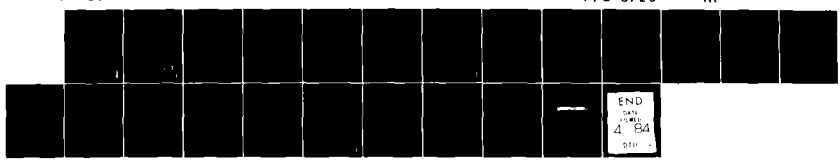
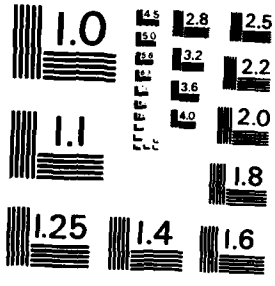
AD-A139 548 THE RESPONSE OF RATS TO CUTANEOUS DOSING WITH TRICHOTHECENE MYCOTOXINS(U) MATERIALS RESEARCH LABS ASCOT VALE (AUSTRALIA) H D CRONE OCT 83 MRL-R-902 1/1

UNCLASSIFIED F/G 6/20 NL





MRL-R-902

AR-003-798

# DEPARTMENT OF DEFENCE

## DEFENCE SCIENCE AND TECHNOLOGY ORGANISATION

## MATERIALS RESEARCH LABORATORIES

MELBOURNE, VICTORIA

AD A139548

## REPORT

## MRL-R-902

# THE RESPONSE OF RATS TO CUTANEOUS DOSING WITH TRICHOTHECENE MYCOTOXINS

H.D. Crone





Approved for Public Release







OCTOBER, 1983

DEPARTMENT OF DEFENCE
MATERIALS RESEARCH LABORATORIES

# REPORT

## MRL-R-902

# THE RESPONSE OF RATS TO CUTANEOUS DOSING WITH TRICHOTHECENE MYCOTOXINS

H.D. Crone

ABSTRACT

T-2 toxin and diacetoxyscirpenol (DAS) formed deep necrotic ulcers on rat skin at doses of 2 μg and 6 μg respectively. Verrucarin A and Roridin A were relatively ineffective at producing skin lesions. The percutaneous $LD_{50}$ of T-2 or DAS was found to be well in excess of 10 mg/kg. DMSO had no marked effect in increasing wound severity or systematic toxicity as opposed to ethyl acetate as the application vehicle. No synergism between T-2 and DAS was found. Histological examination of skin lesions revealed the typical inflammatory reaction that is also caused by thermal burns or other necrotising agents. Thin layer chromatography of the toxins is described, as a method to check purity and stability.



POSTAL ADDRESS: Director, Materials Research Laboratories
P.O. Box 50, Ascot Vale, Victoria 3032, Australia

SECURITY CLASSIFICATION OF THIS PAGE UNCLASSIFIED

DOCUMENT CONTROL DATA SHEET

| REPORT NO. | AR NO. | REPORT SECURITY CLASSIFICATION |
|---|---|---|
| MRL-R-902 | AR-003-798 | UNCLASSIFIED |

TITLE

THE RESPONSE OF RATS TO CUTANEOUS DOSING WITH TRICHOTHECENE MYCOTOXINS


AUTHOR(S)

H.D. CRONE

CORPORATE AUTHOR

Materials Research Laboratories
P.O. Box 50,
Ascot Vale, Victoria 3032


| REPORT DATE | TASK NO. | SPONSOR |
|---|---|---|
| OCTOBER, 1983 | DST 82/160 | DSTO |

CLASSIFICATION/LIMITATION REVIEW DATE

CLASSIFICATION/RELEASE AUTHORITY
Superintendent, MRL
Organic Chemistry Division

SECONDARY DISTRIBUTION

Approved for Public Release

ANNOUNCEMENT

*Announcement of this report is unlimited*


KEYWORDS

Mycotoxins
Trichothecene
Skin Permeation
Inflammatory Reactions
Skin Necrotising Agents

COSATI GROUPS 1502 0620



ABSTRACT

T-2 toxin and diacetoxyscirpenol (DAS) formed deep necrotic ulcers on rat skin at doses of 2 μg and 6 μg respectively. Verrucarin A and Roridin A were relatively ineffective at producing skin lesions. The percutaneous $LD_{50}$ of T-2 or DAS was found to be well in excess of 10 mg/kg. DMSO had no marked effect in increasing wound severity or systemic toxicity as opposed to ethyl acetate as the application vehicle. No synergism between T-2 and DAS was found. Histological examination of skin lesions revealed the typical inflammatory reaction that is also caused by thermal burns or other necrotising agents. Thin layer chromatography of the toxin is described, as a method to check purity and stability.

CONTENTS

# THE RESPONSE OF RATS TO CUTANEOUS DOSING WITH TRICHOTHECENE MYCOTOXINS

## 1. INTRODUCTION

The recent reports [1] of the possible use of trichothecene mycotoxins in S.E. Asia as chemical warfare agents has created great interest in the military aspects of the toxicology of these compounds. Previous research has centred round the presence of the toxins as contaminants of foodstuffs. Thus, for example, studies on skin effects have largely been concerned with the response to small doses as a sensitive indicator of low level contamination [2,3]. By contrast, the military interest is in the effect of large doses which might disable or kill the victim.

In a military context, a toxic chemical is likely to enter the body through the skin or the respiratory tract. We chose initially to examine absorption of the mycotoxins through skin, as this seemed the most likely route of entry for a non-volatile compound that has been described as falling like rain. Drops or particles large enough to fall in air with appreciable velocity cannot be inhaled into the lungs. Some exposure of the respiratory tract may occur by aerosolisation of deposited material, with deposition in the lungs, or more likely in the upper respiratory tract and with consequent diversion to the alimentary tract by swallowing.

Therefore this paper describes the results of the application of purified trichothecene mycotoxins to animal skin, with emphasis on those aspects which relate to possible military use. It is not a comprehensive study of the dermal toxicity of trichothecenes, but was initiated to help answer specific practical questions (e.g. does DMSO increase the dermal toxicity as has been claimed, ref 4). However, it was thought useful to place this work on permanent record, despite its incomplete nature in an academic sense. The paper includes information on the thin layer chromatographic (TLC) examination of the toxins (Appendix A), a study undertaken to assess the purity of the preparations used in the toxicity studies.

## 2. MATERIALS AND METHODS

T-2 toxin was a product of Makor Chemicals Ltd., Jerusalem, Israel, batch no. 9367. Diacetoxyscirpenol (DAS) was obtained from Calbiochem, San Diego, California, lot 702948 B grade. Both compounds were examined by nuclear magnetic resonance (25 mg in 0.6 mL of deuterochloroform). Each had a spectrum compatible with the known structure of the compounds and which agreed with the assignments made by Bamburg [5]. The nmr spectroscopy indicated that each compound had one major component, which was a trichothecene. TLC (Appendix A) confirmed that impurities, if present, were minor. The conclusion is that the compounds are as claimed and in excess of 95 per cent purity, although neither analytical method affords a complete identification and assay. Verrucarin A and Roridin A (both from Makor) were also of a high level of purity (Appendix A).

Female white Porton strain rats were used, of body weight 230 to 280 g. The hair on the back was shaved off with an Oster Animal Clipper, Model A5, No. 80, Size 40. Areas of application were chosen free of visible abrasions caused by the clipper or by scratches and bites from other rats. Each animal received one dose only, except for the initial experiments to establish effective doses, when up to 4 doses were applied simultaneously to different areas of one animal. The dose of trichothecene was applied in 2 µL of ethyl acetate (or as noted in the particular experiment) from a microsyringe with a blunt, bevelled needle. The needle was touched on the skin, the liquid was expelled, and the needle was held against the skin for 2 to 3 seconds. Animals were observed for 7 days at least, often 14 or more depending on the experiment.

## 3. RESULTS

### 3.1 Preliminary Dose-response Studies (T-2 and DAS)

Initial experiments were designed to find the dose of toxin which produced a full-thickness necrotic lesion of the skin, as evidenced by the sloughing-off of affected skin, the presence of a hard scab and the exposure of subcutaneous tissue below the scab. The necessary doses were 2 µg of T-2 and 6 µg of DAS, when applied in 2 µL of ethyl acetate. The effective area of application was 10 to 15 mm in diameter. This response was surprisingly reproducible. The time course of development of the lesion is given in Table 1. This course was not noticeably changed by marked increases in dosage (up to mg quantities). Below the critical dose for a full-thickness lesion, the effects observed were essentially as seen previously, save that a scab did not form. At very low doses (100 ng) the only effect observed was a transient oedema at about 24 hours. Erythema was not a prominent sign, except at large doses (in excess of 100 µg), when the skin of the animal well away from the application area would become blotchy red. DAS did not differ in its effect from T-2 toxin, save in the dose required and in a tendency to form dry white scales over the application area whereas T-2 would produce a single hard scab. This difference could not be used for diagnosis as it was

only apparent when the two toxins were directly compared under the same conditions. In none of the experiments described was any irritant effect of the toxins exhibited, which was surprising considering the nature of some of the wounds. The rats made no obvious attempt to scratch the lesions nor to lick them.

### 3.2 Comparison of DSMO with Ethyl Acetate as the Vehicle, and the Effects of High Toxin Doses

Rats were dosed with 260 µg of T-2 toxin (equivalent to 1 mg/kg body weight) as a solution in either ethyl acetate or DMSO (2 animals for each solvent). The solution was applied as two equal doses of 5 µL on each animal. Each animal developed two lesions of the skin following the course described in Table 1. No difference was seen between those for which DMSO was the vehicle and those receiving ethyl acetate. No systemic effects of the toxin were seen, nor did the body weight of the rats vary significantly over 14 days.

Rats were then treated with the equivalent of 10 mg/kg T-2 or DAS, applied either in ethyl acetate or DMSO (4 rats, each with different solvent/toxin concentration). In order to apply the large amount (2.5 mg) of toxin over a limited skin area, the weighed aliquot of toxin was repeatedly treated with solvent (5 or 10 µL aliquots) which was then applied in successive portions to the same area of skin. DAS was not readily soluble in ethyl acetate and took longer to apply. Ethyl acetate evaporated readily, so that the area of application was smaller than for DMSO. The toxins produced gross skin lesions over the area of application, as described in Table 1. Away from the application area there was intensive erythema over all the visible skin by 4 days. At two days dried blood was visible at the nostrils, and there was a sensitivity of the pads of the forepaws which caused the animals to flex the digits inwards and put weight (if necessary) on the back of the paw. This club-like forepaw posture continued for about 5 days. In one animal (DAS/DMSO combination) the condition persisted permanently on one forepaw due to necrosis of the tissues.

One animal (T-2/ethyl acetate combination) was sacrificed at 5 days and examined internally. The heart, liver and spleen appeared to be slightly enlarged. The lungs were deeper pink than normal; they appeared flushed. The cardiac end of the stomach was contracted and dense, and the caecum was enlarged or very full, although not haemorrhagic, whereas the lower bowel was rather empty. The kidneys, tongue and brain were normal in appearance. No haemorrhage was seen in the body cavity or internal organs.

The 3 remaining animals showed intense lethargy and disinterest in food from 2 days to about 12 days, with a gradual improvement from 6 days onwards. The animals showed a weight loss from the first day (Fig. 1), which became a maximum at 9 days (11 to 15% of initial weight), and slowly recovered to original body weight after 17 to 25 days. The 3 rats at 36 days were healed and perfectly healthy, except for one crippled forepaw.

There was no sign of any difference caused by using DMSO as opposed to ethyl acetate, or between T-2 and DAS. DMSO applied alone to rat skin caused oedema within 20 mins; the blanching and swelling was less obvious in 90 mins and had disappeared after 180 mins. When a toxin was applied in DMSO, there were two cycles of oedema, after 20 mins and 24 hours.

It was concluded that the percutaneous $LD_{50}$ for T-2 and DAS must be well in excess of 10 mg/kg. At this level the dose loses meaning, as it is difficult to define the area of application, and there is a danger of applied toxin falling off the skin or being transferred to other sites (e.g. mouth, paws).

### 3.3 Topical application of macrocyclic trichothecenes

Verrucarin A and Roridin A were used as representatives of the macrocyclic group of trichothecene mycotoxins.

Initial tests with microgramme quantities in ethyl acetate established that the compounds were much less effective than T-2 toxin in producing skin lesions. A test was then made at the 10 mg/kg dose level, using each compound dissolved in DMSO or a 1:1 mixture of ethyl acetate and methanol. The latter mixture was employed because of the very limited solubility of the toxins in pure ethyl acetate. Even so, it was difficult to dissolve the Verrucarin in this mixture, and about 75% of the dose was applied (i.e. approx. 7.5 mg/kg).

The two rats to which Roridin A was applied showed no systemic effects and no change in body weight over 15 days after dosing (Fig. 2). At the area of application lesions were formed which followed the general appearance and time course of those induced by T-2 or DAS. The lesions were smaller and less severe than those produced by 2 µg doses of T-2. It was concluded that the effective dose of Roridin A to produce skin lesions is in the order of 1000 times more than the corresponding dose of T-2, and that death by percutaneous absorption is virtually impossible ($LD_{50}$ >> 10 mg/kg).

The rats dosed with Verrucarin A developed small skin lesions by the fourth day. These were milder than those caused by 2 µg T-2, and differed in that erythema was an obvious component of the syndrome. Both rats passed soft faeces which on the fourth day contained some blood. By the fifth day the condition of the two rats was poor, but then they rallied and rapidly returned to normal condition. The skin lesions were nearly healed by 14 days. These events were paralleled by the body weight of the rats (Fig. 2), which decreased rapidly after dosing to the fourth day, then increased again to the starting value by the seventh day. The rat dosed with DMSO as the solvent was more affected in all respects than that dosed with ethyl acetate. However, the difference was not large, and could be ascribed to the better solvent powers of DMSO. The systemic effects of Verrucarin A at this dose level were much less than those noted for T-2 toxin. No nose, mouth or paw lesions were seen. The effects were confined to anorexia spanning 4 days, and lower bowel haemorrhage for one day.

It was tentatively concluded that Roridin A and Verrucarin A are not skin necrotising agents except at very high dose levels, and that the two compounds do not produce major systemic effects when applied to the skin. Verrucarin A at 10 mg/kg has some effect, but is not life-threatening.

### 3.4 Possible Synergism Between T-2 and DAS

Rats were dosed with 2 µg of toxin in 2 µL of ethyl acetate. One pair was given T-2, another pair DAS and a third pair DAS plus T-2 (i.e. 1 µg DAS + 1 µg T-2 in 2 µL ethyl acetate). The development of skin damage was followed over 12 days. The rats dosed with T-2 developed a scab by the fourth day which began falling off by the eighth, revealing raw dermis without epidermis. Those which received DAS showed a similar result, but the crust which had formed by the fourth day broke up as scaly flakes and fell off (7 to 11 days) revealing an apparently intact epidermis. The rats dosed with the mixture showed an intermediate condition, with some scaliness of the crust and some epidermal damage.

The experiment was repeated, but using 7 µg of pure toxin or mixture in the 2 µL ethyl acetate applied. All six rats exhibited exactly the same lesions with destruction of the epidermis, as described in Table 1. There was no indication that the mixture of toxins produced a more severe wound.

### 3.5 The Removal of T-2 Toxin After Application to the Skin

It was wished to study the removal of applied toxin, to estimate the feasibility of decontamination procedures. Rats were dosed with 7 µg T-2 in 2 µL ethyl acetate, then at set times after dosing the area of application was stripped with an adhesive tape ("Sellotape Regd : Invisible Tape : No. 872" 24 mm x 66 m) which was found to have a mild stripping action on the epidermis. This tape removed loose scales of keratin and any other material without obviously damaging the epidermis. The tape was pressed down over the area of application and then jerked away upwards. Ten stripping actions with fresh tape completed a stripping sequence. Pairs of animals were stripped at 1, 20 and 120 mins after toxin application. All six rats developed lesions of a severity expected from 7 µg of T-2. The wounds on the rats stripped at 1 minute were smaller than those stripped at 120 minutes, but the difference was not great. Any removal of toxin by stripping was minor.

The experiment was then repeated using a tape of greater adhesiveness ("tesa BDF" supplied by Beiersdorf (Aust) Ltd) which removed much of the stratum corneum and left the skin erythemic. There was no difference between the wounds of rats stripped after 1, 20 or 120 minutes and in fact the lesions were all larger than would be expected from the dose. It was concluded that any removal of toxin was more than compensated for by the physical damage caused by the stripping procedure.

### 3.6 Histological Examination of Treated Skin

Four rats were treated topically with 7 µg T-2 toxin in 2 µL of ethyl acetate as before. The rats were sacrificed at 30 hours, 3 days, 5 days and 9 days after treatment. The treated skin area was excised, fixed in buffered formalin, impregnated with paraffin wax (paraplast) and sectioned to 3 µm. The sections were stained with haemotoxylin and eosin. Untreated areas of skin remote to the area of application were removed from the same animals to act as controls.

The specimen at 30 hours showed dermal oedema with separation of collagen bundles and an early mononuclear cell infiltration of the dermis, particularly in the sub-epidermal region and on either side of the panniculus carnosus. There was also patchy lysis of the basement membrane zone and patchy vacuolisation of the basal layer of cells but no clear evidence of epidermal cell necrosis.

The next one in the series (3 days) showed surface fibrino-purulent exudate as well as the dermal oedema and inflammatory cell infiltrate as described before. The epidermis still appeared relatively intact.

After 5 days there was extensive epidermal necrosis and ulceration into the superficial dermis. The ulcer was covered by fibrino-purulent exudate and the base was formed by early granulation tissue containing many mononuclear inflammatory cells. There was also relatively dense mononuclear inflammatory cell infiltrate on the superficial aspect of the panniculus carnosus immediately beneath the ulcer. The adjacent epidermis showed a fibrino-purulent exudate on the surface.

The last one of the series (9 days) showed evidence of healing with much of the ulcerated area (or presumed ulcerated area) covered by a thin, rather atrophic-looking epidermis which was only 1-2 cells thick. Some focal ulceration remained and this was covered by a fibrino-purulent exudate and the underlying dermis contained mononuclear cells. There was also a moderately dense mononuclear cell inflammatory exudate within the deep dermis closely opposed to the superficial aspect of the panniculus carnosus and there was a lesser infiltrate of similar cells on the deep aspect of the panniculus carnosus.

These changes in morphology at the tissue level fit in well with what can be observed by eye on the outside of the intact animal. It confirms that the lesion caused by T-2 toxin in doses above 2 µg extends deep into the dermis, even if it cannot be regarded as necrotising the full skin thickness. There is nothing unusual in the skin response that can be regarded as characteristic of the toxin; these effects could be produced by many other chemical or physical agents, such as thermal burns.

## 4. DISCUSSION

The toxins T-2 and DAS are highly toxic to skin, but are not lethal to the rat when administered percutaneously, except perhaps at massive dosages covering large areas of skin. In the latter case death would be a secondary result of the loss of skin, similar to an extensive thermal burn. In fact the effects on skin of the trichothecenes are very similar to burns. In our experience the effect of 100 ng T-2 can be seen on rat skin and we are willing to believe that 10 ng can be observed by experienced persons, as claimed by others [3]. At the other end of the scale we have found the dose to kill a rat to be in excess of 2.5 mg, giving a ratio between minimal effect and lethality of at least $2.5 \times 10^5$. The skin lesions we have observed are consistent with the known action of trichothecenes as protein synthesis inhibitors [6] and thus as general agents of cell necrosis. No unusual features are present that suggest other than a toxic reaction followed by cell death, that could be caused by other chemical or physical agents. The great ratio between minimal effective dose and lethality suggests that the trichothecenes do not penetrate beyond the skin; if they did direct systemic effects should be obvious at low doses. It is not suggested that the toxins are denied entry by the stratum corneum. The failure of DMSO to increase percutaneous effects rather excludes this. It would appear that the toxins are either trapped in the outer skin by strong absorption to cellular components, or are decomposed, before they can reach the capillaries of the dermis and thus the systemic circulation. The fact that skin lesions are a feature of alimentary toxic aleukia (ATA) resulting from trichothecene ingestion [7] suggests that skin has a peculiar sensitivity to trichothecenes, which may again be due to a specific binding capacity.

In military terms, trichothecenes would have to be rated as casualty-producing agents rather than lethal agents, as far as skin exposure is concerned. The toxins have obvious resemblances to sulphur mustard in this respect and in the fact that the appearance of the lesion is delayed for many hours after exposure. The trichothecenes would appear to be more toxic to skin than sulphur mustard, if we can compare 2 µg of T-2 on rat skin to 20 µg of mustard on human skin, which is about the minimum required to produce a blister.

The presence of some lesions (mouth, paw) on exposed animals not at the site of application (section 3.2) and of general symptoms such as anorexia could indicate that there is some systemic absorption of trichothecenes after dermal application. It is equally possible, however, that these effects arise by direct contamination from the application area to the paws and mouth. The recent work [8] at the United States Army Medical Research Institute of Infectious Diseases (USAMRIID) suggests that the latter is the case. The $LD_{50}$ for topically applied T-2 toxin in methanol on the guinea pig was increased from 4.2 mg/kg to 72.6 mg/kg when a non-occlusive barrier was used. This barrier prevented direct transfer of toxin to the mouth. For all practical purposes these mycotoxins are not lethal when the exposed surface is skin.

The failure of our limited experiments to show any potentiation of percutaneous absorption by administration in DMSO is in agreement with the

more extensive results from USAMRIID [8]. The latter researchers have obtained a decrease in $LD_{50}$ from 72.6 mg/kg to 56.5 mg/kg in the guinea pig when DMSO was used rather than methanol. DMSO appeared to be more effective on rat skin, less so on monkey. This moderate increase of toxicity is of no general significance, and of course the effect of DMSO on trichothecene absorption by human skin is unknown.

We have found no synergism between T-2 and DAS, but to test all aspects of synergism and of solvent effects thoroughly would require much experimentation. Nevertheless, our preliminary results tend to suggest that such effects, if they occur, are not of a magnitude to have practical significance. Synergism between trichothecene toxins and other toxins or components of crude extracts is certainly possible but to our knowledge not proven.

It is surprising that little toxin can be removed from the skin one minute after application. The ethyl acetate runs over the surface and evaporates rapidly, so that the toxin is deposited as a lacquer into all the crevices and depressions of the stratum corneum. Nevertheless the stripping with tesa BDF, which removed much of the stratum corneum, did not remove much toxin. This is consistent with the theory that the stratum corneum is not a great barrier to trichothecene penetration, and also helps to explain the ineffectiveness of known penetration aids such as DMSO. However, others [8] have found value in simple washing with soap. Volatile solvents such as ethyl acetate could not be used in weapons which produce liquid aerosols; trichothecene solutions in solvents of low volatility could be removed from the skin before the drops spread by devices such as the fuller's earth pads or mitts. In view of the great potency of the toxins on the skin, such decontamination would not be much benefit.

This study does not suggest an approach to the therapy of trichothecene intoxication of skin. On the contrary it suggests that as with sulphur mustard burns, little can be done to prevent the development of the lesion, which must then be managed as appropriate to encourage healing and prevent infection.

We conclude that T-2 toxin is very potent locally on skin inducing a necrosis that is ultimately not distinguishable from that caused by thermal burns or by mustard gas. The toxin is not lethal by percutaneous application at any practical dose level in any solvent tested. Therefore any presumed military usage would have had to be aimed at casualty induction after a long delay period (24-48 hours), or to administration by another route. There is no good data on the inhalation toxicity of these toxins, due to the difficulty of aerosolising a sufficient concentration.

## 5. ACKNOWLEDGEMENTS

Dr B.M. Paddle and Mrs J. Paddle-Ledinek performed the histological examinations and Dr A.G. Moritz the n.m.r. spectroscopy. Mr M. O'Connell assisted with the thin layer chromatography. I am grateful to Dr S.E. Freeman for helpful comments on toxicological matters.

## 6. REFERENCES


1. United States Department of State. Special Report No. 98 "Chemical Warfare in Southeast Asia and Afghanistan" March 1982 : Ditto Special Report No. 104 "Chemical Warfare in Southeast Asia and Afghanistan : An Update" November 1982.

2. Wei, R.D., Smalley, E.B. and Strong, F.M. (1972). "Improved skin test for detection of T-2 toxin", Appl. Microbiol. 23, 1029-30.

3. Hayes, M.A. and Schiefer, H.B. (1979). "Quantitative and morphological aspects of cutaneous irritation by trichothecene mycotoxins". Fd. Cosmet. Toxicol. 17, 611-621.

4. Watson, S. (1982). Statements to U.S. House of Representatives Subcommittees on International Security and Scientific Affairs, and on Asian and Pacific Affairs. March 30 1982 pp 68-69. U.S. Govt. Printing Office, Washington, 1982.

5. Bamburg, J.R., Riggs, N.V. and Strong, F.M. (1968). "The structure of toxins from two strains of Fusarium tricinctum", Tetrahedron 24, 3329-36.

6. Busby, W.F. and Wogan, G.N. (1981). "Trichothecenes" in "Mycotoxins and N-nitroso compounds : Environmental Risks" (Ed. Shank, R.C.). CRC Press Inc., Boca Raton, USA.

7. Joffe, A.Z. (1971). "Alimentary Toxic Aleukia" in Microbial Toxins (Ed. Kadis, Ciegler and Ajl) vol. VII, pp 139-189. Academic Press, New York.

8. Wannemacher, R.W., Dinterman, R.E., Pace, J.G. and Bunner, D.L. (1983). "Toxicity of T-2 Mycotoxin when painted on the skin of a quinea pig". Proc. Int. Mycotoxin Symp., Sydney, 12-15 Aug. 1983, Abstract No. 2.8.

9. Scott, P.M. (1980). "Analysis of Mycotoxins other than Aflatoxins in Foodstuffs". in "Thin Layer Chromatography; Quantitative Environmental and Clinical Applications" (Ed. Touchstone and Rogers). Wiley, New York.

10. Yoshizawa, T., Swanson, S.P. and Mirocha, C.J. (1980). "T-2 Metabolites in the Excreta of Broiler Chickens Administered $^{3}H$-Labelled T-2 Toxin". Appl. Environm. Microbiol. 39, 1172-1177.

TABLE 1

Time course of the development of skin lesions in the rat after the application of T-2 or DAS at a dose in excess of 10 µg

| TIME | VISIBLE EFFECTS |
|---|---|
| 20 hrs onwards | Oedema evidenced by local blanching of skin. Later erythema at margin. |
| 48 hrs | Area previously raised by oedema has collapsed, but white area still visible. |
| 48 hrs onwards | Exudate from lesion area forms red-brown patches, which gradually cover whole area. |
| 4 to 5 days | Exudate dries out and forms scab. |
| 6 to 8 days | Scab hardens, lifts and begins to fall off. |
| 10 days | Scab has fallen off. Wound is covered with shiny new epidermis. |
| 10 days onwards | Wound continues to heal by contraction of the margins and consolidation of the epidermis. Skin healed by 14 days, but scar still visible as smooth area of sparse hair regrowth. |

## APPENDIX A

## THIN LAYER CHROMATOGRAPHIC EXAMINATION OF TRICHOTHECENE MYCOTOXIN SAMPLES

### 1. AIMS

The aims of this study were to check the purity of commercial samples as received, and to monitor the purity of the samples during storage and use for toxicity studies.

### 2. METHODS

The plates were pre-coated TLC plates of Silica Gel 60 F-254 from E. Merck of Darmstadt (Cat. No. 5714). These were heated at 110°C for 30 minutes, then allowed to cool to room temperature before samples were applied. Two solvent systems were employed. Solvent System No. 1 was toluene/ethyl acetate/formic acid, 6:3:1 by vol [9]. System No. 2 was chloroform/methanol, 9:1 [10].

Samples were applied from solutions of ethyl acetate or dimethylsulphoxide (DMSO), normally in the concentration range of 1 to 10 mg per mL. Samples of 10 or 20 µg were sufficient. They were applied to the plate as transverse lines 13 mm wide. The plates were developed at 20 ± 1°C until the solvent front had travelled 150 to 160 mm.

The developed chromatograms were examined under UV light at 254 nm, and sample spots were seen as dull red areas against the green fluorescence of the absorbent. The macrocyclic compounds were more readily seen than T-2 toxin or DAS. The limit for the latter two was about 1 µg, whereas a sample of a macrocyclic toxin less than this could be easily seen. Then the plates were exposed to iodine vapour, which detected the compounds at a load of 1 µg, but also formed background colour near the solvent front. Finally, the iodine was aired off and the plates were sprayed with 20% sulphuric acid in methanol. Instead of a short heating period followed by examination under UV light [8,9], we prolonged the heating to char all organic material on the plate, thus allowing observation of any impurities that would not show the blue fluorescence of T-2 toxin and diacetoxyscirpenol (DAS).

To elute a particular zone from a chromatogram, the position of that zone was first established by running test plates. Then the sample of interest was chromatographed alongside a marker sample. The absorbent in the presumed zone was scraped off the plate, then the position checked by spraying the marker and the residue of the sample chromatogram. If correctly located, the absorbent from the plate was packed in a glass column of internal diameter

6 mm, and eluted with 0.5 mL ethyl acetate followed by 0.5 mL ethyl acetate/methanol 1:1 by vol. The eluate was dried down under a stream of air at 20°C, and the residue used for toxicity tests as desired.

## 3. SAMPLES EXAMINED

3.1 T-2 Toxin, Calbiochem-Behring Corp., La Jolla, CA, Cat. No. 616389, Lot 103126.

3.2 T-2 Toxin, Makor Chemicals Ltd, Jerusalem, Israel. Cat. No. 0762, Batch 9367.

3.3 Diacetoxyscirpenol, Calbiochem. Cat. No. 266711, Lot 702948.

3.4 Diacetoxyscirpenol, Makor. Cat. No. 0210, Batch 9050.

3.5 Roridin A, Makor. Cat. No. 0720, Batch 7702.

3.6 Verrucarin A, Makor. Cat. No. 0900, Batch 8322.

## 4. RESULTS

$R_F$ values for the four compounds in two solvent systems are given in Table 1. No difference was found between the products of Makor and Calbiochem. T-2 and DAS were separated in Solvent 1, but not in Solvent 2, in which the two compounds formed a single broad zone at $R_F$ 0.72-0.76. Verrucarin A and Roridin A were separated in both solvents.

The sensitivity of the detection methods was such that 1 µg of a minor component would be detected with certainty, and probably much less. As no minor components could be found in any of the compounds at a load of 20 µg, the presumption is that the compounds were at least 95% pure, and more likely 99%.

Solutions of these compounds in ethyl acetate were stable in storage at 5°, showing neither loss in biological activity, nor the presence of degradation products upon TLC. A sample of T-2 toxin in DMSO was found to have lost biological activity (rat skin test) after 3 months storage at 5°. When chromatographed, a spot corresponding to T-2 toxin was found, but much fainter than expected. No other compounds were observed on the plate. The presumed T-2 spot was eluted from the plate and found to have activity in the rat skin test; a response corresponding to about 4 µg of toxin resulted from a nominal load on the TLC plate of 28 µg. The chromatography indicated that the T-2 had not decomposed but that the concentration in solution had fallen considerably. This result was ascribed to the hygroscopic nature of DMSO. Absorption of water vapour each time the storage vial was opened had led to an increasing concentration of water in the solvent, which resulted in a lower solubility of T-2 toxin in the mixture. It is presumed that the toxin was

deposited on the glass walls of the vial. Simple dilution by water would also decrease the apparent activity of the solution. Such effects are significant when the total volume is small, as was the case here when less than 1 mL of solution was made up. They would have little importance for large storage volumes.

## 5. DISCUSSION

This chromatography is an effective method of detecting contaminants in trichothecene samples, except for the unlikely case where the contaminant has exactly the same mobility as the toxin in both solvents. The high toxicity of the present samples makes it more unlikely that they were contaminated with inactive compounds.

Degradation products would readily be detected by TLC, as the most likely decompositions would involve the loss of ester groups, and hence a decrease in mobility on silica gel due to the freeing of more hydroxyl groups on the molecule.

High performance liquid chromatography (HPLC) would be a superior analytical system, provided an adequate detector for trichothecenes could be found. However, TLC is much cheaper and nearly as quick to operate, but more difficult to quantitate.

## 6. CONCLUSIONS

6.1 Four samples of trichothecene mycotoxins from two commercial sources were found to be better than 95% pure.

6.2 TLC is an adequate method of monitoring the purity and stability of such toxins.

6.3 Storage of the toxins in solution in small volumes of DMSO is not advisable.

TABLE 1

(Appendix A)

$R_F$ values of some trichothecene toxins in two solvent systems

| Compound | Solvent System No. 1 | Solvent System No. 2 |
|---|---|---|
| T-2 | 0.23 | 0.79 |
| DAS | 0.20 | 0.70 |
| Roridin A | 0.22 | 0.79 |
| Verrucarin A | 0.32 | 0.84 |

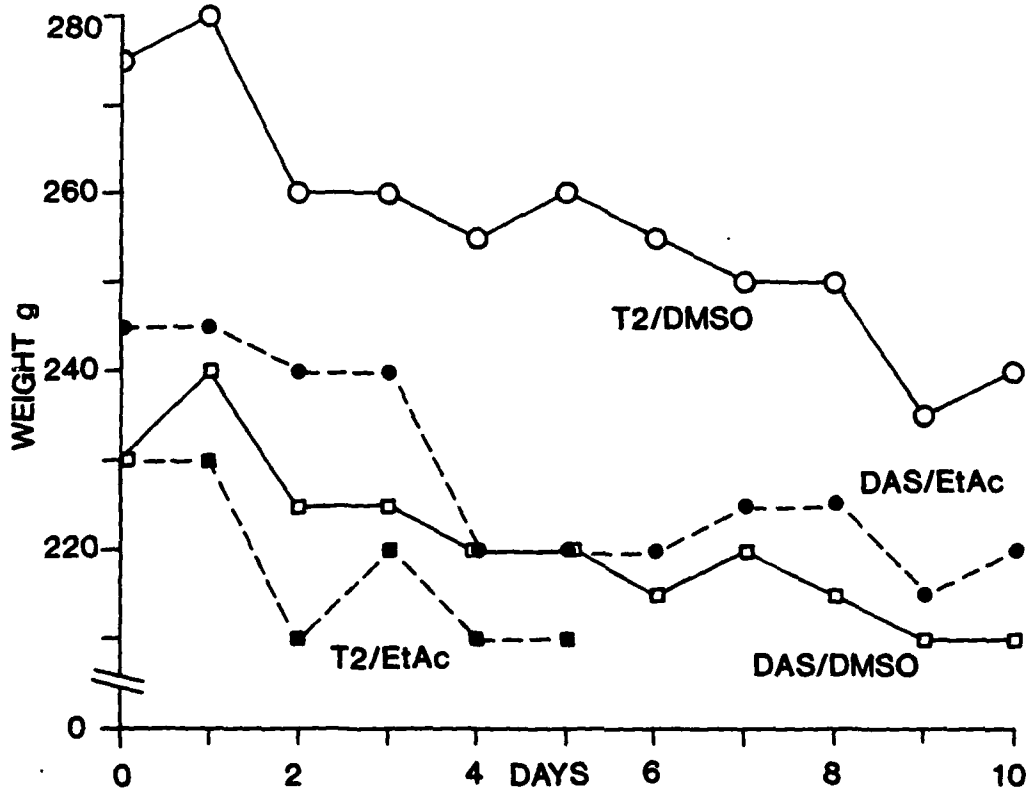



FIGURE 1 Body weight loss of rats dosed cutaneously with 10 mg/kg T-2 toxin or DAS. The toxins were applied in DMSO or ethyl acetate. The T-2/ethyl acetate rat was deliberately sacrificed on day 6 for internal examination.

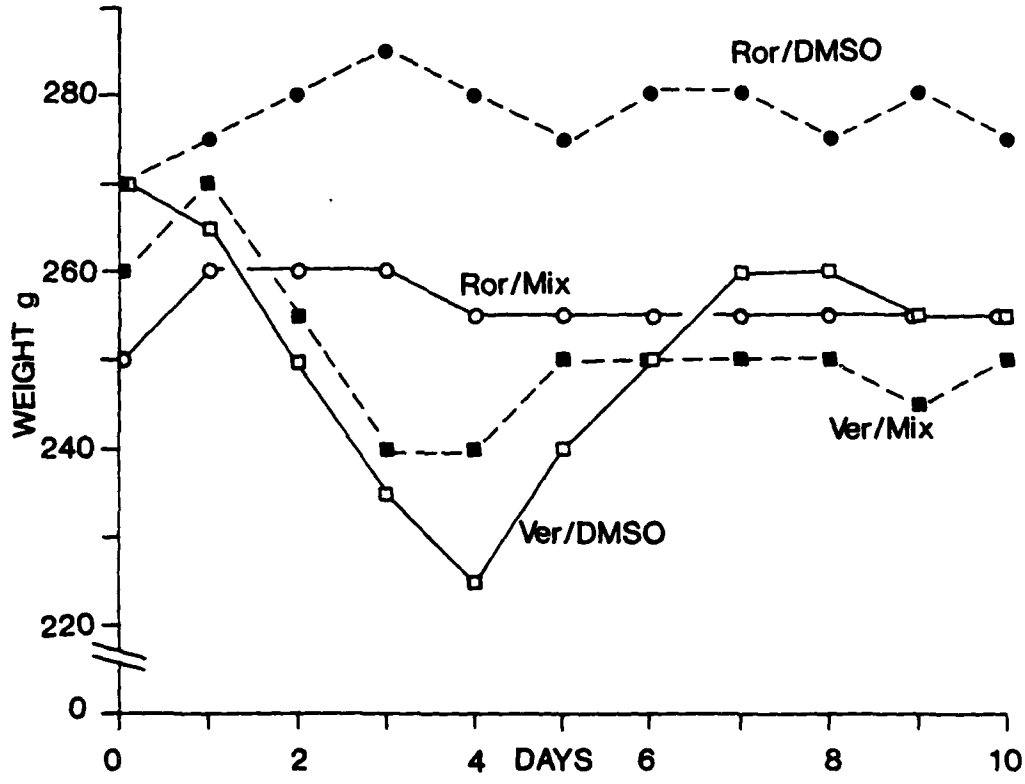



FIGURE 2 Body weight loss of rats dosed cutaneously with Roridin A or Verrucarin A. The toxins were applied in DMSO or a mixture of 1:1 ethyl acetate - methanol.

(MRL-R-902)

DISTRIBUTION LIST

MATERIALS RESEARCH LABORATORIES

Director
Superintendent, Organic Chemistry Division
Library (2 copies)
Dr H.D. Crone

DEPARTMENT OF DEFENCE

Chief Defence Scientist/Deputy Chief Defence Scientist/ (1 copy)
Controller, Projects and Analytical Studies/
Superintendent, Science and Technology Programme
Army Scientific Adviser
Air Force Scientific Adviser
Navy Scientific Adviser
Officer-in-Charge, Document Exchange Centre (17 copies)
Technical Reports Centre, Defence Central Library
Central Office, Directorate of Quality Assurance - Air Force
Deputy Director Scientific and Technical Intelligence,
Joint Intelligence Organisation
Librarian, Bridges Library
Librarian, Engineering Development Establishment
Defence Science Representative, Australia (Summary Sheets only)
High Commission, London
Counsellor Defence Science, Washington, D.C. (Summary Sheets only)
Librarian, (Through: Officer-in-Charge), Materials Testing
Laboratories, Alexandria, NSW
Senior Librarian, Aeronautical Research Laboratories
Senior Librarian, Defence Research Centre, Salisbury, SA

DEPARTMENT OF DEFENCE SUPPORT

Deputy Secretary, DDS
Head of Staff, British Defence Research & Supply Staff (Aust.)

OTHER FEDERAL AND STATE DEPARTMENTS AND INSTRUMENTALITIES

NASA Canberra Office, Woden, ACT
The Chief Librarian, Central Library, CSIRO
Library, Australian Atomic Energy Commission Research Establishment

MISCELLANEOUS - AUSTRALIA

Librarian, State Library of NSW, Sydney NSW
University of Tasmania, Morris Miller Lib., Hobart, Tas.

DISTRIBUTION LIST
(continued)

MISCELLANEOUS

Library - Exchange Desk, National Bureau of Standards, USA
UK/USA/CAN/NZ ABCA Armies Standardisation Representative (4 copies)
[redacted]
Director, Defence Research Centre, Kuala Lumpur, Malaysia
Exchange Section, British Library, UK
Periodicals Recording Section, Science Reference Library,
British Library, UK
Library, Chemical Abstracts Service
INSPEC: Acquisition Section, Institute of Electrical Engineers, UK
Engineering Societies Library, USA
Director, Chemical Defence Establishment, UK
Director, Chemical Research and Development Center, USA
Aeromedical Library, Brooks Air Force Base, Texas, USA
Ann Germany Documents Librarian, The Centre for Research
Libraries, Chicago Ill.
Defense Attache, Australian Embassy, Bangkok, Thailand
(Att. D. Pender)

END

DATE
FILMED

4 84

DTIC