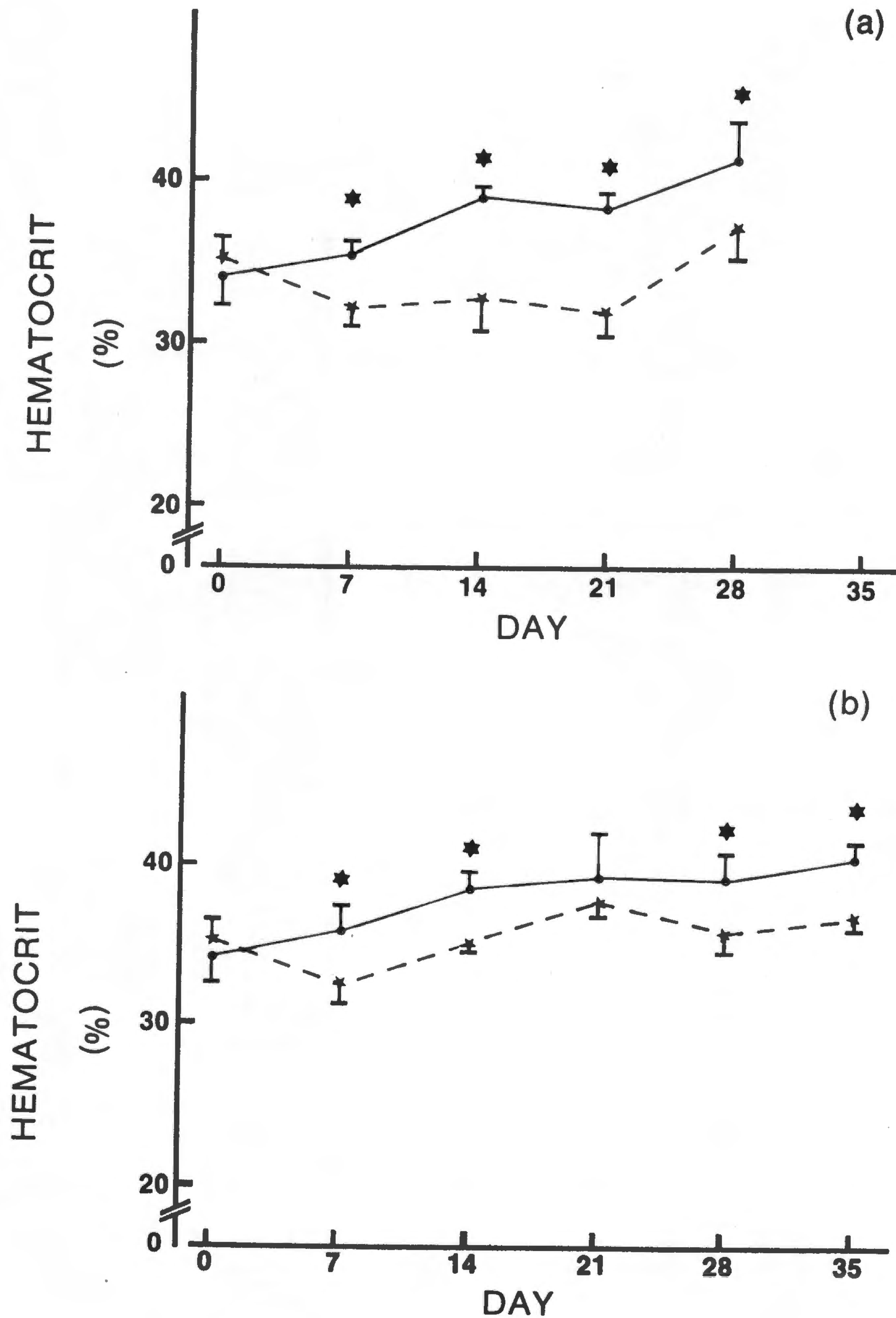


Fig. 3.22 The effect of DAS (a) and NMA (b) on the hematocrit. Statistical differences are indicated by ✱ ($P < 0.05$). Rats were treated as described in Section 2.5.2 (—— control; -----treated).

of myeloid cells and a depletion of megakaryocytes. Similar changes were observed in rats treated with NMA.

The regularity of pathological changes connected with DAS treatment were noted from day 14 and persisted up to day 28. On day 35 the bone marrow, thymus and lymphatic nodes showed signs of recovery. Similar observations were made in rats treated with NMA.

3.4 GLUCURONIDATION OF T-2 TOXIN AND SOME OF ITS METABOLITES

3.4.1 Microsomal UDP-glucuronyltransferase Activity

Liver microsomal UDP-glucuronyltransferase activity of phenobarbital induced rats was determined according to the method of Halac and Bonevardi (125, Section 2.7.2) using phenolphthalein as substrate. The specific activity of the microsomal preparation was found to be 11.2 nmoles phenolphthalein conjugated/min/mg protein.

3.4.2 The Conjugation of T-2 Toxin and its Metabolites

The potential glucuronidation of T-2 toxin, HT-2 toxin and T-2 tetraol was studied using activated hepatic microsomes from a pig and phenobarbital induced rats (Section 3.6.4). The reaction was followed by determining the amount of unconjugated substrate at different time intervals by GC.

As T-2 toxin is converted to HT-2 toxin in the presence of microsomes (Section 3.5) the potential conjugation of both T-2 toxin and HT-2 toxin could be followed when only T-2 toxin was used as substrate.

The microsomal conversion of T-2 toxin to HT-2 toxin was near to completion after 30 minutes of incubation with pig and rat micro= somes, both in the absence and presence of UDP-glucuronic acid. In the presence of UDP-glucuronic acid 0.425 ± 0.009 μmoles HT-2 toxin was formed when T-2 toxin was incubated with rat microsomes, while in the absence of UDP-glucuronic acid 0.452 ± 0.003 μmoles HT-2 toxin was formed. When T-2 toxin was incubated with pig microsomes and UDP-glucuronic acid 0.417 ± 0.109 μmoles HT-2 toxin was formed, while in the absence of UDP-glucuronic acid 0.399 ± 0.049 μmoles HT-2 toxin was formed. No remaining T-2 toxin was detectable in any of the incubations. There were no statistical differences (Section 2.7) between incubations with or without UDP-glucuronic acid.

When T-2 tetraol was incubated with rat microsomes (Section 2.6.4) 0.594 ± 0.020 μmoles T-2 tetraol and 0.578 ± 0.016 μmoles T-2 tetraol remained after incubations without and with UDP-glucuronic acid, respectively. There was no significant change in the T-2 tetraol concentration resulting from the presence of UDP-glucuronic acid.

3.5 DEACYLATION OF T-2 TOXIN

The deacylation of T-2 toxin to HT-2 toxin was studied using rat, human, cat and monkey hepatic microsomes (Section 2.6.5). The effect of temperature and pH on the deacylation reaction was also investig= gated (Sections 2.6.5.1 and 2.6.5.2).

3.5.1 Effect of Temperature

The deacylation reaction catalysed by rat liver microsomes was shown to be dependent on temperature with an optimum at 37°C (Fig. 3.23). Thereafter all kinetic studies were performed at this temperature.

3.5.2 Effect of pH

The pH of the deacylation reaction differed markedly between the various animal species (Fig. 3.24). Rat and human microsomes showed a pH optimum at pH 7.4 and 8.0, respectively, while the rate decreased at higher pH values. The pH optimum for cat microsomes was observed at pH 8.0 to 9.0 while that for monkey microsomes was observed at pH 8.5. The deacylation rate for these two species did not decrease considerably at higher pH values. Non-enzymatic deacylation was found to occur at higher pH values in the absence of microsomes.

3.5.3 Kinetic Studies

In order to study the kinetics of the deacylation of T-2 toxin it was necessary to determine the initial reaction rates. Time course studies were therefore performed to determine the time interval that a linear reaction rate was maintained (Section 2.6.5.3). The deacylation rate was linear with rat and human microsomes over 15 and 20 minutes, respectively (Fig. 3.25). As the deacylation with human microsomes was much faster than with rat microsomes, the reaction for the determination of the

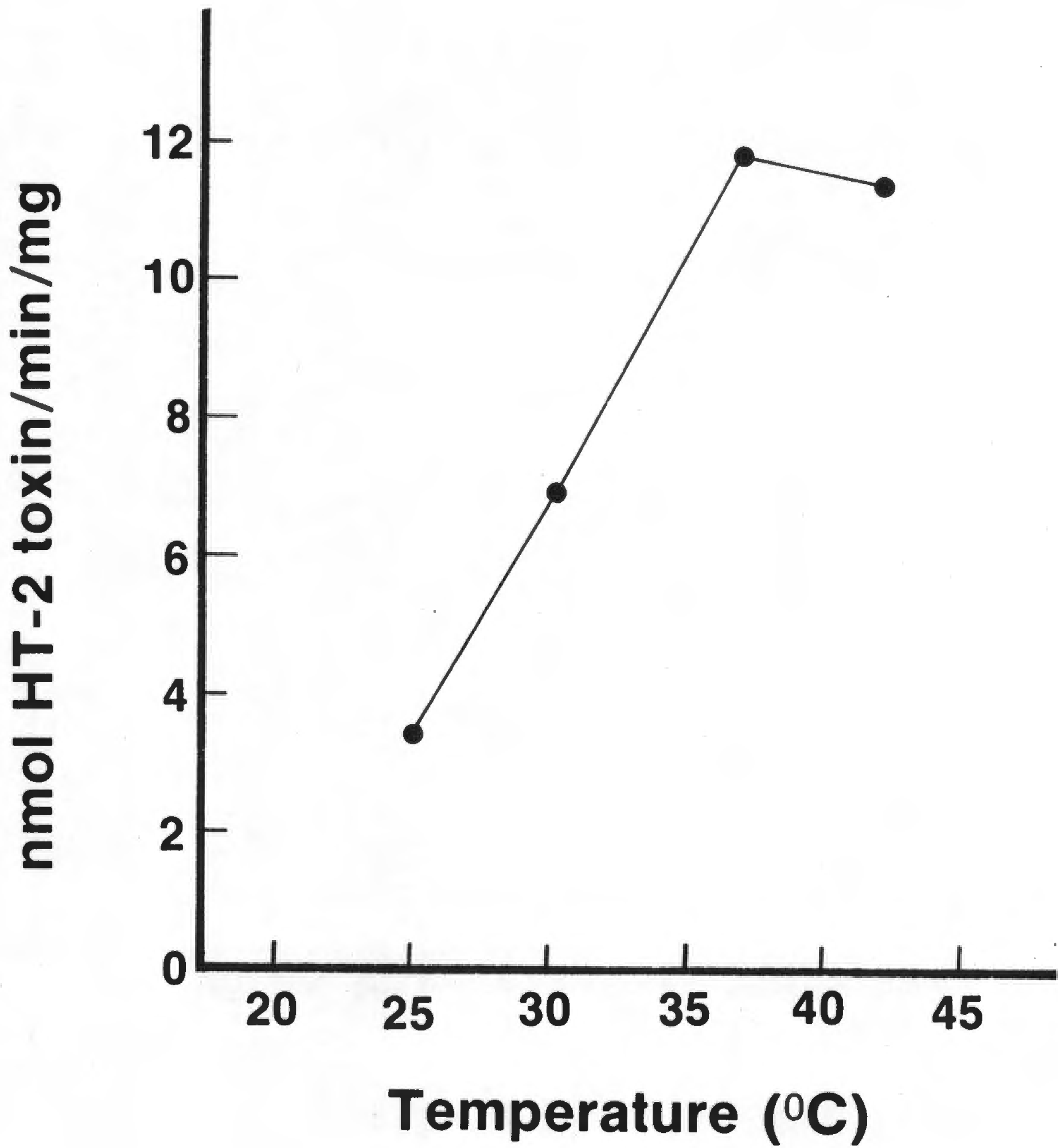


Fig. 3.23 The effect of temperature on the deacylation of T-2 toxin by rat hepatic microsomal esterases (Section 2.6.5.1).

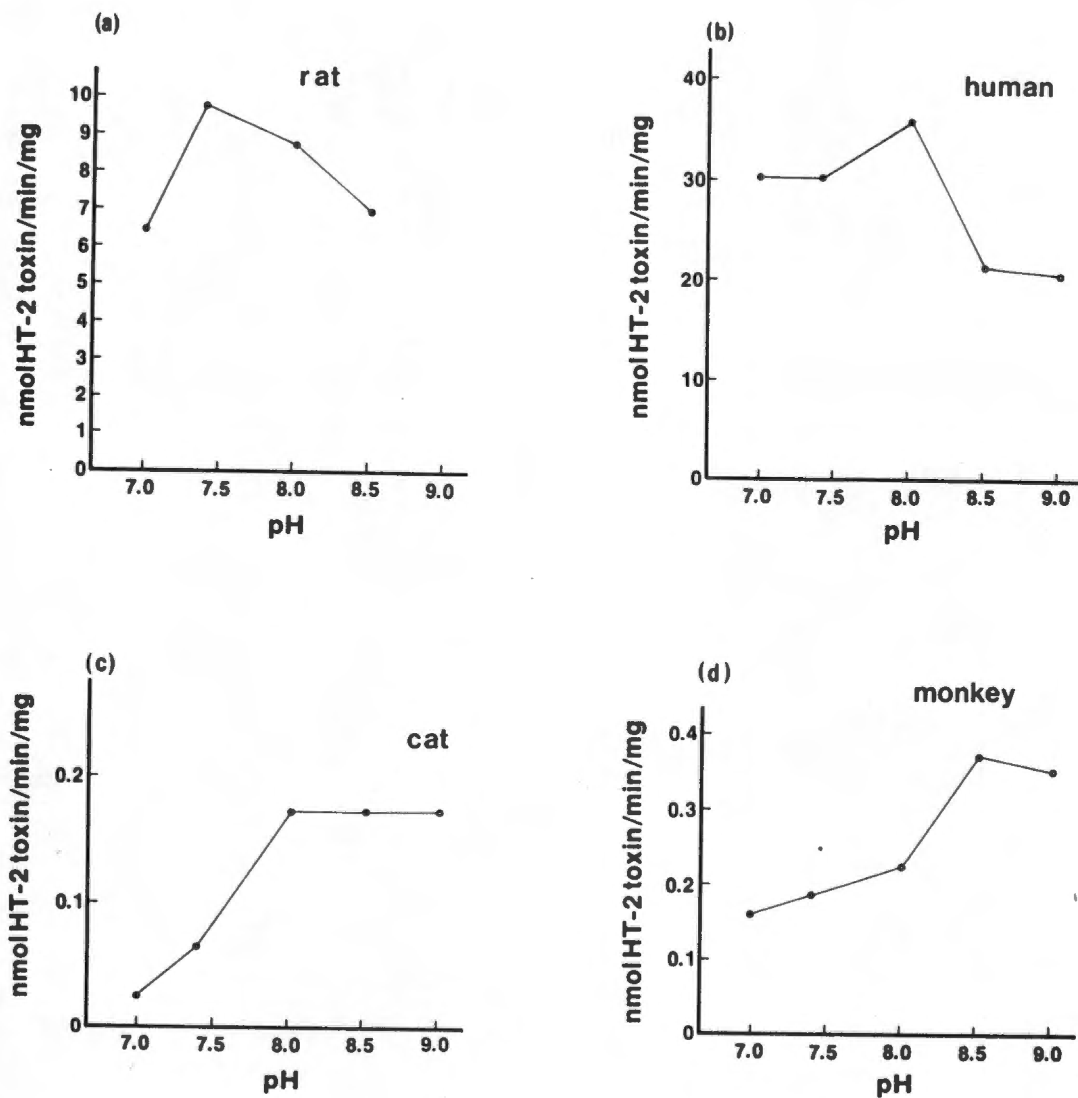


Fig. 3.24 The effect of pH on the deacylation of T-2 toxin by rat, human, cat and monkey hepatic microsomes (Section 2.6.5.2).

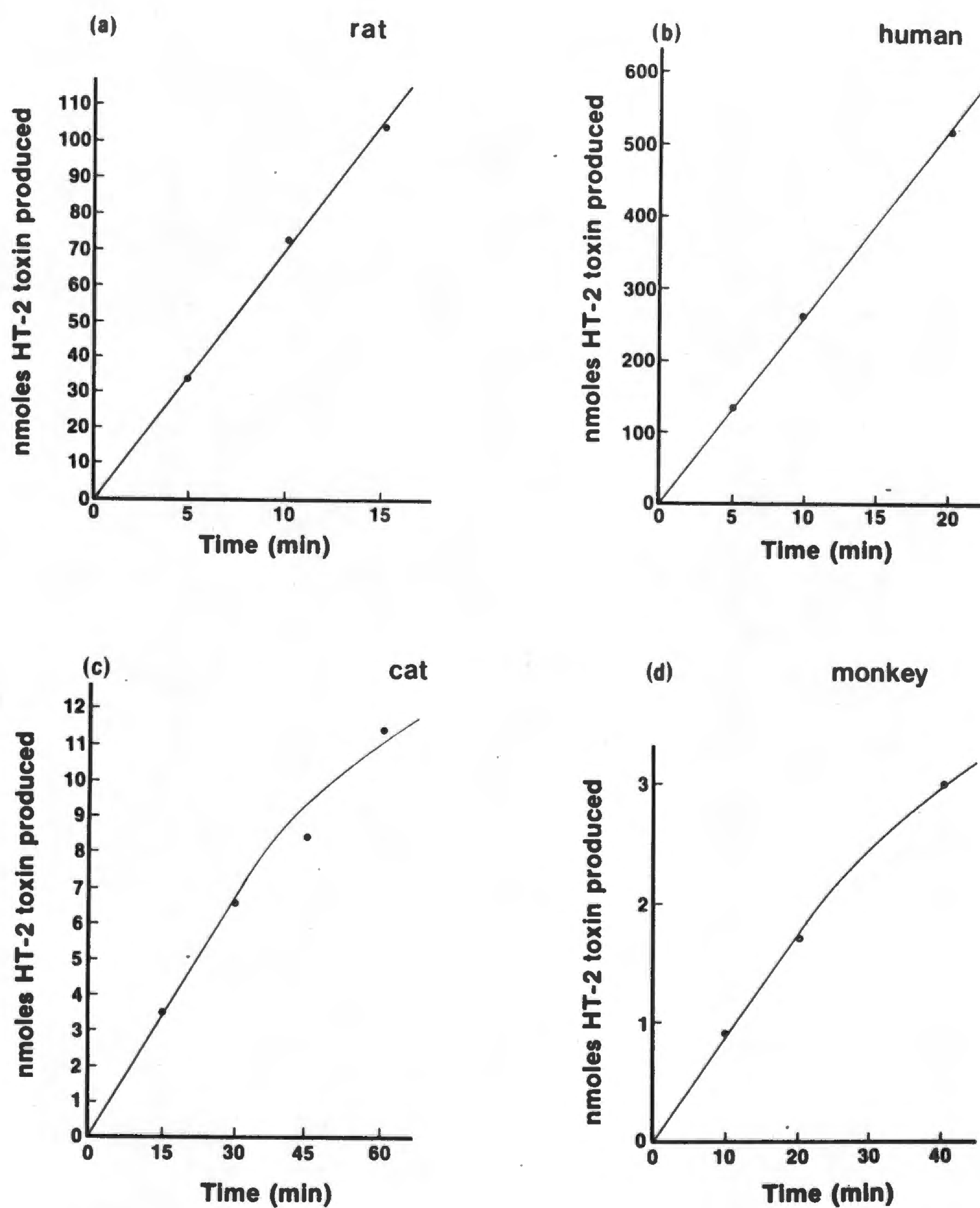


Fig. 3.25 Time course studies of the deacylation of T-2 toxin by rat, human, cat and monkey hepatic microsomes (Section 2.6.5.3).

kinetic parameters were stopped after 5 and 10 minutes when using human and rat microsomes, respectively. Because the deacylation rate was lower with cat and monkey microsomes the time course studies were performed over longer time intervals. The deacylation with cat microsomes was linear for 30 minutes while that for monkey microsomes was linear for 20 minutes. Incubation times of 30 and 15 minutes were used in the kinetic studies with cat and monkey microsomes, respectively.

Kinetic studies as different substrate concentrations (Section 2.6.5.4) indicated that the deacylation rate was the highest with human and rat microsomes while with cat and monkey microsomes the deacylation rates appeared to be similar but about a hundred times lower than the rate with human microsomes (Fig. 3.26). When the data obtained with cat and monkey microsomes were plotted on an expanded scale (Fig. 3.27) it was clear that the plots followed a sigmoidal shape.

The rate constants for the reaction with rat and human microsomes were determined using the Eadie-Hofstee plot (Fig. 3.28). For the rat microsomes the K_m and V_{max} were found to be 0.51 mM and 29.2 nmol HT-2 toxin/min/mg protein, respectively. With the human microsomes the K_m and V_{max} were found to be 1.4 mM and 95.6 nmol HT-2 toxin/min/mg protein, respectively.

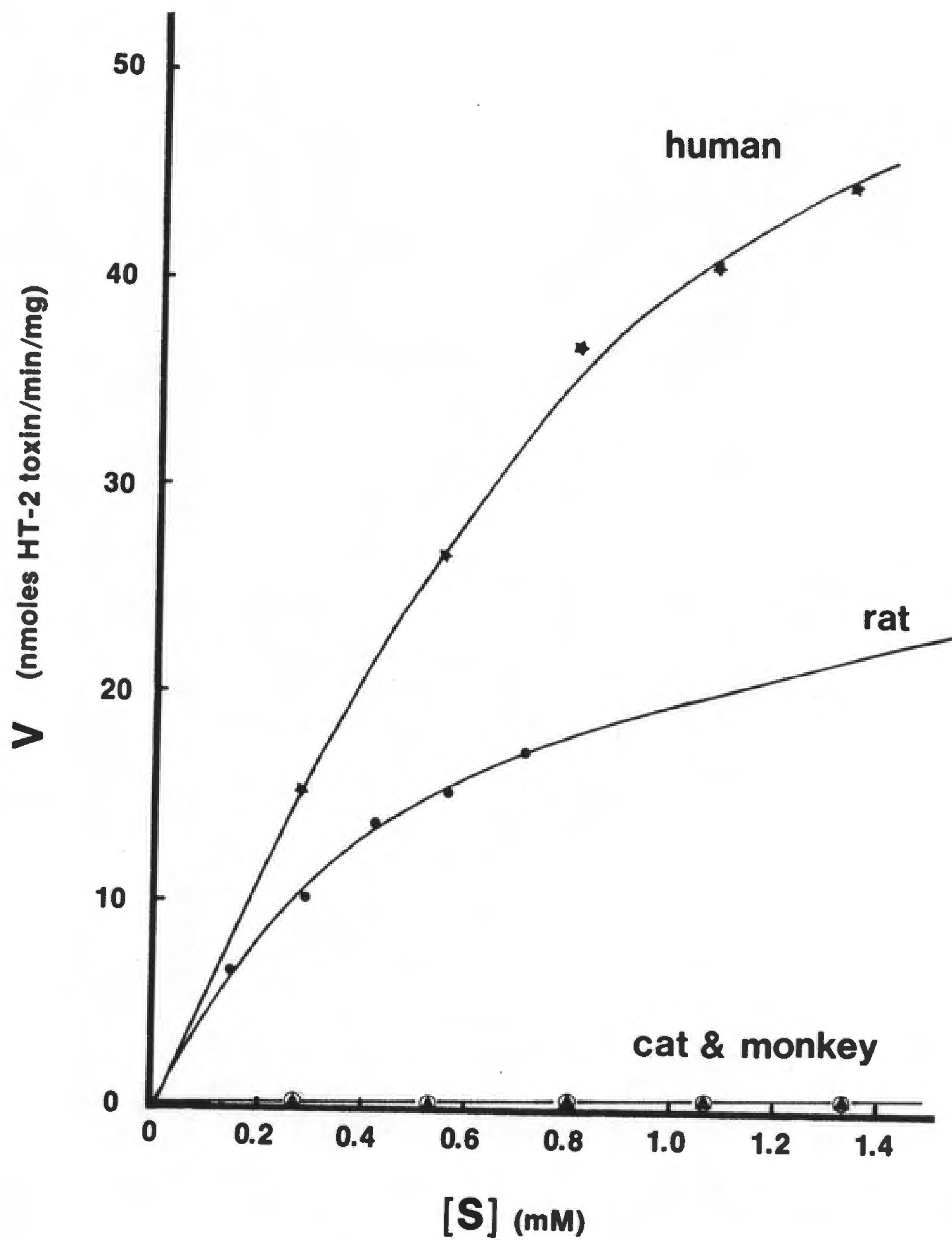


Fig. 3.26 The effect of T-2 toxin concentration on the deacylation rate in human, rat, cat and monkey microsomes (Section 2.6.5.4).

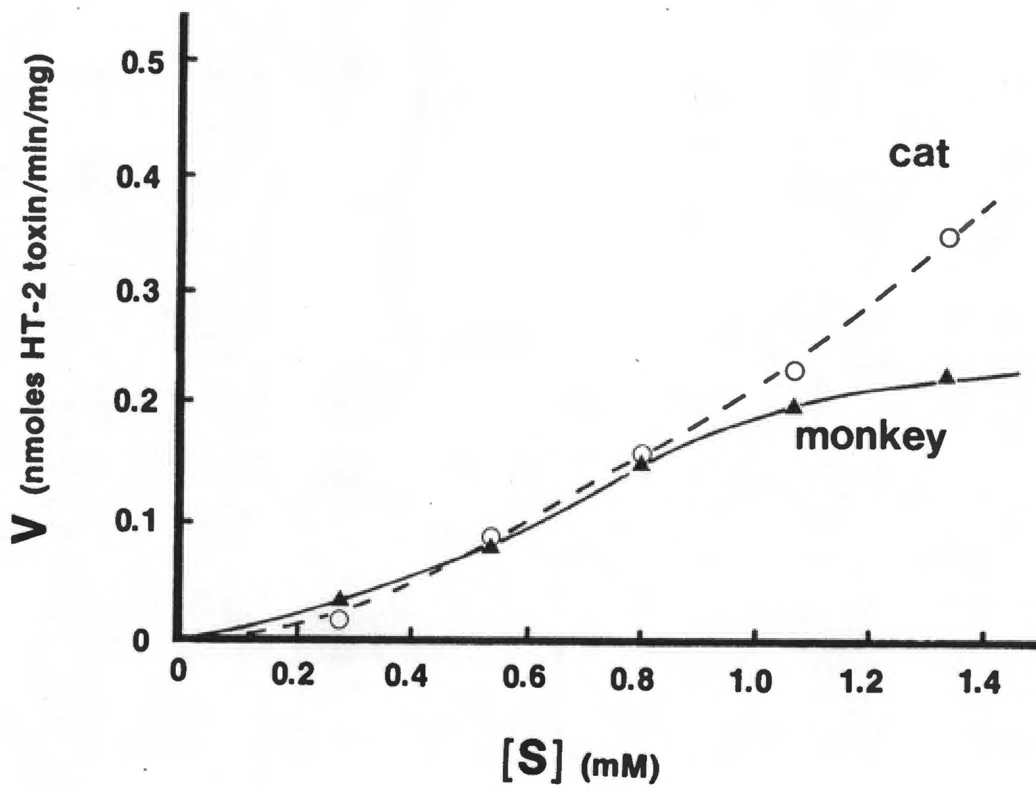


Fig. 3.27 The effect of T-2 toxin concentration on the deacylation rate in cat and monkey microsomes (Section 2.6.5.4).

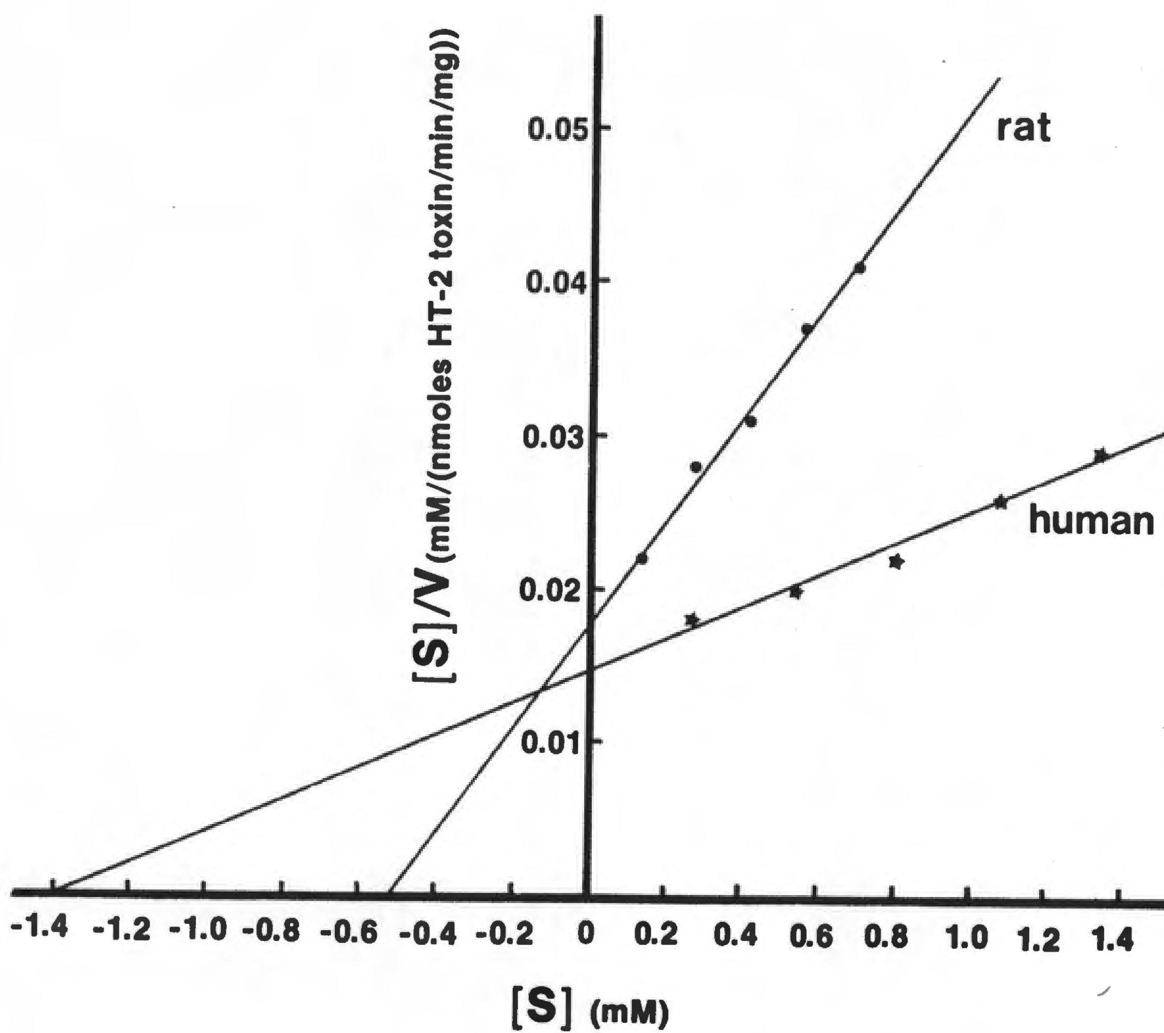


Fig. 3.28 A plot for the determination of kinetic constants for the deacylation of T-2 toxin in rat and human hepatic microsomes (Section 2.6.5.4).

CHAPTER 4

DISCUSSION

4.1 ISOLATION OF NMA

It was necessary to isolate sufficient amounts of neosolaniol mono=acetate (NMA) to perform *in vivo* studies with experimental animals. Two groups independantly isolated NMA from liquid cultures of two different *Fusarium* species. Lansden *et al* (127) isolated NMA from cultures of *F. tricinctum* strain NRRL 23377, identified as *F. chlamydo=sporum* strain MRC 2486 by Nelson *et al* (15), while Ishii *et al* (118) isolated the toxin from cultures of *F. roseum* strain V-18, identi=fied as *F. sambucinum* strain MRC 1903 by Nelson *et al* (15). It is well known that the Fusaria produce larger quantities of toxins on solid substrates than in liquid media (8). Therefore an attempt was made to isolate NMA from maize cultures of *F. compactum* strain MRC 1293, even though the isolation of toxins from liquid cultures is generally easier due to less interfering substances. The brine shrimp toxicity assay, a biological monitoring system previously used for the isolation of several trichothecenes (121), was employed in this study.

Of the three solvent systems tested for extraction, ethyl acetate proved to be more suitable for recovering toxicity (Fig. 3.1). Although MeOH/H_2O and MeOH/$CHCl_3$ extracted only slightly less toxicity these solvent systems are likely to extract far more polar substances that can interfere in the purification of NMA.

The liquid-liquid partitioning of the ethyl acetate residue between acetonitrile and petroleum ether resulted in a five fold purification. Twenty percent of the ethyl acetate residue containing the bulk of the toxicity (Fig. 3.3) was recovered in the acetonitrile phase. The low toxicity detected in the petro= leum ether phase was most probably due to the presence of fatty acids and other fat soluble compounds, which are also toxic to brine shrimps (128).

Column chromatography is frequently used for the fractionation of toxic extracts (118, 129). Fractionations using silica gel and Sephadex LH-20 column chromatography in this study (Section 3.1) were unsuccessful in purifying NMA from maize extracts due to the co-elution of interfering substances with the toxin in both systems. A report by Vesonder et al (130) stated that rice cultures of F. graminearum proved to be more suitable than maize cultures for isolating deoxynivalenol. An attempt was therefore made to isolate NMA from rice cultures of F. compactum. Rice cultures of F. sambucinum strain MRC 1903, one of the first strains from which NMA was isolated, were also included.

TLC could be used to screen the column eluates during the fractio= nation of the acetonitrile extracts from rice cultures, whereas with the maize extracts too many interfering substances were present. The eluates from the first column fractionations of rice cultures of F. sambucinum (Fig. 3.7) and F. compactum contained less interfering substances than the eluate from the

third column fractionation of the extract from the maize culture of F. compactum (Fig. 3.6). This agrees with the observations made by Versonder et al (130). GC analysis revealed that F. sambucinum strain MRC 1903 produced more NMA than F. compactum strain MRC 1293, therefore the former was used for the large scale isolation of NMA.

Based on the small scale isolation of NMA, the yield from a bulk culture of F. sambucinum was 7 times less than expected (Section 3.2). Several factors could have contributed to the lower yield: (1) The culture conditions during bulk cultivation were not optimal for toxin production (2) The inoculum used was not as viable compared to that used during the small scale cultivation (3) The extraction procedure was not as efficient with larger samples as with smaller samples.

The isolated NMA was shown to be pure by using a number of physico-chemical techniques. The melting point of the isolated NMA (190-192°C) compared well with published values (190-190.5°C (127) and 189-190°C (118)) while GC analysis showed a single peak eluting at the same position as a NMA standard. Mass spectral analysis showed the characteristic fragment peaks observed in published spectra (Fig. 3.8). No molecular ion was however detected.

4.2 BIOLOGICAL EFFECTS OF DAS AND NMA

The LD_{50} values of NMA (0.789 mg/kg) and T-2 toxin (1.84 mg/kg)

in day old cockerels suggest that NMA is more toxic than T-2 toxin (127), while DAS (LD_{50} = 7.3 mg/kg) is less toxic to rats than T-2 toxin (LD_{50} = 5.2 mg/kg) (18). It is important that in chronic studies with toxins a dose should be selected that can be administered repeatedly without being lethal to the experimental animals, while still being high enough to induce detectable changes in the animals. Preliminary studies indicated that a dose of 1 mg DAS/kg bodyweight 3 times a week would be suitable for chronic studies in rats. NMA was administered at the same dose so as to compare the chronic effects of these two toxins.

Of all the haematological parameters examined the red blood cell counts appeared to be the most sensitive to DAS and NMA. Since the mean cell volume and the mean cell haemoglobin did not change during the experimental period it would seem that the red blood cells themselves were not affected by the action of the toxins. DAS and NMA treatment resulted in a statistically significant cant decrease in the hematocrit and haemoglobin content, thus agreeing with the decrease in the red blood cell count. This can be expected as the hematocrit and haemoglobin content of blood are directly related to the red blood cell count. Stähelin (21) obtained similar results in a rat experiment performed with DAS. It would appear that a reduction of red blood cells is a general characteristic of the action of the trichothecenes as T-2 toxin also causes decreases in red blood cell counts in cats and monkeys (10, 39).

Blood platelet levels decrease in dogs treated with DAS (21) and in rats there is a very mild decrease in the white blood cell counts (21). As with DAS, T-2 toxin treatment also resulted in a decrease in platelet and white blood cell counts in various animal species (31, 36, 39, 60). In the present study the rats treated with DAS and NMA did not show any significant reduction in the platelet or white blood cell counts. Treatment with NMA did however seem to lower the platelet levels compared to controls (Fig. 3.10) but the difference was not statistically significant. Pathological examination of the bone marrow revealed a decrease in the number of megakaryocytes, the cells from which platelets are formed. A decrease in the platelet counts was therefore expected.

An increase in platelet size was observed with both the NMA and DAS treatments. Circulating platelets are not homogenous with regard to size, density and function and these differences are related to age (131). Younger platelets are larger than platelets that have been in the circulation for a few days. The results obtained from treatment with NMA and DAS seem to indicate an increased proportion of young platelets in the circulation possibly caused by the destruction of circulating blood platelets. However no reduction in the platelet counts was observed which could be ascribed to the short period of exposure.

The atrophy of actively dividing cells of the bone marrow, thymus, spleen and lymph nodes observed after treatment with DAS and NMA

is characteristic of lesions induced by trichothecenes (27-29, 34, 40, 52, 67, 68). DAS caused the greatest changes in the cortex of the thymus similar to the observations with T-2 toxin in mice (30). After five weeks the bone marrow, thymus and lymph nodes showed signs of recovery. Similar recoveries were observed in chickens that received dietary T-2 toxin (69). It is possible that chronic exposure to the toxins could have increased the capability of the detoxification pathways.

4.3 GLUCURONIDATION OF T-2 TOXIN AND SOME OF ITS METABOLITES

UDP-glucuronyltransferases catalyse the transfer of glucuronic acid from UDP-glucuronic acid to hydrophobic, fat soluble molecules thereby increasing their polarity and water solubility to fascilitate their excretion (132, 133). As the glucuronidation of T-2 toxin or its metabolites could play an important role in the detoxification of this toxin, *in vitro* conjugation studies were undertaken.

In freshly prepared microsomes the UDP-glucuronyltransferases exist in a latent state (134) and can be activated by detergents, which are thought to involve the partial solubilization of the enzyme (135, 136). In this study the microsomes were treated with Triton X-100 (Section 2.7.3).

GC was used to monitor enzymatic conjugation by determining the residual substrate concentrations after incubating the toxin

with microsomes in the presence of UDP-glucuronic acid. Prior to GC analysis interfering substances were removed from the reaction mixture. Microsomes were precipitated with TCA and removed by centrifugation. After application of the supernatant to a C_{18} Sep-Pak cartridge the polar constituents, UDP-glucuronic acid, glucuronides (if they were formed) and salts were eluted from the cartridge with water. The toxins, retained on the cartridge, were then eluted with methanol and quantified by GC. The recovery of the toxins from the cartridge was approximately 98%.

No significant glucuronidation could be detected using T-2 toxin or T-2 tetraol as substrates for either pig or rat hepatic micro= somal transferases. The presence of additional hydroxyl groups on the trichothecene molecules, as in T-2 tetraol, did not seem to play a role in the potential conjugation reaction. The fact that glucuronidation of these trichothecenes was not observed with rat microsomes could not be due to a lack of UDP-glucuronyltrans= ferase activity of these microsomes, as active conjugation of phenolphthalein was catalysed by this microsomal preparation (Section 3.3.1). Although T-2 toxin and HT-2 toxin appear not to be glucuronidated under the _in vitro_ conditions employed (Section 3.3.2), glucuronides of T-2 toxin, HT-2 toxin, 3'hydroxy T-2 toxin and 3'hydroxy HT-2 toxin have recently been detected in the bile and urine of pigs treated with T-2 toxin (113). It would thus appear as if the _in vitro_ methods employed for the glucuronidation of T-2 toxin and its metabolites were not suitable

and require further investigation.

4.4 DEACYLATION OF T-2 TOXIN

T-2 toxin is one of the most toxic trichothecenes but it is known that species differ considerably in their susceptibility to T-2 toxin toxicity. LD_{50} values for T-2 toxin vary by more than 10 fold - from 5.2 mg/kg in the rat to <0.5 mg/kg in the cat (18).

T-2 toxin is converted to HT-2 toxin by a microsomal esterase (137) as part of the detoxification pathway (Section 1.5.2). This deacylation rate was studied in microsomes prepared from rat, human, cat and monkey to obtain further information regarding the species differences in detoxification. Human hepatic microsomes were included in this study as T-2 toxin has been associated with the human disease ATA (9).

Kinetic studies with microsomes to determine the rate of deacy= lation were performed at pH 7.4, as non-enzymatic deacylation was found to occur at higher pH values (Section 3.5.2). However, the pH optima for the esterase in cat, monkey and human microsomes were found to be between pH 8 and 9 (Section 3.5.2). These findings would imply that under *in vivo* conditions (pH 7.4) the deacylation of T-2 toxin will not occur under optimal conditions.

The K_m obtained for the deacylation reaction with rat microsomes (0.51 mM) is of the same order as the K_m (0.27 mM) obtained by Ohta _et al_ (106). The K_m of human (1.4 mM) is 3 times higher than that for rat microsomes which implies that the affinity of the human microsomal esterase for T-2 toxin is lower than that of the rat esterase. The sigmoidal curves obtained with cat and monkey microsomes (Fig. 3.27) are characteristic of allosteric enzymes which exhibit positive co-operativity. Main (138) observed that certain mammalian liver and serum esterases show this allosteric behaviour with butyrylthiocholine as substrate. He postulates that the enzyme has two binding sites for the substrate, an allosteric and an active site. At low substrate concentrations with little binding of substrate to the allosteric site, the affinity of the active site for the substrate is low. As the substrate concen= tration increases the substrate will bind to the allosteric site which will in turn increase the affinity of the active site for the substrate, thereby increasing the rate of the reaction. It is possible that the results obtained with cat and monkey microsomes can also be explained by this kind of reaction mechanism.

The high deacylation rate observed with rat and the low rate observed with cat and monkey microsomes correlated with the low sensitivity of rats and the high sensitivity of cats and monkeys to T-2 toxin toxi= city. These findings suggested that the conversion of T-2 toxin to HT-2 toxin could be the important step in the detoxification of T-2 toxin in rats, cats and monkeys and that T-2 toxin is likely to be the toxic entity in these species. In human microsomes the

deacylation rate was very high when compared to the other species investigated, suggesting that human might not be as sensitive to the action of T-2 toxin. This is, however, not the case as T-2 toxin appears to be highly toxic to humans (9). If the toxic mechanism of action of T-2 toxin is the same in cats, humans and monkeys, it would imply that the deacylation of T-2 toxin is not an important detoxification step in humans. It is important to remember that under in vivo conditions, the concentrations of T-2 toxin in the livers of animals might never reach the higher levels reported in Fig. 3.26. To predict the differences in deacylation rate between the different species from the data in Fig. 3.26 comparisons should be made at those concentrations of T-2 toxin which occur in the liver after p.o. administration of similar doses to different species.

Care should be taken in drawing conclusions from these results as the deacylation rate determinations were performed on microsomes obtained from only one monkey, cat and human. As considerable intraspecies variation can occur in esterase activity (139), the results reported here may not necessarily be a true reflection of the activities in these species. The human liver was exposed to dexamethasone and diphenylhydantoin during the two days prior to death. These two drugs are known to be inducers of microsomal enzymes and might have contributed to the high deacylation rate. However a period of 1 to 2 days is probably too short to result in a detectable induction of the esterase activity.

If the deacylation rates observed in this study truly measure the rate of detoxification in each species the results would suggest that a metabolite of T-2 toxin is responsible for the toxic effects in humans. A study of the *in vivo* metabolism of T-2 toxin in chickens indicates that the conversion of T-2 toxin to HT-2 toxin is the main step in the detoxification (114). In a lactating cow, however, it seems that the hydroxylation of T-2 toxin to 3'hydroxy T-2 toxin is the major detoxification step (116). The formation of 3' hydroxy T-2 toxin was not studied in the present investigation so it is not known whether the formation of 3' hydroxy T-2 toxin occurs at a rate similar to or faster than the formation of HT-2 toxin in humans. It is possible that HT-2 toxin and/or 3'hydroxy T-2 toxin or another metabolite could be the major toxic principles in humans.

SUMMARY

The trichothecenes, a group of toxic compounds produced by a variety of fungal species, have been associated with a number of human and animal diseases. They induce a wide spectrum of biological effects in experimental animals, including haemorrhaging, diarrhea, suppression of the immune system and disorders of the haematopoeitic system.

This study involved the investigation of the effects of diacetoxy=scirpenol (DAS) and neosolaniol monoacetate (NMA), two of the most toxic trichothecenes, in rats. In order to conduct these studies, it was necessary to first purify NMA from fungal cultures. The toxin was isolated from rice cultures of *F. sambucinum* strain MRC 1903 and purified using solvent partitioning and silica gel column chromatography. The brine shrimp toxicity test as well as TLC and GC analysis were used for monitoring the toxin. The identity and purity of the NMA were assessed by melting point determination, GC analysis and mass spectroscopy.

Treatment of male Wistar rats with DAS and NMA resulted in a significant decrease in red blood cell counts ($P < 0.05$) and an increase in platelet size ($P < 0.05$). The white blood cell popu=lation and platelet levels were however not significantly affected. Atrophy of the actively dividing cells of the bone marrow, thymus, spleen and lymph nodes was the major pathological lesion, but showed signs of recovery towards the end of the treatment period.

The toxicities of the trichothecenes are known to differ in various animal species. To investigate this phenomenon, the metabolism of another highly toxic trichothecene, T-2 toxin, was studied using rat, cat, human and monkey hepatic microsomes. T-2 toxin was shown to be a substrate for the microsomal esterases. The deacy= lation rate differed considerably between the microsomal prepara= tions from the different species. The highest deacylation rate under similar _in vitro_ conditions was observed with human, followed in decreasing order by rat, cat and monkey microsomes. The latter two exhibited allosteric behaviour. The higher deacylation rate observed with rat compared to that of cat and monkey correlates with the known toxicity of T-2 toxin in these animal species, i.e. the higher the deacylation rate the lower the toxicity.

Studies were conducted to investigate the possible role of the UDP-glucuronyltransferases in the metabolism of T-2 toxin. Neither T-2 toxin nor its metabolites, HT-2 toxin and T-2 tetraol, appear to act as substrates for the enzymes in rat and pig hepatic microsomes under the experimental conditions used in this study.

Studies on the metabolic fate of trichothecenes could contribute to a better understanding of the difference in toxicity in various species. The extrapolation of the toxicity from one species to another should therefore be undertaken with great circumspection.

REFERENCES

1. Schoental, R., Perspect. Biol. Med. 28, 117-120 (1984).

2. Shank, R.C., in "Mycotoxic Fungi, Mycotoxins, Mycotoxicoses - An Encyclopedic Handbook". Wyllie, T.D. and Morehouse, L.G. (eds.), Vol 3, Marcel Dekker, Inc., New York, pp 1-20 (1978).

3. Joffe, A.Z., in "Mycotoxic Fungi, Mycotoxins, Mycotoxicoses - An Encyclopedic Handbook". Wyllie, T.D. and Morehouse, L.G. (eds.), Vol 3, Marcel Dekker, Inc., New York, pp 21-86 (1978).

4. Austwick, P.K.C., in "Mycotoxic Fungi, Mycotoxins, Mycotoxicoses - An Encyclopedic Handbook". Wyllie, T.D. and Morehouse, L.G. (eds.), Vol 2, Marcel Dekker, Inc., New York, pp 279-300 (1978).

5. Rosen, R.T. and Rosen, J.D., Biomed. Mass Spectrom. 9, 443-450 (1982).

6. Ichinoe, M. and Kurata, H., in "Trichothecenes". Ueno, Y. (ed.), Elsevier, Amsterdam, pp. 73-112 (1983).

7. Smalley, E.B. and Strong, F.M., in "Mycotoxins". Purchase, I.F.M. (ed.), Elsevier, Amsterdam, pp 199-228 (1974).

8. Marasas, W.F.O., Nelson, P.E. and Tousson, T.A., in "Toxigenic Fusarium Species". The Pennsylvania State University Press, University Park (1984).

9. Joffe, A.Z., in "Microbial toxins". Kadis, S., Ciegler, A. and Ajl, S.J. (eds.), Vol VII, Academic Press, University Park, pp 139-189 (1971).

10. Lutsky, I., Mor, N., Yagen, B. and Joffe, A.Z., Toxicol. Appl. Pharmacol. 43, 111-124 (1978).

11. Ueno, Y., Adv. Nutr. Res. 3, 301-353 (1980).

12. Kurmanov, I.A., in "Mycotoxic Fungi, Mycotoxins, Mycotoxicoses - An Encyclopedic Handbook". Wylie, T.D. and Morehouse, L.G. (eds.),Vol 2, Marcel Dekker, Inc., New York, pp 85-110 (1978).

13. Jarvis, B.B., Pavanasasivam, G. and Bean, G.A., in "Trichothecenes and other Mycotoxins". Lacey, J. (ed), John Wiley and Sons, Chichester, pp 221-232 (1985).

14. Wollenweber, M.W. and Reinking, O.A., in "Die Fusarien". Paul Parey, Berlin (1935).

15. Nelson, P.E., Tousson, T.A. and Marasas, W.F.O., in "Fusarium Species - An Illustrated Manual for Identification". The Pennsylvania State University Press, University Park (1983).

16. Schmidt, H.R., Ph.D. Thesis, University of Mainz (1982).

17. Cole, R.J. and Cox, R.H., in "Handbook of Toxic Fungal Metabolites". Academic Press, New York (1981).

18. Sato, N. and Ueno, Y., Mycotoxins Hum. Anim. Health Proc. Conf., pp 295-307 (1977).

19. Ueno, Y., Fundam. Appl. Toxicol. 4, 124-132 (1984).

20. Chi, M.S., Robison, T.S., Mirocha, C.J. and Reddy, K.R., Appl. Environ. Microbiol. 35, 363-640 (1978).

21. Stähelin, H., Karlberger-Rüsch, M.E., Sigher, E. and Lazary, S., Arzneimittelforschung 18, 989-994 (1968).

22. Kosuri, N.R., Grove, M.D., Yates, S.G., Tallent, W.H., Ellis, J.J., Wolff, I.A. and Nichols, R.E., J. Am. vet. med. Ass. 157, 938-940 (1970).

23. Ellison, R.A. and Kotsonis, F.N., Appl. Microbiol. 26, 540-543 (1973).

24. Matsuoka, Y., Kubota, K. and Ueno, Y., Toxicol. Appl. Pharmacol. 50, 87-94 (1979).

25. Schoental, R., Joffe, A.Z. and Yagen, B., Cancer Res. 39, 2179-2189 (1979).

26. Huff, W.E., Doerr, J.A., Hamilton, P.B. and Vesonder, R.F., Poultry Science 60, 1412-1414 (1981).

27. Ueno, Y., Ishii, K., Saki, K., Kanameda, S., Tsunoda, H., Tanaka, T. and Enomoto, M., Japan J. Exp. Med. 42, 187-203 (1972).

28. Ueno, Y., Ueno, I., Iittoi, Y., Tsunoda, H., Enomoto, M. and Ohtsubo, K., Japan. J. Exp. Med. 41, 521-539 (1971).

29. Ueno, Y., Sato, N., Ishii, K., Sakai, K. and Enomoto, M., Japan. J. Exp. Med. 42, 461-472 (1972).

30. Rosenstein, Y., Lafont, P. and Frayssinet - Lafarge, C., Collect. Med. Leg. Toxicol. Med. 107, 51-57 (1978).

31. Sato, N., Ito, T., Kumada, H., Ueno, Y., Asano, K., Saito, M., Ohtsubo, K., Ueno, I. and Hatanaka, Y., J. Toxicol. Sci. 3, 335-356 (1978).

32. Richard, J.L., Cysewski, S.J., Pier, A.C. and Booth, G.D., Am. J. Vet. Res. 39, 1674-1679 (1978).

33. Otokawa, M., Shibara, Y. and Egashira, Y., Japan. J. Med. Sci. Biol. 32, 37-45 (1979).

34. Rosenstein, Y., Lafarge-Frayssinet, C., Lespinats, G., Loisillier, F., Lafont, P. and Frayssinet, C., Immunology 36, 111-117 (1979).

35. Weaver, G.A., Kurtz, H.J., Bates, F.Y., Chi, M.S., Mirocha, C.J., Behrens, J.C. and Robison, T.S., Vet. Rec. 103, 531-535 (1978).

36. Rukmini, C., Prasad, J.S. and Rao, K., Fd. Cosmet. Toxicol. 18, 267-269 (1980).

37. Masuda, E., Takemoto, T., Tatsuno, T. and Obara, T., Immunology 45, 743-749 (1982).

38. Sato, N., Ueno, Y. and Enomoto, M., Japan. J. Pharmacol. 25, 263-276 (1975).

39. DeNichola, D.B., Rebar, A.H., Carlton, W.W. and Yagen, B., Fd. Cosmet. Toxicol. 16, 601-609 (1978).

40. Hoerr, F.J., Carlton, W.W. and Yagen, B., Vet. Pathol. 18, 652-664 (1981).

41. Rosenstein, Y., Kretschmer, R.R. and Lafarge - Frayssinet, C., Immunology 44, 555-560 (1981).

42. Tryphonas, H., O'Grady, L., Arnold, D.L., McGuire, P.F., Karpinski, K. and Vesonder, R.F., Toxicology Letters 23, 17-24 (1984).

43. Jagadeesan, V., Rukmini, C., Vijayaraghavan, M. and Tulpule, P.G., Fd. Chem. Toxicol. 20, 83-87 (1982).

44. Friend, S.C.E., Hancock, D.S., Schiefer, H.B. and Babiuk, L.A., Can. J. comp. Med. 47, 291-297 (1983).

45. Friend, S.C.E., Babiuk, L.A. and Schiefer, H.B., Toxicol. Appl. Pharmacol. 69, 234-244 (1983).

46. Lafarge-Frayssinet, C., Lespinats, G., Lafont, P., Loisillier, F., Mousset, S., Rosenstein, Y. and Frayssinet, C., Proc. Soc. Exp. Biol. Med. 160, 302-311 (1979).

47. Rafai, P. and Tuboly, S., Zbl. Vet. Med. 29, 558-565 (1982).

48. Otokawa, M., in "Trichothecenes". Ueno, Y. (ed.), Elsevier, Amsterdam, p 163 (1983).

49. Playfair, J.H.L., in "Immunology at a Glance". Blackwell Scientific Publications, Oxford (1980).

50. Masuko, H., Ueno, Y., Otokawa, M. and Yaginuma, K., Japan. J. Med. Sci. Biol. 30, 159-163 (1977).

51. Wyatt, R.D., Doerr, J.A., Hamilton, P.B. and Burmeister, H.R., Appl. Microbiol. 29, 641-645 (1975).

52. Chi, M.S., Mirocha, C.J., Kurtz, H.J., Weaver, G., Bates, F. and Shimoda, W., Poultry Science 56, 306-313 (1977).

53. Segal, R., Milo-Goldzweig, I., Joffe, A.Z. and Yagen, B., Toxicol. Appl. Pharmacol. 70, 343-349 (1983).

54. Gyongyossy-Issa, M.I.C., Khanna, V. and Khachatourians, G.G., Biochim. Biophys. Acta 838, 252-256 (1985).

55. Gentry, P.A., Ross, M.L. and Chan, P.K-C., Vet. Hum. Toxicol. 26, 24-28 (1984)

56. Weaver, G.A., Kurtz, H.J., Bates, F.Y., Mirocha, C.J., Behrens, J.C. and Hagler, W.M., Res. Vet. Sci. 31, 131-135 (1981).

57. Gentry, P.A., Can. J. comp. Med. 46, 414-419 (1982).

58. Cosgriff, T.M., Bunner, D.P., Wannemacher, R.W., Hodgson, L.A. and Dinterman, R.E., Toxicol. Appl. Pharmacol. 76, 454-463 (1984).

59. Goodwin, W., Haas, C.D., Fabian, C., Heller-Bettinger, I. and Hoogstraten, B., Cancer 42, 23-26 (1978).

60. Gentry, P.A. and Cooper, M.L., Can. J. comp. Med. 45, 400-405 (1981).

61. Kosuri, N.R., Smalley, E.B. and Nichols, R.E., Am. J. Vet. Res. 32, 1843-1850 (1971).

62. Gentry, P.A. and Cooper, M.L., Am. J. Vet. Res. 44, 741-746 (1983).

63. Doerr, J.A., Hamilton, P.B. and Burmeister, H.R., Toxicol. Appl. Pharmacol. 60, 157-162 (1981).

64. Chan, P.K-C. and Gentry, P.A., Fd. Chem. Toxic. 22, 643-648 (1984).

65. Chan, P.K-C. and Gentry, P.A., Fed. Proc. 43, 975 (1984).

66. Yarom, R., More, R., Eldor, A. and Yagen, B., Toxicol. Appl. Pharmacol. 73, 210-217 (1984).

67. Yarom, R., Sherman, Y., More, R., Ginsburg, I., Borinski, R. and Yagen, B., Toxicol. Appl. Pharmacol. 75, 60-68 (1974).

68. Kriegleder, H., Zbl. Vet. Med. 28, 165-175 (1981).

69. Hayes, M.A. and Schiefer, H.B., Can. J. comp. Med. 44, 219-228 (1980).

70. Hayes, M.A., Bellamy, J.E.C. and Schiefer, H.B., Can. J. comp. Med. 44, 203-218 (1980).

71. Chi, M.S., El-Halawani, M.E., Waibel, P.E. and Mirocha, C.J., Poultry Science 60, 137-141 (1981).

72. Chi, M.S., Mirocha, C.J., Kurtz, H.J., Weaver, G., Bates, F., Shimoda, W. and Burmeister, H.R., Poultry Science 56, 103-116 (1977).

73. Pearson, A.W., Res. Vet. Sci. 24, 92-97 (1978).

74. Lilienblum, W., Walli, A.K. and Bock, K.W., Biochem. Pharmacol. 31, 907-913 (1982).

75. Suneja, S.K., Ram, G.C. and Wagle, D.S., Toxicology Letters 22, 113-118 (1984).

76. Trenholm, H.L., Hamilton, R.M.G., Friend, D.W., Thompson, B.K. and Hartim, K.E., J. Am. vet. med. Ass. 185, 527-531 (1984).

77. Matsumoto, H., Ito, T. and Ueno, Y., Japan. J. Exp. Med. 48, 393-399 (1978).

78. Chan, P.K-C. and Gentry, P.A., Toxicol. Appl. Pharmacol. 73, 402-410 (1984).

79. Marasas, W.F.O., Bamburg, J.R., Smalley, E.B., Strong, F.M., Ragland, W.L. and Degurse, P.E., Toxicol. Appl. Pharmacol. 15, 471-482 (1969).

80. Matsuoka, Y. and Kubota, K., Toxicol. Appl. Pharmacol. 57, 293-301 (1981).

81. Feuerstein, G., Goldstein, D.S., Ramwell, P.W., Zerbe, R.L., Lux, W.E., Faden, A.I. and Bayorh, M.A., J. Pharmacol. Exp. Ther. 232, 786-794 (1985).

82. Yarom, R., More, R., Raz, S., Shimoni, Y., Sarel, O. and Yagen, B., Basic. Res. Cardiol. 78, 623-630 (1983).

83. Parker, G.W., Wannemacher, R.W. and Gilman, F.J., Fed. Proc. 43, 578 (1984).

84. Wilson, C.A., Everard, D.M. and Schoental, R., Toxicology Letters 10, 35-40 (1982).

85. Yarom, R., More, R., Sherman, Y. and Yagen, B., Br. J. exp. Path. 64, 570-577 (1983).

86. Wilson, D.J. and Gentry, P.A., Toxicol. Appl. Pharmacol. 79, 159-165 (1985).

87. Hayes, M.A. and Schiefer, H.B., Fd. Cosmet. Toxicol. 17, 611-621 (1979).

88. Ueno, Y., Ishikawa, Y., Amakai, K., Nakajima, M., Saito, M., Enomoto, M. and Ohtsubo, K., Japan. J. Exp. Med. 40, 33-38 (1970).

89. Schoental, R., Joffe, A.Z. and Yagen, B., Br. J. Cancer 38, 171 (1978).

90. Bamburg, J.R., Prog. Mol. Subcell. Biol. 8, 41-110 (1983).

91. Ueno, Y., Ann. Nutr. Aliment. 31, 885-900 (1977).

92. McLaughlin, C.S., Vaughan, M.H., Campbell, I.M., Wei, C.M., Stafford, M.E. and Hansen, B.S., Mycotoxins Hum. Anim. Health Proc. Conf. p263-373 (1977).

93. Mizuno, S., Biochim. Biophys. Acta 383, 207-214 (1975).

94. Cannon, M., Smith, K.E. and Carter, C.J., Biochem. J. 156, 289-294 (1976).

95. Carter, C.J. and Cannon, M., Eur. J. Biochem. 84, 103-111 (1978).

96. Cundliffe, E. and Davies, J.E., Antimicrobial Agents and Chemotherapy 11, 491-499 (1977).

97. Smith, K.E. and Dawid, E.T., Biochem. J. 196, 641-644 (1981).

98. Cannon, M., Jimenez, A. and Vazquez, D., Biochem. J. 160, 137-145 (1976).

99. Liao, L-L., Grollmann, A.P. and Horwitz, S.B., Biochim. Biophys. Acta 454, 273-284 (1976).

100. Carter, C.J. and Cannon, M., Biochem. J. 166, 399-409 (1977).

101. Rosenstein, Y. and Lafarge-Frayssinet, C., Toxicol. Appl. Pharmacol. 70, 283-288 (1983).

102. Willis, E.D., in "Biochemical Basis of Medicine". John Wright and Sons, Ltd., Bristol, Chapter 26, (1985).

103. Chi, M.S., Robison, T.S., Mirocha, C.J., Swanson, S.P. and Shimoda, W., Toxicol. Appl. Pharmacol. 45, 391-402 (1978).

104. Robison, T.S., Mirocha, C.J., Kurtz, H.J., Behrens, J.C., Weaver, G.A. and Chi, M.S., J. Agric. Food Chem. 27, 1411-1413 (1979).

105. Ellison, R.A. and Kotsonis, F.N., Appl. Microbiol. 27, 423-424 (1974).

106. Ohta, M., Ishii, K. and Ueno, Y., J. Biochem. 82, 1591-1598 (1977).

107. Yoshizawa, T. and Sakamoto, T., Proc. Jpn. Assoc. Mycotoxicol. 14, 26-28 (1982).

108. Yoshizawa, T., Sakamoto, T. and Okamoto, K., Appl. Environ. Microbiol. 47, 130-134 (1984).

109. Nakamura, Y., Ohta, M. and Ueno, Y., Chem. Pharm. Bull. 25, 3410-3414 (1977).

110. Yoshizawa, T., Swanson, S.P. and Mirocha, C.J., Appl. Environ. Microbiol. 40, 901-906 (1980).

111. Trusal, L.R. and Watiwat, S.R., Fed. Proc. 43, 578 (1984).

112. O'Brien, J.C., Thompson, W. and Pace, J., Fed. Proc. 44, 1038 (1985).

113. Corley, R.A., Swanson, S.P. and Buck, W.B., J. Agric. Food. Chem. 33, 1085-1089 (1985).

114. Yoshizawa, T., Swanson, S.P. and Mirocha, C.J., Appl. Environ. Microbiol. 39, 1172-1177 (1980).

115. Visconti, A. and Mirocha, C.J., Appl. Environ. Microbiol. 49, 1246-1250 (1985).

116. Yoshizawa, T., Mirocha, C.J., Behrens, J.C. and Swanson, S.P., Fd. Cosmet. Toxicol. 19, 31-39 (1981).

117. Romer, T.R., Boling, T.M. and MacDonald, J.L., J.A.O.A.C. 61, 801-808 (1978).

118. Ishii, K., Pathre, S.V. and Mirocha, C.J., J. Agric. Food Chem. 26, 649-653 (1978).

119. Maes, C.M., Horak, R.M., Marasas, W.F.O., Rabie, C.J., Steyn, P.S. and Vleggaar, R., pp 21, Book of Abstracts, 6th International Symposium on Mycotoxins and Phycotoxins, IUPAC, Pretoria (1985).

120. Mirocha, C.J., Pathre, S.V., Schauerhamer, B. and Christensen, C.M., Appl. Environ. Microbiol. 32, 553-556 (1976).

121. Eppley, R.M., J.A.O.A.C. 57, 618-620 (1974).

122. Lymann and Flemming, J. Marine Res. 3, 134-146 (1940).

123. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J., J. Biol. Chem. 193, 265-275 (1951).

124. Wei, R.D., Strong, F.M., Smalley, E.B. and Schnoes, H.K., Biochem. Biophys. Res. Commun. 45, 396-401 (1971).

125. Halac, E. and Bonevardi, E., Biochim. Biophys. Acta 67, 498-500 (1963).

126. Boutin, J.A., Antoine, B., Batt, A. and Siest, G., Chem. -Biol. Int. 52, 173-184 (1984).

127. Lansden, J.A., Cole, R.J., Dorner, J.W., Cox, R.H., Cutler, H.G. and Clark, J.D., J. Agric. Food. Chem. 26, 246-249 (1978).

128. Curtis, R., Caxon, D.T. and Levitt, G., Fd. Cosmet. Toxicol. 12, 233-235 (1974).

129. Eppley, R.M., J. Am. Oil Chem. Soc. 56, 824-829 (1979).

19 OCT 1987

130. Vesonder, R.F., Ciegler, A., Jensen, A.H., Rohwedder, W.K. and Weisleder, D., Appl. Environ. Microbiol. 31, 280-285 (1976).

131. Ebbe, S., in "The Circulating Platelet "Johnson, S.A. (ed.), Aca= demic Press, London, pp 20-39 (1971).

132. Bock, K.W., Josting, D., Lilienblum, W. and Pfeil, H., Eur. J. Biochem. 98, 19-26 (1979).

133. Inscoe, J.K. and Axelrod, J., J. Pharmac. exp. Ther. 129, 128-131 (1980).

134. Hallinan, T., in "Conjugation reactions in Drug Biotransformation". Aito, A. (ed.), Elsevier/North Holland Biomedical Press, Amsterdam, pp 257-267 (1978).

135. Lucier, G.W., McDaniel, O.S. and Matthews, H.B., Arch. Biochem. Biophys. 145, 520-530 (1971).

136. Bock, K.W., Fröhling, W., Remmer, H. and Rexer, B., Biochim. Biophys. Acta 327, 46-56 (1973).

137. Wei, R.D. and Chu, F.S., Appl. Environ. Microbiol. 50, 115-119 (1985).

138. Main, A.R., J. Environ. Sci. Health B18, 29-63 (1983).

139. Williams, F.M., Clin. Pharmacokinetics 10, 392-403 (1985).

140. Tukey, J.W., in SAS Institute Inc. SAS User's Guide: Statistics, Version 5 Edition. Cary, NC: SAS Institute Inc., p448 (1985).

141. Einot, I. and Gabriel, K.R., J. Am. Stat. Ass. 70, 351 (1975).