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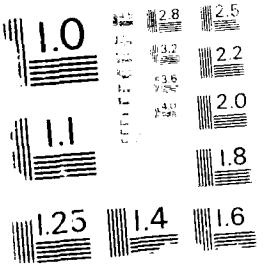
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ON THE TOXICITY AND METABOLISM OF THE TRICHOTHECENE MYCOTOXIN T-2 TOXIN

BY

HELGE JOHNSEN

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The present study deals with toxicological effects and metabolism of the trichothecene mycotoxin T-2 toxin. T-2 toxin was shown to cause a marked decrease in cell production of bone marrow 24 hours after T-2 toxin administration. Furthermore, necrotic injuries in sceletal muscle, heart and probably in the intestine were indicated by an increase in clinically relevant enzymes. The activity of coagulation, fibrinolysis and kallikrein-kinin system was severely depressed after a sublethal dose of T-2 toxin. The maximal effects on the plasma proteases as well as the necrotic injuries were observed 24 hours after administration, corresponding to the time the animals usually die when receiving a lethal dose. The effect of T-2 toxin on plasma protease enzymes

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involved in these systems was suggested to be secondary to cytotoxic effects on the vascular endothelium.

T-2 toxin was rapidly hydrolysed and detoxified by rat liver microsomal fraction into HT-2 toxin as the main metabolite. The enzyme responsible for the hydrolysis of T-2 to HT-2 toxin was identified as a carboxylesterase, and characterized by different substrates for carboxylesterase and specific esterase inhibitors. Rat liver carboxylesterase was separated into five different isoenzymes by isoelectric focusing, and the isoenzyme with pI 5.4 was the only enzyme hydrolysing T-2 toxin to HT-2 toxin. Detoxification of T-2 toxin by hydrolysis was also found to take place in rat and human along two different pathways in the white and red blood cell populations, giving either HT-2 toxin or neosolaniol as primary metabolites, respectively. Neosolaniol formed by the red blood cells represent a metabolic pathway different from that present in liver. The enzymes responsible for hydrolysis of T-2 to HT-2 toxin in white blood cells and T-2 toxin to neosolaniol in red blood cells were identified as carboxylesterases by use of specific inhibitors.

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The thesis is based on the following publications, which will be referred to in the text by Roman numerals:

PAPER I : Johnsen, H., Odden, E., Johnsen, B. A., Bøyum, A. and Amundsen, E. (1988). Cytotoxicity and effects of T-2 toxin on plasma proteins involved in coagulation, fibrinolysis and kallikrein-kinin system. Arch. Toxicol. 61: 237-240.

PAPER II : Johnsen, H., Odden, E., Lie, Ø., Johnsen B. A. and Fonnum, F. (1986). Metabolism of T-2 toxin by rat liver carboxylesterase. Biochem. Pharmacol. 35: 1469-1473.

PAPER III: Johnsen, H., Odden, E., Johnsen, B. A. and Fonnum F. (1988). Metabolism of T-2 toxin by blood cell carboxylesterases. Biochem. Pharmacol. 37: 3193-3197.

ON THE TOXICITY AND METABOLISM OF THE TRICHOTHECENE MYCOTOXIN T-2 TOXIN

SUMMARY

The present study deals with toxicological effects and metabolism of the trichothecene mycotoxin T-2 toxin. T-2 toxin was shown to cause a marked decrease in cell production of bone marrow 24 hours after T-2 toxin administration. Furthermore, necrotic injuries in sceletal muscle, heart and probably in the intestine were indicated by an increase in clinically relevant enzymes. The activity of coagulation, fibrinolysis and kallikrein-kinin system was severely depressed after a sublethal dose of T-2 toxin. The maximal effects on the plasma proteases as well as the necrotic injuries were observed 24 hours after administration, corresponding to the time the animals usually die when receiving a lethal dose. The effect of T-2 toxin on plasma protease enzymes involved in these systems was suggested to be secondary to cytotoxic effects on the vascular endothelium.

T-2 toxin was rapidly hydrolyzed and detoxified by rat liver microsomal fraction into HT-2 toxin as the main metabolite. The enzyme responsible for the hydrolysis of T-2 to HT-2 toxin was identified as a carboxylesterase and characterized by different substrates for carboxylesterase and specific esterase inhibitors. Rat liver carboxylesterase was separated into five different isoenzymes by isoelectric focusing, and the isoenzyme with pI 5.4 was the only enzyme hydrolysing T-2 toxin to HT-2 toxin. Detoxification of T-2 toxin by hydrolysis was also found to take place in rat and human along two different pathways in the white and red blood cell populations, giving either HT-2 toxin or neosolaniol as primary metabolites, respectively. Neosolaniol formed by the red blood cells represent a metabolic pathway different from that present in liver. The enzymes responsible for hydrolysis of T-2 to HT-2 toxin in white blood cells and T-2 toxin to neosolaniol in red blood cells were identified as carboxylesterases by use of specific inhibitors.

1 INTRODUCTION

The trichothecenes are a structurally and chemically closely related group of sesquiterpenoid compounds produced by several species of fungis such as Fusarium, Myrothecium, Trichoderma, Trichothecium, Cephalosporium, Verticimonosporium and Stachybotrys. More than sixty trichothecenes have yet been isolated and characterized. Verrucarins were the first trichothecene compounds found in a search for antibiotics in 1946 (Brian and McGowan, 1946), and they were classified as trichothecenes after the discovery of trichothecin in 1949 (Freeman and Morrison, 1949).

Since Fusarium and the other related fungi infect important foodstuffs, they are associated with human and animal intoxications throughout the world. In 1942-1947, mostly in 1944, more than 10% of the population in Orenburg district of Soviet Union was fatally affected by consumption of moldy grain which overwintered in the fields, with symptoms like vomiting, skin inflammation, diarrhea, leukopenia, multiple hemorrhage, sepsis and exnaustion of bone marrow (Forgacs and Carll, 1962; Joffe, 1965). This serious intoxication was given the name "Alimentary Toxic Aleukia" (ATA) or septic angina. The fungi primarily responsible were identified as Fusarium poae, F.Sporotrichioides and F.tricintum (Joffe,1965), and they were shown to produce trichothecenes such as T-2 toxin, HT-2 toxin and neosolaniol (Ueno et al.,1972). Mirocha and Pathre (1973) identified T-2 toxin in a sample of the toxic extract obtained from V.I.Bilai in Soviet Union. It is, therefore, generally accepted that trichothecene mycotoxins were the toxic factor in the ATA outbreaks in Soviet Union. Human and domestic mycotoxicosis in the last century in which trichothecenes are the suspected causative agents are listed in table 1.

"TAUMELGETREIDE" TOXICOSIS (STAGGERING GRAINS)	SIBERIA	1890 -
ALIMENTARY TOXIC ALEUKIA (ATA)	USSR, EUROPE	1940 - 1960
STACHYBOTRYOTOXICOSIS	USSR, HUNGARY FINLAND, FRANCE, ETC	1931 -
RED-MOLD TOXICOSIS	JAPAN	1900 -
BEAN-HULL POISONING	JAPAN	1937 -
DENDRO-CHIOTOXICOSIS	USSR, EUROPE	1937 -
MOLDY CORN TOXICOSIS	USA	1962 - 1965
"YELLOW RAIN"?	LAOS, KAMPUCHEA, AFHGANISTAN	1976 - ?

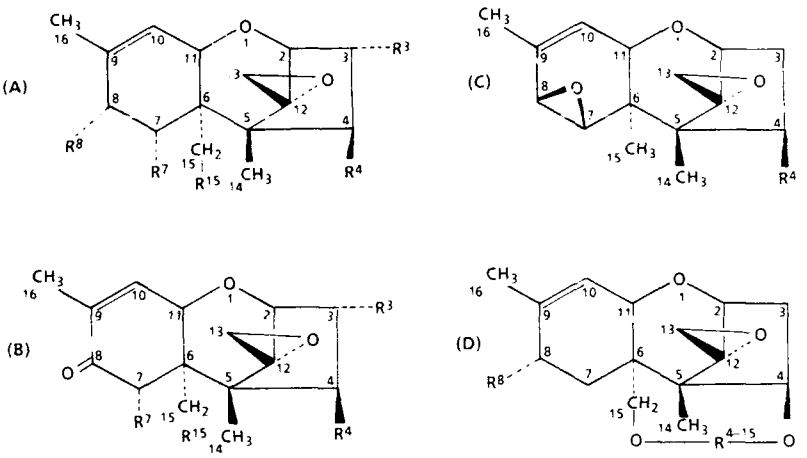
Table 1 *Trichothecene mycotoxicosis affecting human and domestic animals*

In the last ten years trichothecene mycotoxins have attracted international attention because of their alleged use as the chemical warfare agent "Yellow Rain". The term "Yellow Rain" describes a sticky, yellow powder released from munitions used against the Hmong tribesmen in Laos and Kampuchea (Seagrave, 1981). The term "Yellow Rain" is rather artificial since samples after alleged attack had all types of colour and only a few were, in fact, yellow. Some of the reports to U.S. Goverment described symptoms in human that could not be correlated with those produced by any known traditional chemical warfare agents. Symptoms reported included vomiting, diarrhea, hemorrhage, breathing difficulty, itching and skin irritation, nausea, blurred vision, headache, fatigue, dizziness, vertigo and death (Mirocha et al., 1983). These symptoms best fitted those caused by the trichothecenes. A few samples of leaves, water, soil and yellow powder as well as blood, urine and body tissues were collected in areas of alleged attacks and analysed at different laboratories (Mirocha et al., 1983; Rosen and Rosen, 1982; Watson et al., 1984). The samples were found to contain various combinations of T-2 toxin,

diacetoxyscirpenol, nivalenol and zearalenone in small amounts. Although these compounds were not found in control samples from the same regions, it could not be excluded that the positive verification was due to natural occurence of the compounds in these areas. Furthermore, the "Yellow Rain" powder collected at sites of alleged chemical attacks in Laos was found to contain a high percentage of pollen (Nowicke and Meselson, 1984; Own laboratory (not published)). Analysis of this pollen suggested that they were the faeces of honey bees. In conclusion, the question whether trichothecenes have been used as chemical warfare agents still seems to remain open.

1.1 Structure of trichothecenes

The trichothecene compounds comprise a group of closely related sesquiterpenoids and contain a ring system named trichothecane (Gotfredsen et al., 1967). The naturally occuring compounds possess a double bond at C-9,C-10 and an epoxy group at C-12,C-13. Thus, they are characterized as 12,13-epoxytrichothecenes. The structural features of the compounds lent themselves to be categorized in four groups as designed by Ueno (1977a). Class A comprises the hydroxy and acyloxy substituents (T-2 toxin etc), class B the C-8-keto derivatives (nivalenol etc), class C the C-7,C-8-epoxy derivatives (crotocin) and class D the macrocyclic compounds, as summarized in figure 1 (Ueno et al., 1973b). In general, the type A, substituted at C-8 and D trichothecenes are higher in cytotoxicity than the type B and C, indicating the importance of C-8 substitution and macrocyclic ring structure (Ueno, 1977a) (figure 1). The presence of C-12,C-13 epoxide is necessary for biological activity, and reduction of C9,C10 double bond decreased toxicity by 75-80% from the parent compound (Bamburg and Strong, 1971).



	NAME	LD_{50} (MICE) (mg.kg i.p.)	R^3	R^4	R^{15}	R^7	R^8
(A)	T-2 TOXIN	5.2	OH	OAc	OAc	H	$OCOCH_2CH(CH_3)_2$
	HT-2 TOXIN	9.0	OH	OH	OAc	H	$OCOCH_2CH(CH_3)_2$
	NEOSOLANIOL	14.5	OH	Ac	OAc	H	OH
	4-DEACETYL-NEOSOLANOL	-	OH	OH	OAc	H	OH
	DIACETOXY-SCIRPENOL	23	OH	OAc	OAc	H	H
	T-2 TETRAOL	34[1]	OH	OH	OH	H	OH
(B)	DEOXYNIVALENOL	70	OH	H	OH	OH	-
	FUSARENON-X	3.3	OH	OAc	OH	OH	-
	TRICHOTHECIN	300[2]	H	$OCOCH=CHCH_3$	H	H	-
(C)	CROTOCIN	700	-	$OCOCH=CHCH_3$	-	-	-
	CROTOCOL	-	-	OH	-	-	-
				R^{4-15}			R^8
(D)	VERRUCARIN A	0.5[2]	$COCH(OH)CH(CH_3)CH_2CH_2OC(CHOH)CH=CHCH=CHOC$				H
	RORIDIN A	1.5	$COCH(OH)CH(CH_3)CH_2CH_2OCOCH=CHCH=CHOC$				H

Figure 1 The structures of the four different classes of naturally-occurring trichothecenes

1) Chicken, I.V.

2) I.V.

1.2 Biochemical mechanisms of action

The mycotoxicosis described above are characterized by common symptoms as summarized in table 2 (Ueno, 1977b). Toxicological studies with trichothecenes could demonstrate these symptoms in experimental animals such as mice, rats, guinea pigs, rabbits, cats, dogs and avians (Ueno et al., 1971), although toxicological features vary depending on species and toxins (Ueno, 1977a).

VOMITION, EMESIS, TACHYCARDIA, DIARRHEA
HEMORRHAGE, EDEMA, NECROSIS OF SKIN TISSUES
HEMORRHAGE IN MUCOSAL EPITHELIA OF STOMACH AND INTESTINE
DESTRUCTION OF HEMATOPOIETIC TISSUES
IMMUNO SUPPRESSION
DECREASE OF CIRCULATING WHITE BLOOD CELLS AND PLATELETES
MENINGEAL HEMORRHAGE IN BRAIN
NERVOUS DISORDER
REFUSAL OF FEED

Table 2 Toxicological characteristics of trichothecene mycotoxins

All the trichothecene mycotoxins are proved to induce skin necrotization (Ueno et al., 1970b; Chung et al., 1974). The potent cytotoxicity to eukaryotic cells possessed by these toxins are regarded as closely related to their lethal toxicity, dermal toxicity, impairment of immunoresponses and inhibition of macromolecular synthesis (Ueno, 1977b).

1.3 Protein synthesis

The inhibitory effect of trichothecenes on protein synthesis was first demonstrated in rabbit reticulocytes (Ueno et

al., 1968b), and Ehrlich ascites tumor cells (Ueno and Fukushima, 1968a). Since then, this inhibitory effect has been demonstrated in whole animal (Ueno, 1970a; Rosenstein and Lafarge-Frayssinet, 1983), HeLa cells (Liao et al., 1976) and rat liver preparation (Ueno et al., 1973a). The trichothecenes exhibited neither supression of bacterial growth nor inhibition of protein synthesis in microbes, and it was concluded that the trichothecenes are specific inhibitors of protein synthesis in eucaryotic cells (Ueno et al.1973a).

Considerable interest in the trichothecenes as protein synthesis inhibitors was stimulated by the demonstration that trichodermin preferentially inhibited the termination step of protein synthesis as an unique site of action (Stafford and McLaughlin, 1973; Wei and McLaughlin, 1974). A great number of trichothecenes have been tested for inhibition, and the mechanism of this inhibition and the structurefunction relationships have been reviewed (McLaughlin et al., 1977; Carter and Cannon, 1977; Carter and Cannon, 1978). One early evidence was breakdown of polyribosomal profile at the cellular level and different mode of action of the trichothecenes on the polyribosomal behaviour (Wei and McLaughlin, 1974; Ueno et al., 1973a). These accumulated data and consideration of the structure-function relationship led to a hypothesis that the trichothecenes could be subgrouped into two types of inhibitors: initiation site inhibitors (T-2 toxin, diacetoxyscirpenol etc.) and elongation-termination site inhibitors (trichodermin, verrucarol etc.) (Ueno, 1980; McLaughlin et al., 1977).

On the basis of current knowledge, the trichothecenes include the most potent small molecule inhibitors of protein synthesis in eucaryotic cells, and it appears likely that their basic properties as mycotoxins are directly related to this effect (McLaughlin et al., 1977).

1.4 DNA synthesis

It is well established that trichothecene mycotoxins block DNA synthesis in Ehrlich ascites tumor cells (Ueno and Fukushima, 1968a) and HeLa cells (Ohtsubo et al., 1968) without affecting the synthesis of RNA. Other authors have, however, reported partial inhibition of RNA at concentrations of trichodermin completely inhibiting the protein synthesis, a pattern typical for protein synthesis inhibitors (McLaughlin et al., 1977). Since the trichothecenes demonstrated no inhibitory effects on DNA polymerase, thymidine kinase and thymidinylate kinase in vitro, the inhibition of DNA synthesis is probably secondary inhibited by impairment of protein synthesis (Ueno, 1980). Other findings indicate, on the other hand, that impairment of DNA synthesis is not secondary to protein synthesis inhibition, but rather that some damage of cell organization may affect the nucleic acid synthesis (Ueno and Yamakawa, 1970c). Chaetoglobosin A, which is known as a potent inhibitor of membrane function through binding to tubulin, caused a similar inhibitory effect to macromolecule synthesis in protozoan cells as the trichothecenes (Iwakashi et al., 1982).

1.5 Objects of the investigation

The present investigation was carried out to study the cytotoxicity of T-2 toxin in mice and, in particular, relating this to the effects of T-2 toxin on plasma protease enzymes involved in coagulation, fibrinolysis and kallikrein-kinin system (Paper I).

A second aim of this study was to identify and characterize the enzyme responsible for metabolic hydrolysis and detoxification of T-2 toxin (Paper II, III). Previous studies on trichothecene metabolism have shown metabolism in liver preparations. No metabolic activity have ever been detected in blood. First therefore I characterized the

isoenzyme responsible for the liver metabolic activity (Paper II). Since blood cells are known to contain different forms of esterases, indicating that trichothecenes to some extent may be hydrolyzed by these cells, the present study (Paper III) therefore, characterized the metabolic pathway for T-2 toxin in blood cells and characterized the enzymes involved.

2 DISCUSSION OF METHODS

This discussion of methods will be concentrated on the chemical analysis of T-2 toxin and its metabolites. Methods for detection and quantification of trichothecenes implies; a) biological assays based on their cytotoxicity (Ueno, 1980); b) immunochemical methods such as radio-immuno assay (RIA) (Chu et al., 1979), and enzyme-linked immunosorbant assay (ELISA) (Pestka et al., 1981); c) physicochemical methods such as thin layer chromatography (TLC) (Stahr et al., 1979), high-performance liquid chromatography (HPLC) (Schmidt et al., 1981) and gas chromatography (GC) (Ikediobi et al., 1971), with or without combination to mass spectrometer (MS).

The analytical method used in this study is described in detail in paper II. The whole procedure can be divided into:

a): extraction and clean-up
b): derivatisation
c): GC-MS (detection and quantification).

2.1 Extraction and clean-up

Gas chromatographic analysis requires that the sample components (solvent and solutes) are evaporized at the inlet of the separating column. It is, therefore, necessary to extract the polar trichothecenes from the biological sample into an organic solvent prior to the gas chromatography.

The efficient recovery of a range of trichothecenes and their hydrolysis products is complicated by the considerable variation in physicochemical properties such as polarity. The metabolites of T-2 toxin, 4-deacetyl neosolaniol and T-2 tetraol is much more polar than T-2 toxin itself and HT-2 toxin. The first methods available on extracting trichothecene mycotoxins from biological samples were based on liquid/liquid partition with organic solvent such as chloroform and ethylacetate (Ohta et al., 1977; Ellison and Kotsonis, 1974). These methods did, however, not allow extraction of the very polar 4-deacetyl neosolaniol and T-2 tetraol.

The method used in this study utilize extraction by adsorption to Amberlite XAD-2 resin, which provides simultanously extraction and clean-up. Trichothecene metabolites were eluted from the resin by 90% methanol in water or by acetone which is more easily evaporated in order to concentrate the samples. The recoveries obtained for T-2 toxin and HT-2 toxin were about 90% whereas the polar metabolites 4-deacetyl neosolaniol and T-2 tetraol were recovered by only 30-40% (Paper II). The recoveries of these polar metabolites were, however, regarded as sufficient for the detection and quantification.

The Amberlite XAD-2 extraction of trichothecenes is a convenient method frequently used the last ten years for isolation of trichothecenes with a wide range in polarity (Yoshizawa et al., 1980a; Yoshizawa et al., 1980b; Yoshizawa et al., 1984). Other chromatographic methods often used for trichothecene clean-up are reversed phase C_{18} Sep-Pak silica cartridges and Fluorisil column chromatography. The C_{18} reversed phase method is very well fitted to small sample volumes (Swanson et al., 1982) and is perhaps the most frequent method used today. Equipments for automatic sample preparations with C_{18} are available from Analytichem International (Vac-Elute) and Waters (Sep-Pak). These systems are based on vacuum, and are

designed to prepare several samples simultaneously in a short time, making these systems very convenient in use. By combination of these methods, XAD-2, C_{18} reversed phase and Fluorisil, very specific clean-up procedures for trichothecenes with widely differing polarity may be designed with application both to biological and organic samples.

2.2 Derivatisation

The trichothecene compounds are too polar for direct application onto a gas chromatograph (GC). Since all the trichothecenes of interest in this study contain one or more hydroxy groups, they can be derivatized with different trimethyl-silyl (TMS) reagents, thus making the trichothecenes sufficiently nonpolar and volatile for gas chromatographic analysis. There is a number of different TMS reagents available with different affinity for the compounds to be derivatized. An alternative to silylation is acetylation of the trichothecenes with different trifluoroacetylation reagents. In this study silylation of the trichothecenes was chosen because it is more convenient in use as compared to acetylation. Derivatisation of trichothecene mycotoxins has been studied in detail by Kientz and Verweij, (1986) and Kamimura et al., (1981). The reagent used in this study (Sylon BTZ) is a mixture containing three different TMS compounds: N,O-bis(trimethylsilyl,acetamid (BSA), trimethylchlorosilane (TMCS) and trimethylsilylimidazole (TSIM) in the ratio 3:2:3. Sylon BTZ is characterized by the manufacturer to be one of the most potent silylating agents available and the mixture will derivatize all hydroxyl groups in any position (SUPELCO Inc). Sylon BTZ was found to give a complete derivatisation of the trichothecenes involved within a short time (5 min).

2.3 Gas chromatography mass spectrometry (GC-MS)

Gas chromatographic separation is, by far, the most frequently used analytical method for the assay of trichothe-

cenes. Separation of the trichothecenes can be performed either by packed column, such as 3% OV17 or by capillary column like SE-54 or CP-sil 8CB. Packed column was found to be satisfactory for the analytical purposes in the present study with respect to separation and sensitivity for the trichothecenes involved, and was chosen for its convenience, high stability and short running time for each sample injection. Capillary column is, however, recommended for broad range analysis of trichothecenes with great differences in polarity where high requirements to sensitivity and separation is crucial as it would be for trace analysis.

Methods of detection consist of flame ionisation (FID), electron capture (ECD) of fluoro-derivatized trichothecenes and mass spectrometric analysis (MS). Trichothecenes do, however, not respond to nitrogen-phosphor detector (NPD), which is otherwise a sensitive and specific detector for nitrogen/phospor containing compounds. The method used in the present study was gas chromatography combined with mass spectrometer, which is regarded as the most sensitive, specific and versatile method (Scott, 1982). For quantitative analysis the mass spectrometer was operated in a mode of multiple ion detection (MID) in which the instrument is focused on single fragment ions, such as the molecular ion (M^+), specific for the compound to be detected. The mass spectrometer used in this study allow simultaneously monitoring of six different fragment ions. The ionization energy was lowered from 70 eV used to obtain mass spectra to 12 or 14 eV for the purpose to reduce fragmentation of the trichothecenes and thereby achieving higher intensity of fragments with high masses such as M^+, which is more specific and identifying.

3 GENERAL DISCUSSION

3.1 Toxicity

Cytotoxicity of the trichothecenes has been extensively studied and all trichothecene compounds have been shown to induce skin necrosis (Ueno et al., 1970b, Chung et al., 1974). Several different trichothecenes have been tested for cytotoxic activity against cultured cell lines and indeed this class of compounds contains some of the most potent cytostatic agents known. Verrucarin A has an ID_{50} for growth of cultured mouse P-815 cells of 0.6 ng/ml (Harri et al., 1962). Dividing cells were much more affected by the compound than were non-dividing cells and mitosis was completely disrupted (Rusch and Stahelin, 1965). Studies on feeding of animals over a period of days to weeks with diet containing low ppm levels of T-2 toxin have shown morphological evidence of hypoplasia and necrosis of bone marrow (Hayes et al., 1980; Friend et al., 1983). Other authors made the same morphological observations in guinea-pig post mortem after receiving one lethal dose of T-2 toxin P.O. (DeNicola et al., 1978). Furthermore, T-2 toxin is known to cause hematological disorders as leukopenia, anemia and decreased blood coagulation activity as measured by clotting time assays (Ueno, 1977b; Kosuri et al., 1971; Smalley, 1973; Doerr et al., 1974; Gentry and Cooper, 1981; 1983).

The mechanism of how T-2 toxin affect the coagulation system has not yet been established. Pretreatment of animals with vitamin K_1 has no effect, indicating that T-2 toxin does not act as a vitamin K_1 antagonist (Gentry, 1982; Gentry and Cooper, 1983; Cosgriff et al., 1984). Trichothecenes have been shown to be very potent inhibitors of protein synthesis *in vitro*, and T-2 toxin may therefore act by depression of the protein synthesis involved in the coagulation system, as proposed by Cosgriff et al. (1984).

In that case the decrease of clotting proteins should depend on their half lives, which varies from a few hours to 2-3 days for the proteins involved (Austen and Rhymes, 1975). Any decline of these proteins caused by reduced protein synthesis should, therefore, occur in accordance to their half-lives, and therefore with different time courses. Such a correlation between the response of T-2 toxin on clotting proteins and their circulating half-lives was not found in a study of intravenous administration of T-2 toxin to rabbits (Gentry, 1982) and calves (Gentry and Cooper, 1983). These authors proposed that the observed uniformity of response of the various clotting proteins with different half-lives indicates that T-2 toxin cannot function merely as an inhibitor of protein synthesis. The authors proposed that hepatotoxicity may be responsible for some of the effects on coagulation parameters, but they did not exclude the possibility of other mechanisms to be involved. The present study (paper I) also reveals a high degree of uniformity in the time course of the effect on the different plasma protease enzymes involved not only in the coagulation system, but also in the fibrinolytic and kallikrein-kinin systems, with an early onset of the effects (1-4 hours). In this report (Paper I) all effects concerning both cytotoxicity and the different proteolytic enzyme systems reach their maximum 20-24 hours after administration, in accordance to previous studies (Gentry, 1982; Gentry and Cooper, 1983; Cosgriff et al., 1984). This time correspond to the time animals usually die following a lethal dose of T-2 toxin.

The effects of T-2 toxin on plasma protease enzyme systems is very similar to those of bacterial endotoxins causing septicemia with or without complicated shock. Both are associated with marked changes in the plasma kallikrein-kinin, coagulation and fibrinolytic systems, as well as reduction in total plasma protein content (Smith-Erichsen, 1985, Paper I). Also, significant reduction in antithrombin III, which is the main inhibitor of the coagulation

system (Abildgaard, 1979) was found both in T-2 toxicosis and septicemia, (Paper I; Smith-Erichsen et al., 1982), and probably reflect a consumption of this inhibitor subsequent to activation of the coagulation system. Furthermore, reduction in the Hageman factor (Factor XII) have been shown together with increasing amounts of fibrinogen for both T-2 toxicosis and bacterial endotoxicosis (Smith-Erichsen et al., 1982; Gentry, 1982; Cosgriff et al., 1984).

Disseminated intravascular coagulation (DIC) is a syndrome which is often encountered in septic shock, particularly in Gram negative sepsis. This syndrome is characterized by activation of the coagulation, fibrinolytic and plasma kallikrein-kinin systems (Gallimore et al., 1980). Recent research has revealed that these systems are all intimately linked, with Hageman factor (XII) playing a central role (Kaplan et al., 1976). There appear to be a number of conditions that may initiate this reaction, such as release of tissue thromboplastic materials into the blood stream, bacterial endotoxins, activation of certain proteolytic enzyme systems and endothelial damage (Langdell, 1974).

Several mechanisms whereby endotoxin triggers dessiminated intravascular coagulation (DIC) have been suggested. Activation of the intrinsic pathway may occur directly by activation of Hageman factor (Factor XII) (Colman et al., 1972; Morrison and Cochrane, 1974) or indirectly via endothelial damage and then subsequent activation of Hageman factor (XII) (Wilner et al., 1968). The extrinsic pathway may be activated by the release of clot-promoting substances from platelets and other cellular elements of blood (Evensen and Jeremic, 1970) or release of tissue components (Garner and Evensen, 1974).

Hageman factor (XII) has been shown to decline during sepsis in man (Smith-Erichsen et al., 1982) and animals (Gallimore et al., 1978) as well as in T-2 toxicosis (Cosgriff et al., 1984). According to the good correlation

with the cytotoxic effects manifested by alteration in aspartate aminotransferase (ASAT) alanine aminotransferase (ALAT) and creatin phosphokinase (CK) as described in paper I, it is suggested that the effects of T-2 toxin on plasma protease enzymes involved in coagulation, fibrinolytic and kallikrein-kinin systems is secondary to cytotoxic effects in some organs, and not due to reduced protein synthesis. T-2 toxin does not, however, act by cytotoxic effects on liver (Paper I), and vascular endothelial damage is therefore the most likely candidate for activating the plasma protease systems.

3.2 Metabolism

Tritiated T-2 toxin orally administrated to mice and rats was rapidly eliminated into faeces and urine with a ratio of 4-5:1 without specific accumulation in any organ (Matsumoto et al., 1978). About 50% of the total dose administrated was found in faeces and 10 % in the urine during the first 24 hours, partly as T-2 toxin and partly as polar metabolites. Chickens intubated with a single dose of tritiated T-2 toxin excreted 61 % and 82 % of the recovered radioactivity at 24 and 48 hours, respectively (Chi et al., 1978). At 48 hours the bile and gall bladder contained by far the highest specific activity. The patterns of distribution and excretion found in this study suggest that the liver plays a major role in the metabolism and that T-2 toxin and/or its metabolites are excreted into the faeces via the bile. In vivo metabolic studies have been performed for T-2 toxin administrated to chickens (Yoshizawa et al., 1980b) and a lactating cow (Yoshizawa et al., 1981). These studies have shown that T-2 toxin is rapidly transformed into more polar metabolites which is then eliminated in the excreta.

In vitro studies using hepatic homogenates or subcellular fractions have shown that T-2 toxin is rapidly metabolized by ester hydrolysis to HT-2 toxin as the main metabolite

which is further hydrolyzed to 4-deacetyl neosolaniol and T-2 tetraol (Paper II, Ohta et al., 1977; Ellison and Kotsonis, 1974; Yoshizawa et al., 1980a). Subsequent studies have identified two oxidation products, (3'-OH T-2 toxin and 3'-OH HT-2 toxin) hydroxylated in the isovaleryl group and presumably produced by the mixed-function oxidase system. These metabolites were identified in liver homogenates from mice and monkeys (Yoshizawa et al., 1984) and from pigs and rats (Wei and Chu, 1985; Knupp et al., 1986) when these homogenates were supplemented with a reduced NADPH generating system.

Several in vivo studies have confirmed that ester hydrolysis and hydroxylation of the isovaleryl group are the two major pathways involved in metabolism of T-2 toxin (Paper II, Yoshizawa et al., 1980b; Yoshizawa et al., 1981; Corley et al., 1985; Corley et al., 1986; Visconti and Mirocha, 1985). Other mechanisms of trichothecene metabolism including deepoxidation (Yoshizawa et al., 1985) and glucuronid conjugation (Corley et al., 1985; Gareis et al., 1986) have also been recently described. The pathways for metabolism of T-2 toxin are shown in figure 2.

3.3 Carboxylesterase

The importance of carboxylesterase for detoxification of T-2 toxin was shown by the enhanced toxicity of T-2 toxin in mice pretreated with tri-orto-crecyl phosphate (TOCP) (Paper II; Fonnum et al., 1985). TOCP has shown to be a specific inhibitor of carboxylesterase activity (Mendel and Myers, 1953; Sterri et al., 1981). Carboxylesterase (E.C.3.1.1.1.) is a heterogeneous group of enzymes, also called serine hydrolases because of a serine in active site of the enzyme. It is reasonably to assume that all proteins classified as carboxylesterases also are potensial amidases (Heymann, 1980). Histochemical investigations have demonstrated carboxylesterase activity in almost all mammalian tissue with by far the highest activity in liver

(Von Deimling and Boecking, 1976; Junge and Krisch, 1975). Biochemically carboxylesterases in multiple forms with different characteristics have been detected in many tissues (For review: Heymann, 1980; Junge and Krisch, 1975; Krisch, 1971).

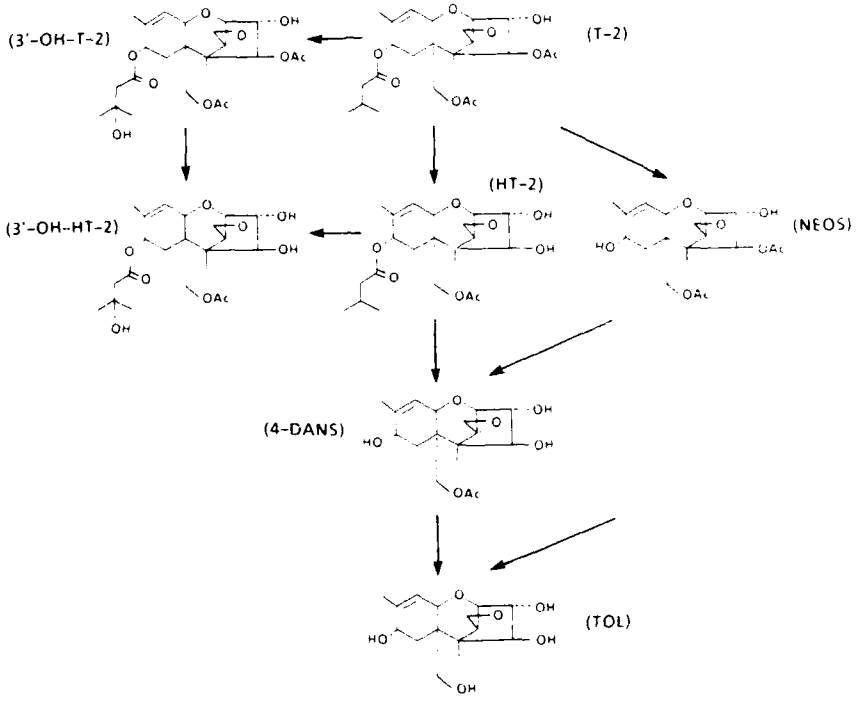


Figure 2 Proposed pathways for metabolism of T-2 toxin. 4-DANS, 4-deacetyl neosolaniol; NEOS, neosolaniol; TOL, T-2 tetraol.

Carboxylesterase as a group is characterized by a broad substrate specificty for aliphatic and aromatic esters as well as for aromatic amides. They have been shown to hydrolyse ester drugs like procain, acetylsalicylic acid, pethidin, steroid hormon esters and amide drugs like pheacetin, lidocaine and chloramphenicol (For review: Junge and Krisch, 1975).

Electrophoretically at least twenty different serine hydrolases have been demonstrated in liver both with an active-site-directed radioactive organophosphate and by esterase staining (Heymann et al., 1979; Heymann and Schwabe, 1977). The most prominent of these are the five isoenzymes termed by their isoelectric point; pI 6.4, 6.2, 6.0, 5.6, 5.2. They do all appear with same subunit weight (60000). The isoenzymes pI 6.4, 6.2, 5.6 and 5.2 behave as monomers, whereas pI 6.0 appear as a trimer with molecular weight about 180000. Enzyme pI 6.0 has by far the highest activity towards short aliphatic esters such as methylbutyrate, and represents the formerly so called "aliesterase" (3.1.1.1.) (Heymann, 1980; Mentlein et al., 1980; Paper II). On the other hand, phenol esters such as 4-nitrophenylacetate and 4-nitrophenylbutyrate are preferably cleaved by pI 6.2 and 6.4. The amide type substrates and drugs are preferably hydrolyzed by the isoenzymes pI 5.6 and pI 6.0 (Mentlein et al., 1980; Mentlein and Heymann, 1984). Isoenzyme pI 6.0 has the highest specific activity for butanilicaine, whereas acetanilid is a relatively specific substrate for the esterase pI 5.6. All substrates used to specify the different isoenzymes are, however, of a relatively simple molecular structure and they do not show absolute specificity among the isoenzymes. The present study (Paper II and III) report on the hydrolysis of the trichothecene mycotoxin T-2 toxin having a very complex and rigid molecular structure (Figure 1). In liver T-2 toxin was shown to be hydrolyzed at the acetyl residue in C-4 position only by the isoenzyme pI 5.4, corresponding to pI 5.6 of the Mentlein, Heymann nomenclature used in this con-

text. No activity was seen by the other isoenzymes. It is reasonably to assume that the complex nature of this toxin makes high requirements for fitting the active site the hydrolysing enzyme, and thereby exhibit a very high degree of substrate specificity. This makes T-2 toxin a suitably substrate for measuring this specific isoenzyme in crude fractions. Ohta et al. have studied the hydrolysis of a number of different trichothecene derivatives by crude liver microsomal fractions to survey the substrate specificity of the enzyme involved (Ohta et al., 1978). It appears that the enzyme attacks the C-4 acetyl residue of the trichothecenes, and that the substituents at C-3 and C-8 play an important role in the enzymatic hydrolysis of the C-4 acetyl residue. To make any conclusive statements regarding substrate specificity of the enzyme involved in this reaction, a study has to be done on the purified and isolated enzyme. Although T-2 toxin was hydrolyzed by isoenzyme pI 5.6, it remains to be shown that this specific enzyme in general is responsible for the hydrolysis for the various trichothecene analogs. It can not be excluded that isoenzymes other than pI 5.6 may be involved in hydrolysis of trichothecenes other than T-2 toxin. In blood cells it was indeed shown that T-2 toxin is hydrolyzed by carboxylesterases along two different pathways in red and white blood cells, respectively (Paper III). In the red blood cells T-2 toxin was hydrolyzed at the isovaleryl group in C-8 position to yield neosolaniol, whereas in white blood cells T-2 toxin was hydrolyzed at C-4 acetyl residue giving HT-2 toxin. These results strongly indicates that different carboxylesterase isoenzymes are involved in trichothecene hydrolysis, at least in blood cells.

A common feature of the carboxylesterase isoenzymes hydrolysing T-2 toxin in liver and blood cells is their low sensitivity towards the esterase inhibitor bis-4-nitrophenyl phosphate. This compound is reported to be a specific carboxylesterase inhibitor (Heymann and Krisch, 1967;

Block and Arndt, 1978). In a study on selective inhibition of rat liver carboxylesterase isoenzymes by various organophosphorous diesters, bis-4-nitrophenyl phosphate (10^{-4}M) was shown to have strong inhibitory effect on isoenzyme pI 5.6 (90-95 % inhibition) as measured by acetanilid, methyl butyrate and 4-nitrophenyl acetate (Brandt et al., 1980). On the other hand, this study did show that T-2 hydrolysis was inhibited by 56 % in liver (Paper II) and 44 % in the white blood cells at 10^{-4}M bis-4-nitrophenyl phosphate, whereas a concentration of 10^{-3}M was necessary to inhibit T-2 hydrolysis in red blood cells by 44 % (Paper III). As was pointed out in paper II (fig 3) there was a slight difference between peak activities of 4-nitrophenyl phosphate and methyl (1-C^{14})butyrate and peak activity for T-2 hydrolysis. This indicates that peak pI 5.6 contains more than one carboxylesterase isoenzyme which have different substrate specificity and different affinity to bis-4-nitrophenyl phosphate. The activity of pI 5.6 towards 4-nitrophenyl butyrate, 4-nitrophenyl acetate, methyl (1-C^{14})butyrate and T-2 toxin was, however, inhibited to the same extent by $5X10^{-6}$M soman (PaperII). Microheterogenity has, indeed, been reported for the purified isoenzyme pI 5.6 (Mentlein et al., 1980). They could demonstrate by 1-naphtyl acetate staining three different bands by analytical gel electrophoresis and five bands by analytical isoelectric focusing.

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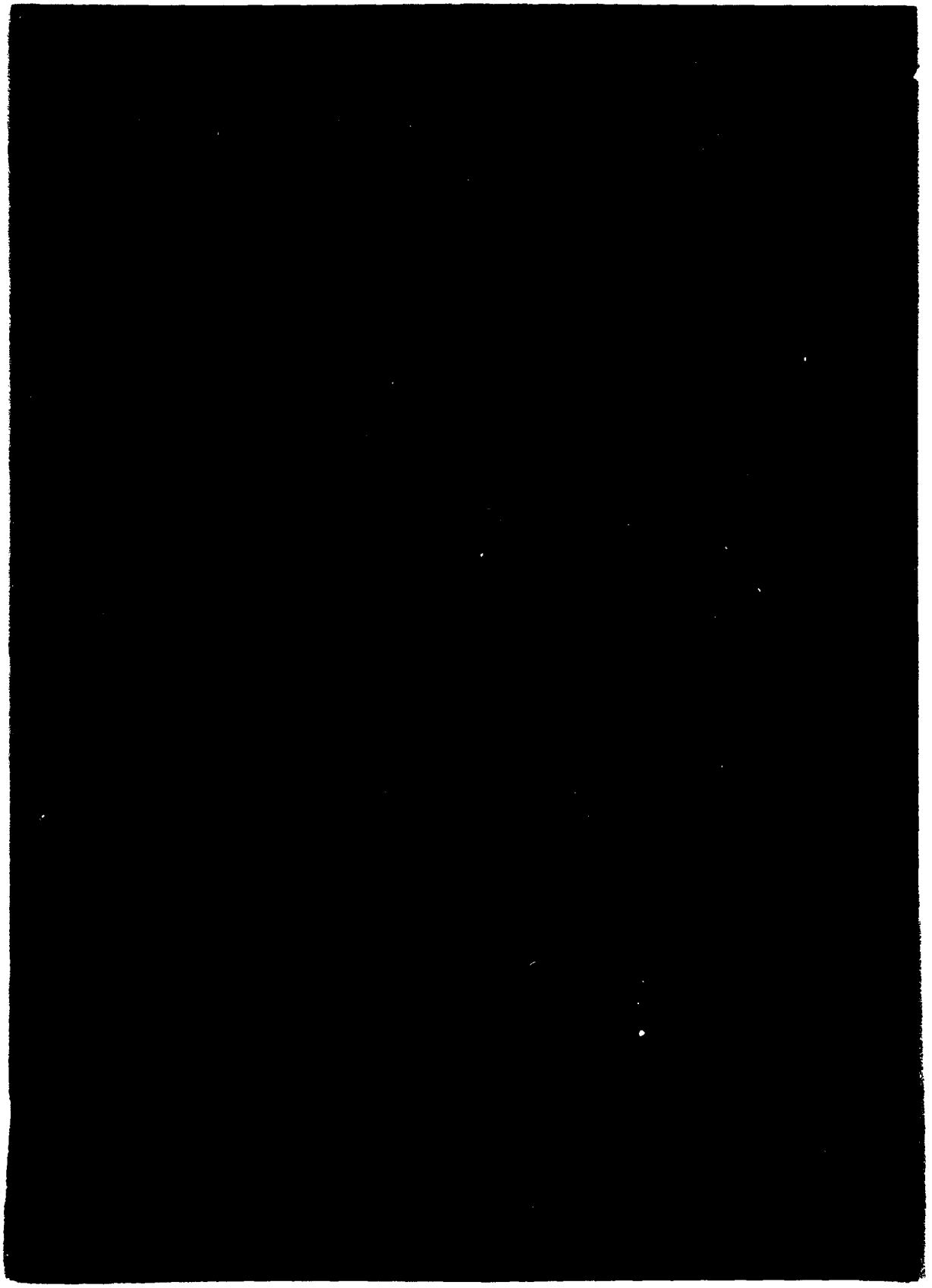

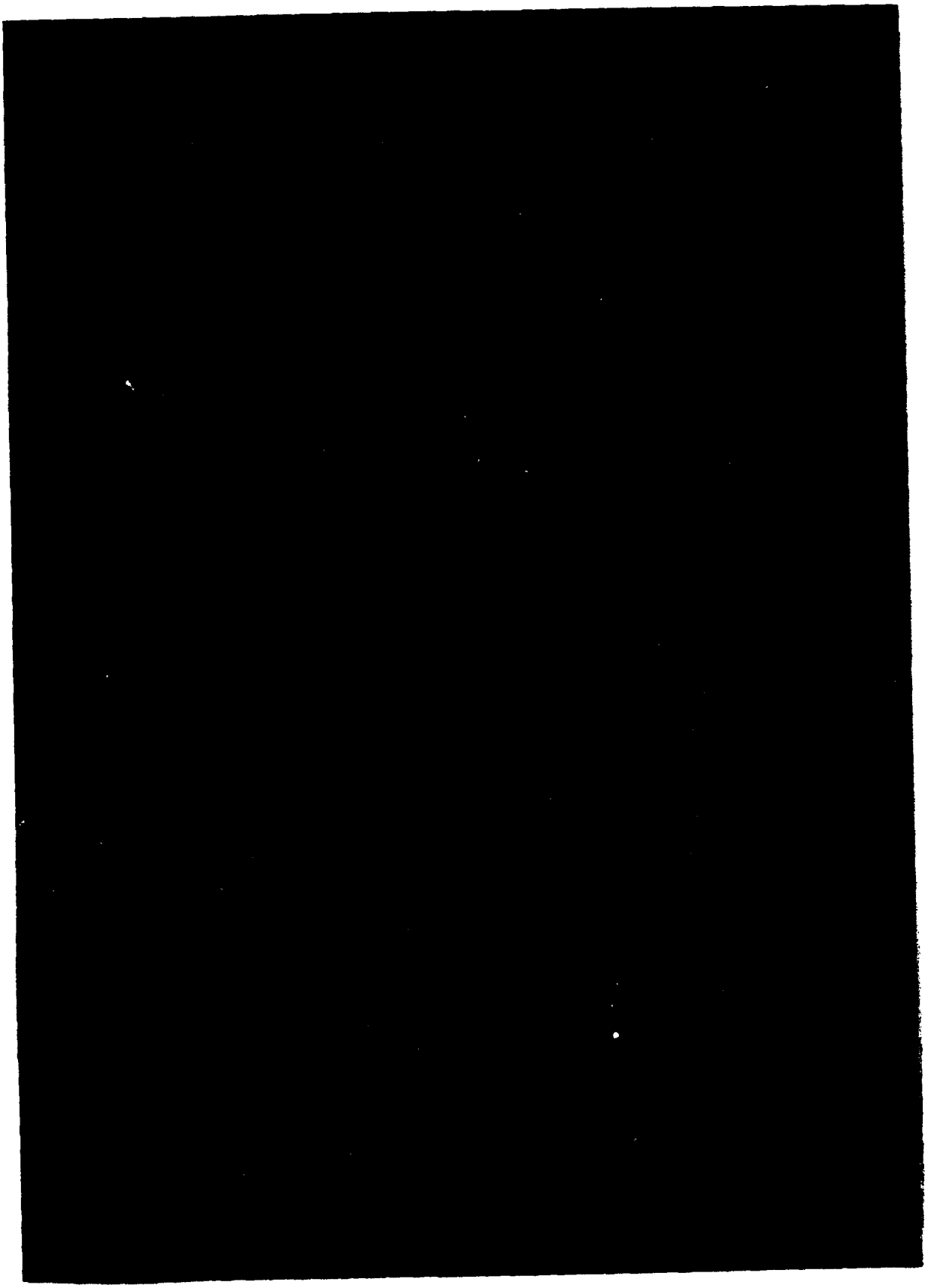

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Cytotoxicity and effects of T2-toxin on plasma proteines involved in coagulation, fibrinolysis and kallikrein-kinin system

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Abstract. The activity of both the coagulation and fibrinolytic systems was markedly depressed 24 h after a sublethal dose of T-2 toxin. T-2 toxin was active as an anticoagulant at low doses, which did not affect the basal state of the animals. The kallikrein-kinin system was also affected by depletion of the prekallikrein, which indicates increased bradykinin levels in plasma. At the same time there was an increased activity of some clinically relevant enzymes in serum, indicating tissue injuries caused by T-2 toxin. All effects observed in this study reached their maximum within 24 h after administration, which corresponds to the time animals usually die when receiving a lethal dose. T-2 toxin does not, however, seem to affect the protease enzymes by reduced protein synthesis, because of early onset of the effects, nor does it act as a trigger itself. The effect of T-2 toxin on plasma protease enzymes is probably secondary to cytotoxic effects in the vascular endothelium.

Key words: *Trichothecene – T-2 toxin* – Cytotoxicity – Coagulation – Kallikrein-kinin system – Serum proteases

Introduction

The trichothecene T-2 toxin is a cytotoxic mycotoxin produced by several species of *Fusarium*, growing in cereals, feeds and vegetables. The biological properties and practical importance of T-2 toxin have been reviewed by Smalley and Strong (1974) and Ueno (1980). In a previous study, we showed that T-2 toxin is rapidly transformed in liver to HT-2 as the main metabolite by the enzyme carboxylesterase isoenzyme pI 5.4 (Johnsen et al. 1986). It is suggested that some of the effect of T-2 toxin is exerted by HT-2 toxin and that the potencies of T-2 and HT-2 toxin are comparable (Ueno et al. 1973; Ellison and Kotsonis 1974). However, blocking hydrolysis by selective inhibition of the carboxylesterase enhances the toxicity of T-2 toxin (Johnsen et al. 1986), indicating the importance of carboxylesterase for detoxification of trichothecenes.

The most prominent biochemical activities in vitro are inhibition of protein and DNA synthesis (McLaughlin et al. 1977; Ueno 1977a). The cytotoxicity of T-2 toxin has been studied by several authors, and all the trichothecene compounds have been shown to induce skin necrosis (Ueno et al. 1970; Chung et al. 1974). The cellular damage is characterized by karyorrhexis and destruction of the actively dividing cells and it is believed that the trichothecenes interfere with the synthesis of macromolecules (Ueno 1977b).

T-2 toxin is known to cause hematological disorders, including leukopenia, anemia (Ueno 1977) and decreased blood coagulation activity as measured by various clotting time assays (Kosuri et al. 1971; Smalley 1973; Doerr et al. 1974; Gentry and Cooper 1981, 1983). The mechanism of action of T-2 toxin on the coagulation system, however, has not yet been established. Possible mechanisms of action on coagulation may be antagonism of vitamin K_1, reduced protein synthesis, trigging of the system by T-2 toxin itself, or the effect may be secondary to cytotoxic effects on liver or some other organs. Pretreatment of animals with vitamin K_1 has no effect (Gentry 1982; Gentry and Cooper 1983; Cosgriff et al. 1984), indicating that T-2 toxin does not act as a vitamin K_1 antagonist. In the present study we have studied the effect of T-2 toxin on plasma proteolytic enzymes in mice using chromogenic substrate techniques. Prothrombin and its inhibitor antithrombin III were used as markers for effects on coagulation, antiplasmin as a marker for effects on fibrinolysis and prekallikrein as a marker for effects on the kallikrein-kinin system. This report also describes the cytotoxic action of T-2 toxin in vivo on actively dividing cells in bone marrow. Tissue injury was studied by means of clinically relevant enzymes. Alanine aminotransferase (ALAT) and alkaline phosphatase (APHOS), mainly synthezised in liver, were used as markers for cell necrosis in liver. Asparagine aminotransferase (ASAT) together with creatine kinase (CK) were used to detect injuries in skeletal and heart muscle.

Materials and methods

Animals and blood sampling. Femal QRF mice weighing 20–30 g were used in this study. They were maintained at constant room temperature (24° C) with 12 h light cycle. Feed and water were provided ad lib. T-2 toxin (1–4 mg/kg) was injected intravenously in 100 µl saline containing less than 2% ethanol. Previous experiments have established that the LD_{50} in mice is between 3 and 4 mg/kg. Blood samples (1 ml) were taken by puncture of the vena cava in animals anesthetized with diethyl ether, and anticoagulated with 3.8% sodium citrate (5:1 v/v). Plasma was

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obtained by centrifugation at 2000 *g* for 10 min. Serum was prepared from blood without anticoagulant, allowing the blood to coagulate.

In vivo studies. To study the effect of T-2 toxin on cell production in vivo whole femurs were ground up and total cell number determined. Bone marrow cells were obtained from the femurs of mice 24 h after subcutanous injection of T-2 toxin at doses varying from 0.1 to 1.0 mg/kg and cultured in methyl cellulose as described by Gjerde et al. (1983). After 7 days of incubation at 37° C in 7.5% CO_2 the stem cell number was determined by counting granulocyte-macrophage colony forming cells (GM-CFC).

Tissue injury was determined by measuring the activity of alanine aminotransferase (ALAT), asparagine aminotransferase (ASAT), alkaline phosphatase (APHOS) and creatine kinase (CK) in serum spectrophotometrically according to standard methods for these enzymes by means of a Cobas Bio multianalyzer. Coagulation activity was measured by "Normo-Test" (Nycomed A/S) according to the manufacturers' procedure (Owren and Strandli 1969). The method is sensitive to changes in activity of factors II, VII and X. Plasma protease activities, their precursors and inhibitors, including prothrombin, antithrombin III, prekallikrein, antiplasmin and fibrinogen, were studied by chromogenic substrate techniques. These substrates are synthesized tripeptides which are blocked at the N-terminal with benzoyl or tosyl and the indicator *p*-nitroanilin (*p*-NA) is linked to the C-terminal by amide linkage. The amino acid sequence determines the substrate specificity and the released *p*-NA determines the enzyme activity. The concentration of liberated *p*-NA is measured at 405 nm, at which wavelength the whole substrate shows no absorbance. The assays were fully automated by use of a Cobas Bio Multianalyzer (Amundsen et al. 1978; Gallimore et al. 1979). These parameters were chosen because they have been proven to be the most reliable prognostic parameters during sepsis in humans (Smith-Eriksen 1985).

In vitro studies. The effects of T-2 toxin on protease enzyme activity, their precursors and inhibitors were studied by incubation of T-2 toxin (2×10^{-4} M or 2×10^{-3} M) with blood, plasma and filtered plasma at 37° C for 2–24 h. Liver slices were incubated overnight with T-2 toxin in isotonic 50 mM Tris buffer pH 7.5 containing the chromogenic substrate (10^{-4} M) for the protease to be studied. No activity was seen when liver slices were incubated without T-2 toxin.

Results

The effect of T-2 toxin administration on bone marrow cells is shown in Fig. 1. Both total number of cells per femur and the number of stem cells were decreased in a dose-dependent manner 24 h after administration. There was an approximately 50% decrease in number of cells after a dose of 0.5 mg/kg, which is 8–10 times lower than the LD_{50} of T-2 toxin.

Single intravenous injection of T-2 toxin was associated with a marked rise in the activities of CK, ASAT and ALAT, whereas APHOS was unaffected (Fig. 2). The peak activities were seen 24 h after injection, with return to normal values within 2 days. The same observations were made following subcutaneous injection, indicating that the cytotoxic effects of T-2 toxin are not dependent on route of administration, and excluding the possibility that the increased enzyme levels reflect local tissue damage.

Coagulation, as measured by the "Normo-Test" clotting time assay, was markedly reduced 10–24 h after i.v.

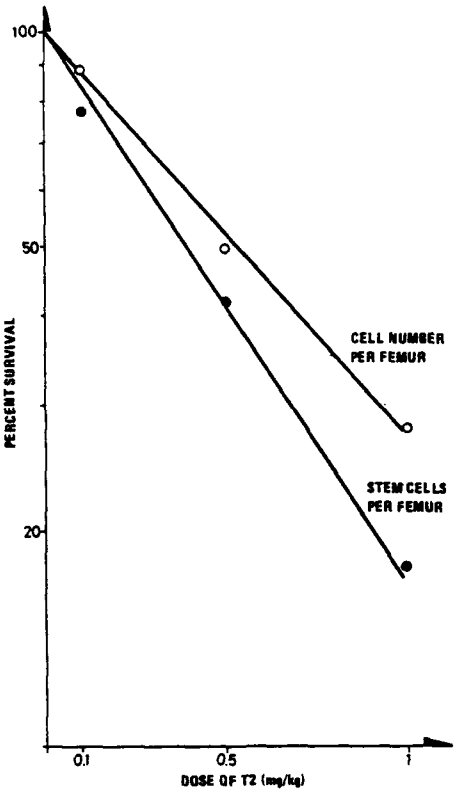

Fig. 1. The effect of T-2 toxin on mouse bone marrow, 24 h after i.v. injection

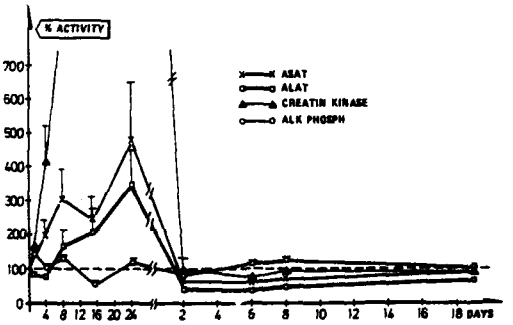

Fig. 2. The effect of T-2 toxin (2 mg/kg i.v.) on the activity of some serum enzymes in mice. The results are presented as mean values ± SEM, $n = 4$

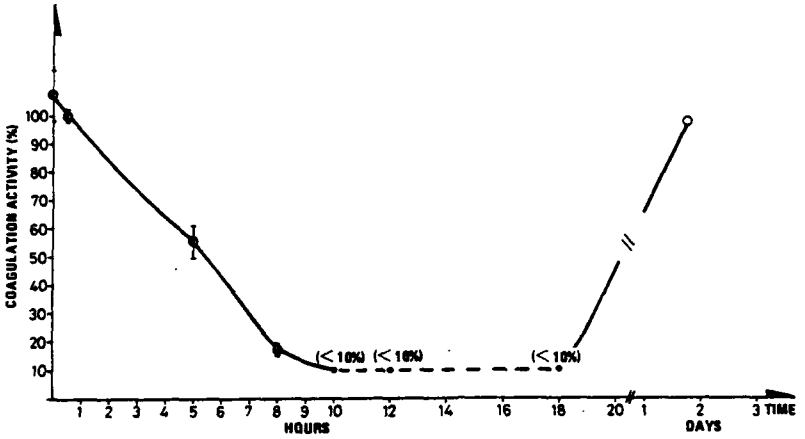

Fig. 3. The effect of T-2 toxin (2 mg/kg s.c.) on coagulation activity in mouse blood measured by the "Normo-Test" clotting time assay. The results are presented as mean values ± SEM, $n = 6$

injection of T-2 toxin (Fig. 3). The effect of a sublethal dose of T-2 toxin on plasma proteases is shown in Fig. 4. It is apparent that coagulation was affected 24 h after administration, as demonstrated by the depletion of prothrombin and its inhibitor antithrombin III. At the same time there was a decline in activity of antiplasmin and prekallikrein, and of total protein content in plasma. The activities were restored in 2–3 days, except for prekallikrein which returned to normal more slowly and without any rebound effect. Significant decreases in plasma protease activity were observed between 1 and 4 h after T-2 toxin administration. T-2 toxin was active as an anticoagulant at low doses, which produced no symptoms of illness in the animals (Table 1).

In vitro incubation of T-2 toxin (2×10^{-4} M) with pooled blood, plasma or filtered plasma had no effect on the level of the proteases measured in this study, indicating that T-2 toxin produces its effects indirectly. Furthermore, T-2 toxin did not affect plasma proteases when incubated with liver slices.

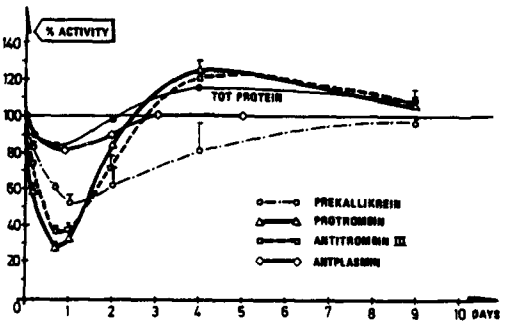

Fig. 4. Alteration in plasma protease enzymes after single i.v. injection of 3 mg/kg T-2 toxin. The results are presented as mean values ± SD, $n = 6$

Discussion

We have found that one single sublethal dose of T-2 toxin administered to mice caused a decrease in the number of bone marrow cells in a dose-dependent manner 24 h after administration. In fact, there was approximately a 50% decrease at a dose 8–10 times lower than the LD_{50} of T-2 toxin. Mice fed a semipurified diet containing 20 ppm T-2 toxin have been reported to develop hypoplasia of bone marrow during 6 weeks of exposure (Hayes et al. 1980). Other authors reported similar findings in sheep fed 0.6 ppm T-2 toxin daily for 12 days (Friend et al. 1983). DeNicola et al. (1978) reported necrosis of bone marrow in guinea pigs receiving one lethal dose of T-2 toxin p.o. Their observations were made post mortem or at the time of death 5–17 h after T-2 toxin administration. They suggested that the injury was radiomimetic.

Necrotic injuries of liver and intestine will induce an increase in ALAT and APHOS, enzymes mainly synthesized in liver. We did find an increase in ALAT 24 h after injection, whereas APHOS seemed to be unaffected. At the same time there was a marked increase in CK and ASAT, as expected with necrotic injuries in skeletal and heart muscles. The results are not conclusive regarding liver as a target organ for the cytotoxic effects of T-2 toxin. On the other hand, it is obvious that skeletal muscle, heart

Table 1. Dose-response of T-2 toxin on coagulation activity 24 h after i.v. injection

T-2 toxin (mg/kg)	% of control	
	Prothrombin	Antithrombin III
1	78 ± 4	68 ± 3
3	33 ± 3	36 ± 2
4	24 ± 3	35 ± 2

The results are presented as mean values ± SD, $n = 6$

and probably the intestine are affected by T-2 toxin. This corresponds to observations made on bovine animals (Gentry et al. 1984).

It is obvious from our findings that in addition to the well-documented effect on coagulation, fibrinolysis is also affected by T-2 toxin, as seen by the consumption of antiplasmin. Of particular interest was the consumption of prekallikrein, suggesting that the kallikrein-kinin system is affected, which may give increased levels of bradykinin and other kinins in plasma. Kinins are potent vasodilators and increase capillary permeability. The kinins may also, like histamine, participate in the normal inflammatory response.

It is noteworthy that all effects observed in this study concerning cytotoxicity and hematological disorders reach their maximum 20–24 h after administration. This corresponds to the time animals usually die following a lethal dose of T-2 toxin.

The mechanism of action of T-2 toxin on coagulation is poorly understood. Cosgriff et al. (1984) proposed that T-2 toxin acts by its depression of protein synthesis, with decreased synthesis of coagulation factors. On the other hand, Gentry and Cooper (1983) proposed that T-2 toxin cannot function merely as an inhibitor of protein synthesis, because of the uniformity in time course of the effects on various factors with half-lives ranging from a few hours to 2–3 days (Austen and Rhymes 1975). The same uniformity in time course of the effect on the proteases was found in the present study. Further evidence that T-2 toxin does not affect the plasma proteases by reduced protein synthesis is the early onset of the effects (1–4 h). However, T-2 toxin does not act as a trigger itself, as indicated by the absence of effects with in vitro incubation. According to the good correlation with the cytotoxic effects manifested by alterations in ASAT, ALAT and CK, we suggest that the effect of T-2 toxin on plasma protease enzymes involved in the coagulation, fibrinolysis and kallikrein-kinin systems is secondary to cytotoxic effects in some organs. T-2 toxin does not, however, act by cytotoxic effects on the liver. There are, of course, a whole series of mediators involved in the inflammatory response. Our studies reveal, however, that plasma protease activation is one factor of relevance. The chromogenic substrate assays have provided us with new tools to study this aspect more closely than was possible before.

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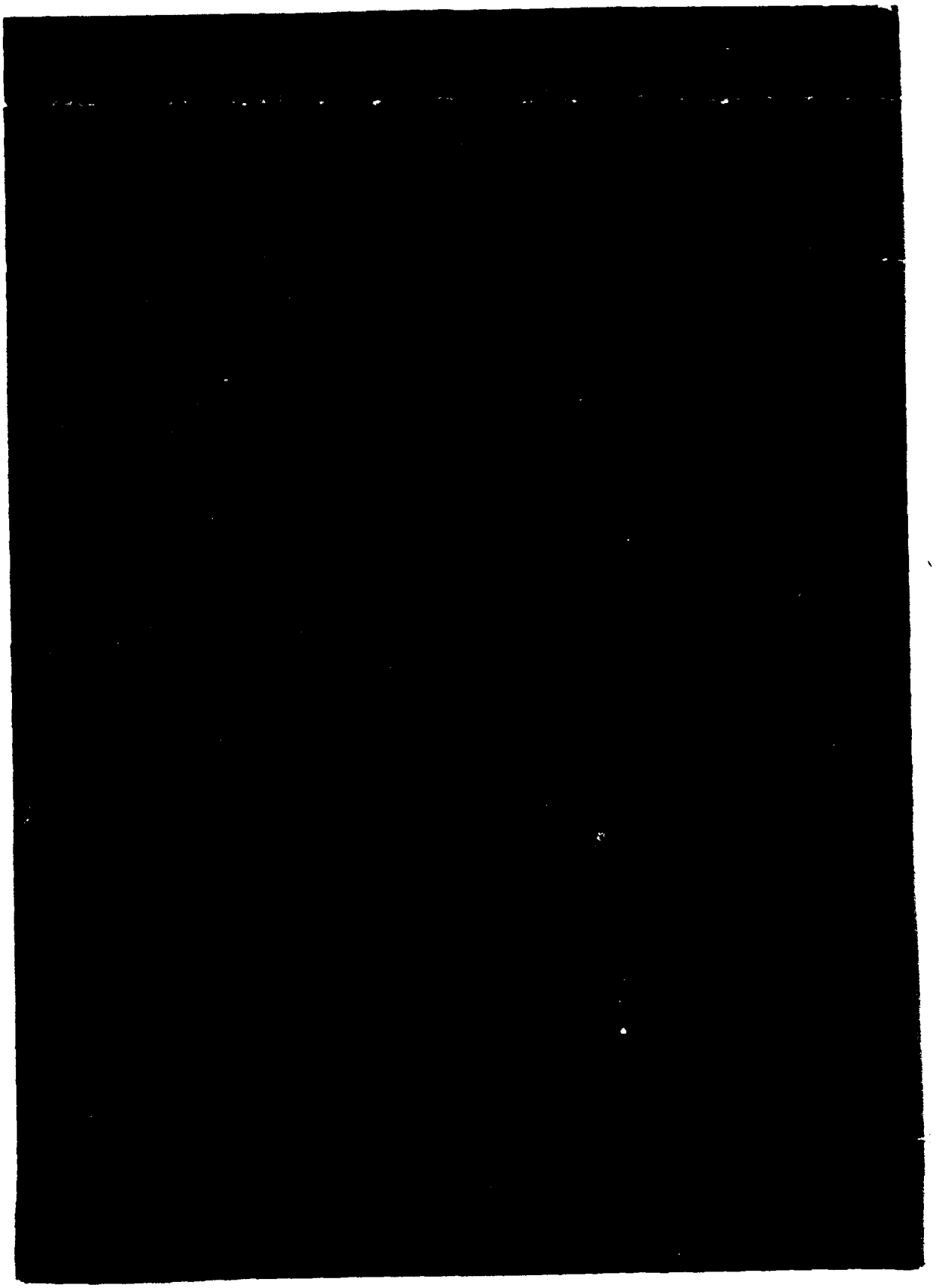

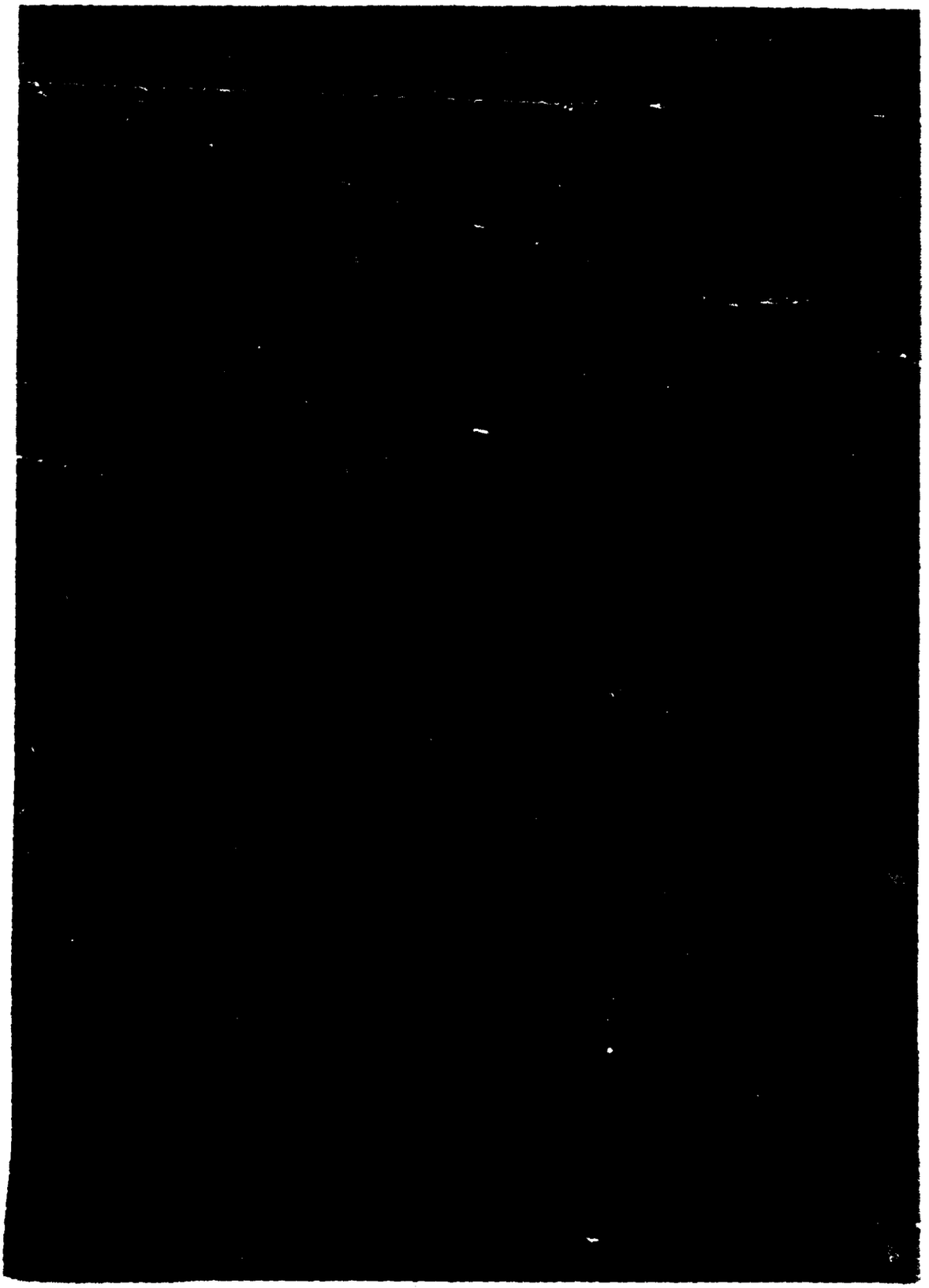

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METABOLISM OF T-2 TOXIN BY RAT LIVER CARBOXYLESTERASE

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(*Received* 5 *July* 1985; *accepted* 4 *November* 1985)

Abstract—The trichothecene T-2 toxin was rapidly hydrolyzed by rat liver microsomal fraction into HT-2 toxin which was the main metabolite. The metabolism was completely blocked by paraoxon, a serine esterase inhibitor, but not affected by EDTA or 4-hydroxy mercury benzoate, inhibitors of arylesterase and esterases containing SH-group in active site, respectively. Among the serine esterases carboxylesterase (EC 3.1.1.1), but not cholinesterase (EC 3.1.1.8) hydrolysed T-2 toxin to HT-2 toxin. Carboxylesterase activity from liver microsomes was separated into at least five different isoenzymes by isoelectric focusing, and only the isoenzyme of pI 5.4 was able to hydrolyse T-2 toxin to HT-2 toxin.

The toxicity of T-2 toxin in mice was enhanced by pre-treatment with tri-o-cresyl phosphate (TOCP), a specific carboxylesterase inhibitor. This confirms the importance of carboxylesterase in detoxification of trichothecenes.

T-2 toxin and related trichothecenes are potent cytotoxic and immunosuppressive mycotoxins produced by various species of *Fusarium*. The toxins may cause dermal necrosis, hemorrhaging, leukopenia and inhibition of protein synthesis in various animals and biological systems [1–3]. T-2 toxin (Fig. 1) is one of the most important trichothecene mycotoxins occurring naturally in various agricultural products [4, 5], and appears to be an important human health problem.

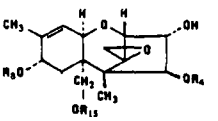
It has been shown that T-2 toxin administered orally to rodents, chickens and a lactating cow, was rapidly transformed into various metabolites and eliminated without accumulation in any organ [6–8].

In mouse and rat liver homogenates, T-2 toxin was rapidly hydrolyzed to HT-2 toxin, which was converted further into T-2 tetraol via 4-deacetyl neosolaniol [10, 11]. Other authors, however, have reported HT-2 toxin as the only metabolite of T-2 toxin produced in rat liver microsomal fraction [12, 13]. *In vitro* formation of 3′-hydroxy T-2 and 3′-hydroxy HT-2 toxins from T-2 toxin by liver homogenates from mice pretreated with phenobarbital and from monkeys by adding NADPH have been reported, indicating that a cytochrome P-450 is catalyzing the hydroxylation at C-3′ position of T-2 and HT-2 toxin [11]. Two of the metabolites found in the lactating cow were also identified as 3′-hydroxy T-2 and 3′-hydroxy HT-2 [9].

Ohta *et al.* [13] found that hydrolysis of T-2 toxin was inhibited by eserine and DFP. They also observed a poor hydrolytic activity in intestinal mucous and serum. On the basis of these findings they suggested that nonspecific carboxylesterase most likely catalyzed the conversion of T-2 toxin to HT-2 toxin. By structure-activity studies they suggested that the esterase selectively hydrolyzed the C-4 acetyl residue of trichothecenes, and that substituents at C-3 and C-8 were essential for the enzymatic hydrolysis of the C-4 acetyl residue [12]. In the present paper the hydrolysis of T-2 toxin by rat liver microsome preparation has been studied in detail. By use of several specific esterase inhibitors, the properties of the enzyme responsible for the hydrolytic activity have been characterized. Such studies strongly indicate that the activity can be accounted for by a carboxylesterase. Isoelectric focusing separated the carboxylesterase activity into five different isoenzymes, and their activity to T-2 toxin was studied.

MATERIALS AND METHODS

Chemicals. 3′-Hydroxy T-2 and 3′-hydroxy HT-2 toxins were generous gifts from C. J. Mirocha, MA, U.S.A. T-2 toxin, acetyl T-2 toxin, HT-2 toxin, diacetoxyscirpenol, T-2 triol, T-2 tetraol (Fig. 1) and 4-nitrophenyl acetate, 4-nitrophenyl butyrate, bis-4-nitrophenyl phosphate and carboxylesterase (EC



TRICHOTHECENE	R_4	R_8	R_{15}
T-2	CH_3CO	$(CH_3)_2CH\ CH_2CO$	CH_3CO
HT-2	H	$(CH_3)_2CH\ CH_2CO$	CH_3CO
4-DEACETYL-NEOSOLANIOL	H	H	CH_3CO
T-2 TETRAOL	H	H	H

Fig. 1. Chemical structure of trichothecene mycotoxins.

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3.1.1.1) from porcine liver were all purchased from Sigma Chemical Company, St. Louis, MO, U.S.A. Horse serum cholinesterase (EC 3.1.1.8) and paraoxon were from Koch-Light Laboratories, U.K., saponin from Merck, Darmstadt, F.R.G. and tri-*o*-cresyl phosphate (TOCP) from K and K, JCN Pharmaceuticals Inc., U.S.A. Sylon BTZ from Supelco Inc., PA, U.S.A. was used as trimethylsilyl derivatizing reagent for gas chromatography of trichothecenes.

Ultrogel AcA34, bead size (swollen) 60–140 μm for gelfiltration was purchased from LKB, France, while polyacrylamide electrofocusing gel (Bio-Lyte) and ampholytes (Bio-Lyte) were from Bio-Rad Laboratories, CA, U.S.A. Soman was synthesized in our laboratory, and methyl-[1-^{14}C]butyrate synthesized as described [14].

Preparation of liver fractions. Livers 10–12 g, from male Wistar rats (250–300 g) were rinsed with ice-cold 50 mM Tris pH 7.5, containing in 0.1 M NaCl and homogenized in 4 vol. of the same buffer with glass–Teflon homogenizer. The homogenate was filtered through glass wool to remove fat and centrifuged at 9000 *g* for 20 min at 4°. The supernatant, S9 fraction, was further centrifuged at 100,000 *g* for 60 min at 4°. The high speed pellet was resuspended in an equal volume of 50 mM Tris pH 7.5 and constituted the microsomal fraction. The high speed supernatant was fractionated by ammonium sulfate precipitation. The hydrolytic activities were mainly precipitated in the fraction from 35–70% ammonium sulfate saturation. This precipitate was resuspended in a small volume of 50 mM Tris pH 7.5, and referred to as the cytosol fraction.

For gelfiltration and isoelectric focusing the microsomal fractions were solubilized by mixing with 1% saponin for 1 hr at 4° and centrifuged at 100,000 *g* for 60 min. The 35–70% ammonium sulfate fraction of the high speed supernatant was resuspended in a small volume of 50 mM Tris pH 8.0 for gelfiltration and 10 mM Tris pH 8.0 for isoelectric focusing. This is called microsomal enzyme fraction.

Enzyme assay. Carboxylesterase assays were performed at pH 7.8 and 30° with the substrates methyl [1-^{14}C]butyrate (1.76 mM), 4-nitrophenyl butyrate (2 mM) and 4-nitrophenyl acetate (0.25 mM) as previously described [14]. Liberated [1-^{14}C]butyrate was measured by scintillation counting and 4-nitrophenol was determined spectrophotometrically at 400 nm [14]. In some experiments we have also used acetanilide as substrate [15].

Gel filtration. The microsomal enzyme fraction containing 100–150 mg protein was applied to a gel filtration column (Ultrogel AcA34, 35 × 25 cm) equilibrated with 50 mM Tris pH 8.0. The sample was eluted at a rate of 0.3 ml/min and fractions collected every 5 min. The fractions were then analyzed for carboxylesterase activity, and peak activities were analyzed for T-2 hydrolysis.

Isoelectric focusing. The microsomal enzyme fraction was desalted by eluting through Sephadex G-25M (Pharmacia PD-10 column, 5 × 1.5 cm) equilibrated with distilled water. Between 5 and 8 ml of the eluate corresponding to 100–150 mg protein was mixed with 90 ml of Bio-Lyte electrofocusing gel slurry containing 3% of Bio-Lyte ampholytes pH 3/10:5/7 (2:3). The slurry was then applied onto a flat bed tray (30 × 12.5 cm) and dried by airstream. The electrolytes were 0.5 M H_3PO_4 for the anode and 0.5 M NaOH for the cathode. A constant power of 25 W was applied for 16 hr at 5°. Harvesting was performed by a grid with 0.7 cm sections, proteins eluted from the gel by mixing with 1 ml distilled water and centrifugation. The fractions were analyzed for carboxylesterase activity and T-2 hydrolyzing activity.

Incubation of T-2 toxin. Aliquots of 1 ml of 10% microsome preparation and 1 ml of cytosol preparation were preincubated with enzyme inhibitors for 30 min at 37° and pH 7.5. To this incubation mixture was added 0.5 μmole T-2, and the incubation continued for 10 and 60 min, respectively. Samples of 1 ml from gel filtration and 0.5 ml from isoelectric focusing mixed with 0.5 ml 0.1 M Tris pH 7.5 were incubated with 0.5 μmole T-2 toxin for 2 hr at 37° and pH 7.5.

The reaction was stopped by adding 2 ml ice-cold 0.6 M perchloric acid and 125 μg diacetoxyscirpenol as internal standard. The sample was centrifuged at 15,000 *g* for 10 min, and the supernatant was treated with 180 μl 7.5 M potassium acetate, recentrifuged and applied onto Amberlite XAD-2 resin column (1 g, 1 × 20 cm). The column was washed with 20 ml of water and eluted by 20 ml of methanol:water (9:1 vol/vol). The methanol eluate was evaporated to dryness and dissolved in 0.5 ml acetone. Aliquots of 50 μl of the acetone extract was mixed with 25 μl Sylon BTZ for trimethylsilyl derivatizing for gas chromatography. Recovery for T-2 toxin and HT-2 toxin by the whole method was about 90% and 30–40% for T-2 tetraol.

In cases where only the metabolism of T-2 and HT-2 was studied, a faster isolation procedure was used by means of Clin-Elute diatomaceous earth column (1 g, 2.5 × 1.2 cm). Recovery of T-2 and HT-2 by this procedure was 70–80%. One millilitre of the sample was applied to the column and it was eluted with 5 ml chloroform. The chloroform extract was evaporated to dryness and dissolved in 500 μl acetone and aliquots of 50 μl were mixed with 25 μl Sylon BTZ for derivatizing.

Gas chromatographic analysis of trichothecenes. Gas chromatography was performed by a Packard Becker 438 equipped with a mass spectrometer (LKB 2091) for identification, or flame ionization detector for quantification of trichothecene metabolites. A glass column (1.2 m × 2 mm I.D.) packed with 3% OV-17 on Supelcoport 80–100 mesh was used under the following conditions: oven temperature programmed from 200° to 275° at 15°/min, injector and detector temperature 300°, flow rate of helium, hydrogen and air; 30, 25 and 250 ml/min respectively. The operating conditions for mass spectrometry were: ionization energy 70 eV or 12 eV, accelerating voltage 3.5 kV. Quantification of metabolites was based on standard curves and diacetoxyscirpenol as internal standard.

RESULTS

T-2 toxin incubated with rat liver microsomal fraction was completely metabolized to HT-2 toxin after

Table 1. Effect of esterase inhibitors on rat liver metabolism of T-2 toxin

Fraction inhibitor	Carboxylesterase activity (μmole 4-NPB*) mg prot × hr		T-2 metabolism (% HT-2 PRODUCED) mg prot × 10 min	
Microsomes (P2)	400 ± 62	(100%)	44 ± 4	(100%)
Microsomes TOCP† (200 mg/kg)	89 ± 18	(22%)	27 ± 1	(60%)
Microsomes BPNP‡ (10^{-4} M)	51 ± 5	(13%)	19 ± 3	(44%)
Microsomes SOMAN (10^{-5} M)	11 ± 2	(3%)	18 ± 5	(40%)
Microsomes SOMAN (10^{-4} M)	9 ± 0	(2%)	2 ± 0	(4%)
Microsomes PARAOXON (10^{-4} M)	0	(0%)	0	(0%)
Microsomes EDTA (10^{-3} M)	409	(100%)	43 ± 1	(100%)
Microsomes MERCURY BENZOATE (10^{-3} M)	338	(84%)	44 ± 1	(100%)
Cytosol (S2)	9 ± 3	(100%)	0.19 ± 0.03	(100%)
Cytosol TOCP (200 mg/kg)	3 ± 0.3	(33%)	0.13 ± 0.02	(70%)
Plasma	3.8 ± 0.3	(100%)	0	(0%)
Plasma TOPC (200 mg/kg)	0.5 ± 0.2	(13%)	0	(0%)

The numbers are mean values ± S.D. of 4–8 rats.
Inhibitors were preincubated at 37° for 30 min before incubation with 0.5 mM and T-2 toxin.
* 4-nitrophenylbutyrate.
† Tri-*o*-cresyl phosphate.
‡ Bis-4-nitrophenylphosphate.

60 min, and no other metabolites were found by GC-MS under the experimental conditions used. In rat liver cytosol fraction the T-2 to HT-2 metabolic activity was much lower, and in plasma no metabolism was found (Table 1).

The metabolic activity was completely blocked by paraoxon (10^{-4} M), which is known to inhibit serine esterases. No effect, however, was seen by EDTA (10^{-3} M), an aryl esterase inhibitor [16, 17], or 4-hydroxy mercury benzoate (10^{-3} M), which inhibit esterases with SH-group in the active site, such as in phosphoryl phosphatases [18]. It is apparent that the metabolism of T-2 toxin is less sensitive than the 4-nitrophenyl butyrate hydrolysis towards organophosphorus inhibitors such as soman (10^{-5} M) and bis-4-nitrophenyl phosphate (10^{-4} M). At higher concentration of soman (10^{-4} M) the metabolic activity was completely inhibited. We therefore concluded that the metabolism of T-2 toxin corresponded to a group of serine-esterases.

Of the commercially available preparations of serine esterases shown in Table 2, carboxylesterase isolated from porcine liver, but not cholinesterase isolated from horse serum, hydrolized T-2 toxin.

Because of the high cost of trichothecenes, the effect of carboxylesterase inhibition on toxicity of T-2 toxin was studied in mice. As shown in Table 3, the toxicity of T-2 toxin is increased by selective inhibition of carboxylesterase *in vivo* by tri-*o*-cresyl phosphate (TOCP) [19], indicating the importance of carboxylesterase for detoxification of T-2 toxin. TOCP is not toxic to mice at this dosage. The carboxylesterase activity in liver homogenates of TOCP-treated animals, measured by the 3 substrates (4-nitrophenyl butyrate, 4-nitrophenyl acetate and methyl [1-^{14}C]butyrate), was only 10–30% of normal activity.

Carboxylesterase is a heterogenous group of enzymes consisting of several isoenzymes [20]. From Table 1, it is apparent that the isoenzyme mainly

Table 2. Hydrolytic activity of commercial serine esterases

Serine esterase	Incubation time (min)	% HT-2 produced of added T-2 toxin
Cholinesterase* – 5 mg/ml	60	0
Carboxylesterase†		
– 1 mg/ml	10	8
– 1 mg/ml	30	26
– 2.5 mg/ml	10	16
– 2.5 mg/ml	30	60

Enzymes were dissolved in 50 mM Tris pH 7.5 and incubated with 0.5 mM T-2 toxin at 37°

* Cholinesterase (horse serum): $\frac{20\ \mu\text{mole butyryl thiocholine}}{\text{mg material} \cdot \text{min}}$

† Carboxylesterase (porcine liver): $\frac{160\ \mu\text{mole ethyl butyrate}}{\text{mg protein} \cdot \text{min}}$

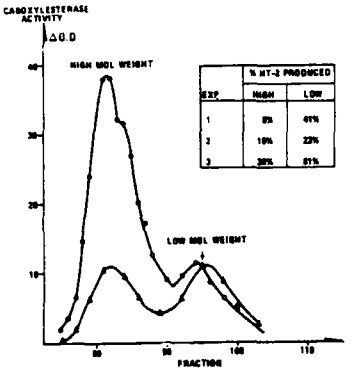

Fig. 2. Gel filtration of rat liver carboxylesterase on Ultrogel AcA34. The column was eluted with 50 mM Tris pH 8.0 at 0.3 ml/min and fractions collected every 5 min. Fractions were analyzed for 4-nitrophenyl butyrate (○——○) and 4-nitrophenylacetate (△——△) activity and peak activities were analyzed for T-2 hydrolyzing activity.

responsible for T-2 to HT-2 metabolism is characterized by being less sensitive both to low concentration of soman (10^{-5} M) and bis-4-nitrophenyl phosphate (10^{-5} M). Separation of carboxylesterase isoenzymes into one high and one low molecular weight fraction identified by the substrates 4-nitrophenyl butyrate and 4-nitrophenyl acetate is shown in Fig. 2. The main hydrolytic activity for T-2 toxin was found in the low molecular weight fraction. The remaining activity in the high molecular weight fraction may be contamination due to incomplete separation of the two peaks.

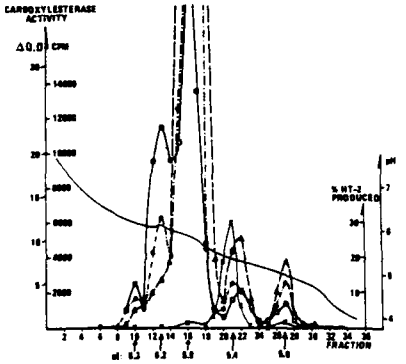

Fig. 3. Preparative flat bed isoelectric focusing of rat liver carboxylesterase on polyacrylamide gel. The fractions were analyzed for carboxylesterase activity by the substrates; 4-nitrophenyl butyrate (●——●), 4-nitrophenyl acetate (□——□) and methyl[1-^{14}C]butyrate (△——△). The five different carboxylesterase isoenzyme were analyzed for T-2 hydrolyzing activity (×——×).

Separation of the isoenzymes by isoelectric focusing is shown in Fig. 3. Carboxylesterase was separated into 5 different isoenzymes according to their isoelectric point (pI), and identified by the 3 different substrates. The isoenzyme pI 5.9 is by far the most active isoenzyme with respect to hydrolysis of all 3 different substrates, but no T-2 hydrolyzing activity was found. It is known that this isoenzyme constitutes the high molecular weight fraction (180,000 MW), whereas the 4 other constitute the low molecular weight fraction (60,000 MW) [21]. It is apparent from Fig. 3 that T-2 to HT-2 metabolism in rat liver is performed by the isoenzyme pI 5.4 only. Isoenzyme pI 5.4 was the only isoenzyme which displayed high activity towards the substrate acetanilide (data not shown). However, as can be seen from the figure, there is a slight difference between the peak activities of 4-nitrophenyl acetate, methyl-[1-^{14}C]butyrate and the peak activity to T-2 hydrolysis, indicating a possible microheterogeneity of isoenzyme pI 5.4. We attempted to resolve this peak by refocusing in a pH gradient 4.5–6, but the peaks achieved were too close to allow any separation by flat bed isoelectric focusing. Separate experiments showed that the enzyme activity of isoenzyme pI 5.4 towards 4-nitrophenyl butyrate, 4-nitrophenyl acetate, methyl[1-^{14}C]butyrate and T-2 toxin was inhibited to the same extent by soman ($5 \cdot 10^{-6}$ M).

DISCUSSION

We have found that in rat liver microsomal fraction, T-2 toxin is metabolized to HT-2 toxin and that the metabolism is performed mainly by a carboxylesterase at pI 5.4. We did not, even after extended incubation, find any other metabolites under our incubation conditions.

We conclude that the metabolism of T-2 to HT-2 is performed by a serine esterase, since it is completely blocked by paraoxon. We could exclude an aryl esterase and phosphoryl phosphatase, since it was not inhibited by EDTA or 4-hydroxy mercury benzoate. Among the serine esterases shown in Table 2, carboxylesterase but not cholinesterase hydrolyses T-2 to HT-2, and carboxylesterase therefore seems to be the most likely candidate for the metabolism of T-2 to HT-2. The results of Ohta *et al.* [13] by use of eserine for inhibition is not conclusive in differentiation between cholinesterase and other esterases.

Table 3. Effect of a carboxylesterase inhibitor on the toxicity of T-2 toxin in mice

T-2 TOXIN (mg/kg I.V.)	% Survival	
	Control	*TOCP (100 mg/kg)*
0.5	100	100
1.0	100	100
2.0	100	0
3.0	70	0
4.0	10	0

Each group consisted of 10 animals

Of particular interest was the fact that much lower activity than expected from carboxylesterase activity was derived from the cytosol fraction and that no metabolic activity was found with rat plasma even after incubation for 60 min. These facts strongly indicate that the metabolic activity could not be accounted for by the whole carboxylesterase group. Thus our conclusion is an extension of the findings by Ohta *et al.* [13], who reached their conclusions only on the basis of results obtained with DFP and eserine. Carboxylesterase is a heterogeneous group of enzymes consisting of several isoenzymes [20, 21]. Carboxylesterase in liver microsomes were separated into 5 different isoenzymes according to their isoelectric point. The isoenzymes revealed different substrate specificity, and the isoenzyme of pH 5.4 was the only isoenzyme able to hydrolyse T-2 toxin to HT-2 toxin. This isoenzyme was found by us to exhibit activity towards acetanilide in agreement with the findings by Mentlein and Heymann [15].

Under our experimental conditions we did not find any 4-deacetyl neosolaniol or T-2 tetraol, the further deacetylated products of HT-2. This is in contrast to the findings of Yoshizawa *et al.* [10] who detected both 4-deacetyl neosolaniol and T-2 tetraol in S9 fractions from rat liver. In their study they stopped the reaction by putting the test tube into a waterbath at 85°. This gradual heating up to 85° will, however, not immediately denaturate the proteins. Since carboxylesterases are heat stable at 50–60° the heating could possibly accelerate the hydrolysis. The heating could also provide sufficient energy to start a further hydrolysis of HT-2. In agreement with our findings Ohta *et al.* [13] and Ellison *et al.* [22] did not find any other metabolites than T-2 and HT-2 although their clean-up procedure could be criticized for being insensitive to the polar metabolites of T-2 [10]. In the presence of NADPH a mixed function oxidase has been reported to give 3'-hydroxy T-2 and 3'-hydroxy HT-2 in mice liver fractions [11]. Our experiments, without addition of NADPH, would not reveal these metabolites, although we used the same isolation procedure for trichothecenes as used by Yoshizawa *et al.* [11].

Toxicity of HT-2 is found to be comparable to that of T-2. HT-2 is 1.7-fold less toxic than T-2 when administered I.P. [23] to mice. With respect to the rapid metabolism of T-2 to HT-2 by various laboratory animals, it has been suggested that the toxic effects of T-2 toxin is exerted by HT-2 toxin mainly and this metabolic change is not considered to be detoxification of T-2 toxin [13, 15].

Our finding of the increased toxicity of T-2 toxin in TOCP treated animals (2–3-fold) can at least in part be explained by the different toxicity of T-2 toxin and HT-2 toxin.

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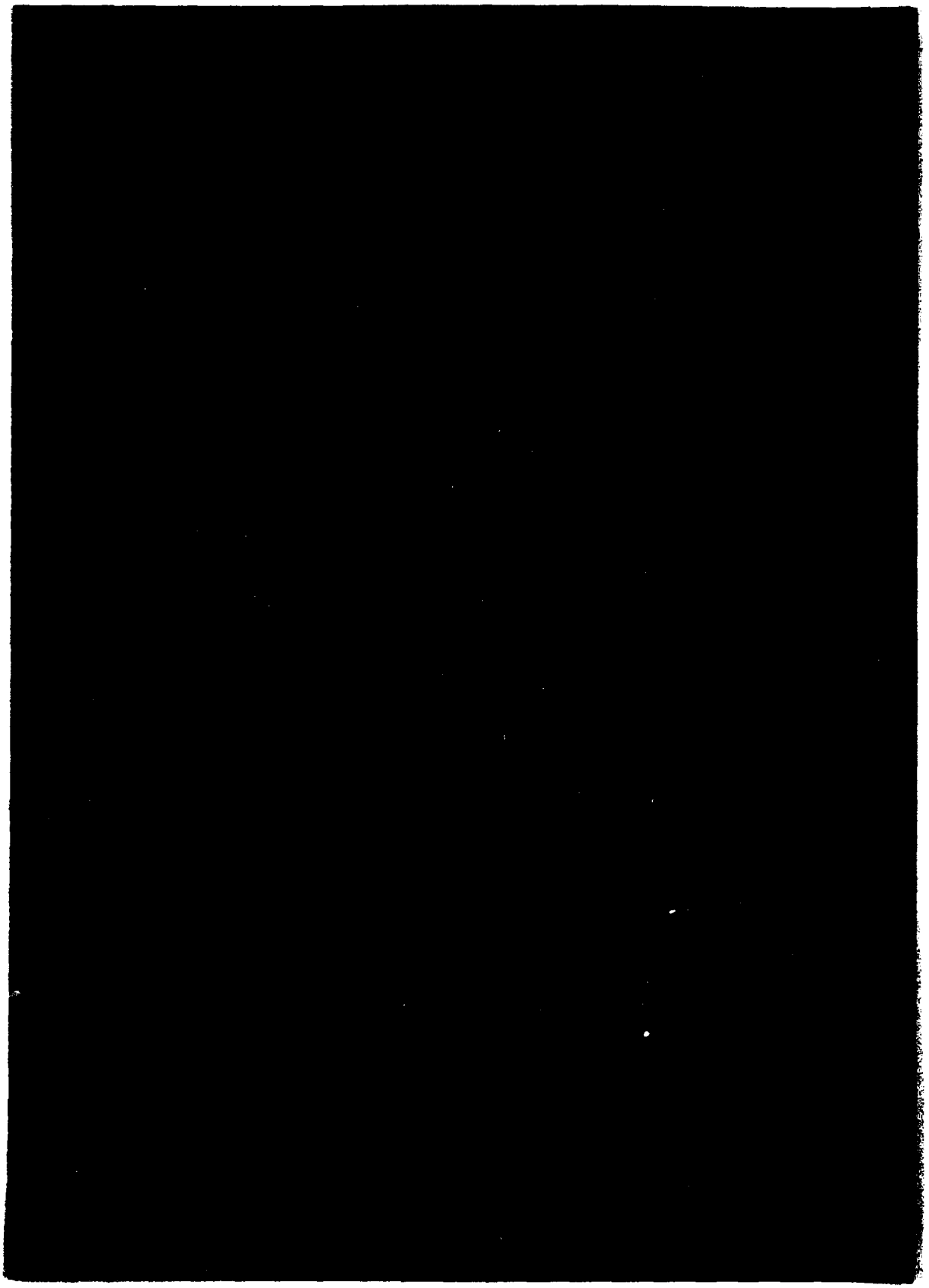

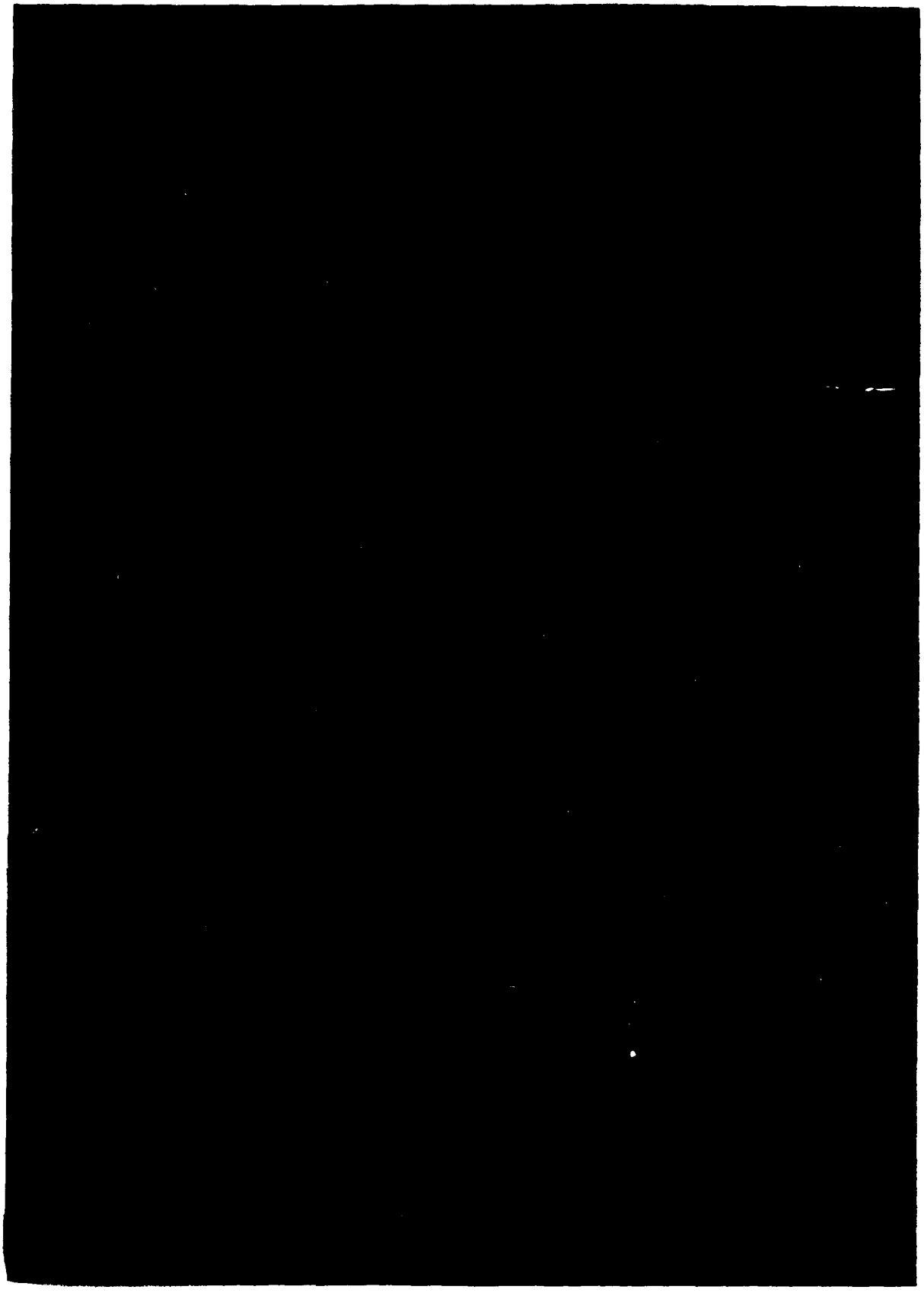

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METABOLISM OF T-2 TOXIN BY BLOOD CELL CARBOXYLESTERASES

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Abstract—Human and rat blood hydrolysed T-2 toxin along two different pathways giving HT-2 toxin and neosolaniol as primary metabolites, respectively. Neosolaniol represents a metabolic pathway different from that obtained by liver. Rat erythrocytes formed neosolaniol as a primary metabolite whereas white blood cells hydrolysed T-2 toxin to HT-2 toxin. Human erythrocytes formed both HT-2 toxin and neosolaniol whereas all human white cells produced only HT-2 as the primary metabolite. The enzymes responsible for hydrolysis of T-2 toxin to HT-2 toxin in white blood cells and T-2 toxin to neosolaniol in red blood cells were all identified as carboxylesterases by use of specific inhibitors. The ratio between trichothecene hydrolysis and 4-nitrophenyl butyrate hydrolysis varied among the different cell fractions indicating that specific isoenzymes are involved.

T-2 toxin is one of the most important trichothecene mycotoxins occurring naturally in agricultural products and is associated with several characteristic mycotoxicoses in both humans and animals. Red mold toxicoses in Japan, moldy corn toxicoses in the U.S.A. and alimentary toxic aleukia (ATA) in Europe are diseases caused by trichothecene mycotoxins [1, 2]. Furthermore, trichothecene mycotoxins have attracted international attention because of their possible use as the chemical warfare agent "Yellow Rain" [3–5].

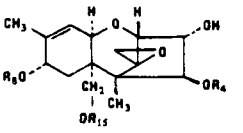
The metabolic activity in different tissues have been studied in rats and rabbits by Ohta *et al.* [6]. In addition to the liver, both kidney and brain were found to hydrolyse T-2 toxin to HT-2 toxin to some extent, whereas no activity was found in plasma or blood cells.

T-2 toxin (Fig. 1) is metabolized in liver mainly by hydrolysis at C-4 position by carboxylesterase to yield HT-2 as its main metabolite [6, 7]. When rat liver carboxylesterases were separated into five different isoenzymes, only isoenzyme pI 5.4 did hydrolyse T-2 to HT-2 toxin [7]. A different pathway possible for hydrolysis of T-2 toxin consists of removal of the isovaleryl group at C-8 position to yield neosolaniol, and this has been reported as a minor pathway in rat and mice intestine [8, 9]. The authors proposed that the hydrolysis to neosolaniol is caused by esterases of dermal microflora. Furthermore, neosolaniol has also been identified as a trace metabolite in mice [9] and swine [10]. T-2 toxin has also been found to be hydroxylated at 3-position of C-8-isovaleryl group by liver cytochrome P-450 [11].

In a previous study at our laboratory we found evidence that T-2 toxin was metabolized by carboxylesterase [7]. Cytochemical methods for identification of monocytes and granulocytes consist of staining for non-specific esterases [12]. Furthermore, multiple forms of esterases with different biochemical characteristics have been identified in the erythrocytes [13, 14]. The presence of esterases in the blood cells, indicates that T-2 toxin to some extent may be hydrolysed in the blood. In the present study we have, therefore, studied the hydrolysis of T-2 toxin by rat and human blood cells.

MATERIALS AND METHODS

Chemicals. T-2 toxin, HT-2 toxin, T-2 tetraol, Neosolaniol, Diacetoxyscirpenol (DAS), bis-4-nitrophenyl phosphate (BPNP), and physostigmine were all purchased from Sigma Chemical Company (St. Louis, MO). Paraoxon came from Koch-Light Laboratories, U.K. 4-Hydroxymercury benzoate-(4OH-MB) were purchased from Aldrich Chemical Company, Inc. (U.S.A.), whereas EDTA came from E. Merck (Darmstadt). Sylon



TRICHOTHECENE	R_4	R_8	R_{15}
T-2	CH_3CO	$(CH_3)_2CH\ CH_2CO$	CH_3CO
HT-2	H	$(CH_3)_2CH\ CH_2CO$	CH_3CO
4-DEACETYL-NEOSOLANIOL	H	H	CH_3CO
T-2 TETRAOL	H	H	H

Fig. 1. Chemical structure of the trichothecene T-2 toxin and its hydrolytic metabolites.

BTZ from Supelco, Inc. (PA, U.S.A.) was used as trimethylsilyl derivatizing reagent for gas chromatography mass spectrometry. Nycodenz (1.090 g/ml, 309 mOsm) from Nycomed A/S, Norway, was used for cell separation.

Animals and blood sampling. Male Wistar rats (200–300 g) of the outbred strain Mol: WIST were used in this study. They were maintained at constant room temperature (24°) with 12 hr light cycle and standard laboratory diet and water *ad libitum*. Blood was collected by puncture of the vena cava in animals anesthetized with diethyl ether, and anticoagulated with 3.8% sodium citrate (5:1 v/v).

Preparation of blood cells. Differential preparation of the blood cells was performed by methods previously developed in our laboratory [15–17]. The purity of the cell fractions was examined microscopically and found to be more than 90% for erythrocytes, white blood cells, lymphocytes and granulocytes. Leucocytes and erythrocytes were virtually absent in the thrombocyte fraction.

Whole blood. Rat and human blood was anticoagulated with 3.8% Na-citrate (5:1 v/v). Plasma was obtained by centrifugation of whole blood at 800 *g* for 15 min.

Red blood cells (RBC). The plasma free cells were washed 4 times with 0.9% NaCl and careful removal of the upper layer with the white blood cells with a pipette. The RBC was resuspended in 50 mM Tris(0.7% NaCl) pH 7.4.

White blood cells (WBC). Anticoagulated whole blood was mixed with Dextran (6%, T500, MW 530 000 in 0.9% NaCl) at a ratio 5:1. The RBC was allowed to sediment at room temperature for 30 min before the plasma residue containing the WBC was collected with a pipette.

Lymphocytes and granulocytes. The dextran treated plasma was layered over an equal volume Nycodenz (1.090 g/l, 309 mOsm) and centrifuged at 600 *g* for 15 min. The mononuclear cells were concentrated in a white layer at the interface region and when isolated from rat blood this layer consisted mainly of lymphocytes together with platelets. The sediment after centrifugation contained the granulocytes, and was resuspended in 1.2 ml 50 mM Tris/0.7% NaCl pH 7.4.

Thrombocytes. Anticoagulated whole blood was centrifuged at 600 *g* for 10 min. The plasma was recentrifuged at 800 *g* to sediment the thrombocyte fraction which was washed once and resuspended in 50 mM Tris/0.7% NaCl pH 7.4 before counting.

Monocytes. Monocytes from rats anesthetized until death with ether were harvested from peritoneal cavity by washing the peritoneum with 10 ml 0.9% NaCl. The ascites fluid was centrifuged at 800 *g* for 10 min and the cells resuspended in 1.2 ml 50 mM Tris/0.7% NaCl pH 7.4. The monocytes/macrophages constitute about 60–80% of the cell population in the ascites fluid.

Incubation of T-2 toxin. To aliquots of 1 ml of blood, plasma or cell preparations were added 0.5 μmole T-2 toxin and incubated for 1 hr at 37° and pH 7.5. Preincubation with enzyme inhibitors was performed at the same conditions for 30 min. The reaction was stopped by adding 2 ml ice-cold 0.6 M perchloric acid and 50 μg DAS as internal standard. Preparation of the samples is described in a previous study [7]. Trimethylsilyl derivatisation for gas chromatography was performed by Sylon BTZ.

Gas chromatographic mass spectrometric analysis. Gas chromatography was performed by a Packard Becker 438 gas chromatograph connected to a LKB 2091 mass spectrometer for identification and quantification of trichothecene metabolites. A glass column (1.2 m × 2 mm I.D.) packed with 3% OV-17 on Supelcoport 80–100 mesh was used under the following conditions: oven temperature programmed from 200° to 275° at 15°/min, injector and interface temperature 300° and flow rate of helium as carrier gas was 30 ml/min. The operating conditions for mass spectrometry were: ion source temperature 270°, ionization energy 70 or 14 eV and 22 eV for multiple ion detection (MID) and acceleration voltage 3.5 kV. Quantification of metabolites was performed by hardware multiple ion detection (MID) with DAS as internal standard. 4-deacetyl neosolaniol was not available as standard and quantification of this metabolite was therefore based on the response factor of neosolaniol.

RESULTS

T-2 toxin was completely hydrolysed to polar metabolites in 24 hr when incubated with rat blood. Table 1 shows the time course of the hydrolysis of T-2 toxin. It is of special interest to note that neosolaniol is one of the major metabolites produced in blood. In fact, the neosolaniol production was 62 ± 3% (SD, N = 3) of that of HT-2 toxin during the first 3 hr of incubation, and at 24 hr the neosolaniol content was 20% higher than HT-2 toxin. Both HT-2 toxin and neosolaniol reached maximum levels around 3 hr. These metabolites were both subjected to further hydrolysis to the more polar compounds 4-deacetyl neosolaniol and T-2 tetraol. T-2 toxin was metabolized to the same extent by human and rat blood. Formation of the polar metabolites 4-deacetyl neosolaniol and T-2 tetraol, however, was more pronounced in the human blood (Table 2).

Both erythrocytes and white blood cells from rat and human blood hydrolysed T-2 toxin. In rat blood, however, the hydrolysis of T-2 toxin was completely separated into the two different pathways by sep-

Table 1. Time course of the hydrolysis of T-2 toxin in rat blood

Time (hr)	Metabolite[a] (molar % of added substrate)				
	T-2[b]	HT-2	NEOS	4-DANS	T4
0.5	87	7	5	2	0
1	74	13	8	6	0.2
3	36	22	14	27	2
24	0	7	8	55	31

[a] Abbreviations: NEOS, neosolaniol; 4-DANS, 4-deacetyl neosolaniol; T4, T-2 tetraol. Values are means of triplicate experiments.

[b] Unmetabolized substrate after incubation of 540 μmole·ml^{-1} T-2 toxin.

Table 2. Hydrolysis of T-2 toxin by rat and human blood cells

Fraction		Cells·ml^{-1}	Metabolite[a] (nmole·ml^{-1}·hr^{-1})				
			T-2[b]	HT-2	NEOS	4-DANS	T4
Whole blood	Rat		358 ± 4	103 ± 2	52 ± 3	53 ± 6	tr[c]
	Human		303 ± 8	85 ± 7	50 ± 3	71 ± 15	16 ± 5
RBC	Rat	$6.4 \pm 0.2 \cdot 10^9$	483 ± 36	0	81 ± 5	11 ± 1	0
	Human	$4.9 \pm 0.4 \cdot 10^9$	315 ± 36	66 ± 9	45 ± 5	44 ± 6	10 ± 1
WBC	Rat	$21.4 \pm 0.1 \cdot 10^7$	365 ± 28	123 ± 28	tr	12 ± 2	0
	Human	$26.9 \pm 1.3 \cdot 10^7$	421 ± 56	28 ± 7	tr	tr	0

[a] Abbreviations: NEOS, neosolaniol; 4-DANS, 4-deacetyl neosolaniol; T4, T-2 tetraol. Values are mean ± SD, N = 3.
[b] Unmetabolized substrate after incubation of 540 nmole·ml^{-1} for 60 min.
[c] tr, trace amount.

aration into the red and white blood cells. Rat erythrocytes formed neosolaniol as the primary metabolite whereas white blood cells hydrolysed T-2 toxin to HT-2 toxin (Table 2). This specific hydrolysis of T-2 toxin in rat erythrocytes to give only neosolaniol as primary metabolite was not found for human erythrocytes, which produced HT-2 toxin and neosolaniol in equal amounts.

Previous studies in liver showed that T-2 toxin was metabolized by carboxylesterases [7]. There were, however, considerable differences towards T-2 toxin by the different carboxylesterase isoenzymes. It was therefore deemed necessary to characterize the metabolic activities possessed by red and white blood cells by use of specific esterase inhibitors which is presented in Table 3. The activities were completely blocked by the serine esterase inhibitors paraoxon (10^{-4} M) and soman (10^{-5} M), but only partly inhibited by the carboxylesterase inhibitor bis-4-nitrophenylphosphate (BPNP). In fact, the T-2 to HT-2 hydrolytic activity in white blood cells were inhibited by 34% by 10^{-4} M BPNP whereas no inhibition of T-2 to neosolaniol hydrolysis in red blood cells was seen. A concentration of BPNP at 10^{-3} M was necessary to inhibit the T-2 to neosolaniol hydrolysis to some degree. This low affinity to BPNP was, however, also found for the carboxylesterase isoenzyme hydrolysing T-2 toxin in liver [7]. Furthermore, no effect was seen with EDTA (10^{-3} M), an arylesterase inhibitor [18, 19] or 4-hydroxy mercurybenzoate (10^{-3} M) which inhibits esterases with SH-group in active site, such as phosphoryl phosphatases [20]. Finally the hydrolysis was not affected by physostigmin at 10^{-5} M, a concentration known to specific inhibit cholinesterase whereas carboxylesterase is not inhibited. The enzyme responsible for the hydrolysis of T-2 toxin in blood cells therefore had similar properties as the carboxylesterase in liver hydrolysing T-2 toxin.

When T-2 toxin was incubated with isolated preparations of monocytes, lymphocytes, granulocytes and thrombocytes, which makes the white cell population in blood, it was found that all these different cell types participated in the hydrolysis of T-2 toxin to HT-2 toxin (Table 4). No neosolaniol was produced by these cells. The different cell populations did also hydrolyse the well defined substrate for carboxylesterase; 4-nitrophenylbutyrate, indicating

Table 3. Effect of esterase inhibitors on hydrolysis of T-2 toxin to HT-2 toxin by white blood cells and T-2 toxin to neosolaniol by erythrocytes

Inhibitor	Metabolite produced (% of control)	
	Neosolaniol[a] (erythrocytes)	HT-2 toxin (white blood cells)
Soman (10^{-5} M)	<5	2.3 ± 0.2
Paraoxon (10^{-4} M)	0	2.6 ± 0.3
BPNP (10^{-3} M)	66 ± 2	—
BPNP (10^{-4} M)	101 ± 3	66 ± 10
Physostigmin (10^{-5} M)	98 ± 2	106 ± 11
EDTA (10^{-3} M)	91 ± 4	—
4OH-MB (10^{-3} M)	96 ± 2	97 ± 17

Values are mean values ± SD, N = 3–4 rats.
Inhibitors were preincubated at 37° for 30 min before incubation with 0.54 mM T-2 toxin.
Abbreviations: BPNP, bis-4-nitrophenyl phosphate; 4OH-MB, 4-hydroxy mercurybenzoate.

Table 4. *Hydrolysis of 0.54 mM T-2 toxin by white blood cells isolated from rat*

Cell fraction	nmole HT-2 produced (mg protein·hr)	CarbE activity μmole 4NPB (mg protein·hr)
Monocytes	34 ± 9	2.7 ± 0.9
Lymphocytes	38 ± 5	1.4 ± 0.3
Granulocytes	49 ± 5	0.16 ± 0.04
Trombocytes	56 ± 2	1601 ± 222

The numbers are mean values ± SD, N = 3–4 animals.

that these cells possess carboxylesterase activity. This activity, however, did not correlate with T-2 toxin hydrolysis, indicating that 4-nitrophenylbutyrate is not a specific substrate for the isoenzyme responsible for T-2 hydrolysis, in agreement with a previous study at our laboratory on the heterogeneity of the carboxylesterases in liver [7].

DISCUSSION

Previous *in vitro* studies on metabolism of T-2 toxin have concentrated on liver preparations. The only report on screening of hydrolytic activity in organs other than liver is made by Ohta *et al.* [6]. In addition to the main metabolic organ which is the liver they found some activity in brain, kidney, spleen and intestine, but they did not find any detectable activity in serum or blood cells. However, because of the evidence for esteratic activity in blood cells stated earlier [12–14], the present study was designed to examine for possible trichothecene hydrolytic activities in blood cells.

In the present work, both human and rat blood cells were shown to convert T-2 toxin to polar and less toxic metabolites more easily excreted, thus indicating that the blood cells take part in the detoxification of T-2 toxin. With respect to the verification of trichothecene mycotoxins in blood samples drawn from animals or humans, it should be brought in mind that hydrolysis does occur even after sampling if not appropriately stored by immediate freezing or otherwise inactivating hydrolysing activity in blood samples.

We conclude that the metabolism of T-2 toxin by red and white blood cells is performed by carboxylesterase since it was completely blocked by the serine esterase inhibitors paraoxon and soman, and that physostigmin at 10^{-5} M did not inhibit the hydrolysis, thus excluding the cholinesterase. Furthermore, we could exclude arylesterase and phosphoryl phosphatase since it was not inhibited by EDTA or 4-hydroxy mercurybenzoate, respectively. The carboxylesterases in both red and white blood cells are characterized by being less sensitive to BPNP, in accordance with the finding in a previous study of T-2 toxin metabolism by rat liver carboxylesterase pI 5.4 [7]. The six different rat liver carboxylesterases have recently been identified by Mentlein *et al.* [21] as alloenzymic forms of ES-3, ES-4, ES-8/ES-10 and ES-15 according to the genetic nomenclature recommended by Van Zutphen [22]. Carboxylesterase pI 5.4 corresponding to pI 5.6 according to

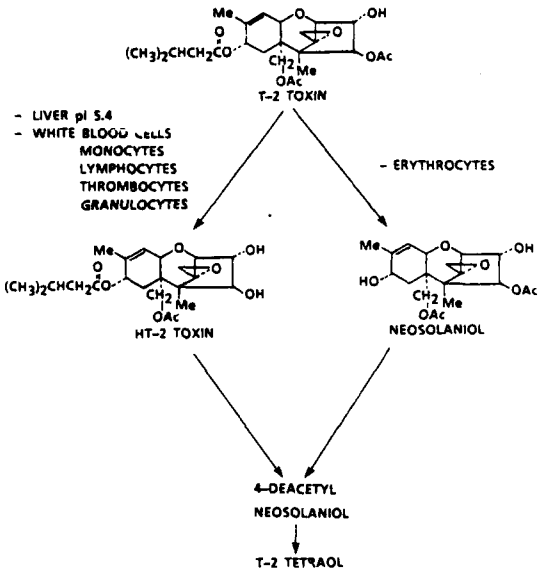


Fig. 2. Different pathways for the hydrolysis of T-2 toxin by carboxylesterases in liver and blood cells.

Mentlein *et al.* [23] was genetically defined as ES-3 [21]. According to the low sensitivity to BPNP for pI 5.4 enzyme hydrolysing T-2 toxin, which was also found for the carboxylesterases in red and white blood cells, and the fact that they all hydrolyse T-2 toxin, it may be suggested that the carboxylesterases present in the blood cells are of the same identity as liver carboxylesterase pI 5.4. On the other hand, small amounts of a nonspecific esterase defined as ES-13 with low affinity to BPNP have been demonstrated in liver and red blood cells with pI around 5.0 [24]. ES-13 can, however, not be responsible for the hydrolysis of T-2 toxin in liver because of their different pI-values, but it cannot be excluded as the enzyme responsible for the hydrolysis of T-2 toxin to neosolaniol in the red blood cells.

Of particular interest was the finding that neosolaniol is a major metabolite in addition to HT-2 toxin in blood. This metabolite is formed by hydrolysis of T-2 toxin at the isovaleryl group in C-8 position whereas HT-2 toxin is formed by hydrolysis of T-2 toxin at the acetyl group in C-4 position (Fig. 1). The C-4 acetyl residue has been found to be the preferential site for microsomal hydrolysis in liver and the substituents at C-3 and C-8 contribute to the selective enzymatic hydrolysis of the C-4 acetyl residue of trichothecenes [25]. Neosolaniol has been considered only as a trace or minor metabolite presumably formed by hydrolysis of T-2 toxin by dermal microflora [9, 10]. However, we provide evidence for erythrocytes as a specific site for enzymatic hydrolysis of T-2 toxin to neosolaniol, thus representing a metabolic pathway different from that of T-2 to HT-2 toxin, which is the pathway for T-2 toxin hydrolysis in liver and white blood cells. In fact, the hydrolytic activity in rat blood was completely separated into a HT-2 toxin producing pathway in white blood cells and another in red blood cells producing neosolaniol as primary metabolite. It was therefore concluded that the carboxylesterase in erythrocytes is different from carboxylesterase isoenzyme pI 5.4 in liver and the carboxylesterase present in the white blood cells. It may well be that the rigid structure of trichothecenes makes these an ideal substrate for characterizing certain groups of carboxylesterases. A summary of the two different pathways for hydrolysing T-2 toxin is shown in Fig. 2.

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