TOXICOLOGIC AND ANALYTICAL STUDIES WITH T-2 AND RELATED TRICHOTHECENE MYCOTOXINS

ANNUAL/FINAL REPORT
FOR THE PERIOD JULY 1, 1982, THROUGH JUNE 30, 1985

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AUGUST 20, 1985

SUPPORTED BY
U. S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-82-C-2179

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SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

Form Approved
OMB No 0704-0188
Exp. Date Jun 30, 1986

1a. REPORT SECURITY CLASSIFICATION: Unclassified	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION/AVAILABILITY OF REPORT: Approved for public release; distribution unlimited
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION: University of Illinois College of Veterinary Medicine; 6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION
6c. ADDRESS (City, State, and ZIP Code): Urbana, Illinois 61801	7b. ADDRESS (City, State, and ZIP Code)
8a. NAME OF FUNDING/SPONSORING ORGANIZATION: U.S. Army Medical Research & Development Command; 8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER: DAMD17-82-C-2179
8c. ADDRESS (City, State, and ZIP Code): Fort Detrick, Frederick, Maryland 21701-5012	10. SOURCE OF FUNDING NUMBERS

PROGRAM ELEMENT NO.	PROJECT NO.	TASK NO.	WORK UNIT ACCESSION NO
63763A	3M263, 763A807	AK	034

11. TITLE (Include Security Classification)
(U) Toxicologic and Analytical Studies With T-2 and Related Trichothecene Mycotoxins

12. PERSONAL AUTHOR(S)
William B. Buck, etc.

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Annual/Final*	FROM 7/1/82 TO 6/30/85	1985 August 20	480

16. SUPPLEMENTARY NOTATION
* Annual for the period 16 August 1984 - 30 June 1985
Final for the period 1 July 1982 - 30 June 1985

17. COSATI CODES		
FIELD	GROUP	SUB-GROUP
06	13	
06	03	

18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
T-2 toxin, T-2 toxicosis, T-2 pathophysiology, histopatholo; skin, therapy, superactive charcoal, T-2 production, T-2 metabolism, T-2 analysis

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

Swine and rats have been used to assess the effects of exposure to T-2 toxin. Acute systemic T-2 toxicosis is a cardiovascular shock syndrome characterized by reductions in cardiac output and blood pressure and increased plasma concentrations of epinephrine, norepinephrine, thromboxane B_2, 6-keto-$PGF_{1\alpha}$, and lactate. In swine, myocardial, brain, renal, splenic, and pancreatic blood flow decreases, while that of the adrenals, liver, and gastrointestinal tract increases or is not affected following T-2 toxin administration. Sublethal and/or lethal intravenous (IV) injections of T-2 toxin produce heart and pancreatic lesions in addition to the well-documented lesions in lymph nodes and gastrointestinal tissues.

Inhalation of T-2 toxin causes clinical signs and lesions which are similar to those in IV dosed pigs. Exposed pigs have lower rates of weight gain and reduced hemagglutination titers. The pulmonary alveolar macrophages

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
☐ UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT ☐ DTIC USERS	

22a. NAME OF RESPONSIBLE INDIVIDUAL	22b. TELEPHONE (Include Area Code)	22c. OFFICE SYMBOL
Mrs. Judy Pawlus	301-663-7325	SGRD-RMI-S

DD FORM 1473, 84 MAR — 83 APR edition may be used until exhausted. All other editions are obsolete

SECURITY CLASSIFICATION OF THIS PAGE

19. Abstract (continued)

of T-2-dosed pigs have a reduced ability to phagocytize bacteria, and their pulmonary lymphocytes have lowered blastogenic responses. Lung tissue alterations are minimal.

Topical application of T-2 toxin in swine produces a severe necrotizing dermatitis with the healing process beginning by day 7. Morphologic changes in internal organs are minimal. Immune responses are altered, and there is neutrophilia, fever, and decreased body weights in treated pigs. There are significant reductions in serum albumin, alkaline phosphatase, and glucose, with increases in serum globulin during the first 2 weeks after exposure.

Numerous potential therapeutic agents were tested in rats and swine for efficacy against T-2 toxicosis. In rats, only methylprednisolone had apparent benefit. In swine, dexamethasone alone or in combination with activated charcoal, fluid support, and/or sodium bicarbonate administration ameliorated some, but not all, of the adverse physiological changes associated with supralethal T-2 toxicosis. A new "superactive" activated charcoal has excellent adsorptive capacity for T-2 toxin. Oral administration of this adsorbent, after oral lethal doses of T-2 toxin, significantly prolonged the survival of rats. Since a major portion of absorbed T-2 toxin and its metabolites are excreted into the gastrointestinal tract, activated charcoal may be beneficial for treatment as well as prevention of systemic trichothecene toxicosis, regardless of the route of exposure.

Scirpenetriol, 15-MAS, de-epoxy MAS, and de-epoxy scirpenetriol were the major metabolites produced in rats orally dosed with DAS. Rats dosed orally, dermally, or intravenously with T-2 toxin excreted 15 to 22 percent of the dose in their urine and 60 to 90 percent in their feces by 7 days after dosing. Four hours after pigs were dosed intravenously with T-2 toxin, the major free metabolites were 3'OH HT-2 and T-2 triol in the bile and urine (20 to 30 percent of administered dose). Glucuronide conjugates represented 63 to 77 percent of the dose, with the major conjugated metabolites being HT-2, T-2 toxin, 3'OH HT-2, and 3'OH T-2. The gastrointestinal tract contained 15.5 to 24.2 percent of the dose, while the remaining tissue contained 4.7 to 5.2 percent. The major free metabolite in tissue has not been characterized.

T-2 toxin is biotransformed by rat liver microsomes to HT-2, neosolaniol, 4-deacetylneosolaniol, T-2 triol, 3'OH T-2, and 3'OH HT-2. Esteratic cleavage is more extensive than hydroxylation of the 3' carbon side chain. In an *in vitro* system utilizing bovine rumen fluid, T-2 toxin, DAS, and DON are converted to a variety of de-epoxide compounds including de-epoxy HT-2, de-epoxy triol, de-epoxy MAS, de-epoxy scirpenetriol, and de-epoxy DON (DOM-1), respectively.

Exposure to trichothecene mycotoxins (T-2, DAS, and DON) can be detected with a rapid and relatively simple analytical procedure based on the total hydrolyzed metabolites in plasma and urine.

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FOREWORD

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

This document was prepared for the period of July 1, 1982, through June 30, 1985, but was updated in April 1987 to reflect the identification of a new metabolite and to provide reference citations that were "In Press" at the time of original submission.

ABSTRACT

Swine and rats have been used to assess the effects of exposure to T-2 toxin. Acute systemic T-2 toxicosis is a cardiovascular shock syndrome characterized by reductions in cardiac output and blood pressure and increased plasma concentrations of epinephrine, norepinephrine, thromboxane B_2, 6-keto- $PGF_{1\alpha}$, and lactate. An initial leukocytosis is followed by a leukopenia. Serum-bound calcium concentrations decrease, while magnesium, phosphorus, and potassium increase. There was greater variation in the levels of selected cardiac bulk electrolytes of rats dosed with T-2 toxin than in control animals. In swine, myocardial, brain, renal, splenic, and pancreatic blood flow decreased, while that of the adrenals, liver, and gastrointestinal tract was increased or not affected following T-2 toxin administration.

Sublethal and/or lethal intravenous injections of T-2 toxin produce heart and pancreatic lesions, in addition to the well-documented radiomimetic lesions. Grossly, there are subendocardial hemorrhages, pinpoint white foci in the myocardium, and pancreatic edema. Microscopic and ultrastructural changes in the heart include myofiber degeneration, vacuolization, necrosis, and mineralization with formation of hypercontraction bands. Pancreatic changes consist of acinar degeneration and necrosis which progress to a diffuse suppurative necrotizing pancreatitis.

Techniques have been developed for using a closed-system inhalation procedure for monitoring dose-response effects of T-2 aerosol exposure. Inhalation of T-2 toxin causes clinical signs and lesions which are similar to those in IV dosed pigs. Exposed pigs have lower rates of weight gain and reduced hemagglutination titers. The pulmonary alveolar macrophages of T-2-dosed pigs have a reduced ability to phagocytize bacteria, and their pulmonary lymphocytes had lowered blastogenic responses. Lung tissue alterations were minimal or not observed.

Topical application of T-2 toxin in swine produces a severe necrotizing dermatitis with the healing process beginning on day 7. Morphologic changes in internal organs are minimal. Responses of purified peripheral blood lymphocytes to the T-cell mitogens, phytohemagglutinin, and concanavalin A are decreased between days 20 and 31 after dosing. Neutrophilia and fever are apparent during the first 2 weeks of exposure. Body weights of treated pigs decrease after the first week. There are significant reductions in serum albumin, alkaline phosphatase, and glucose but increases in serum globulin in the first 2 weeks.

Numerous potential therapeutic agents have been tested in rats and swine for efficacy against T-2 toxicosis. In rats, only methylprednisolone had apparent benefit. In swine, dexamethasone alone or in combination with activated charcoal, fluid support, and/or sodium bicarbonate administration ameliorated some, but not all, of the adverse physiological changes associated with

supralethal T-2 toxicosis. A new "superactive" activated charcoal has excellent adsorptive capacity for T-2 toxin in rats. Since a major portion of absorbed T-2 toxin and its metabolites are excreted into the gastrointestinal tract, activated charcoal may be beneficial for treatment, as well as prevention, of systemic trichothecene toxicosis, regardless of the route of exposure.

Substantial quantities of purified T-2 toxin (80 g) were produced by culturing a strain of Fusarium fungus and isolating and purifying the toxin. A number of T-2 metabolites were produced by using the S-9 fraction from rat livers (3'OH T-2 and 3'OH HT-2) and an in vitro system utilizing bovine rumen fluid (de-epoxy compounds).

Scirpenetriol, 15-MAS, de-epoxy MAS, and de-epoxy scirpenetriol were the major metabolites produced in rats orally dosed with DAS. Rats dosed orally, dermally, and intravenously with T-2 toxin excreted 15 to 22 percent in their urine and 60 to 90 percent in their feces 7 days after dosing. In pigs dosed intravenously with T-2 toxin, after 4 hours the major free metabolites were 3'OH HT-2 and T-2 triol in the bile and urine (20 to 30 percent of administered dose). Glucuronide conjugates represented 63 to 77 percent of the dose with the major conjugated metabolites being HT-2, T-2 toxin, 3'OH HT-2, and 3'OH T-2. The gastrointestinal tract contained 15.5 to 24.2 percent of the dose, while the remaining tissue contained 4.7 to 5.2 percent. The major free metabolite in tissue has not been characterized.

T-2 toxin is biotransformed by rat liver microsomes to HT-2 neosolaniol, 4-deacetylneosolaniol, T-2 triol, 3'OH T-2, and 3'OH HT-2. Esteratic cleavage is more extensive than hydroxylation of the 3' carbon side chain. In an in vitro system utilizing bovine rumen fluid, T-2 toxin, DAS, and DON are converted to a variety of de-epoxide compounds including de-epoxy HT-2, de-epoxy triol, de-epoxy MAS, de-epoxy scirpenetriol, and de-epoxy DON (DOM-1), respectively.

Trichothecene exposure (any of T-2, DAS, DON) can be determined with a rapid and easy procedure with analyzes for total hydrolyzed metabolites in plasma and urine.

EXECUTIVE SUMMARY

I. Pathophysiology of T-2 Toxicosis

Swine and rats were used to study toxic effects of T-2 toxin, diacetoxyscirpenol (DAS), and deoxynivalenol (DON), common trichothecene fungal toxins. Acute toxicosis from T-2 and DAS is a cardiovascular shock syndrome similar to, but distinct from, that of an endotoxin. The syndrome is similar following exposure by oral, inhalation, or intravascular routes. As documented after intravenous administration, effects include (1) rapid drop in cardiac output, metabolic acidosis, increased plasma levels of prostaglandins, as well as epinephrine and norepinephrine and (2) decreased blood flow to vital organs including heart and pancreas. The parent toxins, T-2 and DAS, have plasma and tissue half-lives of 14 to 17 minutes, while DON has a slightly longer half-life. Lesions of T-2 toxin and DAS toxicosis included cardiac and pancreatic necrosis, in addition to radiomimetic lesions. Methylprednisolone was of benefit in treating T-2 toxicosis in rats, while dexamethasone, activated charcoal, and fluid support were useful in swine. A new "superactive" activated charcoal was effective in preventing oral toxicosis in rats. T-2 toxin, once absorbed, is primarily metabolized by the liver. The major metabolites include 3'OH T-2, HT'-2, 3'OH HT-2, and especially their glucuronide conjugates (in swine), which are excreted via bile (feces) and urine. Because of probable enterohepatic recycling of toxin and metabolites, activated charcoal may be an effective therapeutic agent in toxicosis induced by a variety of routes of administration.

A,B. Hemodynamics, Vasoactive Mediators, Hematology and Serum Biochemistries

T-2 toxin was given as a single intravascular dose at either 0.6 or 4.8 mg/kg to different groups of 50 kg, female swine. Shock was characterized by reductions in cardiac output and blood pressure and increased plasma concentrations of epinephrine, norepinephrine, thromboxane B_2, 6-keto-$PGF_{1\alpha}$, and lactate. Total peripheral resistance was unchanged in the high-dose group but decreased in the low-dose group. Pulmonary vascular resistance increased in both groups. Decreases occurred in arterial pH and the partial pressure of arterial oxygen. No alterations occurred in plasma concentrations of histamine or serotonin.

Blood samples were taken at hourly intervals for determination of concentrations or activities of the following substances in serum or plasma: creatinine, blood urea nitrogen, inorganic phosphorus, total calcium, ultrafilterable calcium, magnesium, sodium, potassium, chloride, total protein, albumin, cholesterol, glucose, alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase, and total bilirubin. Coagulation analyses included prothrombin time, partial thromboplastin time, activated coagulation time, and fibrin degradation products. The serum concentration of bound calcium decreased, while phosphorus, magnesium, and potassium increased. Clinical screening tests detected no evidence of a coagulopathy.

Red blood cell, white blood cell, and platelet counts; hemoglobin concentrations; and hematocrits were determined from whole blood samples. An initial leukocytosis was followed by a leukopenia. The numbers of red blood cells, hemoglobin concentration, and hematocrit were increased. Nucleated red blood cells were seen in the blood smears.

C. Systemic Distribution of Blood Flow During T-2 Toxicosis

Additional studies were conducted with swine dosed intravascularly with T-2 toxin at 0, 0.6, and 2.4 mg/kg to determine if alterations occurred in blood flow to vital tissues of the body. Hemodynamic measurements were also performed.

Cardiac output declined in both T-2 toxin-treated groups for up to 3 hours, after which the high-dose group continued to decline and the low-dose group began to rise. Aortic mean blood pressure of the high-dose group did not begin decreasing until 1-1/2 hours after exposure. The heart rate and pulmonary vascular resistance were increased in both groups. Left and right ventricular work decreased following administration of T-2 toxin at both the high and low doses. Blood gas values indicated maintenance of arterial oxygen tension, slight decreases in arterial carbon dioxide tensions, and intense reductions in pH.

Myocardial and brain blood flow had similar patterns of changes following T-2 administration. Both decreased following administration of 2.4 mg/kg, and both had marked fluctuations in blood flow

following administration of 0.6 mg/kg. Blood flow to the adrenals, liver and the overall gastrointestinal tract of animals dosed with T-2 toxin was increased or similar to predosing and vehicle control values. Renal, splenic, and pancreatic blood flows decreased following T-2 administration, with blood flow to the pancreas and spleen being the most severely compromised.

D. Inhalation Studies

1. A method of administration of aerosols to anesthetized or unanesthetized swine.

Considerable effort has been invested in developing a system for studying the effects of inhaling an aerosol of T-2 toxin on swine. Techniques have been worked out, and protocols have been developed for the following: (1) animal restraint and monitoring, (2) toxin handling and administration, (3) aerosol generation and clean-up, and (4) aerosol characterization. These efforts have resulted in the ability to produce sublethal or lethal T-2 toxicosis by the inhalation route of exposure. Although we are able to estimate the dose of toxin retained by the pigs, a more reproducible and accurate method, using a radioactive isotope, is under development. This technique will allow more meaningful assessment of dose-reponse relationships and comparisons with the other routes of administration.

Two inhalation studies were performed. The first study was designed to evaluate the effects of inhaled T-2 toxin at

nebulized doses of 0 and 8 mg/kg (calculated dose of retained T-2 less than 3 mg/kg) on the clinical signs, clinical pathology, and systemic immunity of swine. All T-2 treated pigs experienced vomition, hyperemia, lethargy, and anorexia, but no significant changes in the mean rectal temperature. The T-2 treated pigs had significantly lower mean body weight gains. Significantly lower hemagglutination titers to sheep red blood cells were noted between days 3 and 7 after the first immunization in T-2 treated pigs. The data for clinical pathology and mitogen-induced blastogenic responses of peripheral blood lymphocytes have been entered on a computer, and the analyses are currently underway.

The second swine inhalation study was designed to evaluate the effects of inhaled T-2 toxin at nebulized doses of 0 and 9 mg/kg (calculated dose of retained T-2 less than 3 mg/kg) on lung morphology and both local pulmonary and systemic immunity. Two T-2 treated pigs died between 8 and 16 hours after treatment. One T-2 treated pig was killed in a moribund state approximately 16 hours after exposure. All other T-2 treated pigs survived the exposure and experienced similar clinical signs as the pigs in the first study. The T-2 treated pigs that died spontaneously or were killed on day 0.33 after inhalation exposure had typical radiomimetic changes in the lymphoid organs and GI tract. The changes, however, were minimal in the pigs surviving the T-2

treatment when killed on postdosing days 1, 3, and 7. Mild to moderate, multifocal, acute injury with cellular infiltration, mainly neutrophils, were seen in the alveoli of the expired T-2 treated pigs. Alveolar injury was also seen in pigs surviving the dosing and killed on days 1, 3, and 7; however, the lesions were patchy and lass severe than those of the pigs which died or were killed on day 0.33 and the cellular infiltration was mainly macrophages. The alveolar macrophages of T-2 treated pigs had a lower capacity for bacterial phagocytosis than those of control pigs, especially those of pigs that died or were killed on days 0.33 and 1. The pulmonary lymphocytes of T-2 treated pigs also showed lower blastogenic responses to various mitogens than those of control pigs. The data of mitogen-induced blastogenic responses of peripheral blood lymphocytes have been entered on a computer, and the analyses are currently underway.

E,F. Ultrastructural Study of Acute T-2 Toxicosis

The pathologic effects of T-2 toxicosis were evaluated in 8 pigs. They were administered T-2 toxin in intravenous doses of 0.0 mg/kg (2 pigs) and 0.6 mg/kg (6 pigs) dissolved in 2.5 mL of 50 percent ethanol and were killed 24 and 48 hours later. On gross examination, pancreatic edema, multifocal subendocardial hemorrhages, and pinpoint white foci were present scattered throughout the myocardium of one pig killed at 48 hours. Myofiber degeneration and necrosis with contraction bands were seen in all T-2-dosed pigs, mainly in the

subendocardial region. Although the lesions were present throughout the heart, they were predominant in the atria, papillary muscles of the left ventricle and lower left and upper right ventricles. In addition, myofiber vacuolization was another morphological alteration observed in some affected muscle bundles. Vacuolization was more often detected in papillary muscles of the left ventricle. Ultrastructural changes consisted of areas of sarcoplasmic edema with myofibrillar disorganization and loss of Z and M bands, as well as glycogen accumulation in mildly affected myocytes. In severely damaged myocytes, hypercontraction bands with myofibrillar lysis or marked distension of sarcoplasmic reticulum with myofibrillar lysis was evident.

Pancreatic changes consisted of multifocal acinar degeneration and necrosis. These changes became a suppurative necrotizing pancreatitis in the pigs killed at 48 hours. Early ultrastructural changes consisted of dilation of the membranous portion of the rough endoplasmic reticulum and disorganization, as well as mitochondrial swelling and loss of cristae.

Tissue lesions in pigs exposed to T-2 toxin by the inhalation route of exposure were, in general, similar to the IV-dosed animals. No ultrastructural changes have been observed in sections of the lung or pancreas. Lesions, including endothelial damage, were observed in the livers of two pigs.

Evaluation of tissues is still in progress and will be completed by the end of this year.

G. Cardiac Bulk Electrolytes

Cardiac bulk electrolytes (sodium, potassium, magnesium, and calcium) were determined in both a T-2 toxin dosed and a control group of rats. Those rats which received T-2 toxin appeared to show larger intergroup variations in the levels of those electrolytes than did the control rats.

H. Subacute Toxicity and Immunotoxicity of Topically Applied T-2 Toxin in Swine

Topical application of 0 and 15 mg/kg T-2 toxin in 0.75 mL DMSO was performed in swine. Representative animals were subsequently killed for pathologic examination on days 1, 3, 7, and 14. Grossly, the areas of skin exposed to T-2 toxin were swollen and progressively became dark red to dark purple. The affected skin became ulcerated after day 7 and was focally separated from the underlying tissue at the border of the exposure area on day 14. Histologically, the initial lesion consisted of degeneration of the stratum germinativum of the epidermis and mild neutrophilic infiltration of the upper dermis. The extent and severity of the changes then increased gradually with severe, necrotizing dermatitis apparent on days 7 and 14. Hyperkeratosis, hyperplasia, and fibroplasia began to appear on day 7 and became more prominent on day 14. The changes in the internal organs were minimal.

The immunotoxicity of dermally-applied T-2 toxin was evaluated in a group of 14 pigs which received the same treatment as described

above and were bled on days -2, 1, 3, 5, 7, 10, 14, 20, 22, 24, 26, 28, and 31. The purified peripheral blood lymphocytes of pigs exposed to T-2 toxin had significantly lower responses to the T-cell mitogens phytohemagglutinin and concanavalin A approximately 20 days after dosing, but the differences became nonsignificant by day 31. Significantly higher responses to the B-cell mitogen, lipopolysaccharide, were seen in the treated group during the first 10 days or so. No differences were found in anti-sheep red blood cell antibody titers between treated and control groups. Significant neutrophilia was seen in the T-2 toxin-treated group during the first 2 weeks. The test group had significantly lower levels of serum albumin after 1 week of exposure, but significantly higher levels of serum globulin after 5 days of exposure. Serum alkaline phosphatase activities in the test group were significantly lower than those of the control group after day 3 of exposure. Blood glucose of the treated group was significantly lower during the first 2 weeks. A significant increase in the rectal temperatures of T-2-treated pigs occurred in the first 2 weeks. Significant differences between mean body weights of T-2-treated and control groups were noted after the first week of treatment.

II. Therapeutic Studies

A-C. Rat and Swine Therapeutic Studies

The water-soluble glucocorticosteroid, methylprednisolone sodium succinate, and dexamethasone sodium phosphate appear to be

efficacious in the treatment of acute T-2 toxicosis in rats and swine, respectively. In swine, dexamethasone sodium phosphate when combined with other therapy such as activated charcoal, fluid support, and/or sodium bicarbonate administration ameliorated some but not all of the adverse physiological changes noted following the induction of supralethal acute T-2 toxicosis. Certain other therapeutic approaches utilized in either rats or swine have not proven to significantly enhance survival duration or rate following T-2 toxin administration.

D. Studies with Oral "Superactive" Charcoal

Studies with oral "superactive" charcoal have shown that it is effective in preventing toxicosis in rats given an oral LD_{100} dose of T-2 toxin. Since our laboratory has found that T-2 toxin and its metabolites are excreted into the gastrointestinal tract following intravascular exposure, activated charcoal may be beneficial for treatment, as well as prevention, of systemic trichothecene toxicosis, regardless of the route of exposure. To date, studies with swine appear to support that hypothesis.

E. Therapeutic Oral Activated Charcoal Studies

Efforts have been directed in three areas: (1) determining the lowest dose of superactivated charcoal which is effective in preventing deaths in rats due to oral T-2 toxin administration; (2) assessing the benefit, if any, that would be gained from concomitant use of activated charcoal and different cathartics after oral T-2

toxin administration to rats; and (3) determining how long after oral dosing with T-2 toxin an activated charcoal regimen can be delayed and still be effective in alleviating toxicosis.

F. Prevention and Treatment of T-2 Toxicosis in Dermally Exposed Swine

Attempts have been made to induce clinical signs of T-2 toxicosis in swine exposed to the toxin dermally. Exposure to up to 45 mg/kg body weight T-2 toxin did not result in classic signs of acute T-2 toxicosis or death. The results and the quantities of toxin necessary for these investigations have suggested that the pig is not a good animal model for studying systemic signs of T-2 toxicosis after dermal application of the toxin. However, it does cause intense local necrosis of the skin of pigs, and we are investigating methods of preventing these effects.

III. Production of Toxins

We have isolated Fusarium fungi from naturally contaminated field samples and are examining them for potentially toxigenic strains. At this time, we have about 40 stains and have tested some of them for the ability to produce toxin.

We have produced over 80 g of purified T-2 toxin along with milligram quantities of several trichothecene metabolites since September of 1982 for use in a variety of experiments. T-2 toxin was produced by culturing a strain of Fusarium (3299) on a rice or vermiculite media. The cultures were extracted with ethyl acetate, and the extract was cleaned up using Florisil columns followed by multiple recrytallizations. T-2 toxin of greater than 99 percent purity has been produced by this method.

3' OH-T-2 is produced from T-2 toxin by using the liver S-9 fraction from rats pretreated with phenobarbital combined with the addition of paraoxon to inhibit enzymatic hydrolysis of the T-2 substrate. It is difficult to obtain standards of the 3'hydroxylated compounds; the only other procedure presently available includes extracting these metabolites from the excreta of animals dosed with T-2 toxin or lengthy synthesis reactions.

De-epoxide trichothecenes have been produced by incubating T-2 toxin, DAS, and DON in vitro with rumen fermentation preparations using rumen fluid obtained from a fistulated cow. After incubation in bovine rumen fluid, the compounds were extracted using an XAD resin column, a Florisil column, and HPLC. Milligram quantities were obtained with greater than 95 percent purity.

IV. Analytical Methods

A technique was developed which results in a rapid and easy method to screen for several trichothecenes (T-2 toxin, DAS, and DON) in plasma and urine. The compounds were extracted using a Clin Elute column followed by hydrolysis under basic conditions. The hydrolyzed samples were cleaned up with a silica cartridge and derivatized for analysis using gas chromatography. Detection of the parent alcohol would be an indication of trichothecenes in the plasma or urine.

A method was developed to analyze for DON and its de-epoxy metabolite DOM-1 in bovine urine and feces to aid in diagnosis of DON exposure in cattle. The mycotoxin compounds were extracted using Clin Elute columns

and disposable silica cartridges. Trimethylsilyl derivatives of extracts were formed and examined by gas chromatography with electron capture detection.

V. Metabolism: In vivo

Rats were orally dosed with DAS at 2.8 mg/kg. The metabolites found in the urine and feces were 15-MAS, scirpenetriol, and two de-epoxide compounds determined to be de-epoxy MAS and de-epoxy scirpenetriol by GC/MS and NMR. The metabolites 15-MAS, scirpenetriol, de-epoxy MAS, and deepoxy scirpenetriol were detected in urine at 3.5 percent, 4.9 percent, 9.5 percent, and 7.2 percent, respectively. The latter two compounds were also detected in feces at 9.5 percent and 18.9 percent, respectively. No parent compound was found in the urine or feces.

Rats were dosed with tritium labeled T-2 toxin at 0.60 mg/kg and 0.15 mg/kg by oral, dermal and intravenous routes. Urine and feces were collected for a total of 7 days at 24-hour intervals. After 7 days, 15 to 22 percent of the administered dose was found in the urine, and 60 to 90 percent was found in the feces. Separation of the metabolites was done by HPLC after extraction of the urine and feces. Hydrolyzed metabolites were the primary compounds isolated along with several unknowns which have not been characterized at this time.

Metabolite profiles in the bile and urine of two swine were determined following intravascular administration of tritium labeled T-2 toxin. A total of 13.1 percent and 1.3 percent of the dose was found in the gallbladders in addition to 17.9 and 42.5 percent in the urine of the

two swine 4 hours after dosing. Free metabolites represented less than 20 and 30 percent of the total metabolite residues in bile and urine, respectively, with the parent compound never exceeding 0.25 percent. The major free metabolites were 3'OH HT-2 and T-2 triol. Glucuronide conjugates represented 63 and 77 percent of the metabolite residues in urine and bile, respectively. The major conjugated metabolites were glucuronides of HT-2, 3'OH T-2, 3'OH HT-2 and T-2 toxin. Neosolaniol, 4-deacetylneosolaniol, and T-2 tetraol were also identified in addition to three unknown metabolites.

Metabolite profiles in plasma and tissues of two swine were determined following intravascular administration of tritium labeled T-2 toxin. The plasma elimination phase half-life was 90 minutes for total tritium residues. The greatest amount of radioactivity was located in the gastrointestinal tract (15.5 and 24.1 percent of the dose for the two swine) 4 hours after administration. The remaining tissues represented 5.2 and 4.7 percent of the dose for the two swine. Twenty-one metabolites were identified following reverse phase HPLC. Approximately 55 percent of the extractable radioactivity in the tissues and gastrointestinal tract of both swine corresponded to T-2 toxin, HT-2, de-epoxy HT-2, T-2 triol, deepoxy T-2 triol, 3'OH T-2, 3'OH HT-2, T-2 tetraol, and de-epoxy T-2 tetraol. The major metabolite in tissues did not correspond to any standard and represented an additional 27 percent of the extractable radioactivity. Overall, identified metabolites in urine, tissues, and the gastrointestinal tract accounted for 55 to 75 percent of the total radioactivity administered.

VI. Metabolism: In vitro

T-2 toxin was biotransformed by rat liver microsomes to a variety of metabolites including HT-2, neosolaniol, 4-deacetylneosolaniol, T-2 triol, 3'OH T-2, and 3'OH HT-2. Metabolism resulting from esteratic cleavage of the parent T-2 toxin was more extensive than hydroxylation of the 3' carbon side chain by mixed function oxidases. Pretreatment of rats with phenobarbital enhanced hydroxylation of T-2 toxin at the 3' carbon position, and the addition of paraoxon to the microsomes inhibited hydrolysis of the C3' oxidized product.

DAS, DON, and T-2 toxin were incubated in vitro for 0, 12, 24, and 48 hours with rumen microorganisms obtained from a fistulated dairy cow. Gas chromatograph and GC/MS analysis of the extracts indicated that all three toxins were biotransformed to a variety of de-epoxy and deacylated products. DON was partially converted to a single product identified as de-epoxy DON or DOM-1. DAS was completely converted to 15-MAS, scirpenetriol, and two new products identified as de-epoxy MAS and de-epoxy scirpenetriol. T-2 toxin was biotransformed to HT-2, T-2 triol, and two new products identified as de-epoxy HT-2 and de-epoxy triol. Determination of the rumen microflora responsible for the de-epoxidation of the toxins was attempted; however, at this point, the samples have not been evaluated to determine the microfloral identities.

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I. PATHOPHYSIOLOGY OF T-2 TOXICOSIS

A. EXPERIMENTAL T-2 TOXICOSIS IN SWINE. I. CHANGES IN CARDIAC OUTPUT, AORTIC MEAN PRESSURE, CATECHOLAMINES, 6-KETO-$PGF_{1\alpha}$, THROMBOXANE B_2 AND ACID-BASE PARAMETERS

--Roseanne M. Lorenzana, Val R. Beasley, William B. Buck, Arthur W. Ghent, Gregg R. Lundeen, Robert H. Poppenga

ABSTRACT

Experimental T-2 Toxicosis in Swine. I. Changes in Cardiac Output, Aortic Mean Pressure, Catecholamines, 6-keto-$PGF_{1\alpha}$, Thromboxane B_2 and Acid-Base Parameters. Lorenzana, R.M., Beasley, V.R., Buck, W.B. (1984). Fundam. Appl. Toxicol. T-2 Toxin given as a single intravascular dose to swine produced a shock syndrome. Dosages of 0.6 or 4.8 mg/kg were administered to different groups of swine. Shock was characterized by reductions in cardiac output and blood pressure, and increased plasma concentrations of epinephrine, norepinephrine, thromboxane B_2 and 6-keto-$PGF_{1\alpha}$ and lactate. Total peripheral resistance was unchanged in the high dose group but decreased in the low dose group. Pulmonary vascular resistance increased in both groups. Decreases occurred in arterial pH and arterial oxygen partial pressure. No alterations occurred in plasma concentrations of histamine or serotonin.

INTRODUCTION

T-2 toxin (3a-hydroxy-4β, 15-diacetoxy-8a-(3-methylbutyryloxy)-12, 13-epoxytrichothec-9-ene) is a secondary metabolite of several species of Fusarium. Trichothecene production by Fusaria tends to occur in grain overwintered in the field and in high-moisture grains that have been improperly stored. Although T-2 toxin is not frequently detected in grains used for

animal feed in the United States, when T-2 toxin is identified, it is associated with serious detrimental effects (Hsu et al., 1972; Greenway and Puls, 1976). As early as the 1940's when a widespread human disease called alimentary toxic aleukia (ATA) was recognized in the USSR (Forgacs and Carll, 1962), clinical syndromes in both man and animals were associated with the consumption of moldy grain. Fusarium producers of T-2 toxin were later found to be among the predominant fungi apparently responsible in that outbreak of ATA (Joffe and Yagen, 1977). Historically, Fusarium mycotoxins have been associated with moldy corn toxicosis in cattle in the USA, Akakabibyo (red-mold) disease in humans and animals in Japan, and bean hull poisoning in horses in Japan (Pathre and Mirocha, 1979; Saito and Ohtsubo, 1974; Ueno, 1977). Most controversial of the toxicoses produced by the trichothecenes involves their alleged use by communist forces in chemical weapons in Laos, Cambodia (Kampuchea) and Afghanistan. The popularized pseudonym, "Yellow Rain", refers to the yellowish cloud sprayed from planes or burst from shells in the form of sticky yellow drops. Reported effects include skin rashes, difficult respiration, emesis, hemorrhage and death within a brief period of time in humans, as well as death in exposed animals and plants (Schiefer, 1982). Samples taken from foliage revealed 48 ppm T-2 toxin and the presence of other Fusarium mycotoxins (Rosen and Rosen, 1982). HT-2, a metabolite of T-2 toxin was found in the blood of two alleged victims (Congressional special report, 1982).

In our previous work with swine given T-2 toxin intravascularly, it became apparent that the animals died following a shock syndrome induced by the toxin. At an intravascular LD50 dose (1.2 mg/kg), shock progressed to death within 12-16 hours (Beasley, 1983; Weaver, et al., 1978).

Since the cardiovascular and pulmonary physiology of swine is very similar to that of man, and swine are of sufficient size to allow collection of multiple blood specimens, the pig is considered an appropriate model (Tenney and Remmers, 1963; Dodds, 1982). It was the purpose of this study to characterize the pathophysiology of acute T-2 toxicosis in swine. In this report, the changes in hemodynamic function, blood gases and pH, and vasoactive mediators in response to T-2 toxin given intravascularly are described.

MATERIALS AND METHODS

Chemical and animals

Purified T-2 toxin[3] was dissolved in 6.5 mL of 70 percent ethanol. White, crossbred female swine ranging from 40-60 kilograms were immunized against erysipelas,[4] given an intramuscular injection of selenium and allowed to acclimate to their housing and feed for four to seven days prior to surgical catheterization. After surgery, the animals were allowed to recover for two to four weeks, and were active and in good health at the time of the study. Swine were fed a commercial diet which contained no detectable concentrations of vomitoxin (deoxynivalenol), zearalenone, T-2 toxin, diacetoxyscirpenol (DAS), or aflatoxins B1, B2, G1 and G2.[5]

Surgical preparation

Anesthesia was induced in fasted swine using 5 percent halothane in a mixture of oxygen and nitrous oxide via a nose cone. After endotracheal intubation, anesthesia was maintained with 0.75-1.0 percent halothane in a mixture of oxygen and nitrous oxide using a closed circuit system[6] and intermittent positive pressure ventilation.[7] The heart rate and rhythm were monitored while a left lateral thoracotomy was performed. Non-compliant fluid-filled catheters[8] were implanted in the pulmonary artery and left

atrium via pericardiotomy. Another catheter was placed in the ascending aorta via the internal thoracic artery (Tranquilli et al., 1982).

These catheters were filled with heparin, exteriorized through the lateral thoracic wall and buried in the subcutis until the day of the study. Each animal was given antibiotics post-surgically, and the skin sutures were removed after ten days.

Experimental protocol

In a preliminary group, T-2 toxin was given intravascularly to evaluate procedural protocols, instrumentation, the appearance of the vasoactive substances histamine, serotonin, epinephrine, norepinephrine, and the hydrolysis products of thromboxane A_2 and prostacyclin, thromboxane B_2 (TXB_2), and 6-keto-$PGF_{1\alpha}$, respectively. In this group, two pigs received T-2 toxin at 5.4 mg/kg and one pig received T-2 toxin at 1.2 mg/kg.

In the formal study, pigs were divided into three groups. A lethal dose of 4.8 mg/kg, was given to five swine (high dose group), and a sublethal dose of 0.6 mg/kg was administered to five other pigs (low dose group). Five control swine received only the 70 percent ethanol vehicle intravascularly in addition to undergoing identical surgical and handling procedures.

On the day of the study, the conscious animal was restrained in a webbed stanchion which allowed the pig to rest on its sternum and abdomen while its legs hung below the webbing. All animals quickly adapted to this metnod of restraint and appeared to rest comfortably. After the overlying skin was infiltrated with 2 percent lidocaine HCl, the catheters were exteriorized through small skin incisions and attached via manifolds to measuring and sampling devices.

Prior to dosing two blood samples were withdrawn from the aortic catheter for determination of arterial concentrations of oxygen (PaO_2) and carbon dioxide ($PaCO_2$), as well as arterial pH (pH_a). Multiple cardiac output determinations were made and aortic mean pressure was recorded prior to the collection of blood samples for baseline clinical chemistry determinations. The toxin solution was then administered into the pulmonary artery by a continuous infusion over a five-minute period.[9] The beginning of the infusion was considered to be the starting time of the experiment.

All catheters were frequently flushed with heparinized saline to ensure patency and the absence of clots in the samples. During the course of the experiment, each pig received four liters of fluid due to this flushing. The amount of blood taken for clinical determinations was less than 10 percent of the animal's total blood volume (i.e., 500 mL or less).

In the high dose group, on the day before the study, a self-retaining catheter was passed into the urinary bladder. During the study, blood samples and hemodynamic measurements were taken up to the time of death. In the low dose and control groups, samples and measurements were made from predosing through 24 hours after toxin administration. The animals were then subjected to euthanasia by exsanguination after receiving an anesthetic dose of sodium thiamylal.

Hemodynamic measurements

Pressures in the left atrium, pulmonary artery and aorta were measured every 30 minutes using noncompliant fluid filled systems and transducers[10] on a multichannel physiograph.[11] Transducers were zeroed at the level of the scapulohumeral joint which was considered to correspond to the level of the right atrium.

Cardiac output was determined every 30 minutes by an indicator dilution technique (Manohar, 1978). Indocynanine green[12] was injected into the left atrium and blood was withdrawn from the aorta at a known constant rate through a linear densitometer.[13] The resultant curve area was determined by semilogarithmic plotting of the downslope on a computer.[14]

Total peripheral resistance (TPR) was calculated as the quotient of aortic mean pressure (AOM) divided by cardiac output (CO). Pulmonary vascular resistance (PVR) was calculated by subtracting the left atrial mean pressure (LAM) from pulmonary artery mean pressure (PAM) and dividing the difference by CO.

Clinical chemistry determinations

Arterial blood was anaerobically collected every 30 minutes from the aortic catheter for determination of PaO_2, $PaCO_2$, and pH_a.[15] Blood gas tensions and pH_a were corrected to the animal's rectal temperature, using temperature coefficients published for human blood (Severinghaus, 1966).

Plasma lactic acid concentrations were determined from aortic blood. Blood was collected every 30 minutes in chilled syringes, immediately added to chilled perchloric acid and agitated in a vortex for 30 seconds. The protein precipitate was removed by centrifugation, and the plasma was maintained for no longer than seven days at 4°C before analysis.[16]

Ten mL of aortic blood was collected hourly for catecholamine analysis in chilled, heparinized syringes, immediately transferred to chilled, heparinized glass tubes and gently mixed for 15 seconds before being centrifuged at 2000 g at 4°C for ten minutes. The plasma was transferred to plastic vials and quick-frozen in a dry ice and alcohol bath. The samples were stored at -70°C until analyzed as previously described (Chou et al., 1983).[17]

Aortic blood was collected into chilled syringes every 30 minutes for the first two hours, and thereafter on an hourly basis for the determination of concentrations of 6-keto-$PGF_{1\alpha}$ and TXB_2. The blood was immediately added to siliconized glass tubes containing 2 percent EDTA and 0.4 percent aspirin in saline, gently mixed for 15 seconds and then centrifuged at 2000 g at 4° for ten minutes. Plasma was decanted into plastic vials using plastic pipettes, and the samples were frozen and maintained at -20°C until analysis. The concentrations of 6-keto-$PGF_{1\alpha}$ and TXB_2 were determined by radioimmunoassay.[18] The procedure was previously validated for use with unextracted porcine plasma.

Duplicate samples were included in each assay for the concentrations of 6-keto-$PGF_{1\alpha}$ and TXB_2. If the coefficient of variation for the replicates was greater than 12 percent, the sample was rerun in a subsequent assay. Individual samples were frozen and thawed only once prior to assay and were assayed within a few hours of thawing.

The intra-assay coefficients of variation for TXB_2 and 6-keto-$PGF_{1\alpha}$, respectively, were 4.7 percent and 5.7 percent. The inter-assay coefficient of variation for TXB_2 was 7.4 percent for a high concentration pooled plasma sample and 3.8 percent for a low concentration sample. The inter-assay coefficient of variation for 6-keto-$PFG_{1\alpha}$ was 6.8 percent for a high concentration pooled plasma sample and 15.3 percent for a low concentration sample. The TXB_2 and 6-keto-$PGF_{1\alpha}$ recovery rates were 91.2 percent and 86.1 percent, respectively. The sensitivity limits of the assays were considered to occur at 90 percent binding and the mean values were 6.3 pg/mL for TXB_2 and 17.6 pg/mL for 6-keto-$PGF_{1\alpha}$.

Plasma serotonin and histamine concentrations were determined in the three preliminary swine. Blood for serotonin analysis was collected in chilled

syringes, added to chilled plastic vials containing EDTA and ascorbic acid, and gently mixed for ten seconds. The samples were then centrifuged at 2000 g at 4°C for ten minutes, and the plasma was removed using plastic pipettes, placed in plastic vials and quick-frozen in a dry ice and alcohol bath. The samples were stored at -70°C until analyzed as previously described (Chou et al., 1983).''

Blood for histamine analysis was collected in chilled syringes, added to plastic vials containing potassium oxalate and gently mixed for ten seconds. The samples were then handled in a manner similar to the procedure for serotonin and the plasma was stored at -70°C until analyzed by a flurometric technique.''

Statistical evaluation

Widely differing predose values and postdose variances in these data negated standard analyses of variance methods. As an alternative approach, each animal in the three (High, Low and Control) groups was tested for significant upward or downward trend in each parameter, these trends being assessed for statistical significance by both Pearson's r and Kendall's tau correlation analyses, with the times of the sequence of successive observations serving as the independent variates. By this approach, all animals were individually tested against the hypothesis that treatment had no nonrandom effects upon the monitored parameters. Intergroup differences were then judged by comparing the sets of intragroup tests with each other. In this way, for example, five high dose animals, all showing a significant increase in a particular parameter, were clearly behaving differently than a population of five low dose animals all showing either no change or the opposite change in the same parameter.

A value of P less than 0.05 (two-sided) was considered statistically significant. During the early time period when the high dose animals were alive, these intergroup comparisons were buttressed by the Kruskal-Wallis nonparametric one-way analysis of variance (Ghent, 1974; Nie et al., 1975). Only those increases and decreases in the monitored parameters that proved statistically significant are hereafter discussed, the qualification "statistically significant" being often omitted in the interest of brevity.

RESULTS

Clinical observations

Clinical signs were readily apparent in swine given intravascular T-2 toxin. Both the high and low dose animals reacted similarly in the first several hours. They began to chew and salivate within 15 to 30 minutes. This was followed by persistent vomition and, in some animals, watery diarrhea and flatulence. All the swine were notably restless. Within the first hour most animals exhibited abdominal straining associated with prolonged exhalation, and purplish mucous membranes began to be apparent. Urine output was negligible at two hours after administration of T-2 toxin at the high dose, while swine in the low dose and vehicle groups continued to produce urine. The skin was noticeably reddened, and the ears and limbs were cold to the touch by two to three hours. After three to four hours, the animals appeared drowsy but could be aroused. Animals in the high dose group died as early as five hours after administration of the toxin. The signs of the animals in the low dose group began to regress between five and six hours and at 12 hours, the animals appeared clinically normal except for apparent reductions in alertness and activity. The alterations in body temperature were unusual (Figure 1). In the high dose group, every animal's temperature either

increased (two animals) or decreased (three animals) significantly. In the low dose group, there was an early decrease in body temperature between predosing and one hour in all animals.

Hemodynamics

The rapid onset of shock induced by T-2 toxin was reflected in an early decline in CO and AOM in both the high and low dose groups (Figures 2 and 3). In the high dose group, the value of both parameters continued to decline through death. The CO in the low dose group stabilized at about four hours, continued to be stable through eight hours, and by 24 hours was restored to the predosing level. The AOM in the low dose group stabilized between three and four hours. In contrast to the stable nature of CO between four and eight hours, there was an increase in AOM during that time period and thereafter no further increase was detected. At 24 hours AOM was still reduced by 23 to 25 percent of the predosing value.

There was a significant increase in heart rate in both the high and low dose groups. It can be noted in Figure 4; however, that the rate did not increase appreciably until two hours postdosing. No significant trend was detected in the TPR in the high dose group, but the TPR of the low dose group decreased (Figure 5).

Both high dose and low dose groups exhibited elevations in PVR (Figure 6). Due to the manner in which PVR is calculated (PAM - LAM/CO), an increase in PAM or a decrease in LAM may result in an increase in the resistance value. This mathematic relationship is demonstrated by the high dose group in which PAM increased but then decreased while LAM decreased at each time point (Figures 7 and 8). In contrast, the elevated PVR in the low dose group occurred because PAM did not increase until after 3-4 hours, while LAM was

decreasing in the zero through four hour period. After four hours, the PAM and LAM of the low dose group increased while CO was stable. After 8 hours, the PVR of the low dose pigs decreased as CO increased, LAM did not change and PAM decreased.

Plasma catecholamines

Results from the preliminary intravascularly dosed swine revealed that plasma histamine and serotonin concentrations did not increase during T-2 toxicosis, although a shock syndrome and death ensued in all the preliminary animals. In contrast, the concentrations of norepinephrine and epinephrine were strikingly elevated in these preliminary swine. In the formal study, tremendous oscillating responses occurred in epinephrine and norepinephrine in the high dose group (Figures 9 and 10). There was an elevated but attenuated response in the low dose group. There were no significant changes in the plasma concentrations of dopamine in either the high or the low dose group.

6-keto-$PGF_{1\alpha}$ and Thromboxane B_2

The concentrations of TXB_2 and 6-keto-$PGF_{1\alpha}$ were significantly increased in the T-2 toxin dosed animals (Figures 11 and 12). The concentra- tion of 6-keto-$PGF_{1\alpha}$ never exceeded the limit of detection in the control group. The plasma concentration of TXB_2 rose before 6-keto-$PGF_{1\alpha}$ in both the high and low dose groups.

Acid-base and blood gas parameters

Although the PaO_2 decreased between zero and 2 1/2 hours in both groups, the decline was significant only in the low dose group. Nevertheless the arterial oxygen tension remained functionally adequate in all groups until immediately prior to death (Figure 13). After 3 hours PaO_2 began to rise toward the predosing value and the $PaCO_2$ decreased.

Acidosis became severe early in both groups (Figure 14). After 2 1/2 hours the acidosis in the low dose group began to resolve. The decline in pH_a in animals that received 4.8 mg/kg T-2 toxin was accompanied by an increase in plasma lactic acid from a concentration of 0.49 $\pm$ .09 to 7.63 $\pm$ .59 mmol/L (mean $\pm$ SEM).

DISCUSSION

There is limited documentation that T-2 toxin has the ability to induce shock. We have investigated numerous parameters in an effort to elucidate the nature of this toxic syndrome. Additional cardiovascular and pathologic determinations were made in these swine and will be reported subsequently.

Between predosing and 4 hours, the rates of decline in CO and AOM in the high dose group were not statistically different from one another. In other words, while both CO (Figure 2) and AOM (Figure 3) were declining rapidly during the first 4 hours in the high dose group, they declined along essentially parallel slopes, so the systemic resistance (TPR, Figure 5, calculated as AOM/CO) did not depart significantly from a flat trend. However, our clinical observations of skin and limb perfusion strongly suggested that peripheral vascular flow was in fact reduced. Our resistance values (TPR, Figure 5) are calculated from parameters monitored only in the heart and aorta. If peripheral capillary vasculature closed suddenly and intensely, and very early after dosing, our parameters may already have reflected resistance to flow through only the limited circuit of heart, lungs, and brain as early as the first (30 minutes) observations.

In contrast, TPR in the low dose group decreased from predosing through 4 hours. Again both CO (Figure 2) and AOM (Figure 3) decreased, but the much steeper AOM decrease resulted in a significant AOM/CO (TPR, Figure 5) downward trend.

The rates of decline of CO in the high and low dose groups were not statistically different from one another. Apparently the cardiac effects were similar in both T-2 dosed groups but the peripheral pressor responses resulted in greater reductions in aortic pressure in the low dose group. The microcirculation retained responsiveness in the low dose group as demonstrated during the 4 to 8 hour period. While CO was fairly constant, the vasculature responded by increasing resistance, bringing the TPR back toward the predosing value and increasing the blood pressure.

While these cardiovascular changes were occurring, the concentration of catecholamines was elevated. Inspite of these chronotropic substances, the heart rate did not increase until after a delay. Normally, catecholamines rapidly increase arterial blood pressure, cardiac output, heart rate and total systemic resistance (DeQuattro and Campese, 1981). The action of T-2 toxin apparently did not permit expression of these effects. Thus, many of the effects could be attributable to primary cardiac and vascular factors.

Another hypothesis may also be considered. The early and rapid decline in CO and AOM, the biphasic responses of CO and AOM in the low dose group, the intense restriction of peripheral flow in the high dose group, the high concentrations of catecholamines reflecting tremendous autonomic activity, the presence of cholinergic symptoms (vomiting, diarrhea, flatulence, salivation) and later, the apparent depression or lethargy of the survivors could be evidence for a significant nervous system component involved in this toxic syndrome. The disturbances in thermoregulation may suggest central mediation.

These effects could also be mediated peripherally. The occurrence of elevated levels of norepinephrine in the presence of decreased blood pressure could support a peripheral mediation theory since the major source of plasma

norepinephrine is peripheral sympathetic synapses. It has been debated that elevated concentrations of the α and ß agonist, norepinephrine, coincide with hypertension (Lake, 1984; DeQuattro et al., 1984). However, in our experiments blood pressure was consistently depressed. This could suggest an alteration in the response of peripheral vasoactive receptors.

Several years ago it was determined that catecholamines induce platelet aggregates to liberate vasoconstrictor substances (Neil, 1975). Later it was found that thromboxane, in addition to other vasoactive compounds, is produced by platelets and has the ability to enhance shock by causing pulmonary hypertension, coronary vasospasm and the constriction of bronchial and damaged vascular smooth muscle (Granstrom et al., 1982; Terashita et al., 1978; Frolich et al., 1980). Moreover, norepinephrine potentiates the in vitro biosynthesis of thromboxane (Wolfe, 1976). Feedback mechanisms between prostaglandins and catecholamines have been described that involve the α and ß receptors, and it has been proposed that modulation of adrenergic responses may be accomplished by prostaglandins, including prostacyclin (Ziegler and Lake, 1984; Nasjletti and Malik, 1982; Stjarne, 1973).

Elevations in the concentrations of 6-keto-$PGF_{1\alpha}$ and thromboxane B2, the stable hydrolysis products of prostacyclin and thromboxane A_2 respectively, were detected in all T-2 toxin dosed animals and the increase was concurrent with the rise in catecholamines. This observation is compatible with an interaction between these substances. Generally, however, the increases in these humoral substances were delayed as compared to the early decline in CO and AOM and the peripheral vascular responses, suggesting that the former were not the ultimate cause for these alterations although they contributed to the hemodynamic changes.

Thromboxane A_2, as well as its metabolite, TXB_2, have been shown to induce pulmonary hypertension (Huttemeier et al., 1982; Watkins, et al., 1982; Friedman et al., 1979; Frolich et al., 1980). In this experiment the rise in the concentration of TXB_2 compares well with the rise in PVR. PVR increased substantially after 2-3 hours, and remained elevated after 4 hours, whereas TXB_2 concentrations were declining by this time in both groups. In addition to thromboxane, acidosis and catecholamines may have contributed to the increase in pulmonary resistance (Berk et al., 1977; Webb and Brunswick, 1982).

The early decrease in PaO_2 in both groups may indicate that the source of the elevated PVR was primarily arteriolar. The subsequent increase in PaO_2 in the low dose group in the face of continued elevation of PVR may indicate a shift to primarily venule constriction. In the presence of severe acidemia, immediate respiratory compensation in the form of hyperventilation and a substantial reduction in $PaCO_2$ would have been expected. However, the fact that $PaCO_2$ of the low dose group decreased, remaining within the range of normal, may indicate an ineffective attempt at respiratory compensation. The absence of appropriate respiratory response may indicate an effect of T-2 toxin on the nervous system. The effect could have been enhanced by the abdominal straining that occurred in these animals and consequent impairment of respiratory exchange.

The concentration of 6-keto-$PGF_{1\alpha}$ increased after TXB_2 perhaps as an attempt to balance the effects of thromboxane A_2. This increase in 6-keto-$PGF_{1\alpha}$ probably augmented the decrease in systemic blood pressure, since systemic hypotension can be produced by prostacyclin (Collins et al., 1982; Moore et al., 1982).

In summary, acute T-2 toxicosis in swine is characterized by an early and simultaneous decrease in CO and AOM, and increases in 6-keto-$PGF_{1\alpha}$, catecholamines and heart rate that were accompanied by a decrease in TPR in the low dose group but the absence of an obvious trend in TPR in the high dose group. An increase in PVR occurred along with increases in norepinephrine, TXB_2 and acidemia. Evidence for possible nervous system mediation of this toxic syndrome include: the sudden decrease in CO and AOM, the differing responses of the microcirculation in the high and low dose groups, the delayed increase in heart rate, the erratic adrenergic response in the high dose group and the sustained norepinephrine concentrations accompanied by decreased AOM in the low dose group, the presence of cholinergic clinical signs, signs suggestive of mental depression, lack of appropriate respiratory stimulation and altered thermoregulation. Further studies must be performed before a theory of nervous system mediation of T-2 toxicosis can be substantiated.

ACKNOWLEDGEMENTS

The authors express their appreciation to Mr. Steve Swanson for his supervision of the analytical chemistry procedures, to Dr. Art Siegel for assistance in data analysis, to Mr. Dick Manuel for technical assistance.

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FOOTNOTES

[1] Supported by the US Army Medical Research and Development Command, Contract No. DAMD17-83-C-2179

[2] Presented at the FASEB Research Conference, "Diagnosis, toxicity, and therapy of trichothecene mycotoxicosis," June 1984

[3] MycoLab Company, Chesterfield, MO

[4] E. rhusiopathiae bacterin, Dellen Lab, Omaha, NB

[5] Thin layer and gas chromatography, Analytical Toxicology Laboratory, College of Veterinary Medicine, University of Illinois Diagnostic Lab, Urbana, IL

[6] Modulus anesthetic machine with Vernitrol vaporizer, Ohio Med Prod, Madison, WI

[7] Bird Mark 4A ventilator, 3M Corp., Minneapolis, MN.

[8] Tygon tubing, 16 ga., I.D. = 0.05 in., O.D. = 0.09 in., 18 ga., I.D. = 0.04 in., O.D. = 0.07 in., A. Daigger and Co., Chicago, IL

[9] IV infusion pump 2681, Harvard Apparatus Med Prod, Millis, MA

[10] P23ID, Statham Medical Instruments, Gould Inc., Oxnard, CA

[11] Gilson Medical Electronics Inc., Middleton, WI

[12] Cardiogreen, Hynson, Westcott and Dunning Inc., Baltimore, MD

[13] DTL, Gilson Medical Electronics Inc., Middleton, WI

[14] DTCCO-07, Electronics for Medicine, Honeywell, New York, NY

[15] Radiometer BM3MK2, The London Co., Cleveland, OH

[16] Sigma Chemical Co., St. Louis, MO

[17] American Medical Lab, Fairfax, VA

[18] RIA kits, NEK-007A and NEK-008A, New England Nuclear, Boston, VA

[19] Bio-Science Lab, Bellwood, IL

FIGURE 1. Rectal temperature of swine given the high (top) and low (middle) dose of T-2 toxin and vehicle (bottom) intravascularly. Each line represents an individual animal. The alterations in temperature was significant in all animals that received T-2 toxin. The * denotes significant trends at $p \leq 0.05$.

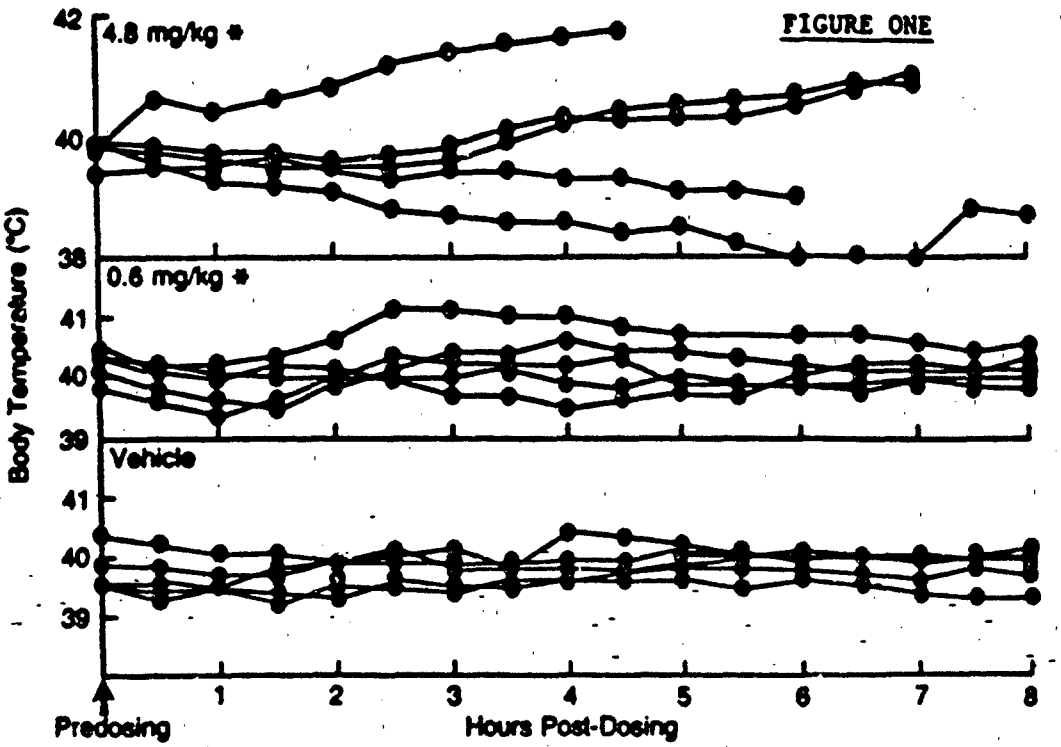

FIGURE 2. Alterations in cardiac output (CO) over time in swine given T-2 toxin intravascularly and in swine given the vehicle, only. In the high dose group (o----o), there were only three survivors at seven hours postdosing. (Mean ± SEM) The * denotes significant trends at $p \leq 0.05$.

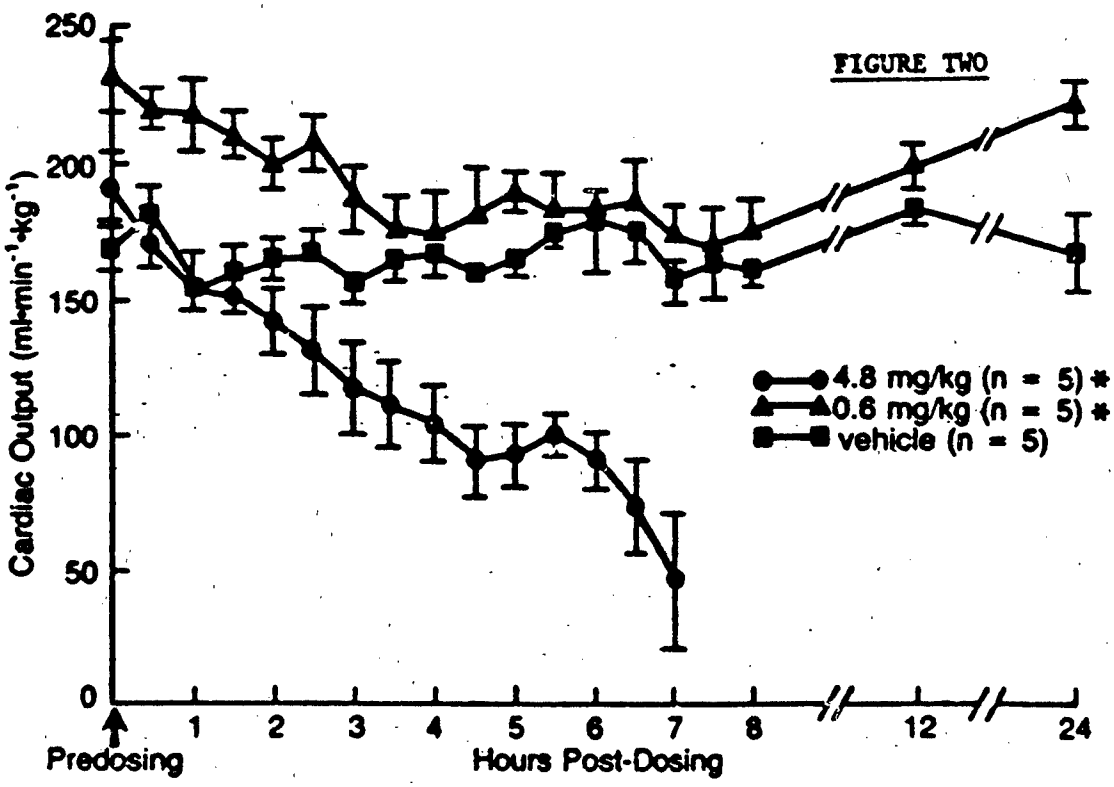

FIGURE 3. Changes in mean aortic blood pressure (AOM) over time in swin given T-2 toxin or vehicle intravascularly. (Mean ± SEM) The * denotes significant trends at $p \leq 0.05$.

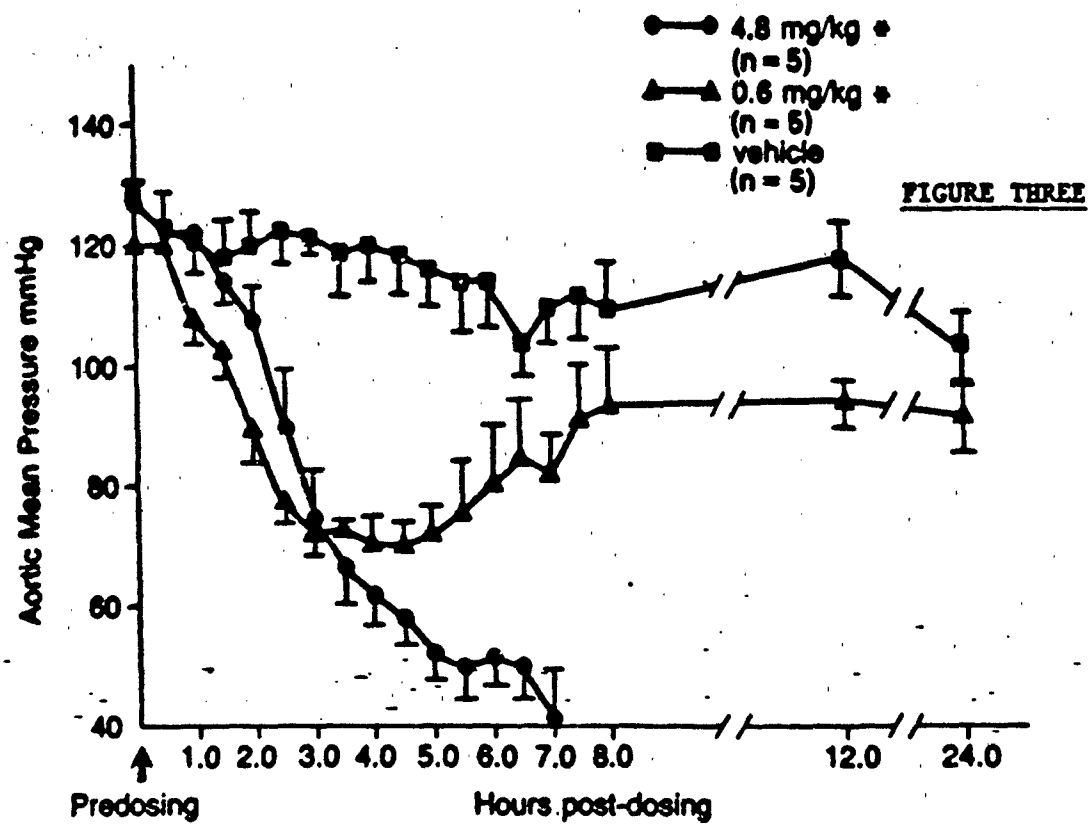

FIGURE 4. Changes in heart rate (beats per minute) of swine given T-2 toxin intravascularly. (Mean ± SEM) The * denotes significant trends at $p \leq 0.05$.

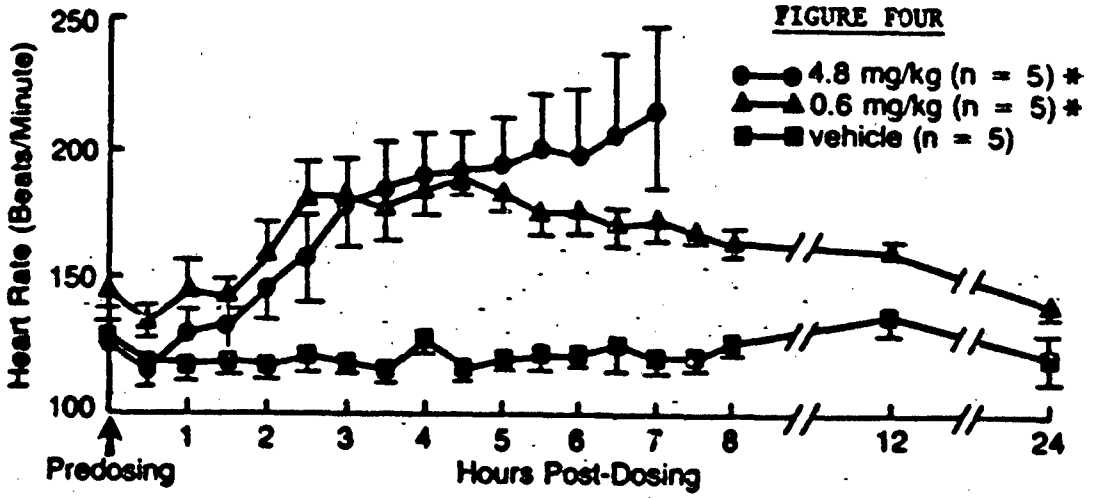

FIGURE 5. Total peripheral resistance (TPR) expressed as a percent of t predosing value in swine given T-2 toxin intravascularly. (Mean SEM) The * denotes significant trends at $p \leq 0.05$.

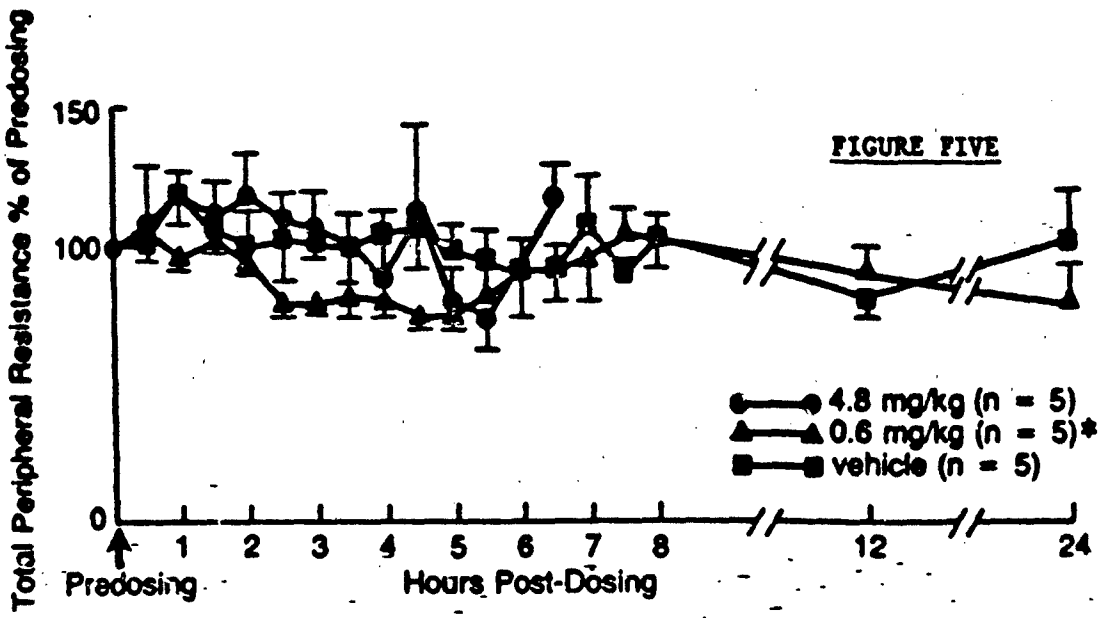

FIGURE 6. Pulmonary vascular resistance (PVR) expressed as a percent of the predosing value in swine receiving T-2 toxin intravascularly. (Mean ± SEM) The * denotes significant trends at $p \leq 0.05$.

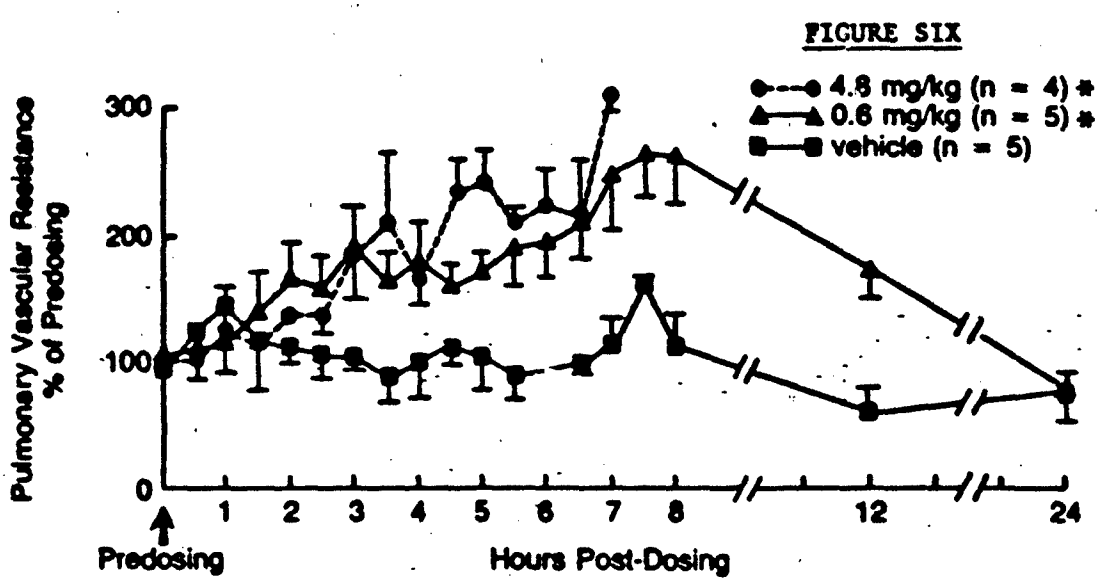

FIGURE 7. Mean pulmonary artery pressure (PAM) in swine receiving T-2 toxin intravascularly. (Mean ± SEM) The * denotes significant trends at $p \leq 0.05$.

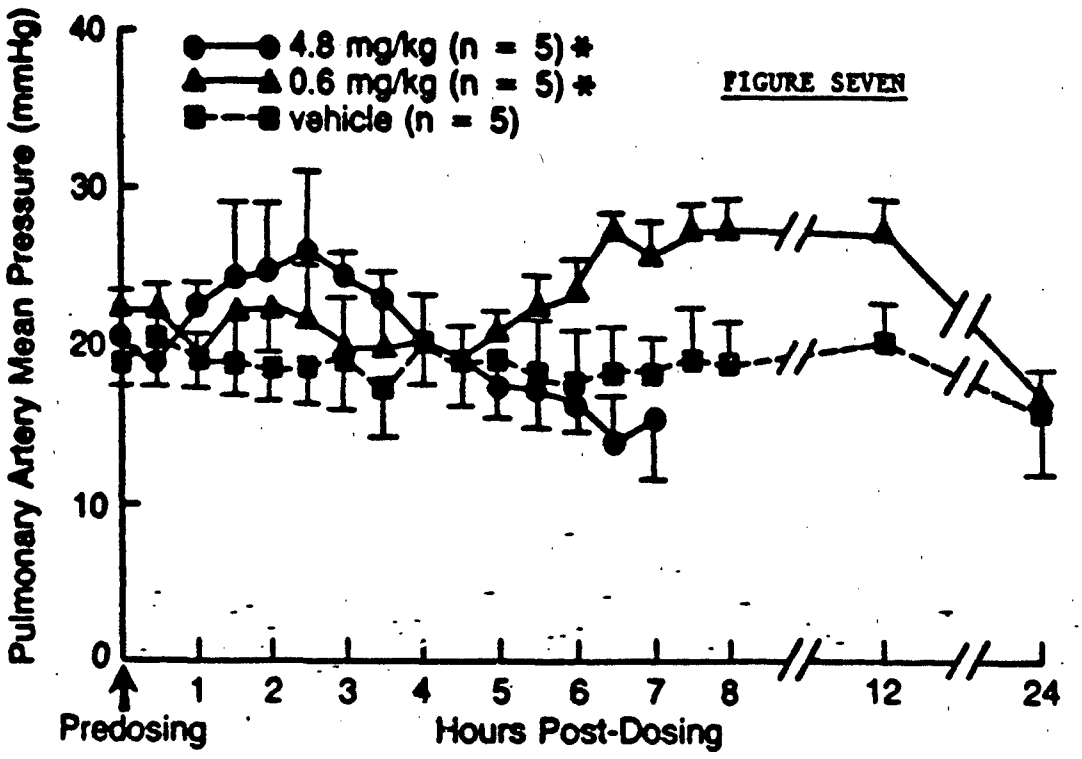

FIGURE 8. Mean left atrial pressure (LAM) in swine receiving T-2 toxin intravascularly. (Mean ± SEM) The * denotes significant trends at $p \leq 0.05$.

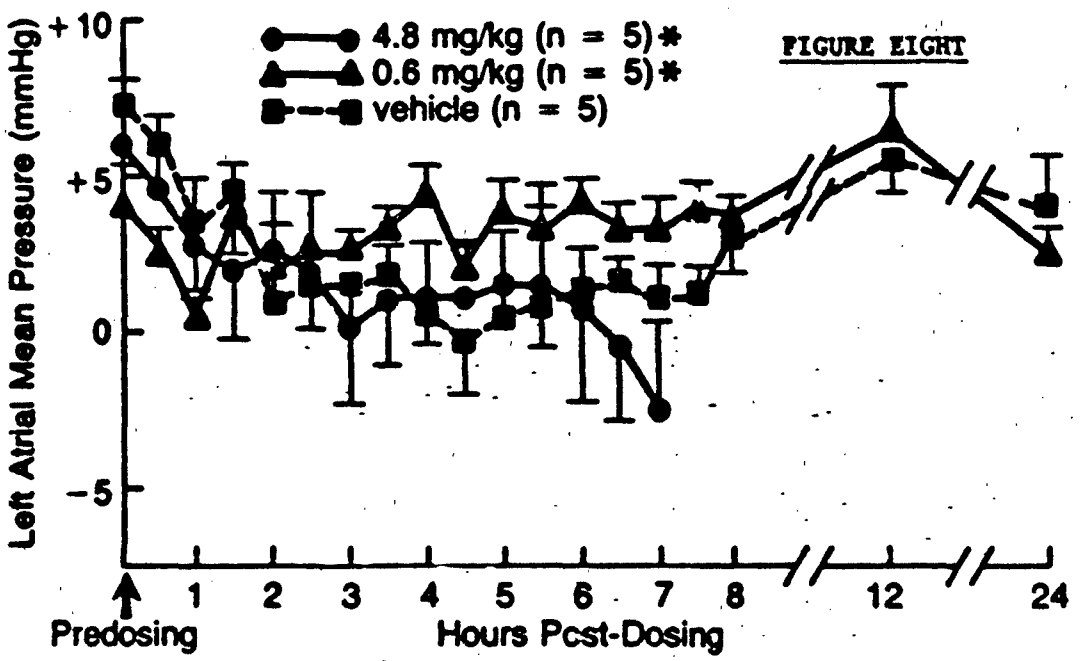

FIGURE 9. Concentration of plasma epinephrine in swine given the high (top) and low (middle) dose of T-2 toxin and vehicle (bottom) intravascularly. Each line represents an individual animal. The * denotes significant trends at $p \leq 0.05$.

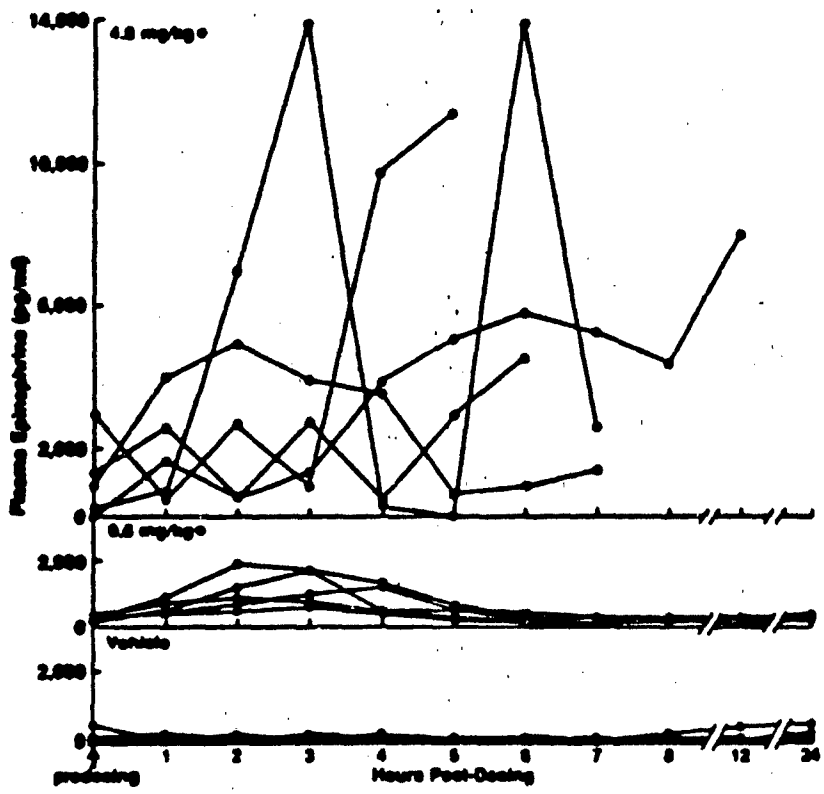

FIGURE NINE

FIGURE 10. Concentration of plasma norepinephrine in swine given the high (top) or low (middle) dose of T-2 toxin and vehicle (bottom) intravascularly. Each line represents an individual animal. The * denotes significant trends at $p \leq 0.05$.

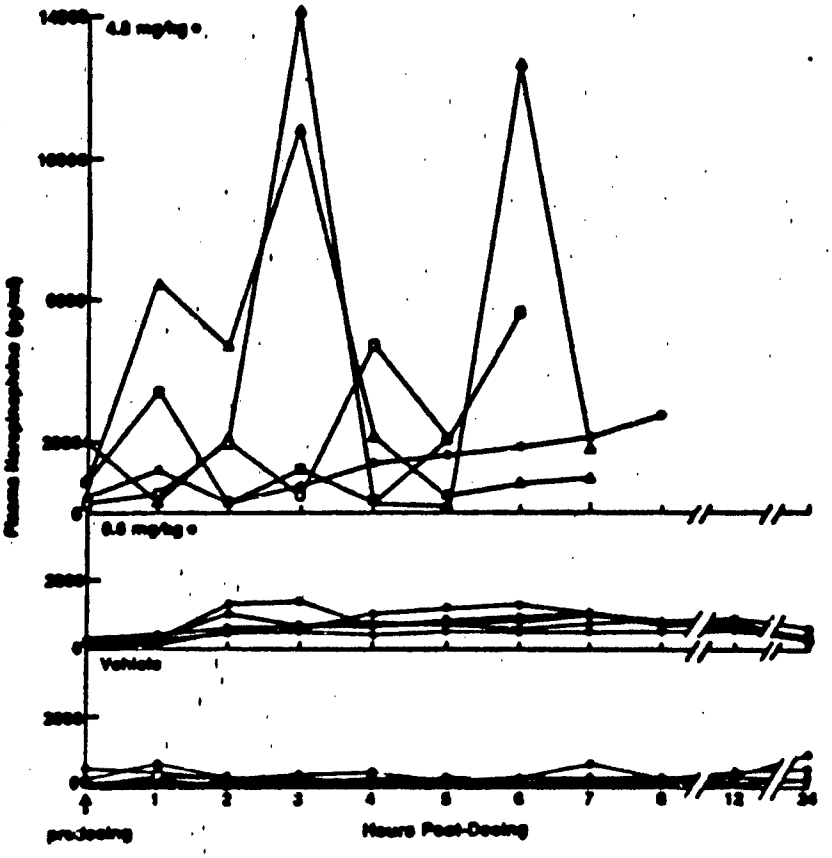

FIGURE TEN

FIGURE 11. Plasma concentration of thromboxane B_2 (TXB_2) in swine given the high (top) or low (middle) dose of T-2 toxin and vehicle (bottom) intravascularly. Each line represents an individual animal. The * denotes significant trends at $p \leq 0.05$.

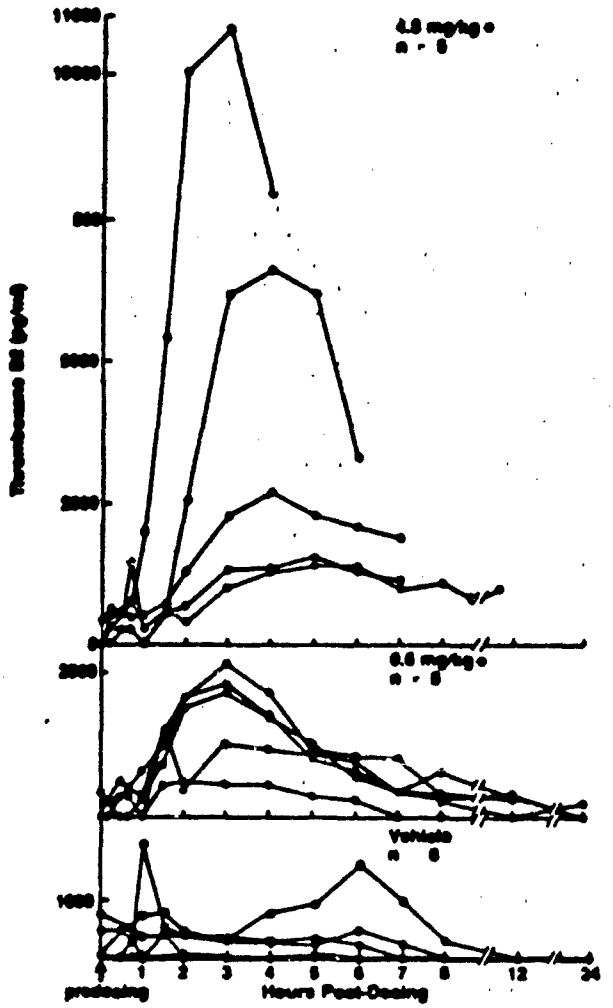

FIGURE ELEVEN

FIGURE 12. Plasma concentration of 6-keto-$PGF_{1\alpha}$ in swine given the high (top) or low (bottom) dose of T-2 toxin intravascularly. Each line represents an individual animal. The concentration of 6-keto-$PGF_{1\alpha}$ never exceeded the limit of detection in the vehicle group. The * denotes significant trends at $p \leq 0.05$.

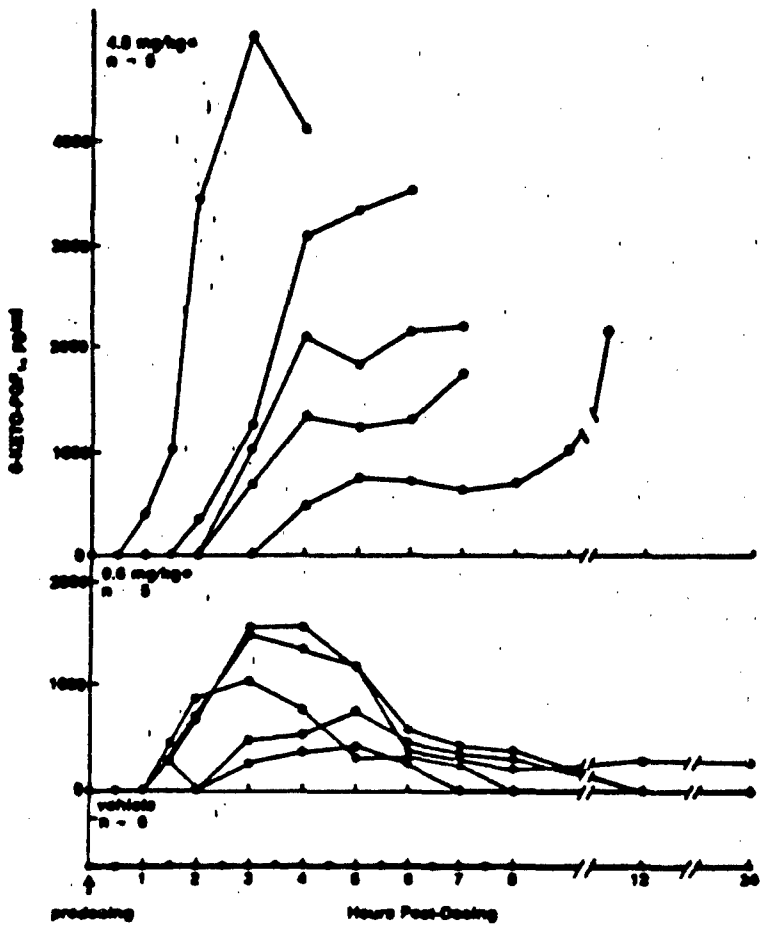

FIGURE TWELVE

FIGURE 13. Concentrations of arterial blood oxygen (PaO_2) and carbon dioxide ($PaCO_2$) in swine given T-2 toxin or vehicle intravascularly. (Mean ± SEM) The * denotes significant trends at $p \leq 0.05$.

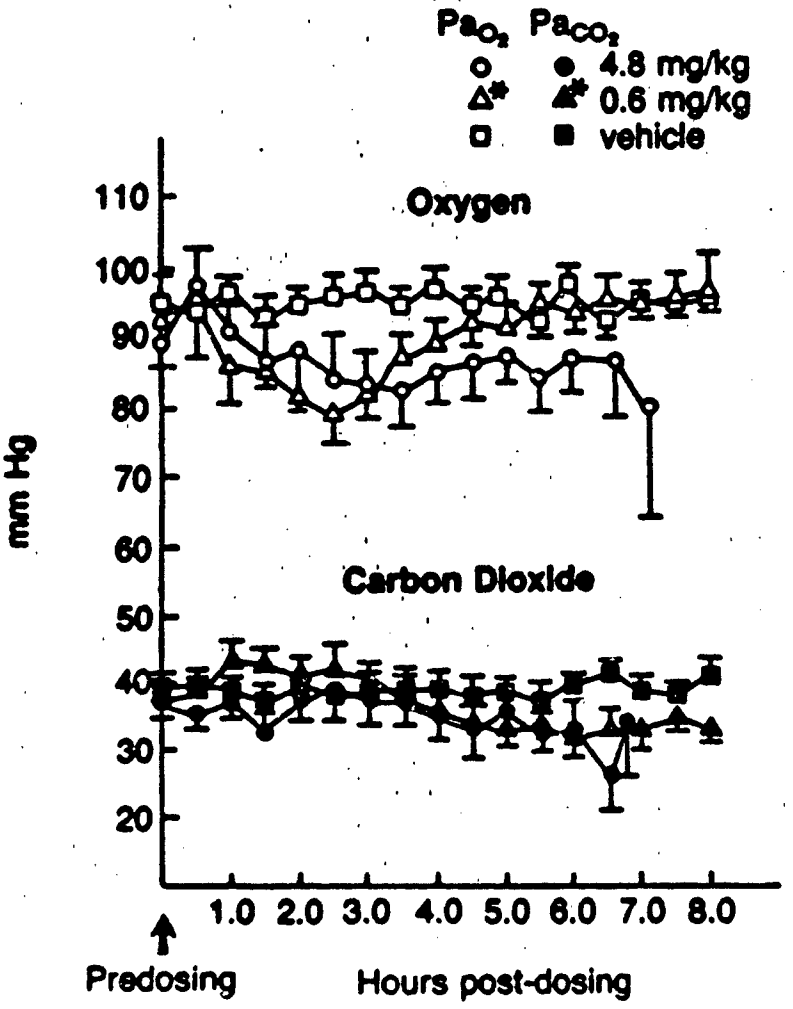

FIGURE THIRTEEN

FIGURE 14. Alterations of arterial pH (pH_a) over time in swine given T-2 toxin or vehicle intravascularly. (Mean ± SEM) The * denotes significant trends at $p \leq 0.05$.

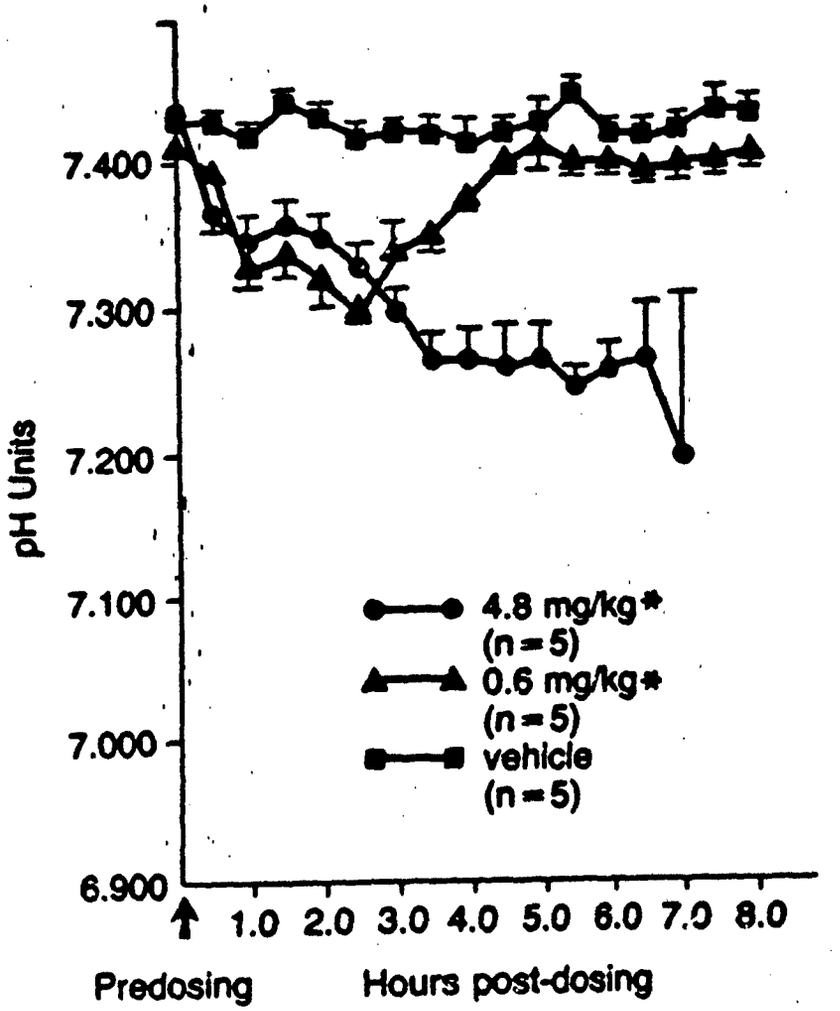

FIGURE FOURTEEN

B. EXPERIMENTAL T-2 TOXICOSIS IN SWINE. II. EFFECT OF INTRAVENOUS T-2 TOXIN ON SERUM ENZYMES AND BIOCHEMISTRY, BLOOD COAGULATION AND HEMATOLOGY--

Roseanne M. Lorenzana, Val R. Beasley, William B. Buck, Arthur W. Ghent

ABSTRACT

Experimental T-2 Toxicosis in Swine. II. Effect of Intravenous T-2 Toxin on Serum Enzymes and Biochemistry, Blood Coagulation and Hematology. Lorenzana, R. M., Beasley, V. R., Buck, W. B. Ghent, A. W. (1985). Fundam. Appl. Toxicol. T-2 toxin was given as a single intravascular dose at either 0.6 or 4.8 mg/kg to different groups of fifty kilogram, female swine. Blood samples were taken at hourly intervals for determination of concentrations or activities of the following substances in serum or plasma: creatinine, blood urea nitrogen, inorganic phosphorus, total calcium, ultrafilterable calcium, magnesium, sodium, potassium, chloride, total protein, albumin, cholesterol, glucose, alkaline phosphatase, aspartate aminotransferase, and total bilirubin. Coagulation analyses included prothrombin time, partial thromboplastin time, activated coagulation time, and fibrin degradation products. Red blood cell, white blood cell, and platelet counts, hemoglobin concentrations and hematocrit, were determined from whole blood samples. An initial leukocytosis was followed by a leukopenia. The numbers of red cells, the hemoglobin concentration, and the hematocrit were increased. Nucleated red blood cells were seen in the blood smears. The serum concentration of bound calcium decreased, while phosphorus, magnesium and potassium increased. Clinical screening tests detected no evidence of a coagulopathy in swine given T-2 toxin intravascularly.

INTRODUCTION

T-2 toxin, a potent trichothecene mycotoxin produced primarily by members of the genus Fusarium, has been reported to cause infertility (Weaver et al., 1978a), hemorrhagic "moldy corn disease" (Bamburg et al., 1969), vomiting, paresis and death (Weaver et al., 1978b) in swine. In this study, T-2 toxin was given intravascularly to characterize the pathophysiology of the shock syndrome that can be a lethal as well as a sublethal effect in swine (Beasley, 1983; Lorenzana et al., 1985). T-2 toxin has recently been identified as a component of the "Yellow Rain" chemical warfare agent (Rosen and Rosen, 1982). Swine were used as a model to study the possible effects of "Yellow Rain" toxicosis in man. In this report, the enzymatic and biochemical changes detected in serum, the effect on blood coagulation and alterations in the hemogram induced by intravenous T-2 toxin are described. Hemodynamic parameters were also determined in these animals and are described in a companion report (Lorenzana et al., 1985).

MATERIALS AND METHODS

Chemical and Animals

Purified T-2 toxin[3] was dissolved in 6.5 mL of 70 percent ethanol at room temperature.

White, crossbred, female swine ranging from 40-60 kg were immunized against erysipelas,[4] given an intramuscular injection of selenium and allowed to acclimate to their housing and feed for four to seven days prior to surgical catheterization of the pulmonary artery, aorta, and left atrium. After surgery, the animals were allowed to recover for two to four weeks, and were active and in good health at the time of the study. They were fed a standard ration which contained no detectable concentrations of deoxynivalenol

(vomitoxin), zearalenone, T-2 toxin, diacetoxyscirpenol (DAS) or aflatoxins B1, B2, G1 and G2.[5]

Surgical Preparation

The manner in which catheters were placed in the pulmonary artery, aorta and left atrium, have been previously described (Lorenzana, et al., 1985).

Experimental Protocol

Pigs were divided into three groups. A lethal dose of 4.8 mg/kg T-2 toxin which was four-fold higher than the approximate pigs intravascular LD50 (Weaver et al., 1978b) was given to five swine (high dose group), and a sublethal dose of 0.6 mg/kg was administered to five other swine (low dose group). T-2 toxin was given as a single intravascular dose in 6.5 mL of 70 percent ethanol as a vehicle. The six animals in the control group received only the vehicle intravascularly in addition to undergoing identical surgical and handling procedures.

On the day of the study, the conscious animal was restrained in a webbed stanchion which allowed the pig to rest on its sternum and abdomen while its legs hung below the webbing. All swine quickly adapted to this method of restraint and appeared to rest comfortably. After the overlying skin was infiltrated with 2% lidocaine HCl, the catheters were exteriorized through small skin incisions and attached via manifolds to measuring and sampling devices.

Blood samples for baseline (predosing) clinical determinations were collected and the toxin was then administered into the pulmonary artery by a continuous infusion over a five minute period.[6] The beginning of the infusion was considered to be the starting time of the experiment. In the evaluation of the acute toxicity of a new compound, intravenous administration is

generally the initial route of exposure in experimental animals. This ensures that 100 percent of the dose has been absorbed and, therefore, simplifies the dose-response relationship. In our experiments the catheter in the root of the pulmonary artery gave access to the venous circulation.

Blood samples were collected via the aortic catheter at hourly intervals. All catheters were frequently flushed with heparinized saline to ensure patency and the absence of clots in the samples. Throughout the experiment, each pig received four liters of fluid due to this flushing. The amount of blood taken for clinical determinations was less than ten percent of the animal's total blood volume (i.e.: 500 mL or less). The heparinized saline and an aliquot of blood were withdrawn from the catheter and discarded prior to the collection of a blood sample for analysis. Urine was collected from all experimental animals; however, it was quantitated only in the high dose group.

In the high dose group, samples were taken hourly until the time of death. In the low dose and control groups, samples were taken at 0, 1, 2, 3, 4, 5, 6, 7, 8, 12 and 24 hours after toxin administration. The swine were anesthetized with sodium thiamylal and then killed by exsanguination.

Laboratory Determinations

All blood samples were collected through the aortic catheter. Serum for biochemical and enzymatic evaluations was collected after allowing the blood to clot at 37°C for two hours. The concentrations of creatinine, total protein, albumin, cholesterol, glucose, phosphorus, calcium, blood urea nitrogen, sodium, chloride, potassium, and total bilirubin and the activities of alkaline phosphatase (SAP), and aspartate aminotransferase (AST) were determined by an autoanalyzer.[7] When the determined values were above the working range of the autoanalyzer, samples were diluted and reanalyzed. Bound and unbound calcium

in serum samples were separated by ultracentrifugation. Four cc of serum were placed in a membrane cone[8] and centrifuged at 1000 g for 20 minutes. The calcium content of the ultrafiltrate was determined as described above. Magnesium concentrations were determined by atomic absorption spectrophotometry.[9]

Whole blood was collected in tubes containing EDTA for hematologic evaluation. The hemoglobin concentrations were determined by the cyanmethemoglobin method.[10] Cell and platelet numbers were determined by an electronic particle counter,[11,12] and the white blood cell differential was determined from blood smears. One hundred cells were observed for every 1 x 10^4 white blood cells per uL counted in the total. Plasma prothrombin time and partial thromboplastin time were determined from blood samples to which sodium oxalate had been added.[13] Activated coagulation time was determined by immediately adding freshly collected blood to a premeasured amount of diatomaceous earth and determining the number of seconds that transpired from the moment the blood contacted the earth until the first signs of clotting were visible.[14] Serum for quantitation of fibrin degradation products was obtained by adding blood to tubes containing thrombin and an enzyme inhibitor.[15] All serum and plasma samples were frozen and stored at -20°C prior to analysis. Analyses were per- formed within five days of collection.

Statistical Evaluation

Widely differing predose values and postdose variances in these data negated standard analyses of variance methods. As an alternative approach, each animal in the three (high, low, and control) groups was tested for significant upward or downward trends in each parameter, these trends being assessed for significance by both Pearson's r and Kendall's tau two-tailed correlation analyses, with the time sequence of successive observations serving

as the independent variate. In this approach, all animals were individually tested against the hypothesis that treatment had no nonrandom effects upon the monitored parameters. Inter-group differences were then judged by comparing the sets of intra-group tests with each other. In this way, for example, five high dose animals, all showing a significant increase in a particular parameter, are clearly behaving differently than a population of five low dose animals, all showing either no change or the opposite change in the same parameter.

A value of P less than 0.05 was considered statistically significant. During the early time period when the high dose animals were alive, these inter-group comparisons were buttressed by Kruskal-Wallis nonparametric one-way analysis of variance (Ghent, 1974; Nie et al., 1975).

RESULTS

Serum Enzymes and Biochemistry

The high dose group had the greatest changes in serum ion concentrations (Figure 1). Potassium and magnesium progressively increased while calcium decreased. In the low dose group, a decrease in calcium also occurred but the concentration later began to increase toward the predosing concentration. At various time points, the concentrations (mg/dL) of ultrafilterable calcium in the high dose group ranged from 4.0 $\pm$ .2 to 4.7 $\pm$ .2, in the low dose group from 4.5 + .4 to 5.4 $\pm$ .8 and the control group from 3.3 $\pm$ .5 to 4.7 $\pm$ .7 (mean $\pm$ SEM). No trend was observed in the ultrafilterable calcium in any group during the experiment. It was, therefore, the concentration of bound calcium that significantly decreased in both high and low dose groups. This decline in concentration of bound calcium occurred along with a decline in blood pH (Figure 2).

In the high dose group, serum concentrations of inorganic phosphorus increased over time (Table 1), whereas there were no trends in the low dose and vehicle groups. The concentration of blood urea nitrogen became increased in both the high and low dose groups (Table 1). An increase in serum creatinine from 1.2 ± .06 mg/dL to 2.3 ± .4 mg/dL (mean ± SEM) occurred in the high dose group only. No significant trends were detected in the low dose and control groups.

The activity of SAP was significantly increased in the high dose group only (Table 1). AST activity increased significantly from 34.4 ± 3.7 U/L at predosing to 84.3 ± 15 U/L at 7 hours postdosing in the high dose group and from 32.2 ± 3.5 U/L at predosing to 78.6 ± 30.2 at 24 hours postdosing in the low dose group (mean ± SEM). No significant trend occurred in the concentrations of sodium, chloride, total protein, albumin, cholesterol, glucose, and bilirubin.

Hematology

Although the total white blood cell count (WBC) was increased in both high and low dose groups at one hour, thereafter a significant decrease occurred in both of these groups (Figure 3). In the low dose group, there was a subsequent increase and by 12 hours postdosing the WBC was similar to the predosing number.

The early leukocytosis and the latter leukopenia was due to increases followed by reductions in absolute numbers of both neutrophils and lymphocytes (Figures 4 and 5). In the low dose group at 24 hours postdosing the number of lymphocytes was approximately 37 percent of the predosing number. Significant decreasing trends occurred in the absolute numbers of monocytes and eosinophils in the high dose group, but no significant variation occurred in the low dose

group. In the high dose group, the number of monocytes per microliter of blood decreased from 631 ± 217 at predosing to zero at 7 hours postdosing (mean ± SEM), and the number of eosinophils per microliter of blood decreased from 837 ± 174 at predosing to 152 ± 55 at 7 hours postdosing (mean ± SEM). No significant trend occurred in any group in the number of bands and basophils.

In the high dose group, between predosing and 7 hours postdosing, significant elevations occurred in the hematocrit, the number of red blood cells and the hemoglobin concentration. The hematocrit (%) increased from 33.4 ± 2.1 to 38.5 ± 2.1, the number of red blood cells (1 x 10^6 per microliter) increased from 7.6 ± .6 to 9.0 ± .7 and the concentration of hemoglobin (g/dL) increased fom 10.9 ± .6 to 12.8 ± .9 (mean ± SEM). No significant trend in these parameters occurred in the low dose or control groups.

Nucleated red blood cells (metarubricytes) were seen in the peripheral blood smears in both the low and high dose groups (Figure 6). In the high dose group, the number continued to increase at each successive observation. In the low dose group, their incidence began to decrease after six hours, and by 24 hours, no nucleated cells were observed.

Blood Coagulation Parameters

No significant trends occurred in any of the groups in prothrombin time, partial thromboplastin time, activated coagulation time, platelet number and fibrin degradation products.

Urine Production

The high dose animals produced an average of 94 mL of urine during the first four hours. No urine was produced by these pigs after four hours. The low dose and control animals produced urine (greater than 300 mL at one urination) and continued to voluntarily micturate throughout the course of the experiment.

DISCUSSION

There is limited documentation that T-2 toxin has the ability to induce shock. We have investigated numerous parameters in an effort to elucidate the nature of this toxic syndrome. A companion report discusses several of these parameters. Pathologic (Pang et al., 1983) and blood flow studies conducted in these animals will be reported in future publications.

Alterations in hemodynamics during the shock syndrome resulted in diminished perfusion and contributed to organ dysfunction (Lorenzana et al., 1985). In the high dose group at 4 hours postdosing, when systemic blood pressure fell below an adequate renal filtration pressure of 60 mm Hg, urine production ceased (Guyton, 1976). The serum data would suggest decreased renal clearance of blood urea nitrogen, creatinine, inorganic phoshorus, and potassium. It is likely that the increased serum concentrations of these substances occurred not only as a result of inadequate blood pressure but also from renal vasoconstriction due to elevated circulating catecholamines and thromboxane.

An additional source of the elevated blood urea nitrogen may have been from the breakdown of structural proteins and the increased activity of AST in both groups may suggest toxic or ischemic damage to visceral or muscular tissues. In the low dose group, systemic pressure was reduced but remained adequate for renal filtration; organ dysfunction was not as severe; and concentrations of cathecholamines and thromboxane were not as great as in the high dose group. Consequently, the rate of increase in the concentration of blood urea nitrogen and AST in the low dose group was attenuated and there was no significant increase in the concentrations of creatinine, inorganic phosphorus or potassium.

Other circumstances probably contributed to the change in serum ion concentrations in the high dose group. A severe metabolic acidosis occurred in these animals (Lorenzana et al., 1985). The increase in serum potassium could have been augmented by an exchange of intracellular potassium for extracellular hydrogen ions. The increased inorganic phosphorus concentration may have been enhanced by blockade of the production of ATP and/or excessive destruction of ATP.

In the high dose group, tissue damage was the most probable source of the increases in serum magnesium and the activity of SAP. In soft tissue, the intracellular concentration of magnesium is greater than the concentration in extracellular fluid. SAP could have escaped fom damaged cells in the liver during the metabolism of T-2 toxin (Beasley, 1986) or from the gastrointestinal tract where necrosis was histologically apparent (Pang et al., 1983).

A probable initiating impetus for the decline in bound calcium was acidic blood pH. Hydrogen ions competitively displace ionic calcium from albumin making it unbound. Homeostatic mechanisms work to maintain a constant concentration of unbound calcium. Since this occurs within minutes, the expected measured effect is a decrease in bound calcium and this is what was observed in these experiments. It has been proposed that a solubility equilibrium exists between bone and the extracellular fluid compartment (Popovtzer and Knochel, 1980). An inverse relationship between serum calcium and serum phosphorus is present during extreme, acute changes in serum phosphorus, so that the product of both ions remains constant. Thus, elevations in phosphorus and magnesium may have exerted an additional hypocalcemic effects through equilibria and hormonal mechanisms, respectively (Massry et al., 1970; Gitelman et al., 1968).

Excessive concentrations of plasma epinephrine and norepinephrine, diminished cardiac output and histological evidence of myocardial damage were observed in these animals (Lorenzana et al., 1985; Pang et al., 1983). Adrenergic agents promote myocardial calcium ion influx as a pharmacologic action (Balaza and Bloom, 1982) and an increase in the calcium content of the myocardium has been associated with myocardial lesions (Fleckenstein, 1970; Lehr, 1981). The physiologic alterations induced by direct or indirect action of T-2 toxin may have caused an excessive calcium ion influx sufficient to cause myocardial injury.

Although focal areas of hemorrhage were observed in these swine (Pang et al., 1983), the single intravascular doses of T-2 toxin in this study did not cause hemostatic deficiencies as determined by clinically employed screening tests and numbers of circulating platelets. Other reports have associated T-2 toxin with coagulopathies identified through assessment of individual clotting factors (Gentry and Cooper, 1983; Gentry, 1982).

The pattern of the leukogram of animals that received T-2 toxin reflected a stress response (Schalm et al., 1975). The initial leukocytosis followed by leukopenia resembled an epinephrine response in which cells are shifted from the marginal to the circulating pool of cells. In the low dose group, the neutrophilia, lymphopenia, and eosinopenia at 24 hours postdosing were compatible with the effects of endogenous steroids in response to stress.

The leukopenic period in both high and low dose groups may have been due to sludging in peripheral or pulmonary vessels, margination, destruction, or excessive utilization. Our blood samples were collected from the aorta and, therefore, represent an evaluation of the white blood cell population in major vessels, only. However, during histopathologic examination increased numbers of leukocytes were noted in the liver and lung (Pang et al., 1983).

T-2 toxin is known to have suppressive effects on leukocyte production and to cause lymphoid degeneration in several species (Weaver et al., 1978b; DeNicola et al., 1978; Gentry, 1982; Murphy et al., 1978; Friend et al., 1983; Ueno, 1984). Necrosis and cellular debris in lymphoid tissue and in the bone marrow were observed in these animals (Pang et al., 1983). Lymphocytes have the ability to return to lymphoid tissue after being released into the circulation (Schalm et al., 1975). It is possible that actual lysis of lymphocytes occurred and contributed to the sustained lymphopenia observed in the low dose group.

The appearance of circulating nucleated red blood cells without a concommitant decrease in numbers of mature red blood cells is suggestive of injury to the bone marrow endothelium. Since no nucleated red blood cells were observed in the low dose group at 24 hours, it can be assumed that the damaging influence, perhaps ischemia or a cytotoxic effect of T-2 toxin or its metabolites, was no longer present. Diacetoxyscirpenol and deoxynivalenol, other trichothecene mycotoxins, also cause the appearance of nucleated red blood cells in the peripheral circulation of swine and cattle (Coppock, 1983).

As expected in a multisystem syndrome such as T-2 toxin induced shock, there are combined and complex interactions. In this discussion of numerous biochemical and hematologic parameters, we have examined the effects of T-2 toxin and suggested possible causes; however, further research is needed. In the elucidation of this pathologic state, we have described the result of physiologic alterations and the primary clinical problems. The mode of action of the agent must be further characterized in efforts to reach an ultimate goal of control and therapy.

ACKNOWLEDGEMENTS

The authors express their appreciation to Dr. Walter Hoffmann for his consultation in clinical pathology, to Mr. Steve Swanson for his supervision of the analytical chemistry procedures, to Dr. Art Siegel for assistance in analysis and to Mr. Dick Manuel for technical assistance.

FOOTNOTES

[1]Supported by the US Army Medical Research and Development Command, Contract No. DAMD17-83-C-2179.

[2]Presented at the Gordon Research Conference on Trichothecene Mycotoxins, June 1983

[3]MycoLab Company, Chesterfield, MO

[4]E. rhusiopathiae bacterin, Dellen Lab, Omaha, NB

[5]Thin layer and gas chromatography, Analytical Toxicology Laboratory, College of Veterinary Medicine, University of Illinois Diagnostic Lab, Urbana, IL

[6]IV infusion pump 2681, Harvard Apparatus Med Prod, Millis, MA

[7]Hycel Super Seventeen Autoanalyzer, Houston, TX

[8]Centriflo membrane cone, type CF25, Amicon Corp, Danvers, MA

[9]305A Perkin-Elmer spectrophotometer, Norwalk, CT

[10]Cyanmethemoglobin method, Sigma Chemical Co., St. Louis, MO

[11]Model αZBI, Coulter Electronics, Hialeah, FL

[12]Camco platelet count, Cambridge Chemical, Ft. Lauderdale, FL

[13]Dade Diagnostic, Inc., Miami, FL

[14]Diatomaceous earth, Becton-Dickinson, Rutherford, NJ

[15]Thrombo-Wellcotest, Wellcome Reagents Limited, England

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FIGURE 1: Serum concentrations of total calcium, potassium and magnesium in swine given T-2 toxin intravascularly. The high dose group demonstrated the greatest alterations in these ions. (mean ± SEM)

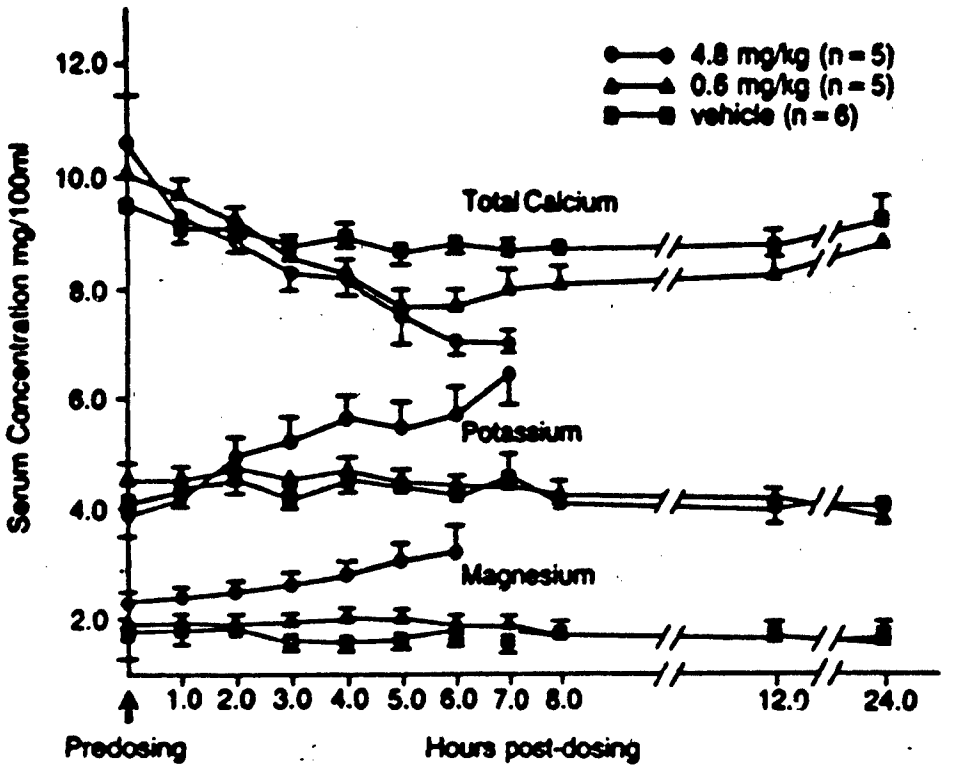

FIGURE ONE

FIGURE 2: Serum concentration of bound calcium and arterial blood pH in swine receiving 4.8 mg/kg T-2 toxin intravascularly. Both serum calcium and blood pH demonstrated decreases over time. (mean ± SEM)

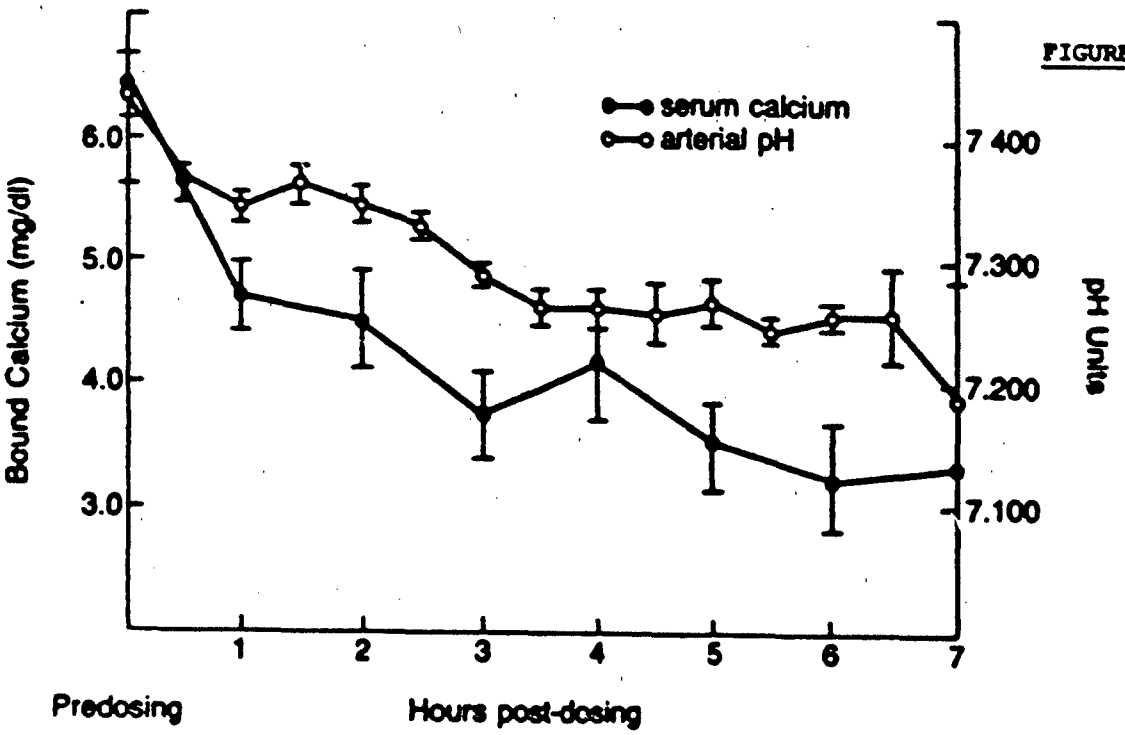

FIGURE 3: Total white blood cell concentrations in blood of swine given T-2 toxin intravascularly. Blood samples were collected from the ascending aorta. An initial leukocytosis was followed by a leukopenia. (mean ± SEM)

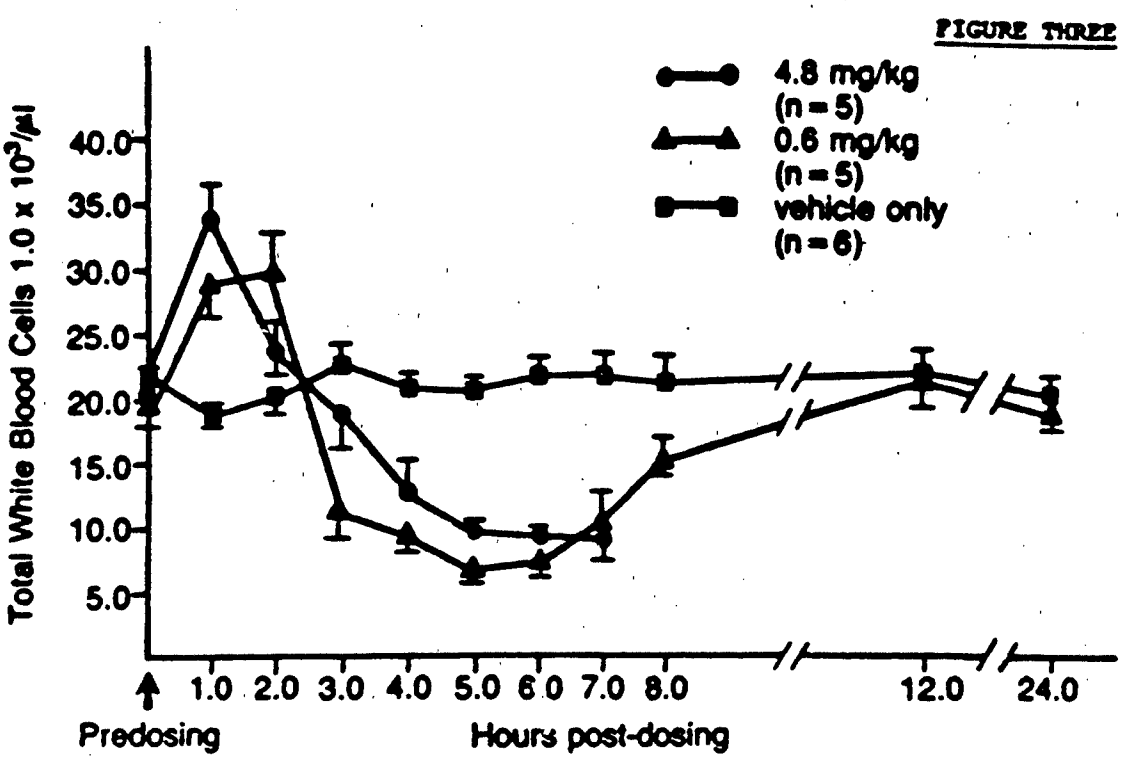

FIGURE 4: Absolute number of circulating segmented neutrophils in swine given T-2 toxin intravascularly. (mean ± SEM)

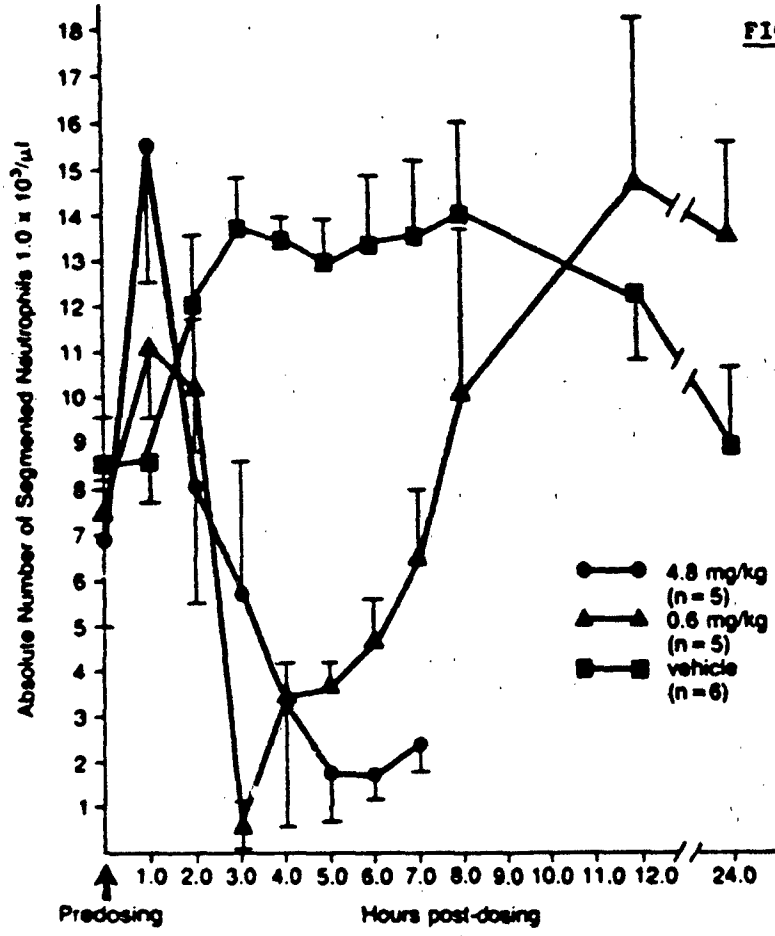

FIGURE 5: Absolute number of circulating lymphocytes in swine given T-2 toxin intravascularly. (mean ± SEM)

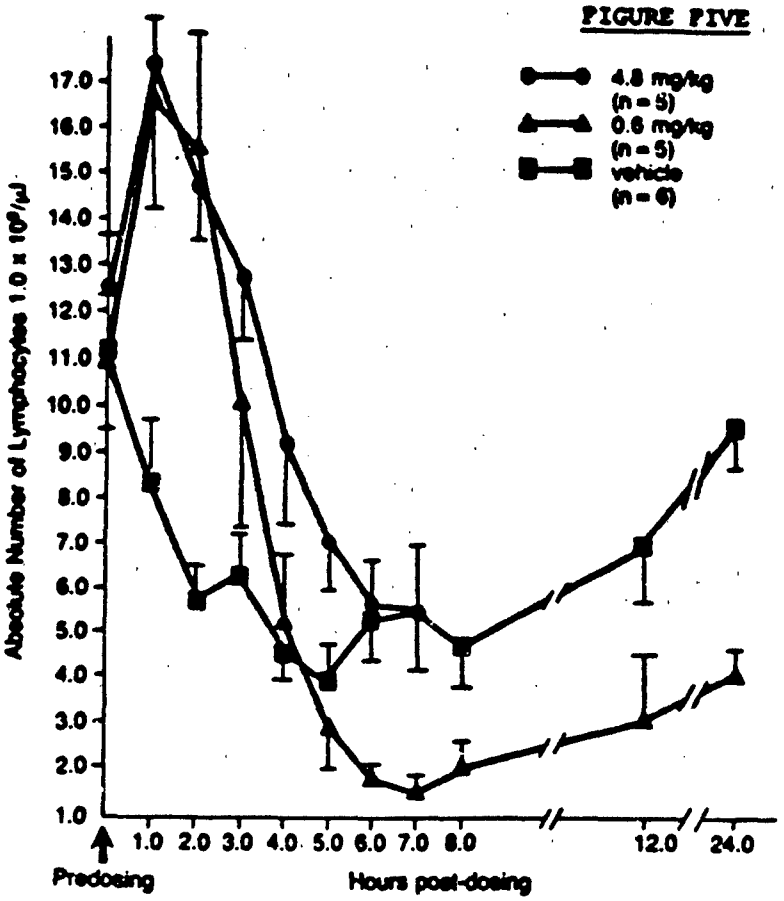

FIGURE 6: Nucleated red blood cells in peripheral blood of animals given T-2 toxin intravascularly. No nucleated red blood cells were observed in blood smears of the vehicle dosed animals. (mean ± SEM)

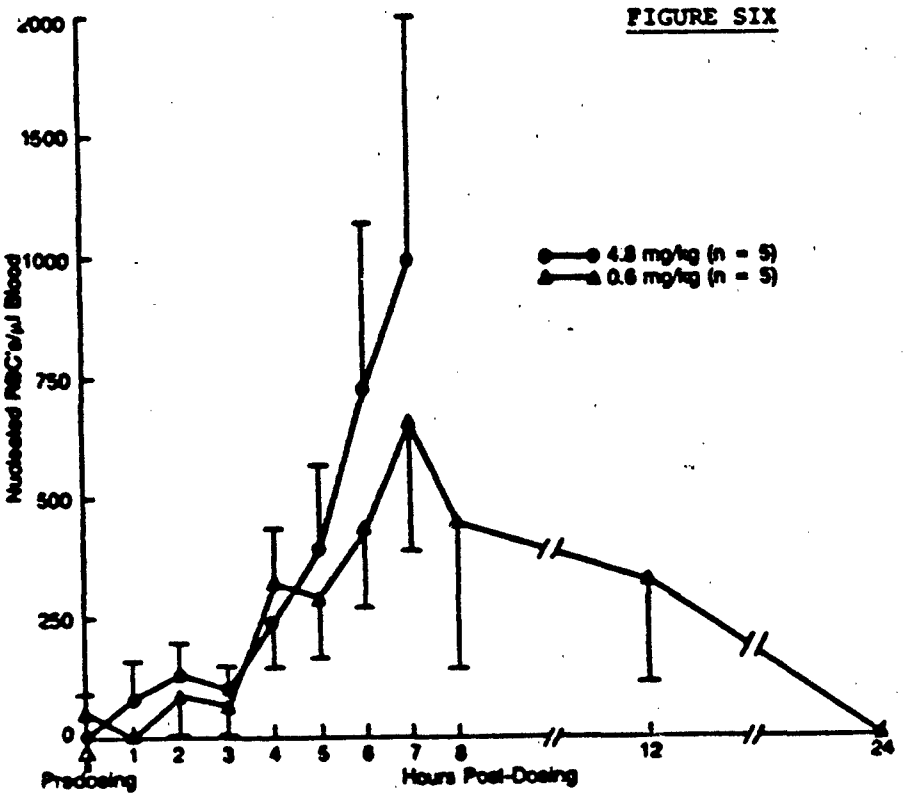

TABLE 1

Table 1
Serum Biochemical Values in Swine Given T-2 Toxin Intravascularly
Mean (± SEM)

Hours Post-Dosing	0	1	2	3	4	5	6	7	8	12	24
Phosphorus (mg/dl)											
Dose mg/kg											
4.8 (n = 5)	8.4	7.2	7.5	8.6	10.3	12.3	11.5	13.9*			
	(.5)	(.2)	(.4)	(.7)	(1.1)	(2.0)	(1.2)	(3.0)			
0.6 (n = 5)	8.6	8.8	7.1	7.9	8.2	8.6	7.9	8.4	9.3	10.3	8.6
	(.1)	(.2)	(.3)	(.3)	(.3)	(.4)	(.4)	(.6)	(.5)	(.3)	(.5)
vehicle (n = 6)	8.2	7.6	8.3	8.1	8.9	8.9	8.8	9.2	8.8	8.8	8.4
	(.3)	(.3)	(.3)	(.2)	(.3)	(.2)	(.1)	(.3)	(.2)	(.3)	(.3)
Blood Urea Nitrogen (mg/dl)											
Dose mg/kg											
4.8 (n = 5)	11.4	11.4	13.8	17.2	18.8	24.8	26.5	31.6*			
	(1.2)	(1.2)	(1.4)	(1.7)	(3.5)	(2.1)	(3.0)	(3.8)			
0.6 (n = 5)	10.8	10.8	13.8	15.6	17.2	18.4	19.4	21.2	21.8	21.2*	10.4
	(1.5)	(1.3)	(1.3)	(1.1)	(1.2)	(1.2)	(1.4)	(1.1)	(1.7)	(2.1)	(4.0)
vehicle (n = 6)	13.8	11.5	11.5	10.3	9.7	10.1	9.3	9.5	8.8	8.5	11.3
	(2.7)	(1.3)	(1.6)	(.9)	(.3)	(1.2)	(1.0)	(.9)	(1.1)	(.9)	(4.0)
Alkaline Phosphatase (U/l)											
4.8 (n = 5)	56.4	62.6	67.8	71.8	102.2	121.8	99.8	116.6*			
	(5.0)	(13.6)	(13.4)	(11.0)	(16.8)	(28.4)	(7.0)	(11.5)			
0.6 (n = 5)	56.0	66.8	66.4	61.2	69.8	69.2	69.2	64.6	62.3	67.8	72.4
	(6.1)	(7.6)	(7.8)	(7.5)	(7.4)	(8.1)	(8.4)	(12.1)	(11.7)	(7.3)	(6.9)
vehicle (n = 6)	49.5	48.3	47.3	44.3	46.0	43.7	43.1	43.5	42.2	46.5	49.3
	(5.0)	(5.5)	(5.1)	(5.0)	(4.7)	(5.0)	(4.6)	(4.7)	(4.3)	(5.0)	(7.2)

n = number of individuals per group
*statistically significant trend from predosing through indicated time, $P < 0.05$

PATHOLOGY REPORT: EXPERIMENTAL T-2 TOXICOSIS IN SWINE--Victor F. Pang

1. Summary of gross findings from pigs described in previous two studies

a. Group 2 (4.8 mg/kg; n = 5)

The snout and abdominal skin were slightly to moderately purple. The lateral aspect of the left apical and cardiac lung lobes were tightly adhered to the thoracic wall in each pig. There was approximately 40 to 50 mL of a straw-colored clear fluid in the thoracic cavity of one pig. Adhesions due to catheterization were noted between the pericardial sac and the epicardium in four pigs. Five-hundred ml of blood-tinged fluid was present in the pericardial sac of the remaining pig. Areas of moderate to severe subendocardial hemorrhage were seen in the left ventricle, especially in the area adjacent to the mitral valve.

The peripheral and visceral lymph nodes were moderately swollen, mottled red and slightly to moderately edematous. The spleen was soft and the white pulp was not as prominent as normal.

The mucosa of the gastric fundus was diffusely dark red to purple and was covered by a layer of thick mucus. The mucosa of the ileum, especially in the terminal portion, was diffusely red. The contents of the small intestine and spiral colon were semifluid. There were no formed feces in the terminal colon.

The liver was mottled red. The wall of the gall bladder was severely edematous. Occasional petechial hemorrhages were present on the surface of the adrenal gland and the parenchyma was mottled red. The mucosa of the uterus was red. The meninges were congested, and

in some cases, there were large bright red areas grossly indistinguishable from hemorrhages. Occasionally, the thyroid gland was mottled red.

b. Group 3 (0.6 mg/kg; n = 5)

Fibrotic adhesions due to surgical manipulation and catheterization were seen between the left lung lobes and thoracic wall in all pigs of this group as observed in group 2. In addition, four pigs had fibrotic adhesions between the pericardium and epicardium. Hydropericardium was seen in one pig. Mild linear erosions were found in the fundic portion of the stomach of two pigs. One pig had prominent edema in the capsule and adjacent connective tissue of the pancreas. Focally extensive hemorrhage (approximately 5 percent of the organ) was noted in the subcapsular region of the pancreas of one pig. Focal necrosis of the mucosa was observed in the cecum of two pigs.

c. Control pigs (vehicle only; n = 2 in each group)

Aside from fibrotic adhesions between the left lung lobes and thoracic wall, as well as between the pericardium and epicardium, no other abnormalities were found.

2. Summary of histopathologic findings

a. Group 2 (4.8 mg/kg; n = 5)

Thymus. There was mild to moderate lymphoid depletion and a small to large number of tingible body macrophages in the cortex. Mild to moderate lymphocytic necrosis was noted in the medulla with some small eosinophil aggregates randomly present.

Palatine tonsil. Diffuse, severe lymphocytic necrosis, mainly in the germinal centers, was accompanied by areas of mild to moderate

neutrophil infiltration. Multifocal, mild to severe degeneration and necrosis occurred in the crypt epithelium. A moderate to large number of transepithelial lymphocytes were undergoing degeneration and necrosis.

Submandibular, cervical, mediastinal, bronchial, mesenteric and inguinal lymph nodes. There were diffuse, moderate to marked lymphocytic depletion and lymphocytic necrosis, primarily in the germinal centers. Mild to moderate reticuloendoethelial hyperplasia and areas of edema and small neutrophil aggregates were seen in the sinuses and were accompanied by areas of hemorrhages, primarily in the subcapsular region.

Spleen. Diffuse, moderate to severe lymphocytic necrosis and lymphoid depletion were observed in the white pulp with scattered mild perifollicular hemorrhage. The red pulp was moderately to severely congested with mild to moderate ieukocytosis (neutrophils) in the sinuses.

Stomach. Areas of epithelial sloughing were present on the mucosal surface. Diffuse, moderate to marked congestion, moderate edema and areas of mild hemorrhage were noted in the luminal phase of the lamina propria. Occasionally, fibrin thrombi were seen in capillaries. Necrosis was observed in the small lymphoid nodules of the submucosa and in the lymphocytes of the lamina propria.

Intestine. Mild to severe villous necrosis, primarily in the small intestine, was observed. Congestion, occasional mild hemorrhage and fibrin thrombi were also present. Moderate to severe crypt epithelial necrosis was diffusely present, especially in the jejunum

and ileum. The number of mononuclear cells in the lamina propria was moderately increased throughout the intestine. Severe necrosis was noted in the lymphocytic population. Prominent necrosis was seen in Peyer's patches and lymphoid nodules throughout the submucosa.

Gall bladder. Moderate to severe subserosal edema was present.

Liver. Areas of mild to moderate congestion and sinusoid leukocytosis (neutrophils and lymphocytes) were noted. Occasionally, there was a mild neutrophil and eosinophil infiltration in the perilobular fibrous tissue. The hepatocytes were slightly swollen.

Pancreas. Occasionally, individual cell degeneration and necrosis were present in the islets of Langerhans.

Lung. The bronchus-associated lymphoid nodules and the peribronchiolar and perivascular lymphoid aggregates were moderately to severely necrotic. Areas of congestion were noted in the interalveolar septa.

Heart. Diffuse, subepicardial fibrosis, edema, neovascularization and minimal mononuclear cell infiltration associated with catheterization were seen in four pigs. Areas of mild to severe conges- tion and hemorrhage occurred in the subendocardial myocardium. Focal hyalinization and fragmentation were present in the affected muscle bundles.

Kidney. Scattered mild fibrosis and mononuclear cell infiltrates were seen in the interstitium. Cellular necrosis was noted in the mononuclear cell infiltrates and subpelvic lymphoid nodules. Congestion was present in the glomeruli and interstitium.

Adrenal. Individual cell degeneration and necrosis were randomly distributed in the cortex. Mild leukocytosis was present in capillaries.

Ovary. Degeneration and necrosis occurred in the ova of primary follicles.

Uterus. The lamina propria of the endometrium was moderately congested.

Brain. The meninges as well as parenchyma were moderately congested.

Bone marrow. Mild cellular necrosis was observed.

Rib (costochondral junction). There were only a small number of osteoblasts along the cartilagenous spicules of the calcification zone. Moderate cellular necrosis was noted in the marrow cavity.

Spinal cord, eye, esophagus, trachea, aorta, bladder, skeletal muscle, tongue, parotid salivary gland, skin, mammary gland, pituitary gland, sciatic nerve. No significant lesions.

b. Group 3 (0.6 mg/kg; n = 5)

Thymus. There was scattered individual cell necrosis and a moderate to marked increase in the number of tingible body macrophages in the cortex.

Lymph Nodes. Minimal lymphocytic necrosis as well as prominent lymphoid and RE hyperplasia were noted. Occasional moderate hemosiderin deposition was also present.

Spleen. Minimal lymphocytic necrosis in the germinal centers of the follicles and mild to moderate neutrophilic infiltration in the red pulp were observed in two pigs.

Palatine Tonsil. Minimal lymphoid necrosis and moderate lymphoid hyperplasia were seen in two pigs.

Heart. Four pigs had moderate to marked diffuse subepicardial fibrosis which was associated with catheterization. Occasional deeply eosinophilic myofibers with pyknotic nuclei were noted in the myocardium.

Pancreas. There was moderate, multifocal degeneration and necrosis of single or small groups of acinar cells characterized by vacuolization, globular condensation of the cytoplasm as well as nuclear pyknosis and karyorrhexis.

c. Control pigs (vehicle only; n = 2 in each group)

Aside from prominent subepicardial fibrosis of the heart, no other lesions were observed in any internal organs.

C. SYSTEMIC DISTRIBUTION OF BLOOD FLOW DURING T-2 TOXICOSIS--Gregg R. Lundeen

OBJECTIVE:

To determine how the distribution of cardiac output is altered as acute T-2 toxicosis progresses over time.

HYPOTHESIS TO BE TESTED:

The fraction of cardiac output supplying the viscera, musculature, skin and kidneys is vastly reduced in T-2 toxicosis as cardiac output declines and the animal attempts to conserve blood flow to the brain and heart.

ABSTRACT:

Three groups of swine (6 per group) were used to determine hemodynamic and blood flow alterations induced by T-2 toxin. Two groups were dosed at 0.6 or 2.4 mg/kg T-2 toxin, and one group served as a vehicle control (70 percent ethanol). Organ blood flow was determined at 0 hour (predosing) and at 90-minute intervals for 6 hours postdosing using 15 ?m diameter radionuclide-labeled microspheres injected into the left atrium. Hemodynamic parameters were obtained at the same time points.

The infusion of T-2 toxin resulted in reductions in cardiac output. This trend appeared to reverse itself in the low-dose animals after 3 hours (72 percent of predosing at 3 hours; 85 percent at 6 hours), whereas in the high-dose group, cardiac output continued to decline (58 percent of predosing at 3 hours; 36 percent at 6 hours). Mean aortic pressure (MAP) declined in a dose-dependent fashion which tended to parallel the reduction observed in cardiac output (Low dose MAP: 64 percent of control at 3 hours; 72 percent at 6 hours. High dose MAP: 62 percent of control at 3 hours; 37 percent at 6 hours). Heart rate was increased in both groups treated with T-2 toxin. While

total peripheral resistance decreased in the low-dose group, it remained unchanged at the high dose. Pulmonary vascular resistance was increased in both T-2 groups. Left and right ventricular work decreased following administration of T-2 toxin at both high and low doses. Blood gases showed maintenance of arterial oxygen tension, slight decreases in arterial carbon dioxide tensions and intense reductions in pH.

Blood flow to the brain, heart and kidneys decreased following exposure to the toxin. However, the relative percentage of cardiac output received by these organs was maintained despite the drop in blood flow. Pancreatic and splenic blood flows were the most severely compromised as a result of T-2 toxicosis. Consequently, the percentage of cardiac output going to the pancreas and spleen was reduced dramatically. Adrenal, hepatic and total gastrointestinal blood flows increased or did not change from control values. As a result, the percentage of cardiac output supplying these organs increased.

INTRODUCTION

T-2 toxin is a trichothecene mycotoxin produced by several species of Fusarium. Debilitating disease states and even death have been reported in humans and livestock following consumption of grain contaminated with these fungi (26). More recently, this toxin has been implicated as a chemical warfare agent (5).

Because T-2 toxin has been shown to cause alterations in cardiovascular function, there is a need to characterize the shock-like syndrome associated with it. Although some research has been performed in this area, conflicting reports exist as to its effect on the cardiovascular system in different animal species. The effect of the shock syndrome induced by T-2 upon the systemic distribution of blood flow in swine has not been previously reported.

The use of radionuclide-labeled microspheres allows determination of cardiac output as well as distribution of blood flow to the various organs. We employed this technique to examine the cardiovascular effects of T-2 toxin in pigs at 0.6 mg/kg and 2.4 mg/kg and compared them to vehicle control (70 percent ethanol) values. Swine were chosen as our experimental model because of similar physiologic and anatomic characteristics with humans (6).

MATERIALS AND METHODS

This experiment was conducted on 18 healthy female pigs weighing 43.5 to 97.5 kg (54.7 $\pm$ 4.4 kg; mean $\pm$ SEM). Each animal was surgically instrumented several weeks prior to beginning the study in order to avoid the effects of acute surgical trauma.

Surgical Preparation of Animals

Four to eight weeks prior to the study, all pigs were subjected to a left lateral thoracotomy. Anesthesia was induced by administration (via a nose cone) of 5 percent halothane in oxygen using a Fortec® vaporizer. Following endotracheal intubation, anesthesia was maintained with a mixture of halothane (0.5 to 1 percent) and oxygen delivered via a closed circuit anesthetic system and a ventilator. Saline-filled Tygon catheters were implanted in the left atrium and pulmonary artery. The ascending aorta was also catheterized via the left internal thoracic artery. A fourth catheter, used for reference blood withdrawal for organ blood flow determinations, was advanced into the lumbar region of the descending aorta via the right femoral artery. These catheters were then heparinized to maintain patency and the distal ends housed subcutaneously near the spine.

Procedures

At the time of the study, all animals were completely recovered from surgery. Food was withheld 12 hours prior to the experimental protocol but H_2O was provided ad libitum. On the day of the study, the animal was placed in a sling such that its ventral surface was supported while the limbs hung freely. The animals quickly adapted to this mode of restraint. Previously implanted catheters were exteriorized following local infiltration of 2 percent lidocaine.

Phasic and mean pressures in the aorta, pulmonary artery and left atrium were recorded on a multichannel physiograph using noncompliant fluid-filled systems and pressure transducers. The transducers were zeroed at the level of the scapulohumoral joint which was considered to be the level of the right atrium.

Left atrial injection of 15 ?m radiolabeled microspheres (^{141}Ce, ^{113}Sn, ^{103}Ru, ^{95}Nb, ^{46}Sc) was used for organ blood flow determina- tions. The microspheres were randomized among the time points of the study. Three to five million microspheres were injected for each blood flow deter- mination. Reference arterial blood was withdrawn from the descending aorta (14 ml $\cdot$ min^{-1}) beginning just prior to microsphere injection and continu- ing for 90 seconds post-injection. This procedure was carried out with careful hemodynamic monitoring. Adequate mixing of microspheres with blood was demonstrated by similar blood flow values obtained for some of the bilateral organs in the body (i.e., each half of the cerebrum, adrenals and kidneys) in all animals. All criteria for organ blood flow and cardiac output determinations by the microsphere technique (1,3) were satisfied

At the end of the experiment, the animals were anesthetized with Sodiu Thiamylal (Surital®) and killed by exanguination. Brain, heart, adrena glands, kidneys, spleen, liver, pancreas, stomach, small intestine and larg intestine were removed. The brain was cleared of the meninges and large pia vessels and divided into the right and left cerebral hemispheres, cerebellu and brain stem using anatomic landmarks. The heart was stripped of the atria, epicardial fat, large vessels, valves and chordae tendinae and divided int right ventricle, left ventricle and interventricular septum. The capsule wa removed from the kidneys and the gastrointestinal tract was cleared of extra- neous tissue. The various organs were minced into small pieces and placed i preweighed vials, weighed again and counted in a gamma well scintillatio counter along with the reference arterial blood.

At each 1.5-hour interval (see protocol), the following measurements were obtained sequentially during steady state conditions: 1) arterial and mixed venous blood gas tensions and pH, 2) pressures in the ascending aorta, pulmon- ary artery and left atrium and cardiac output using the dye dilution technique, 3) cardiac output and organ blood flow using the microsphere technique and 4) follow-up blood gas tensions and pH as described for 1). The similarity of blood gas parameters between 1) and 4) was part of the criteria for determining the existence of a hemodynamic steady state.

For CO determination using the dye dilution technique, a CO computer was used. Dye was injected into the pulmonary artery while blood was being withdrawn at a constant rate from the aorta.

PROTOCOL

Each animal was studied as described above at 1.5-hour intervals over a 6-hour period after one of the following three treatments: (1) ethanol

control, (2) low dose T-2 toxin (0.6 mg/kg) and (3) high dose T-2 toxin (2.4 mg/kg). The animals were randomly assigned to the above treatments.

A. Ethanol Control (n = 6)

Predosing (0 hour) organ blood flow and hemodynamic parameters were obtained in pigs resting quietly in the sling. A hemodynamic steady state was determined by the stability of the heart rate, arterial and pulmonary artery pressures and blood gas tensions.

Upon completion of the predosing measurements, 7 mL of 70 percent ethanol was infused into the pulmonary artery over a 2-minute period. Organ blood flow and hemodynamic parameters were then measured at 1.5-hour intervals for 6 hours.

B. Low Dose T-2 Toxin (0.6 mg/kg) (n = 6)

Predosing measurements were obtained as described for ethanol controls. The animals were then subjected to infusion of 0.6 mg/kg T-2 toxin dissolved in 7 mL of 70 percent ethanol over a 2-minute period. Measurements were then made at 1.5-hour intervals as stated above.

C. High Dose T-2 Toxin (2.4 mg/kg) (n = 6)

This treatment was carried out in the same manner described for A and B except that 2.4 mg/kg T-2 toxin was dissolved in 7 mL of 70 percent ethanol.

Measurements and Calculations

Organ blood flow (F_u) was calculated from the equation:

$$F_u = F_r/N_r \times N_u$$

were F_r represents the known withdrawal rate of the reference blood. N_r is the number of microspheres in the reference blood, and N_u is the number of

microspheres in the tissue sample. The cardiac output (F_T) at the time of microsphere injection was determined using the equation:

$$F_T = F_r/N_r \times N_T$$

where N_T represents the total number of microspheres injected. The cardiac outputs determined using the microsphere technique were within 4 to 6 percent of cardiac outputs determined simultaneously using the dye dilution technique.

To determine the number of microspheres in an unknown sample, the on-board computer of the gamma counter used a least squares fitting technique in order to calculate the number of counts contributed by each radioisotope in the sample. During the least squares fit, a constant (K_u) was determined which represented the ratio of the number of counts for an isotope in the unknown sample, C_u, to the number of counts in a standard spectrum of the isotope, C_s, using the formula $K_u = C_u/C_s$.

Hemodynamic parameters were obtained over several cardiac cycles and recorded simultaneously with organ blood flow determinations. Stroke volume and cardiac output are expressed on a body weight basis. Blood gas variables were corrected to the animal's body temperature using nomograms (4,13,14,22) built into the blood-gas analyzer. Calibration of the blood-gas analyzer was checked after each set of blood samples using pig blood tonometered at 38° C with gases of known oxygen and carbon dioxide tensions.

Total peripheral resistance (TPR) and pulmonary vascular resistance (PVR) were determined from the quotient of mean arterial or pulmonary artery pressure (mmHg) and cardiac output ($mL \cdot min^{-1} \cdot kg^{-1}$). The calculation of pulmonary vascular resistance included subtraction of the mean left atrial pressure from the mean pulmonary artery pressure. Vascular resistance within the respective tissues was calculated by dividing mean arterial pressure

(mmHg) by tissue blood flow (mL · min^{-1} · g^{-1}). Ventricular work (kg · mm · min^{-1}/kg body weight) was computed from the formula cardiac output (ml · min^{-1} · kg) x mean pressure (aortic or pulmonary arterial [mmHg]) x 1.36 x 10^{-2}; where 1.36 is the conversion factor for changing pressure in mmHg to g/cm^2.

Statistical Analysis

The experiment was set up as a completely randomized design. The data was, therefore, analyzed using a one-way analysis of variance. When significant F values were found, Fisher's protected least significant difference method was used to determine differences between treatment means (23). A probability level of 5 percent ($P < 0.05$) was considered statistically significant. Data are presented as mean $\pm$ 1 SEM.

RESULTS

Arterial blood gas tensions and pH, mixed venous blood gas tensions, body temperatures and hematocrit values are shown in Table 1. Predosing (0 hour) values of blood-gas variables were similar between the three treatment groups. Oxygen tension (PaO_2) and body temperature were unchanged throughout the study in all groups. Arterial carbon dioxide tension ($PaCO_2$) in animals dosed at 0.6 mg/kg T-2 toxin was decreased from vehicle control values at 4.5 and 6 hours. The arterial pH in this group was decreased following infusion of T-2 toxin and remained below control (vehicle) values throughout the time course of the study. Animals dosed at 2.4 mg/kg T-2 toxin showed more severe changes in $PaCO_2$ and pH (Table 1). Mixed venous oxygen tension (P_vO_2) increased in both groups of animals treated with T-2 toxin. The packed cell volume of the low dose group was similar to that of the control animals, whereas the high dose group increased.

Hemodynamic variables are presented in Table 2. Predosing values (0 hour) were similar between the three treatment groups. Heart rates were significantly increased above control values in both groups. Cardiac outputs in T-2 treated animals were maintained near control values for the first 1.5 hours following toxin infusion, after which it began to decline, with the extent of the reduction being more severe in the high dose group. Mean aortic pressure and stroke volume paralleled changes in cardiac output. Mean pulmonary artery pressure was unchanged by T-2 toxin administration. Total peripheral resistance was decreased with the 0.6 mg/kg dose, while the high dose group remained unchanged. Pulmonary vascular resistance was increased while left and right ventricular work was decreased in both groups.

Administration of T-2 toxin caused dose dependent decreases in brain blood flow (Figure 1). However, the percentage of cardiac output received by the brain (Table 3) was higher than or equal to values for control animals. The increases were most evident in the high dose group.

Myocardial blood flow (Figure 2) to all regions of the myocardium was altered in a fashion similar to that described for brain blood flow. The fraction of cardiac output going to the myocardium (Table 3) in the low dose group remained unchanged from the control group. In the high dose group, percent cardiac output was unchanged from control at 1.5 and 3 hours post-dosing but increased at 4.5 and 6 hours post-dosing.

Adrenal blood flow (Figure 3) and the percent of cardiac output (Table 3) received by the adrenal glands was increased significantly in animals dosed at 2.4 mg/kg. In animals given 0.6 mg/kg T-2 toxin, adrenal blood flow did not show a statistically significant increase, whereas the fraction of cardiac output received did increase. Blood flow to the kidneys (Figure 4) was

decreased from control values in both groups of animals treated with T-2 toxin. However, the renal fraction of cardiac output (Table 3) remained unchanged. Pancreatic and splenic blood flow (Figures 5 and 6, respectively) were decreased with both levels of T-2 toxin administration. Similarly, the pancreatic and splenic fractions of cardiac output (Table 3) were severely reduced.

Hepatic arterial blood flow (Figure 7) and percentage of cardiac output (Table 3) was increased in animals given 0.6 mg/kg T-2 toxin. In the high dose group, hepatic arterial blood flow was increased at 1.5 and 3 hours post-dosing and then declined towards control values. However, the percentage of cardiac output to the liver remained elevated above respective temporal control values.

Total gastrointestinal blood flow (Figure 8) (stomach, small intestine and large intestine) in animals dosed at either level of T-2 toxin remained unchanged from control values 1.5 hours after dosing. At 3 and 4.5 hours post-dosing, both T-2 treated groups had significantly increased blood flow. The high dose group had returned towards the control value at 6 hours, while the low dose group remained elevated. The percentage of cardiac output perfusing the GI tract (Table 3) mimicked changes in blood flow. Further discussion of the effects of T-2 toxin on individual organs of the digestive tract will be forthcoming. The percentage of cardiac output to the vessel rich group of organs (Table 3) demonstrated dramatic increases at 3 and 4.5 hours post-dosing. This was particularly evident in the high dose group.

DISCUSSION

The PaO_2, $PaCO_2$ and arterial pH changes were similar to those previously reported in swine (19), rats (7) and guinea pigs (7). However, these findings

are in contrast to those reported for primates (12). Despite increased ventilation, pigs were unable to compensate for metabolic acidosis. The increase in mixed venous oxygen tension (P_vO_2) may be the result of decreased oxygen extraction by the tissues and/or increased shunting.

Hemodynamic Parameters

T-2 toxin caused a dose-dependent decrease in mean aortic pressure. This is in agreement with our previous findings in swine (19) and observations in guinea pigs (7) and primates (12). Similar results have been observed in calves (24), dogs (2) and primates (2) during endotoxic shock. In contrast, our results differ from those reported for rats which indicated an increase in arterial pressure following exposure to T-2 toxin (7,27).

The decreases in cardiac output and stroke volume following administration of T-2 toxin are consistent with previous findings (7,12,19). These reductions are evident despite significant increases in heart rate (i.e., low dose: 30 56, 73 and 56 percent increases from control values at 1.5, 3, 4.5 and 6 hours post-dosing; high dose: 10, 59, 85 and 84 percent increases from respective control values, see Table 2). This increase in heart rate is consistent with previous findings in pigs (19), primates (12) and rats (7). However, this contrasts with the decrease in heart rate observed in guinea pigs treated with T-2 toxin (7). Increased heart rate in conjunction with decreased arterial pressure and cardiac output supports the contention that T-2 toxin obtunds myocardial contractility as reported in conscious primates (12). Yarom et al. (29), using isolated perfused rat heart preparations, also found T-2 to cause a decrease in contractility, although the toxin concentrations used were extremely high. These observations are similar to those reported for alteration in myocardial function during endotoxic shock (9).

Left ventricular work significantly decreased from control values in both T-2 treated groups beginning at 3 hours after dosing. This decrease, which was attributable to the dose-related decrease in cardiac output and mean aortic pressure, has not been previously reported. However, the reduction in cardiac output and increase in arterial pressure reported for rats (7) would indicate that calculated left ventricular work remained unchanged or increased which is opposite of our findings. The decrease in calculated right ventricular work can be explained by a decrease in cardiac output and maintenance of mean pulmonary artery pressure.

In animals dosed at 0.6 mg/kg T-2 toxin, total peripheral resistance was decreased from control values. This is consistent with the findings in primates (12). In contrast, these findings do not agree with total peripheral resistance values reported in rats following exposure to T-2 toxin. Because total peripheral resistance remained unchanged from control values in the high-dose group (2.4 mg/kg T-2 toxin), it is apparent that the decrease in blood pressure was proportional to reductions in cardiac output. Pulmonary vascular resistance was increased from respective ethanol control values in both groups of swine following T-2 administration. In the low-dose group, this was not evident until 3 hours post-dosing. This is similar to that previously reported in swine (19), but has not been reported in other species. The increases in pulmonary vascular resistance observed in swine following T-2 administration are strikingly similar to those reported in the dog (10), primate (11), calf (24) and cat (15) during endotoxic shock. Our findings indicate that T-2 toxin may play a major role in causing pulmonary vasoconstriction accounting for the lack of change in mean pulmonary artery pressure. A concomitant decrease in calculated right ventricular work and

increased pulmonary vascular resistance would indicate a deterioration of myocardial performance (i.e., contractility).

Organ Blood Flow

Blood flow to the various organ systems of the body during T-2 toxicosis has not been previously reported. However, blood flow values obtained at pre-dosing in our 3 treatment groups compare favorably with awake, unanesthetized values previously reported for swine using identical techniques (20,25).

Brain blood flow (Figure 1) showed marked variation in animals dosed at 0.6 mg/kg T-2 toxin (i.e., significantly lower at 1.5 and 4.5 hours post-dosing; not significant at 3 and 6 hours post-dosing). Despite these fluctuations, the percentage of cardiac output received by the brain (Table 3) remained unchanged from control levels. In the high-dose group (2.4 mg/kg), brain blood flow remained below control values throughout the time course of the study. In spite of lower absolute blood flows to the brain, the fraction of blood flow (percent CO) received by the brain remained unchanged up to 3 hours post-dosing and eventually increased at 4.5 and 6 hours post-dosing. This finding indicates that the reduction in blood flow to the brain did not continually parallel T-2 induced reductions in cardiac output. As T-2 toxin has been shown to cause a dose-dependent vasoconstriction in a bovine ear preparation (28), the decrease in brain blood flow may be due to both a lower level of perfusion pressure and a given extent of vasoconstriction. However, as shown in Table 4, the calculated vascular resistance for brain tissue may indicate a local compensatory vasodilatation brought about by intact autoregulation in an attempt to preserve blood flow to the brain, particularly in the latter stages of the experiment.

Alterations in myocardial blood flow (Figure 2) induced by T-2 toxin are similar to those observed for the brain. The percentage of cardiac output received by the heart (Table 3) and coronary vascular resistance (Table 4) in the low-dose group did not change from respective control values, whereas animals in the high-dose group showed an increase in the fraction of cardiac output going to the cardiac ventricles at 4.5 and 6 hours. Coronary vascular resistance in this group decreased from respective ethanol control values in the later stages of the study. This reduction in vascular resistance would be expected as a compensatory local response to the decreased blood flow coupled with intense tachycardia in an attempt to meet an increased myocardial oxygen demand.

Adrenal blood flow (Figure 3) increased above control levels in a dose-related fashion. This observation correlates well with previous findings which show increases in endogenous catecholamines following exposure to T-2 (7,19). A likely explanation for increased adrenal blood flow is that it parallels enhanced adrenal activity and oxygen consumption which occurs as catecholamine production increases in an attempt to overcome hypotension and reduced cardiac output.

The drop in renal blood flow (Figure 4) paralleled the decrease in mean aortic pressure. This may help explain the findings of decreased urinary output during the later stages of the toxicosis (19). Since renal blood flow paralleled the reductions in total cardiac output induced by T-2 toxin, the percentage of cardiac output received by the kidneys (Table 3) remained unchanged from respective control values.

Blood flow to the pancreas (Figure 5) was severely affected by T-2 toxin administration. This reduction may be due, in part, to direct action of T-2

and/or its metabolites on the pancreatic vasculature. It is probable that this severe hypoperfusion and concomitant ischemia results in the release of proteolytic enzymes. Hypoxia is a potent stimulus for lysosomal membrane disruption (18). These enzymes may then enhance the production of myocardial depressant factor (MDF) (17). Myocardial depressant factor has a negative inotropic effect on both isolated heart preparations (16) and intact hearts (8). Hence it is suggested that because of the severe decrease in pancreatic blood flow, the release of these factors during T-2 toxicosis plays an important role in the deterioration of myocardial performance.

In both groups of T-2 treated swine, there was a drastic reduction in splenic blood flow (Figure 6). However, this decrease was not evident in the high-dose group until 3 hours post-dosing. Intense splenic contraction may be caused by the increased levels of circulating catecholamines previously reported in T-2 dosed swine (19). Splenic contraction could also explain the observed increase in hematocrit (Table 1). However, the data may also be explained by a local damaging effect of T-2 toxin on lymphoid tissue which could result in compromised circulation in this T-2 sensitive organ.

Blood flow to the liver (via the hepatic artery) (Figure 7) remained unchanged or increased in both groups administered T-2 toxin. Consequently, the percentage of cardiac output received by the liver (Table 3) in these two groups was much higher than respective control values. The maintenance or increased blood flow may be mediated locally and may influence the detoxification and/or clearance of T-2 toxin and its metabolites.

Blood flow to the gastrointestinal tract (Figure 8) (stomach, small intestine, large intestine) was maintained near or above control values in spite of the decline in mean aortic pressure. As a result, the fraction of

total cardiac output (Table 3) received by this group of tissues was higher than the associated control values. The reason for this is not clearly understood. The increased blood flow to the GI tract may be a result of severe local cytotoxic effects and secondary reflex responses to T-2 toxin. Since T-2 toxicosis causes shock, the increase in blood may coexist with a pooling of blood in the splanchnic region similar to that reported in endotoxic shock (2).

The percentage of cardiac output accounted for in this study in the low-dose group was higher than control values at 3, 4.5 and 6 hours post-dosing. Similarly, these values in the high-dose group were higher than control levels at 3 and 4.5 hours post-dosing but declined towards control at 6 hours. Neither T-2 treated group exhibited a difference from control at 1.5 hours post-dosing. The dramatic increase in the percentage of cardiac output received by the organs observed in this study indicates an intense shunting of blood away from the periphery. It appears that this may be an attempt to preserve blood flow to the vital organs at the expense of the peripheral tissues. This may also account for the cyanotic mucous membranes and cold extremities previously reported by Lorenzana et al. (19), and observed in the present study.

In conclusion, it appears that the extent of T-2 induced alterations on cardiovascular function is dose-dependent. Animals observed in this study, particularly the high-dose group, demonstrated classic signs of circulatory shock including reduced cardiac output, hypotension and tissue hypoperfusion. The compensatory responses to T-2 toxicosis observed in this syndrome (i.e., increased heart rate and increased catecholamine levels) were similar to those for other types of shock. However, these compensatory responses did not alter

the deterioration of hemodynamic and blood flow parameters measured in this study. The shock syndrome associated with T-2 may be manifested in two ways. There is an apparent direct effect of T-2 toxin on organ/tissue vasculature and the heart itself. Secondly, and perhaps more importantly, there may be a release of lysosomal enzymes and myocardial depressant factor from ischemic tissue (i.e., pancreas). These compounds may further precipitate cardiovascular deterioration leading to irreversible shock. The reduction in blood flow and consequently tissue perfusion was less in the 0.6 mg/kg group than in the 2.4 mg/kg group. Therefore, it follows that the extent of the release of these deleterious compounds was likely lower in the low dose group which would explain the difference between the two T-2 groups in severity of toxicosis.

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TABLE 1. Blood gas variables. Data are presented as the mean ± SEM.

	Vehicle (70 percent ethanol) (n = 6) Hours Post-Dosing					Low Dose (0.06 mg/kg) (n = 6) Hours Post-Dosing					High Dose (2.4 mg/kg) (n = 6) Hours Post-Dosing				
	0	1.5	3	4.5	6	0	1.5	3	4.5	6	0	1.5	3	4.5	6
Body temperature (° C)	39.7 ± .2	39.8 ± .1	40.0 ± .2	39.8 ± .2	39.9 ± .2	39.1 ± .1	39.2 ± .2	39.5 ± .2	39.9 ± .2	39.8 ± .2	39.0 ± .2	38.9 ± .2	39.7 ± .2	39.9 ± .5	39.4 ± .7
Arterial oxygen tension (mmHg)	91.4 ±2.1	96.8 ±3.7	94.4 ±3.3	96.0 ±3.4	94.0 ±3.1	93.4 ±2.9	88.9 ±5.0	90.9 ±4.4	98.0 ±3.7	97.7 ±2.9	92.8 ±1.8	95.9 ±3.5	96.8 ±2.3	96.5 ±1.5	97.3 ±3.0
Arterial carbon dioxide tension (mmHg)	38.3 ± .9	36.0 ± .8	37.2 ± .6	37.7 ± .6	35.9 ±1.0	37.4 ±1.2	38.9 ±1.7	39.4 ±1.5	34.9 ± .7	33.0 ±1.0	38.0 ± .3	35.2 ± .4	36.4 ± .8	33.6 ±1.1	28.6 ±1.0
Arterial pH	7.387 ± .014	7.398 ± .012	7.400 ± .009	7.388 ± .008	7.397 ± .017	7.407 ± .005	7.312 ± .022	7.300 ± .010	7.323 ± .016	7.347 ± .014	7.400 ± .018	7.318 ± .017	7.252 ± .017	7.227 ± .026	7.21 ± .02
Mixed venous oxygen tension (mmHg)	46.3 ± .9	45.2 ±2.1	43.6 ±2.6	45.8 ±1.8	45.4 ±2.2	49.1 ±1.3	53.0 ±1.5	50.2 ±1.1	50.3 ±1.8	46.6 ±2.2	48.2 ±1.5	52.2 ±2.1	54.2 ±1.9	53.0 ±1.6	47.3 ±1.5
Mixed venous carbon dioxide tension (mmHg)	43.4 ± .8	43.8 ±1.1	44.0 ±1.2	43.4 ±1.3	41.8 ±1.1	43.4 ±1.5	45.1 ±1.9	47.5 ±2.9	42.7 ±2.1	40.8 ±2.1	41.7 ±1.5	42.0 ±1.1	44.0 ±1.5	43.9 ±2.3	43.4 ±2.2
Packed cell volume (percent)	34 ±1	32 ±2	30 ±1	31 ±2	30 ±2	32 ±1	33 ±1	34 ±1	33 ±1	33 ±1	34 ±1	37 ±1	38 ±1	40 ±2	41 ±2

TABLE 2. Hemodynamic parameters. Data are presented as the mean ± SEM.

	Vehicle (70 percent ethanol) (n = 6) Hours Post-Dosing					Low Dose (0.06 mg/kg) (n = 6) Hours Post-Dosing					High Dose (2.4 mg/kg) (n = 6) Hours Post-Dosing				
	0	1.5	3	4.5	6	0	1.5	3	4.5	6	0	1.5	3	4.5	6
Heart rate (beats min^{-1})	110 ± 4	114 ± 5	107 ± 7	103 ± 5	113 ± 7	111 ± 7	148 ±16	167 ±13	178 ± 9	176 ±12	113 ± 3	125 ± 4	170 ±12	191 ± 8	208 ± 8
Cardiac output (ml · min^{-1} · kg^{-1})	160 ± 8	150 ±11	144 ±11	155 ± 7	166 ±14	165 ±15	178 ±13	118 ± 8	127 ± 6	140 ± 7	164 ± 7	147 ±14	96 ± 9	68 ±10	60 ±10
Stroke volume (ml · $beat^{-1}$ kg^{-1})	1.5 ±.1	1.3 ±.1	1.3 ±.1	1.5 ±.1	1.5 ±.2	1.5 ±.1	1.2 ±.1	.7 ±.1	.7 ±.1	.8 ±.1	1.4 ±.1	1.2 ±.1	0.6 ±.1	0.4 ±.1	0.3 ±.1
Mean aortic pressure (mmHg)	121 ± 3	122 ± 8	125 ± 6	122 ± 7	121 ± 5	117 ± 4	90 ± 6	72 ± 4	67 ± 4	84 ± 8	122 ± 5	113 ± 7	78 ± 8	51 ± 4	45 ± 2
Pulmonary artery mean pressure (mmHg)	22 ± 2	19 ± 2	19 ± 2	19 ± 2	19 ± 1	22 ± 2	16 ± 2	16 ± 2	19 ± 1	22 ± 1	21 ± 3	20 ± 4	22 ± 3	18 ± 2	12 ± 1
Total peripheral resistance (mmHg/ml · min^{-1} kg^{-1}	.785 ±.045	.838 ±.084	.898 ±.84	.798 ±.061	.579 ±.083	.734 ±.062	.500 ±.050	.610 ±.028	.530 ±.029	.605 ±.062	.753 ±.047	.825 ±.130	.860 ±.135	.830 ±.115	.811 ±.095
Pulmonary vascular resistance (mmHg/ml · min^{-1} kg^{-1}	.110 ±.015	.107 ±.008	.114 ±.004	.104 ±.012	.106 ±.010	.112 ±.013	.090 ±.015	.139 ±.017	.151 ±.009	.154 ±.011	.112 ±.023	.164 ±.052	.288 ±.073	.391 ±.042	.287 ±.067

TABLE 2 Continued.

	Vehicle (70 percent ethanol) (n = 6) Hours Post-Dosing					Low Dose (0.06 mg/kg) (n = 6) Hours Post-Dosing					High Dose (2.4 mg/kg) (n = 6) Hours Post-Dosing				
	0	1.5	3	4.5	6	0	1.5	3	4.5	6	0	1.5	3	4.5	6
Left ventricular work ($kg \cdot mm \cdot min^{-1} \cdot kg^{-1}$)	270 ±20	248 ±20	244 ±16	256 ±13	272 ±21	263 ±28	219 ±25	117 ±13	117 ±12	160 ±18	271 ±14	223 ±18	101 ±12	48 ± 8	38 ± 7
Right ventricular work ($kg \cdot mm \cdot min^{-1} \cdot kg^{-1}$)	47 ± 5	39 ± 3	39 ± 6	40 ± 5	45 ± 6	51 ± 9	37 ± 3	26 ± 2	33 ± 2	39 ± 7	46 ± 6	38 ± 3	27 ± 2	15 ± 1	10 ± 2

Data are presented.

TABLE 3. Percentage of cardiac output received by the brain, heart, adrenals, kidneys, pancreas, spleen, liver and GI tract. Data are presented as the mean ± SEM.

	Vehicle (70 percent ethanol) (n = 6) Hours Post-Dosing					Low Dose (0.06 mg/kg) (n = 6) Hours Post-Dosing					High Dose (2.4 mg/kg) (n = 6) Hours Post-Dosing				
	0	1.5	3	4.5	6	0	1.5	3	4.5	6	0	1.5	3	4.5	6
Brain	0.74 ±.09	0.86 ±.14	0.96 ±.12	0.86 ±.14	0.85 ±.11	0.96 ±.14	0.62 ±.12	1.27 ±.25	0.79 ±.07	0.92 ±.08	0.85 ±.03	0.61 ±.10	0.82 ±.13	1.48 ±.29	1.72 ±.48
Heart	2.69 ±.39	3.14 ±.46	3.33 ±.44	3.16 ±.52	2.95 ±.43	3.36 ±.40	2.40 ±.27	4.18 ±.79	3.57 ±.44	3.38 ±.33	3.26 ±.25	3.40 ±.49	4.12 ±.78	4.88 ±.29	5.98 ±.45
Adrenals	0.081 ±.010	0.116 ±.007	0.109 ±.008	0.095 ±.009	0.091 ±.009	0.092 ±.009	0.100 ±.018	0.263 ±.049	0.238 ±.051	0.165 ±.047	0.093 ±.005	0.145 ±.017	0.340 ±.066	0.452 ±.044	0.458 ±.059
Kidneys	10.40 ±1.36	12.55 ±1.75	13.72 ±1.14	12.48 ± .80	11.41 ± .87	12.25 ±1.84	8.93 ±1.57	10.74 ±2.08	9.74 ±1.36	10.63 ±1.25	9.54 ±.80	9.58 ±1.15	8.90 ±1.67	10.23 ±1.35	6.52 ±1.19
Pancreas	0.679 ±.051	0.625 ±.035	0.662 ±.068	0.566 ±.056	0.594 ±.081	1.037 ±.161	0.241 ±.071	0.315 ±.042	0.142 ±.037	0.296 ±.064	0.764 ±.088	0.706 ±.101	0.203 ±.032	0.350 ±.078	0.376 ±.074
Spleen	3.36 ±.14	±4.16 ±.47	5.73 ±.48	5.50 ±.41	5.31 ±.82	3.35 ±.38	2.78 ±.79	2.02 ±.31	0.87 ±.34	0.51 ±.12	2.98 ±.37	3.68 ±.88	0.59 ±.26	0.17 ±.09	0.14 ±.07
Liver	2.88 ±.51	3.74 ±1.35	4.84 ±1.78	5.34 ±1.96	4.70 ±1.79	4.80 ±1.38	7.56 ±.50	13.59 ±2.12	10.47 ±1.72	12.45 ±1.98	2.88 ±1.08	10.18 ±2.32	16.92 ±3.35	15.91 ±1.61	9.04 ±1.71
Gastrointestinal tract	14.61 ±1.94	15.95 ±.90	15.60 ±1.49	14.66 ±1.77	12.00 ±2.11	17.86 ±1.75	20.68 2.52	34.03 ±3.19	29.89 ±2.78	22.45 ±2.87	16.01 ±2.66	13.98 ±2.15	33.30 ±2.13	37.41 ±8.60	20.05 ±4.30
Total percentage of cardiac output received by these organs	35.435 ±3.266	41.642 ±2.982	44.539 ±3.548	42.652 ±3.043	37.910 ±4.311	43.702 ±5.406	43.585 ±2.896	61.400 ±7.750	55.709 ±4.231	51.061 ±4.142	36.352 2.512	42.291 ±4.625	65.198 ±6.175	70.592 ±10.19	49.784 ±7.265

TABLE 4. Brain and myocardial vascular resistance (mmHg/ml · min^{-1} · 100 g^{-1}). Data are presented as the mean ± SEM.

	Hours Post-Dosing				
	0	1.5	3	4.5	6
Brain:					
Vehicle	1.654 ±.162	1.607 ±.255	1.466 ±.148	1.560 ±.208	1.352 ±.145
Low Dose	1.438 ±.113	1.646 ±.179	1.017 ±.185	1.222 ±.108	1.219 ±.181
High Dose	1.378 ±.060	2.172 ±.267	1.690 ±.186	.922 ±.075	.898 ±.182
Myocardium:					
Vehicle	.738 ±.048	.666 ±.027	.673 ±.042	.694 ±.077	.654 ±.040
Low Dose	.675 ±.022	.681 ±.081	.490 ±.051	.464 ±.026	.616 ±.092
High Dose	.679 ±.017	.728 ±.090	.628 ±.032	.403 ±.048	.409 ±.062

FIGURE 1. Alterations in blood flow to the brain of swine dosed with T toxin.

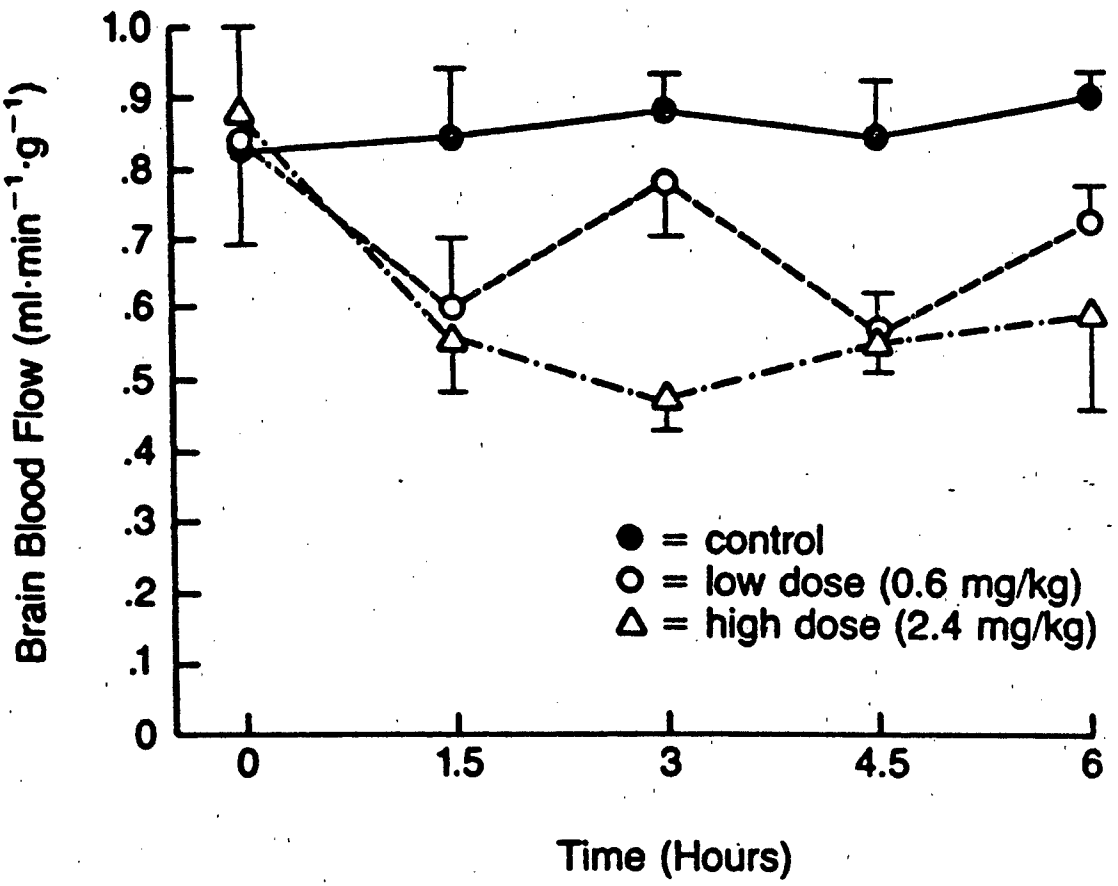


FIGURE 2. Changes in blood flow to the myocardium of swine following the administration of 70 percent ethanol, 0.6 mg/kg T-2 toxin and 2.4 mg/kg T-2 toxin.

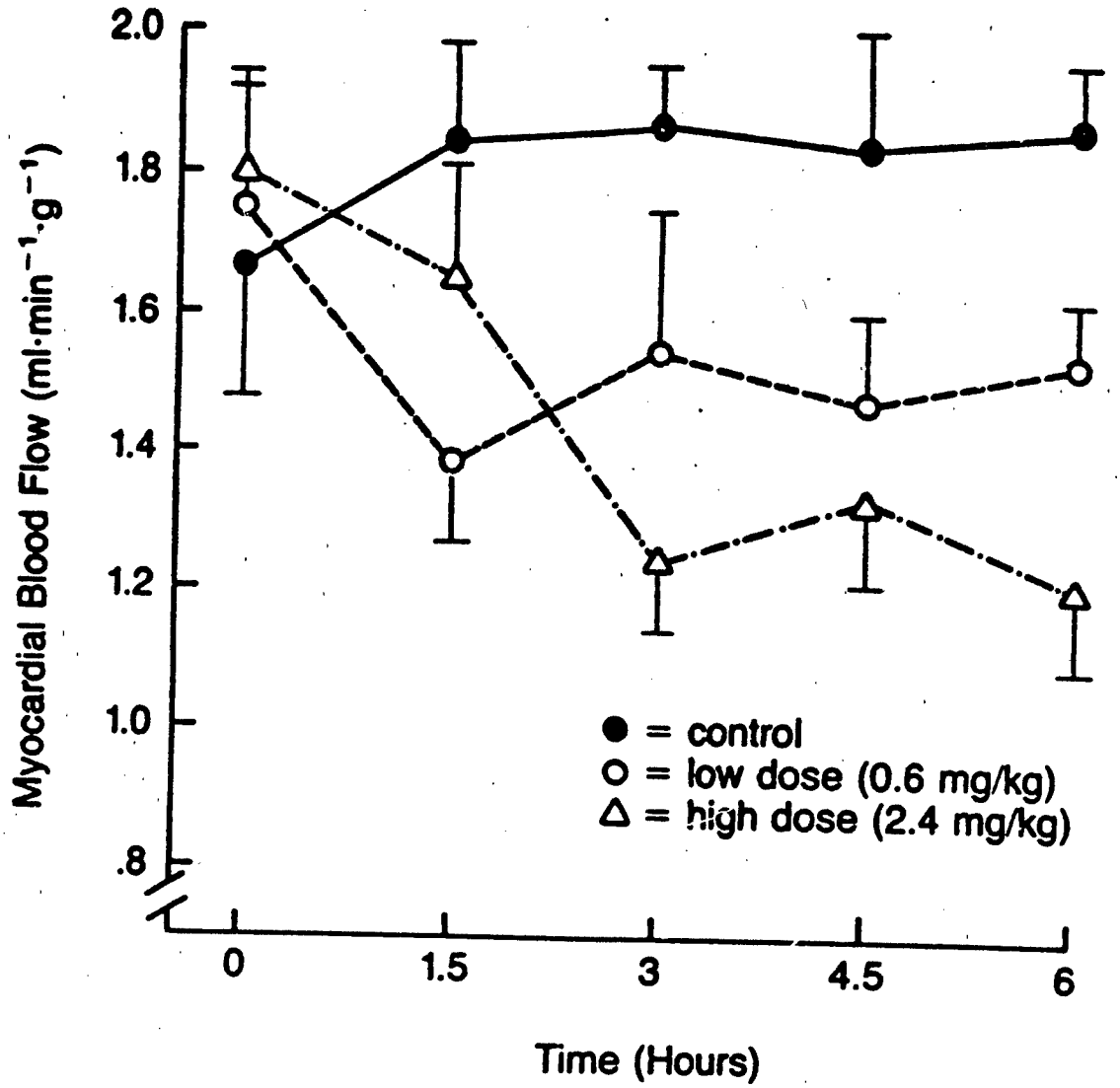


FIGURE 3. Blood flow to the adrenal glands of pigs after being dosed with T-2 toxin. Note the increase in blood flow in the high-dose group.

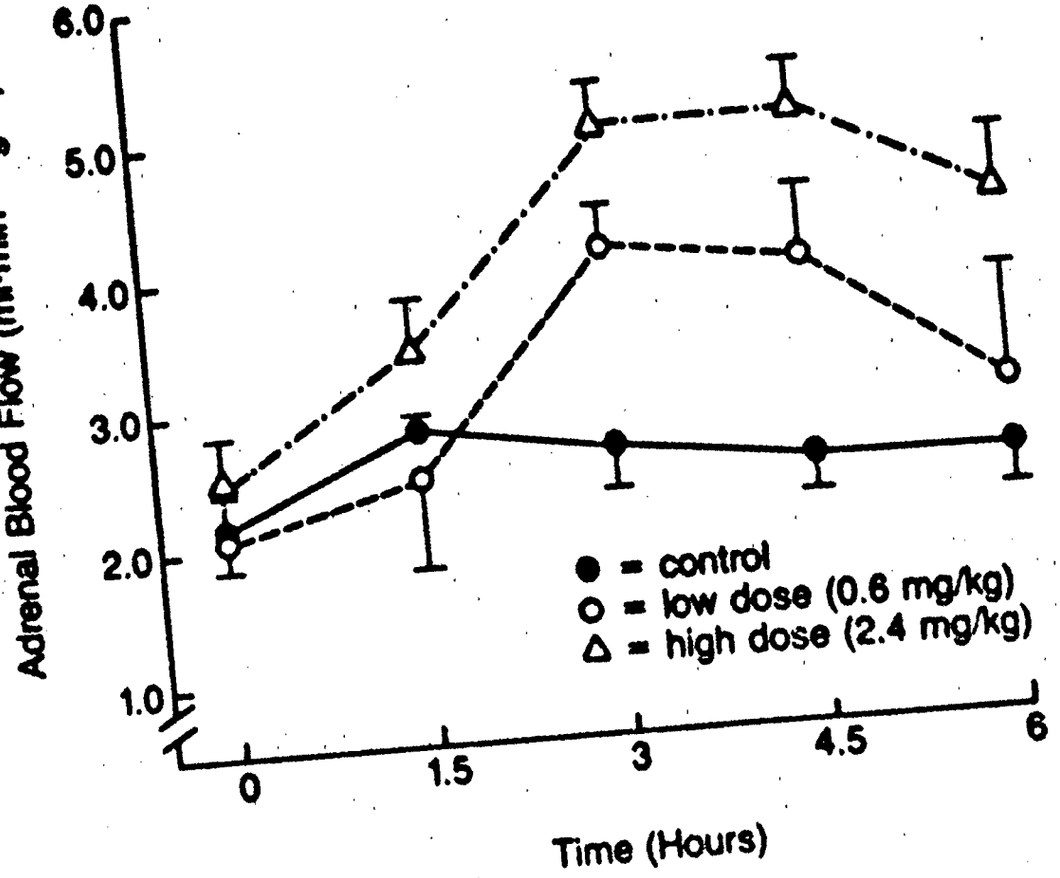


FIGURE 4. Renal blood flow in swine dosed with 70 percent ethanol, 0.6 mg/kg T-2 toxin and 2.4 mg/kg T-2 toxin.

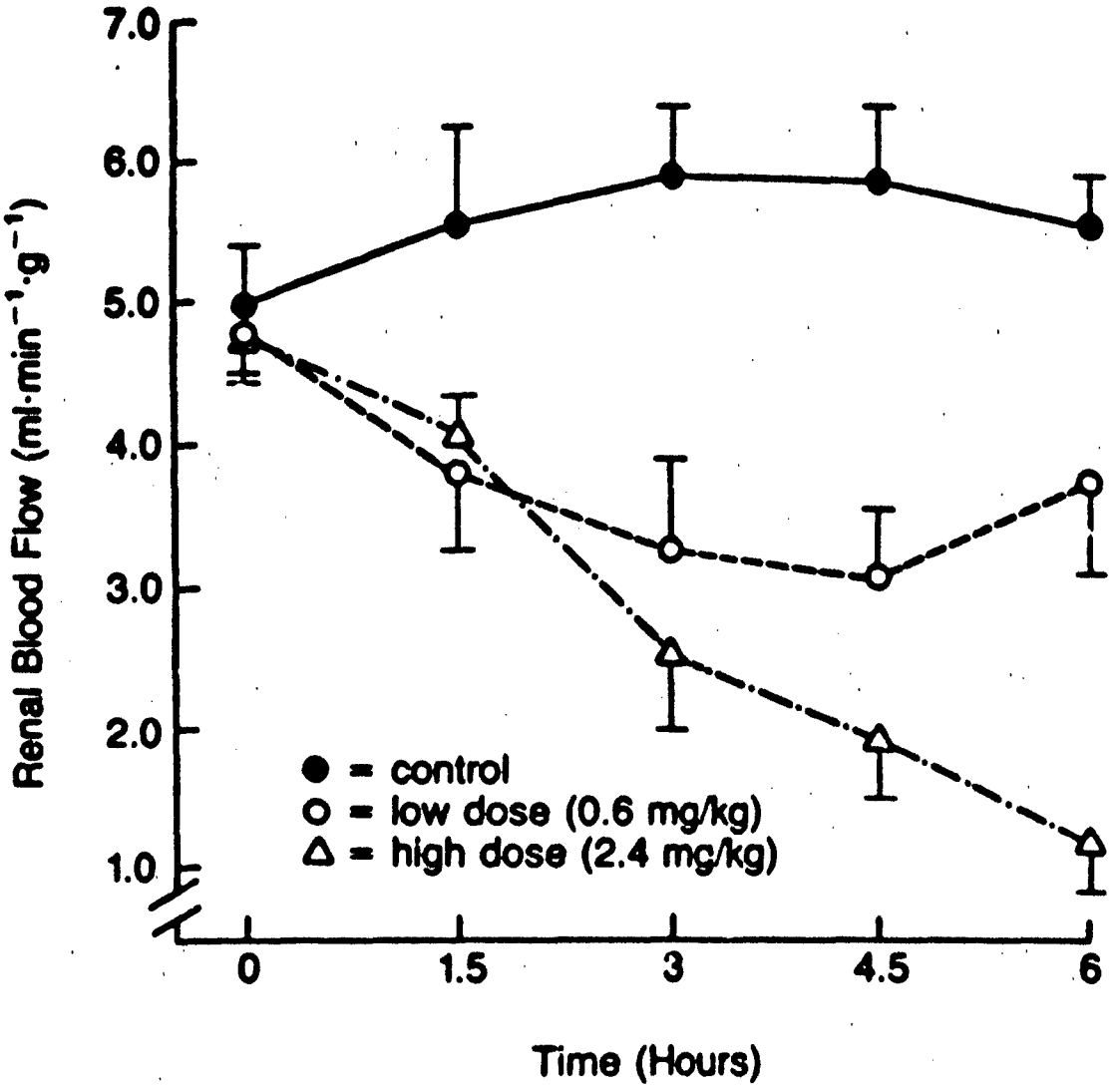


FIGURE 5. Alterations in pancreatic blood flow following administration of T-2 toxin.

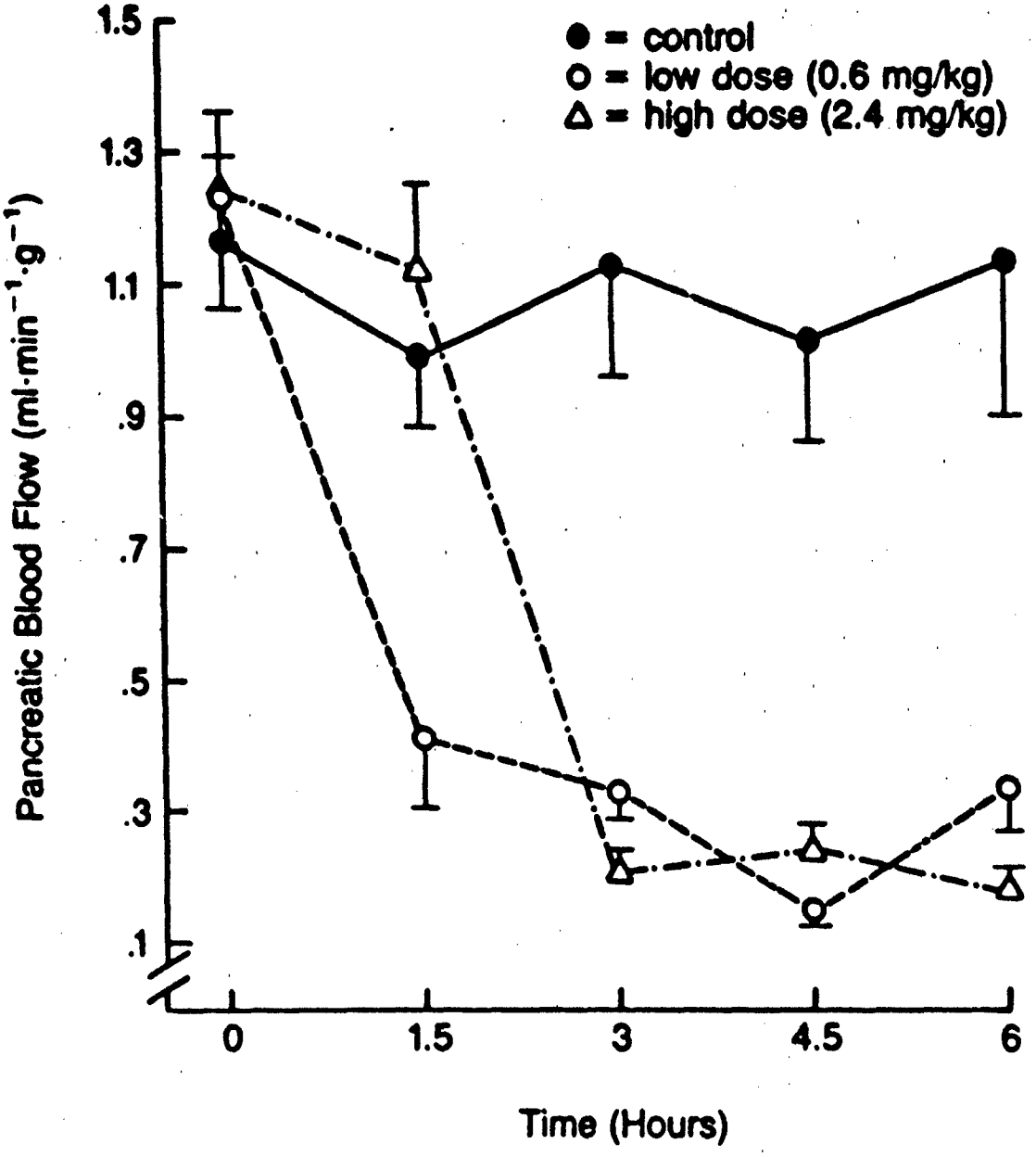


FIGURE 6. Changes in blood flow to the spleen. Note the tremendous decrease in splenic blood flow of both groups dosed with T-2 toxin.

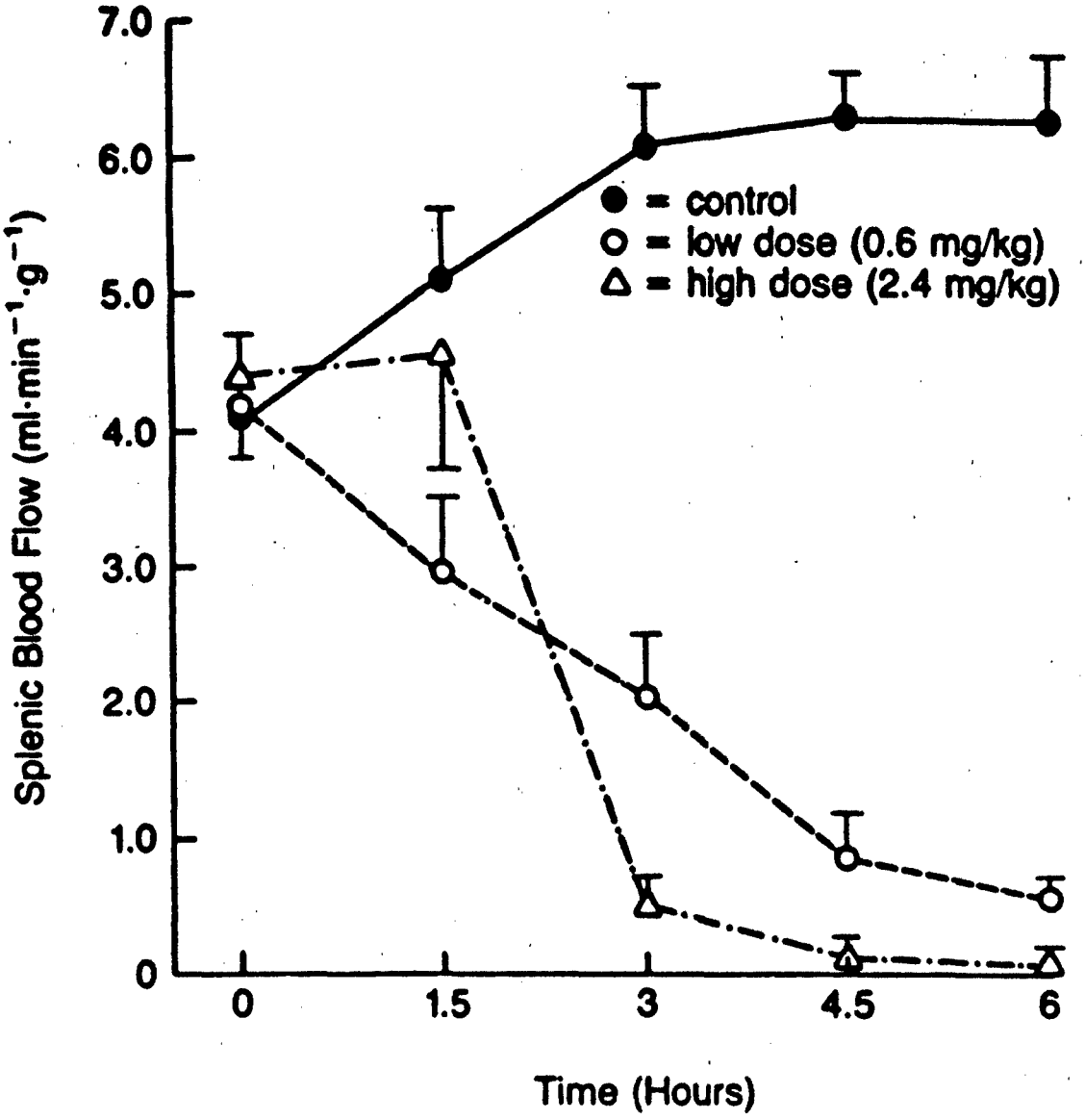


FIGURE 7. Hepatic blood flow (via the hepatic artery). Note the increase in blood flow at 1.5 and 3 hours post-dosing.

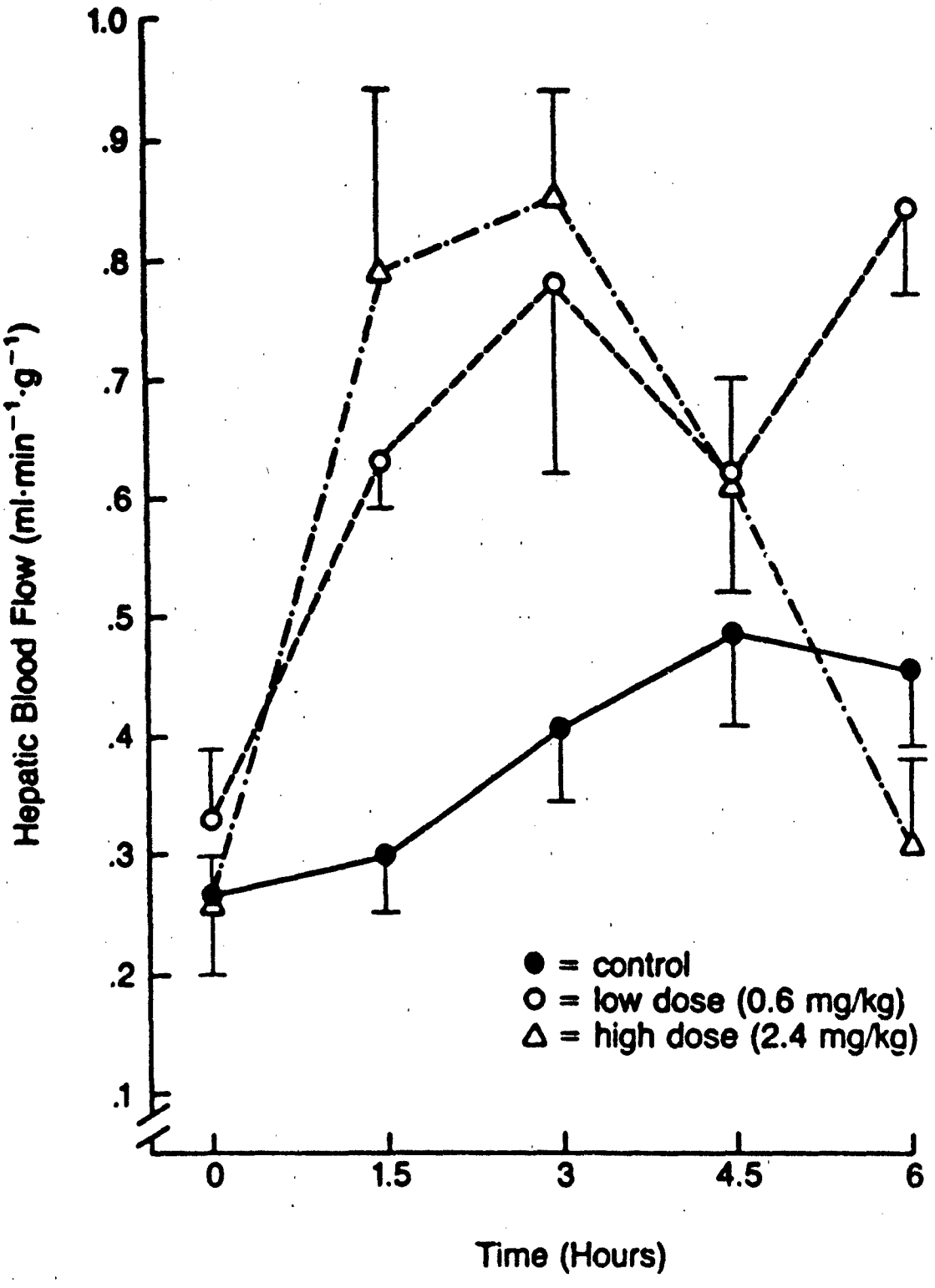
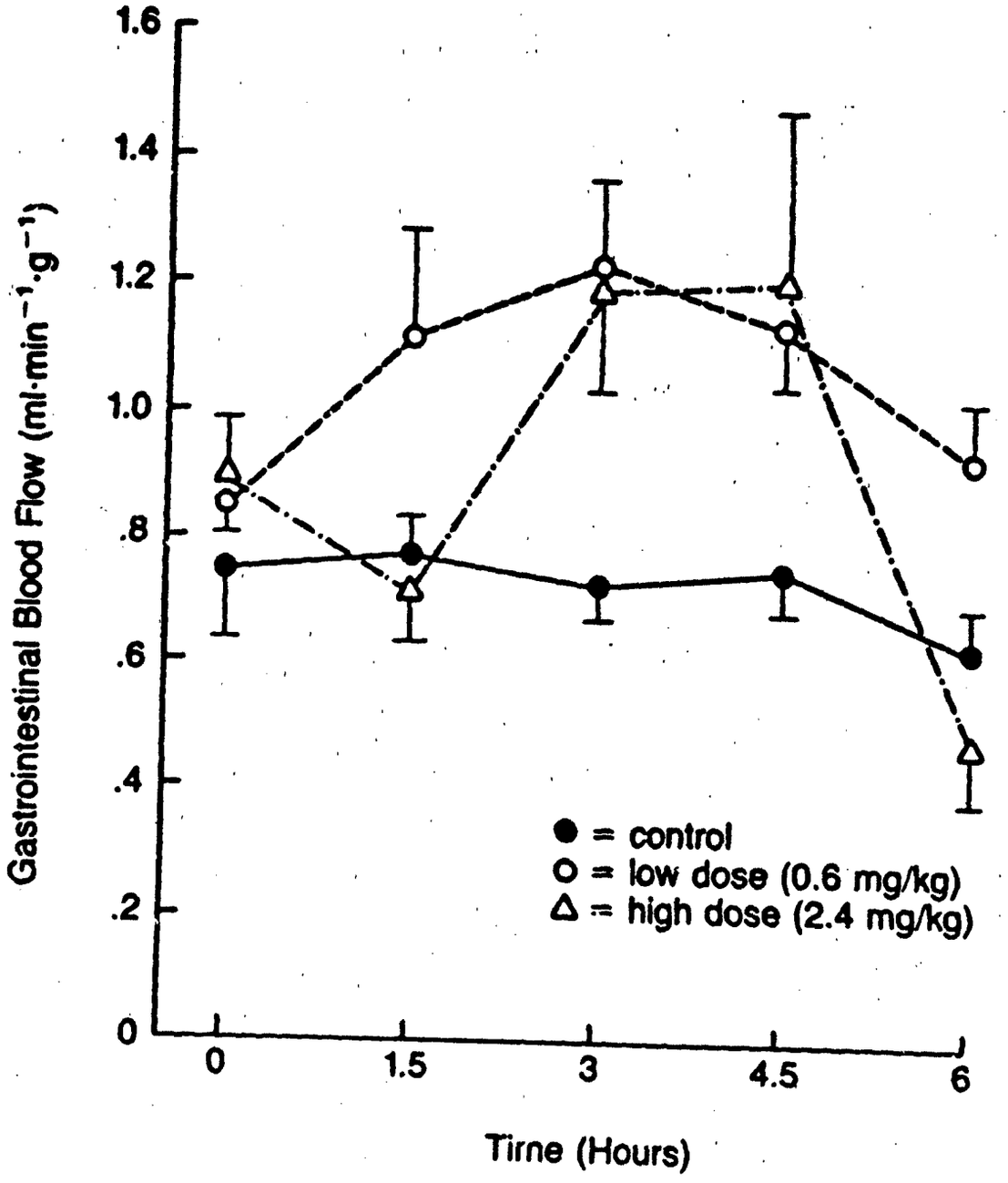


FIGURE 8. Alterations in total gastrointestinal blood flow (stomach, small intestine, large intestine) induced by T-2 toxin. Note the elevated blood flow in both groups dosed with T-2.



D. A METHOD FOR ADMINISTRATION OF AEROSOLS TO ANESTHETIZED OR UNANESTHETIZED SWINE--R. J. Lambert, DVM, PhD, V. R. Beasley, DVM, PhD, B. L. Kindler, BS, R. H. Poppenga, DVM, G. R. Lundeen, MS, M. L. Biehl, DVM, R. M. Lorenzana, DVM

INTRODUCTION

Swine have been used as biomedical models for the study of diseases because they share with humans many anatomical and physiological similarities with respect to the cardiovascular system, gastrointestinal tract, integument, metabolic function and nutritional requirements (3, 10). They are readily available in a variety of breeds and strains which can be obtained pathogen-free. They are easily handled, and their size permits repeated biological sampling and a variety of surgical manipulations.

Swine of from 20 to 150 kilograms have resting respiratory rates (10-30/min) and tidal volumes (400-800 mL) similar to humans (2,6,12). With respect to the structure and distribution of mucus glands in the tracheo-bronchial tree (5) and the subgross (mesoscopic) structure of the lungs (7), the pig resembles the human more closely than other species, e.g., rat, cat, dog and monkey, commonly used to study respiratory pollutants (9). The pig shares with the human a common pattern of lung development (11,13,14). The porcine pulmonary blood-air barrier and alveolar structure have many similarities with those of other animals (1,4,13). Despite these similarities, swine have rarely been used as models for studying respiratory function or the consequences of inhalation of air contaminants or other substances.

By-passing the elaborate nasal conchae of swine would enhance the delivery of aerosols to the lungs and allow their effects to be studied without the potentially confounding variables of nasal and oral exposure and subsequent

ingestion of impacted aerosolized particles. In this report, we describe techniques and equipment which have been used for administering aerosols to swine. The method is particularly useful in instances where the material to be studied is expensive and available in limited amounts.

MATERIALS AND METHODS

Female or castrated male specific pathogen-free crossbred swine of from 15 to 52 kg have been used in these studies. After arrival, pigs are allowed a one-week acclimatization period. They are housed in groups of up to 10 pigs per pen in a confinement building kept at 20-23°C and approximately 50% relative humidity. They are allowed free access to a commercial swine ration and water.

On the day prior to treatment, the animals are moved to the building housing the inhalation facilities and caged individually with free access to water. Food is removed 12 to 16 hours before dosing.

The swine are transported with a two-tiered cart (Fig. 1). The lower, wheeled portion serves as a transport device and is set at a height which allows the upper part, which is also on wheels, to roll directly into the animal chamber. The upper portion of the cart supports a vinyl sling which has openings for the limbs. The sling provides ventral body support and holds the animal in an upright position. It can be compressed or expanded lengthwise to accommodate different sized pigs. The anterior portion is extended in order to provide support for the pig's head. The animal is restrained by cushions which are taped to the sling and placed over the neck just behind the ears and over the caudal lumbar region (Fig. 1). In addition, the limbs are hobbled to decrease their range of motion and minimize the possibility of injury.

Prior to the inhalation exposure, the animal is given an intravenous injection of atropine[1] (0.05 mg/kg body weight) via an ear vein. It is then anesthetized with a mixture of halothane and oxygen delivered via an anesthetic machine[2] and nose cone. The larynx is desensitized with a lidocaine spray,[3] and the trachea is intubated with the largest cuffed endotracheal tube which can be comfortably introduced. The endotracheal tube is modified prior to use by inserting a length of tubing (3 mm outer diameter) through the side, into the lumen and up to the tip (Fig. 2A). The interior tube is fenestrated at the tip, and the external end is connected to a suction device which then provides a means for intermittent aspiration of secretions (5 to 10 seconds approximately every 15 minutes) that may accumulate at the end of the endotracheal tube. The endotracheal tube is tied to a wooden chock which is placed in the mouth and taped to the snout (Fig. 2B). Anesthesia can be discontinued before aerosol administration, or it can be maintained during exposure provided that a method other than inhalation of a gaseous anesthetic is used.

The pig is placed into the animal chamber (Fig. 3) and blindfolded to reduce external stimulation. The endotracheal tube is then connected to the equipment used for aerosol generation and collection of the expired material. The animal breathes room air or dilution air prior to the onset of dosing.

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[1]Frank Veterinary Laboratories, Inc., Edina, MN

[2]The Foregger Company, Inc., Smithtown, L.I., NY

[3]The Butler Company, Columbus, OH

[4]H. L. Moore, New Berlin, CT

The material which we are studying is soluble in alcohols but not in water. It is also expensive and available in limited quantities. In an effort to maximize delivery to the pig, we have chosen a nebulizer[5] with a relatively low output which generates a suitable particle mass median aerodynamic diameter. Dried and filtered medical grade air is used as the source of both nebulization and dilution air. Air at a pressure of 20 psi with a constant flow rate of about 0.5 liters per minute is used to drive the nebulizer, while the dilution air flow rate is set to meet the respiratory needs of the animal (6 to 10 liters per minute). The high ratio of dilution air to nebulization air evaporates the ethanol droplets in which the test material is dissolved, yielding dry particles. In the glovebox containing the aerosol generation equipment, there is a collapsible air reservoir (Figure 3) which provides additional inspiratory air without significantly increasing the pig's inspiratory efforts. A filtered vent prevents pressure buildup within the glovebox.

The aerosol is delivered to the endotracheal tube by flexible plastic tubing and a low pressure two-way nonrebreathing valve.[6] The small amount of dead space in the valve minimized the mixing of inspiratory and expiratory air. The exhaled air and test material are directed to the collector of the scrubber system via the exhalation tube. Due to negative pressure generated by the system at that point, air rushes around the tube carrying the exhaled air and test substance into the scrubber unit.[7] The particles are then knocked

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[5]Model 01-100, In-Tox Products, Albuquerque, NM

[6]Model 2600, Hans Rudolph, Inc., Kansas City, MO

[7]Duall Industries, Inc., Owosso, MI

down on glass fiber and polypropylene filters[8] by a water spray. A vacuum pump generates a negative pressure below the filters while a separate pump recycles the water.

The chamber containing the pig is sealed during the exposure period, and a separate vacuum unit draws filtered air into the top of the chamber and out of the bottom, creating a slight negative pressure (one inch of water) in the unit. The chamber exhaust is passed through two activated charcoal beds and vented into a fume hood. These factors would minimize exposure of the room or personnel to aerosolized particles released accidentally within the chamber due to disconnection of, or a leak in, the administration tubing.

The animal chamber[9] is made of stainless steel, and the entry door and two sides have large plexiglass windows which enable the pig to be monitored visually. Two glove ports on each side of the pig allow limited manipulation of the animal and equipment in the chamber.

Upon completion of the inhalation exposure, the chamber is opened, the administration tubing is disconnected from the endotracheal tube and the animal is extubated. It can then be monitored or transported back to a cage or holding area. The generation and scrubber systems are then partially dismantled and rinsed. The rinses can be collected in order to recover the material which remains in the system.

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[8]Gelman Sciences, Inc., Ann Arbor, MI

[9]Basic 48 in. knock-down inhalation chamber with ramps and rack, Young and Bertke, Cincinnati, OH

RESULTS

Using these techniques, more than sixty crossbred female or castrated male pigs weighing from 15 to 52 kg have been successfully exposed to aerosols for up to two hours. If the endotracheal tube is held in place and kept patent and the air reservoir is not allowed to empty, then the system is adequate for supplying the respiratory needs of the unanesthetized pig. Respiratory rates and tidal volumes can be maintained at pre-exposure levels if the aerosol particles are not physiologically reactive.

The animals may briefly resist the restraint several times during a one-hour exposure period. Some animals seem quite comfortable, will not struggle and appear to sleep during the exposure. There have been two animals, both cryptorchid males, which did struggle frequently during the treatment period.

The plexiglass enclosure for the aerosol generation equipment and the closed animal chamber have effectively prevented exposure of the room or personnel to the aerosol after infrequent separation of chamber tubing connections or a single incident where a tear had occurred in the air reservoir bag. Isolation of the animal in the chamber also apparently reduces auditory stimulation. When the dismantled portions of the aerosol generation and transport systems, as well as the scrubber system, are rinsed, about 80 percent of the material remaining in those parts can be collected in the washes for later recovery.

DISCUSSION

The techniques described have evolved over a period of time as a result of efforts to devise a means to administer aerosols of materials which are costly and available in limited amounts to anesthetized or unanesthetized swine. A

previous report (6) describing a method for intranasal exposure of anesthetized swine to radioactive aerosols revealed retention of a significant portion of the aerosolized particles in the nasal conchae of one of four pigs. Inhalation of the aerosol directly into the trachea eliminates that possibility. While it can be argued that this is not a normal physiological route of exposure, it does provide a means of studying the effects of direct lung exposure to particles or gases while limiting the secondary effects caused by oral or nasal exposure. It also minimizes the possibility of ingestion of the aerosolized material (9).

A rolling sling apparatus has been used previously to transport and maintain anesthetized swine in an upright position (6), and it proved to be convenient for use with unanesthetized animals as well.

Unanesthetized swine generally will not sit still for a one- or two-hour exposure period. The addition of restraint pads and leg hobbles is necessary to prevent an alert pig from placing its front legs on the sling and attempting to sit upright. The restraint pad behind the head limits the animal's ability to jerk its head caudally or laterally, thereby reducing the likelihood of an outward tug on the endotracheal tube or the tubing connections. Shaking the head, with the two-way nonrebreathing valve attached, tends to displace the cuffed end of the endotracheal tube toward the larynx. The resulting tension on the trachea or the presence of the cuff at the level of the larynx will usually cause the pig to struggle due to apparent discomfort. Tying the endotracheal tube to the maxilla with roll gauze was found to be an inadequate means of controlling this problem. Securing the endotracheal tube to the mouth chock and securely taping it to the snout effectively limits distal movement of the apparatus. The mouth chock also

holds the tube in the center of the mouth and keeps the jaws from closing fully and causing mastication damage to the tubing.

Topical application of lidocaine to the glottis desensitizes the area and facilitates intubation. Intravenous administration of atropine prior to the induction of anesthesia helps to counteract the depressive effects of halothane on the heart (8) and reduces glandular secretions.

An accumulation of tracheal secretions at the distal end of the endotracheal tube can impair both air and particle delivery to the pig. The ability to periodically aspirate those secretions is essential to prevent the stress caused by an insufficient air supply. It also increases the efficiency of aerosol delivery. Struggling is thus minimized by padded restraints, limiting external visual and auditory stimuli and assuring a patent airway.

During the exposure period, the dilution air flow rate is set at a level which matches the respiratory needs of the pig. If there is a sudden change in the respiratory requirements, the air accessory hose (Figure 3) can be used to rapidly add air to the distensible reservoir without substantially altering the aerosol system pressure. If the change is not transient, the dilution air flow rate can be altered. However, it is difficult to respond to rapid changes in the minute volume demands of the pig by adjusting the dilution air flow rate since that system adds air directly to the delivery tubing. If the air supply is more than the animal needs, the excess tends to go through the nonrebreathing valve rather than into the air reservoir. This decreases the delivered dose.

The glovebox enclosure for the aerosol generation system and the animal chamber, with its internal negative pressure, prevents exposure of the laboratory or personnel in case of accidental aerosol leakage. The only

external hoses carrying aerosol are monitored carefully and are not subject to stresses which may result in leaks.

The system is designed to facilitate recovery of nonwater-soluble particles. At the conclusion of a run, all of the tubing surfaces exposed to the aerosol can be rinsed. These washes as well as the scrubber filters and water are collected for later drying and recovery of as much as 80% of the aerosolized material remaining in the system. Since it is possible to recover the scrubber water used to generate the spray which knocks the aerosol particles out of the air, water soluble substances can also be recovered using this system.

With the described techniques, it is possible to administer aerosols directly to the trachea of anesthetized or unanesthetized female or castrated male swine. This will enable them to be used as biomedical models for human exposure to air contaminants, aerosolized toxins or medications. Though normal intact or cryptorchid male swine could also be used, further refinements in the technique or anesthesia will be necesesary to control their tendency to struggle.

ACKNOWLEDGEMENTS

The authors are grateful for the technical assistance of Dr. B. Babka, Ms. L. Keyes, Mr. D. Manuel, Dr. R. Lovell, Mr. S. Dugan and Ms. E. Fortenberry.

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FIGURE 1. The transport base, cart with sling and devices used to restrain the swine.

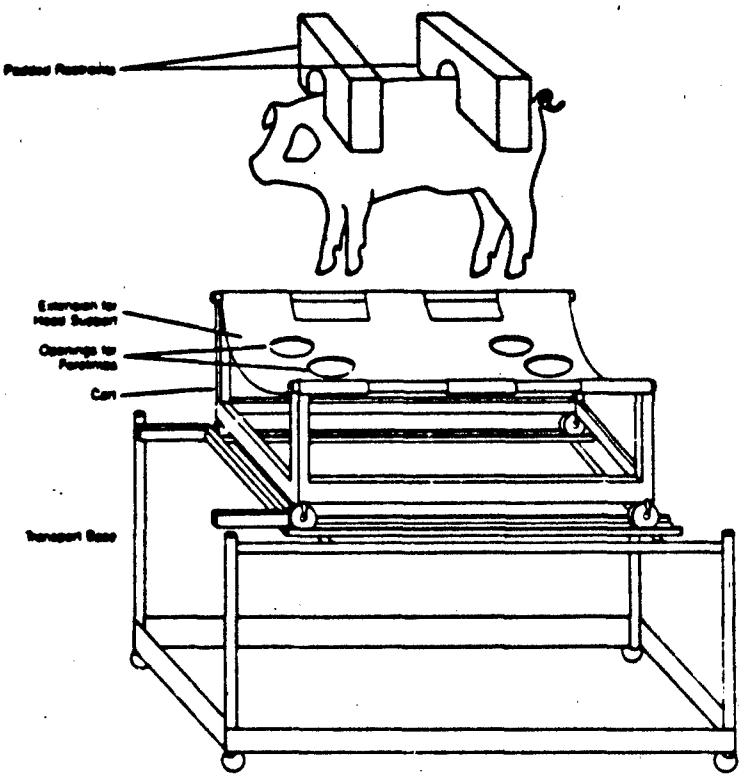
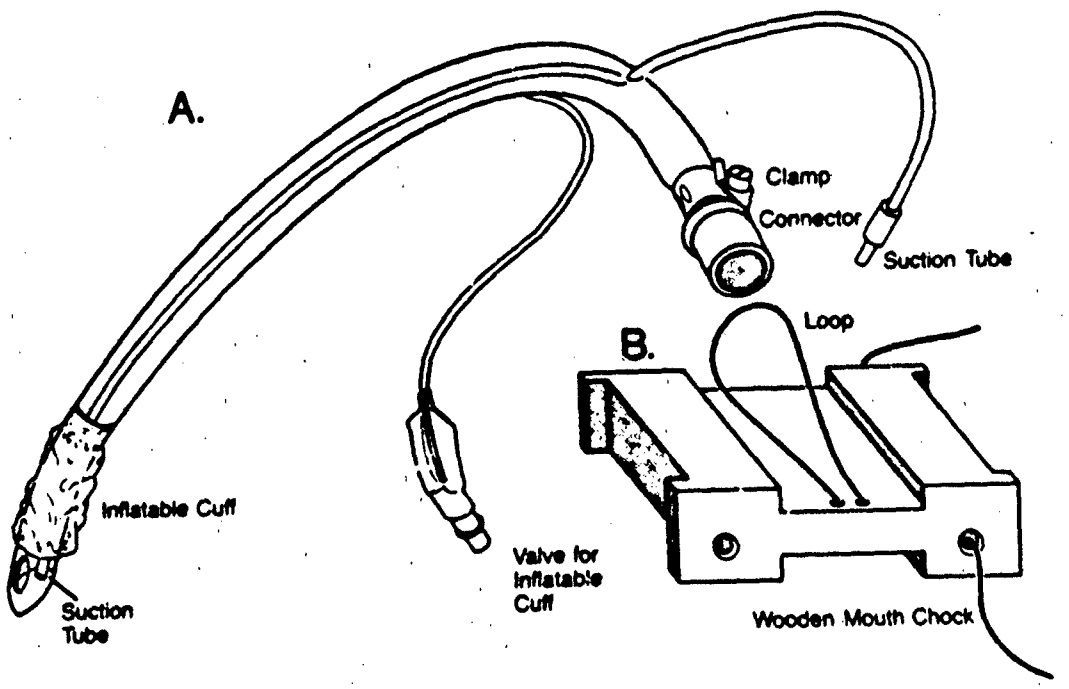


FIGURE 2. (A) Modified cuffed endotracheal tube. The addition of the internal suction tube allows intermittent aspiration of secretions from the distal end of the endotracheal tube, (B) wooden mouth chock which is fastened to the endotracheal tube by the loop which is placed between the connector and clamp. The chock is placed in the mouth and taped to the snout.



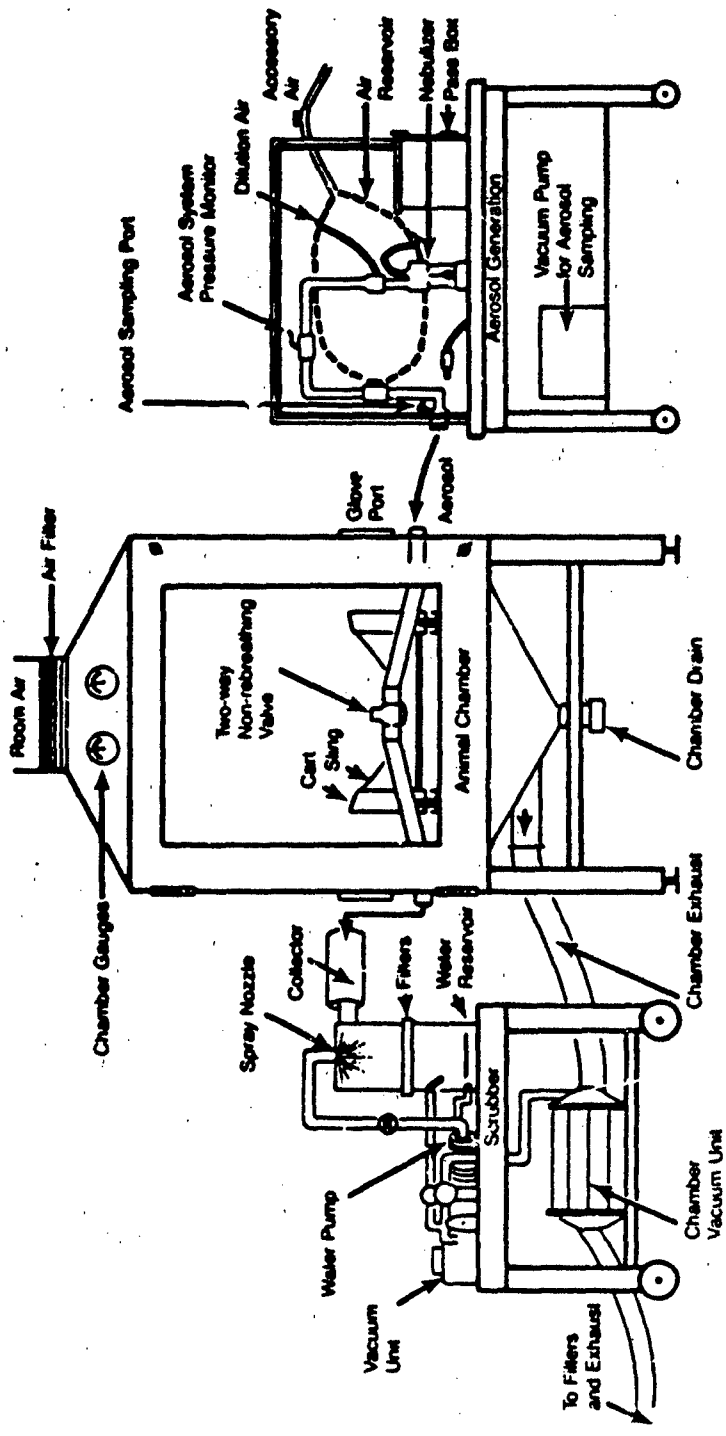


FIGURE 3. Swine inhalation exposure chamber with the aerosol generation equipment and scrubber unit.

2. SUBACUTE TOXICITY OF INHALED T-2--Victor Pang

INTRODUCTION AND OBJECTIVES

Exposure to T-2 toxin via inhalation is rare in nature, although it is possible in an environment with a large quantity of contaminated grain dust. If T-2 toxin was used in biochemical warfare as suspected in southeast Asia, inhalation would be one of the most direct and important routes of exposure. Therefore, we examined the effects of T-2 toxin following inhalation exposure.

Swine were chosen as a suitable model for these studies since: (1) pigs share many anatomical and physiological similarities with humans, particularly in the cardiovascular, integumentary and gastrointestinal systems, (2) appropriately sized pigs have respiratory rates (10 to 30/min) and tidal volumes (400 to 800 mL) similar to humans, (3) pigs resemble the human more closely than other species (e.g., rats, cats, dogs and monkeys) commonly used in inhalation studies, with respect to the structure and distribution of mucous glands in the tracheobronchial tree and the microscopic structure of the lungs and (4) their size permits repeated biological sampling and multiple surgical manipulations.

The objectives of these studies were to characterize the sequential effects of a single sublethal dose of T-2 toxin administered by the inhalation route on: (1) clinical signs, (2) clinical pathology, (3) both systemic and local immunity and (4) morphology of the lungs and other internal organs.

METHODS AND RESULTS

A. Subacute Toxicity of Inhaled T-2 Toxin on the Clinical Signs, Clinical Pathology and Systemic Immunity

Eleven crossbred, male castrated, SPF-derived pigs (six T-2 treated and five vehicle control) were used. T-2 toxin was dissolved in absolute ethanol at a concentration of 50 mg T-2 toxin per mL of ethanol. The T-2 treated pigs were exposed to a dose of 8 mg/kg body weight nebulized T-2 toxin admixed with 100 to 200 μCi Tc-99m. According to a previous study, it was estimated that the pigs retained approximately 1/3 of the amount of nebulized toxin. The control pigs were exposed to an ethanol and Tc-99m solution, with the amount of nebulized ethanol equivalent to that of the T-2 treated pigs on a mL/kg body weight basis. The exposure periods for both treatments ranged from 46 to 60 minutes. All pigs were immunized subcutaneously with sheep red blood cells (SRBC) (10^9 SRBC in 1 ml of phosphate buffered saline) immediately after exposure on day 0 and again 21 days later. Blood was collected from the anterior vena cava on days -3, -1, 1, 3, 5, 7, 10, 14, 20, 22, 24, 26, 28 and 31 for hematology and immunology studies. Serum samples collected during the first 2 weeks were used for blood chemistry analyses. Body temperature was measured daily throughout the study. Pigs were weighed before dosing and weekly thereafter for 4 weeks. The effect of inhaled T-2 toxin on the systemic immunity was evaluated at the cellular level using the same lymphocyte transformation assay previously used in the dermal study with four different mitogens (phytohemagglutinin [PHA], concanavalin A [Con A], pokeweed mitogen [PWM] and lipopolysaccharide [LPS]). The humoral response was evaluated by quantitation of anti-SRBC antibody titers using the

hemagglutination test. Data are being analyzed currently with the Statistical Analysis System (SAS) statistical package.

1. Clinical Signs

All T-2 treated pigs vomited either just before or at the end of the dosing period, or immediately after being returned to their cage. The skin turned from a pre-exposure color of pale pink to pinkish red to red in the first 3 to 4 hours after exposure. It then gradually turned purple during the next 20 hours and returned to normal (pale pink) after 1 day. The extremities, particularly the ears, were cold to the touch in the first 20 to 24 hours. The T-2 treated pigs were restless in the first 3 to 4 hours after treatment and became lethargic or even laterally recumbent in the following 12 to 24 hours. Thereafter, gradual recovery occurred. The T-2 treated pigs were anorexic in the first 12 to 18 hours after exposure and water consumption was also markedly decreased. Although the feed intake increased gradually, it did not return to normal levels until 3 to 4 days later. All control pigs ate and drank normally after the exposure and were clinically normal throughout the study.

Although the mean rectal temperature of the T-2 treated group was slightly higher than that of the control group on days 2 and 3 after exposure, the differences were not statistically significant.

The mean body weight of the T-2 treated group was significantly lower than that of the control group before treatment (3.32 kg, $p < .006$), but the difference became more prominent in the following 4 weeks after T-2 administration (7.19 to 8.3 kg, $p < .001$) (Figure 1).

2. Clinical Pathology

The data have been computerized and the analysis is currently underway.

3. Systemic Immunity

The effect of inhaled T-2 toxin on humoral immunity was evaluated by the hemagglutination (HA) titers specifically against SRBC (Figure 2). The T-2 treated group had significantly ($p < .05$) lower mean HA titers on days 5 and 7 after primary immunization. Thereafter, the differences were not statistically significant and gradually diminished. No significant differences were observed after the secondary immunization.

The effect of inhaled T-2 toxin on cellular immunity was evaluated by nonspecific mitogen-induced blastogenesis with the enriched peripheral blood lymphocytes. The data of the lymphocyte transformation assay have been computerized and analysis is currently underway.

B. Immunological (Both Local and Systemic) and Morphological Effects of Inhalation Exposure to A Single Dose of T-2 Toxin

The objectives of this study were (1) to evaluate the effects of inhaled T-2 toxin on the function of pulmonary macrophages and lymphocytes, as well as peripheral blood lymphocytes and (2) to evaluate the sequential morphological effects of inhaled T-2 toxin on the lungs and other organs at 0.33 (8 to 10 hours), 1, 3 and 7 days after exposure. A total of 18 pairs (1 T-2 treated and 1 vehicle control as a pair) of 36 crossbred, male castrated, SPF-derived pigs were used. One T-2 pig died between 9 and 16 hours after exposure. Because of severe autolysis of the T-2 treated pig, this pair was not included in the data analyses. Four pigs

(two T-2 treated and two vehicle control) died or were killed respectively at approximately 8 to 10 hours after exposure. Ten pigs (five T-2 treated and five vehicle control) were killed at each of the other three time points. The T-2 treated pigs were exposed to a nebulized dose of 9 mg/kg of T-2 toxin administered with Tc99m in absolute ethanol. According to our previous calculation, approximately 1/3 of the nebulized T-2 toxin was retained by the pigs. The control pigs received ethanol and Tc99m only.

The pigs were killed by electrocution, and the trachea and lungs were immediately removed in toto. The major bronchi of the right lung lobes were tied off, and lavage was performed on the left lung lobes. Fifty mL of cold sterile phosphate buffered saline (PBS) containing 2 percent EDTA was placed into the left bronchus, and after a generalized gentle massage of the lung lobes, the recovered fluid was poured into a siliconized bottle. A total of 500 mL of PBS (10 washes) was used in each pig.

The cellular component was separated from the fluid by centrifugation. The supernatant was frozen immediately for protein and immunoglobulin analysis. The cell pellet was resuspended in 10 mL of RBC lysing buffer and incubated in ice for 10 minutes to remove the erythrocytes. The cell suspension was then diluted by adding 40 mL of cold sterile PBS (without EDTA). The cells were spun down again and resuspended in 10 ml of Eagle's essential medium supplemented with 20 percent fetal bovine serum. The cell viability was checked with a vital stain, 0.2 percent trypan blue in PBS.

To evaluate the function of the pulmonary macrophages, a phagocytosis assay using bacteria was performed. A small portion of the lavage cell suspension was adjusted to contain 10^6 live macrophages per mL. Two mL of

a suspension of an 18-hour culture of a virulent strain of Staphylococcus aureus (approximately 10^7 bacterial per mL) were added to an equal amount of the adjusted alveolar macrophage suspension to achieve a 1 to 10 ratio of macrophages to bacteria. The phagocytic ability of the macrophages was then evaluated at 15, 30, 45, 60, 90, 120, 150 and 180 minutes after mixing the cell suspension with bacteria and incubating at 37° C. At each time point, small aliquots of the suspension, approximately 80 µL, were used for cytospin smears. The smears were stained with a modified Wright's stain.

The phagocytic ability of the alveolar macrophages (AM) was evaluated by the percentage of AM engulfing 1 or more S. aureus organisms in 200 randomly selected cells. The remaining unadjusted lavage cell suspension was used for the enrichment of pulmonary lymphocytes. In order to condense the pulmonary lymphocyte population, a carbonyl iron powder and magnet method was used to remove the majority of the pulmonary macrophages from the lavage cell suspension. The remaining unadjusted lavage cell suspension was first diluted to 50 mL by adding Eagle's essential medium supplemented with 10 percent fetal bovine serum. Aliquots of the diluted cell suspension, approximately 5 mL, were added to approximately 1 g of prewashed (with PBS) and autoclaved carbonyl iron powder in a 50 mL flask. The cell suspension and the carbonyl iron powder were mixed well and incubated at 37° C for 2 hours. The flasks were shaken every 10 to 15 minutes during the incubation. The alveolar macrophages that engulfed the carbonyl iron powder and the remaining free carbonyl iron powder were held at the bottom of the flasks by the magnets that were placed beneath the flasks. Lymphocytes comprised 80 to 95 percent of the cells contained in

the supernatant. It was withdrawn with Pasteur pipettes. The cell viability and differentiation were checked again with the vital stain (.2 percent trypan blue in PBS). The cell suspension was readjusted to contain 10^6 viable pulmonary lymphocytes per mL.

To study the function of pulmonary lymphocytes, the lymphocyte transformation assay was performed with the concentrated pulmonary lymphocyte suspension using mitogens PHA, ConA, PWM and LPS. To study the function of peripheral blood lymphocytes, blood was collected from the anterior vena cava before dosing and immediately after electrocution. For histopathologic study, the right lung lobes which were not used for lavage were perfused with Karnovsky's fixative. Representative tissue samples from other organs were also collected and fixed in 10 percent formalin.

1. Clinical Signs

Two T-2 treated pigs died, one approximately 8 hours after exposure and one between 9 to 16 hours after exposure. One T-2 treated pig was killed in a moribund state approximately 10 hr after exposure. These three pigs had severe vomiting toward the end of exposure and were extremely lethargic during the first 3 to 4 hours after exposure. They then became laterally recumbent and gradually developed labored breathing as well as cyanosis until death or killing. The skin was hot to the touch while they were laterally recumbent but it was cold to touch at the terminal stage. The remaining T-2 treated pigs survived the dosing, and during the first few days, showed similar clinical signs to the pigs in the first formal inhalation study. In addition, one of the T-2 treated pigs killed on day 3 developed severe yellow watery diarrhea 1 day after

exposure. No adverse effects were noted in any of the control pigs; they ate and drank immediately after being returned to their cage.

2. Immunology Study

In this study the effects of inhaled T-2 toxin on the immune system, both local and systemic, were evaluated. The function of alveolar macrophages, pulmonary lymphocytes and peripheral blood lymphocytes was examined.

a. Alveolar macrophages. Bacterial phagocytosis was used to test the function of alveolar macrophages (AMS). The data were expressed both as mean percentage of bacterial uptake by AMS in each group (T-2 treated and vehicle control) and as mean percentage of control.

In the pigs which were killed or died 8 to 10 hours after exposure (two T-2 treated and two vehicle controls), the mean percentage of AM containing bacteria in the T-2 treated group was always lower than the control group throughout the 3-hour incubation period. Statistically significant differences were present up to 90 minutes (Figure 3). When expressed as a mean percentage of the control pigs, phagocytosis by T-2 pigs was 48.2 to 52.2 percent in the first 90 minutes and 68 to 68.4 percent at the remaining time points (Figure 3).

In the pigs killed on day 1 (five T-2 treated and five vehicle controls), the mean percentage of AMS containing bacteria in the T-2 treated group also was lower than in the control group throughout the 3-hour incubation period. However, statistically significant differences were present only at 15,

30 and 60 minutes of incubation (Figure 4). When expressed as a mean percentage of the control pigs, phagocytosis by T-2 pigs was 72.4 to 77.3 percent in the first 30 minutes, 86.4 to 89.2 percent in the next 30 minutes and 92.8 to 96.6 percent in the following 2 hours (Figure 4).

In both the 3 and the 7 day groups, although no statistically significant differences were present at any time, the mean percentage of AMS which contained bacteria in the T-2 treated pigs was still lower than the control pigs throughout the 3-hour incubation period and, hence, values as the mean percentage of control pigs also never reached 100 percent.

b. Pulmonary lymphocytes. The nonspecific mitogen-induced (PHA, ConA, PWM and PLS) lymphocyte transformation assay measured by [^{3}H] thymidine incorporation was used to test the function of enriched pulmonary lymphocytes. The data were expressed in two different ways, as mean delta counts per minute (MDCPM) = (mitogen induced CPM - background CPM) divided by number of pigs in each group, and as mean percentage of control values.

Response to PHA - In both optimal (20 µg/ml) and suboptimal (2 µg/mL) concentrations of PHA, the MDCPM of the T-2 treated pigs at all four time points were lower than the control pig values (Figure 5). The differences were most prominent at days 0.33 (8 to 10 hours) and 1. Because of marked individual variation and limited sample size, the differences were not statistically significant. If the data were expressed as mean percentage of control, they were 29.8 $\pm$ 21.1 (mean percent

control ± SEM), 21.2 ± 8.9, 85.2 ± 31.3 and 15.3 ± 0.8 in the optimal concentration (20 µg/mL) (Figure 9) and 27.1 ± 18.5, 36.1 ± 16.8, 59.4 ± 20.6 and 39.4 ± 21.6 in the suboptimal concentration (2 µg/mL) at days 0.33, 1, 3 and 7, respectively (Figure 10).

Response to Con A - Similar to PHA, the MDCPM of the T-2 treated pigs of all 4 time points were lower than the control pigs in both optimal (50 µg/mL) and suboptimal (10 µg/mL) concentrations, although the differences were more evident in suboptimal concentration (Figure 6). These differences were not statistically significant. If the data were expressed as a mean percentage of the control pigs, they were 43.4 ± 27.4 (mean percent control ± SEM), 37.4 ± 30.6, 55.4 ± 27.4 and 3 ± 2.3 in the optimal concentration (50 µg/mL) (Figure 9) and were 44.5 ± 31.4, 22.1 ± 11.4, 59.3 ± 16.3 and 18.4 ± 4.2 in the suboptimal concentration (10 µg/mL) at days 1/3, 1, 3 and 7, respectively (Figure 10).

Response to PWM - The MDCPM of the T-2 treated pigs also were lower than the control pigs in both optimal (1:200) and suboptimal (1:400) concentrations at all four time points, but the differences were not statistically significant (Figure 7). If the data were expressed as a mean percentage of the control pigs, they were 33.8 ± 20.9 (mean percent control ± SEM), 12.0 ± 9.3, 90.4 ± 5.0 and 9.4 ± 4.9 in the optimal concentration (1:200) (Figure 9) and were 25.1 ± 17.7, 18.2 ± 14.9, 61.3 ± 24.6 and 26.7 ± 14.7 in the suboptimal concentration (1:400) at days 0.33, 1, 3, and 7, respectively (Figure 10).

Response to LPS - The MDCPM of the T-2 treated pigs were lower than the control pigs in the optimal concentration (20 µg/mL) at all four time points. In the suboptimal concentration (2 µg/mL), the MDCPM of the T-2 treated pigs were higher than the control pigs at days 0.33 and 3 but were lower than the control pigs at days 1 and 7. The differences were not statistically significant (Figure 8). If the data were expressed as a mean percentage of control pigs, they were 0 ± 0, 3.2 ± 2.0, 225.0 ± 157.6 and 18.6 ± 16.4 in the optimal concentration (20 µg/mL) and were 167.7 ± 0, 373.4 ± 209.3, 318.7 ± 139.2 and 11.6 ± 10.4 in the suboptimal concentration (2 µg/mL) at days 0.33, 1, 3, and 7, respectively.

c. Peripheral blood lymphocytes. The function of enriched peripheral blood lymphocytes was also evaluated by the non-specific mitogen-induced lymphocyte transformation assay measured by [^{3}H] thymidne incorporation. The data have been computerized, and analysis is currently underway.

3. Pathology Study

Gross Findings - The T-2 treated pigs that died spontaneously or were killed at 8 to 10 hours after inhalation exposure had prominent changes in the gastrointestinal tract, gall bladder and heart. The gastric mucosa was diffusely dark red (congestion and hemorrhage), particularly in the fundic portion, along with multiple, variably sized linear or irregularly shaped erosions and ulcers. The stomach contained a small to moderate amount of thick bile-stained mucus that was occasionally mixed with some blood. The mucosa of the entire

small intestine was diffusely dark red, especially in the distal jejunum and ileum. The small intestine contained a small amount of thick, dark-brown mucus. The contents of the cecum and spiral colon were yellow and watery. The mesenteric lymph nodes were enlarged and mottled red. There were areas of extensive subendocardial hemorrhage in the left ventricle. Occasionally, focal subepicardial hemorrhage was found in the left atrium. The wall of the gall bladder was markedly thickened by edema; marked centrilobular congestion was seen in the liver. Occasionally, small, dark-red foci, approximately 2 to 3 mm in diameter, were randomly scattered in the lung lobes, mainly in the diaphragmatic and accessory lobes. The cerebral meninges were markedly congested.

The gross changes in the T-2 treated pigs which survived until scheduled kill days (1, 3 and 7) were subtle and mild. Foci similar to those present in the lungs of the T-2 treated pigs that were killed or died at approximately 8 to 10 hours were also observed in a few T-2 treated pigs killed at days 1, 3 and 7. Occasionally, small, linear erosions and ulcers, approximately 2 to 3 cm in length, were found in the gastric mucosa of T-2 treated pigs killed at day 1. Although watery diarrhea developed in 1 T-2 treated pig killed at day 3, no changes were noted in the intestine apart from the yellow watery contents.

Histopathologic Findings

Lungs: A patchy slight to moderate cellular infiltrate was present in the alveolar spaces of the T-2 treated pigs that were killed or died 8 to 10 hours after inhalation exposure. The infiltrate consisted mainly of neutrophils

along with a few macrophages. In addition, areas of mild to moderate fibrin deposition, a small amount of cellular debris and occasional mild edema were also seen in the alveolar spaces. The alveolar septa were slightly thickened due to congestion and mild infiltration of neutrophils and macrophages. Prominent lymphoid necrosis was noted in the peribronchial and peribronchiolar lymphoid nodules. Cellular debris (possibly lymphocytes) was occasionally observed in the epithelium and lamina propria as well as in the lumina of the small airways.

Microscopic changes were also found in the lungs of some of the T-2 treated pigs killed at days 1, 3 and 7; however, the changes were subtle and mild. The cellular infiltrates in the alveolar spaces consisted mainly of macrophages, although scattered neutrophils were still present. Occasionally, small focal areas of alveolar edema were seen in pigs killed at day 1, but there was no fibrin deposition. Mild thickening of the alveolar septa due to infiltration by mononuclear cells was only found in the areas where the alveolar spaces contained cellular infiltrates.

Thymus. Moderate to marked lymphoid necrosis along with various numbers of tingible body macrophages was seen in the cortex of the T-2 treated pigs that were killed or died around 8 to 10 hours after treatment. Mild to moderate lymphoid necrosis was noted in the medulla as well.

There was a mild to moderate increase in the number of tingible body macrophages in the cortex of a few T-2 treated pigs killed at days 1, 3 and 7, but no lymphoid necrosis was observed.

Tonsil, Spleen and Lymph Nodes. Massive lymphoid necrosis, mainly in the germinal centers of the lymphoid follicles, along with prominent lymphoid depletion was seen in the T-2 treated pigs that were killed or died around 8 to 10 hours after exposure.

A few of the T-2 treated pigs killed at days 1, 3 and 7 had scattered individual cell necrosis.

Stomach. Extensive necrosis, congestion, edema, areas of hemorrhage and fibrinoid degeneration of the vascular wall as well as formation of fibrin thrombi were diffusely present in the luminal portion of the mucosa in T-2 treated pigs that were killed or died approximately 8 to 10 hours after dosing.

Similar but focal changes were found in the pigs killed at day 1 that had gross gastric lesions. Mild degeneration and necrosis of the parietal cells and various amounts of cellular debris were seen in the epithelia and lumina of the crypts adjacent to the severely affected regions.

Intestine. In the T-2 treated pigs that were killed or died approximately 8 to 10 hours after exposure, there were massive lymphoid necrosis and lymphoid depletion in the Peyer's patches and the lymphoid nodules in the submucosa. Areas of mild to moderate necrosis of the mucosal epithelium and the crypt epithelium were seen in all segments of the intestinal tract, especially in the ileum and cecum. Flattening of the epithelial lining was often present in the mucosal epithelium adjacent to the necrotic regions. There were small to large amounts of cellular debris admixed with varying numbers of neutrophils in the lumen of the intestine. Occasionally, cellular debris was also present in the crypt lumena.

No lesions were found in the intestine of the pigs killed at days 1, 3 and 7.

Pancreas. Multifocal degeneration and necrosis of single or small groups of acinar cells of varying severity were seen in all of the T-2 treated pigs at all four time points. The changes were characterized by vacuolization and condensation of the cytoplasm as well as pyknosis and karyorrhexis of the nuclei.

CONCLUSIONS

Although the data have not been analyzed completely, the following conclusions can be made based on the information available.

1. Exposure of swine to T-2 toxin via inhalation resulted in death of several pigs at a nebulized dose of 9 mg/kg but not at a nebulized dose of 8 mg/kg. Vomiting occurred in all treated pigs.
2. The massive necrohemorrhagic gastritis, intestinal necrosis, necrosis of the lymphoid organs and tissues as well as degeneration and necrosis of exocrine pancreas which occurred in pigs that were killed or died approximately 8 to 10 hours after inhalation exposure are similar to those observed in our previous studies involving intravenous administration of a lethal dose of T-2 toxin.
3. Because of the marked cytotoxic effects of T-2 toxin, massive necrosis of the lungs was originally anticipated to occur in pigs following inhalation of T-2 toxin. However, the morphological changes in the lungs were mild to moderate and appeared to be transient.
4. A significantly lower humoral immune response, evaluated by measuring antibody titers against SRBC, was present in the T-2 treated group during the first week following primary immunization.
5. Significantly lower cellular immune responses were observed as both non-specific mitogen-induced blastogenesis of pulmonary lymphocytes and bacterial uptake by alveolar macrophages in T-2 treated groups, at least in the first 24 hours after inhalation exposure.

Therefore, inhalation exposure of swine to T-2 toxin can result in significant clinical signs and an adverse effect on the immune system as well as the morphology of the lung and other organs. The altered local and systemic

immunity, both specific and nonspecific, in the exposed animals may make them more susceptible to infection.

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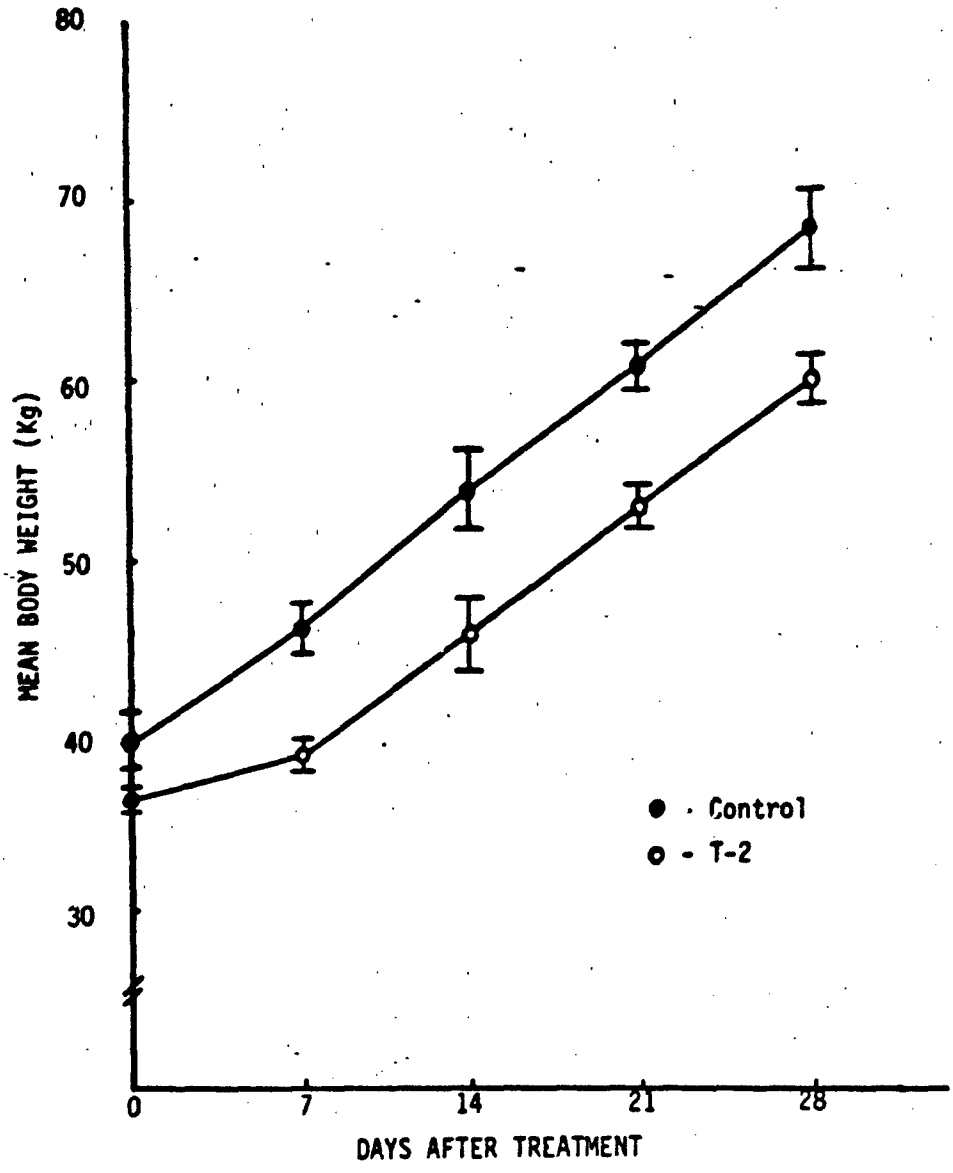

Fig.1 The body weight of T-2 treated pigs and vehicle controls via inhalation exposure. Vertical bars represent SEM in each group.

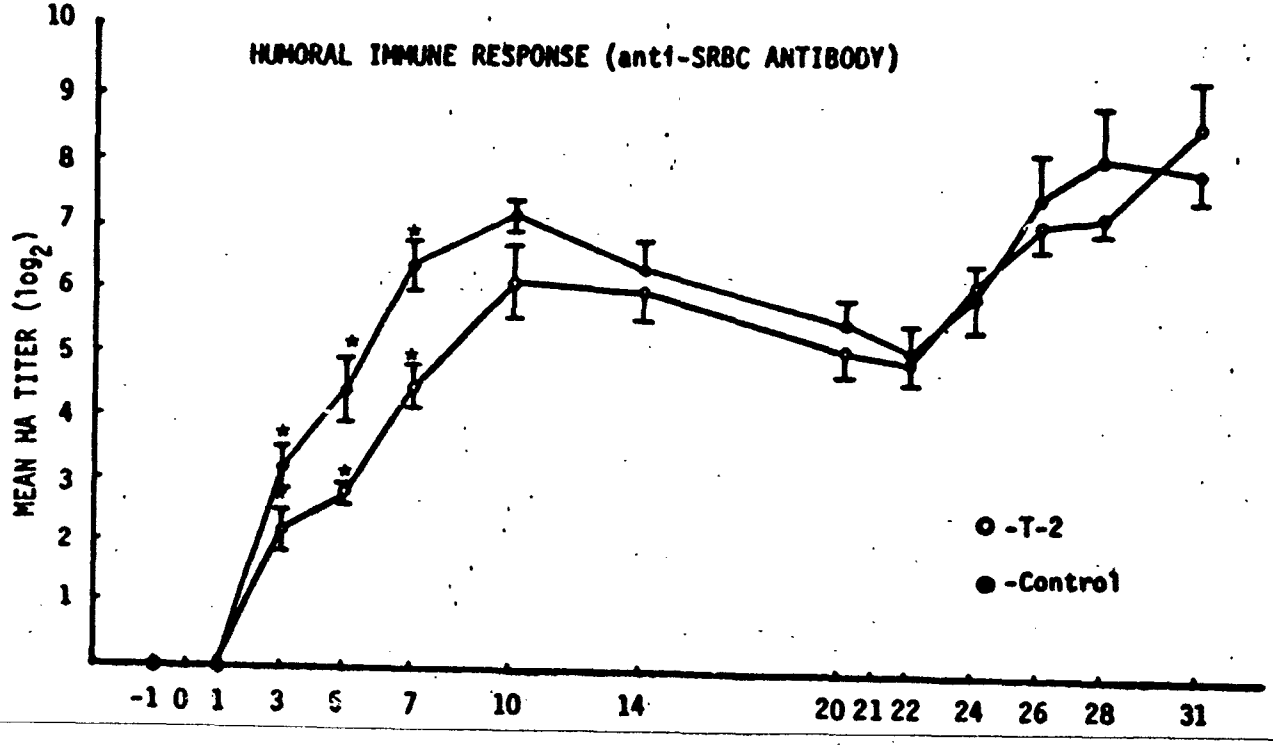

Fig.2 The HA titers of T-2 treated pigs at a nebulized dose of 8 mg/Kg and vehicle controls via inhalation exposure. Vertical bars represent SEM in each group. Statistically significant differences (*;P<0.05) are present at days 3,5,and 7.

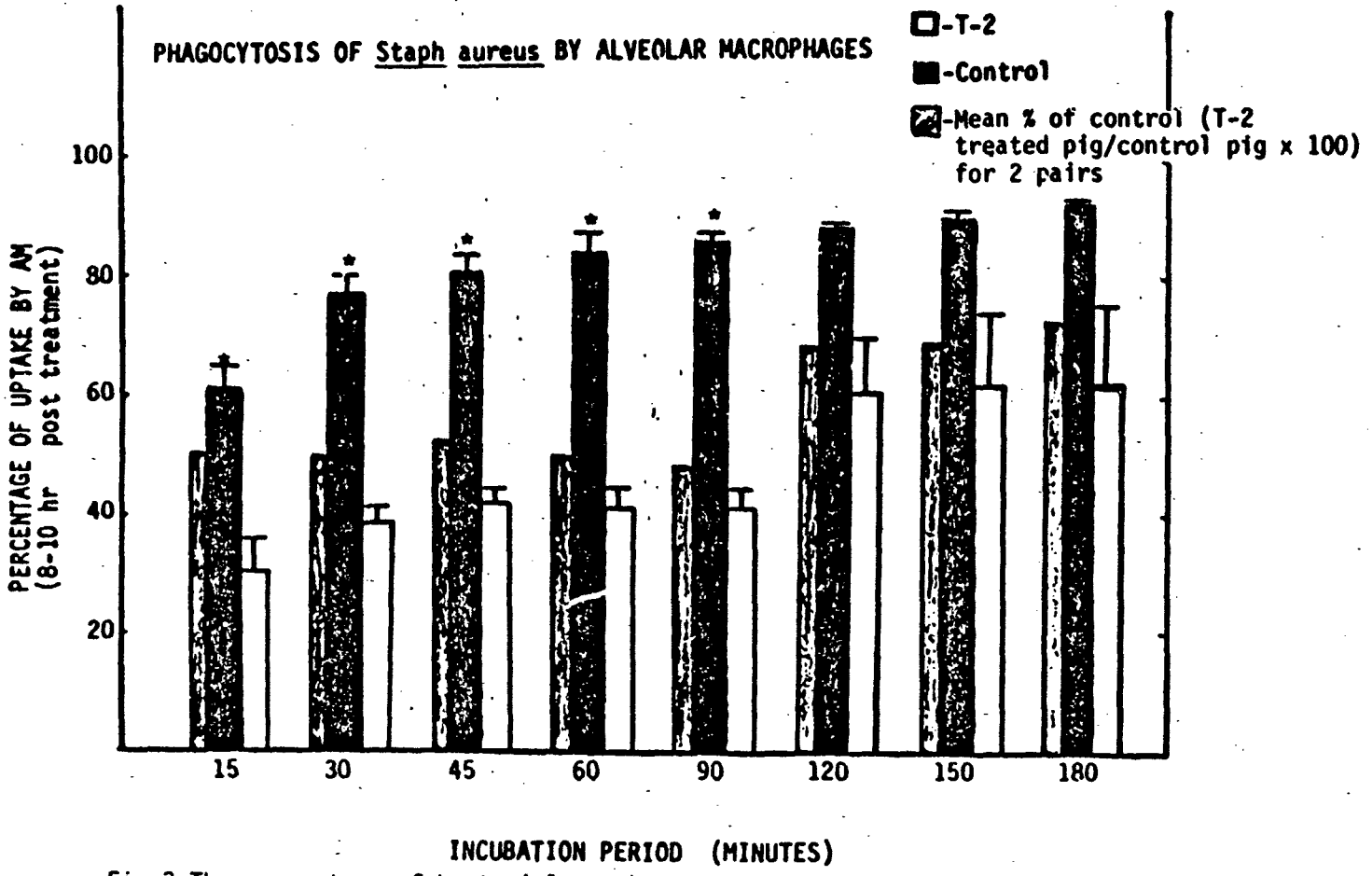

Fig.3 The percentage of bacterial uptake by AMs of T-2 treated pigs and vehicle controls,and the percentage of control of bacterial uptake by AMs of T-2 treated pigs at 8-10 hr after inhalation exposure. Vertical bars represent SEM in each group. Statistically significant differences

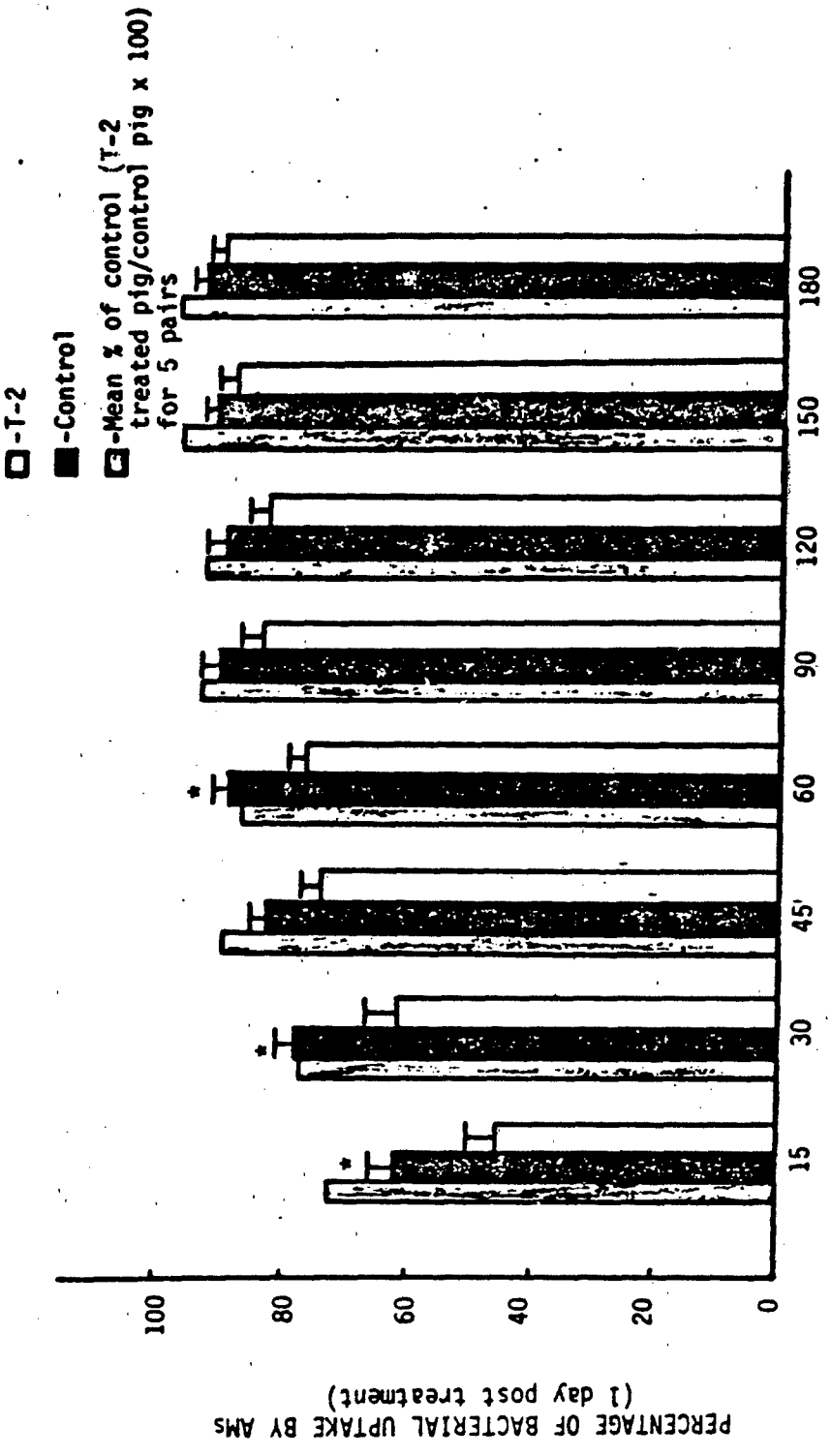

Fig.4 The percentage of bacterial uptake by AMs of T-2 treated pigs and vehicle controls,and the percentage of control of bacterial uptake by AMs of T-2 treated pigs at day 1 after inhalation exposure. Vertical bars represent SEM in each group. Statistically significant differences (*; P<0.05) are present at 15,30,and 60 min. afetr incubation.

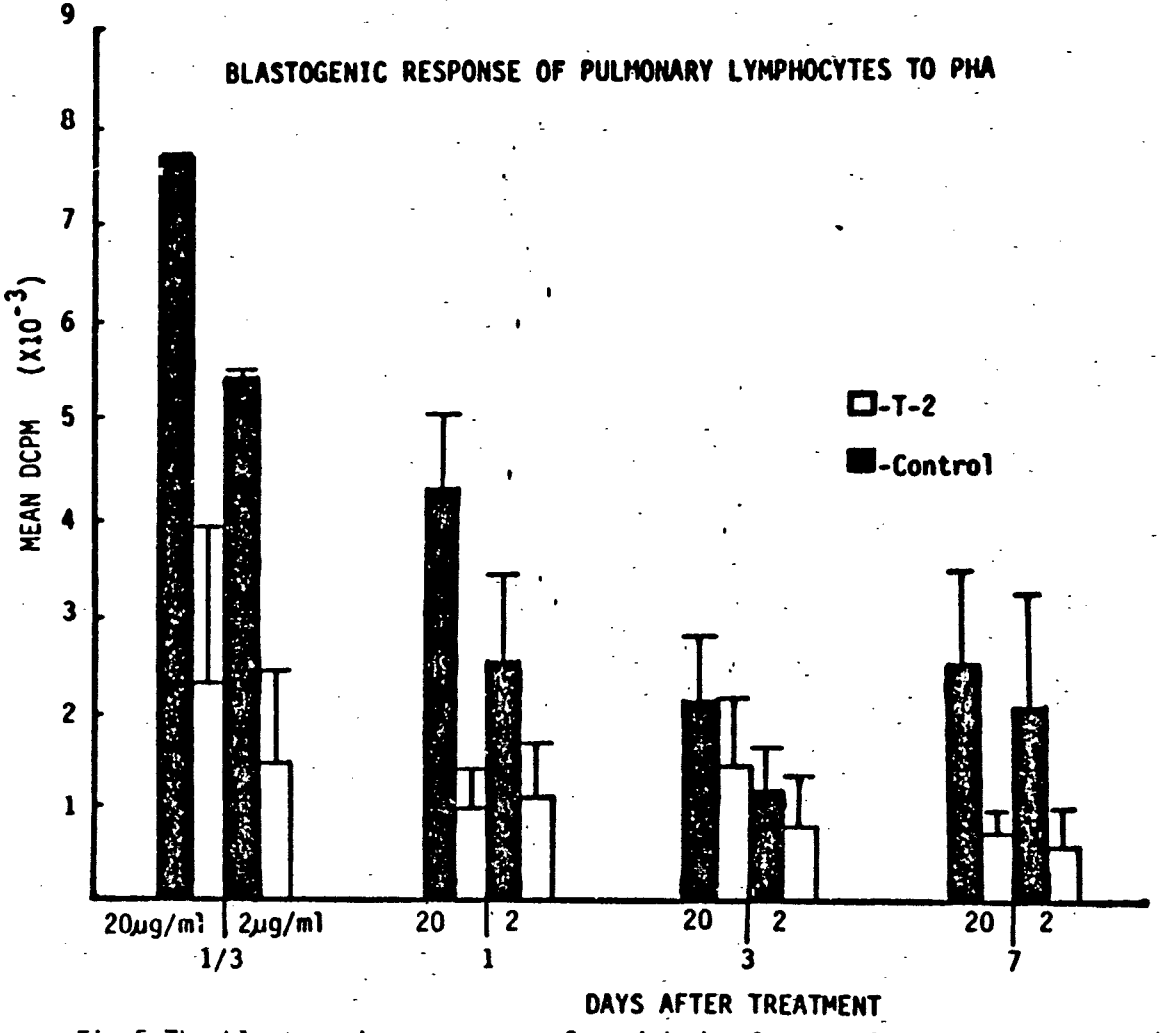

Fig.5 The blastogenic responses of enriched pulmonary lymphocytes to PHA (at conc. of 20 ug/ml and 2 ug/ml) of T-2 treated pigs and vehicle controls via inhalation exposure. Vertical bars represent SEM in each group.

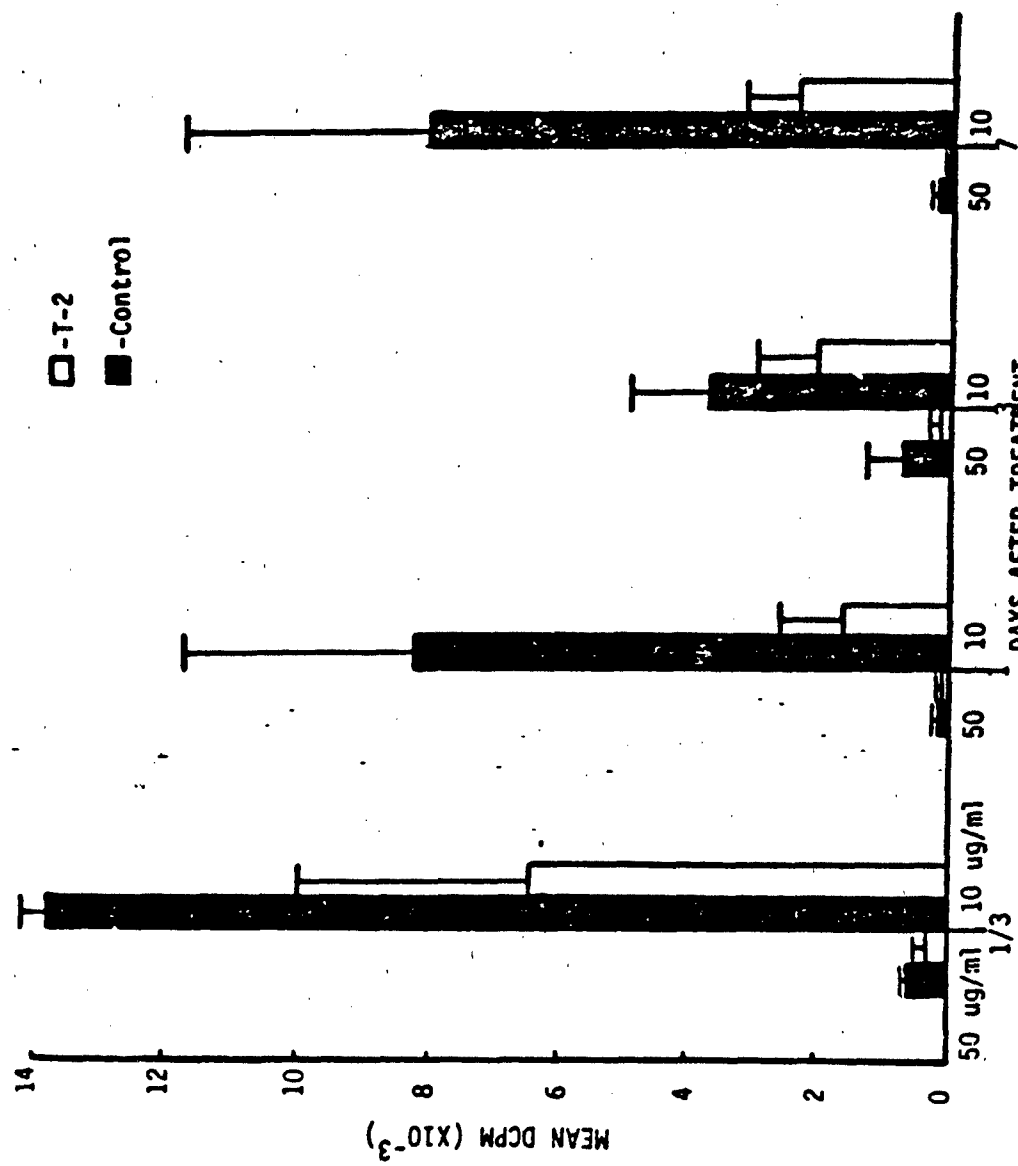

Fig.6 The blastogenic responses of enriched pulmonary lymphocytes to Con A (at conc. of 50ug/ml and 10ug/ml) of T-2 treated pigs and vehicle controls via inhalation exposure. Vertical bars represent SEM in each group.

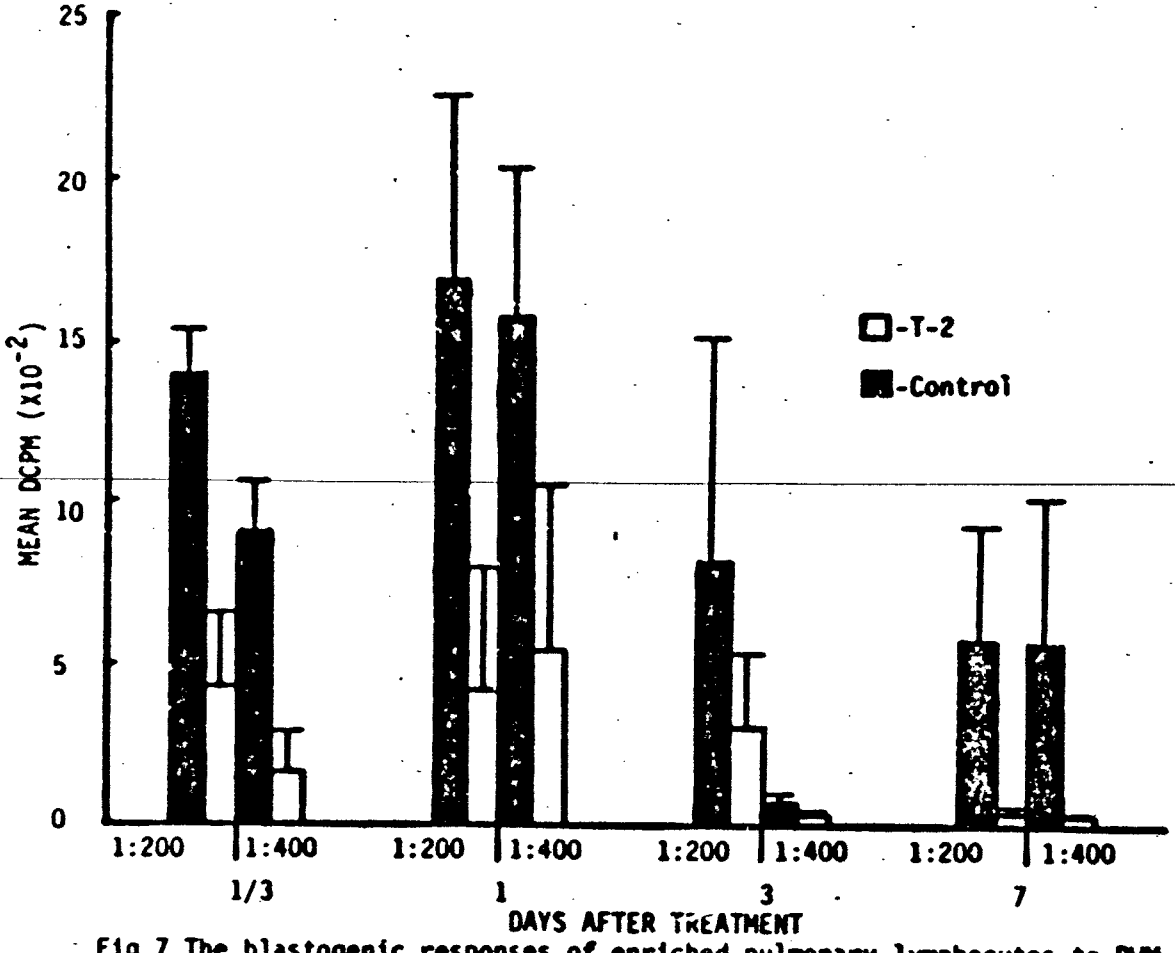

Fig.7 The blastogenic responses of enriched pulmonary lymphocytes to PWM (at conc. of 1:200 and 1:400) of T-2 treated pigs and vehicle controls via inhalation exposure. Vertical bars represent SEM in each group.

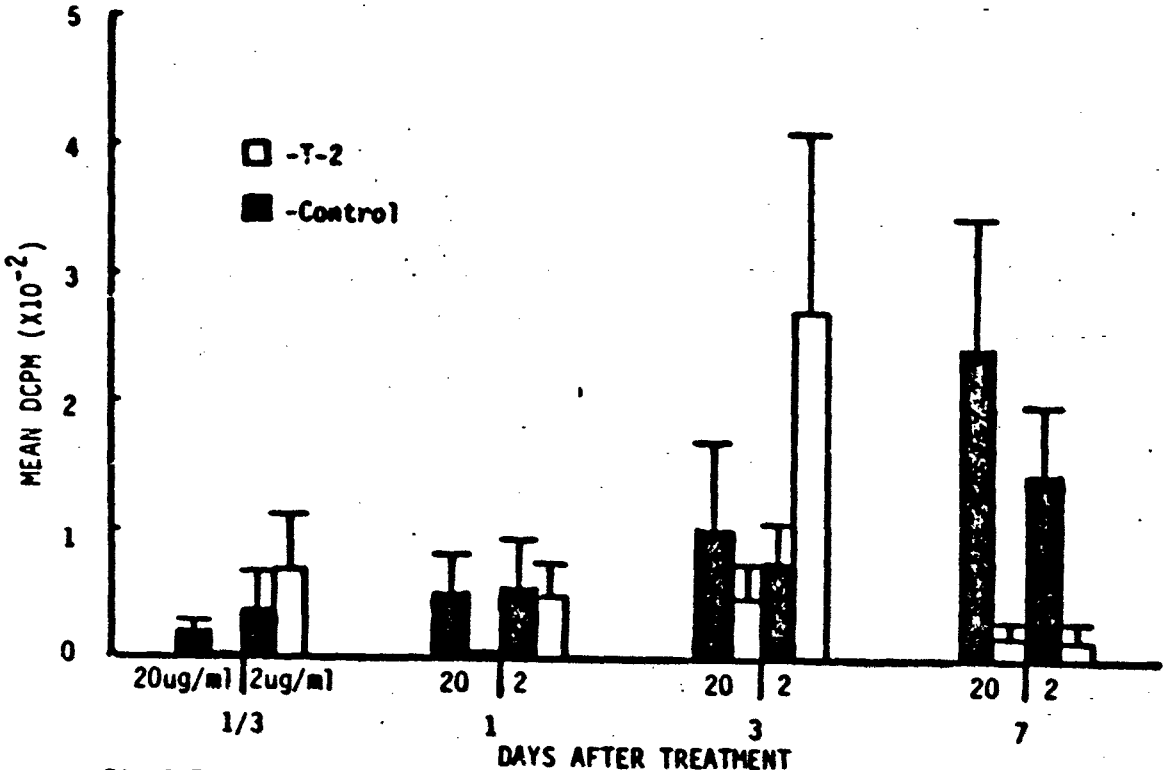

Fig.8 The blastogenic responses of enriched pulmonary lymphocytes to LPS (at conc. of 20ug/ml 2ug/ml) of T-2 treated pigs and vehicle controls via inhalation exposure. Vertical bars represent SEM in each group.

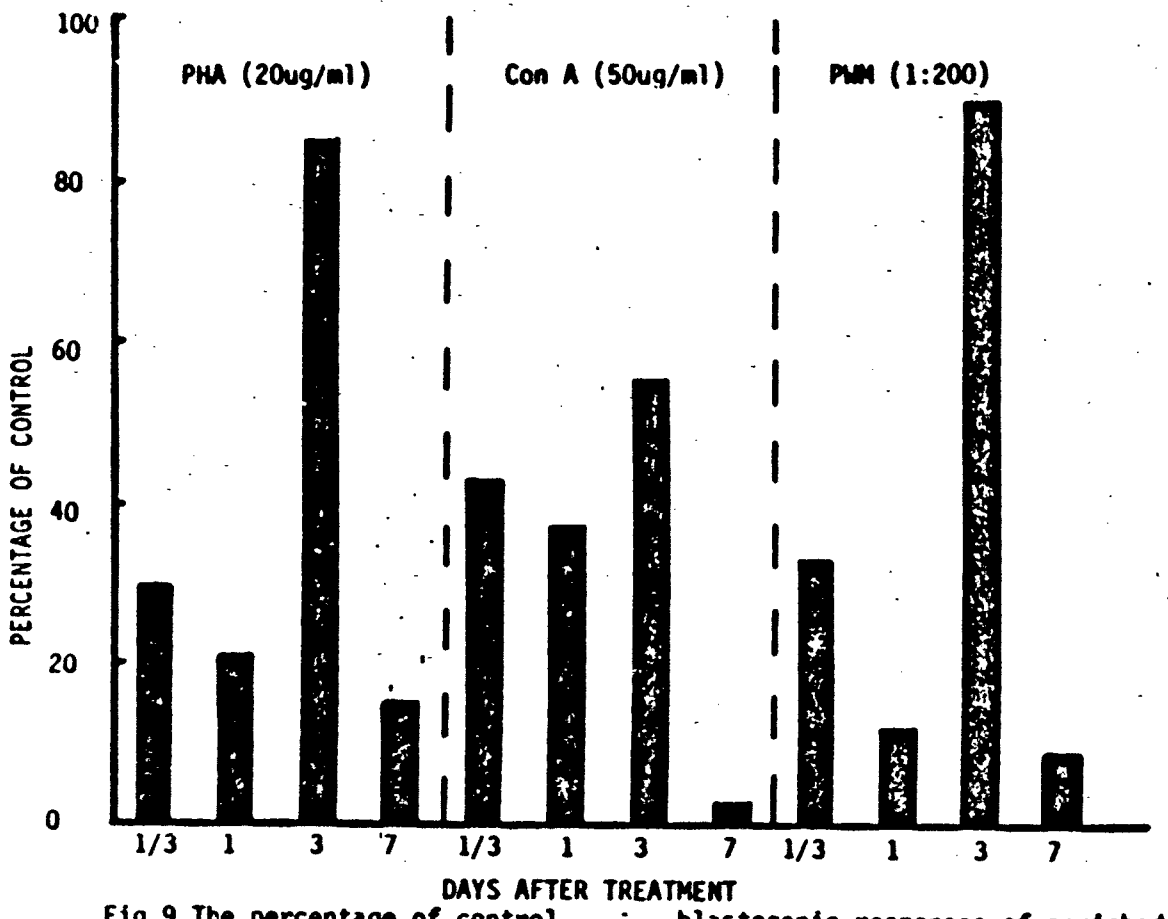

Fig.9 The percentage of control : blastogenic responses of enriched pulmonary lymphocytes to PHA (20ug/ml),Con A (50ug/ml),and PWM (1:200) of T-2 treated pigs via inhalation exposure.

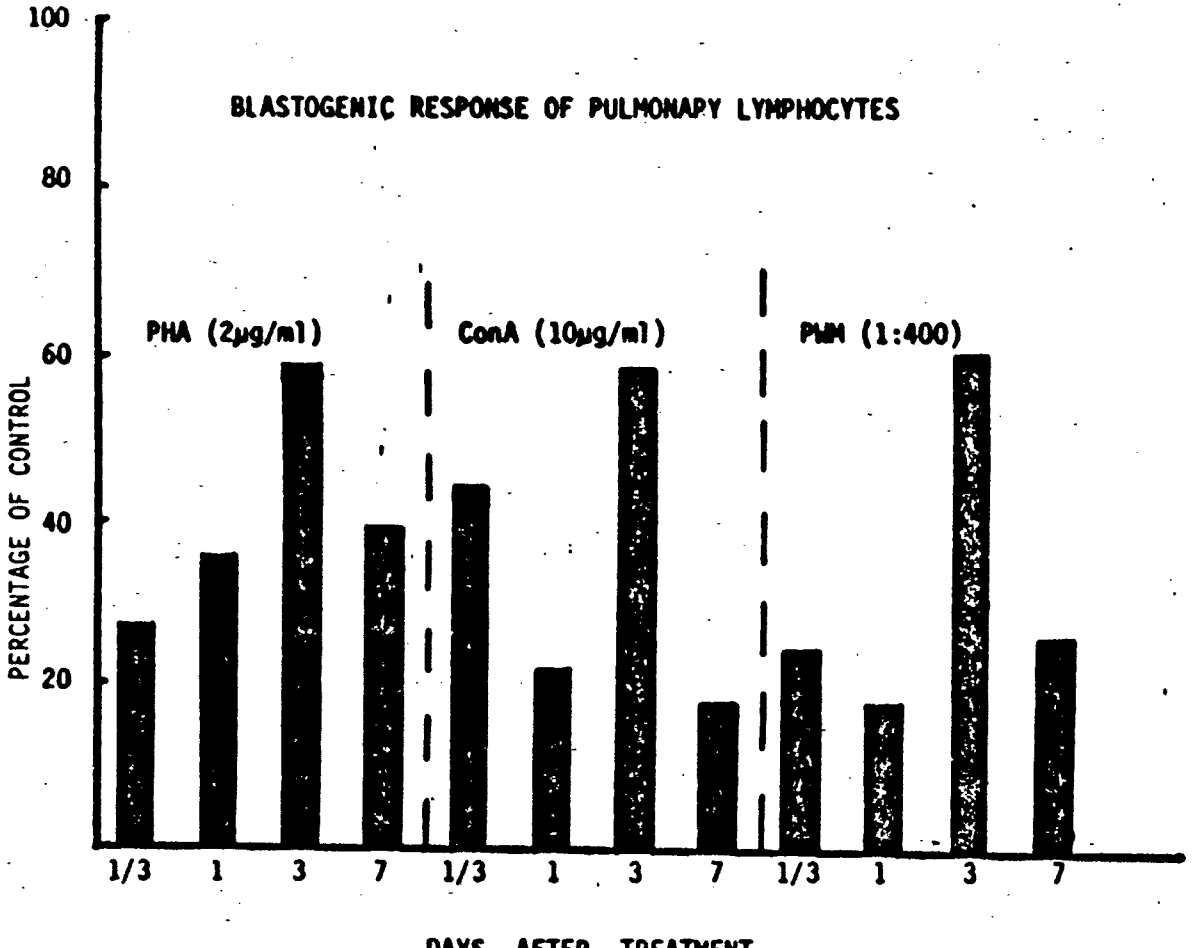

Fig.10 The percentage of control blastogenic responses of enriched pulmonary lymphocytes PHA (2ug/ml),Con A (10ug/ml),and PWM (1:400) of T-2 treated pigs via inhalation exposure.

E. ULTRASTRUCTURAL STUDY OF ACUTE T-2 TOXICOSIS IN SWINE: INTRAVENOUS AND INHALATION ROUTES OF EXPOSURE--W. M. Haschek

INTRODUCTION AND OBJECTIVES

T-2 toxin is a radiomimetic agent which cases damage to the actively dividing cells of the gastrointestinal, lymphoid, reproductive and hematopoietic systems of animals and birds (Tatsuno, 1968; Saito et al., 1969; Ueno et al., 1969, 1972; Hayes et al., 1980; Hoerr et al., 1981). Cardiovascular changes, characterized by fibrin thrombi, myocardial degeneration, cellular infiltration and fibrosis, were initially reported in rats surviving multiple doses of T-2 toxin (Schoental et al., 1979). Acute myocardial changes were later described both in vivo and in perfused heat preparations from the rat (Yarom et al., 1983a,b).

In our previous studies, acute T-2 toxicosis in pigs was characterized by massive lymphoid necrosis; severe congestion of the gastrointestinal tract, meninges and uterus; and hemorrhages in the subendocardial region of the heart and in lymph nodes. Variable, but severe, necrosis of enterocytes was noted, especially in the crypts of the small intestine. Necrosis was also present in the bone marrow. Myocardial degenerative changes characterized by myofiber granularity, eosinophilia and necrosis were consistently present.

Although the morphologic effects of T-2 toxin have been extensively studied at the light microscopic level, ultrastructural studies are few. Mycocardial changes consisting of edema and disorganization of myofibrils as well as hypercontraction of myofibrils with accumulation of mitochondria have been described in the rat (Yarom et al., 1983a,b). In an in vitro system using human fibroblast cultures, significant ultrastructural damage was found

in nuclei, rough endoplasmic reticulum and associated ribosomes, the respective locations of DNA and protein synthesis, while damage to plasma membranes and lysosomes was not detected (Oldham et al., 1980).

The present study examines the early ultrastructural changes and their progression following acute intravascular and inhalation administration of a single dose of T-2 toxin to pigs. Knowledge of the development of the lymphoid, myocardial and intestinal lesions will lead to a better understanding of the pathogenesis of T-2 toxicosis and thus may aid the development of therapeutic regimens.

Specific objectives were to:

1. Characterize the early ultrastructural changes and their progression following a lethal intravascular dose of T-2 toxin in swine.
2. Similarly, characterize the early ultrastructural lesions and their progression following T-2 toxin given by inhalation.
3. Compare the lesions induced by T-2 toxin when given by different routes of administration.
4. Carefully examine myocardium, pancreas and endothelial cells for possible T-2 induced damage.

METHODS

1. Intravenous T-2 Toxin Study Protocol

   a. High dose (2.4 mg/kg)

      Eight, 8-week-old male castrated crossbred SPF-derived piglets were administered purified T-2 toxin (99 percent pure) at 0 (2 control pigs) or 2.4 mg/kg (6 treated pigs). The T-2 toxin was dissolved in 2.5 mL of 50 percent ethanol and administered through a catheter into the ear vein. The catheter was then flushed with 5 mL

of normal saline. Similarly, the control pigs received 2.5 mL of 50 percent ethanol followed by 5 mL of normal saline. Pigs were killed by electrocution at 0.5 (1 T-2 pig), 1 (2 T-2 and 1 control p'g), 4 (2 T-2 and 1 control pig) and 8 (1 T-2 pig) hours after exposure.

b. Low dose (0.6 mg/kg)

T-2 toxin was administered iv at 0 (2 control) and 0.6 mg/kg (6 treated pigs) to eight 17- to 18-week-old SPF-derived crossbred gilts as described above. The pigs were killed 24 or 48 hours after treatment and selected tissues, heart and pancreas were prepared for electron microscopy.

2. Inhalation T-2 Toxin Study Protocol

Nine, 12-week-old male castrated crossbred SPF-derived pigs were administered 0 (2 control pigs) or 10 mg/kg (seven treated pigs) nebulized T-2 toxin (as described under inhalation studies) mixed with Tc 99 m in absolute ethanol. Preliminary calculations indicate that approximately 1/3 of the nebulized dose was retained in the lungs. Pigs were killed by electrocution at 0.5 (one T-2 pig), 1 (two treated and one control pig), 4 (two treated and one control pig) and 8 (two treated pigs) hours postdosing.

3. Tissue Preparation

Sections from pancreas, spleen, splenic and bronchial lymph nodes, duodenum, ileum, colon, liver, heart, trachea and bone marrow were diced and fixed by immersion in 2 percent glutaraldehyde in 0.1 M phosphate buffer overnight at 4° C. Lungs were first fixed by intratracheal instillation of fixative.

In pigs used for the inhalation study, the left lobe was tied off and the right perfused with fixative. Sections were taken uniformly from the periphery of the cranial, middle and caudal lobes. Additional samples which contained airways were taken from a deeper portion of the same lobes. Thus, 6 sections were taken from each pig in a consistent manner and from the same location. Samples from the left lung lobe and a nasal swab were taken from random pigs and cultured. No significant organisms were isolated.

After the initial fixation, tissue samples were washed in 0.1 M phosphate buffer, postfixed in 1 percent osmium tetroxide, dehydrated in a series of graded ethanols, infiltrated by propylene oxide and embedded in epoxy resins. Semi-thin sections were stained with methylene blue or toluidine blue and examined by light microscopy. Ultrathin sections were stained with saturated alcoholic uranyl acetate and lead citrate and then examined with a JOEL® 100 CX transmission electron microscope at 80 KV.

RESULTS AND DISCUSSION

1. Study 1 (IV): As anticipated, necrosis of lymphocytes, hematopoietic and crypt epithelial cells was observed within lymphoid tissues, bone marrow and intestines, respectively. Additional changes were found in the lung, heart and pancreas. Marked changes were present in the lung at 4 hours postdosing. The capillaries were markedly dilated and contained large numbers of neutrophils and macrophages which contained phagocytosed debris (Figure 1).

At the same time, in the heart there were scattered foci of degeneration characterized by the formation of contraction bands within myofibers. In the pancreas, 8 hours after T-2 toxin, early degenerative

changes in the membranous compartment of the rough endoplasmic reticulum were noted.

The ultrastructural changes observed in the heart and pancreas progress to lesions which are observable at the light microscopic and even gross level as demonstrated in the accompanying manuscript entitled "Myocardial and Pancreatic Lesions Induced by T-2 Toxin in Swine" which has been submitted for publication to Veterinary Pathology. In this study (1b), on gross examination we noted scattered subendocardial hemorrhages, multifocal pinpoint white foci in the myocardium and severe pancreatic edema in one treated pig killed at 48 hours. Histologic changes in the myocardium of all treated pigs consisted of multifocal edema, mononuclear cell infiltration, myofiber hyalinization and formation of contraction bands with nuclear pyknosis. Ultrastructurally, there were areas of edema, myofibrillar disorganization, dilation of sarcoplasmic reticulum and formation of hypercontraction bands. Myocardial mineralization was seen in the pig with gross lesions. Pancreatic changes in all treated pigs consisted of multifocal acinar degeneration and necrosis. Ultrastructural changes included irregular dilation of rough endoplasmic reticulum (RER) and irregularity, smudging and a decrease in electron density, of some zymogen granules. In addition, the pig with severe pancreatic edema grossly had a severe suppurative necrotizing pancreatitis.

2. Study 2 (inhalation). On gross examination, T-2 treated pigs killed 4 and 8 hours after exposure had an increased amount of clear fluid within the pericardial sac, and in several pigs there was severe congestion and hemorrhage in the glandular portion of the stomach.

No ultrastructural changes have been observed in sections of the lung and pancreas examined to date. However, myocardial degeneration was evident 4 hours after treatment. Myocardial degeneration was multifocal and characterized by the presence of hypercontraction bands (Figure 2a) similar to those observed following intravascular T-2 toxin. In addition, by 8 hours, there was severe focal loss and disorganization of myofibrils in some myofibers (Figure 2b). In the bone marrow, by 4 hours, there were many necrotic cells (Figure 3a); preliminary examination suggesting that these are hematocytoblasts, red blood cell precursors. By 8 hours, eosinophils were observed to be undergoing necrosis with degranulation into the surrounding area (Figure 3b).

Changes were also noted in the liver of two pigs 8 hours after inhalation exposure. There was dramatic necrosis and loss of sinusoidal endothelial cells (Figure 4). The remaining endothelial cells showed cytoplasmic edema and granular material as well as small numbers of neutrophils were present in the sinusoids. In addition, there was loss of the smooth endoplasmic reticulum (SER) within the cytoplasm and increased vesiculation along the sinusoidal border of the hepatocytes. These changes were diffusely present in one pig and multifocal in the second pig.

The lesions observed in the IV treated pigs corresponded to the light microscopic changes previously described. The changes in the myocardium had been described previously in rats but not in swine. The pancreatic lesions have not been previously described in any species, although recent information indicates that similar lesions have been seen in the cat following T-2 toxin administration (R. Gunther, personal communication). These lesions are described and discussed extensively in the accompanying

manuscript. The finding of large numbers of neutrophils in pulmonary capillaries correlates with the disappearance of these cells from the circulation early after IV T-2 toxin administration. The presence of macrophages in the pulmonary capillaries is a normal finding in pigs. However, after T-2 treatment, these cells contained phagocytosed debris, presumably from circulating necrotic cells from other tissues, such as bone marrow.

It is interesting that similar changes were not observed in the lungs of pigs exposed by inhalation to T-2 toxin. In fact, no lesions were detected in sections examined so far from the lungs of these pigs. The finding of liver lesions is of interest. We have seen severe liver necrosis in a few pigs following inhalation exposure to T-2 toxin and hepatocyte proliferation in several animals following IV administration of 0.6 mg/kg T-2 toxin. However, these changes have not been consistently present. We are presently evaluating liver ultrastructure from additional pigs. It is of interest that the liver is the only organ to date in which we have seen endothelial cell damage. No endothelial lesions were seen in the myocardium as have been described in rats (Yarom, 1983a).

Evaluation of remaining tissues is in progress and will be completed by the end of this year.

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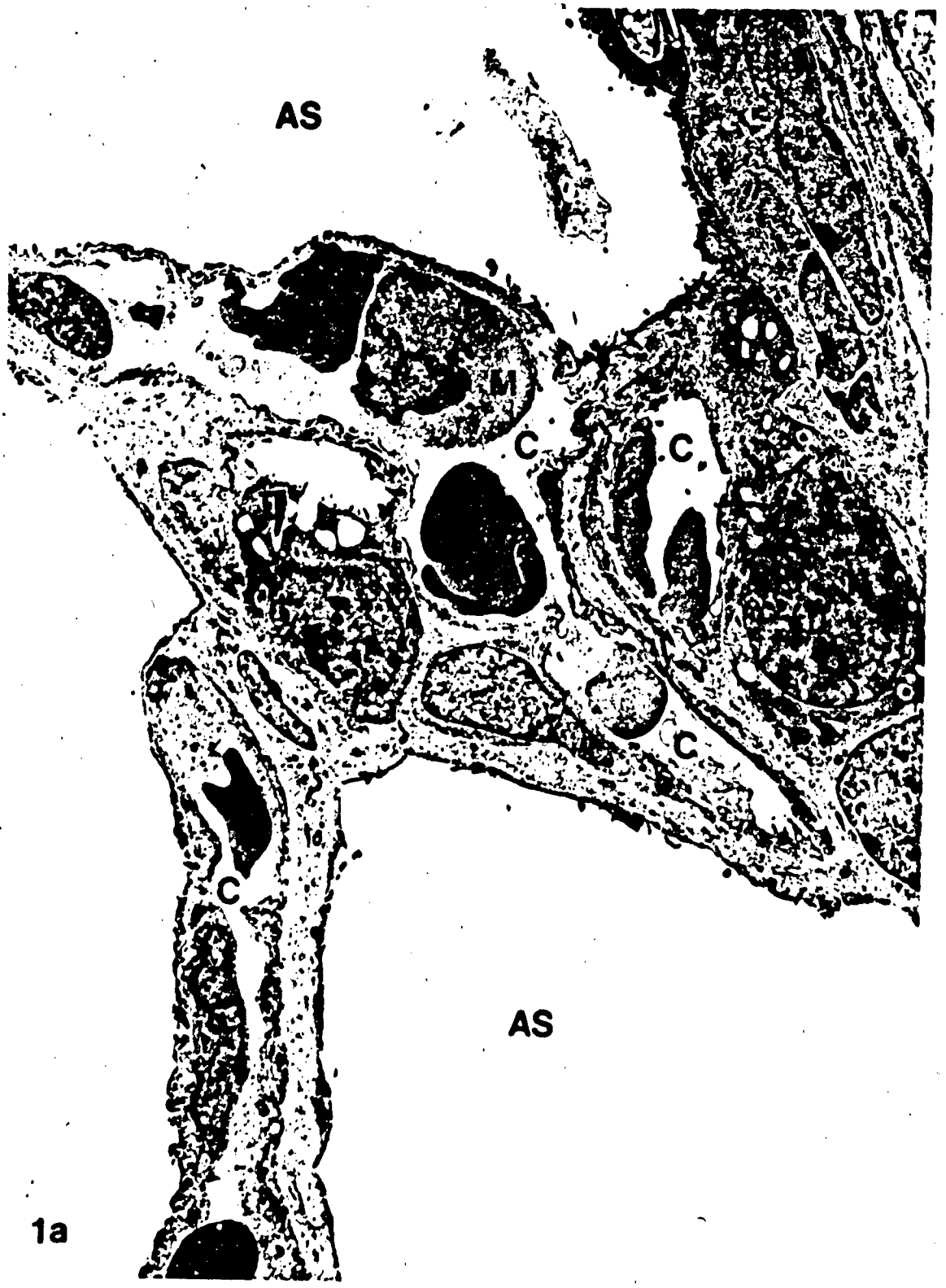
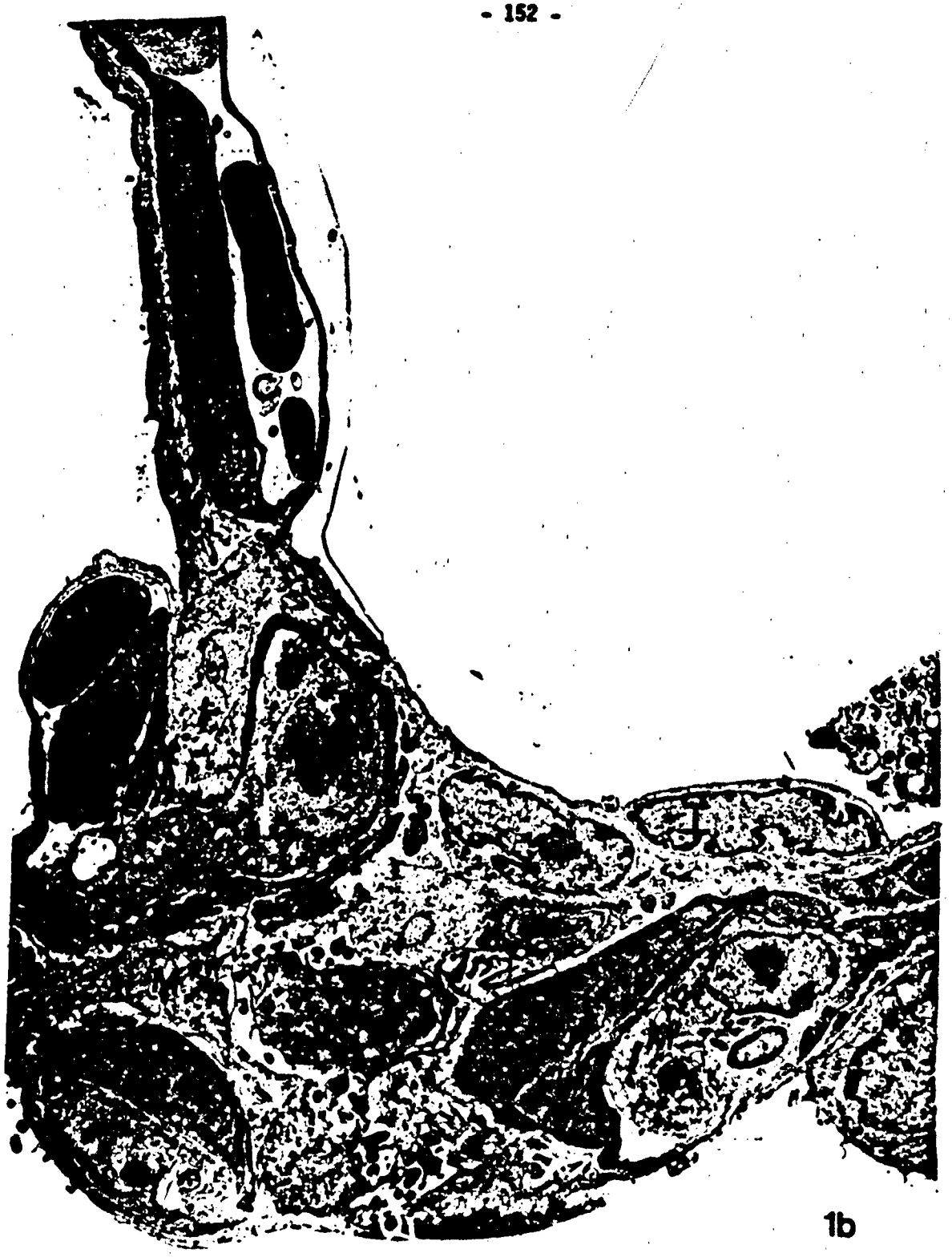
Figure 1. Lung from a pig 4 hours after IV administration of 2.4 mg/kg T-2 toxin.

a. Vehicle control. An alveolar septum present within the alveolar space (AS) is lined by thin cytoplasm of a type I cell. Capillaries (C), lined by endothelial cells (E), contain red blood cells and macrophages (M) which are a normal feature of the porcine lung. Several type II epithelial cells (II) and fibroblasts (F) can be seen. x 7,000.

b. T-2 treated. An alveolar macrophage (M), undergoing disintegration, abuts the thin cytoplasm of a type 1 cell (I) which lines the alevolar wall. Capillaries (C) contain neutrophils or macrophages (M), many of them containing phagocytosed debris. A neutrophil (N) is migrating through the interstitium. x 7,300.



1a

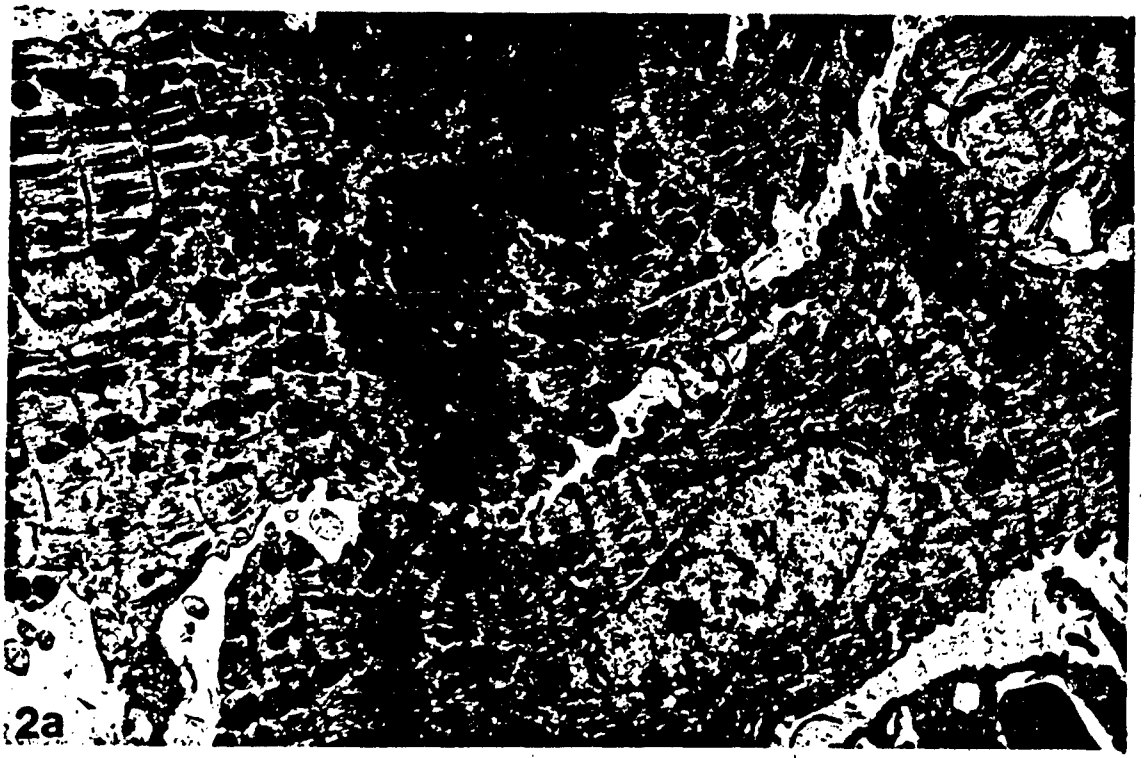


1b

Figure 2. Heart from pigs exposed to T-2 toxin by inhalation.

a. Four hours after exposure. Prominent contraction bands (CB) characterized by electron dense contractile material are noted in adjacent myofibers. Mitochondria are accumulated adjacent to the contraction bands. Nuclei (N) appear normal. x 6,700.

b. Eight hours after exposure. An area of severe myofibril disorganization (MD) lies adjacent to the nuclei (N) which shows peripheral clumping of chromatin. x 13,000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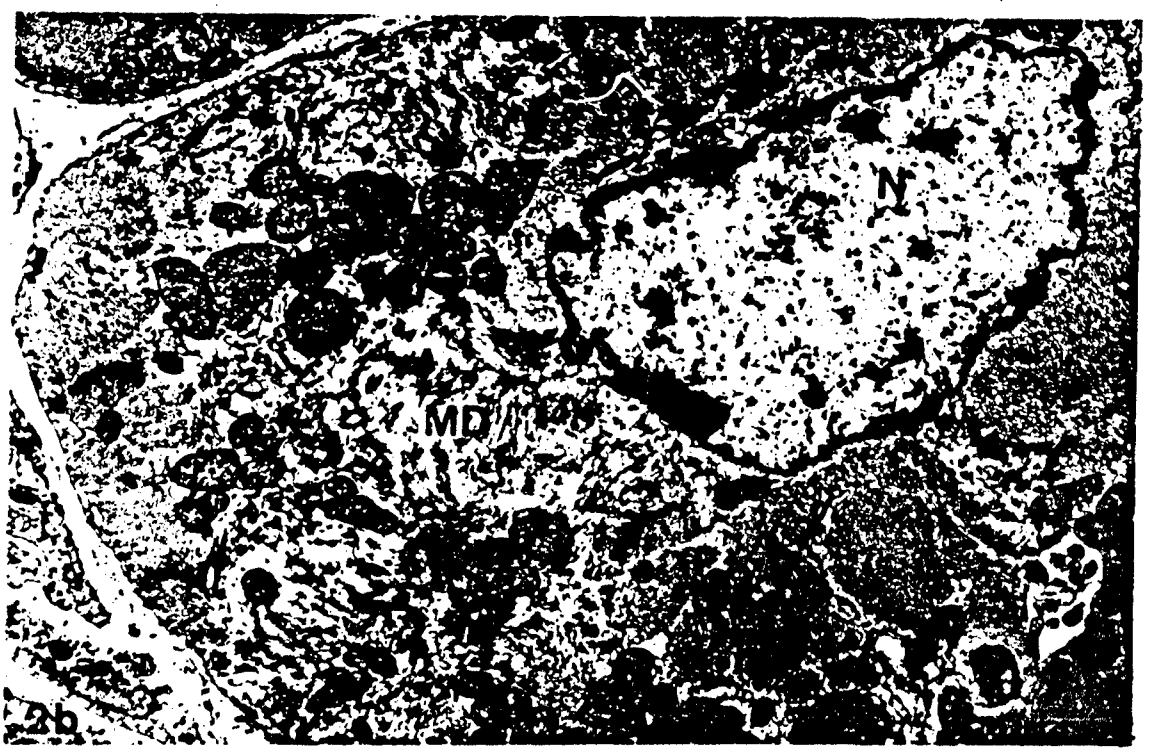
N
MD
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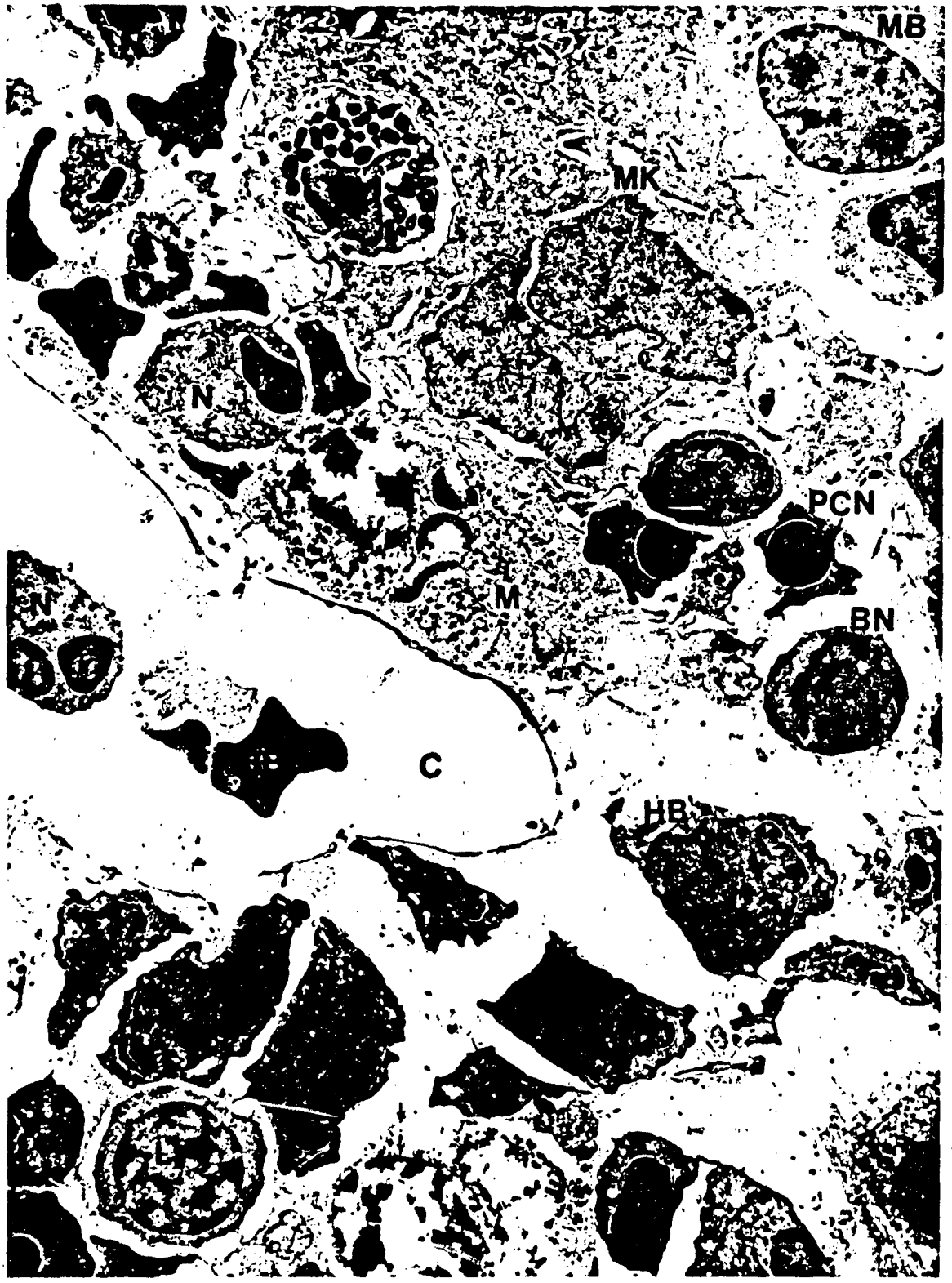
Figure 3. Bone marrow from pigs exposed to T-2 toxin by inhalation.

a. Four hours after exposure. Normal bone marrow cells including a megakaryocyte (MK), myeloblast (MB), neutrophils (N), lymphocytes (L), polychromatophilic normoblasts (PCN), and other erythrocyte precursors are noted around a sinusoidal capillary (C). Several hematocytoblasts (HB) show condensation of nuclear chromatin and cytoplasm while undergoing degeneration. Three cells are undergoing degeneration and necrosis (arrows). One has been phagocytosed by a macrophage (M). x 5,700.

b. Eight hours after exposure. Two degenerating eosinophils (DE) are releasing their granules (arrow) into the adjacent tissue. Myeloblasts (MB) appear normal. x 10,800.


MB
MK
N
PCN
M
BN
C
HB

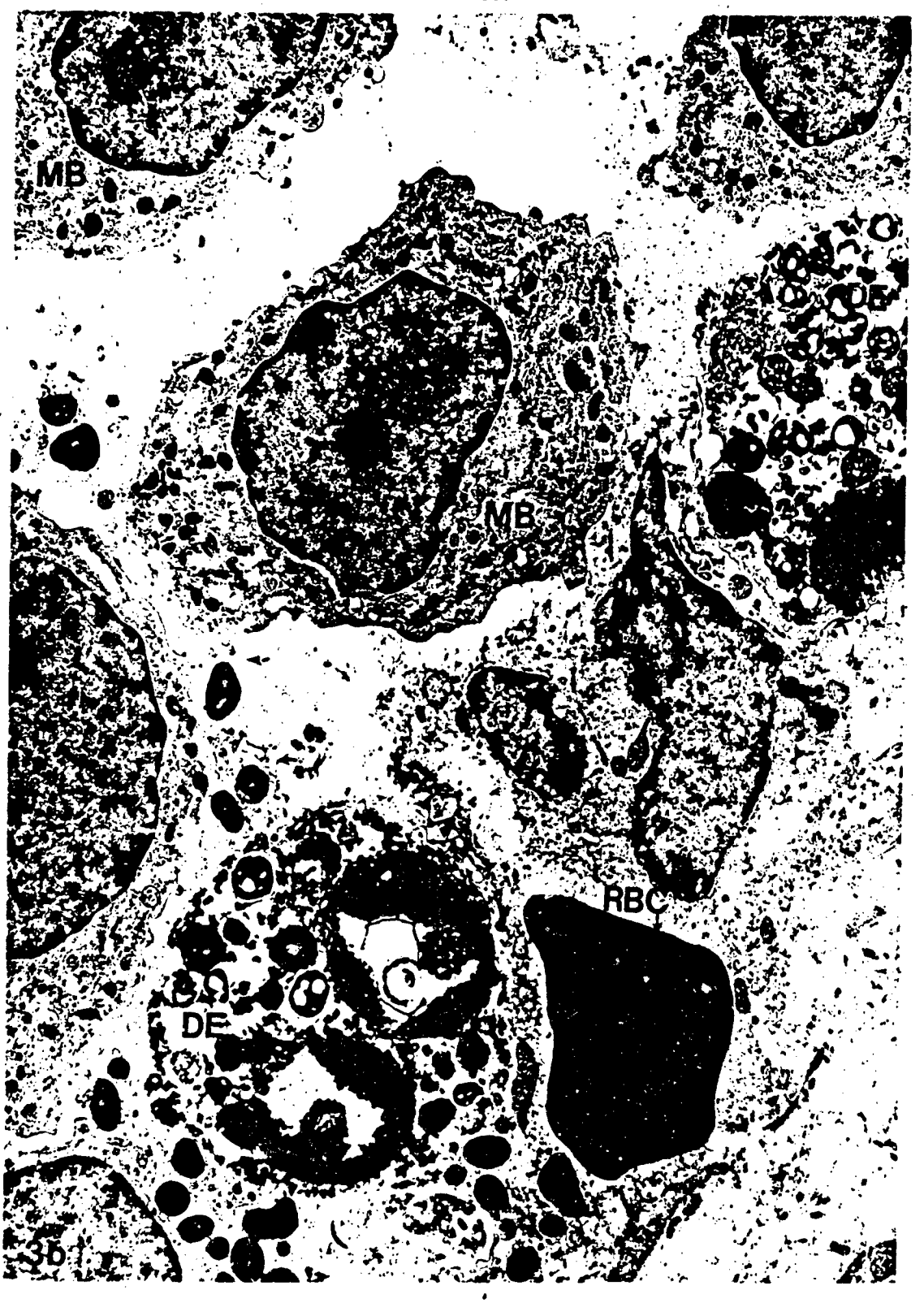
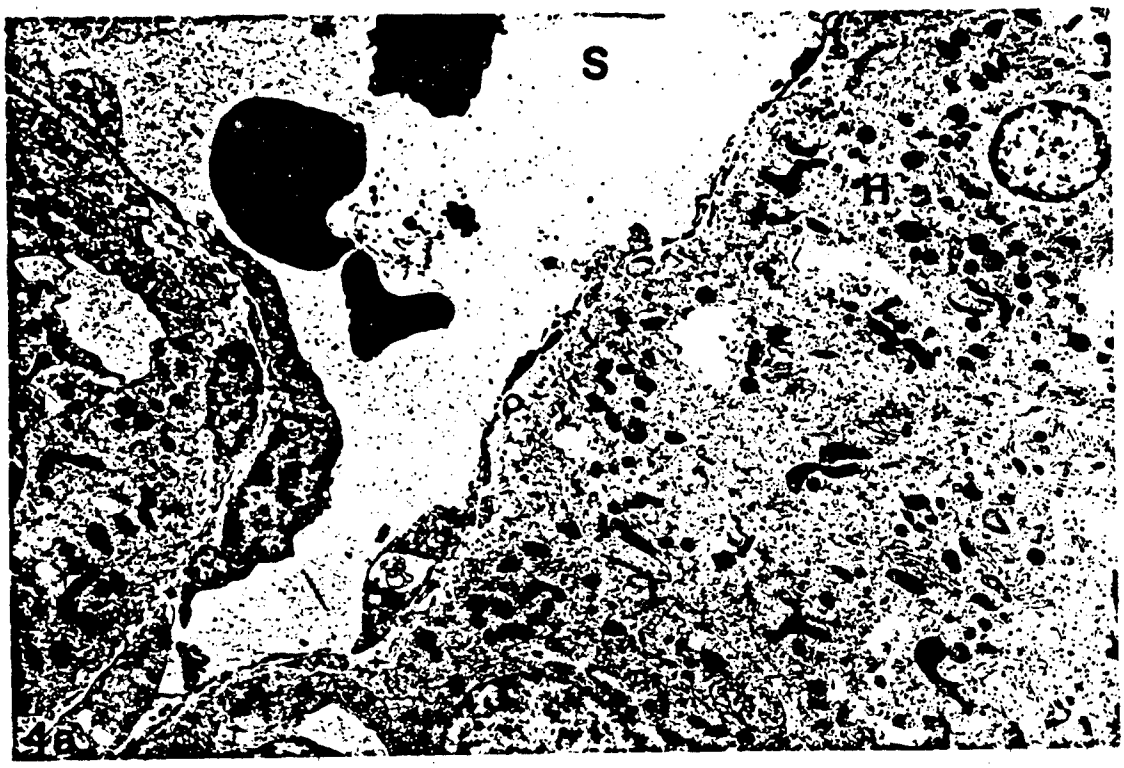

MB
MB
RBC
DE
3b

Figure 4. Liver from pigs 8 hours after treatment.

a. Vehicle control. Normal endothelial cell (E) lines a sinusoid (S). Hepatocytes (H) appear normal. x 4,400.

b. T-2 treated. The hepatocyte (H) and bile canuliculus (BC) appear normal. In the sinusoid (S), a neutrophil (N) is present and there is an increased amount of dense granular material. The normal endothelial cell lining is lost and only reticulum fibers (arrows) outline the sinusoid (S). x 10,600.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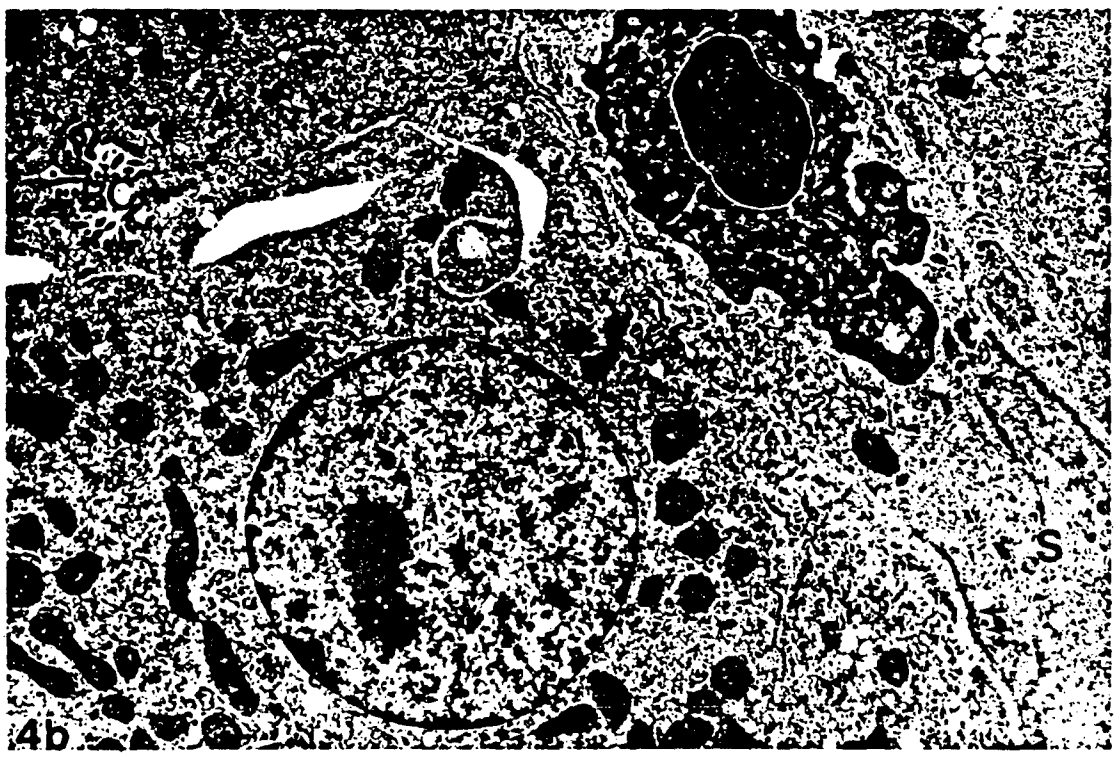
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4b

F. MYOCARDIAL AND PANCREATIC LESIONS INDUCED BY T-2 TOXIN, A TRICHOTHECENE MYCOTOXIN, IN SWINE--V. F. Pang, J. H. Adams, V. R. Beasley, W. B. Buck and W. M. Haschek

ABSTRACT

The myocardial and pancreatic lesions induced by a sublethal dose of T-2 toxin in swine were characterized at the light and ultrastructural levels. T-2 toxin was administered iv at 0 (two vehicle control pigs) and 0.6 mg/kg (six T-2 treated pigs) to 17- to 18-week-old SPF-derived crossbred gilts. The pigs were killed 24 or 48 hours after treatment and selected tissues were examined by light and electron microscopy. On gross examination, we noted scattered subendocardial hemorrhages, multifocal pinpoint white foci in the myocardium, and severe pancreatic edema in one treated pig killed at 48 hours. Histologic changes in the myocardium of all T-2 treated pigs consisted of multifocal edema, mononuclear cell infiltration, myofiber hyalinization, and vacuolation, as well as formation of contraction bands with nuclear pyknosis. Ultrastructurally, there were areas of edema, myofibrillar disorganization, dilation of sarcoplasmic reticulum, and formation of hypercontraction bands. In addition, myocardial mineralization was seen in the pig with gross lesions. Pancreatic changes were present in all T-2 treated pigs and consisted of multifocal acinar degeneration and necrosis. Ultrastructural changes included irregular dilation of rough endoplasmic reticulum (RER), and irregularity, smudging, and a decrease in electron density of some zymogen granules. The pig with gross severe pancreatic edema also had a severe suppurative necrotizing pancreatitis. Thus, in addition to the well documented radiomimetic lesions of the gastrointestinal tract and lymphoid organs, the heart and pancreas are target organs of T-2 toxin in swine.

INTRODUCTION

T-2 toxin is one of several trichothecene mycotoxins produced by Fusarium species. Field outbreaks of T-2 mycotoxicosis in animals and man have been reported in the United states,[18] Canada,[35] Japan,[44] and the Soviet Union.[54] More recently, T-2 toxin has gained international attention by being implicated as a biochemical warfare agent in southeast Asia and Afghanistan.[17,38,40]

T-2 toxin is markedly cytotoxic to epithelial cells of skin and mucous membrane, including the gastrointestinal tract,[14,30,34,53] and to lymphoid and hematopoietic tissues of many species.[15-16,19,29,51] Because of its ability to cause marked damage to actively dividing cells of the thymus, lymph node, spleen, bone marrow and crypts of the small intestine, T-2 toxin has been called a radiomimetic agent.[40] Effect on other organ systems have also been reported.

Cardiovascular lesions have been observed in suspected field cases of T-2 toxicosis associated with moldy bean-hull poisoning of horses in Japan,[45] in geese experimentally fed a diet containing T-2 toxin for 5 to 10 weeks,[33] and in rats surviving more than a year after several intragastric doses.[41] However, the cardiovascular toxicity of T-2 toxin has only recently been studied. Increased blood pressure and severe cardiovascular abnormalities were reported in a long-term rat study in which multiple doses of T-2 toxin were administered intragastrically.[52] A dose-related decrease of contractility, ECG changes and ultrastructural damage to myofibers were found in isolated preparations of rat heart.[55] Histologic and ultrastructural myocardial changes occurred in hearts of rats given single large, or repeated small doses of T-2 toxin intraperitoneally.[56] Pancreatic lesions characterized by degenerative

and reparative changes in the pancreatic ducts, stromal vessels and associated connective tissue, as well as tumors of the endocrine and exocrine pancreas have only been reported in one long-term rat study of T-2 toxicosis.[41] These changes have not been studied at the ultrastructural level.

In our own studies, we observed subtle myocardial degeneration in swine and cattle in a toxicokinetic and toxicodynamic study of T-2 toxin.[4] In addition, we have seen similar myocardial degeneration as well as exocrine pancreatic degeneration and necrosis in pigs during studies of T-2 toxicosis via intravascular administration (unpublished data).

The purpose of this study was to characterize the histologic and ultrastructural changes induced by T-2 toxin in the myocardium and pancreas of swine. Characterization of these changes may allow a better understanding of the pathogenesis of T-2 toxicosis and thus influence the choice of therapeutic regimens for the treatment of this toxicosis.

MATERIALS AND METHODS

Eight 17-18-week-old SPF-derived (specific pathogen free) crossbred gilts, weighing between 54 and 66 kg, were randomly assigned to 2 groups. Each group consisted of four pigs, three T-2 treated and one vehicle control. Purified T-2 toxin (99 percent pure) at a sublethal dose of 0.6 mg/kg, was dissolved in 2.5 mL of 50 percent ethanol and administered through a catheter into the ear vein. The catheter was then flushed with 5 mL of normal saline. Similarly, the control pigs received 2.5 mL of 50 percent ethanol followed by 5 mL of normal saline. The pigs were immobilized with ketamine (Ketaset®, Bristol-Myers Co., Syracuse, NY), and then killed by electrocution either 24 or 48 hours after treatment.

The heart and pancreas were removed immediately and representative tissue samples for ultrastructural evaluation were taken from the head, body and tail of the pancreas, and from the papillary muscles of the left ventricle, the septum and free walls of the right and left ventricles and atria. The tissue samples were diced and fixed in 2.5 percent glutaraldehyde in 0.1 M phosphate buffer overnight at 4°C. They were then washed in buffer, postfixed in 1 percent osmium tetroxide, dehydrated in a series of graded ethanols, infiltrated by propylene oxide and embedded in epoxy resins. Semi-thin sections were stained with methylene blue or toluidine blue and examined by light microscopy. Ultrathin sections were stained with saturated alcoholic uranyl acetate, then lead citrate and examined with a Joel® 100 CX transmission electron microscope at 80 KV.

Sections of heart (11-14 from each pig) and pancreas immediately adjacent to those collected for electron microscopy as well as tissues from other organs (spleen, thymus, lymph node, gastrointestinal tract, liver, kidney, thyroid, adrenal, bone marrow, brain) were fixed in 10 percent neutral buffered formalin, embedded in paraffin, sectioned at 6 μm, stained with hematoxylin and eosin (HE) and examined by light microscopy. Selected myocardial sections were stained with phosphotungstic acid hematoxylin (PTAH).

Some of the tissues that had been prepared for light microscopy were reprocessed for electron microscopy. The desired areas were trimmed from the paraffin blocks and the embedding material was dissolved with six changes of xylene for 5 minutes each. The xylene was changed to absolute ethanol with several changes overnight. The tissues were rehydrated with ethanol at ascending grades, and then fixed for one hour in 1 percent osmium tetroxide. These tissues were then dehydrated and embedded as described above for electron microscopy.

Samples of liver were collected from all pigs and analyzed for selenium content by a gas chromatographic assay using an electron-capture detector.[21]

RESULTS

Clinical signs were readily apparent in pigs treated with T-2 toxin. Chewing and salivation followed by several bouts of vomition occurred within 5 to 30 minutes after treatment. The pigs were lethargic and had labored breathing, purplish discoloration of the oral mucosa and snout, as well as marked congestion of the conjunctiva and sclera. The skin became diffusely reddened during the first few hours after dosing, and then turned to purple. The ears and limbs were cold to the touch. After 5 to 6 hours, the signs began to regress. Aside from being less alert and active, the pigs appeared clinically normal at the time of killing. Liver selenium concentrations of both treated and control pigs ranged from 224 to 488 ppb which were within the normal range.

No gross lesions were seen in the T-2 treated pigs killed at 24 hours or in the control pigs. One of the three T-2 treated pigs killed at 48 hours had scattered small subendocardial hemorrhages in the left ventricle, mainly in the papillary muscles. In addition, there were multiple pinpoint white foci randomly scattered throughout the myocardium of the entire heart, but they appeared most prominent in the left ventricle. In the same pig, diffuse severe subcapsular and interlobular edema was present in the pancreas. Areas of slight interlobular pancreatic edema were also noted in the other two T-2 treated pigs killed at 48 hours.

Histologically, the principal myocardial lesions consisted of multifocal degeneration and necrosis together with interstitial edema with or without mononuclear cell infiltration. The changes were noted in all T-2 dosed pigs, although individual variation in the severity and frequency of the lesion was

present in pigs killed at both 24 and 48 hours. Two distinct types of degenerative changes were identified and these two changes usually did not occur concomitantly in the same myofiber. The first type of degenerative change was widely distributed and was characterized by affected myofibers becoming deeply eosinophilic or completely hyalinized with pyknotic nuclei (Figure 1a). In many of the severely damaged myofibers, the normal longitudinal striations were replaced by multiple highly eosinophilic and transversely oriented contraction bands (Figure 1a) which were stained intensely with PTAH (Figure 1b). Granulation, fragmentation, and mineralization with variable loss of myofibers were apparent in some areas (Figure 1c). Interstitial edema and mononuclear cell infiltration were usually associated with this type of alteration. Although the changes were randomly scattered throughout the heart, they were more prominent in the atria, papillary muscles of the left ventricle, and lower left and upper right ventricle, particularly in the subendocardial region. There was no correlation between severity of lesions and length of survival but the intensity of mineralization and loss of myofibers were most severe in the pig which had grossly visible heart lesions when killed at 48 hours.

The second type of degenerative change was vacuolation of myofibers (Figure 1d). The affected myofibers had either single large, or multiple variably sized vacuoles which frequently displaced the nuclei laterally. The vacuoles were clear or contained pale staining, finely granular to fibrillar material or occasionally contained well defined large hyaline droplets. This type of alteration appeared to have a predilection for the papillary muscles and the free wall of both ventricles. As with the first type of degeneration, no correlation between severity of vacuolation and kill time was found.

Ultrastructural changes in myocardial tissue fixed directly for electron microscopy consisted of intermyofibrillar edema with some separation of myofibrils, and mild dilation of sarcoplasmic reticulum. Since changes consistent with the transverse contraction bands, vacuolation or necrosis observed at the light microscopic level were not found in the plastic embedded sections, samples from the paraffin embedded tissues were prepared for electron microscopy. Numerous dense necrotic myofibers were seen in the areas that had contraction bands on light microscopy. These myofibers contained clumps of disrupted, electron-dense, contractile material which was usually surrounded by large numbers of mitochondria (Figure 2a). Loss of filaments, edema, and accumulation of a finely granular and fibrillar material were noted between these dense clumps of disrupted contractile material. Focal distension of the sarcoplasmic reticulum was seen in some affected myofibers.

Myofibers which were vacuolated at the light microscopic level ultrastructurally contained variably dilated elements of the sarcoplasmic reticulum (Figure 2b) and membrane bound vacuoles containing variable amounts of finely granular and fibrillar material. At the margins of the very large vacuoles, aggregates of membranous fragments were often observed. Connections appeared to be present between the membranous aggregates and the lining membrane of the space, suggesting formation of these vacuoles by coalescence of dilated elements of sarcoplasmic reticulum. Destruction and loss of the myofibrils with accumulation of large quantities of disorganized fibrillar substance were evident in the injured myofibers; however, no hypercontraction bands were observed next to the large vacuoles. Mitochondria of these affected myofibers occasionally contained amorphous electron-dense granules.

Histologic pancreatic changes in all T-2 treated pigs killed at 24 hours consisted of multifocal degeneration and necrosis of single or grouped acinar cells (Figure 3). Affected cells contained either a single large vacuole, or occasionally, multiple variable-sized vacuoles. The nuclei were laterally displaced and often pyknotic or undergoing karyorrhexis. Globular cytoplasmic condensation or hyalinization surrounded by a halo was seen in many of the degenerating cells. Areas of interlobular edema and fibrin deposition along with fat necrosis were observed in two pigs killed at 24 hours. The islets of Langerhans were relatively unaffected, although occasional single cell degeneration consisting of vacuolation and increased cytoplasmic granularity was observed. Similar, but more severe changes were seen in two of the T-2 treated pigs killed at 48 hours. In the third pig, which grossly had shown prominent pancreatic edema, a diffuse, suppurative necrotizing pancreatitis was present (Figure 3c). This was characterized by indistinct lobules, and disorganized and disrupted acini. Edema, fibrin deposition, and infiltration of neutrophils and macrophages were prominent along with fat necrosis and scattered small focal intralobular hemorrhages. Epithelial hypertrophy and hyperplasia, characterized by epithelial evagination and frequent mitotic figures, were present in the ducts.

Ultrastructurally, the degenerating acinar cells had variable dilation of the rough endoplasmic reticulum with eventual disruption and coalescence to form large membrane bound spaces (Figure 4). Small irregular, membrane-lined vacuoles, myelin-like membranous aggregates and small membranous fragments were often present in these large membrane bound spaces. The remaining area of these spaces either was clear or contained electron lucent granular substance. Some zymogen granules were irregular, smudgy, and electron-lucent

(Figure 4c). In necrotic cells, the nuclear remnant was displaced laterally by large cytoplasmic vacuoles and surrounded by a disintegrated nuclear envelope with markedly dilated perinuclear cisternae (Figure 4c).

Minimal to moderate multifocal lymphocytic necrosis was observed in the cortex of the thymus of five T-2 treated pigs, three killed at 24 hours and two killed at 48 hours. A moderate number of hepatocytes in mitosis were noted in the liver of two T-2 treated pigs killed at 48 hours. The gastric mucosa of two T-2 treated pigs killed at 24 hours had mild multifocal necrosis mainly at the luminal phase, together with mild congestion, hemorrhage, and neutrophilic infiltration. Fibrinoid degeneration and fibrin thrombi were noted in scattered capillaries.

DISCUSSION

Based on the histologic and ultrastructural findings, two types of myocardial lesions were induced by T-2 toxin in swine. In the first type, mild dilation of sarcoplasmic reticulum, sarcoplasmic edema, and malaligned myofibrils were followed by myofiber necrosis with formation of hypercontraction bands, myofibrillar loss, and finally by mineralization and loss of myofibers. In the second type of lesion, sarcoplasmic vacuolation was characterized by distention of the sarcoplasmic reticulum as well as myofibillar loss and occasional amorphous electron-dense mitochondrial deposits.

In other studies, most rats given a single large dose of T-2 toxin (2 mg/kg) intraperitoneally and killed 1, 2, or 3 days later had myocardial lesions. These lesions included interstitial edema, focal cellularity, and groups of edematous and disorganized myofibers.[56] Large areas of cellular infiltration and patchy fibrosis, mainly in the subendocardial region of the

left ventricle, were described in rats killed 1 or 2 months after a series of small intraperitoneal doses of T-2 toxin (0.5 mg/kg or 0.3 mg/kg).[56] In isolated perfused rat hearts, T-2 toxin caused decreased contractility and electrocardiographic abnormalities without much effect on heart rate, or QRS and QT intervals.[55] Ultrastructurally, the affected myofibers and their mitochondria were swollen. The sarcomeres were hypercontracted and malaligned with poorly staining Z lines.

Necrosis with contraction band formation occurs in many forms of cardiac injury in which excessive amounts of calcium enter the myofibers and trigger a number of events, including decreased mitochondrial energy production, formation of intramitochondrial calcific deposits, and activation of sarcoplasmic phospholipase and proteases.[6,43,46] This type of injury can be induced by a number of agents including catecholamines.[2,10,47] Dramatically increased plasma concentrations of catecholamines, both epinephrine and norepinephrine, were seen in a previous study during which swine were given iv T-2 toxin at lethal (4.8 mg/kg) and sublethal (0.6 mg/kg) doses.[28]

There is a striking tendency for localization of the myocardial lesion induced by catecholamines, such as isoproterenol, to the subendocardial third or half of the myocardium.[2] The lesions are most pronounced in the left ventricle.[3,47-48] It is well known that perfusion pressure is lowest in the subendocardium because coronary pressure decreases, while tissue pressure increases, as one moves from the epicardium toward the endocardium.[32] It has been suggested that the myocardial lesions induced by catecholamines are probably attributable to their exaggerated pharmacologic effects including positive chronotropic and inotropic actions as well as myocardial hypotension.[2] Tachycardia decreases the period of perfusion, whereas

hypotension may lower coronary perfusion. Positive inotropic actions result in greater expenditure of energy, and therefore greater requirements for ATP production. All of these events, then, lead to a condition of relative coronary insufficiency which is one hypothetical mechanism by which the focal necrosis occurs.[2]

In the present study, myocardial degeneration, necrosis, and mineralization were also present mainly in the subendocardial region, although lesions were observed in both ventricles and atria. The changes in affected myofibers were compatible with those induced by catecholamines.

Extensive sarcoplasmic vacuolization has also been observed in the atrial myocardium of pigs with acute monensin toxicosis.[46] Profound cytoplasmic vacuolation, mainly due to pronounced swelling of the tubules and cisternae of the sarcoplasmic reticulum, is one of two forms of degeneration occurring in the cardiac myofibers in anthracycline toxicosis.[9] Peroxidative damage of membranes was cited as one possible mechanism in both instances. Although T-2 toxin is not known to exert direct peroxidative effects, accelerated phospholipid degradation and lipid peroxidation of plasma membranes have been speculated to be one mode of action of T-2 toxin.[55]

In the present study, the papillary muscles of the left ventricle had the most severe myofiber vacuolation. The oxygen demand of the left ventricular papillary muscles is high because of their great mechanical work supporting the mitral valves during systole.[2] Because the capillary bed in the papillary muscles is utilized at near capacity even at rest, hypoxia develops when demand increases, and this hypoxia is probably more severe and of longer duration than that of other subendocardial tissues.[7,37] These anatomic and functional specificities may make the papillary muscles of the left ventricle more

susceptible to membranous damage with resultant development of distension of the sarcoplasmic reticulum in T-2 treated pigs.

Ischemia,[50] toxic chemicals or drugs,[26] as well as viruses, endotoxin, nutritional deficiency, and trauma can induce direct pancreatic acinar cell damage. Cytotoxic effects induced by chemical agents include changes in RER and mitochondria, enhanced autophagy, loss of secretory granules and necrosis.[26] Aflatoxin B_1 causes dilation of Golgi and RER with disaggregation of ribosomes, as well as changes in zymogen granules in the rat exocrine pancreas.[36] In our previous study when T-2 toxin was given as a single intravascular dose to swine (4.8 mg/kg) a shock syndrome was produced.[28] It was characterized by a decline in cardiac output and blood pressure, increased plasma concentrations of epinephrine, norepinephrine, thromboxane B2, 6-keto-PGF-1α and lactate as well as decreased arterial pH and arterial oxygen partial pressures. The pancreatic vasculature appears to be sensitive to alterations in cardiovascular status, the hypotension associated with shock usually resulting in hypoperfusion of the splanchnic region.[23] Pancreatic blood flow decreases abruptly in hemorrhagic shock.[11,24,42] Recently acquired data show that blood flow to the pancreas in swine is markedly reduced following iv administration of T-2 toxin (G. R. Lundeen, personal communication). It has been suggested that defects in the cell membrane, presumably due to activation of endogenous phospholipases and/or inhibition of the reacylation of lysophospholipids, are an early feature or irreversible ischemic cell injury.[1,42] Studies using hepatocytes and animals with induced myocardial ischemia indicated that the plasma membrane, and the membranes of the endoplasmic reticulum in liver cells and of the sarcoplasmic reticulum in myocardial cells, are preferentially affected in ischemia.[5,8] Distension of

the RER was the most evident morphological alteration of the pancreatic acinar cells present in our pigs.

Hypoxia and ischemia are potent stimuli for disruption of lysosomal membranes.[23] Proteolytic lysosomal enzymes can activate pancreatic proenzymes.[27] The release and activation of these enzymes can enhance preexisting injury and thus cause acinar cell necrosis. It is speculate that ischemia secondary to hypoperfusion might play an important role in pancreatic acinar cell injury in T-2 treated pigs, although one cannot rule out the possibility of a direct cytotoxic effect of T-2 toxin. Release of enzymes from necrotic acinar cells into the interstitium can cause injury to adjacent cells and thus extend the damage. In massive and severe acinar cell necrosis, the release of enzymes and cell debris forms a powerful stimulus for the initiation of inflammation. This autolytic process might be the mechanism responsible for the severe necrotizing pancreatitis which occurred in one of the T-2 treated pigs killed at 48 hours.

The proteolysis associated with the zymogenic and lysosomal proteases released during the shock process may also stimulate the production of myocardial depressant factor (MDF).[22,25] MDF, a peptide released from acinar cells through damaged cell membranes, may bind to large carrier proteins in the extracellular fluid or remain as a free peptide.[21] It is then taken up by lymphatic capillaries or is transported directly by capillaries to the systemic circulation.[12] The best known action of MDF is its negative inotropic effect in both the isolated papillary muscle[20] and the intact animal.[13,49] In this study, the pig which had the most severe pancreatic damage was the only one which showed gross myocardial lesions. MDF released from damaged pancreatic acinar cells, may have augmented the T-2 induced

cardiac dysfunction or, conversely, severe myocardial injury may have increased the severity of pancreatic ischemia.

ACKNOWLEDGEMENT

The authors wish to thank Mr. R. K. Manuel for technical assistance, Mr. D. H. Fritts for assistance with photomicrography, and Mr. Steve Swanson for supplying T-2 toxin. Facilities for electron microscopy were provided by the Center for Electron Microscopy, School of Life Sciences, and Electron Microscopy Laboratory, College of Veterinary Medicine, University of Illinois, Urbana.

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Figure 1. Heart from swine treated with T-2 toxin.

a. 24 hours. Increased staining, pyknotic nuclei and transversely oriented contraction bands are noted in the centrally located myofibers. There is a mild interstitial infiltration of mononuclear cells. HE. Bar = 40μ

b. 24 hours. Higher magnification of a. Prominent transverse contraction bands and granular sarcoplasm are present in affected myofibers. PTAH. Bar = 20μ

c. 48 hours. Degeneration and mineralization of myofibers are prominent. HE. Bar = 40μ

d. 24 hours. Severe myofiber vacuolation with peripheral nuclear displacement (lower middle) as well as myofiber fragmentation and early karyorrhexis (upper right) are evident. HE. Bar = 20μ.

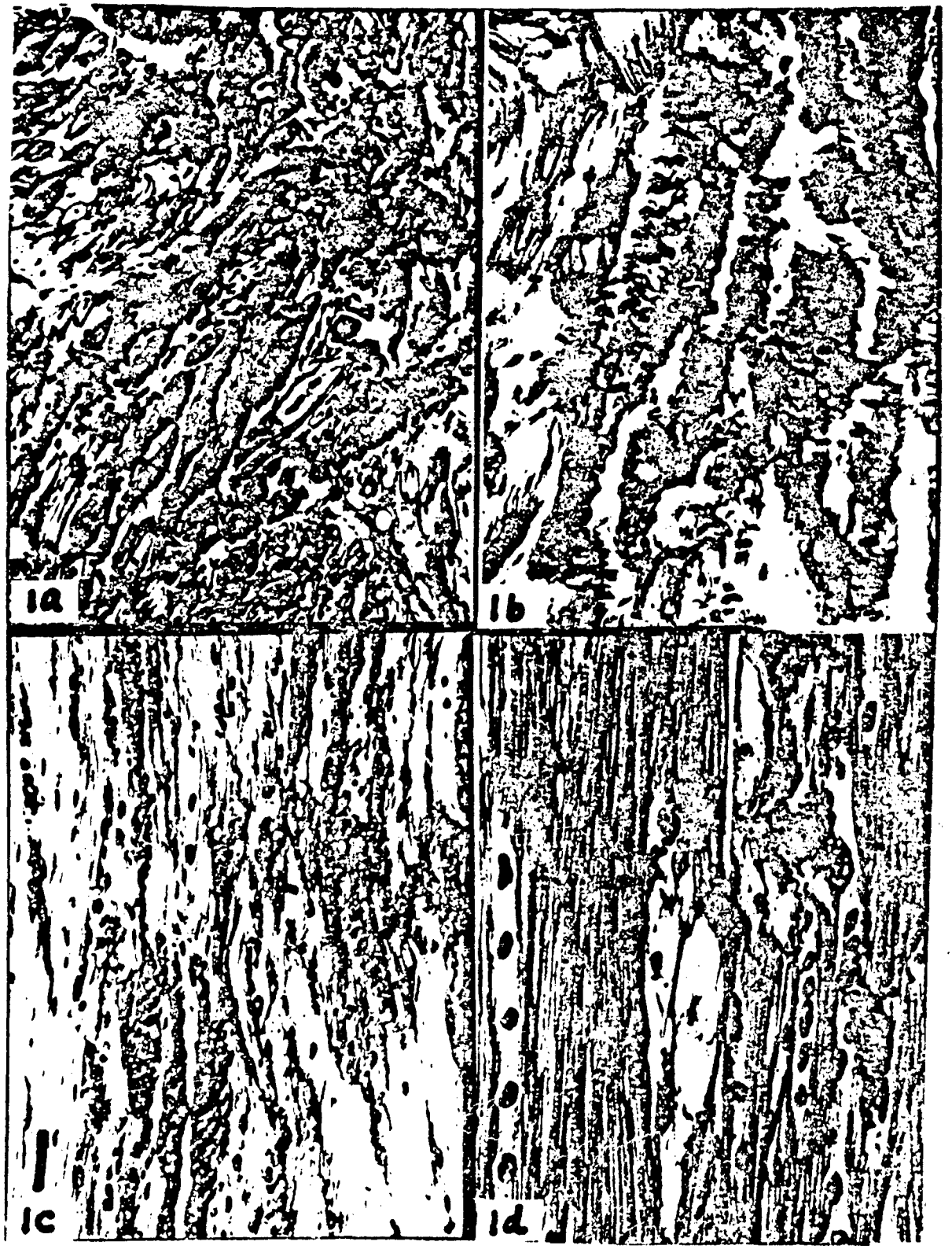
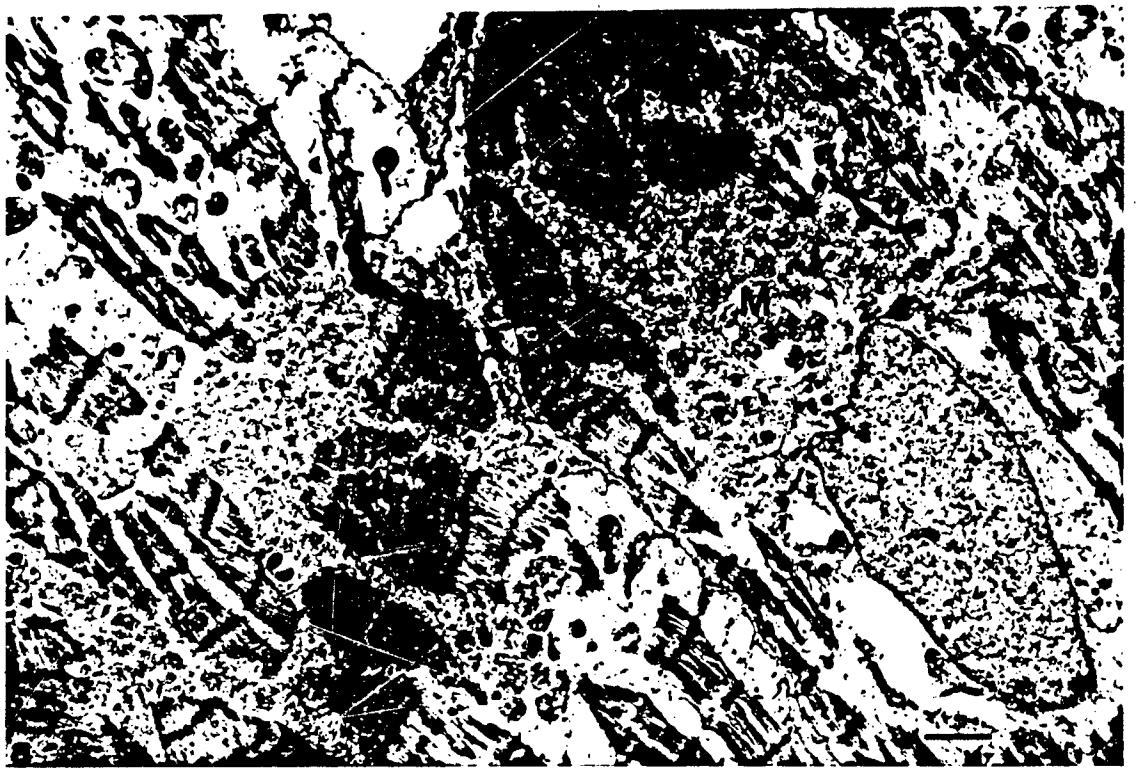

1a
1b
1c
1d

FIGURE 2. Heart from swine 24 hours after treatment with T-2 toxin.

a. Prominent contraction bands, characterized by electron dense contractile material, are surrounded by large numbers of mitochondria (M). In adjacent areas edema separates sarcoplasmic contents. Bar = 3μ.

b. Two myofibers are widely separated by interstitial edema (center). Mild sarcoplasmic edema is present in one myofiber (bottom) while the other myofiber (top) contains remnants of normal sarcomeres (arrows), however the majority of myofilaments are in the dispersed state. Severe sarcoplasmic vacuolar degeneration is noted in this myofiber. A membranous fragment (arrow head) is present within one such vacuole. Bar = 1μ.



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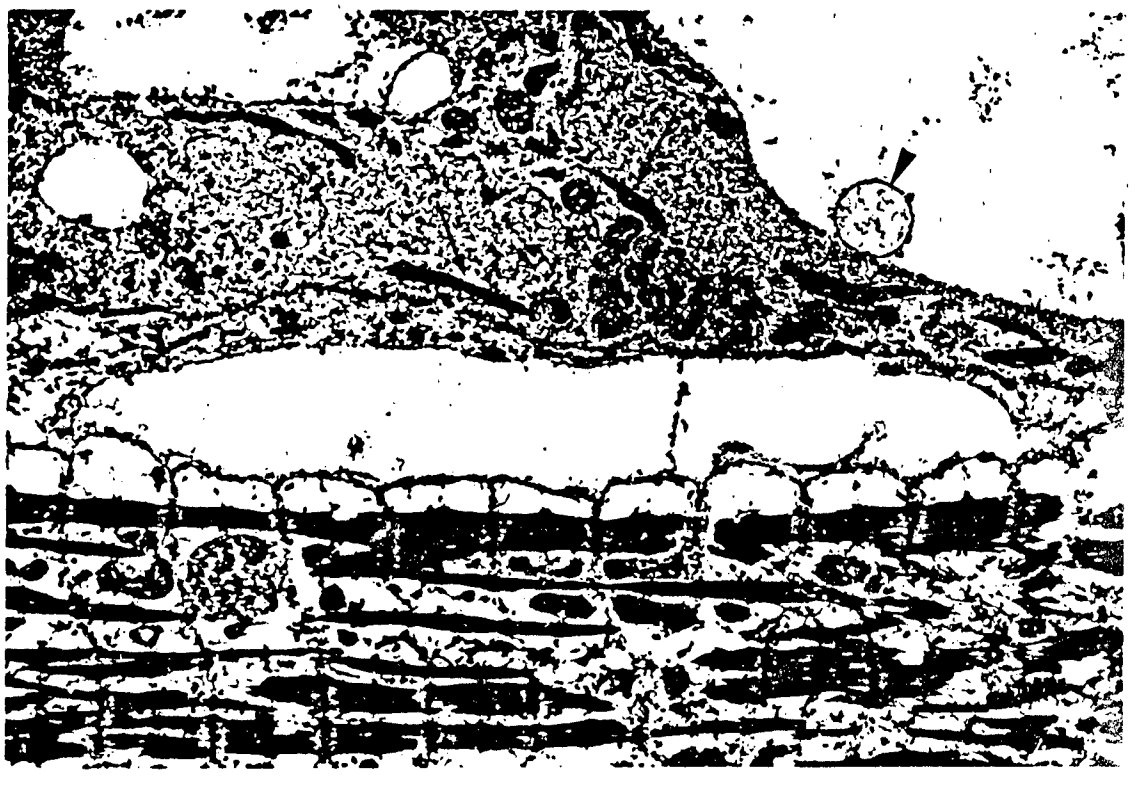

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FIGURE 3. Pancreas from swine treated with T-2 toxin. HE.

a. 24 hours after vehicle (control). Normal acinar cells. Bar = 20μ.

b. 24 hours after T-2 toxin. There is severe vacuolar degeneration and individual cell necrosis (arrows) of acinar cells. Bar = 20μ.

c. 48 hours after T-2 toxin. Severe disorganization and necrosis of acinar cells are present. Note the hypertrophy and hyperplasia of ductal epithelium with several mitotic figures (arrow). Bar = 40μ.

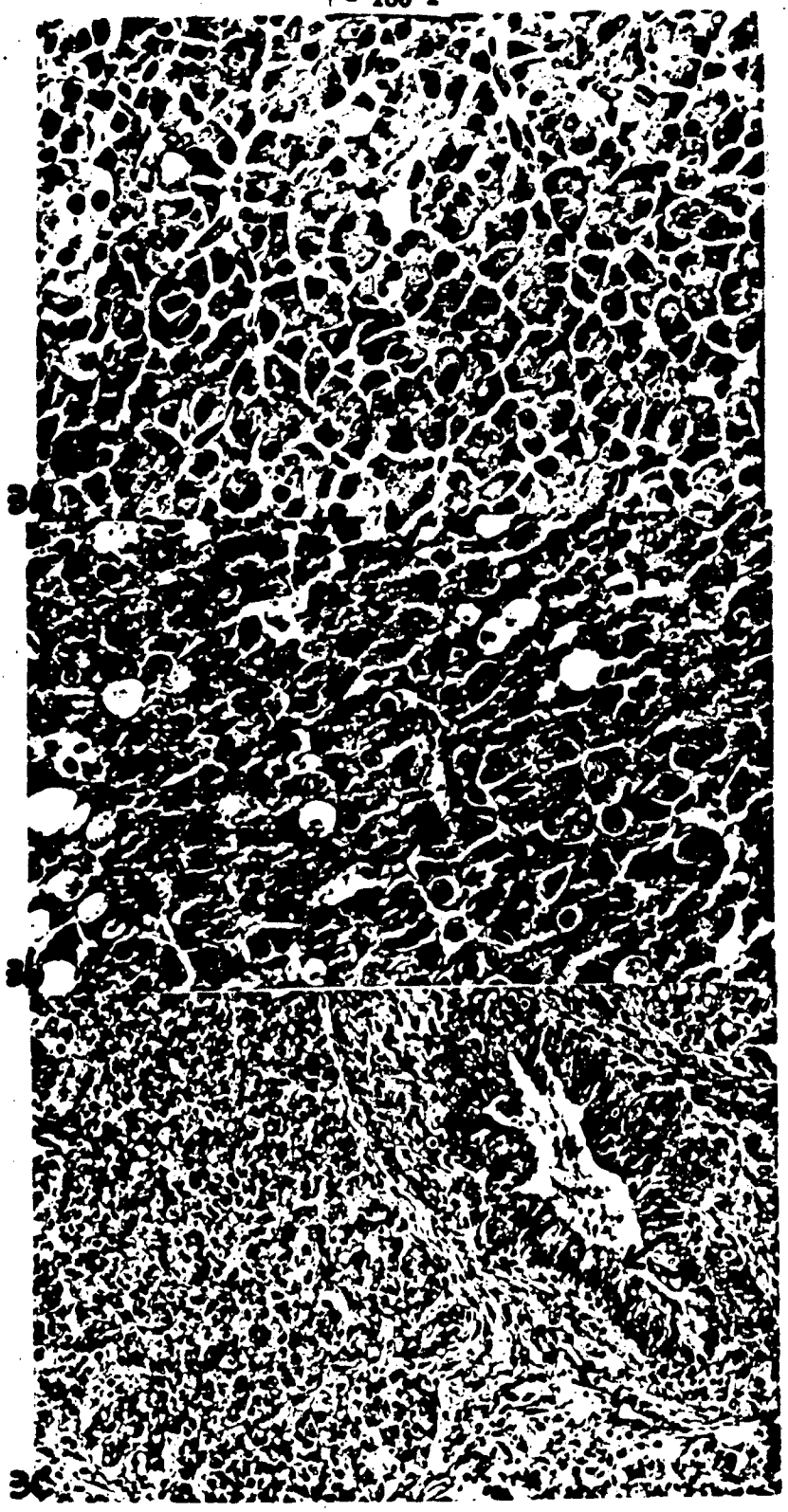
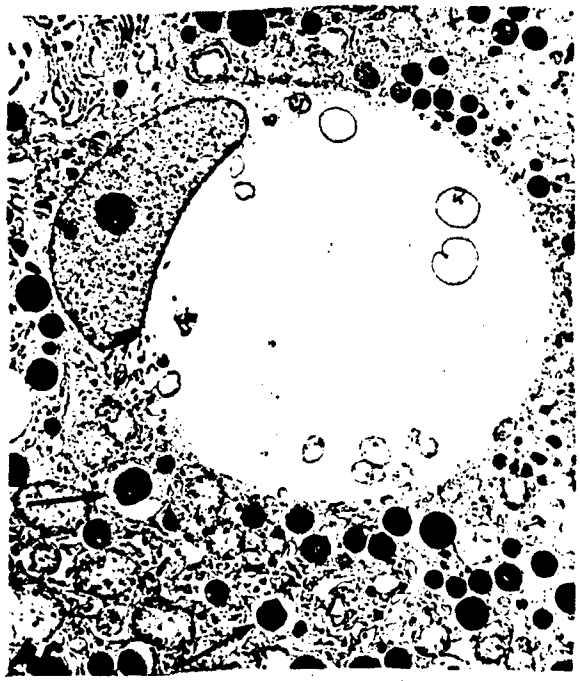


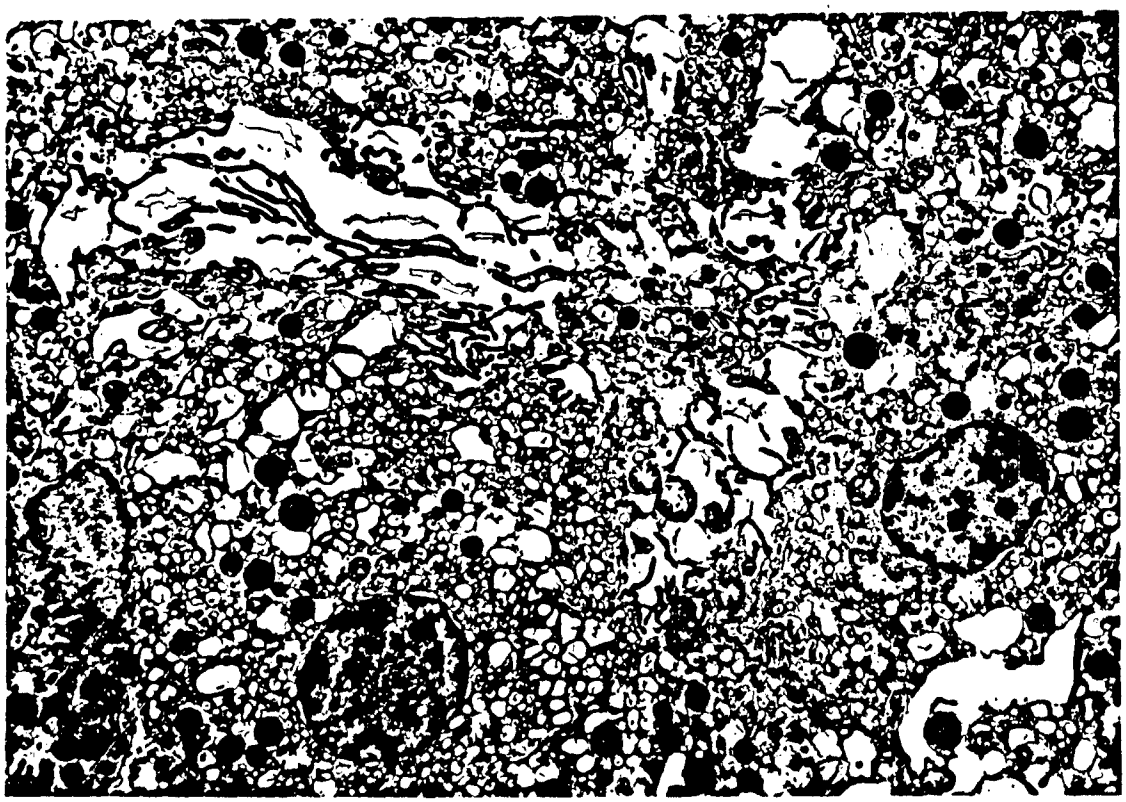
FIGURE 4. Pancreas from swine 24 hours after treatment.

a. Vehicle control. Parallel arrays of RER and electron-dense zymogen granules are present in pancreatic acinar cells. Bar = 7μ.

b. T-2 treated. There is disorganization of RER with variable and irregular dilatation. Bar = 7μ.

c. T-2 treated. In more severely affected cells, large membrane bound vacuoles displace the nucleus or nuclear remnant peripherally and contain small membranous fragments. Some zymogen granules are irregular, smudgy and have decreased electron-density with widening of the perigranular space (arrows). Bar = 8μ.





G. CARDIAC BULK ELECTROLYTIC STUDY--Bob Poppenga

SUMMARY

Cardiac bulk electrolytes (sodium, potassium, magnesium and calcium) were determined in both a T-2 toxin dosed and a control group of rats. Those rats which received T-2 toxin appeared to show larger intergroup variations in the levels of these electrolytes compared to the control rats. The significance of these variations has not yet been determined.

MATERIALS AND METHODS

Twenty, approximately 330 to 340 gram, female, Sprague-Dawley rats were randomly divided into 1 of 2 experimental groups following a 1-week acclimatization period. After an overnight fast, one experimental group received 3 mg/kg T-2 toxin IP dissolved in a 50 percent ethanol vehicle (5 mg T-2 toxin per ml of vehicle). The control group received a comparable amount of the 50 percent vehicle IP.

Following T-2 toxin administration, the rats were returned to their cages and observed for 8 hours. Water was available ad libitum. After the 8-hour observation period, all the rats were sacrificed by decapitation. The heart from each rat was immediately removed, rinsed with deionized water to remove any residual blood, dried, placed in individual whirlpac bags and frozen. Several days later, the hearts were thawed, weighed and put into individual crucibles. The crucibles were placed in a dessicating oven for 4 hours. Following dessication, the samples were ashed in a murrle furnace.

Each ashed sample was next dissolved in 10 mL of a 25 percent hydrochloric acid solution. Ten mL or a 2 percent canthanium oxide solution was then added to each sample bringing the total volume of each sample to 20 mL. One-hundred

µ/L aliquots from each solution were transferred to 10 mL volumetric flasks and brought to volume with a one percent canthanium oxide solution.

Sodium, potassium and magnesium levels were determined by an atomic absorption spectrophotometer following aspiration from the volumetric flasks. Calcium determinations were made in a similar manner with the exception that spectrophotometer aspirations were made directly from the crucibles. Appropriate standards were run to assure accuracy.

RESULTS

The electrolyte concentrations from each rat are shown in Tables 1 and 2. Concentrations are reported in ppm. The data from one control rat was discarded due to an apparent error in dilution of the sample.

A relatively large difference in cardiac tissue mean sodium levels occurred between the control and treatment groups, 881.0 ppm vs 629.3 ppm, respectively; a drop of 28.6 percent. The range was more variable in the treatment group (261 ppm to 883 ppm) vs. the control group (748 to 1089). Differences were also noted in mean potassium, magnesium and calcium levels (for K^{+}, 3862.5 ppm in the control group vs. 4035.8 ppm for the treatment group, an increase of 4.5 percent; for Mg^{++}, 235.7 ppm in the control group vs. 220.1 ppm in the treatment group, a drop of 6.6 percent; and for Ca^{++}, 25.4 ppm in the control group vs. 29.6 ppm in the treatment group, an increase of 16.5 percent. Ranges were more variable in the treatment group than in the control group for all 3 electrolytes (see Tables 1 and 2).

DISCUSSION

Unfortunately, whole tissue electrolyte content without plasma electrolyte levels does not allow for determination of changes in the intracellular vs. extracellular levels of the individual electrolytes. Whole tissue levels may

provide an insight into whole body levels, however, assuming no significant dehydration is present. In the absence of severe vomiting, diarrhea or diuresis significant dehydration would not have been expected to occur within the 8 hours between T-2 toxin administration and sacrifice time as was the case in this experiment. Detailed discussion of the above results will await appropriate statistical analysis. At least in individual animals, significant changes appear to have taken place when compared to control animals.

TABLE 1.

Control No.	Rat Weight	Wet Weight Heart (Gram)	Na^{+} (ppm)	K^{+} (ppm)	Mg^{++} (ppm)	Ca^{++} (ppm)
2	299	.89	773	3396	237	23.9
4	330	.95	893	4304	243	26.3
6	277	.79	932	3601	227	25.8
8	320	.86	930	3927	246	24.7
10	332	.94	1089	3877	238	29.0
12	365	1.16	938	3908	237	25.5
14	368	1.04	785	3675	228	24.5
16	386	1.07	748	4154	221	23.3
18	348	1.18	841	3917	244	25.5
EK	3025	8.88	7929	34759.4	2121	228.5
$\bar{x}$ $\pm$ SD	336.1 $\pm$ 34.8	.99 $\pm$ .13	880.0 $\pm$ 106.5	3862.1 $\pm$ 276.2	235.7 $\pm$ 8.6	25.9 $\pm$ 1.7
Ranges	277 to 386	.79 to 1.18	748 to 1089	3396 to 4304	221 to 246	23.3 to 29.0

TABLE 2.

Control No.	Rat Weight	Wet Weight Heart (Gram)	Na^{+} (ppm)	K^{+} (ppm)	Mg^{++} (ppm)	Ca^{++} (ppm)
1	321	.98	261	4082	235	23.6
3	270	.96	883	4352	260	30.3
5	388	1.05	792	4233	238	23.8
7	403	1.34	860	4710	244	48.0
9	404	1.15	668	4792	228	51.5
11	338	1.00	592	4267	230	27.3
13	360	1.07	314	2409	138	13.0
15	329	1.07	688	4237	239	22.0
17	329	.99	857	4579	252	21.5
19	280	.89	378	2697	137	35.3
E	3430	10.5	6293	40358	2201	296.3
x ± S.D.	343.0 ± 45.6	1.05 ± .12	629.3 ± 235.8	4035.8 ± 816.2	220.1 ± 44.6	29.6 ± 12.1
Ranges	278 to 404	.89 to 1.34	261 to 883	2409 to 4792	137 to 260	13.0 to 51.5

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H. SUBACUTE TOXICITY AND IMMUNOTOXICITY OF TOPICALLY APPLIED T-2 TOXIN IN SWINE--Victor F. Pang

INTRODUCTION AND OBJECTIVES

Interest in the group of trichothecene mycotoxins, particularly T-2 toxin, has been greatly increased as accumulating evidence points towards their use in biochemical warfare by Soviet-supplied forces in southeast Asia and elsewhere. Dermal exposure is one of the most direct and important routes of exposure. Since the permeability properties of their skin and the general physiology, particularly the cardiovascular system, of pigs are comparable to man, swine were chosen as a suitable model for the study of dermal exposure to T-2 toxin.

The objectives of this study were: 1) to characterize the sequential morphologic changes of skin and other organs after a single sublethal topical dose of T-2 toxin and 2) to assess the effects of topically applied T-2 toxin on the immune system, hematologic parameters, serum enzymes, blood chemistries, body temperature and body weight.

PRELIMINARY STUDIES

Two preliminary studies were carried out in order to determine an appropriate sublethal but toxic dose, to establish procedural protocols and to evaluate experimental techniques of topical application and immunoassays for use in the formal study.

Four pigs were used in the first preliminary study. In order to determine whether sheep red blood cells (SRBC) were a suitable immunogen in swine, two doses (each 10^9 SRBC suspended in 1 mL of phosphate buffered saline) 21 days apart were administered subcutaneously in two pigs. The response to this

immunizaton was measured by the lymphocyte transformation assay and hemagglutination titers. Adequate immunization was achieved by this route. For dose range determination, T-2 toxin was dissolved in 0.75 mL of dimethyl sulfoxide (DMSO) and applied at doses of 20 mg/kg and 15 mg/kg to one pig each. The dissolved T-2 toxin was applied topically to a shaved area on the back, approximately 10 x 15 cm^2. A protective nonocclusive foam pad was used to prevent loss of the toxin from the dosed area and, at the same time, to allow indirect exposure to the air through an elevated layer of gauze. The pig dosed with 20 mg/kg died within 24 hours due to causes unrelated to T-2 toxin. The dose of 15 mg/kg caused severe dermal ulceration at the site of application with relatively good recovery of the animal after three weeks. The pig dermally dosed with 15 mg/kg T-2 toxin was immunized subcutaneously with 1 mL of 10^9 SRBC at the time of dosing. This was followed by a second immunization 21 days later. Data obtained from the lymphocyte transformation assay in this pig showed a poor response of the peripheral blood lymphocytes to both phytohemagglutinin (PHA) and concanavalin A (Con A), but a slight increase in the responses to lipopolysaccharide (LPS) and pokeweed (PWM) mitogens when compared with the 2 pigs immunized with SRBC only. Hemagglutination titers of this treated pig during the period of the primary immune response were slightly higher than those of the two untreated pigs.

Four pigs were used in the second preliminary study. T-2 toxin was applied topically at doses of 10 mg/kg (two pigs) and 15 mg/kg (two pigs) in order to evaluate survival, clinical signs and immunologic effects. All pigs survived in this study. Pigs exposed to 15 mg/kg of T-2 toxin had more severe clinical signs (fever, anorexia, lethargy) than pigs dosed at 10 mg/kg. No immunologic effects were seen in pigs dosed at 10 mg/kg. Effects on the immune system of

pigs dosed at 15 mg/kg were similar to those seen in the pig used in the first preliminary study, although individual variation in immune response was evident.

FORMAL STUDIES

A. Histologic Effects of Subacute Dermal Exposure to T-2 Toxin

In this study, histologic effects of dermally applied T-2 toxin were evaluated in 26 11-12 week old, crossbred, female, specific-pathogen-free (SPF) pigs. T-2 toxin was dissolved in 0.75 mL DMSO and applied topically as described in the preliminary studies, at doses of 0 (eight control pigs) and 15 mg/kg (18 pigs). Animals were killed sequentially on 1, 3, 7 and 14 days postdosing. A complete necropsy was performed and representative tissue samples were fixed for histologic examination.

Grossly, the areas of skin exposed to T-2 toxin were swollen and bright to dark red on days 1 (Figure 1) and 3 (Figure 2), then becoming purple to dark purple on day 7 (Figure 3) and day 14 (Figure 4), respectively. Prominent scale formation was noted on day 3, and it became progressively worse on days 7 and 14. On day 7, the affected skin was necrotic and covered with serosanguinous exudation at the margin of the exposed regions. These lesions became bloody and covered by a thick, crusty scab on day 14. The affected skin and the underlying tissue were focally separated at the border of the exposed area.

On day 1, the histologic changes were located primarily in the epidermis and upper dermis, although occasional mild involvement of lower dermis and superficial subcuticular fat was present. The lower dermis and subcuticular fat were affected in all pigs by day 3. The severity and depth of the lesions in the three layers of skin increased progressively from day 1 to day 14.

The changes in the epidermis on day 1 were characterized by multifocal mild to moderate ballooning degeneration and cellular dissociation (possibly due to intercellular edema) of epithelial cells of the strata spinosum and basale, along with formation of vesicles and mild infiltration by neutrophils and eosinophils (Figure 5). Some of the infiltrating inflammatory cells were undergoing degeneration and necrosis. In addition, severe epithelial necrosis, marked neutrophilic infiltration and multifocal intraepidermal abscesses were present in the mildly to moderately acanthotic epidermis on day 3 (Figure 6). In some of the severely involved regions, suprabasilar intraepidermal bullae and clefts were observed. Locally extensive necrosis and subcorneal accumulations of neutrophils accompanied by marked parakeratosis and pseudoepitheliomatous hyperplasia were noted on days 7 and 14 (Figure 7), but ballooning degeneration and vesicular formation had become less evident than on day 3.

The dermis on day 1 was infiltrated, mainly around the vessels, by a moderate number of neutrophils admixed with some eosinophils. Degeneration and necrosis were seen in many of the inflammatory cells. Mild edema, fibrin deposition and disruption of collagen were present in the papillary layer and the perivascular regions of the upper reticular layer. Fibrinoid degeneration and formation of fibrin thrombi occurred in some of the capillaries of the upper dermis. These changes, especially the infiltration of inflammatory cells, became more diffuse and prominent on day 3. The infiltrate consisted of neutrophils, macrophages, lymphocytes and eosinophils. Degeneration and necrosis of the inflammatory cells were evident in the upper dermis. Small perivascular cuffs of mixed inflammatory cells with mild perivascular edema and fibrin deposition were

constantly observed in the superficial subcuticular fat. In addition to an increase in the intensity of the changes occurring in the dermis and subcutis on day 3, mild to moderate fibroplasia began to appear on day 7. Although fibroplasia became more evident, extensive necrosis was still present in the dermis on day 14. Hyperplasia with papillary folding of the lining epithelium of sweat glands in the lower dermis was seen more often on day 14 than on day 7. Although the healing process was recognized on day 7, the necrotizing dermatitis persisted during the first 2 weeks after dosing.

The changes in the internal organs were minimal. Minimal to mild increases in the number of tingible body macrophages were found in the cortex of the thymus. Occasional single cell necrosis was noted in the lymphoid follicles. There were scattered single cell vacuolization and necrosis in the acinar cells of the pancreas. These changes were randomly present on all four days, although they were more often seen on days 1 and 3.

This study demonstrated that T-2 toxin has a profound, direct, cytotoxic effect on skin. Based on the occurrence and severity of lesions, it appears that the cells of stratum germinativum (including strata basale and spinosum), especially the basal layer, were the main targets of the T-2 toxin. It is well known that cells of the basal layer of skin are highly metabolically active. They synthesize both protein and DNA. T-2 toxin also has been documented to be a strong inhibitor of protein synthesis with subsequent inhibition of RNA and DNA synthesis. Endothelial cells might be another target of T-2 toxin, because vasculitis and microthrombi were evident on day 1. The extensive necrotizing dermatitis

and cellulitis that subsequently developed might be a result of release of lysosomal enzymes from the large numbers of degenerating and necrotic inflammatory cells.

Significant quantities of T-2 toxin and several of its metabolites were detected in the skin and subcuticular fat of the exposed and adjacent tissues in all animals (Table 1). It has been suggested that the dermis may provide a significant barrier or reservoir for lipid soluble compounds. It is speculated that skin may act as such a reservoir for T-2 toxin from which a small amount of the toxin and its metabolites are constantly released into the blood stream. This may also explain why dramatic damage occurred in the dosed skin, but the histologic changes in the internal organs were minimal.

B. Subacute Toxicity of Topically Applied T-2 Toxin on the Immune System, and Clinical Pathology Parameters

The effects of a single, sublethal dose of topically applied T-2 toxin on the immune system, hematology, serum enzymes, blood chemistries, body temperature and body weight were evaluated in 14, 11-12 week old, crossbred, male castrated, SPF pigs. T-2 toxin was applied topically as described previously at doses of 0 (six control pigs) and 15 mg/kg (eight test pigs) on day 0. The pigs were immunized subcutaneously with 1 mL of 10^9 SRBC at the time of dosing and 21 days later. Blood was collected from the anterior vena cava on days -2, 1, 3, 5, 7, 10, 14, 20, 22, 24, 26, 28 and 31 for hematology and immunology studies. Serum samples during the first 2 weeks were used for analysis of serum proteins, enzymes, glucose, cholesterol and electrolytes. Body temperature was measured daily during the first 2 weeks and on days 16, 18, 21, 23, 27 and 30.

Pigs were weighed before dosing and thereafter weekly for 5 weeks. The split-plot design with repeated measurements was used as the statistical model for analyzing the data. The analysis was done with the Statistical Analysis System (SAS) statistical package. Differences were considered statistically significant when P-values were less than 0.05.

1. Immunology Study

In this study, the effects of T-2 toxin on the immune system were evaluated at the cellular level on purified peripheral blood lymphocytes with four different mitogens (phytohemagglutinin [PHA], concanavalin A [Con A], pokeweed mitogen [PWM] and lipopolysaccharide [LPS]) by the lymphocyte transformation assay. Humoral responses were evaluated by determining the hemagglutination titers of sera.

The ability of swine peripheral blood lymphocytes to respond to mitogens was measured by their ability to incorporate [^{3}H] thymidine. The data were compared in two different ways: as mean counts per minute (M CPM) where

M CPM = (mitogen-stimulated CPM)/number of pigs in each group,

and a mean stimulaton index (MSI) where

MSI = (mitogen-stimulated CPM/background CPM)/number of pigs in each group.

a. Response to Con A. At a concentration of 50 µg/mL (optimal concentration), significantly lower responses were found in the test group at day 22 when expressed as M CPM and at days 22, 26 and 28 when expressed as MSI (Figures 8, 9). No significant differences were found at a concentration of 10 µg/mL (sub-optimal concentration) when expressed as either M CPM or MSI.

b. Responses to PHA. At a concentraton of 20 µg/mL (optimal concentration), significantly lower responses were found in the test group at days 5, 22 and 26 when expressed as M CPM and at days 20, 22, 24, 26 and 28 when expressed as MSI (Figures 10, 11). No significant differences were found at a concentration of 2 µg/mL (suboptimal concentration) when expressed as either M CPM or MSI.

c. Responses to LPS. At a concentration of 2 µg/mL (suboptimal concentration), significantly higher responses were found in the test group at days 3, 5, 7 and 10 when expressed as M CPM and at day 14 when expressed as MSI. At the optimal concentration, 20 µg/mL, a significantly higher response was found in the test group only at day 10 when expressed as M CPM, but no significant differences were seen in MSI (Figures 12, 13).

d. Responses to PWM. At an optimal concentration of 1:200, significantly lower responses were noted in the test group at day 5 when expressed as M CPM and at days 22 and 26 when expressed as MSI. Significantly lower responses were found at day 5 when expressed as M CPM and at day 22 when expressed as MSI (Figures 14, 15).

The lymphocyte transformation assay is useful for assessing the gross immunological competence of humans and other animals. Lectin-induced mitogenesis can involve macrophages, T-lymphocytes and B-lymphocytes. The lectins that were used in this study are somewhat lymphocyte-specific. For example. Con A is a broad T-cell mitogen, PHA is mostly a T-effector cell mitogen, PWM is

a mitogen activates both B and T-cells and LPS is a B-cell mitogen. The study showed that a single, topically applied, sublethal dose (15 mg/kg) of T-2 toxin in pigs caused significantly lower responses of peripheral blood lymphocytes to mitogens, PHA, Con A and, possibly, PWM. Although no significant changes were observed in the lymphoid system in the morphological study, it is reasonable to speculate that T-2 toxin might cause functional depression of T-cell populations. Most of these significant reductions in T-cell function occurred between days 20 and 28 and were reversible. Based on these findings and the results of dermal residue analyses for T-2 toxin and metabolites, the functional depression may have been due to cumulative effects of constantly released T-2 toxin and metabolites from this dermal reservoir. Conversely, significantly higher responses to the B-cell mitogen LPS (at least when expressed as M CPM) occurred mainly within the first 2 weeks after dosing.

The ability of specific antibody production in both treated and control pigs was measured by the titers of anti-SRBC antibody (Ab) with the hemagglutination test. Although the mean titers of anti-SRBC Ab of the treated group were approximately 2-fold higher than those of the control group at days 10, 14, 22 and 24, no statistically significant differences were present at these time points (Figure 16). SRBC are a T-cell dependent immunogen. Anti-SRBC Ab production is controlled by helper T-cells and suppressor T-cells, although macrophages also participate by presenting antigenic determinants of SRBCs to

both T-cells and B-cells. According to the data from both the lymphocyte transformation assay and the hemagglutination test, it appears that topically applied T-2 toxin in pigs had no effects on the specific population of helper and suppressor T-cells, or on the ability of B-cells to produce anti-SRBC Ab. Thus, the effects on these cells were not sufficient to significantly influence Ab production.

Aside from helper and suppressor cells, T-cells consist of at least two more populations, the cytotoxic and delayed hypersensitivity T-cells. Although Ab is an important arm of the immune system against many infectious agents, it often is insufficient to provide full protection for the host without the involvement of cellular immunity. It is well known that cellular immunity plays a very important role in viral, intracellular bacterial and protozoal infections and in tumor cell killing. Therefore, the functional suppression of T-cells shown in this study might be of importance in disease defense, in spite of the absence of specific effects on anti-SRBC Ab production. This is an important area which needs further elucidation.

2. Clinical Pathology Study

a. Hematology

The mean total white blood cell count of the test group was significantly higher in the first 2 weeks after dosing (Figure 17). It increased gradually after dosing, reaching a peak at day 10 and returning to control levels by day 20. The leukocytosis was mainly due to an absolute increase in neutrophils

(Figure 18) and also due to an apparent increase in the absolute number of monocytes at days 3 and 14 (Figure 19). The neutrophilia coincided with the severe necrotizing dermatitis induced by T-2 toxin. Before dosing, the mean total lymphocyte count of the treated group was relatively higher than that of the control group, but it was significantly lower at days 7 and 14 after dosing (Figure 20).

The mean total erythrocyte count of the test group was significantly lower than that of the control group at days 1, 3 and 5 (Figure 21). It dropped sharply at day 1, then gradually increased but was still significantly depressed at days 3 and 5. No differences were present after day 5. Both mean hemoglobin and mean packed cell volume of the test group were significantly lower than those of the control group at days 10 and 14 (Figures 22, 23).

b. Serum Proteins, Enzymes, Glucose, Cholesterol and Electrolytes

The mean total serum protein of the test group was significantly higher than that of the control group at days 5 and 7 (Figure 24). The mean serum albumin of the test group was significantly lower than that of the control group at days 7, 10 and 14 (Figure 25). Anorexia during the first week after dosing with T-2 toxin might play an important role. Conversely, the mean serum globulin of the test group gradually increased after dosing, became significantly higher than that of the control group at day 5 and reaching a plateau after day 7 (Figure 26). The source of the increased globulin is uncertain, but it could

be (at least partially) due to the severe inflammation. Significantly lower mean serum alkaline phosphatase activities were present in the treated group at days 3, 5, 7, 10 and 14 (Figure 27). The decline in both serum alkaline phosphatase activities and albumin might also be related to decreased protein synthesis, caused directly by T-2 toxin. No significant differences were noted in SGOT, SGPT, and LDH.

The mean blood glucose values of the T-2 group were significantly lower than that of the control group throughout the first 2 weeks (Figure 28). A significantly lower mean serum cholesterol concentration was present at days 1, 3 and 10 in the treated group. This might be associated with decreased feed intake.

The mean serum phoshorus and calcium concentrations of the treated group were significantly lower than that of the control group at days 3, 7 and 14. In addition, mean serum calcium levels were also significantly reduced at day 10. No differences were noted in sodium, chloride or potassium.

c. Body Temperature and Body Weight

A significant increase in mean rectal temperature of the treated group, as compared to the control group, occurred during the first 2 weeks, but differences were not significant at other points (Figure 29). Neutrophils and macrophages are the two main sources of intrinsic pyrogens and they were two of the major inflammatory cells present in the skin exposed to T-2

The mean body weights of the treated and control groups were

similar at the day of dosing. The rate of gain of the treated group was lower than that of the control group throughout the entire study. Significant differences were seen at weeks 2, 3, 4 and 5 (Figure 30).

TABLE 1. Concentration of T-2 toxin and metabolites in skin and subcuticular fat at 1, 3, 7 and 14 days after topical administration of T-2 toxin at 15 mg/kg body weight.

	Concentration in ppm			
Toxin	1 day[a]	3 days[b]	7 days[b]	14 days[c]
Skin				
T-2	220.00 ± 23.00	247.00 ± 78.00	224.0 ± 38.0	40.00 ± 21.00
HT-2	2.20 ± 0.27	2.60 ± 0.60	75.0 ± 30.0	6.40 ± 1.10
TRIOL	0.06 ± 0.01	0.21 ± 0.03	7.4 ± 6.0	8.70 ± 6.70
NEO	0.90 ± 0.45	1.20 ± 1.30	12.0 ± 2.0	0.45 ± 0.11
4-DN	0.73 ± 0.18	0.44 ± 0.14	24.0 ± 0.6	5.00 ± 1.30
TETRAOL	ND[d]	0.02 ± 0.02	2.2 ± 0.6	5.60 ± 3.50
Fat				
T-2	34.00 ± 23.00[a]	28.00 ± 18.00[a]	32.0 ± 9.0[a]	3.00 ± 1.00[a]

NEO: neosolaniol; 4-DN: 4-deacetylneosolaniol.

[a]Mean ± SD of at least 3 animals
[b]Mean ± SD of 4 animals
[c]Mean ± SD of 5 animals
[d]None detected

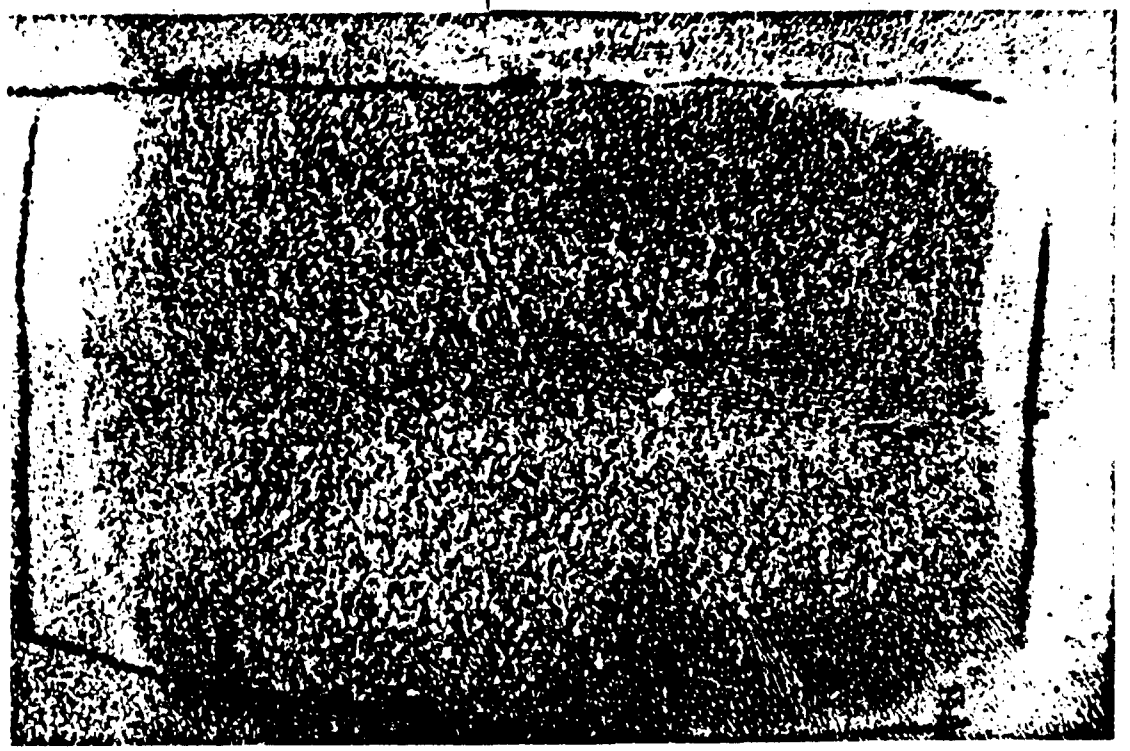


Fig. 1 The area of skin exposed to T-2 toxin (15 mg/kg) from a pig killed on day 1. It shows swelling and bright red discoloration.

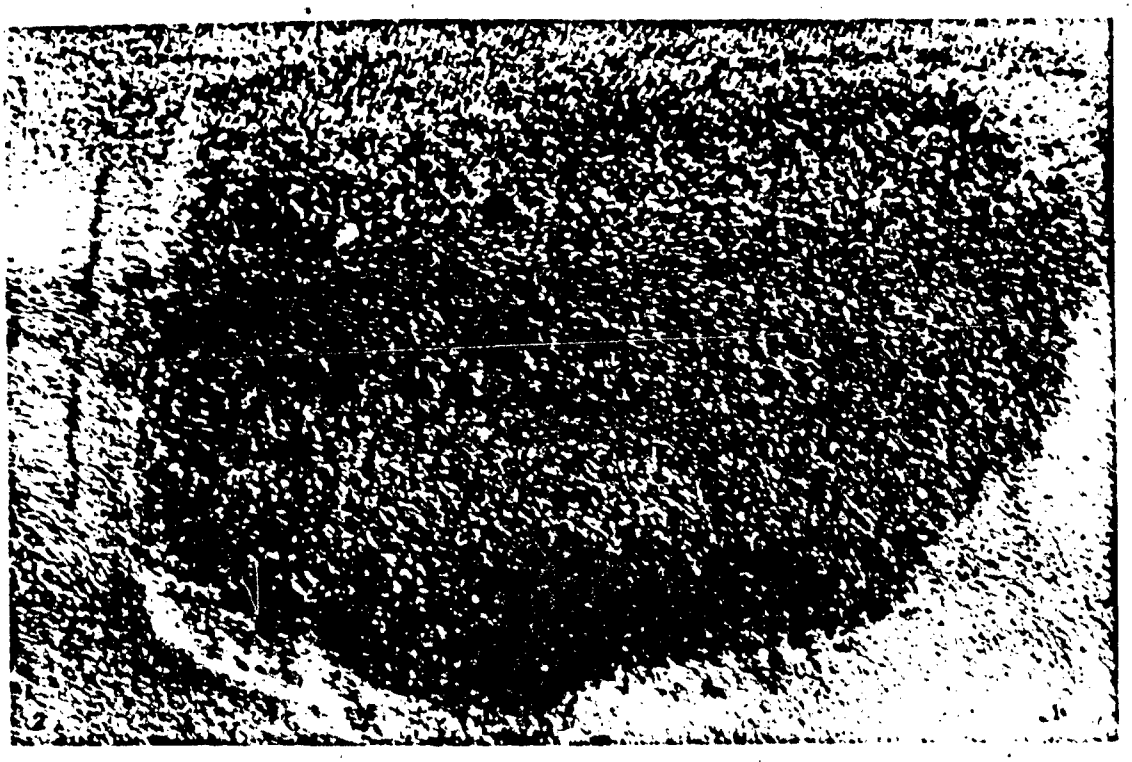


Fig. 2 The area of skin exposed to T-2 toxin (15 mg/kg) from a pig killed on day 3. It shows marked swelling and dark red discoloration with scale formation.

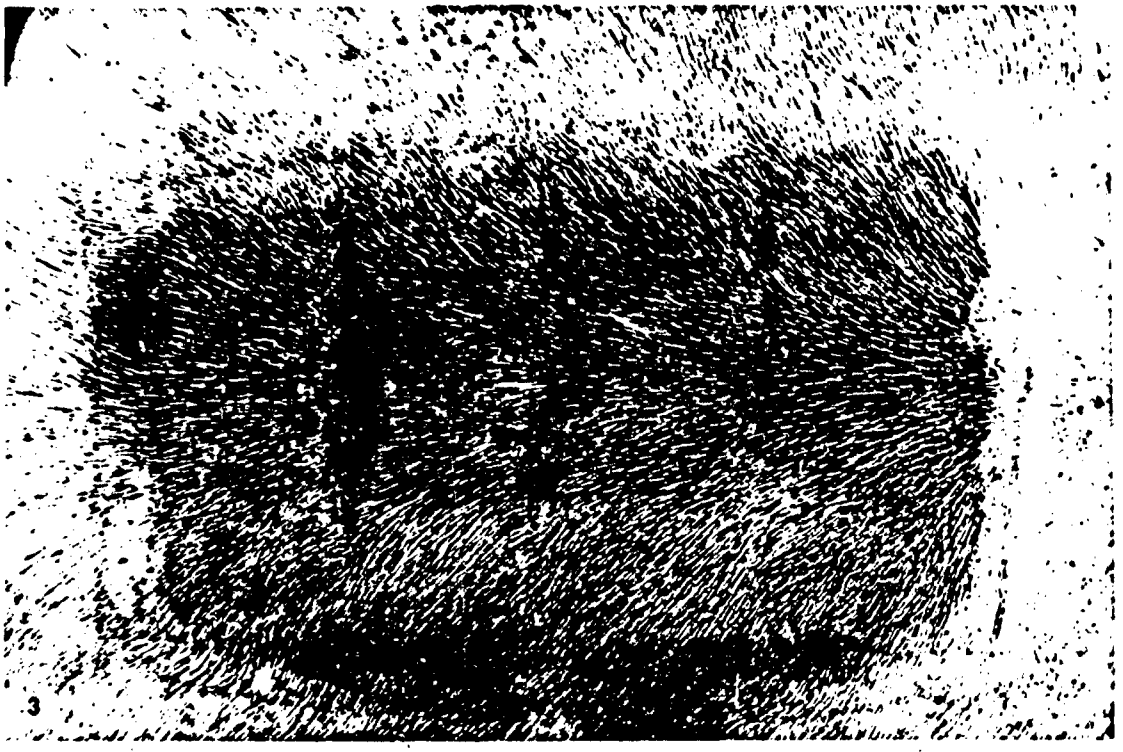


Fig. 3 The area of skin exposed to T-2 toxin (15 mg/kg) from a pig killed on day 7. It shows purple discoloration and ulcers with serosanguinous exudation, mainly at the margin of the exposed region.

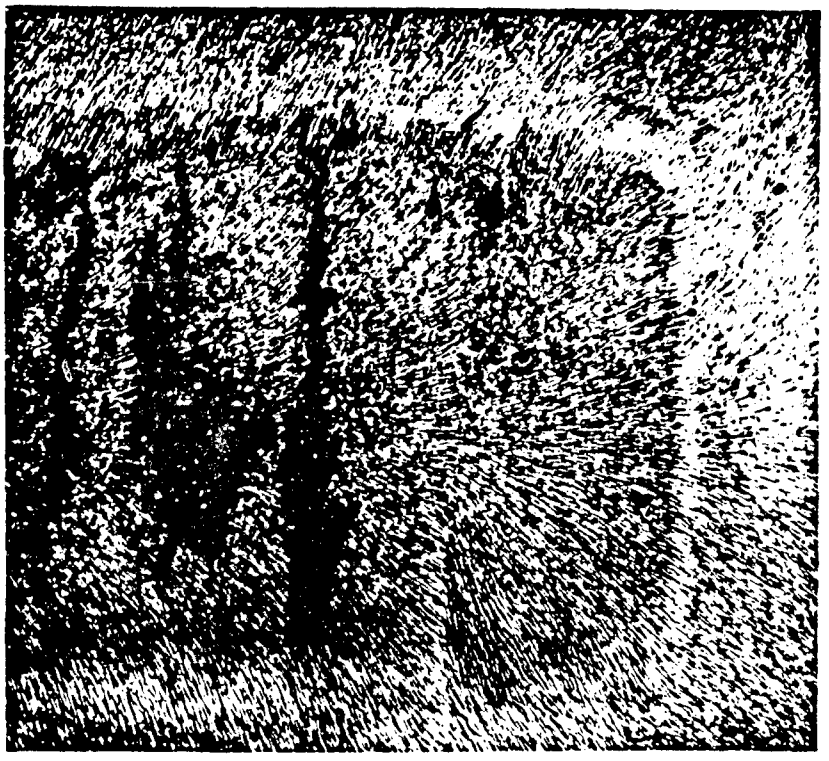
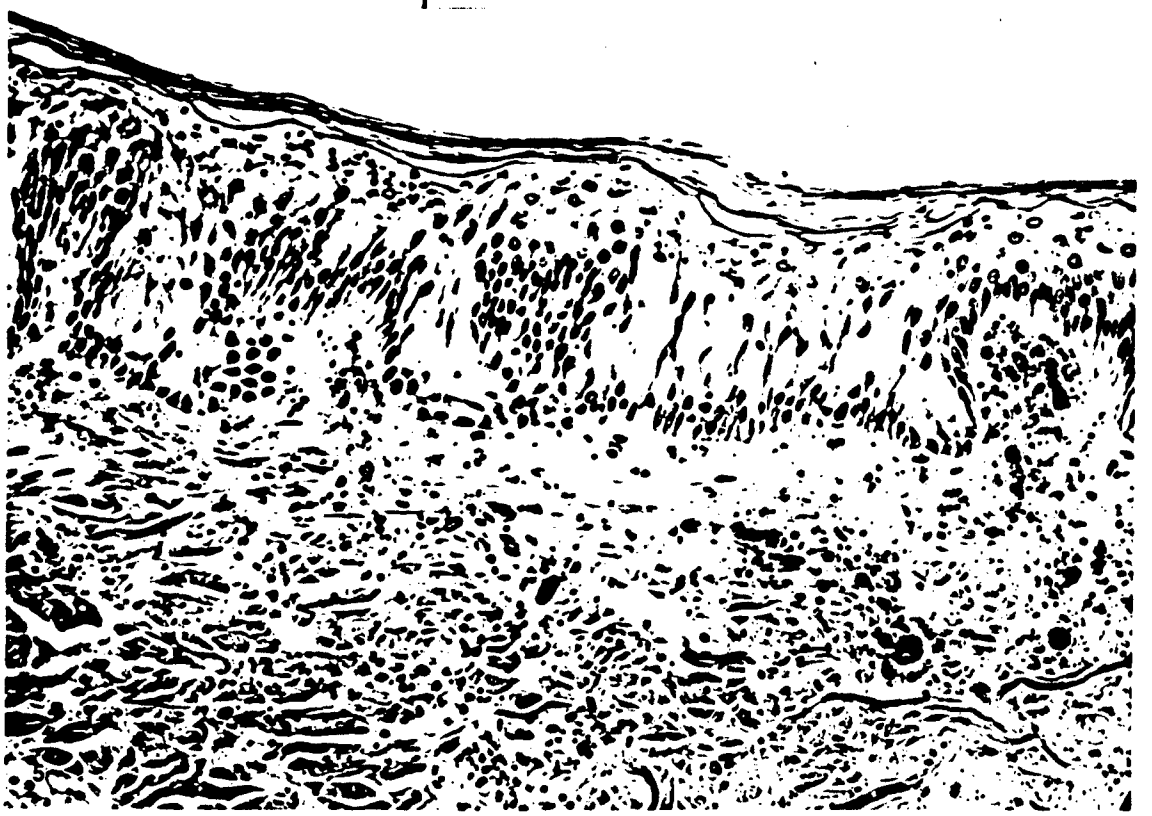


Fig. 4 The area of skin exposed to T-2 toxin (15 mg/kg) from a pig killed on day 14. It shows dark purple discoloration, ulcers with hemorrhage, formation of a thick crusty scab and separation from the underlying tissue at the margin of the exposed region.

FIGURE 5. Skin from a pig killed at day 1. Multifocal mild to moderate ballooning degeneration and cellular dissociation of stratum germinativum with formation of vesicles and mild infiltration by neutrophils and eosinophils in the epidermis. The dermis also has congestion, edema and small perivascular cuffs of neutrophils. HE stain, 560 x.

FIGURE 6. Skin from a pig killed at day 3. Extensive cellular necrosis and formation of suprabasilar intraepidermal clefts in the epidermis. There are severe diffuse inflammatory cell infiltration, edema, fibrin deposition and disruption of collagen of the dermis. HE stain, 560 x.



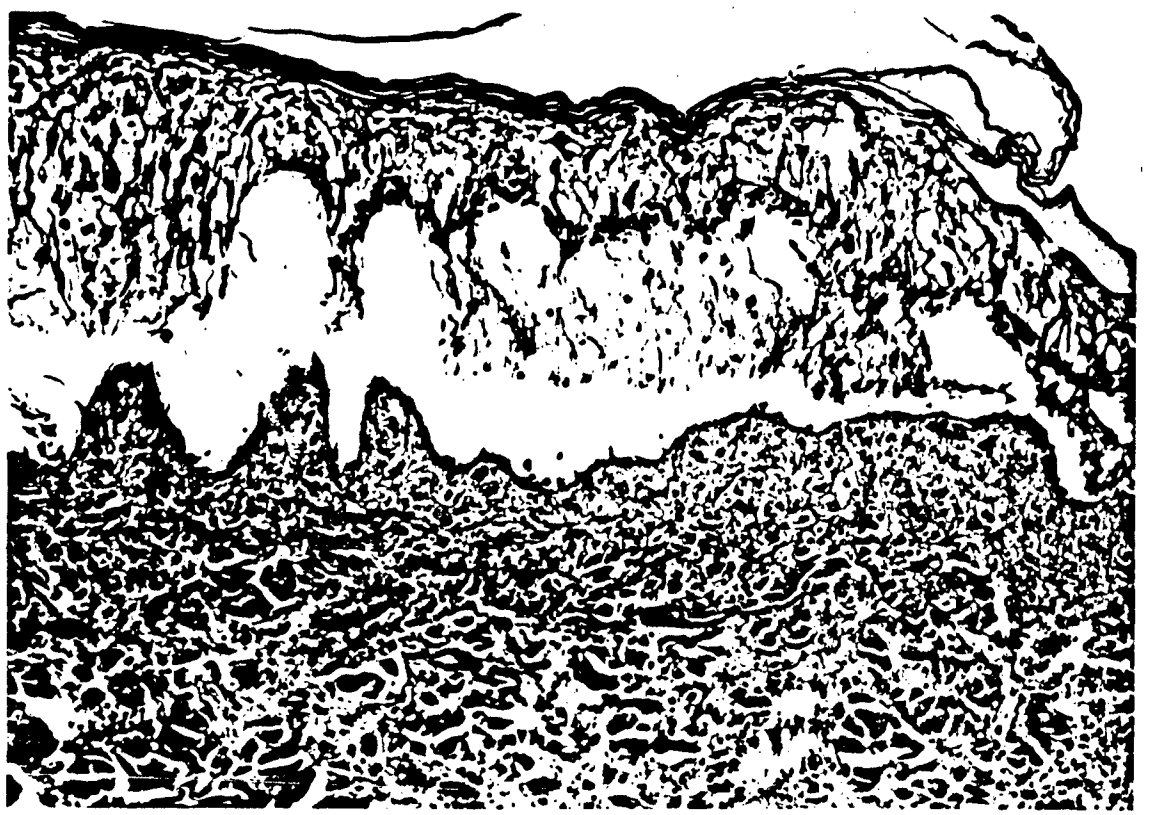


FIGURE 7. Skin from a pig killed at day 7. Subcorneal accumulation of neutrophils and prominent pseudoepitheliomatous hyperplasia of the epidermis. There are edema, fibroplasia and severe inflammatory cell infiltration in the dermis. HE stain, 225 x.

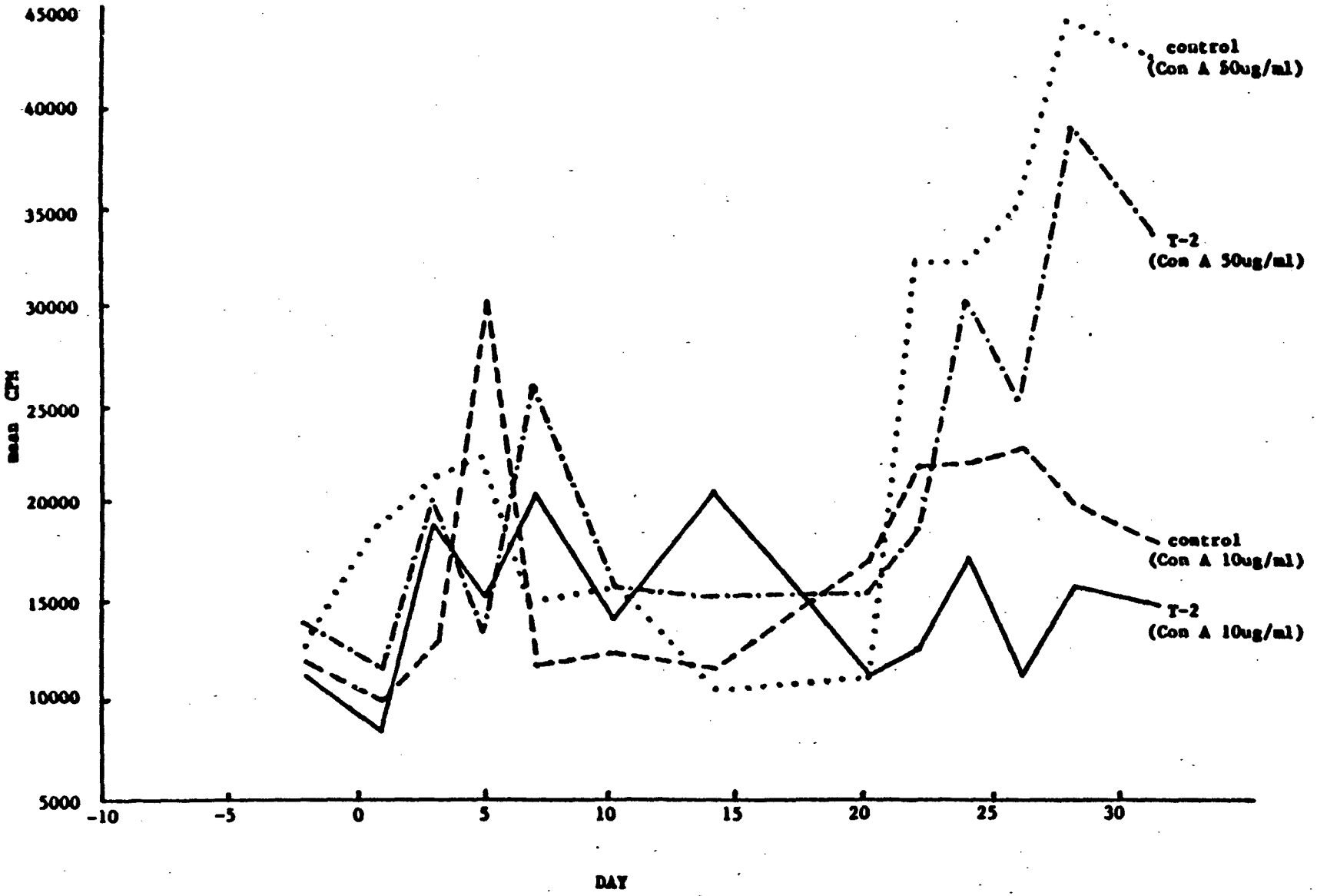


Fig. 8 Blastogenic responses (M CPM) of peripheral blood lymphocytes to the optimal (50 ug/ml) and suboptimal (10 ug/ml) concentration of Con A of pigs exposed dermally to T-2 toxin (15 mg/kg) and the group exposed to vehicle only.

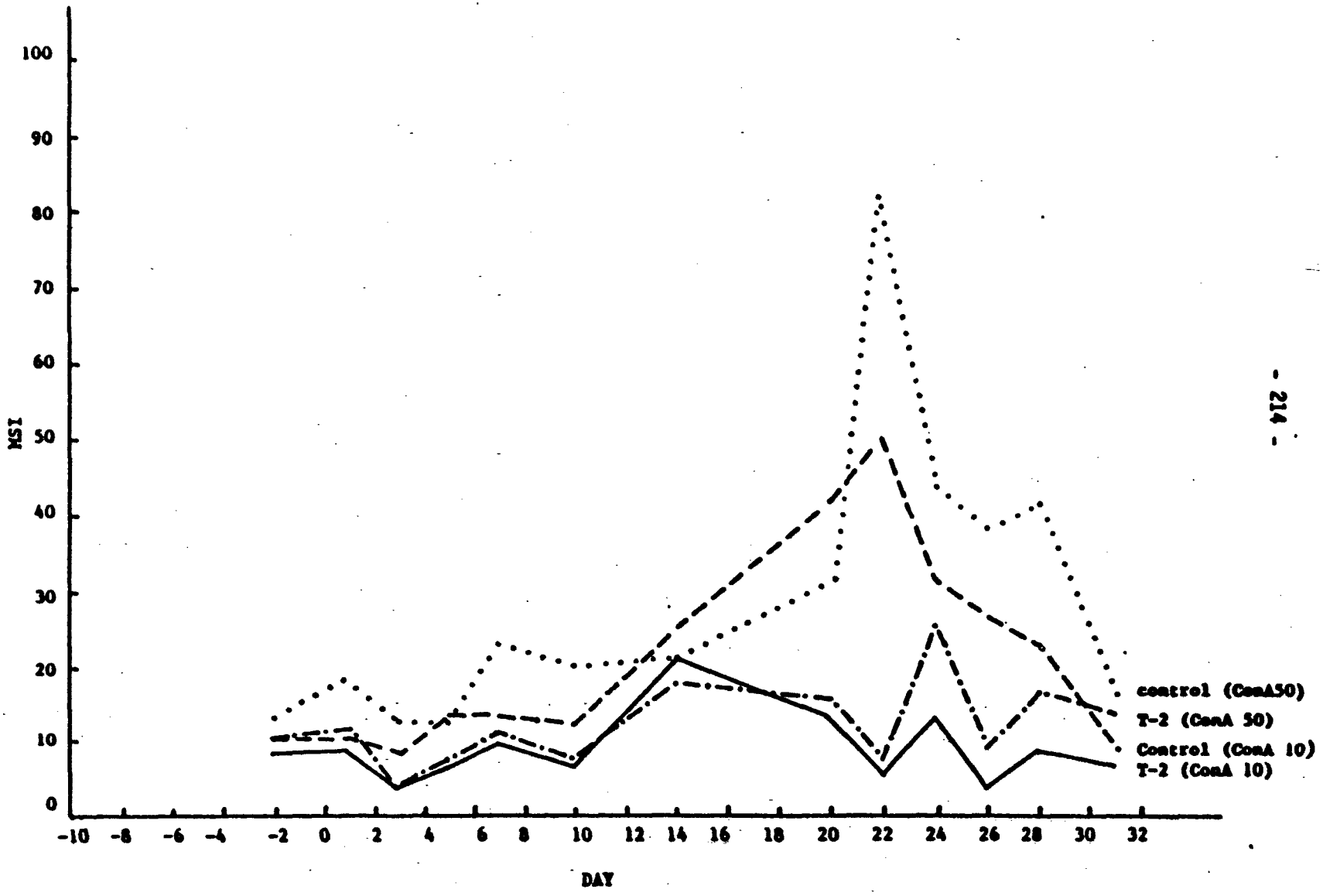


Fig. 9 Blastogenic responses (MSI) of peripheral blood lymphocytes to the optimal (50 ug/ml) and suboptimal (10 ug/ml) concentrations of Con A of pigs exposed dermally to T-2 toxin (15 mg/kg) with vehicle and the group exposed to vehicle only.

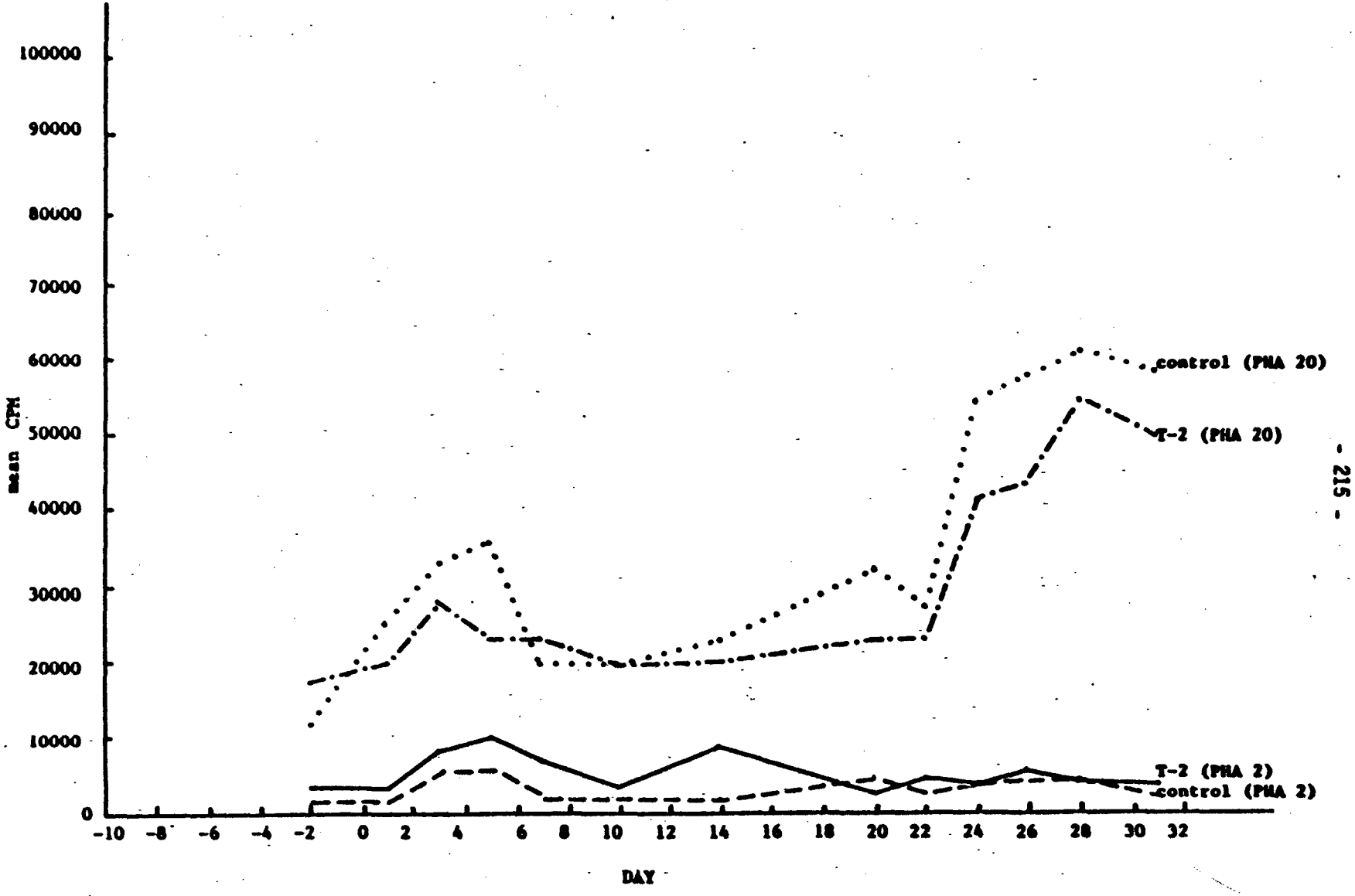


Fig. 10 Blastogenic responses (M CPM) of peripheral blood lymphocytes to the optimal (20 ug/ml) and suboptimal (2 ug/ml) concentrations of PHA of pigs exposed dermally to T-2 toxin (15 mg/kg) with vehicle and the group exposed to vehicle only.

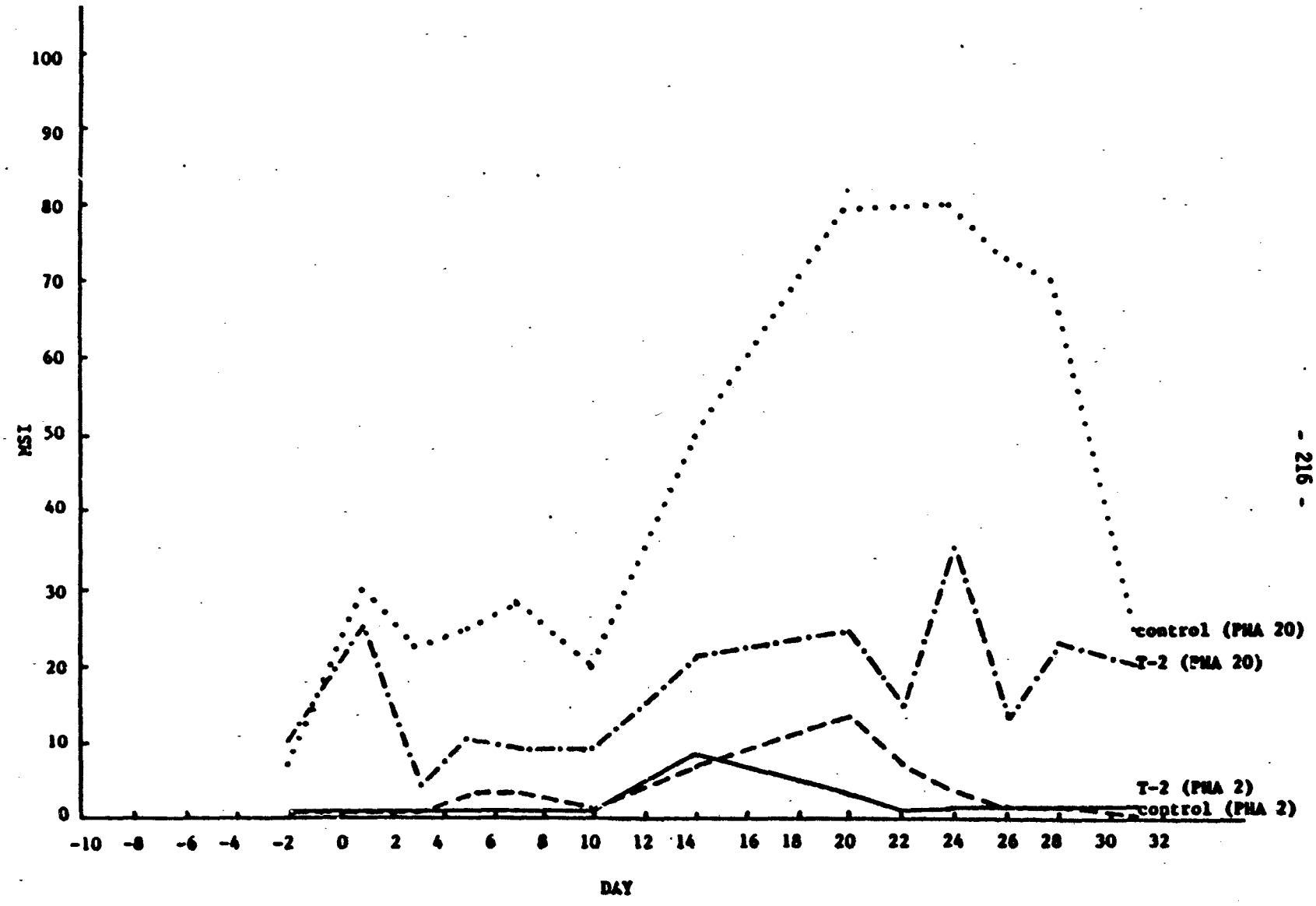


Fig. 11 Blastogenic responses (MSI) of peripheral blood lymphocytes to the optimal (20 ug/ml) and suboptimal (2 ug/ml) concentration of PHA of pigs exposed dermally to T-2 toxin (15 mg/kg) with vehicle and the group exposed to vehicle only.

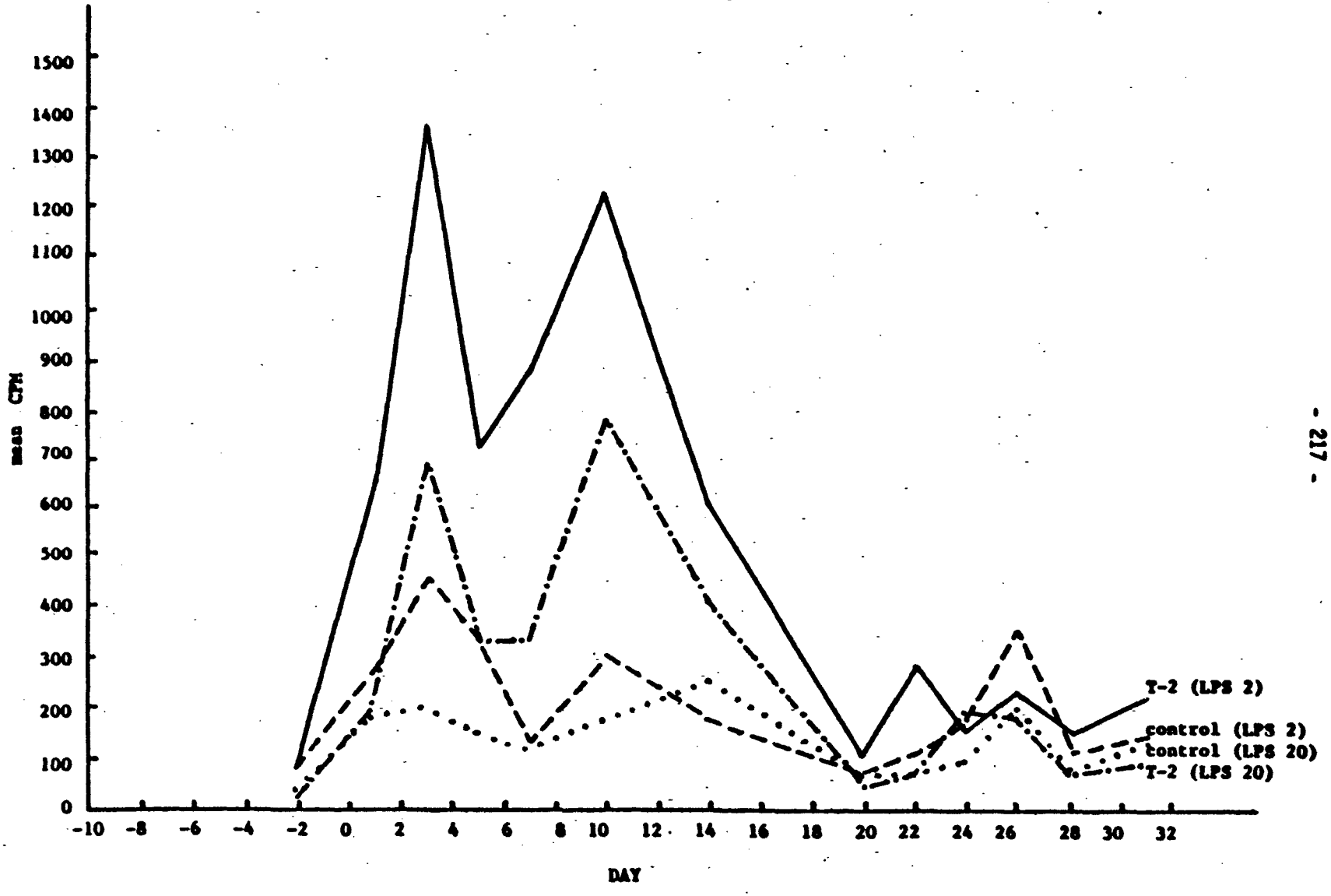


Fig. 12 Blastogenic responses (M CPM) of peripheral blood lymphocytes to the optimal (20 ug/ml) and suboptimal (2 ug/ml) concentration of LPS of pigs exposed dermally to T-2 toxin (15 mg/kg) with vehicle and the group exposed to vehicle only.

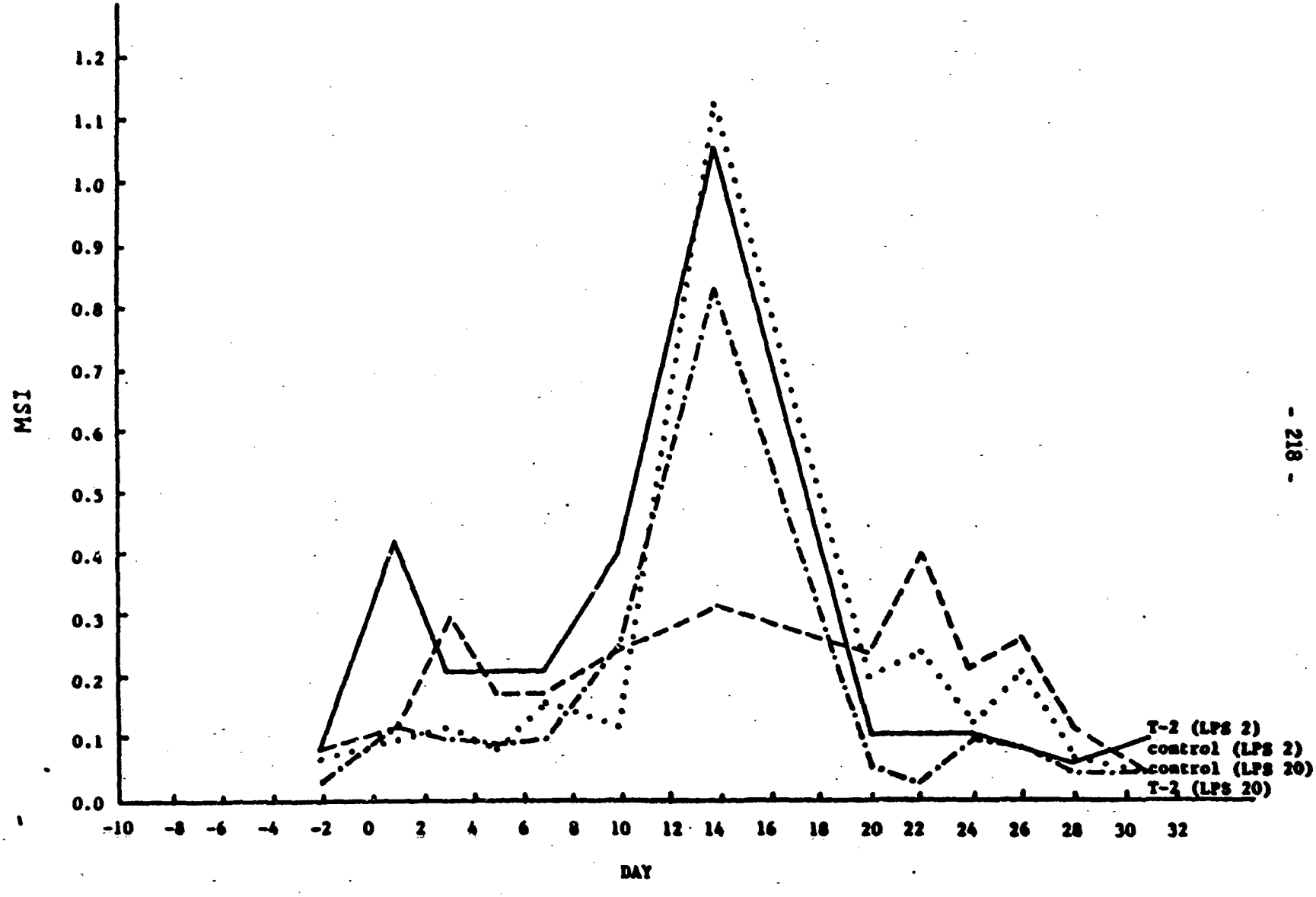


Fig. 13 Blastogenic responses (MSI) of peripheral blood lymphocytes to the optimal (20 ug/ml) and suboptimal (2 ug/ml) concentration of LPS of pigs exposed dermally to T-2 toxin (15 mg/kg) with vehicle and the group exposed to vehicle only.

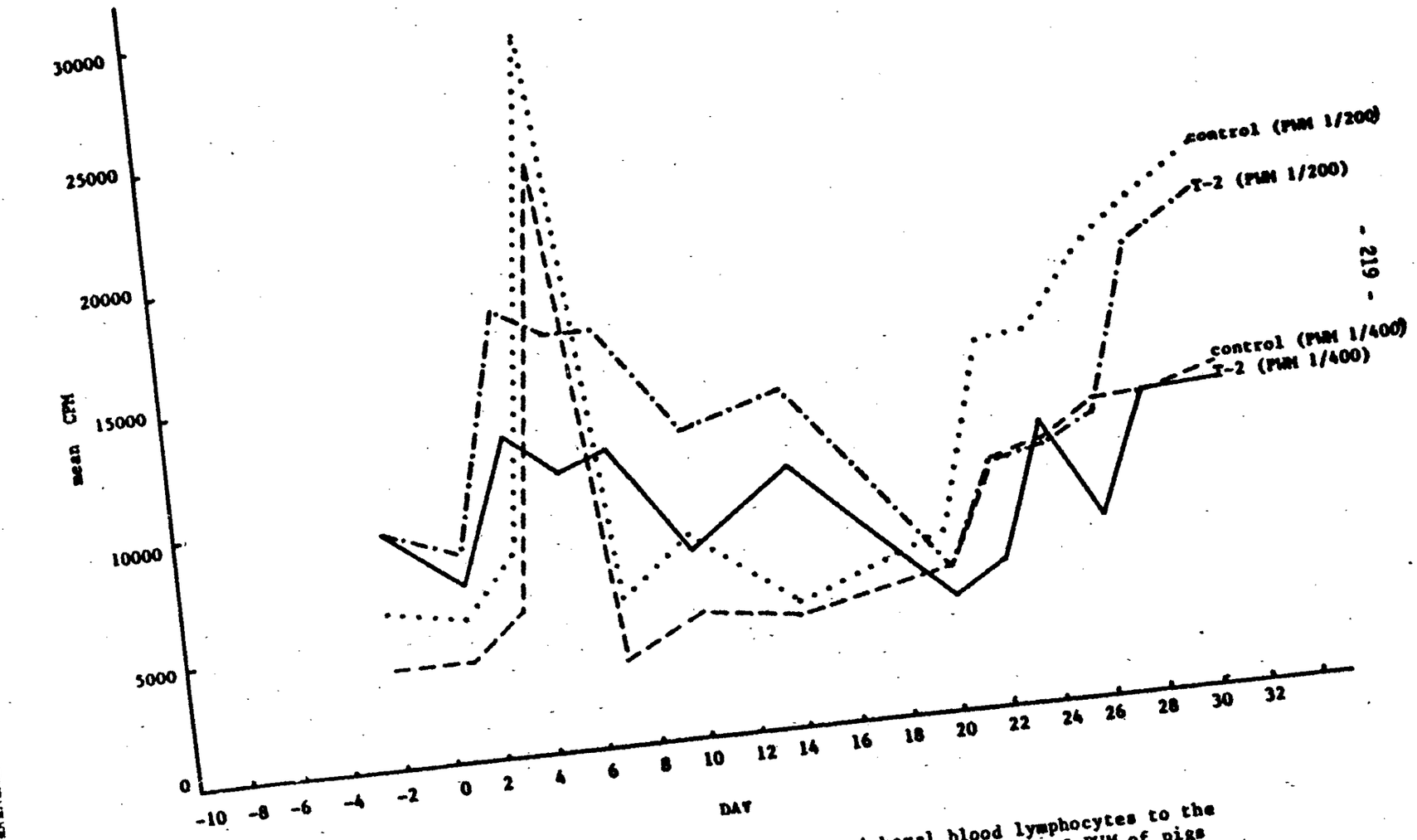


Fig. 14 Blastogenic responses (M CPM) of peripheral blood lymphocytes to the optimal (1:200) and suboptimal (1:400) concentration of PWM of pigs exposed dermally to T-2 toxin (15 mg/kg) with vehicle and the group exposed to vehicle only.

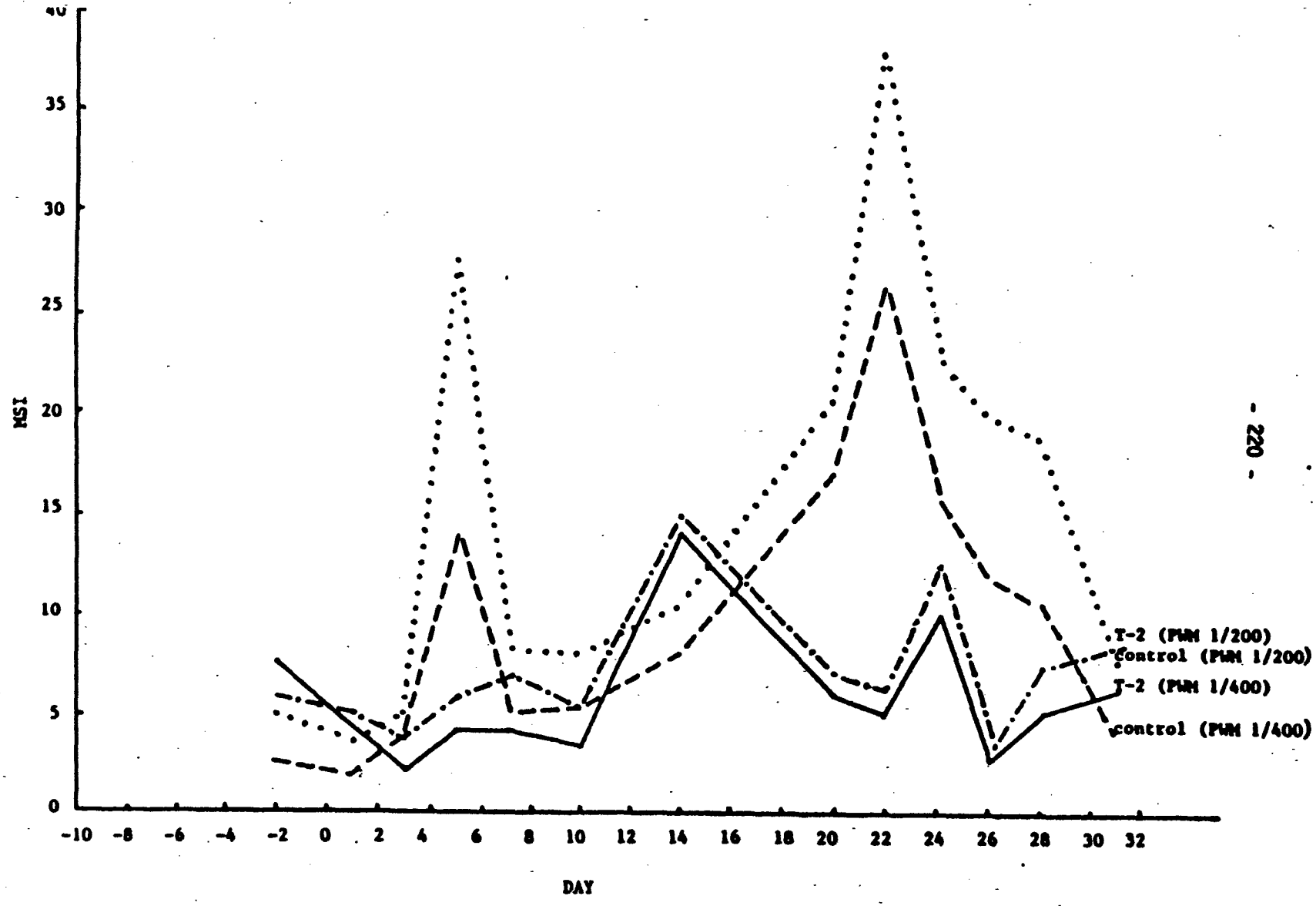


Fig. 15 Blastogenic responses (MSI) of peripheral blood lymphocytes to the optimal (1:200) and suboptimal (1:400) concentrations of PWM of pigs exposed dermally to T-2 toxin (15 mg/kg) with vehicle and the group exposed to vehicle only.

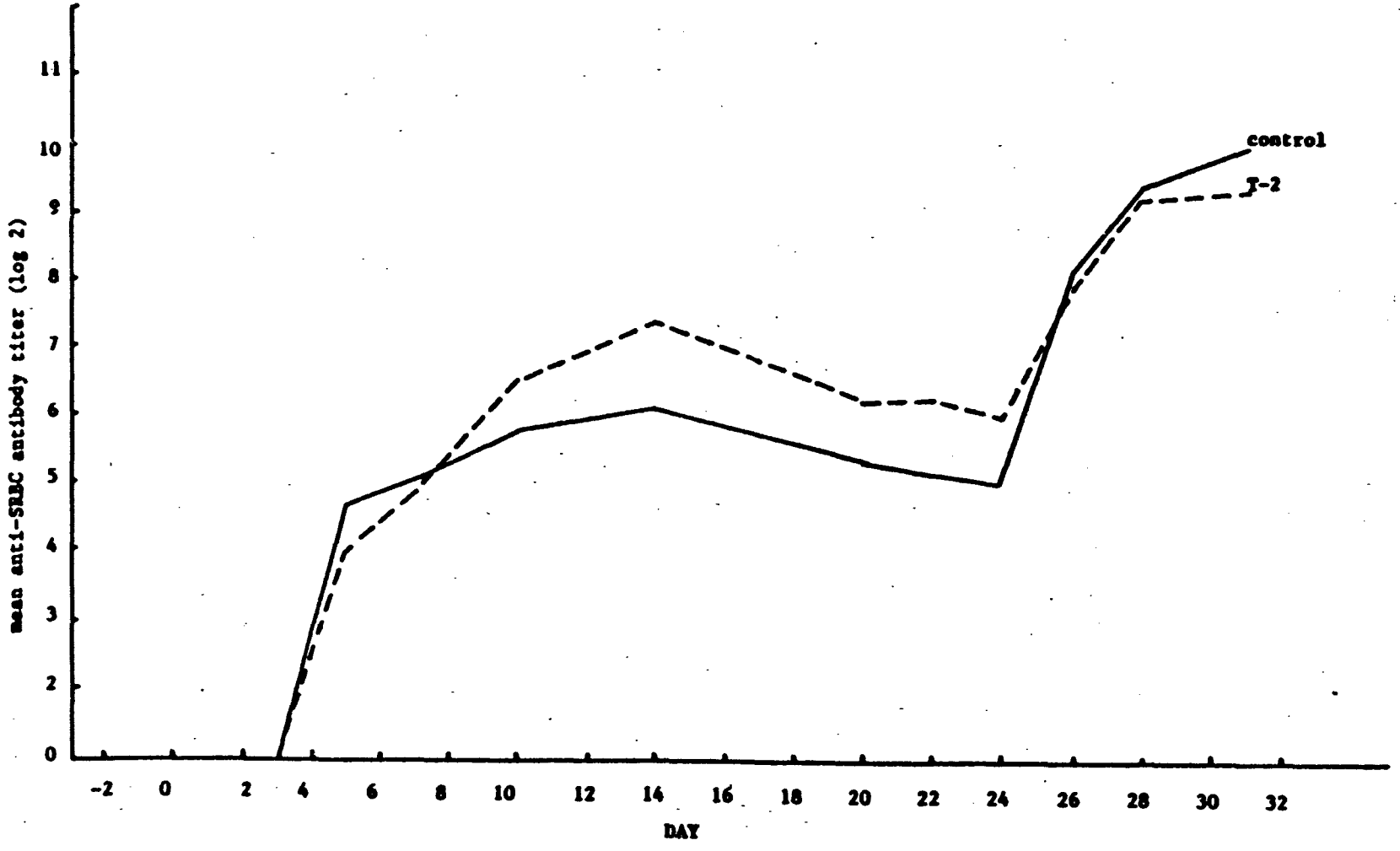


Fig. 16 Mean anti-SRBC Ab titers of pigs exposed dermally to T-2 toxin (15 mg/kg) with vehicle and the group exposed to vehicle only.

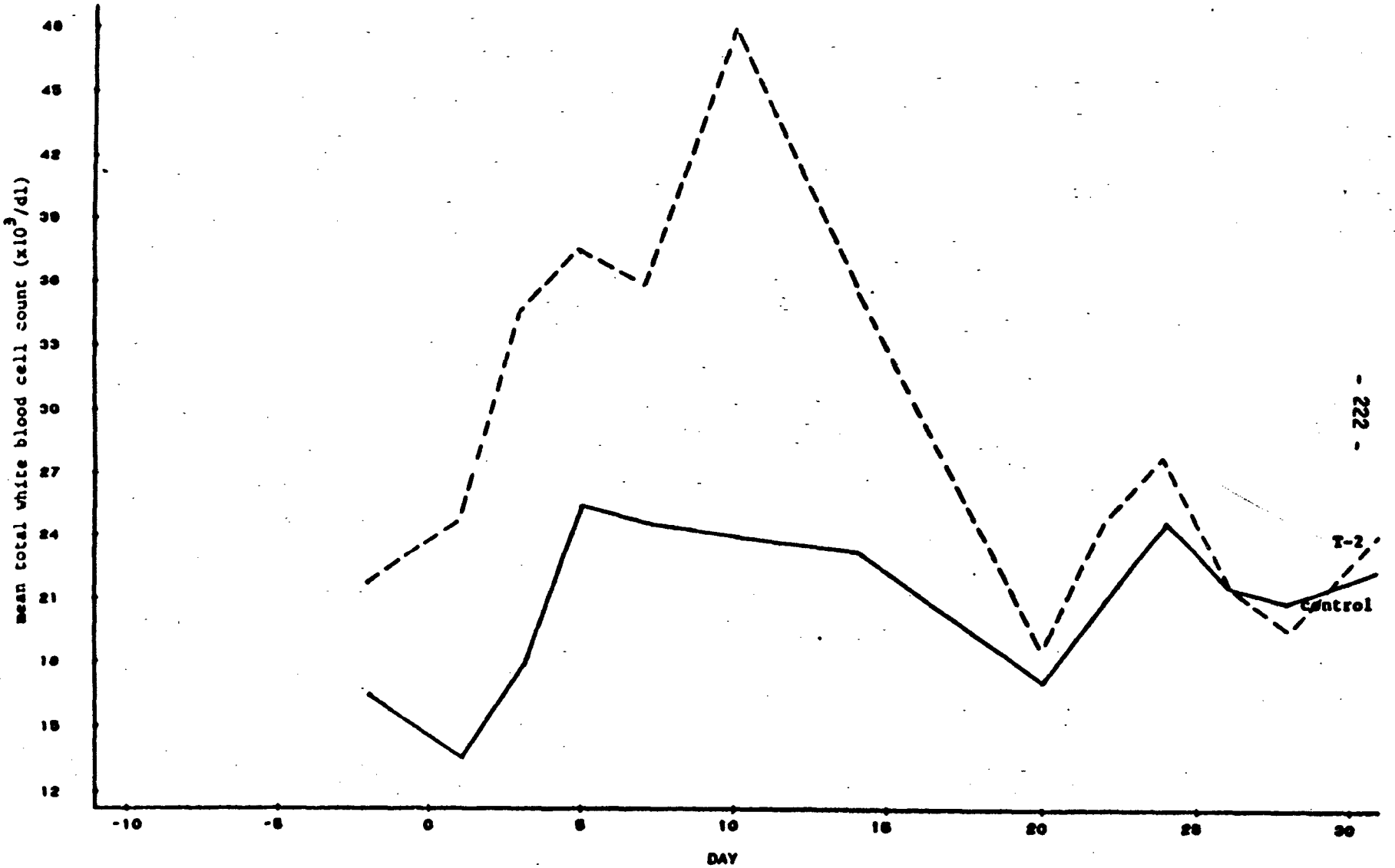


Fig. 17 Mean total white blood cell counts of pigs exposed dermally to T-2 toxin (15 mg/kg) with vehicle and the group exposed to vehicle only.

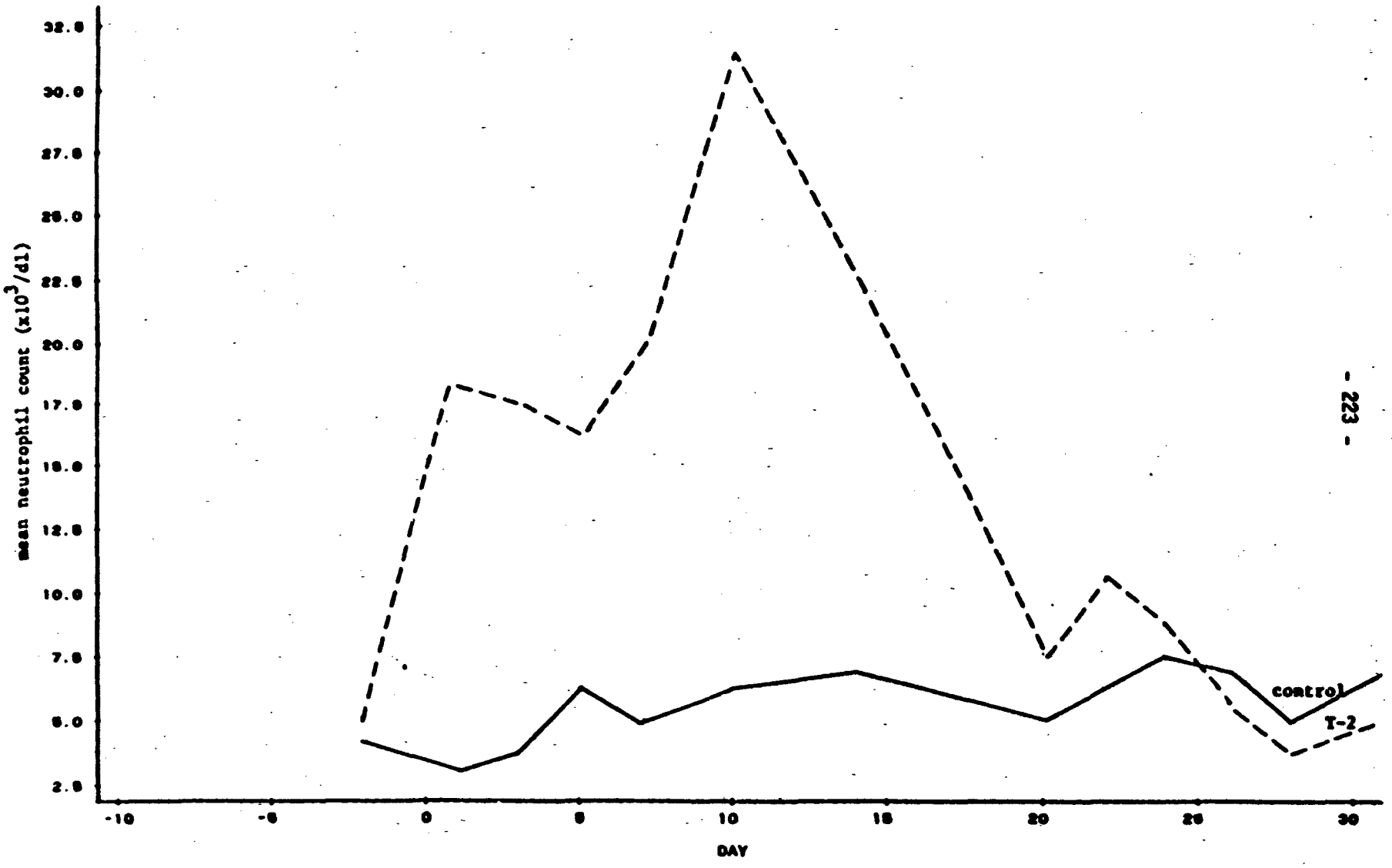


Fig. 18 Mean total neutrophil counts of pigs exposed dermally to T-2 toxin (15 mg/kg) with vehicle and the group exposed to vehicle only.

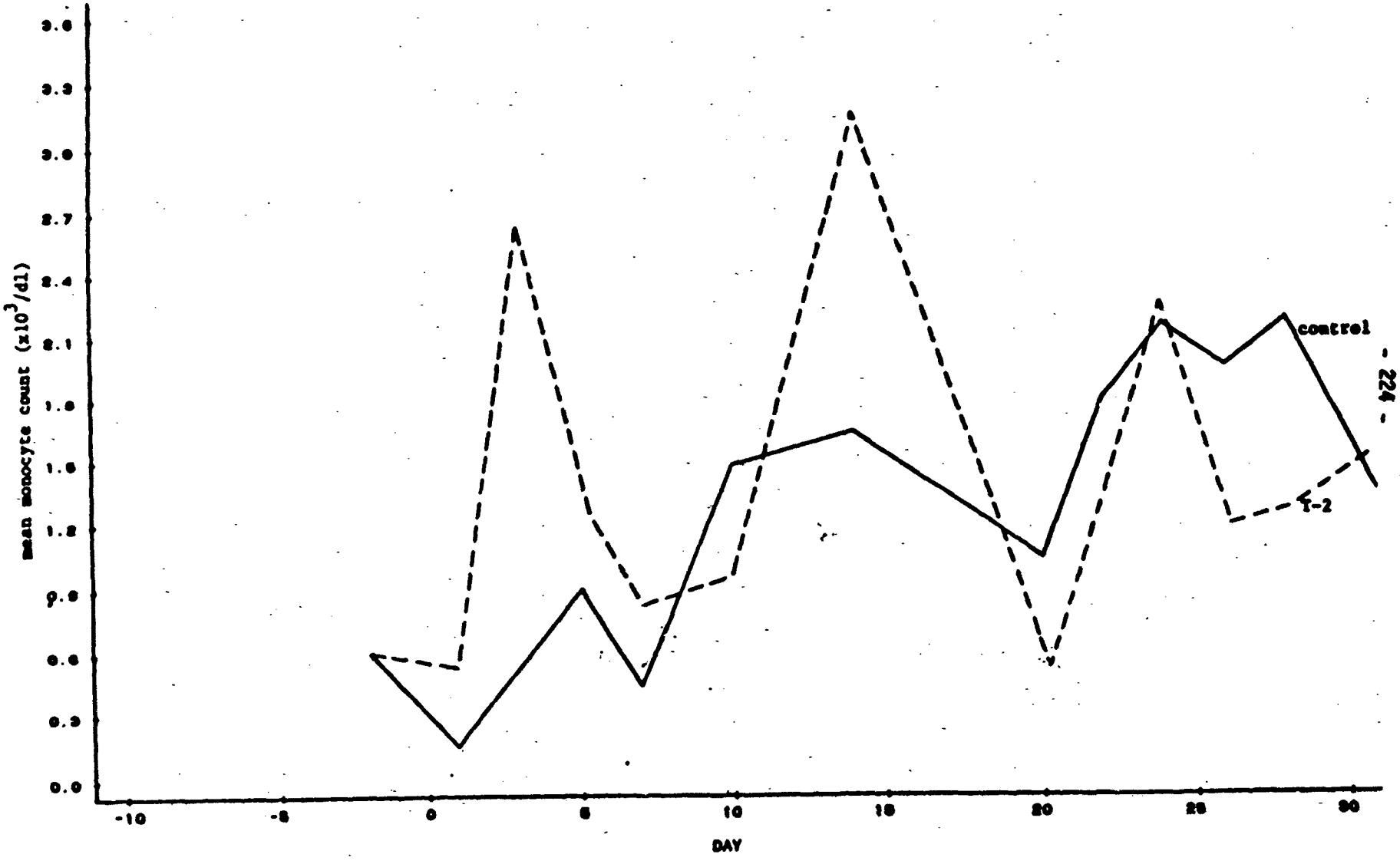


Fig. 19 Mean total monocyte counts of pigs exposed dermally to T-2 toxin (15 mg/kg) with vehicle and the group exposed to vehicle only.

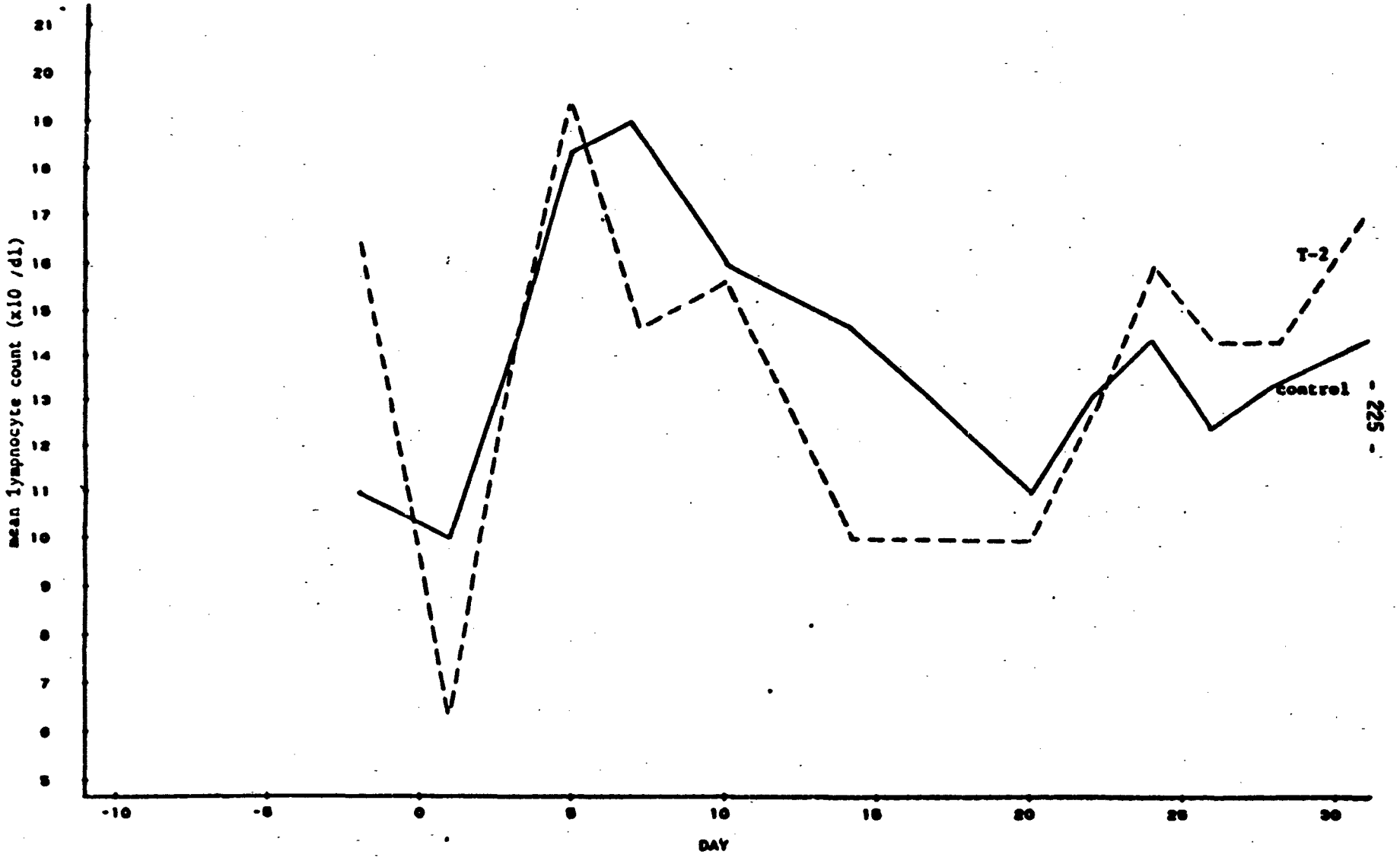


Fig. 20 Mean total lymphocyte counts of pigs exposed dermally to T-2 toxin (15 mg/kg) with vehicle and the group exposed to vehicle only.

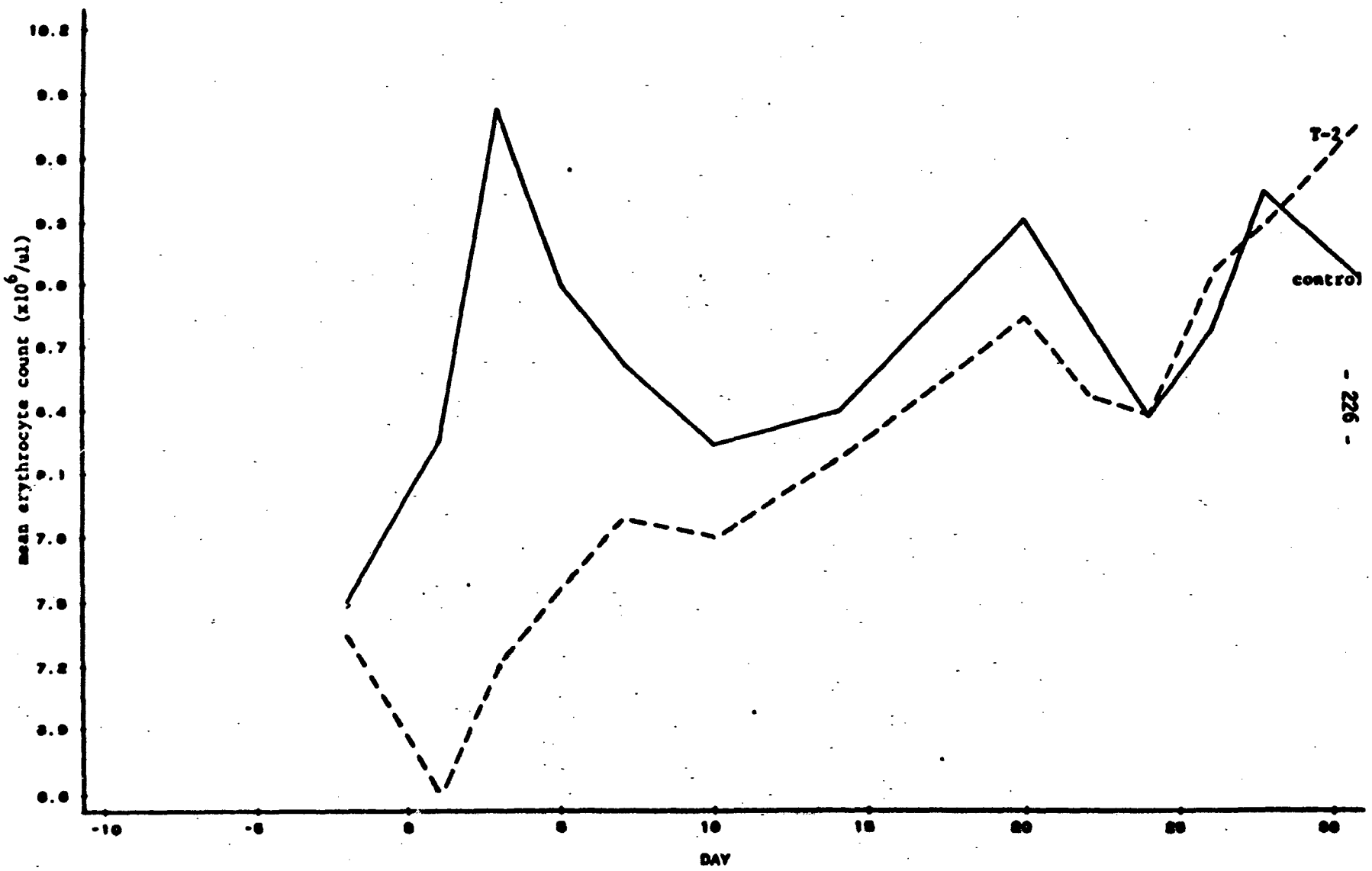


Fig. 21 Mean total erythrocyte counts of pigs exposed dermally to T-2 toxin (15 mg/kg) with vehicle and the group exposed to vehicle only.

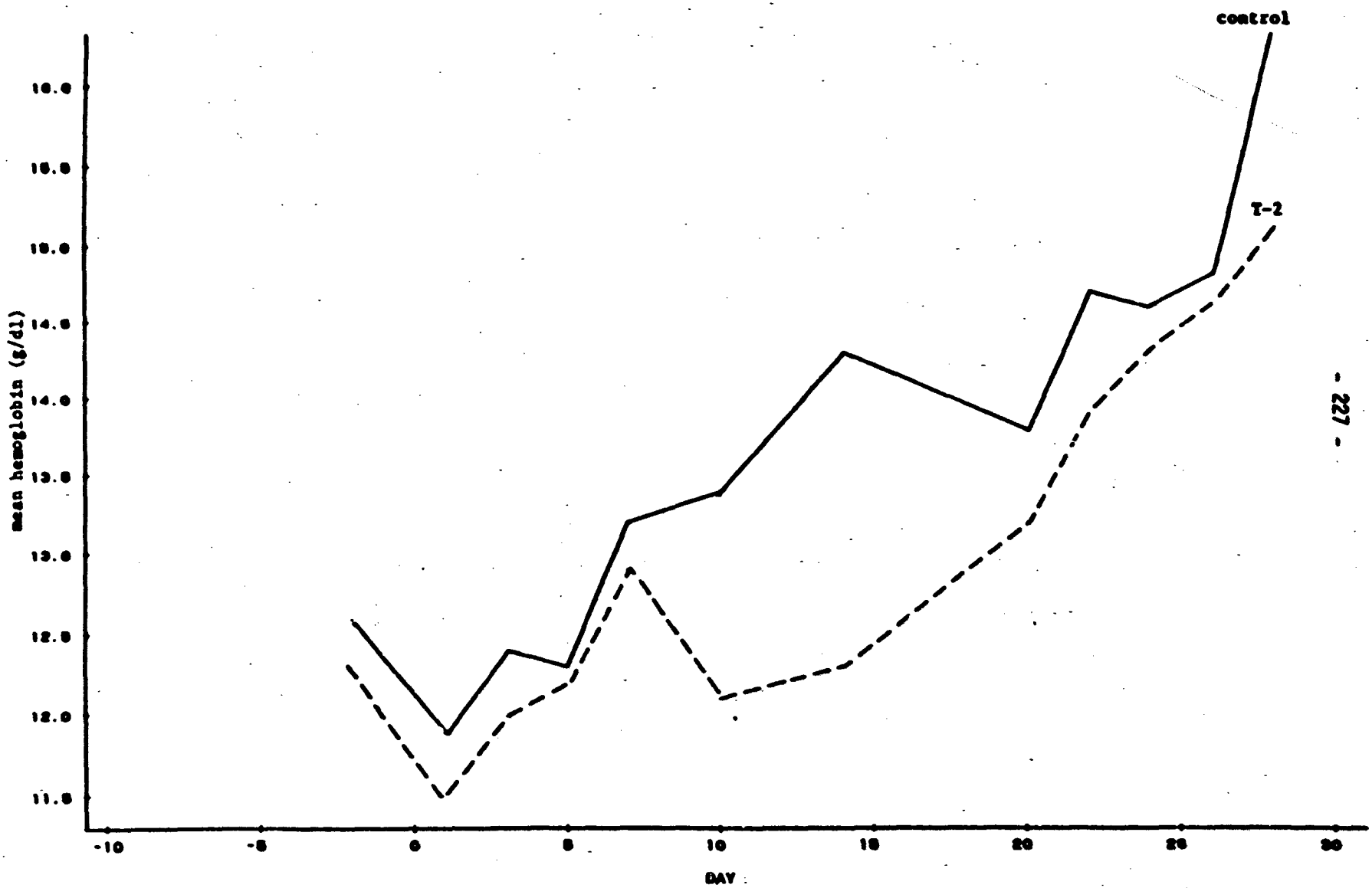


Fig. 22 Mean hemoglobin of pigs exposed dermally to T-2 toxin (15 mg/kg) with vehicle and the group exposed to vehicle only.

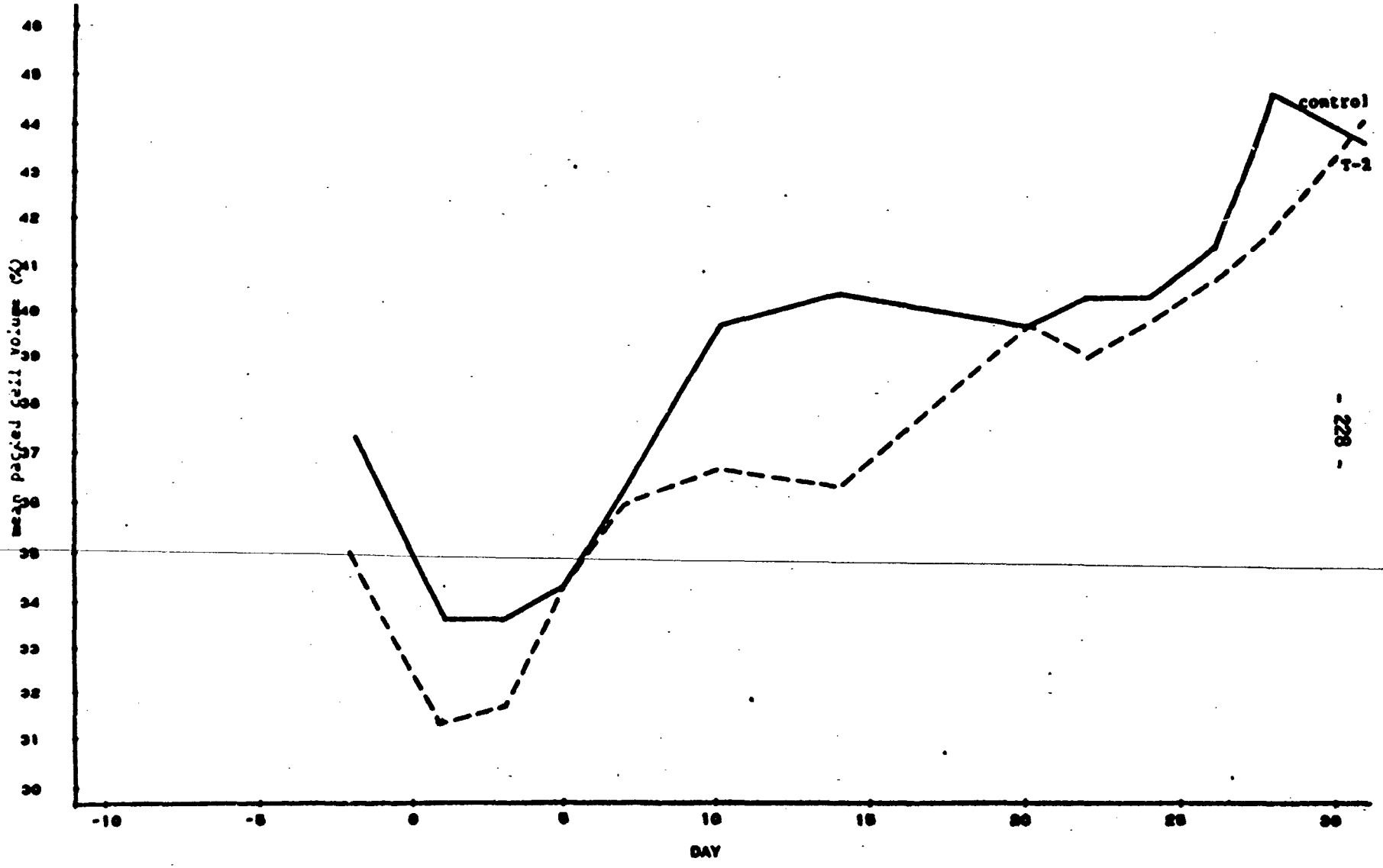


Fig. 23 Mean packed cell volume of pigs exposed dermally to T-2 toxin (15 mg/kg) with vehicle and the group exposed to vehicle only.

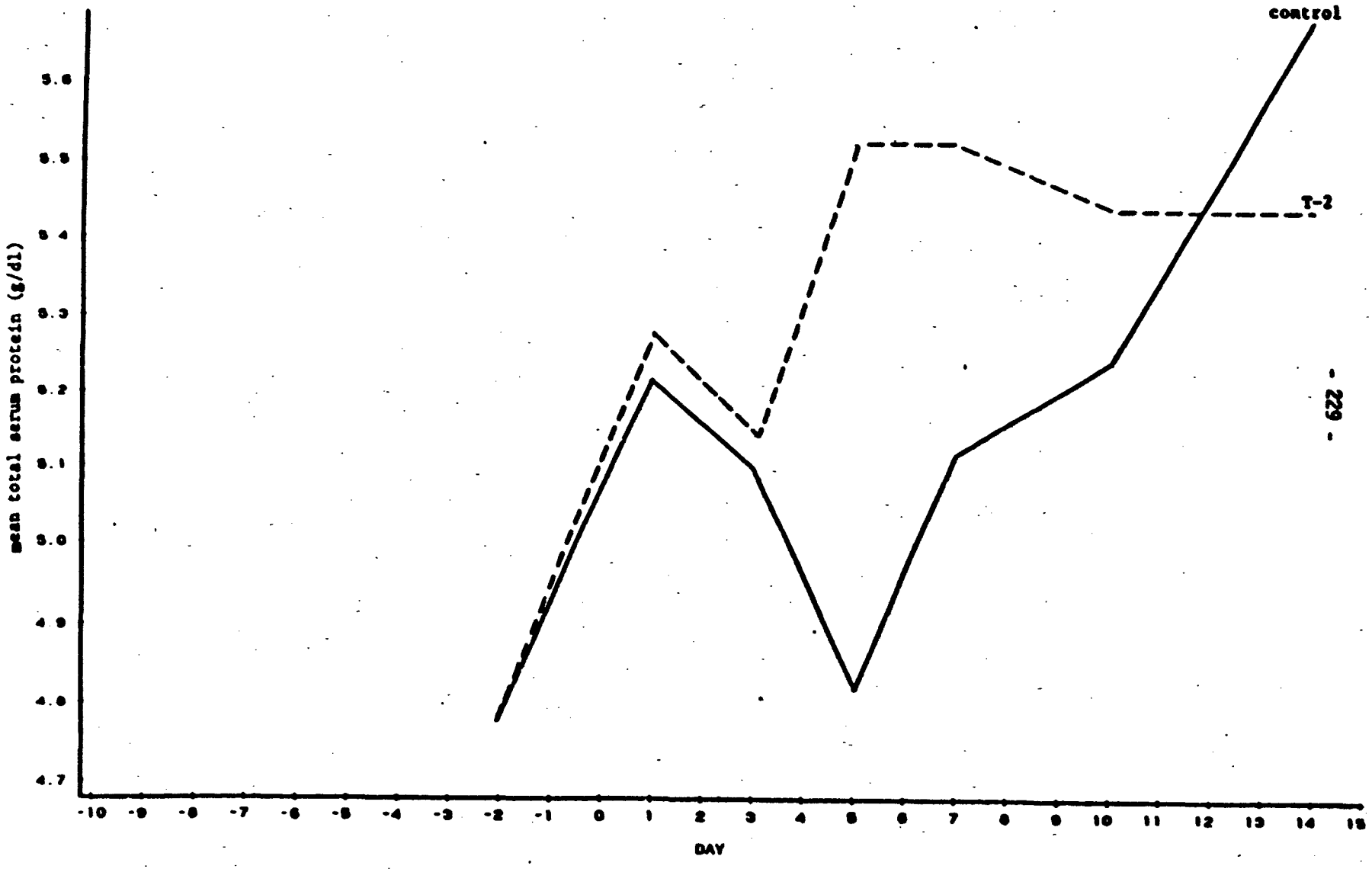


Fig. 24 Mean total serum protein of pigs exposed dermally to T-2 toxin (15 mg/kg) with vehicle and the group exposed to vehicle only.

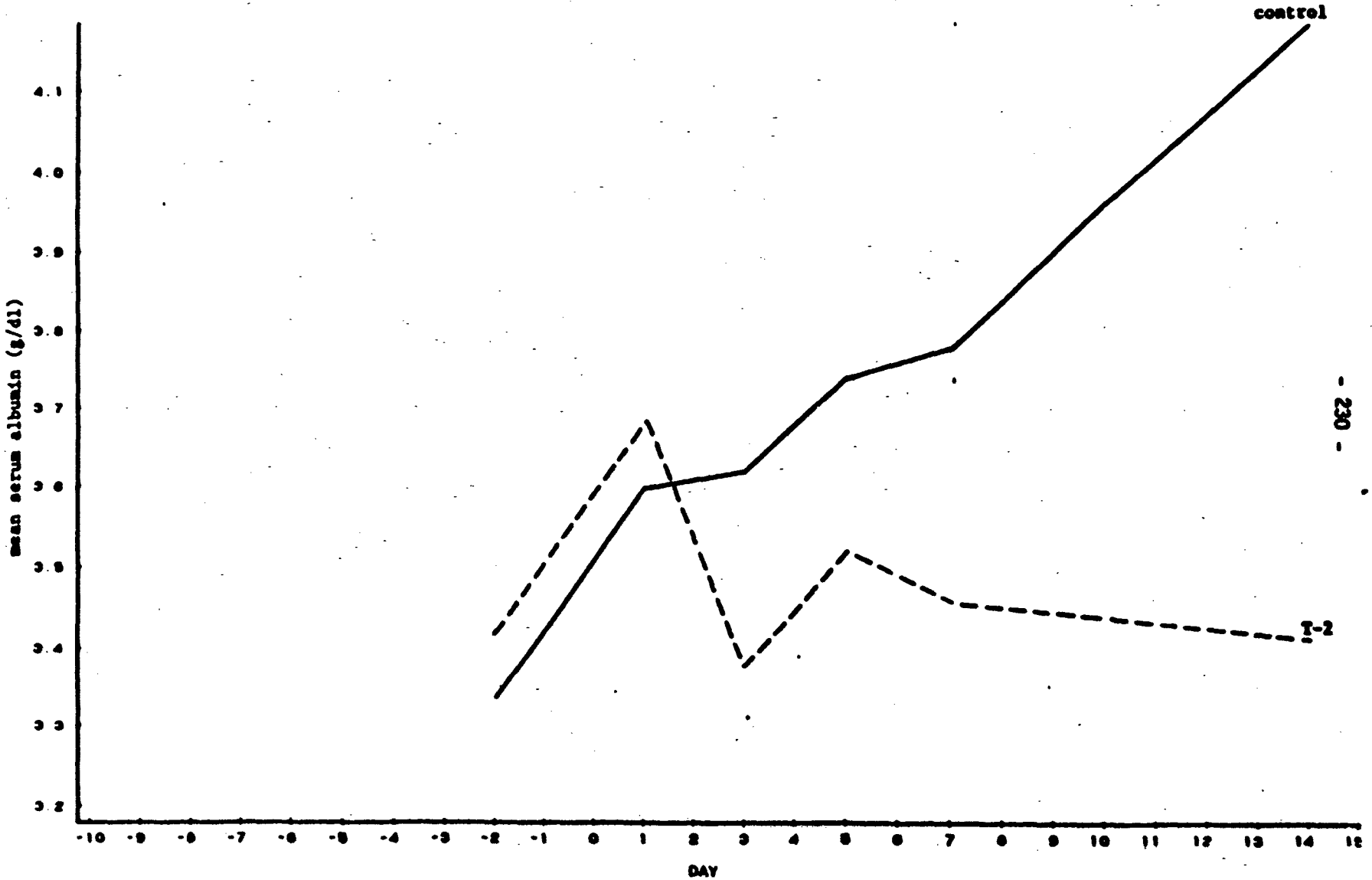


Fig. 25 Mean serum albumin of pigs exposed dermally to T-2 toxin (15 mg/kg) with vehicle and the group exposed to vehicle only.

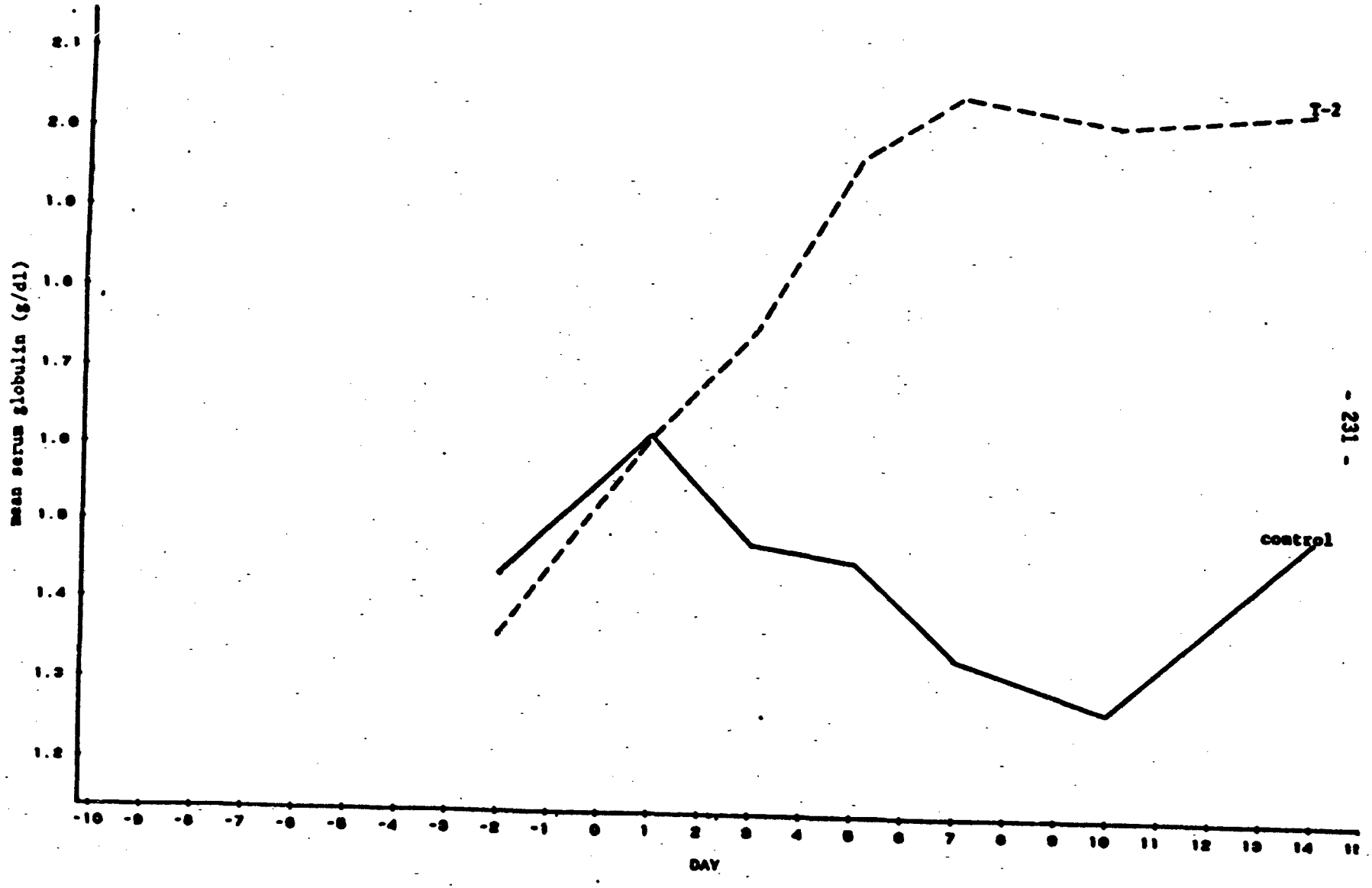


Fig. 26 Mean serum globulin of pigs exposed dermally to T-2 toxin (15 mg/kg) with vehicle and the group exposed to vehicle only.

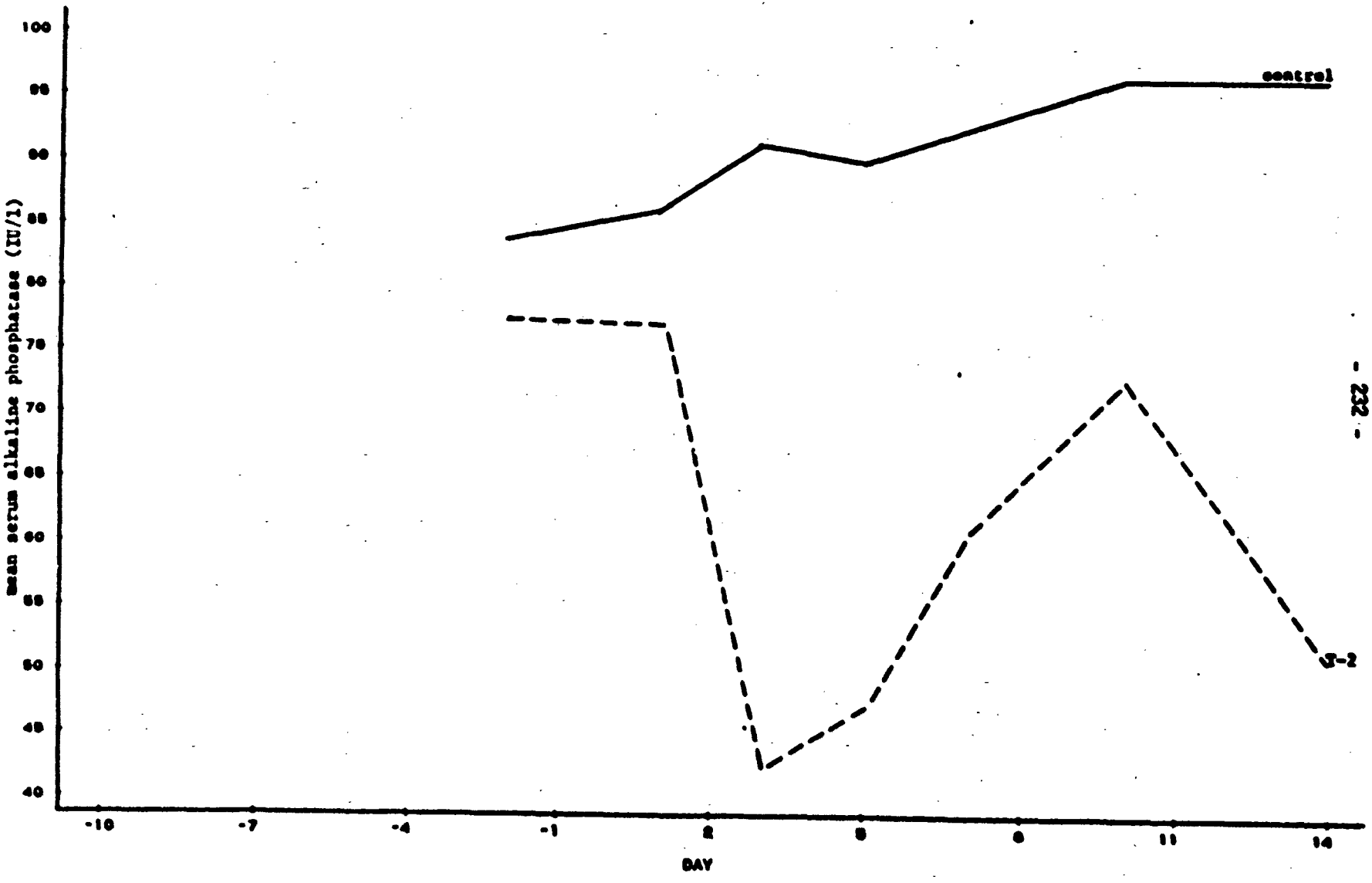


Fig. 27 Mean serum alkaline phosphatase of pigs exposed dermally to T-2 toxin (15 mg/kg) with vehicle and the group exposed to vehicle only.

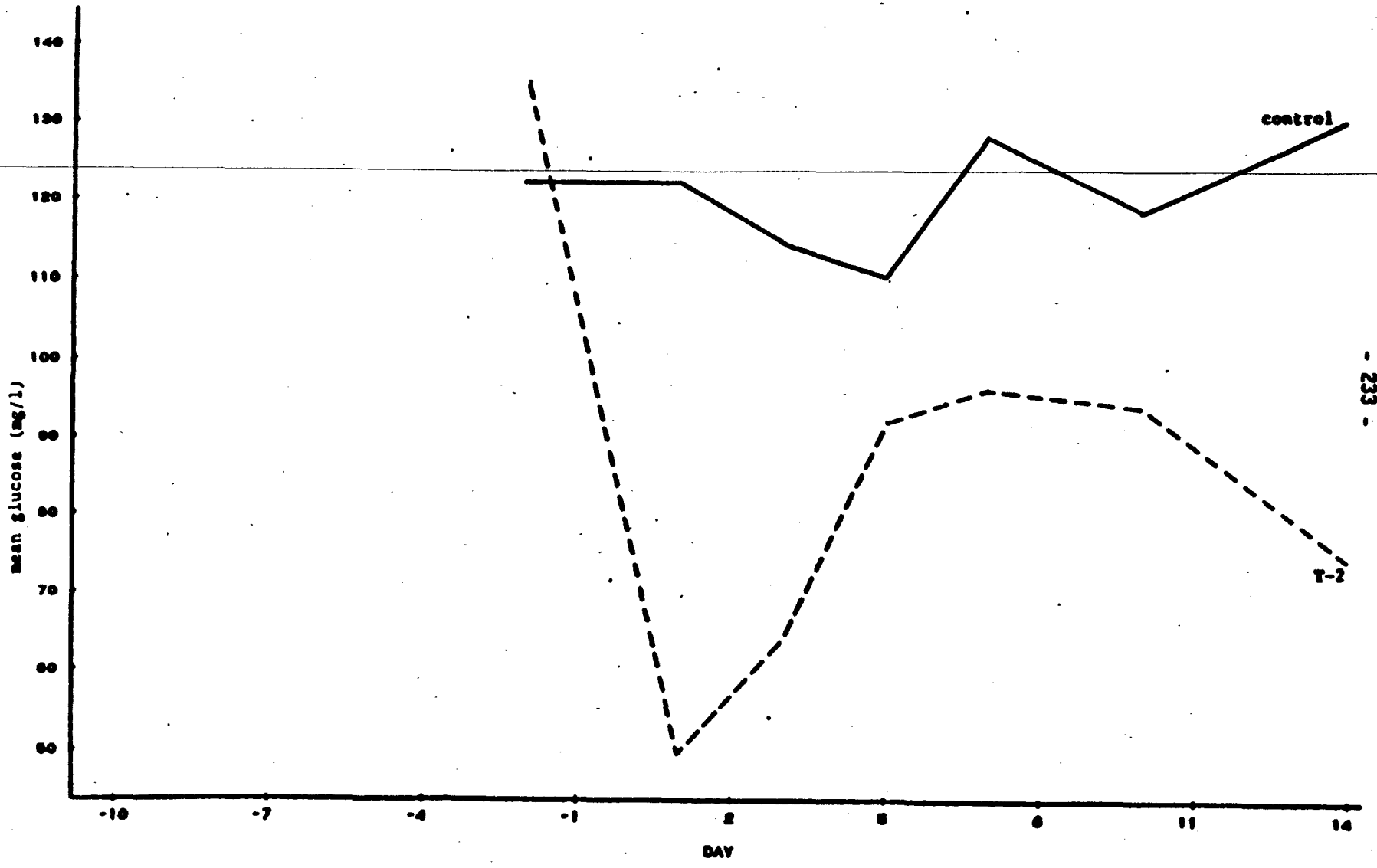


Fig. 28 Mean blood glucose of pigs exposed dermally to T-2 toxin (15 mg/kg) with vehicle and the group exposed to vehicle only.

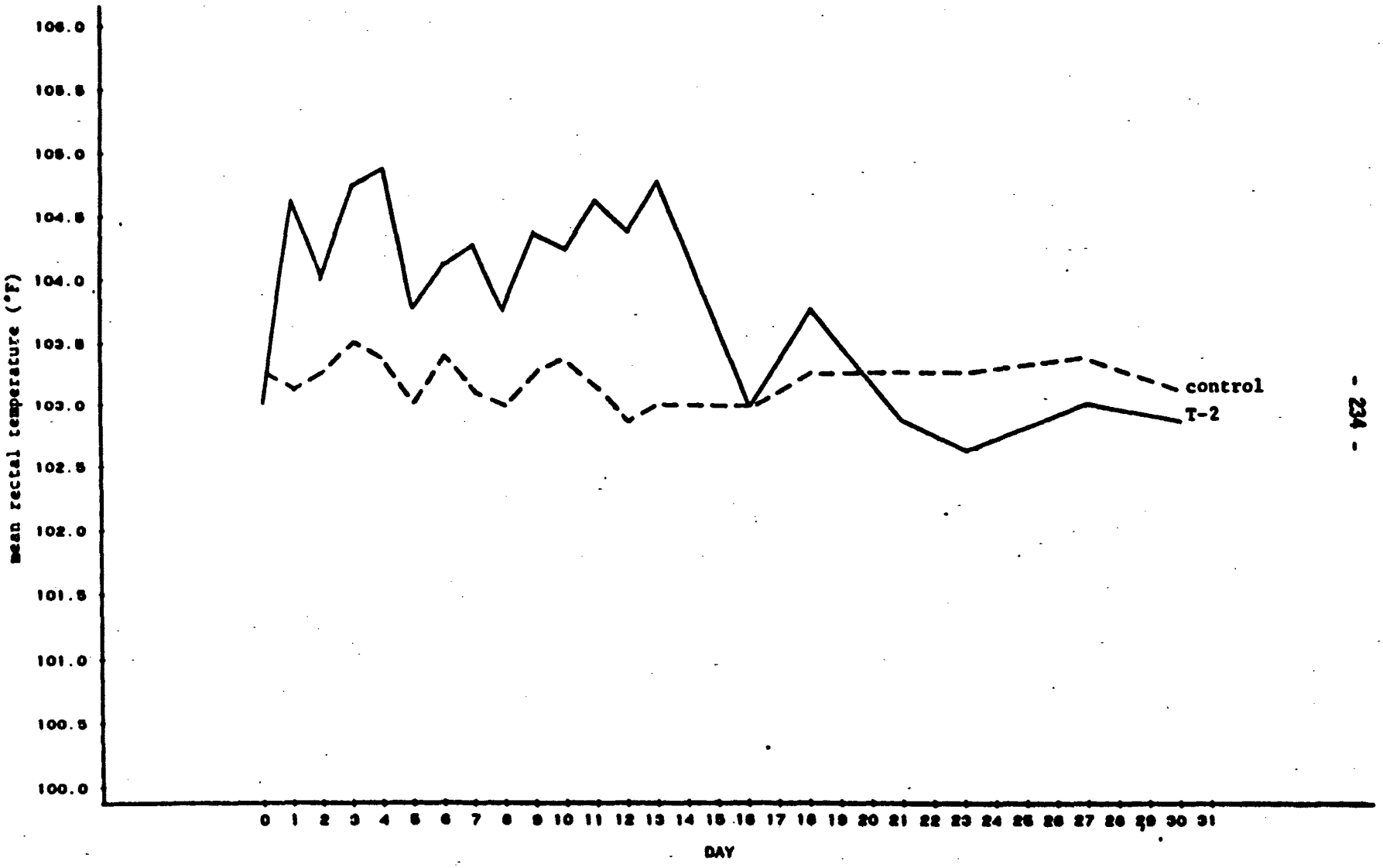


Fig. 29 Mean rectal temperature of pigs exposed dermally to T-2 toxin (15 mg/kg) with vehicle and the group exposed to vehicle only.

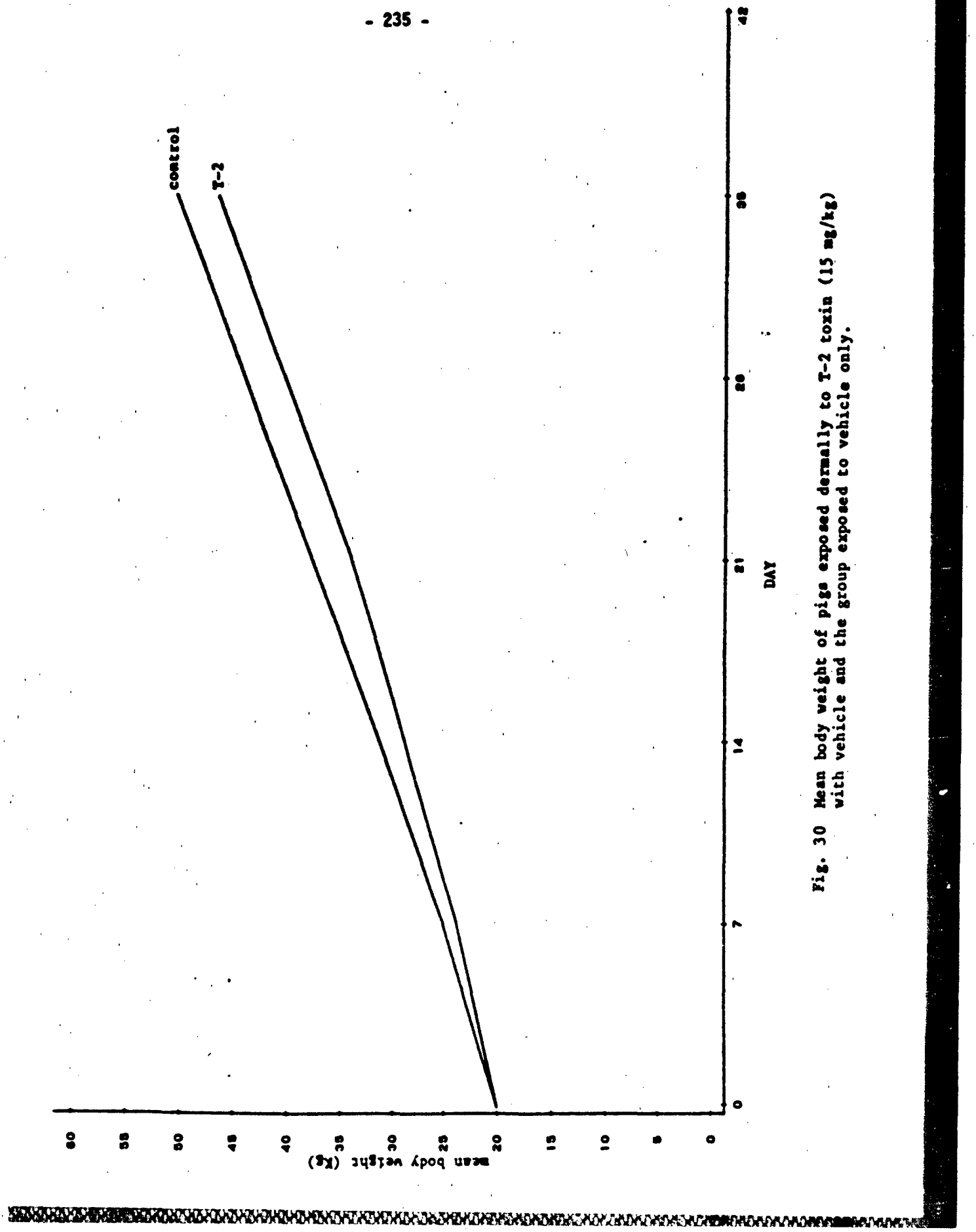


Fig. 30 Mean body weight of pigs exposed dermally to T-2 toxin (15 mg/kg) with vehicle and the group exposed to vehicle only.

II. THERAPEUTIC STUDIES

IIA. RAT THERAPEUTIC STUDIES--Bob Poppenga

A. SUMMARY

The efficacy of a variety of approaches for the treatment of acute T-2 toxicosis were assessed utilizing young, female rats. Superphysiologic doses of the water soluble salt of methylprednisolone (Solu-Medrol®) significantly ($p < .05$) enhanced survival time in T-2 toxin-treated animals as compared to nontreated, positive T-2 controls. Trichodermin, given to rats either 1 hour prior to or immediately after administration of T-2 toxin, significantly ($p < .05$) decreased mean survival time compared to positive T-2 control animals. Other agents screened in rats did not enhance mean survival times with the dosage regimens used. These agents included naloxone hydrochloride, diltiazem hydrochloride, dazemgrel, N-acetylcysteine, dimethylsulfoxide, adenosine triphosphate (ATP), ATP combined with magnesium chloride ($ATP\text{-}MgCl_2$), ascorbic acid and aprotinin (see Table 1)

B. MATERIALS AND METHODS

A series of five experiments was conducted using different agents in each experiment. For each individual experiment, approximately 225 gram, female Sprague-Dawley rats were randomly assigned to either treatment or control groups following appropriate acclimation periods. Each experiment in the series had a positive control group; and where multiple drug dosings were required (experiments 1 and 2), an additional positive control group was included which received sham saline injections IP as often as the most frequently treated group within that particular experiment. This second

control group was included in order to assess the effect, if any, of handling stress on survival. In some cases, where only a single administration of a compound was required, the difference in handling stress between the control and treatment groups was considered to be minimal and an additional "handling stress" control group was not included (experiments 3 through 5).

Twenty-four hours prior to T-2 toxin and drug administration, rats were weighed and appropriate doses of T-2 toxin and the various therapeutic agents were prepared. A 50 percent ethanol solution was used as the vehicle for the T-2 toxin (1 mg T-2 toxin per ml vehicle). Other appropriate vehicles were used for the treatment agents.

Following an overnight fast, all rats were dosed intravenously via the tail vein with T-2 toxin at the rate of 1 mg/kg body weight, an approximate LD_{50} determined from a previous study. Therapy with the various agents was begun 15 minutes after T-2 toxin administration with the exception of 1 treatment group in experiment 3 which was treated with trichodermin 1 hour prior to T-2 toxin administration. All therapeutic agents were given IP. Dosage rates and intervals were selected for individual compounds based on a literature review of their uses in other experimental studies with the exception of trichodermin and ascorbic acid whose doses were empirically formulated. The dosage regimens are summarized in Tables 2 through 6.

After dosing, all rats were returned to their cages and periodically observed. All had food and water available ad libitum following dosing. Survival times were recorded for each rat. Those animals surviving 48 hours were killed with ether. A postmortem was done on each rat and

tissues saved in ten percent buffered formalin for subsequent histopathologic examination.

Mean survival times and standard errors of the mean were calculated. Statistical significance between group means was determined using the Statistical Analysis System's (SAS) t-test procedure.

C. RESULTS

Mean survival times for the various control and drug treatment groups are given in Tables 2 through 6. As can be seen in Table 2, the administration of methylprednisolone sodium succinate (Solu-Medrol,® Upjohn) at 30 mg/kg body weight IP 15 minutes after T-2 toxin administration nearly tripled survival time compared to the T-2 control group. Six of the eight rats in this group survived the 48-hour observation period, and all six appeared to be in good health just prior to the kill time. The longest lived rat in the T-2 control group survived 19 hours. Interestingly, there was a substantial difference in the mean survival times between the two T-2 control groups in this experiment. The group being handled more frequently and given sham saline injections IP survived an average of approximately 11 hours longer. The variability in survival times for this group was fairly large, however, ranging from a low of approximately 10-1/2 hours to the full 48 hours. Three of the eight rats in this group survived 48 hours.

The other significant finding was the negative effect that trichodermin had on survival when given either 1 hour prior to or 15 minutes after T-2 toxin. The control group mean survival time was 22.47 $\pm$ 4.84 hours, whereas the trichodermin pretreatment group and the trichodermin post T-2 treatment group mean survival times were 11.26 $\pm$ 1.15 and 10.41 $\pm$ .63 hours, respectively.

Other therapeutic approaches did not prove to be of significant benefit in terms of clinical symptomatology or survival time and rate.

D. DISCUSSION

Superphysiologic doses of glucocorticosteroids have proven to be of benefit in the treatment of various forms of shock. Since irreversible shock is a manifestation of acute T-2 toxicosis following administration of a lethal dose of T-2 toxin, it is not surprising that glucocorticosteroids have a beneficial effect on survival. The mechanism underlying this beneficial effect in T-2 toxin induced shock is not entirely clear.

It was hoped that trichodermin, being a much less acutely toxic trichothecene than T-2, would serve as a competitive inhibitor of T-2 at cellular or subcellular target sites. It is evident from the data presented here that trichodermin has at least an additive, if not synergistic, adverse effect on survival time when given IP either before or following T-2 toxin administration.

Other agents tested cannot be definitively ruled out as being able to play a beneficial role in treating acute T-2 toxicosis. The possibility exists that larger doses or doses administered over a longer period of time than was the case in these experiments could be beneficial. It is also possible that while individual agents when used alone may not increase survival in acute T-2 toxicosis, they may be useful therapeutic adjuncts in an overall multiple agent treatment protocol.

TABLE 1. Agents screened in rats for efficacy in treating acute T-2 toxicosis.

Agent	Action	Source
Naloxone hydrochloride	Endogenous opioid antagonist	Raw material obtained courtesy of DuPont Pharmaceuticals
Diltiazam hydrochloride	A slow calcium channel blocker	Raw material obtained courtesy of Marion Laboratories, Inc.
Methylprednisolone sodium succinate	Glucocorticosteroid	Purchased as a commercially available preparation (Solu-Medrol® from UpJohn
Dazemgrel (UK-38,485)	A thromboxane synthetase inhibitor	Raw material obtained courtesy of Pfizer Central Research
N-acetyl cysteine, sodium salt	Source of cysteine, the limiting amino acid in the manufacture of glutathione	Purchased as a commercially available preparation (Mucomyst®) from Mead Johnson Pharmaceutical
Dimethyl sulfoxide	Anti-inflammatory	Purchased as a commercially available preparation (90 percent DMSO) from Burlington Biomedical Corporation
Trichodermin	Less toxic trichothecene mycotoxin	
ATP ATP and $MgCl_2$ (ATP-disodium salt, crystalline, from equine muscles, low Ca^{++} content)	Source of high energy phosphate for cell metabolic functions	Purchased both ATP and $MgCl_2$ from Sigma Chemical in raw form
Ascorbic acid	Antioxidant	Purchased as a commercial preparation (Scorbate® from Burns-Biotec Laboratories
Aprotinin (from bovine lung, affinity purified, lyophilized powder, 10-20 TIU per mg solid)	Protease inhibitor	Purchased raw material from Sigma Chemical

TABLE 2. Rat therapeutic study. Experiment 1.

Group	Dosage Regimen	Mean Survival Time (Hour ± SEM)
T-2 Control	1 mg/kg T-2 toxin IV; no therapy	14.11 ± 0.96 n = 8
T-2 + Saline	0.25 cc normal saline IP each hour for 5 hours	25.63 ± 6.57 n = 8
T-2 + Naloxone	10 mg/kg IP each hour for 5 hours	13.68 ± 1.48 n = 8
T-2 + Diltiazem	18 mg/kg IP every 2 hours for 3 treatments	13.56 ± 0.56 n = 8
T-2 + Methyl-prednisolone	30 mg/kg IP given once	41.57 ± 4.24* n = 8

*Animals surviving the entire 48-hour observation period were assigned a survival time of 48 hours.

TABLE 3. Rat therapeutic study. Experiment 2.

Group	Dosage Regimen	Mean Survival Time (Hour ± SEM)
T-2 Control	1 mg/kg T-2 toxin IV; no therapy	12.91 ± 1.13 n = 6
T-2 + Saline	0.25 cc normal saline each hour for 5 hours	12.79 ± 1.43 n = 7
T-2 + Dazemgrel	25 mg/kg IP initial dose followed by 10 mg/kg every hour for 5 hours	11.95 ± 0.33 n = 10
T-2 + N-acetyl cysteine	140 mg/kg IP initial dose followed by 70 mg/kg 5 hours later	11.37 ± 0.17 n = 6
T-2 + DMSO	1 gram/kg IP given once	14.49 ± 2.62 n = 7

TABLE 4. Rat therapeutic study. Experiment 3

Group	Dosage Regimen	Mean Survival Time (Hour ± SEM)
T-2 Control	1 mg/kg T-2 toxin IV; no therapy	22.47 ± 4.84 n = 7
Trichodermin Pretreatment	1 mg/kg trichodermin IP 1 hour prior to T-2 toxin	11.26 ± 1.15 n = 8
Trichodermin Post T-2	1 mg/kg trichodermin IP immediately following T-2 toxin	10.41 ± 0.63 n = 8

TABLE 5. Rat therapeutic study. Experiment 4

Group	Dosage Regimen	Mean Survival Time (Hour + SEM)
T-2 Control	1 mg/kg T-2 toxin IV followed by 0.25 cc normal saline 15 minutes later	14.85 ± 2.70 n = 8
ATP	ATP solution (200 μ mol/ml) 0.125 mL given IP immediately after T-2 and again 15 minutes later	12.30 ± .37 n = 8
ATP + $MgCl_2$	ATP + $MgCl_2$ (100 μ mol ATP and 100 μ mol $MgCl_2$ per mL) 0.125 mL given IP immediately after T-2 and again 15 minutes later	14.06 ± 1.57 n = 7

TABLE 6. Rat therapeutic study. Experiment 5

Group	Dosage Regimen	Mean Survival Time (Hour + SEM)
T-2 control	1 mg/kg T-2 toxin IV; no therapy	11.76 ± 0.62 n = 7
T-2 + Vitamin C	500 mg/kg IP given once	13.32 ± 1.02 n = 8
T-2 + Aprotinin	15,000 KIU/kg IP initial dose, then 10,000 KIU/kg IP 2 hours later	14.01 ± 1.62 n = 8

IIB. PRELIMINARY SWINE THERAPEUTIC STUDIES

A. SUMMARY

Swine were treated with several different therapeutic agents either alone or in combinations following administration of 2.4 to 3.6 mg/kg T-2 toxin IV. Phenoxybenzamine, a nonspecific α-blocker, administered by IV drip, appeared to improve peripheral perfusion and delay the onset of diarrhea in two swine compared to a positive T-2 control animal. Propranolol, a ß-blocker, hastened the decline in cardiac output and decreased the time to death in two swine as compared to a control animal. Survival time was enhanced in two pigs receiving either dexamethasone or methylprednisolone sodium succinate along with bicarbonate and fluid therapy. Metoclopramide appeared to be an effective antiemetic in swine following T-2 toxin administration.

B. MATERIALS AND METHODS

These preliminary studies were designed to allow for familiarization with surgical and monitoring techniques and to allow for formulation of an appropriate sampling protocol.

A summary of the preliminary swine treatment protocols is included in Table 1. A more definitive materials and methods discussion is included in the following section.

C. RESULTS

Tables 2 through 9 have been included in this section providing data on several key parameters monitored during the conduct of these preliminary studies. All cardiac catheters implanted in P5 dopamine were inoperative; therefore, it was decided to forego in-depth physiological monitoring although the treatment protocol was continued. Animal survival times are noted in Table 1.

In general, it can be seen that serum glucose, total bound serum calcium, cardiac output, stroke volume, mean arterial blood pressure and arterial blood pH all decline, while heart rate and lactic acid values increase.

D. DISCUSSION

Since statistical analysis for determining the significance of the changes in the parameters mentioned was not possible, discussion is somewhat limited. Many of the trends noted in Tables 2 through 9 would be expected based on previous pathophysiology studies in our laboratory; e.g., progressive declines in total serum calcium and cardiac output or increases in heart rate and lactic acid values.

From the above data, survival times are probably the best indication of a particular therapeutic agent's promise in treating acute T-2 toxicosis. Average survival time was decreased by 33 percent in the two animals treated with the ß-blocker propanolol as compared to the control. It was hoped that if excessive circulating catecholamine levels were adversely impacting on cardiac function over time, propranolol would provide a protective effect. This did not appear to be the case.

Phenoxybenzamine, a nonspecific α-blocker (both an α_1 and an α_2 antagonist), appeared to clinically improve peripheral circulation (as assessed by capillary refill time) and delay the onset and severity of diarrhea compared to a control animal. Survival time may or may not have been affected by the administration of phenoxybenzamine since both treatment animals and the control animal were killed if they survived the 24 hour observation period. A longer observation period may have allowed differences in survival time to be measured. One consequence of improved

peripheral circulation would be improved venous return. If the decline in cardiac output in T-2 toxin-treated animals is due to poor venous return, it might be anticipated that if phenoxybenzamine enhanced venous return this would be reflected in improved cardiac output. Indeed, cardiac index data appears to show more stability over time in the two phenoxybenzamine treated swine than in the control animal. More selective α-blockers (prazosin) along with appropriate fluid support may warrant future attention.

As mentioned in the section on rat therapeutic studies, death from T-2 toxin is associated with the occurrence of a shock syndrome. Based upon other studies investigating the usefulness of glucocorticosteroids in the treatment of shock, it was anticipated that duration and rate of survival would be enhanced when utilizing these drugs in cases of T-2 toxicosis. Both swine receiving steroids (in addition to $NaHCO_3$ and Ringer's) survived a 3.6 mg/kg IV dose of T-2 toxin (3 times the LD_{50}) for 24 hours; a four-fold increase in survival time compared to a control animal (6 hours).

Intuitive assessment of the effects of glucocorticosteroids on various measured physiologic parameters can be gleaned from tabulated data. They appeared to have little effect on changes in serum glucose and calcium. They tended to moderate the large increases in heart rate noted in other animals and to help maintain cardiac output and stroke volume (due to improved venous return), although they did not prevent arterial blood pressure from falling. Lastly, their action may have resulted in improved peripheral organ perfusion as reflected in a moderation of the rise of plasma lactic acid compared to other animals.

Based upon these preliminary studies and others conducted by the DOD, it was concluded that glucocorticosteroids should form the cornerstone of treatment in more definitive therapeutic studies.

TABLE 1. Summary of preliminary swine therapeutic study

Drug	Number of Animals And Survival Time	T-2 Dose	Treatment Protocol
Phenoxybenzamine, raw material courtesy of Smith, Kline and French.	n = 2 control = 1 All survived a 24-hour observation period prior to killing	2.4 mg/kg body weight (100 percent ETOH vehicle)*	1 mg/kg body weight administered by slow IV drip over 2 hour period. This was combined with a gravity drip of Ringer's solution.
Propranolol HCL injectable, Inderal® (1 mg propranolol per mL), Ayerst	n = 2** Mean survival time 4 hours	3.6 mg/kg body weight (100 percent ETOH vehicle)*	1 mg IV drip over first 5 minutes post T-2, then 1 mg/min to reach a total dose of .15 mg/kg body weight, then a maintenance IV infusion of .05 mg/min (one animal received metoclopramide).
Dopamine HCL, Intropin® (40 mg dopamine per mL), American Critical Care.	n = 2** Mean survival time 6 hours and 30 minutes	3.6 mg/kg body weight (100 percent ETOH vehicle)*	5 mcg/kg body weight/ minute via IV drip. If MAP*** declined, this rate was adjusted in an attempt to maintain adequate perfusion pressure (one animal received Ringer's).
Dexamethasone Azium®, (2 mg/mL) Schering	n = 1** Surival time 24 hours	3.6 mg/kg body weight (100 percent ETOH vehicle)*	8 mg/kg body weight IV immediately following T-2, then 4 mg/kg body weight IV at 4 hours and 2 mg/kg body weight at 8 hours. This was combined with sodium bicarbonate therapy and a gravity drip of Ringer's solution.
Methylprednisolone sodium succinate Solu-Medrol® (125 mg vial) Upjohn	n = 1** Surival time 23 hours	3.6 mg/kg body weight (100 percent ETOH vehicle)*	15.2 mg/kg body weight IV immediately following T-2 toxin and again at 4 hours post T-2. This was combined with sodium bicarbonate therapy and a gravity drip of Ringer's solution.

*Total of 0.1 mL vehicle per kg body weight.
**One control animal was treated with 3.6 mg/kg body weight T-2 IV. The animal survived 6 hours and 5 minutes.
***Mean arterial blood pressure.

TABLE 2. Preliminary swine therapeutic study: Blood glucose concentrations.

Pig Number	Time 1 hour Pre	Glucose (mg/dL) 0	1	2	3	4	5	6	7	8	12	24	48
Preliminary T-2 Control	109	109	144	151	203	170	145						
P2β-Blocker	108	120	122	101	85								
P3 MP*	108	100	88	106	85	64	46	33	38	37	52	43	
P4β-Blocker	106	100	N/A	130	N/A	N/A							
P5 Dopamine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A					
P6 Azium	80	89	67	74	63	56	37	34	27	29	55	41	
P7 Dopamine	105	113	153	114	95	40							

N/A = not available
*MP = methylprednisolone
β-Blocker = Propranolol

TABLE 3. Preliminary swine therapeutic study. Total serum calcium/ionized serum calcium concentrations.

Pig Number	Time 1 hour Pre	Total Ca^{++}/Ionized Ca^{++} (mg/dL) 0	1	2	3	4	5	6	7	8	12	24	48
Preliminary T-2 Control	9.8/ 5.0	9.5/ 3.7	9.3/ 4.9	8.5/ 5.1	8.1/ 4.6	7.9/ 6.0	7.9/ 6.3						
P2ß-Blocker	9.1/ 5.3	8.1/ 5.3	9.0/ 5.5	9.0/ 5.3	8.8/ 5.4	8.2/ 4.9							
P3 MP*	9.4/ 5.4	9.1/ 3.9	8.6/ 2.2	8.1/ 5.0	7.5/ 5.1	7.5/ 5.3	7.4/ 6.1	7.3/ 5.5	7.2/ 5.8	7.1/ N/A	6.7/ 5.5	6.2/ 4.7	
P4ß-Blocker	9.6/ 6.1	9.4/ 6.1	10.0/ 6.3	8.5/ 5.1	8.4/ 5.2	8.3/ 4.8							
P5 Dopamine	NA/ NA	NA/ NA	NA/ NA	NA/ NA	NA/ NA	NA/ NA	NA/ NA	NA/ NA					
P6 Azium	9.4/ 4.0	9.1/ 3.5	8.5/ 3.1	8.1/ 4.7	7.8/ 3.0	6.5/ 4.1	6.0/ 3.1	5.8/ 4.1	6.2/ 4.0	5.9/ 3.0	4.6/ 3.4	5.1/ 3.5	
P7 Dopamine	9.6/ 5.1	9.2/ 4.9	9.0/ 4.9	8.3/ 4.1	8.3/ 4.3	7.2/ 4.2							

N/A = not available
*MP = methylprednisolone
ß-Blocker = Propranolol

TABLE 4. Preliminary swine therapeutic study. Heart rate.

Pig Number	Time 1 hour Pre	Heart Rate (Beats x Min^{-1}) 0	1	2	3	4	5	6	7	8	12	24	48
Phenoxybenzamine	N/A	111	141	168	189	180	177	178	201	204	N/A	150	
Phenoxybenzamine 2	N/A	66	135	147	168	168	147	162	171	174	N/A	108	
Phenoxybenzamine	N/A	99	147	168	N/A	216	222	210	234	N/A	N/A	189	
Preliminary T-2 Control	90	138	84	120	144	218	214						
P2ß-Blocker	126	132	84	114	168								
P3 MP	120	114	N/A	114	75	168	148	150	168	144	120	N/A	
P4ß-Blocker	112	108	111	84	180	198							
P5 Dopamine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A					
P6 Azium	96	90	114	114	114	108	120	138	141	132	147	231	
P7 Dopamine	90	118	99	96	174	234							

N/A = not available.
*MP = methylprednisolone.
ß-Blocker = Propranolol

TABLE 5. Preliminary swine therapeutic study: Cardiac index.

Pig Number	Time 1 hour Pre	Cardiac Index* (C.O. in ml x min^{-1} x kg)$^{-1}$ 0	1	2	3	4	5	6	7	8	12	24	48
Phenoxy-benzamine	N/A	168	163	160	153	199	150	147	121	120	N/A	131	
Phenoxy-benzamine	N/A	156	140	156	118	115	126	144	111	110	N/A	72	
Phenoxy-benzamine	N/A	125	131	153	N/A	79	72	83	84	93	N/A	103	
Prelim-inary T-2 Control	141	139	97	67	52	38	37	N/A					
P2ß-Blocker	183	156	99	59	31								
P3 MP**	156	144	N/A	80	80	115	115	146	95	163	97		
P4ß-Blocker	180	163	162	80	88	71							
P5 Dopamine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A					
P6 Azium	143	130	133	136	136	153	192	249	150	171	49	71	
P7 Dopamine	153	131	93	64	51	34							

N/A = not available.
*Cardiac index = cardiac output in ml x minutes^{-1} x kg^{-1}.
**MP = methylprednisolone.
ß-Blocker = Propranolol

TABLE 6. Preliminary swine therapeutic study: Stroke volume.*

Pig Number	Time 1 hour Pre	Stroke Volume (mL · beat^{-1} x kg^{-1}) 0	1	2	3	4	5	6	7	8	12	24	48
Phenoxy-benzamine 1	N/A	1.51	1.16	.95	.81	1.11	.85	.83	.60	.59	N/A	.87	
Phenoxy-benzamine 2	N/A	2.36	1.04	1.06	.70	.68	.86	.89	.65	.63	N/A	.67	
Phenoxy-benzamine	N/A	1.26	.89	.91	N/A	.37	.32	.40	.36	N/A	N/A	.54	
Prelim-inary T-2 Control	1.57	1.01	1.15	.56	.36	.17	.17						
P2ß-Blocker	1.45	1.18	1.18	.52	.18								
P3 MP**	1.30	1.26	N/A	.70	1.07	.68	.78	.97	.57	1.13	.81	N/A	
P4ß-Blocker	1.61	1.51	1.46	.95	.49	.44							
P5 Dopamine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A					
P6 Azium	1.49	1.44	1.17	1.19	1.19	1.42	1.60	1.80	1.06	1.30	.33	.31	
P7 Dopamine	1.70	1.11	.94	.67	.29	.15							

N/A = not available
*Stroke volume = heart rate (beats · min^{-1}) divided by cardiac index (mL · min^{-1} · kg^{-1})
**MP = methylprednisolone
ß-Blocker = Propranolol

TABLE 7. Preliminary swine therapeutic study: Mean arterial blood pressure.

Pig Number	Time 1 hour Pre	Mean Arterial Pressure (mm Hg) 0	1	2	3	4	5	6	7	8	12	24	48
Phenoxy-benzamine 1	N/A	120	104	76	62	52	51	45	48	46	N/A	80	
Phenoxy-benzamine 2	N/A	109	100	76	64	56	54	60	48	52	N/A	60	
Phenoxy-benzamine	N/A	120	112	96	N/A	69	58	56	54	N/A	N/A	68	
Prelim-inary T-2 Control	130	115	97	67	52	38	37						
P2ß-Blocker	124	100	124	116	73								
P3 MP*	N/A due to inoperative catheter												
P4ß-Blocker	102	109	94	62	52	58							
P5 Dopamine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A					
P6 Azium	124	122	112	110	58	60	64	67	72	78	114	112	
P7 Dopamine	116	116	100	92	84	72							

N/A = not available.
*MP = methylprednisolone.
ß-Blocker = Propranolol

TABLE 8. Preliminary swine therapeutic study: Arterial blood pH/lactic acid determinations.

Pig Number	Time 1 hour Pre	0	Arterial Blood pH/Lactic Acid (mmoles/L) 1	2	3	4	5	6	7	8	12	24	48
Phenoxy-benzamine 1	N/A/ N/A	7.435/ 1.6	7.407/ 2.9	7.372/ 3.8	7.305/ 2.5	7.255/ 4.2	7.224/ 6.3	7.226/ 3.6	7.250/ 0.9	7.336/ 2.3	N/A/ N/A	7.391/ 1.5	
Phenoxy-benzamine 2	N/A/ N/A	7.428/ 2.6	7.316/ 4.7	7.269/ 2.4	7.279/ 2.1	7.252/ 4.6	7.292/ 4.3	7.301/ 5.5	7.283/ 5.2	7.261/ 5.8	N/A/ N/A	7.324/ 3.1	
Phenoxy-benzamine	N/A/ N/A	7.428 N/A	7.307 N/A	7.336 N/A	N/A N/A	7.285 N/A	7.289 N/A	7.312 N/A	7.328 N/A	7.320 N/A	N/A N/A	7.437 N/A	
Preliminary T-2 Control**	/.85	/1.10	/3.14	/3.02	/5.58	/7.87	/9.08	/16.51					
P2β-Blocker**	/1.71	/1.45	/2.83	/3.72	/6.56								
P3 MP*	/1.53	/1.56	N/A	/3.48	/2.91	/3.14	/3.98	/4.05	/3.86	/3.38	/2.35	N/A	
P4β-Blocker**	/.60	/.57	/2.10	/2.91	/3.05	/6.41							
P5 Dopamine**	N/A/ N/A	N/A/ N/A	N/A/ N/A	N/A/ N/A	N/A/ N/A	N/A/ N/A	N/A/ N/A	N/A/ N/A					
P6 Azium**	/.86	/1.05	/1.55	/1.75	/1.65	/1.87	/1.40	/2.10	/2.51	/2.25	/1.89	/4.88	
P7 Dopamine**	N/A/ N/A	/.84	/7.61	/3.30	/4.12	/6.69							

N/A = not available.
MP* = methylprednisolone
** = blood pH data not available due to malfunctioning blood-gas machine.
β-Blocker = Propranolol

TABLE 9. Preliminary swine therapeutic study: Arterial blood gas determinations.

Pig Number	Time 1 hour Pre	0	Arterial Blood Gases $PaO_2/PaCO_2$ (mm Hg) 1	2	3	4	5	6	7	8	12	24	48
Phenoxy-benzamine 1	N/A/ N/A	77.4/ 45.1	N/A N/A	86.3/ 19.0	96.8/ 37.7	94.2/ 40.7	101.4/ 35.4	92.3/ 37.7	84.6/ 34.0	97.9/ 26.5	N/A/ N/A	99.7/ 26.6	
Phenoxy-benzamine 2	N/A/ N/A	96.5/ 36.1	99.7/ 41.5	91.6/ 34.8	87.2/ 46.6	94.0/ 45.3	101.0/ 39.1	97.8/ 37.5	98.7/ 37.6	99.1/ 37.1	N/A/ N/A	94.2/ 34.1	
Phenoxy-benzamine	N/A/ N/A	99.2/ 39.2	105/9/ 35.3	99.8/ 34.4	N/A/ N/A	101.7/ 31.0	106.1/ 29.7	111.7/ 25.2	104.7/ 21.6	106.5/ 27.2	N/A/ N/A	N/A/ 25.1	
Prelim-inary T-2 Control	98.7/ 35.0	98.7/ 36.9	100.1/ 34.6	101.5/ 31.8	106.6/ 29.9	106.0/ 28.7	119.7/ 23.0	104.6/ 33.6					
P2ß-Blocker	91.2/ 36.0	92.8/ 35.4	97.4/ 37.3	72.5/ 41.8	60.3/ 39.7								
P3 MP*	94.8/ 38.5	75.7/ 33.4	N/A/ N/A	81.9/ 32.0	86.8/ N/A	82.0/ N/A	95.6/ N/A	90.0/ N/A	98.8/ 29.5	95.0/ 38.0	91.8/ 28.2		
P4ß-Blocker	94.3/ 35.7	82.7/ 40.7	95.5/ 34.7	72.5/ 36.7	75.6/ 36.2	67.0/ 30.5							
P5 Dopamine	N/A/ N/A	N/A/ N/A	N/A/ N/A	N/A/ N/A	N/A/ N/A	N/A/ N/A	N/A/ N/A	N/A/ N/A					
P6 Azium	109.4/ 37.4	106.1/ 33.9	99.6/ 32.4	91.0/ 36.0	99.4/ 32.2	97.1/ 36.8	103/ 38.6	107.8/ 36.9	95.9/ 37.0				
P7 Dopamine	N/A/ N/A	90.6/ 41.2	80.9/ 43.3	88.5/ 41.8	82.6/ 39.5	104.9/ 30.9							

N/A = not available.
MP* = methylprednisolone
ß-Blocker = Propranolol

IIC. DEFINITIVE SWINE THERAPEUTIC STUDIES--Bob Poppenga

A. SUMMARY

Four different treatment protocols were assessed for efficacy in the treatment of acute T-2 toxicosis in swine. A variety of physiologic parameters were monitored in order to determine which treatment(s) most effectively maintained homeostasis. All four treatment groups showed improved survival times as compared to a positive T-2 control group.

B. MATERIALS AND METHODS

Approximately 3 to 4 weeks prior to inclusion in this study, 25 kg, female, cross-bred swine were obtained from a nearby commercial swine operation. After a 3- to 4-day acclimation period, fluid-filled catheters were surgically implanted in the main pulmonary artery, left atrium, aorta via the internal thoracic artery and left jugular vein of each animal. All catheters were heparinized and placed subcutaneously for later exteriorization. The swine were allowed an appropriate recovery period.

On the day prior to dosing, each animal was anesthetized with halothane and the subcutaneously placed catheters were located and exteriorized. In addition, one or two 18 gauge, 3-inch Angiocaths® were secured in ear vein(s) and a foley catheter placed in the bladder and secured in place. The exposed catheters were bandaged for protection and the animal was allowed to recover from anesthesia. Water but not food was provided ad libitum overnight.

On the morning of dosing, the individual animal was placed in a restraining apparatus which allowed a certain degree of full movement. Appropriate monitoring equipment was attached to the animal at this time.

Parameters to be assessed included hemodynamics, electrocardiogram, blood-gas variables, hematology, serum chemistries and clinical variables (respiratory rate, urine production and capillary refill time). Each of the above parameters was to be assessed at hourly intervals beginning 1 hour prior to dosing with T-2 toxin and continuing through 8 hours post-dosing and, when survival permitted at 12, 24 and 48 hours post-dosing.

Each animal had been previously randomly assigned to 1 of 5 experimental groups (n = 3). These are listed in Table 1. The individual therapeutic agents utilized in this study and their administration protocols are given in Table 2. Doses employed were either based on previous recognized uses of the particular agents (such as activated charcoal or magnesium sulfate) and were thus the same for each individual on a body weight basis, or were based on trying to maintain certain physiologic parameters, e.g., blood pH and vascular pressures within normal limits using sodium bicarbonate and normal saline. In the latter case, the amounts administered varied among individuals depending on a particular animal's needs.

A maximum observation period of 48 hours was selected after which surviving animals were killed with a barbiturate. A longer observation period was considered but discarded due to the difficulty of keeping exposed catheters clean and free from infections which might have altered physiologic parameters.

At death, all animals were necropsied and tissues saved for histopathologic examination. Fresh bone marrow smears were made for later examination, and cardiac muscle and pancreatic tissue samples were frozen for determination of tissue electrolyte levels (sodium, potassium, magnesium and calcium).

Many measured parameters have yet to be examined in detail. Appropriate statistical analyses will be forthcoming at a later date.

C. RESULTS

As mentioned above, a large number of parameters have not been examined in depth. Several key parameters have been tabulated and are included in this report.

Group survival times can be seen in Table 1. All treatment groups survived a minimum of twice as long as the control group. Two groups, number 2 and number 5, survived approximately four times as long. The ranges within each group are also given.

Tables 3 through 11 tabulate several important variables including blood glucose, total and ionized serum calcium, serum magnesium, heart rate, cardiac index, stroke volume, mean arterial blood pressure, blood pH, lactic acid, PaO_2 and $PaCO_2$.

D. DISCUSSION

The common denominator among all T-2-treated groups was the administration of metoclopramide (Reglan®, AH Robins Company) and dexamethasone sodium phosphate (Azium S/P®, Schering) to all animals. Metoclopramide was also given to the 3 animals in the T-2 control group.

Metoclopramide was included as an antiemetic with the hope that animals given activated charcoal and magnesium sulfate PO would retain a significant portion of the administered dose. Partial success was achieved, vomiting still occurred although at a much reduced rate based upon previous experience. Vomiting generally did not occur until shortly after the administration of the activated charcoal-magnesium sulfate slurry via stomach tube. Gravity flow was not sufficient for administering

the slurry. Therefore, a hand pump was used to speed the process. The quick expansion of the stomach with a volume of approximately 1/2 liter appeared to be enough to stimulate the onset of emesis which continued for approximately 30 to 45 minutes. Also, the metoclopromide was given in three discrete IV doses (see Table 2) which, in retrospect, resulted in excessive intervals between doses. A continuous IV drip would probably have been more appropriate based upon its very short t-1/2 following intravenous administration in man.

It is evident from the group survival data that dexamethasone probably contributed in a major way to enhanced survival times. One interesting point, however, is the difference between group number 3's mean of 17.8 hours and group 2, 4 and 5's means of 35.16 hours, 27.30 hours and 39.80 hours, respectively. While the statistical significance of this difference has not been determined, it does appear relatively large. Group 3 received all the therapeutic agents with the exception of activated charcoal and magnesium sulfate given PO. One hypothesis that these results may support is that following IV administration of T-2 toxin, there is a significant enterohepatic circulation of T-2 toxin or its metabolites. Recirculating parent T-2 or T-2 metabolites may adversely impact on animal survival if not trapped in the gut by activated charcoal. Another hypothesis that this survival data may support is that endotoxin may play a secondary role in the pathophysiology of acute T-2 toxicosis following its systemic absorption through T-2 mediated severely compromised gut wall defenses.

Whichever hypothesis, if either, is true, one thing can be said about giving activated charcoal and magnesium sulfate: it moves very slowly through the gastrointestinal tract. Despite the early occurrence of

diarrhea, only those animals surviving 48 hours passed charcoal-tainted feces. Those animals which lived less than 24 hours seldom had significant amounts of charcoal beyond the cecum on postmortem examination. This fact combined with the observation of significant bloating in live animals and postmortem gas-distended bowels implies a gut stasis is occurring despite early and often severe diarrhea.

Individual animals showed rather large increases in serum magnesium levels with time (see Table 5), although data means do not appear to reflect this. Absorption of magnesium from the gut following administration of magnesium sulfate is a possible explanation for this observation, as is release of intracellular magnesium following cell injury.

The administration of normal saline by gravity flow was not effective in maintaining arterial blood pressure or, it would appear, venous return. In fact, much of the administered fluid probably found its way into the gastrointestinal tract. Occasionally, significant amounts of subcutaneous edema and fluid in the pleural and peritoneal cavities were found on postmortem despite no evidence of fluid overloading as judged by central venous pressure and pulmonary auscultation. In several instances, with aggressive fluid therapy, urine output could be maintained despite low arterial blood pressures. In other instances, anuria would occur despite administration of large volumes of fluid over a period of time.

Administration of sodium bicarbonate was effective in maintaining a tolerable blood pH (see Table 10). Despite cessation of sodium bicarbonate therapy when blood pH went above 7.350, animals surviving 48 hours often had a slight alkalosis with a blood pH slightly above 7.500 (see Table 10).

By and large, the ability to control the acidosis with sodium bicarbonate was reflected in the blood-gas variables (see Table II).

Once all data is analyzed, more definitive conclusions will be drawn.

E. OVERALL COMMENTS

1. Glucocorticosteroids will enhance survival in swine and rats dosed intravenously with T-2 toxin. Combinations of glucocorticosteroids and other anti-shock drugs (such as prostanglandin E) need to be investigated.
2. Ancillary treatments are important in maintaining physiologic homeostasis. Sodium bicarbonate is an example. Maintenance of blood glucose levels would also appear to be an important adjunctive therapy.
3. Adequate arterial blood pressure cannot be maintained with fluid therapy alone. Plasma expanders such as dextran should be investigated.
4. Oral activated charcoal may be of benefit following parenteral exposure to T-2 toxin.

TABLE 1. Definitive swine therapeutic study.

Group (n = 3)	Treatment	Mean Weight (kg)	Survival (Hours ± SEM)
1	Control T-2 + Metoclopramide	51.0	8.58 ± 0.84 Range 7 hours and 45 minutes to 9 hours and 25 minutes
2	Metoclopramide Dexamethasone Normal saline and $NaHCO_3$ Activated charcoal + $MgSO_4$ (All Therapy)	53.3	35.1 ± 22.23 Range 9 hours and 30 minutes to 48 hours
3	Metoclopramide Dexamethasone Normal saline and $NaHCO_3$ (No Activated Charcoal)	45.5	17.94 ± 2.17 Range 15 hours and 45 minutes to 20 hours and 5 minutes
4	Metoclopramide Dexamethasone Activated Charcoal + $MgSO_4$ Normal Saline (No $NaHCO_3$)	47.8	27.15 ± 18.89 Range 11 hours and 16 minutes to 48 hours
5	Metoclopramide Dexamethasome Activated Charcoal + $MgSO_4$ $NaHCO_3$ (No Saline)	53.6	39.76 ± 14.26 Range 23 hours and 30 minutes to 48 hours

TABLE 2. Definitive swine therapeutic study: Drug administration protocol

Drug	Source	Dosage Regimen
Metoclopramide	Injectable form (5 mg/mL) courtesy of A. H. Robins Company	1 mg/kg body weight IV immediately prior to T-2 toxin administration and 1/4 and 1-1/4 hour post T-2 toxin.
Activated Charcoal	SuperChar® courtesy of Gulf BioSystems, Inc., Dallas, TX	2 grams activated charcoal (dry weight) per kg body weight in 420 mL tap water PO 1/2 hour and 4 hours post T-2.
Magnesium Sulfate	Epsom salt, magnesium sulfate USP, purchased from Dow Chemical Company	1/2 gram/kg body weight PO mixed with activated charcoal slurry and administered 1/2 hour and 4 hours post T-2
Dexamethasone Sodium Phosphate	Azium S/P® (4 mg dexamethasone sodium phosphate/mL), purchased from Schering Corporation	6 mg/kg body weight IV immediately and 4 hours post T-2, then 4 mg/kg 8 and 12 hours post T-2, followed by 2 mg/kg 16 and 20 hours post T-2 and 1 mg/kg 24 hours post T-2.
Sodium Bicarbonate	5 percent sodium bicarbonate injection, USP. Purchased from Abbott Laboratories.	Variable speed drip based on hourly blood pH measurements. Started if pH < 7.350 and stopped if pH > 7.350.
Normal Saline	0.9 percent sodium chloride injection, USP. Purchased from Abbott Laboratories.	Rapid IV drip (gravity flow) as MAP* begins decline. Administration slowed to maintenance levels if MAP does not respond or if CVP** > 10 mmHg.

*Mean arterial blood pressure.
**Central venous pressure.

TABLE 3. Definitive swine therapeutic study: Blood glucose concentrations.

Group	Pig No.	Time 1 Hour Pre	Glucose (mg/dL) 0	1	2	3	4	5	6	7	8	12	24	48
Control	380	110	130	115	109	113	73	49	48	35	36			
	394	115	109	109	128	116	43	58	43	33	34			
1	420	105	118	135	259	252	117	52	54	59	173			
	Mean	110	119	120	165	160	78	53	48	42	81			
	± SEM	±2.9	±6.0	±7.9	±47.2	±45.8	±21.5	±2.7	±3.2	±8.4	±46.0			
All therapy	396	114	110	115	103	81	70	49	44	61	66	96	99	95
	410	114	109	83	112	134	133	77	49	23	23			
2	424	108	99	99	99	108	95	65	63	72	76	90	99	90
	Mean	112	106	99	105	108	99	64	52	52	55	93	99	93
	± SEM	±2.0	±3.5	±9.2	±3.9	±15.3	±18.3	±8.1	±5.7	±14.8	±16.3	±3.0	±0.0	±3.5
No Activated	386	105	108	76	85	84	66	63	62	75	99	66		
Charcoal	414	129	151	118	92	78	70	66	66	65	60	79		
3	428	102	111	88	109	102	77	69	43	39	36	24		
	Mean	112	123	94	95	88	71	66	57	60	65	56		
	± SEM	±8.5	±13.9	±12.5	±7.1	±7.2	±3.2	±1.7	±7.1	±10.7	±18.4	±16.6		
No HCO_3	400	92	95	92	83	79	55	50	50	53	61	55		
	418	93	109	98	91	86	57	44	46	53	58	58		
4	426	95	108	93	112	110	117	78	67	69	67	82	71	93
	Mean	95	104	94	95	92	76	57	54	58	62	65	71	93
	± SEM	±1.7	±4.5	±1.8	±8.7	±9.4	±20.3	±10.5	±6.4	±5.3	±2.7	±8.5		
No Saline	398	135	105	102	102	106	97	62	68	67	62	73	93	98
	416	96	103	68	92	94	79	77	132	82	65	46	78	
5	422	104	116	83	91	103	84	56	51	59	63	77	95	95
	Mean	112	108	84	95	101	87	65	84	69	63	65	89	97
	± SEM	±11.9	±4.0	±9.8	±3.5	±3.6	±5.4	±6.2	±24.7	±6.8	±0.9	±9.8	±5.4	±1.5

TABLE 4. Definitive swine therapeutic study: Total serum calcium/ionized serum calcium concentrations.

Group	Pig no.	Time 1 hr Pre	Total Calcium/Unbound Calcium (mg/dL) 0	1	2	3	4	5	6	7	8	12	24	48
Control	380	8.5/ 5.8	8.9/ 5.7	8.5/ 5.9	8.0/ 4.9	7.1/ 4.4	6.7/ 4.6	6.1/ 4.2	5.7/ 4.1	5.8/ 4.3	5.4/ 4.0			
	394	9.3/ 4.6	9.5/ 5.6	9.2/ 3.9	8.6/ 4.8	8.0/ 3.3	7.7/ 4.9	7.0/ 4.8	6.8/ 4.2	6.7/ 4.6	7.4/ 4.4			
1	420	9.2/ 5.7	9.2/ 6.3	7.8/ 5.2	7.5/ 5.4	7.5/ 4.8	6.9/ 3.7	6.3/ 4.5	6.2/ 4.0	5.9/ 3.2	6.1/ 4.1			
	Mean	9.0/ 5.4	9.2/ 5.9	8.5/ 5.0	8.0/ 5.0	7.5/ 4.2	7.1/ 4.4	6.5/ 4.5	6.2/ 4.1	6.1/ 4.0	6.3/ 4.2			
All therapy	396	9.5/ 4.4	9.3/ 4.2	9.2/ 3.6	8.3/ 4.2	6.8/ 4.6	6.0/ 3.0	5.7/ 4.7	5.7/ 4.1	5.2/ 3.6	5.1/ 3.5	N/A/ 2.7	N/A/ 3.4	9.0/ 4.3
	410	9.6/ 3.7	9.2/ 3.8	8.2/ 4.0	8.0/ 4.1	7.1/ 4.0	5.5/ 1.8	4.7/ 1.9	5.1/ 2.0	4.9/ 2.0	5.3/ 3.8			
2	424	9.9/ 4.8	10.0/ 5.8	10.1/ 5.1	9.2/ 4.8	9.7/ 4.6	9.7/ 4.8	9.6/ 3.8	9.8/ 3.3	9.1/ 3.5	9.0/ 3.3	9.1/ 3.1	8.9/ 4.3	9.0/ 5.0
	Mean	9.7/ 4.3	9.5/ 4.6	9.2/ 4.2	8.5/ 4.4	7.9/ 4.4	7.1/ 3.2	6.7/ 3.5	6.9/ 3.1	6.4/ 3.0	6.5/ 3.5	9.1/ 2.9	8.9/ 3.9	9.0/ 4.7
No Activated Charcoal	386	9.4/ 5.0	9.1/ 4.2	9.1/ 5.7	7.8/ 5.4	6.6/ 4.3	5.5/ 3.9	5.3/ 3.7	4.9/ 3.1	4.4/ 3.0	4.5/ 2.8	N/A/ 1.9		
	414	9.6/ 1.6	9.1/ 1.35	9.0/ 1.4	7.9/ 2.6	6.6/ 1.39	6.0/ 2.07	5.6/ 1.98	5.3/ 2.11	4.1/ 1.85	4.0/ 1.68	4.1/ 0.63		
3	428	9.1/ 5.0	9.3/ 5.6	9.0/ 4.9	7.9/ 4.6	6.9/ 4.9	6.5/ 4.5	6.0/ 4.1	5.5/ 3.7	5.4/ 3.6	5.5/ 3.7	5.6/ 3.67		
	Mean	9.4/ 3.9	9.2/ 3.72	9.0/ 4.0	7.9/ 4.2	6.7/ 3.5	6.0/ 3.49	5.6/ 3.26	5.2/ 2.97	4.6/ 2.82	4.7/ 2.72	4.9/ 2.07		

TABLE 4. CONTINUED

Group	Pig no.	Time 1 hr Pre	Total Calcium/Unbound Calcium (mg/dL) 0	1	2	3	4	5	6	7	8	12	24	48
No HCO_3	400	9.3/ 5.8	9.4/ 4.5	9.4/ 4.1	8.7/ 3.6	6.7/ 4.8	5.2/ 5.6	5.6/ 5.3	5.0/ 5.1	N/A/ 4.8	5.7/ 3.1	3.7/ 3.4		
	418	9.7/ 4.3	9.4/ 4.3	9.2/ 5.3	9.0/ 5.2	7.1/ 4.3	6.7/ 4.6	6.8/ 4.8	6.7/ 4.9	6.7/ 4.2	6.6/ 4.3	8.2/ 4.7		
4	426	9.1/ 5.5	9.7/ 7.9	8.7/ 5.1	7.3/ 5.25	7.1/ 4.8	6.9/ 4.4	6.4/ 4.7	6.2/ 4.1	6.2/ 3.7	5.9/ 3.6	5.5/ 3.8	6.8/ 4.4	8.9/ 6.0
	Mean	9.4/ 5.2	9.5/ 5.6	9.1/ 4.8	8.3/ 4.68	7.0/ 4.6	6.3/ 4.9	6.3/ 4.9	6.0/ 4.7	6.5/ 4.2	6.1/ 3.7	5.8/ 4.0	6.8/ 4.4	8.9/ 6.0
No Saline	398	9.5/ 2.5	9.3/ 2.1	9.3/ 4.8	8.6/ 4.4	8.3/ 2.8	6.2/ 2.5	6.0/ 2.5	5.8/ 2.7	5.6/ 2.0	5.5/ 2.3	4.8/ 3.1	6.1/ 2.6	8.6/ 3.1
	416	9.6/ 5.4	9.5/ 3.0	8.8/ 5.0	8.1/ 5.0	7.2/ 3.75	6.6/ 3.0	6.2/ 3.4	5.7/ 3.2	5.3/ 2.6	4.0/ 2.3	3.7/ 1.8	3.9/ 2.5	
5	422	9.3/ 5.0	8.9/ 5.2	9.1/ 6.2	8.3/ 7.6	7.6/ N/A	6.5/ 5.3	6.0/ 4.0	5.5/ N/A	5.0/ 4.5	4.9/ 3.4	N/A/ N/A	N/A/ N/A	8.3/ N/A
	Mean	9.5/ 4.3	9.2/ 3.4	9.1/ 5.3	8.3/ 5.7	7.7/ 3.28	6.4/ 3.6	6.1/ 3.3	5.7/ 3.0	5.3/ 3.0	4.8/ 2.7	4.3/ 2.5	5.0/ 2.6	8.5/ 3.1

N/A = not available.

TABLE 5. Definitive swine therapeutic study: Serum magnesium concentrations.

Group	Pig No.	Time 1 Hour Pre	Serum Magnesium (µg/mL) 0	1	2	3	4	5	6	7	8	12	24	48
Control	380	19.4	17.9	22.7	21.6	20.5	20.1	21.8	21.6	23.5	24.2			
	394	20.3	22.1	22.1	22.8	25.0	23.2	25.4	25.4	26.9	32.2			
1	420	17.4	19.1	17.4	18.1	19.1	18.8	19.4	21.8	21.4	23.4			
	Mean	19.0	19.7	20.7	20.8	21.5	20.7	22.2	22.9	23.9	26.6			
	± SEM	±.9	±1.3	±1.7	±1.4	±1.8	±1.3	±1.7	±1.2	±1.6	±2.8			
All therapy	396	17.4	20.7	19.6	21.4	19.6	18.8	23.0	23.6	25.8	26.1	27.6	19.9	17.7
	410	20.3	19.5	17.8	20.3	19.1	21.2	22.5	39.0	36.4	37.3			
2	424	21.9	22.2	21.9	20.0	19.4	18.9	20.5	23.7	24.7	24.4	24.3	20.4	N/A
	Mean	19.9	20.8	19.8	20.6	19.4	19.6	22.0	28.8	29.0	29.3	26.0	20.2	17.7
	± SEM	±1.3	±.8	±1.2	±.4	±.2	±.8	±.8	±5.1	±3.7	±4.0	±1.6	±.3	--
No Activated	386	17.3	17.7	16.6	16.2	15.2	13.9	14.3	15.0	14.7	16.6	18.1		
Charcoal	414	18.8	18.8	18.4	16.8	16.0	16.8	18.8	15.2	15.6	16.8	18.4		
3	428	21.7	18.7	19.6	20.4	19.6	19.2	19.6	19.2	19.6	20.4	N/A		
	Mean	19.3	18.4	18.2	17.8	16.9	16.6	17.6	16.5	16.6	17.9	18.3		
	± SEM	±1.3	±.3	±.9	±1.3	±1.3	±1.6	±1.7	±1.4	±1.5	±1.2	±.1		
No HCO_3	400	17.3	17.3	18.1	19.0	18.1	18.1	26.4	29.0	26.4	26.9	31.5	32.3	
	418	20.1	20.9	20.9	23.3	18.5	21.7	24.8	30.4	28.1	31.2	43.5		
4	426	24.7	22.8	21.7	19.8	20.2	19.8	19.0	18.9	20.6	19.4	20.6	18.0	N/A
	Mean	20.7	20.3	20.2	20.7	18.9	19.9	23.4	26.1	25.0	25.8	31.9	25.2	
	± SEM	2.1	±1.6	±1.1	±1.3	±.6	±1.0	±2.3	±3.6	±2.3	±3.5	±6.6	±7.1	
No Saline	398	22.3	21.5	20.6	21.5	22.3	20.6	23.1	24.8	24.0	24.0	N/A	21.5	17.3
	416	20.1	22.5	17.7	19.3	20.9	19.3	30.2	30.4	31.2	31.2	30.4	32.0	
5	422	21.7	19.2	19.9	21.4	21.7	20.6	26.3	28.7	28.9	28.4	N/A	N/A	N/A
	Mean	21.4	21.1	19.4	20.7	21.6	20.2	26.5	28.0	28.0	27.9	30.4	26.8	17.3
	± SEM	±.6	±1.0	±.9	±.7	±.4	±.5	±2.1	±1.7	±2.1	±2.1	--	±5.2	--

TABLE 6. Definitive swine therapeutic study: Heart rate.

Group	Pig No.	Time 1 Hour Pre	Heart Rate (Beats · min^{-1}) 0	1	2	3	4	5	6	7	8	12	24	48
Control	380	102	99	99	108	174	168	195	168	177	192			
	394	114	111	120	168	192	216	225	237	246	N/A			
1	420	87	81	81	123	192	195	228	189	216	N/A			
	Mean	101	97	100	133	186	193	216	198	213	192			
	± SEM	±7.8	±8.7	±11.3	±18.0	±6.0	±13.9	±10.5	±20.4	±20.0	--			
All therapy	396	72	75	87	102	126	150	147	150	153	162	213	162	138
	410	108	123	108	111	120	162	168	168	162	168			
2	424	75	84	84	129	123	153	122	144	146	180	N/A	99	N/A
	Mean	85	94	93	114	123	155	146	154	154	170	213	131	138
	± SEM	±11.5	±14.7	±7.6	±7.9	±1.7	±3.6	±13.3	±7.2	±4.6	±5.3	--	±31.5	
No Activated	386	144	117	141	138	147	216	219	222	192	204	210		
Charcoal	414	132	93	96	111	126	222	216	207	201	180	171		
3	428	102	123	129	207	204	207	180	177	183	165	144		
	Mean	126	111	122	152	159	215	205	202	192	183	175		
	± SEM	±12.5	±9.7	±13.5	±28.6	±23.3	±4.3	±12.5	±13.2	±5.2	±11.4	±19.2		
No HCO_3	400	102	99	99	93	129	135	135	150	135	132	132		
	418	111	102	111	111	138	156	174	174	168	165	N/A		
4	426	102	93	108	114	135	150	174	156	144	174	120	120	114
	Mean	105	98	106	106	134	147	161	160	149	157	129	120	114
	± SEM	±3.0	±2.7	±3.6	±6.6	±2.7	±6.2	±13.0	±7.2	±9.9	±12.8	±3.0		
No Saline	398	90	117	117	114	117	159	156	156	177	174	150	96	126
	416	120	87	87	111	144	174	147	144	168	165	135	162	
5	422	102	108	93	117	129	177	180	174	177	168	141	120	117
	Mean	104	104	99	114	130	170	161	158	174	169	142	126	122
	± SEM	±8.7	±8.9	±9.2	±1.7	±7.8	±5.5	±9.9	±8.7	±3.0	±2.7	±4.3	±19.3	±4.5

*N/A = not available.

TABLE 7. Definitive swine therapeutic study: Cardiac index.

Group	Pig No.	Time 1 Hour Pre	Cardiac Index (mL · min^{-1} · kg^{-1}) 0	1	2	3	4	5	6	7	8	12	24	48
Control	380	160.7	161.5	259.3	258.0	203.4	160.0	126.2	107.4	112.8	96.8			
	394	181.8	167.0	176.9	173.6	146.8	125.9	101.5	100.9	113.4	N/A			
1	420	186.3	129.3	134.2	136.7	127.0	112.3	81.8	82.5	76.9	67.6			
	Mean	176.3	152.6	190.1	189.4	159.1	132.7	103.2	96.9	101.0	82.2			
	± SEM	±7.9	±11.8	±36.7	±35.9	±22.9	±14.2	±12.8	±7.4	±12.1	±14.6			
All therapy	396	128.7	138.2	144.1	152.1	166.9	170.6	180.6	179.3	170.3	158.1	134.1	174.9	121.2
	410	190.1	218.4	238.0	205.1	127.2	113.7	96.7	98.5	90.7	48.7			
2	424	107.7	138.1	167.2	198.5	183.4	152.1	177.7	182.9	N/A	N/A	N/A	N/A	N/A
	Mean	142.2	164.9	183.1	185.2	159.2	145.5	151.7	153.6	130.5	103.4	134.1	174.9	121.2
	± SEM	±24.7	±26.7	±28.2	±16.7	±16.7	±16.7	±27.5	±27.5	±39.8	±54.7			
No Activated	386	185.3	143.2	210.1	201.7	190.1	159.6	148.0	165.5	167.5	195.7	167.3		
Charcoal	414	153.6	137.7	161.2	208.2	259.7	234.2	209.6	181.8	190.1	134.6	118.2		
3	428	169.6	142.0	152.8	150.0	152.8	149.4	149.6	155.4	156.8	192.8	158.6		
	Mean	169.5	141.0	174.7	186.6	200.9	181.1	169.1	167.6	171.5	174.4	148.0		
	± SEM	±9.2	±1.7	±17.8	±18.4	±31.3	±26.7	±20.3	±7.7	±9.8	±19.9	±15.1		
No HCO_3	400	186.4	140.0	156.3	152.7	171.5	202.0	187.2	176.1	175.3	167.3	146.8		
	418	184.9	139.1	136.2	151.3	175.3	159.6	164.6	128.3	120.3	98.9			
4	426	140.2	120.8	N/A	N/A	N/A	N/A	N/A	N/A	N/A	136.8	140.9	144.6	150.1
	Mean	170.5	133.3	146.3	152.0	173.4	180.8	175.9	152.2	147.8	134.3	143.9	144.6	150.1
	± SEM	±15.2	±6.2	±10.0	±0.7	±1.9	±21.2	±11.3	±23.9	±27.5	±19.8	±3.0		
No Saline	398	152.8	181.8	156.7	170.7	125.2	141.2	149.2	130.6	124.6	131.2	136.6	137.8	286.7
	416	189.2	166.0	171.5	163.3	148.8	150.9	143.2	152.5	129.5	156.4	144.1	N/A	
5	422	136.5	134.4	141.2	145.7	130.7	123.2	113.5	115.6	102.5	113.1	125.7	153.1	140.2
	Mean	159.5	160.7	156.5	159.9	134.9	138.4	135.3	132.9	118.9	133.6	135.5	145.5	213.5
	± SEM	±15.6	±13.9	±8.8	±7.4	±7.1	±8.1	±11.0	±10.7	±9.6	±12.5	±5.4	±7.6	±73.3

TABLE 8. Definitive swine therapeutic study: Stroke volume.

Group	Pig No.	Time 1 Hour Pre	Stroke Volume (mL · beat^{-1} · kg^{-1}) 0	1	2	3	4	5	6	7	8	12	24	48
Control	380	1.58	1.63	2.62	2.39	1.17	0.95	0.65	0.64	0.64	0.50			
	394	1.59	1.56	1.47	1.03	0.76	0.58	0.45	0.43	0.46	N/A			
1	420	2.14	1.60	1.66	1.11	0.66	0.58	0.36	0.44	0.36	N/A			
	Mean	1.77	1.58	1.92	1.51	0.86	0.70	0.49	0.50	0.49	0.50			
	± SEM	±.18	±.04	±.36	±.44	±.16	±.12	±.09	±.07	±.08				
All therapy	396	1.79	1.84	1.66	1.49	1.32	1.14	1.23	1.20	1.11	0.98	0.63	1.08	0.88
	410	1.76	1.78	2.20	1.85	1.06	0.70	0.58	0.57	0.56	0.29			
2	424	1.44	1.64	1.99	1.54	1.49	0.99	1.46	1.27	N/A	N/A	N/A	N/A	N/A
	Mean	1.66	1.75	1.95	1.63	1.29	0.94	1.09	1.01	0.84	0.64	0.63	1.08	0.88
	± SEM	±.11	±.06	±.16	±.12	±.13	±.13	±.27	±.23	±.28	± 35			
No Activated Charcoal	386	1.39	1.22	1.49	1.46	1.29	0.74	0.68	0.75	0.87	0.96	0.80		
	414	1.16	1.48	1.58	1.88	2.06	1.05	0.97	0.88	0.95	0.75	0.69		
3	429	1.66	1.15	1.18	0.72	0.75	0.72	0.83	0.88	0.86	1.17	1.10		
	Mean	1.37	1.28	1.45	1.35	1.37	0.84	0.83	0.84	0.89	0.96	0.86		
	± SEM	±.14	±.10	±.24	±.34	±.38	±.11	±.09	±.05	±.03	±.12	±.12		
No HCO_3	400	1.83	1.41	1.58	1.64	1.33	1.50	1.39	1.17	1.30	1.27	1.11		
	418	1.67	1.36	1.23	1.36	1.27	1.02	0.95	0.74	0.72	0.60			
4	426	1.37	1.30	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.79	1.12	1.21	1.32
	Mean	1.62	1.36	1.41	1.50	1.30	1.26	1.17	0.96	1.01	0.89	1.12	1.21	1.32
	± SEM	±.13	±.03	±.19	±.14	±.03	±.24	±.22	±.21	±.29	±.20			
No Saline	398	1.70	1.55	1.34	1.50	1.07	0.89	0.96	0.84	0.70	0.75	0.91	1.44	2.28
	416	1.58	1.91	1.97	1.47	1.03	0.87	0.97	1.06	0.77	0.95	1.07	N/A	
5	422	1.34	1.24	1.52	1.25	1.01	0.70	0.63	0.66	0.50	0.67	0.89	1.28	1.20
	Mean	1.54	1.57	1.61	1.41	1.04	0.82	0.85	0.85	0.68	0.79	0.96	1.36	1.74
	± SEM	±.10	±.20	±.18	±.08	±.02	±.06	±.11	±.12	±.06	±.08	±.06	±.08	±.54

N/A = not available.

TABLE 9. Definitive swine therapeutic study: Mean arterial blood pressures.

Group	Pig No.	Time 1 Hour Pre	Mean Arterial Pressure (mmHg) 0	1	2	3	4	5	6	7	8	12	24	48
Control	380	111	111	100	86	69	62	48	56	52	56			
	394	116	110	106	86	66	56	50	58	54	N/A			
1	420	141	106	108	111	81	62	53	48	48	50			
	Mean	123	109	105	94	72	60	50	54	51	53			
	± SEM	±9.3	±1.5	±2.4	±8.3	±4.6	±2.0	±1.4	±3.1	±1.8	±3.0			
All therapy	396	118	119	105	96	72	57	53	58	54	55	82	78	82
	410	110	102	90	102	72	58	52	48	40	52			
2	424	70	113	134	122	112	105	87	82	139	116	N/A	96	N/A
	Mean	99	111	110	107	85	73	64	63	78	74	82	87	82
	± SEM	±14.8	±5.0	±12.9	±7.9	±13.3	±15.8	±11.5	±10.1	±30.9	±20.8			
No Activated	386	106	99	94	82	68	62	60	66	66	80	85		
Charcoal	414	108	116	106	100	72	56	62	57	56	58	66		
3	428	128	116	114	82	54	52	63	64	76	66	114		
	Mean	114	110	105	88	65	57	62	66	66	68	88		
	± SEM	±7.0	±5.7	±5.8	±6.0	±5.5	±2.9	±0.9	±2.7	±5.8	±6.4	±14.0		
No HCO_3	400	118	114	118	97	94	58	64	50	54	54	56		
	418	124	119	128	100	74	66	62	58	56	58			
4	426	138	129	135	112	98	79	80	78	81	79	104	97	101
	Mean	127	121	127	103	89	68	69	62	64	64	80	97	101
	± SEM	±5.9	±4.4	±4.9	±4.6	±7.4	±6.1	±5.7	±8.3	±8.7	±7.7	±24.0		
No Saline	398	119	106	114	100	86	64	62	60	62	60	78	92	94
	416	120	110	102	90	54	52	52	50	44	50	54	30	
5	422	118	118	116	96	74	60	54	48	46	52	76	84	80
	Mean	119	111	111	95	71	57	56	53	51	54	69	69	87
	± SEM	±0.6	±3.5	±4.4	±2.9	±9.4	±3.5	±3.1	±3.7	±5.7	±3.1	±7.7	±19.5	±7.0

N/A = not available

TABLE 10. Definitive swine therapeutic study: Arterial blood pH/lactic acid determinations.

Group	Pig No.	Time 1 hr Pre	pH/Lactic Acid (mmoles/L) 0	1	2	3	4	5	6	7	8	12	24	48
Control	380	N/A/ .77	N/A/ .79	N/A/ 1.12	N/A/ 1.32	N/A/ 2.75	N/A/ 3.84	N/A/ 5.42	N/A/ 5.65	N/A/ 6.88	N/A/ 8.23			
	394	7.448/ .71	7.448/ .66	7.417/ 2.02	7.315/ 2.27	7.245/ 2.13	7.238/ 2.20	7.261/ 1.98	7.216/ 2.91	7.249/ 4.92	6.977/ 1.11			
1	420	7.460/ .86	7.426/ .86	7.425/ 1.35	7.319/ 2.42	7.274/ 1.46	7.230/ 3.74	7.251/ 3.85	7.244/ 3.91	7.234/ 3.75	7.185/ 6.88			
	Mean	7.454/ .78	7.437/ .77	7.421/ 1.50	7.317/ 2.00	7.260/ 2.11	7.234/ 3.26	7.256/ 3.75	7.230/ 4.16	7.242/ 5.18	7.081/ 8.74			
All Therapy	396	7.442/ .74	7.430/ .69	7.398/ 1.48	7.373/ 1.08	7.299/ 1.11	7.314/ 1.19	7.291/ 2.05	7.319/ 2.36	7.340/ 2.10	7.357/ 1.89	7.400/ 2.06	7.524/ 1.55	7.504/ 1.38
	410	7.476/ 1.62	7.480/ 2.02	7.382/ 2.49	7.314/ 2.62	7.272/ 2.86	7.270/ 3.25	7.271/ 3.15	7.275/ 3.57	7.335/ 3.21	7.012/ 15.14			
2	424	7.498/ .94	7.489/ 1.07	7.422/ 1.61	7.350/ 1.62	7.336/ 1.22	7.325/ 1.90	7.358/ 1.19	7.374/ .87	7.431/ 1.73	N/A/ 5.30	N/A/ 1.72	7.496/ .94	7.519/ 1.87
	Mean	7.472/ 1.10	7.466/ 1.26	7.401/ 1.86	7.346/ 1.77	7.302/ 1.73	7.303/ 2.11	7.307/ 2.13	7.323/ 2.17	7.369/ 2.35	7.185/ 7.44			
No Activated Charcoal	386	N/A/ .59	N/A/ .67	N/A/ 2.47	N/A/ 1.23	N/A/ .89	N/A/ 1.08	N/A/ 1.81	N/A/ 1.43	N/A/ /1.46	N/A/ 1.58	N/A/ 9.00	N/A/ 3.60	
	414	7.422/ .52	7.443/ .48	7.363/ 1.16	7.321/ .95	7.301/ 1.66	7.263/ 2.25	7.325/ 2.44	7.327/ 1.55	7.316/ 1.33	7.347/ 1.27	7.339/ 3.40		
3	428	7.418/ 1.19	7.429/ 2.27	7.321/ 3.18	7.331/ 2.59	7.295/ 1.94	7.280/ 1.23	7.347/ 1.33	7.419/ 1.51	7.381/ 1.14	7.397/ .95	7.413/ 1.27		
	Mean	7.420/ .77	7.436/ 1.14	7.342/ 1.27	7.326/ 1.59	7.298/ 1.50	7.262/ 1.52	7.336/ 1.86	7.373/ 1.50	7.349/ 1.31	7.372/ 1.27	7.376/ 4.56		

TABLE 10 CONTINUED

Group	Pig No.	Time 1 hr Pre	pH/Lactic Acid (mmoles/L) 0	1	2	3	4	5	6	7	8	12	24	48
No HCO_3	400	7.469/ .74	7.461/ .68	7.354/ 2.57	7.324/ 1.94	7.241/ 1.51	7.232/ 2.28	7.192/ 2.80	7.208/ 2.29	7.231/ 1.32	7.288/ 1.13	7.310/ .92		
	418	7.460/ 2.12	7.426/ .95	7.384/ 1.94	7.281/ 2.37	7.229/ 2.00	7.228/ 1.44	7.185/ 3.35	7.234/ 2.57	7.225/ 3.04	7.267/ 2.84	N/A/ 10.12		
4	426	7.451/ .83	7.477/ .75	7.371/ 1.48	7.344/ 1.22	7.275/ 1.82	7.273/ 1.89	7.258/ 2.13	7.272/ 1.21	7.333/ 2.13	7.337/ 1.26	7.377/ 1.00	7.449/ 1.03	7.543/ 1.17
	Mean	7.460/ 1.23	7.455/ .79	7.370/ 2.00	7.316/ 1.84	7.248/ 1.78	7.244/ 1.87	7.212/ 2.76	7.238/ 2.02	7.263/ 2.16	7.297/ 1.74	7.344/ 4.01		
No Saline	398	7.434/ 1.03	7.457/ .65	7.362/ 2.59	7.348/ 1.30	7.328/ 1.45	7.281/ 2.05	7.338/ 2.17	7.345/ 3.19	7.364/ 2.57	7.390/ 3.19	N/A/ 2.26	7.516/ 1.31	7.540/ 1.29
	416	7.454/ .92	7.452/ 1.23	7.400/ 2.02	7.342/ 2.77	7.280/ 2.00	7.291/ 1.64	7.271/ 2.18	7.298/ 1.44	7.373/ 1.56	7.380/ 2.39	7.398/ 2.36	7.308/ 10.44	
5	422	7.455/ .70	7.444/ .91	7.371/ 1.97	7.367/ 2.88	7.355/ 2.00	7.377/ 1.75	7.390/ 1.82	7.394/ 1.59	7.416/ 1.74	7.421/ 2.62	7.431/ 2.08	7.465/ 1.37	7.472/ 2.10
	Mean	7.448/ .90	7.451/ .93	7.378/ 2.19	7.352/ 2.32	7.321/ 1.82	7.316/ 1.81	7.333/ 2.06	7.346/ 2.07	7.384/ 1.96	7.397/ 2.73	7.415/ 2.23	7.430/ 4.37	

N/A = ot available.

TABLE 11. Definitive swine therapeutic study: Arterial blood gas determinations.

Group	Pig No.	Time 1 hr Pre	P_aO_2/P_aCO_2 (mmHG) 0	1	2	3	4	5	6	7	8	12	24	48
Control	380	99.1/ 35.3	96.1/ 37.9	103.5/ 32.6	100.2/ 36.8	101.1/ 35.7	112.4/ 29.9	104.8/ 31.0	104.6/ 29.9	115.0/ 27.9	119.9/ 19.3			
	394	104.4/ 38.2	95.9/ 37.9	104.3/ 32.7	100.7/ 34.0	97.8/ 38.7	106.8/ 32.6	109.1/ 28.8	111.2/ 25.0	113.6/ 20.2	91.0/ 33.6			
1	420	95.6/ 39.7	99.1/ 37.6	104.5/ 37.1	100.8/ 36.6	96.7/ 37.6	106/ 35.7	106.4/ 32.7	111.8/ 32.1	112.4/ 31.0	118.3/ 24.9			
	Mean	99.7/ 37.7	97.0/ 37.8	104.1/ 34.1	100.6/ 35.8	98.5/ 37.3	108.4/ 32.7	106.8/ 30.8	109.2/ 29.0	113.7/ 26.4	109.7/ 25.9			
All Therapy	396	97.5/ 37.8	88.6/ 39.2	99.2/ 32.3	99.6/ 30.8	99.2/ 29.3	93.4/ 34.2	107.1/ 26.9	102.4/ 28.2	106.7/ 30.9	N/A/ 32.2	102.3/ 30.6	99.7/ 32.2	95.9/ 34.7
	410	100.9/ 37.0	104.0/ 39.4	110.0/ 40.0	99.0/ 36.5	84.4/ 36.8	95.3/ 35.9	93.4/ 34.3	96.9/ 35.5	84.6/ 35.1	87.1/ 46.6			
2	424	94.2/ 38.5	97.5/ 35.8	102.1/ 34.8	93.8/ 36.2	94.2/ 35.4	99.5/ 36.9	102.2/ 31.8	102.4/ 35.8	100.4/ 35.8	N/A/ N/A	N/A/ N/A	86.9/ 31.8	94.9/ 28.1
	Mean	97.5/ 37.8	96.7/ 38.1	103.8/ 35.7	97.5/ 34.5	92.6/ 33.8	96.1/ 35.7	100.9/ 31.0	100.5/ 33.2	97.2/ 33.9	87.1/ 39.4			
No Activated Charcoal	386	104.8/ 38.1	101.3/ 37.9	98.0/ 39.2	104.5/ 36.2	95.8/ 41.9	91.6/ 40.0	94.2/ 39.1	96.1/ 40.7	98.5/ 37.9	101.2/ 31.9	90.8/ 37.6		
	414	98.9/ 36.4	97.5/ 37.4	90.1/ 35.8	99.7/ 36.8	99.7/ 36.7	91.5/ 37.2	93.1/ 40.5	87.0/ 40.8	94.7/ 37.0	89.1/ 38.3	89.3/ 37.5		
3	428	84.2/ 41.7	93.2/ 38.6	102.3/ 37.8	102.6/ 34.3	79.8/ 43.1	82.5/ 36.3	79.0/ 35.6	88.0/ 30.1	91.9/ 30.6	86.2/ 32.9	76.0/ 30.7		
	Mean	95.9/ 38.7	97.3/ 38.0	96.8/ 37.6	102.3/ 35.8	91.8/ 40.6	88.5/ 37.8	88.8/ 38.4	90.4/ 37.2	95.0/ 35.2	92.2/ 34.4	85.4/ 35.3		

TABLE 11 CONTINUED

Group	Pig No.	Time 1 hr Pre	P_aO_2/P_aCO_2 (mmHg) 0	1	2	3	4	5	6	7	8	12	24	48
No HCO_3	400	93.9/ 38.6	98.4/ 39.6	98.3/ 36.7	92.6/ 36.8	90.8/ 36.1	81.1/ 34.4	101.2/ 38.0	95.6/ 36.1	98.7/ 32.5	105.5/ 27.6	101.9/ 26.1		
	418	96.7/ 38.8	93.1/ 40.7	106.4/ 36.5	94.4/ 38.0	96.3/ 35.0	88.2/ 33.5	101.2/ 34.4	102.3/ 31.8	108.9/ 27.7	111.3/ 24.1			
4	426	92.1/ 36.8	90.7/ 34.2	94.7/ 35.2	97.1/ 33.5	98.7/ 34.0	101.8/ 33.2	105.1/ 34.6	103.7/ 30.9	113.7/ 25.3				
	Mean	94.2/ 38.1	94.1/ 38.2	99.8/ 36.1	94.7/ 36.1	95.3/ 35.0	90.4/ 33.7	102.5/ 35.7	100.5/ 32.9	107.0/ 28.5/	109.1/ 25.7			
No Saline	398	92.7/ 39.7	97.1/ 35.4	99.4/ 35.5	98.0/ 35.0	89.7/ 37.5	91.1/ 38.3	92.5/ 42.8	88.4/ 36.3	93.6/ 34.7	92.1/ 31.1	N/A/ N/A	94.9/ 35.1	95.3/ 34.9
	416	103.6/ 37.4	91.2/ 39.3	96.8/ 35.9	93.7/ 37.5	86.7/ 37.7	90.1/ 38.4	91.8/ 32.1	92.5/ 29.0	94.7/ 29.4	97.9/ 31.5	85.7/ 38.2	82.5/ 29.5	
5	422	99.1/ 32.7	104.6/ 34.7	99.8/ 36.1	103.0/ 32.4	98.7/ 32.2	86.5/ 37.4	100.2/ 32.8	95.3/ 33.2	95.4/ 30.3	103.7/ 30.0	105.6/ 27.1	100.1/ 27.4	111.1/ 19.3
	Mean	98.5/ 36.6	97.6/ 36.5	98.7/ 35.7	98.2/ 35.0	91.8/ 35.8	89.2/ 38.0	94.8/ 35.9	92.1/ 32.8	94.6/ 31.5	97.9/ 30.9	95.7/ 32.7	92.5 30.7	

N/A = not available.

D. STUDIES OF THE ADSORPTIVE CAPACITY OF ACTIVATED CHARCOALS FOR T-2 TOXIN AND THEIR EFFICACY IN PREVENTING T-2 TOXICOSIS--P. M. Bratich

OBJECTIVES:

1. To determine the <u>in vitro</u> adsorptive capacity of SuperChar,[1] Amoco PX-21,[2] Calgon activated charcoal,[3] Toxiban,[4] Norit A,[5] a Super- Char + sorbitol mixture, a SuperChar + sorbitol + preservative mixture and cholestryamine[6] for T-2 toxin by using high pressure liquid chromatog- raphy and gas chromatography to measure unbound T-2 toxin.
2. To verify the use of a refractive index detector with high pressure liquid chromatography for assaying the adsorptive capacity of different charcoal suspensions.
3. To determine the <u>in vivo</u> efficacy of different charcoal suspensions in rats given supralethal oral doses of T-2 toxin.

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[1]SuperChar, Distributed exclusively by Gulf Bio-Systems, Inc., Dallas, TX 75230

[2]Amoco PX-21, Distributed exclusively by Gulf Bio-Systems, Inc., Dallas, TX 75230

[3]Calgon Activated Charcoal, Calgon Corporation, P.O. Box 1346, Pittsburg, PA 15230

[4]Toxiban, Vet-A-Mix, Inc., Shenandoah, IA 51601

[5]Norit A, American Norit Company Inc., Jacksonville, FL 32200

[6]Cholestyramine, Mead-Johnson, Evansville, IN 47700

METHODS:

1. In Vitro Analysis Examining the Adsorptive Capacity of Various Activated Charcoals for T-2 Toxin by High Pressure Liquid Chromatography (HPLC).

The T-2 toxin used was produced in our laboratory at the University of Illinois. Three concentrations of T-2 toxin and three concentrations of charcoal suspensions were used (see Table 1). The volumes of charcoal suspensions and T-2 solutions that were mixed together corresponded to the volumes intended for the 250 g rats that were to be used for the in vivo study. The treatment suspensions and T-2 dilutions were rocker-mixed for 30 minutes, and a 0.25 mL aliquot of the mixture was removed, diluted with 1 mL methanol, filtered and assayed by HPLC. The samples were injected into a 10 μ Alltech C18 column connected to a Perkin Elmer Series 10 HPLC. The solvent system was methanol and water (7:1) with a flow rate of 1.4 mL/min. Due to the lack of any significant UV absorbance by T-2 toxin, a refractive index detector was used to examine the column effluent. The percentages of the total T-2 toxin adsorbed by the various activated charcoals and cholestyramine were calculated (Table 1).

2. In Vitro Analysis Examining the Adsorptive Capacity of Various Activated Charcoals for T-2 Toxin by Gas Chromatography (GC).

An in vitro analytical method for examining the adsorptive capacity of activated charcoals for T-2 toxin using gas chromatography was developed. A mixture of the highest concentration of T-2 toxin (3 mg/mL) and the intermediate concentration of charcoal (104 mg/mL) utilized in the HPLC study was assayed. The volumes of toxicants and adsorbants used for the GC experiments were the same as those used for the HPLC studies. The T-2 toxin used was from the same lot as that used in the HPLC studies.

The charcoal and cholestyramine suspensions and T-2 dilutions were rocker-mixed for 30 minutes, and a 0.25 mL aliquot of the mixture was removed, diluted with 1 mL methanol, filtered, derivatized (using heptaflouro-butyrlimidazoie) and assayed by GC. Dieldrin was used as an internal standard to compensate for the number of pipetting steps involved. The samples were injected into a Hewlett Packard 5840A GC equipped with a 6 ft OV-17 column and an electron capture detector at 240°C. The percent of T-2 toxin adsorbed by various charcoal preparations was compared to Super-Char using both analytical techniques (HPLC and GC).

3. In Vivo Study Examining the Efficacy of Charcoal for T-2 Toxin.

Sixty-five female Harlan Sprague Dawley rats were used in this study to compare the efficacy of SuperChar, Calgon and Norit-A activated charcoals, Toxiban and a SuperChar + sorbitol mixture in preventing oral T-2 toxin induced deaths. A dose of T-2 toxin that killed 100 percent of the positive control rats but allowed differentiation between the different charcoal suspensions was used (25 mg T-2/kg body weight). The charcoal dose administered was 9 mL of suspension/kg body weight at a concentration of 104 mg of charcoal/mL.

Individual rats were weighed to the nearest gram and placed in individual cages. The doses of toxicant and the prescribed treatment for each rat were calculated and measured into 3 mL syringes. The doses of T-2 toxin and charcoal were administered consecutively with an 18 gauge 2 inch gavage needle. Positive control rats received tap water at 9 mL/kg in addition to T-2 toxin. Food and water were available ad libitum before and during the study.

The survival rate and duration were recorded until 14 days after the administration of the T-2 toxin and treatment. All rats were continually observed the first 24 hours after treatment and four times per day thereafter. Each of 5 T-2 control rats were necropsied at the time of death and a paired SuperChar-treated rat was killed concurrently and also necropsied (three SuperChar alone, two SuperChar + sorbitol). Tissues from the brain, heart, thymus, lungs, liver, spleen, kidneys, adrenal glands, uterus, stomach, duodenum, jejunum, ileum and cecum were placed in ten percent buffered formalin for histologic examination.

RESULTS AND DISCUSSION:

In Vitro Studies:

1. The different charcoal suspensions and their relative adsorptive capacities for T-2 toxin as measured by HPLC are given in Table 1. SuperChar was the most effective in adsorbing T-2 toxin. Toxiban and cholestyramine were the least effective. The preservatives and sorbitol did not change the adsorptive capacity of the SuperChar suspension.
2. The different charcoal suspensions and their adsorptive capacity for T-2 toxin as assayed by HPLC and GC are given in Table 2. SuperChar was the most effective in adsorbing T-2 toxin as measured by both methods. Toxiban and cholestyramine were the least effective. Although differences were observed between the HPLC and GC studies, the trends were identical. Thus, the HPLC method for examining the adsorptive capacities of different charcoal suspensions for T-2 toxin was confirmed.

In Vivo Study:

The results of the in vivo study are summarized in Table 3. SuperChar was superior when compared to the other charcoal suspensions in preventing deaths

from T-2 toxin administration. As predicted by the in vitro study, sorbitol did not change the efficacy of the SuperChar suspension. The surviving SuperChar-treated rats had minimal or no clinical signs of toxicosis.

The necropsy results of the five control rats and the five paired positive control comparisons of SuperChar-treated rats are summarized in Table 4. Evaluation of SuperChar-treated rats revealed mild necrosis or no lesions present in the various tissues examined. Lymphoid necrosis was present in the spleen, thymus and lymph nodes S of all T-2 toxin positive control rats. In addition, there was necrosis of superficial and glandular epithelial cells of the stomach along with crypt epithelial necrosis in the small intestine. Individual hepatocyte necrosis was seen in all T-2 toxin positive control livers. Severe necrosis of adrenal cortical cells as well as mild necrosis of individual pancreatic acinar cells were also seen. The lungs of these animals had neutrophilia along with mild perivascular edema and lymphocyte/eosinophil necrosis. In all of the T-2 positive controls, there was mild myofiber lysis in the heart and mild necrosis in the stroma of the uterus.

TABLE 1. The adsorptive capacity of different adsorbants for three different concentrations of T-2 toxin.

Concentration of Charcoal (mg/mL) (volume = 2.5 mL)	Brand	Concentration of T-2 Toxin (volume = 1 mL)		
		1 mg/mL	2 mg/mL	3 mg/mL
		Mean percent of T-2 toxin adsorbed		
52	Calgon	100	82	72
	Amoco PX-21	100	88	77
	Norit A	100	70	66
	SuperChar	100	95	92
104	Calgon	100	82	79
	Amoco PX-21	100	85	84
	Toxiban	60	60	58
	Norit A	100	78	68
	SuperChar	100	95	92
	SuperChar + sorbitol	100	100	89
	Cholestyramine	10	5	3
	SuperChar + sorbitol + preservatives	100	92	92
250	Calgon	100	98	97
	Amoco PX-21	100	100	95
	Norit A	100	97	95
	SuperChar	100	100	100

TABLE 2. The adsorptive capacity of adsorbants for T-2 toxin as measured by high pressure liquid chromatography and gas chromatography.

Brand of adsorbant[a]	Assay method	
	HPLC	GC
	Mean percent T-2 toxin adsorbed[b]	
SuperChar	100	100
SuperChar + sorbitol + preservatives	100	93
SuperChar + sorbitol	97	93
Amoco PX-21	91	85
Calgon	86	77
Norit A	74	68
Toxiban	63	32
Cholestyramine	3	11

[a]volume = 2.5 mL at a concentration of 104 mg/ml

[b]Volume = 1.0 mL at a concentration of 3 mg/ml

The formula for the percentages is $\frac{\text{Percent of T-2 adsorbed}}{\text{Percent of T-2 adsorbed by SuperChar}} \times 100$

TABLE 3. Efficacy of various oral charcoal treatments in preventing deaths in rats given lethal (6 X LD_{50}) oral doses of T-2 toxin.

	T-2 Toxin (25 mg/kg)	
	Survivors/Animals Dosed	Charcoal/T-2 Toxin
SuperChar*	6/10	37/1
Calgon	0/10	37/1
Toxiban	0/10	37/1
Norit A	0/10	37/1
SuperChar + Sorbitol	7/10	37/1
Control	0/10	--

*An additional five rats were given T-2 toxin plus SuperChar and were killed and necropsied as paired comparisons to five of the control rats that were necropsied.

TABLE 4. Severity of lesions associated with T-2 toxicosis in control and SuperChar-treated rats killed and necropsied as paired comparisons.

Organ	Lesion Severity									
	C	C	C	C	C	SC	SC	SC	SC	SC
Spleen	+++	+++	+++	+++	+++	-	-	-	-	-
Thymus	++	++	++	+++	++	-	+	+	+	+
Lymph Node	++	++	++	++	+++	-	NE	NE	NE	+
Peyer's Patch	+++	-	-	-	-	NE	NE	-	NE	NE
Stomach	+++	+++	++	++++	+++	+	++	+	++	+
Small Intestine	++	+++	+++	+++	+++	-	-	-	-	-
Liver	++	++	+++	+++	++	-	-	+	-	-
Adrenal	++	+++	+++	+++	+++	-	-	-	-	-
Pancreas	-	+	+	+	+	-	-	-	-	-
Lung	++	++	++	++	NE	+	+	+	+	NE
Heart	+	+	+	+	+	+	-	-	-	-
Uterus	+	+	+	+	+	-	-	-	NE	-

C = control rat.
SC = Superchar-treated rat killed and necropsied as a paired comparison.
Lesion severity: + = mild
++++ = very severe
- = examined, no lesion
NE = not examined

E. THERAPEUTIC ORAL ACTIVATED CHARCOAL STUDIES IN RATS--Frank Galey

Rat Study--ED_{50} Dose Activated Charcoal To Prevent T-2 Toxicosis

A three-part study is now in progress evaluating orally administered superactivated charcoal for the treatment of 250 gram, female, Sprague-Dawley rats given an oral lethal dose of 8 mg/kg body weight T-2 toxin via stomach gavage. The first portion of this study was designed to determine an estimate of an ED_{50} (effective dose) dosage for the superactivated charcoal (Superchar) in treating the above rats when given simultaneously with the T-2 mycotoxin. This ED_{50} superchar dose was than used to assess different com- monly used cathartics as a possible adjunct to oral superactivated charcoal treatment in the second study. A third study will be done to assess the efficacy of a 100 percent effective dose of superactivated charcoal (when given right after T-2 toxin) when combined with the best cathartic (if any) when given orally to rats at varying periods of time following administration of a lethal dose of 8 mg/kg body weight T-2 toxin.

Survival data obtained in the first study are shown in Table 1. In addition to this information, records of the onset and duration of clinical signs were kept along with the survival times for each rat. Feed intake and average daily weight gains were also recorded. Ante- and postmortem observations were made regarding the time of passage of the activated charcoal and the location of the charcoal in the gastrointestinal tract, respectively. Although the bulk of the data has yet to be scrutinized statistically, an estimate of the ED_{50} was made using the log-probit analysis shown in Figure 1. From this graph, an estimated ED_{50} of 0.175 $\pm$ 0.01 g/kg oral superactivated charcoal was obtained.

Activated Charcoal plus Cathartic Studies

In the second study, the ED_{50} of 0.175 g/kg body weight superactivated charcoal was given immediately post oral T-2 administration via gavage to rats. The difference in the second study was that the charcoal was mixed with various cathartics prior to administration. This was done in order to determine what differences there were, if any, between giving the superactivated charcoal alone or administering cathartics with the superactivated charcoal on survival rates and times.

The data obtained are shown in Table 2. From this information, it appears evident that most of the cathartics tested had no positive effect on survival rates. Only superactivated charcoal plus magnesium sulfate or sodium sulfate compared equally with the use of oral superactivated charcoal alone, keeping in mind the small size of treatment groups.

In order to further discern which oral regimen was the most efficacious in treating oral T-2 toxicosis in the rat, survival times were considered. Using this preliminary data (Table 3), it appeared that only the administration of sodium sulfate with the superactivated charcoal resulted in mean survival times longer than those of the positive control group that received oral superactivated charcoal alone.

Average daily weight gains before and after dosing were recorded as were the other parameters mentioned above for the preliminary portion of the study. These data have not been analyzed completely. A finding worth mentioning at this time is that those rats that died after receiving charcoal plus cathartic tended to have extremely distended bowels and were very dehydrated. This may be a result of gut stasis induced by the effects of T-2 toxin and could be a potential problem when some T-2 is allowed to be

absorbed. Finally, all rats examined in this study have lost weight during the first 2 to 4 days postadministration of the T-2 toxin except those given the largest doses of superactivated charcoal immediately following oral gavage with T-2 toxin. This information awaits further analysis.

The information collected so far further documents the efficacy of oral activated charcoal in adsorbing T-2 toxin. It also indicates that the best regimen for the third portion of this study, which will determine the period of time postadministration of a lethal oral dose of T-2 mycotoxin at which oral superactivated charcoal will be efficacious, may include a sodium sulfate cathartic.

TABLE 1. Study 1. The Efficacy of Superactivated Charcoal and the Determination of an ED_{50} Dose of Activated Charcoal in the Prevention of Toxicosis and Death in Rats Administered Oral T-2 Toxin

Group	Description	T-2 Dose	No. Rats	Superchar Treatment	Ratio Charcoal: T-2	Survival (Percent)
(1)	Control	8 mg/kg	5	Water placebo		0/5 (0)
(2)	Test	8 mg/kg	5	1 g/kg PO	125:1	5/5 (100)
(3)	Test	8 mg/kg	5	0.5 g/kg PO	62.5:1	5/5 (100)
(4)	Test	8 mg/kg	8	0.2 g/kg PO	25:1	5/8 (62.5)
(5)	Test	8 mg/kg	8	0.175 g/kg PO	22:1	5/8 (62.5)
(6)	Test	8 mg/kg	8	0.15 g/kg PO	19:1	2/8 (25)
(7)	Test	8 mg/kg	5	0.10 g/kg PO	12.5:1	0/5 (0)
(8)	Test	8 mg/kg	5	0.04 g/kg PO	5:1	0/5 (0)

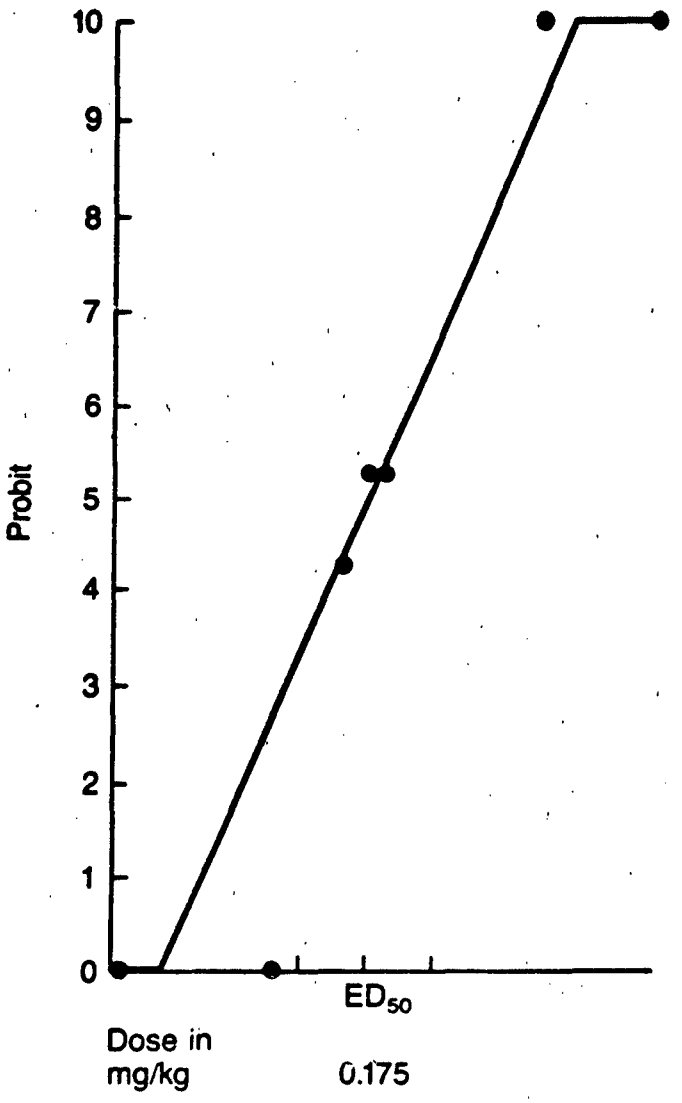
TABLE 2. Efficacy of Cathartics in Aiding Activated Charcoal in Preventing Orally Induced T-2 Deaths in Rats

Group	Description	T-2 Dose	Treatment	Survival Rate
(1)	Control	8 mg/kg	0.175 mg/kg superchar (dry weight) as a slurry	2/10 (20 percent)
(2)	Test	8 mg/kg	0.175 mg/kg superchar (dry weight) as a slurry + sorbitol cathartic in a 2:1 ratio cathartic to charcoal (1/6 the recommended dose of sorbitol)	0/10 (0 percent)
(3)	Test	8 mg/kg	0.175 mg/kg superchar (dry weight) as a slurry + sorbitol cathartic (recommended dose of sorbitol)	0/10 (0 percent)
(4)	Test	8 mg/kg	0.175 mg/kg superchar (dry weight) as a slurry + 1 g/kg magnesium sulfate cathartic	1/11 (9 percent)
(5)	Test	8 mg/kg	0.175 mg/kg superchar (dry weight) as a slurry + 1 g/kg sodium sulfate cathartic	2/11 (18 percent)

TABLE 3. Survival Time of Rats Given T-2 Toxin Followed by Oral Activated Charcoal/Cathartic Mixtures (all rats were given 8 mg/kg T-2 orally).

Group	Number of Rats	Oral Treatment	Survival Time ± Standard Error of the Mean (Hours:Minutes)
1	(8)	0.175 g/kg superchar	21:05 ± 2:33
2	(10)	0.175 g/kg superchar + 0.35 g/kg sorbitol	23:00 ± 1:36
3	(10)	0.175 g/kg superchar + 1 g/kg sorbitol	18:39 ± 3.28
4	(10)	0.175 g/kg superchar + 1 g/kg $MgSO_4$	24:22 ± 1:58
5	(9)	0.175 g/kg superchar + 1 g/kg $NaSO_4$	33:56 ± 7:41

FIGURE 1. Log-dose probit estimation of median effective dose (ED_{50}) for superactive charcoal in rats given a lethal oral dose of 8 mg/kg T-2 toxin. Lines on each side of the ED_{50} indicate the 95% fiducial limits of 0.139 and 0.247 mg superactive charcoal/kg.



F. PREVENTION AND TREATMENT OF T-2 TOXICOSIS IN DERMALLY EXPOSED SWINE--Mike Biehl

OBJECTIVES

1. To determine the dosage of T-2 toxin which produces systemic signs and death when applied dermally to the pig.
2. To compare the application of an activated charcoal paste with soap and water wash for the reduction of local and systemic effects of topically applied T-2 toxin.

SEQUENCE OF APPROACH

1. Determination of an acutely lethal dermal dose.
2. Comparison of activated charcoal to soap and water in a therapeutic regimen.

PRELIMINARY STUDIES

Our goal was to characterize the systemic effects caused by dermally applied T-2 toxin. To achieve these effects, we utilized a dermal dosage of approximately 45 mg/kg, which is three times the dosages used in prior experiments. In the prior studies, it was the opinion of the investigators that the pigs experienced intractable pain. As a result, it was thought that a dosage of 50 mg/kg might produce even more discomfort so protocols for sedation and main- tenance of analgesia were formulated.

Initially, one female, crossbred, specific-pathogen-free (SPF) pig (D-1) weighing 22.3 kg was dosed. One gram of T-2 (44.8 mg/kg) was dissolved in 3.0 mL of 75 percent dimethyl sulfoxide (DMSO) and applied topically to a shaved, 20 x 15 sq. cm area on the back of the pig. A protective nonocclusive, foam pad was utilized to prevent toxin loss while allowing air flow.

Clinically, none of the major, classic, systemic signs of acute T-2 toxicosis occurred. Capillary refill time and perfusion of the extremities remained normal throughout the study, and nausea and vomition were not observed. The pig did become hyperthermic, partially anorexic and slightly depressed. At 24 hours after dosing, the body temperature was 106.7° F (predose 103.2° F) and remained elevated until necropsy 7 days later. By 48 hours feed consumption was slightly decreased and the animal appeared slightly depressed and less active. The expected pain was never exhibited by the subject, even when pressure was directly applied to the exposed area.

On day 2, gross examination of the area exposed to T-2 toxin revealed a swollen, dark red dermal surface. Some epithelial scale formation was present in addition to minor erosions. On day 7, the skin was thickened, crusted, purple and focally ulcerated.

A second pig (D-2) weighing 23.0 kg was subsequently dosed with 1 gram (43.5 mg/kg) of T-2 dissolved in 2.0 mL of acetone. It was thought that acetone might increase the systemic absorption of the toxin. The clinical observations of pig D-2 were essentially identical to D-1. A slight decrease in feed intake appeared at day 1 in this pig. Rectal temperature at necropsy (day 13) was 107.8° F. Grossly, dermal changes induced by the T-2 toxin were similar to the first pig.

Histological evaluation of the tissues is presently underway and will be related in subsequent reports.

Based on these results, it was decided to curtail any further studies designed to produce systemic reactions by dermal dosing with T-2 toxin. These findings substantiate previous reports of the cytotoxic action of T-2 toxin on skin. Apparently, in the pig, the toxin is very slowly absorbed into the

systemic circulation, even at extreme dosing rates. Previous researchers have suggested that the dermis serves as a reservoir for lipid soluble sub- stances such as T-2 toxin. These substances and their metabolites may be gradually released for subsequent systemic absorption. Increasing the dosing level above 50 mg/kg would probably result in more dramatic skin lesions, without substantial systemic effects. For our purposes, it is not economically practical to attempt to cause death or systemic signs in pigs dosed dermally with T-2 toxin unless a more suitable vehicle or effective dosing procedure is forthcoming. Our research efforts will focus on the treatment of localized dermal effects caused by topically applied T-2 toxin.

Studies are presently underway to determine an effective therapeutic regimen for dermal exposure to T-2 toxin. A standardized dose of T-2 toxin is being applied to multiple areas on individual pigs. These areas are then individually treated with activated charcoal and soap and water washes in various combinations and at different times post-dosing. The results of these efforts will be presented in future reports.

It is believed that detoxification steps which reduce local effects on an animal's skin would be similarly effective in reducing systemic effects in a species capable of more rapid cutaneous absorption.

WBB:sfb/674

02/26/87

III. PRODUCTION OF TOXINS

A. IN VITRO PRODUCTION OF 3'-HYDROXY T-2 TOXIN FROM T-2 TOXIN BY RAT LIVER MICROSOMES--Catherine A. Knupp, Steven P. Swanson, William B. Buck

INTRODUCTION

T-2 toxin (4ß, 15-diactoxy-8-(3-methylbutyryloxy)-3-hydroxy-12,13-epoxytrichothec-9-ene) is a trichothecene mycotoxin produced by species of Fusarium. It is rapidly biotransformed to many different metabolites in all species. 3'-hydroxyT-2 (TC-1) and 3'-hydroxy HT-2 (TC-3) have been identified as major metabolites of T-2 toxin following oral (chicken and lactating dairy cow) and intravascular (swine) administration of ^{3}H labeled T-2 (1-5). The C3' hydroxylated products have also been identified in vitro using monkey, mice, and rat liver homogenates supplemented with a NADPH generating system (6,7). It is presently difficult to obtain standards of the 3'hydroxylated compounds other than extracting these metabolites from the excreta of animals dosed with T-2 or through lengthy synthesis reactions. This paper describes the production of 3'OH T-2 using the liver S-9 fraction from rats pretreated with phenobarbital combined with the addition of paraoxon to inhibit enzymatic hydrolysis of the T-2 substrate.

EXPERIMENTAL

Chemicals. T-2 toxin was extracted and purified from Fusarium cultures grown in our laboratories and was greater than 98 percent pure. All solvents were HPLC grade from Fischer Scientific (Itasca, IL). Tris(hydroxymethyl) aminomethane, B-NADP (95 percent purity), glucose-6-phosphate, glucose-6-phosphate dehydrogenase, $MgCl_2$ and sodium phenobarbital were obtained from

the Sigma Chemical Company (St. Louis, MO). Male, 400-500 gram, Spraque-Dawley rats were purchased from the Harlan Sprague-Dawley Company (St. Louis, MO). Trifluoroacetic anhydride (TFAA) was purchased from Pierce Chemical (Rockford, IL). Paraoxon was a gift from the laboratory of Dr. L. Hanson, College of Veterinary Medicine at the University of Illinois at Urbana-Champaign.

Apparatus. A Series-4 Perkin-Elmer liquid chromatograph equipped with an Alltech silica column (10 μ, 25 cm, 10 mm ID) was used to separate the metabolites formed during the S-9 incubation. A solvent gradient beginning at 25 percent hexane plus 75 percent $CHCL_3$ was programmed linearly over a 60-minute time period to 10 percent hexane, 15 percent acetone and 75 percent $CHCl_3$. The column flow rate was 2.0 mL per minute. Fractions (2.0 mL) were collected with an automatic fraction collector and analyzed using silica thin layer chromatography plates (250 μ, Whatman Company). The metabolites were confirmed by comparing to known standards using a Hewlett-Packard 5790 gas chromatograph with a .25 mm ID (.25 μ film coating) fused silica capillary column with a ^{63}Ni electron-capture detector. The gas chromatographic operating conditions were as follows: injector 275° C, detector 340° C, hydrogen carrier gas at 45 cm/sec. The column temperature was programmed from 90° C to 260° C at 10 degrees per minute and held at 260° C for 5 minutes.

Analytical Procedure. Two male Spraque-Dawley rats were pretreated for three consecutive days with intraperitoneal injections of 75 mg per kg sodium pheno- barbital dissolved in physiologic buffered saline. On the fourth day, the animals were sacrificed, exsanguinated and the individual livers perfused with a cold sucrose solution (0.25 M containing 0.05 mM EDTA) through visible blood vessels. The livers were then combined and homogenized with three volumes of the same medium. The homogenate was centrifuged at 10,000 x g for

10 minutes at 0° C and the supernatent filtered through glass wool. This supernatant was the S-9 fraction of the liver homogenate. Tris buffer (0.05 M, pH 7.4) containing 0.15 M KCl was added to adjust the volume of the S-9 to five times the original liver weight. A cofactor solution containing B-NADP (0.667 mM), glucose-6-phosphate (3.33 mM), $MgCl_2$ (10.0 mM) and glucose-6-phosphate dehydrogenase (0.443 units/mL) dissolved in the Tris buffer was prepared such that there was four times the volume of the diluted S-9 fraction. The S-9 fraction was mixed with the cofactor solution, then divided equally into 16 x 125 mL erlenmeyer flasks (final volume 80 mL each) and placed in a water bath shaker warmed to 37° C. Paraoxon (200 nmoles per mL of S-9 per flask) was mixed with T-2 toxin (14.3 mg per flask), dissolved in EtOH (1.0 mL per flask) and added to the erlenmeyer flasks. The flasks were agitated gently at 37° C for 3 hours, then removed from the bath for extraction.

The contents of the 16 incubation flasks were combined into 2 liter erlenmeyers, and the liver proteins precipitated with acetone. The solution was filtered through analytical filter pulp and transferred to 500 mL separatory funnels. Saturated NaCl (10-20 ml) was added and the aqueous layer extracted three times with $CHCl_3$. The $CHCl_3$ extract was dried with Na_2SO_4, filtered into a 1,000 mL round bottom flask and concentrated on a rotary evaporator. The residue was transferred with 3 x 4.0 mL 9+1 $MeCl_2$: MeOH into a 20 mL glass vial and concentrated to dryness under air and gentle heat. The residue was redissolved in 6.0 mL 25+75 hexane:$CHCl_3$, filtered through a 0.2 µ HPLC filter and split into three aliquots for injection onto the liquid chromatograph.

An aliquot from each LC fraction (5 µL/2.0 mL) was spotted on silica thin layer chromatrophy plates. The plates were developed in 9+1 $CHCl_3$:MeOH,

allowed to air dry, then sprayed with 30 percent H_2SO_4 in MeOH and heated for 5 minutes at 110° C. Following observation under long wave ultraviolet light and comparison with the Rf of known standards (T-2 = .81; 3'OH T-2 = 0.77), fractions containing one metabolite were combined by transferring to a round bottom flask and concentrating with a rotary evaporator.

Derivatization. Further confirmation of a metabolite was achieved through use of gas chromatography. An aliquot was removed from the combined LC fractions of each metabolite, concentrated, then redissolved in 1.0 mL 9+1 toluene: acetonitrile containing 2.0 mg/mL dimethylaminopyridine (DMAP). Fifty µL of TFAA was added and the sample incubated at 60° C for 20 minutes. After cooling at room temperature for 5 minutes, 1.0 mL of 5 percent bicarbonate solution was added and the mixture vortexed until the top layer was clear. The sample was centrifuged at 1,500 rpm for 3 minutes, 200 µL removed from the top layer, diluted with 800 µL isooctane and 1 µL was injected into the gas chromatrograph.

RESULTS AND DISCUSSION

Under the conditions described in this paper, 40 to 50 percent of the added T-2 was converted to 3'OH T-2 in the 3-hour incubation period. Longer incubation time (up to 20 hours) did not affect the amount of 3'OH T-2 formed, probably due to the limited supply of cofactors necessary for mixed function oxidase activity. Pretreatment of the rats with phenobarbital was used to stimulate the mixed function oxidase activity of the S-9 fraction and, therefore, promote the formation of C3' hydroxylated product. Paraoxon was used at a concentration that would effectively inhibit the majority or the esterase activity contained in the liver homogenate without significantly depressing the oxidative pathway. A small amount of HT-2 (less than one percent) was also formed during the reaction.

The advantage of using the S-9 fraction for metabolite production rather than a pure microsomal system was that an ultracentrifugation step was not required. The disadvantage was that more paraoxon had to be added to inhibit the esterase activity associated with the higher protein levels. This additional protein did not interfere with extraction, since it was easily precipitated with acetone and filtered, producing a very clean $CHCl_3$ extract. Since the 3'OH T-2 and T-2 were very soluble in $CHCl_3$, no further extraction of the aqueous fraction was necessary. The T-2 that was not metabolized was saved and could be recrystallized for use in another incuba- tion. We have produced a total of 120 mg of > 95 percent pure 3'OH T-2 using this procedure.

This method was readily adapted to the production of metabolites other than 3'OH T-2. We have produced Iso-3'OH T-2 (confirmed by GC-MS) from Iso-T-2 and 4-deacetylneosolaniol from T-2 (no paraoxon or cofactors were necessary for the later reaction) and plan on incubating HT-2 to produce 3'OH HT-2 as well as the de-epoxy metaboiites to form the C3' hydroxylated de-epoxy compounds. These metabolites can be used for standard preparation and further toxicologic evaluation since until now studies investigating the toxicity of the 3'-hydroxy metabolites have been limited due to the lack of appreciable quantities of these compounds.

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B. T-2 TOXIN PRODUCTION--

T-2 toxin production has been scaled up to meet the demand created by inhalation and metabolism experimentation. Cultures of Fusarium strain 3299 have yielded 41.6 g of purified crystalline T-2 toxin since September 1984, as well as mg quantities of several metabolites that are now in use as analytical standards. During the three-year period of this contract, a total of 80 grams of purified T-2 toxin have been produced by our laboratory.

T-2 toxin production has been accelerated through introduction of new inoculation techniques, as well as careful manipulation of the culture media to optimize nutrients and water requirements of the fungus. Flash chromatograhy has been introduced to speed purification of extracts and larger columns have been utilized to aid in initial purification of raw extracts.

Crystallization of toxin has been accomplished in batches of approximately 10.0 g T-2, yielding toxin of purity greater than 98 percent. Additional T-2 toxin has been produced of purity greater than 99.5 percent by fraction collection of an HPLC system and this ultrapure toxin has been used in metabolism work, as well as in preparation of standards.

C. FUNGUS ISOLATION, CULTURE COLLECTION AND CULTURE SCREENING--Jean Nicoletti

We are now isolating Fusarium from naturally contaminated field samples which come in through the diagnostic lab. Multiple samples of fungi are removed from feedstuffs such as corn. Samples are transferred to pentachloronitrobenzene agar plates (PCBN), a medium which inhibits bacteria and carnation leaf agar (CLA), a medium which selects for fungus (Table 1). The inoculated plates are incubated at room temperature. After 2 weeks, hyphal tips are microscopically examined under wet mount for characteristic micro- and macroconidia. Those samples demonstrating Fusarium macroconidia are transferred (hyphal tips) and grown at room temperature on PCNB or PDA. The process was repeated until purified isolates were obtained. Approximately 1 cm^2 mycelial plugs from the agar plates are then transferred to rice and vermiculite media (Table 2) to be examined for toxin production. These cultures are also transferred to sterile soil for prolonged storage. Following soil inoculation, the Fusarium is maintained on PDA for 3 weeks or until the soil culture has been shown to be viable by growth on PDA. At this point, we have isolated four potentially toxogenic Fusarium strains from field samples.

Several additional laboratories with Fusarium culture libraries have been contacted and have or are sending us toxogenic strains of Fusarium. These labs include the Northern Regional Research Center, USDA, Peoria, IL; USDA, Ames, Iowa; Paul Nelson Plant Pathology, Penn State; and Plant Pathology, University of Illinois. The Northern Regional Research Center has given us four cultures and the USDA, Ames, Iowa has given us 12 cultures. We presently have 35 strains of Fusarium spp. in long-term maintenance on soil, plus five strains in preparation. We are actively culturing for toxin production 25 strains on rice-water medium and five strains on potato-dextrose-agar.

These cultures are being grown both on rice and vermiculite media (Table 2). Extracts of cultures are being initially screened for trichothecene parent alcohols utilizing a hydrolysis similar to that described in the analysis section. We are monitoring for nivalenol, verrucarol, deoxynivalenol, T-2 tetraol, scirpenetriol and their deepoxy equivalents. In this manner, we can screen the cultures for the basic trichothecene groups without having to monitor for each individual trichothecene myotoxin. This screening technique was only recently instituted and results are not yet complete.

TABLE 1

Pentachloronitrobenzine Medium (PCNB)

	Percent
Difco peptone	1.5 w/v
$KH_2 PO_4$	0.1 w/v
$MgSO_4$ 7 H_2O	0.05 w/v
Agar	2.0 w/v
Pentachloronitrobenzene	0.1 w/v
Water	97.8 v/v
*Streptomycin sulfate stock solution	2.0 v/v
*Neomycin sulfate stock solution	1.2 v/v

*Add stock solution after autoclaving. The stock solution is prepared by combining 5 g of streptomycin sulfate to 100 µl water and 1 g of neomycin sulfate to 100 ml water.

Potato Dextrose Agar (PDA), Difco

	Percent
Potatoes, Infusion	20.0 g w/v
Bacto - dextrose	2.0 g w/v
Bacto - agar	1.5 g w/v
Water	100.0 µl v/v

Carnation Leaf Agar (CLA)

	Percent
Agar	3.0 g w/v
Water	100.0 ml v/v

Sterile carnation leaf dropped on medium surface.

TABLE 2

Rice Medium

Rice	200 percent wt/v
Water	100 percent v/v

Combine and autoclave in Erlenmeyer flasks with foam stoppers.

Vermiculite Medium

		Percent
Vermiculite		1:3.5 fluid
Glucose		15.00 w/v
KCl		0.05 w/v
$MgSO_4 \cdot 7\ H_2O$	0.10 w/v	
$NaNO_3$		0.20 w/v
NH_4NO_3	1.00 w/v	
$K_2\ HPO_4$	0.10 w/v	
Yeast extract		0.10 w/v
Water		100.00 v/v

Combine vermiculite with fluid medium in a ratio of 1:3.5 and autoclave in Erlenmeyer flasks with foam stoppers.

D. ISOLATION AND PURIFICATION OF DE-EPOXIDE TRICHOTHECENE COMPOUNDS FROM RUMEN FLUID--Harold D. Rood, Jr.

It is apparent from several studies (see in vitro metabolism, in vivo metabolism) that deepoxidation of trichothecenes occurs not only in ruminants but also nonruminants including rats and swine. Since standards of these deepoxy metabolites are not available, it was necessary to develop a system for production.

The deepoxy metabolite of DON (DOM-1), deepoxy HT-2, deepoxy Triol, deepoxy MAS and deepoxyscirpenetriol were produced by incubating T-2, DAS and DON with rumen microorganisms. Larger (mg) quantities of deepoxy trichothecenes were wanted for analytical standards and toxicological evaluation. The in vitro rumen incubation system developed was, therefore, scaled up in attempts to produce larger quantities of these compounds. Using this procedure, we have been obtaining almost 100 percent conversion of DON, DAS, and T-2 to their deepoxy metabolites.

Culture Conditions

The total volume was scaled up to 700 mL. The nutrient solutions used were as described in Tables 1 and 2. We have performed several large scale incubations using 50 to 100 mg toxin per flask. We are now in the process of isolating and purifying metabolites.

EXTRACTION

DAS and T-2. The rumen fluid was filtered through a bed of celite using a Buchner funnel, and the bed was washed with 200 mL of water thus giving a total volume of 1 liter. Approximately 500 cc of purified XAD-4 resin beads were added to the fluid, and the solution was allowed to stand overnight. The

entire mixture was transferred to a 25 mm x 125 mm glass column. The rumen fluid was drained at a rate of 4 to 5 drops per second, then the column was rinsed with 500 mL of water. The aqueous solutions were discarded. One liter of acetone was used as the elution solvent which was collected and concentrated to dryness.

DON. The rumen fluid was extracted three times with ethyl acetate in a separatory funnel. The ethyl acetate was combined and concentrated to dryness.

FLORISIL COLUMN

DAS, DON and T-2. The residue was loaded onto a 25 mm x 125 mm Florisil column (60 to 100 mesh) packed in dichloromethane. The column was rinsed with 100 mL of dichloromethane-acetone (97:3) at a rate of 2 to 3 drops per second. Four 100 mL fractions of dichloromethane-methanol (9:1) were used for elution, with each 100 mL fraction collected separately. A final elution with 100 mL dichloromethane-methanol (8:2) was employed and collected.

Each fraction was spotted on a TLC plate, developed in dichloromethane-methanol (9:1) and sprayed with 30 percent sulfuric acid in methanol to determine the toxin profile.

Results

T-2: Combined all five elution fractions.

DAS: Combined the first two elution fractions which contained MAS/de-MAS.
Combined the last three elution fractions which contained STR/de-STR.

DON: Combined the first four elution fractions which contained DON/DOM-1.

The appropriate elution fractions were combined, concentrated to dryness and subjected to normal phase HPLC.

HPLC: NORMAL PHASE SILICA COLUMN

An Alltech 10 micron 25 cm x 10 mm, column was utilized with a mobile phase flow rate of 2 mL/min. One mL fractions were collected.

MAS/de-MAS. A chloroform-acetone (95:5) solvent system was utilized. De-MAS eluted in fractions 42 to 70 mL.

STR/de-STR. A chloroform-acetone (97:3) solvent system was used and de-STR eluted in 28 to 44 mL.

HT-2/de-HT-2, TRI/de-TRI. An initial isocratic chloroform-acetone (96:4) solvent system was used for 40 minutes then programmed linearly to chloroform-acetone (9:1) over 5 minutes with a final hold of 20 minutes of chloroform-acetone (9:1). HT-2/de-HT-2 eluted in a volume of 36 to 70 mL and TRI/de-TRI eluted in 80 to 120 mL.

DOM-1. A chloroform-acetone (97:3) solvent system was used with DOM-1 eluting in 30 to 48 mL.

The appropriate fractions containing the semipurified toxins were combined and concentrated.

HPLC: C-18 COLUMN

Appropriate fractions from the normal phase HPLC were chromatographed by reverse phase HPLC. Conditions were as follows:

An Alltech 10 micron, 25 cm x 10 mm column was used at a flow rate of 2 mL/min. One ml fractions were collected. Mobile phases and gradient programs are given below.

De-MAS. Mobile phase, initially 50 percent methanol for 40 minutes then program linearly to 100 percent methanol over 10 minutes with a final hold of 10 minutes. De-MAS eluted in 70 to 94 mL.

De-STR. Forty percent methanol mobile phase for 40 minutes then program linearly to 100 percent methanol over 10 minutes. De-STR eluted in 50 to 62 mL.

De-HT-2. Sixty percent methanol mobile phase, hold 25 minutes then program linearly to 100 percent methanol over 25 minutes. De-HT-2 eluted in 58 to 76 mL.

De-TRI. Fifty percent methanol mobile phase, hold 25 minutes then program linearly to 100 percent methanol over 30 minutes. De-TRI eluted in 79 to 92 mL.

DOM-1. Thirty percent methanol mobile phase, hold 40 minutes then program linearly to 100 percent methanol over 10 minutes. DOM-1 eluted in 46 to 62 mL.

RECRYSTALLIZATION

De-STR, De-TRI. The residue was dissolved in the minimal amount of ethyl acetate. The ethyl acetate solution was slowly dripped into hexane not exceeding four times the volume of ethyl acetate. A white precipitate was formed and allowed to settle to the bottom of the vial. The mother liquor was care- fully decanted and saved. The remaining solid material was dried overnight in a vacuum dessicator and transferred to a vial.

De-HT-2. The same procedure was followed as for de-TRI/de-STR except that a glassy material was formed instead of a white precipitate. This material was dried in a vacuum dessicator for approximately 2 weeks. The material subsequently turned into a hard disc-like mass which was easily broken. A fine, white powder was obtained and transferred to a vial.

At this time, solid forms of DOM-1 and de-MAS were not obtained. A vacuum oven would greatly enhance the possibility of recrystallizing these compounds into a solid material of appropriate form.

FINAL YIELDS (approximate weights > 95 percent pure)

De-HT-2: 20 mg

De-TRI: 7 mg

De-STR: 5 mg

De-MAS: 7 mg (glassy material)

DOM-1: 25 mg (glassy material)

CONFIRMATION

Mass spectra of these metabolites have been obtained. We are in the process of obtaining NMR spectra as well.

CONCLUSION

Deepoxytrichothecenes have been detected as metabolites in a variety of metabolism studies, both *in vitro* and *in vivo*. DOM-1, deepoxy DON, has been detected in both rat and cow urine and feces. Both de-DAS and deepoxy T-2 metabolites have recently been detected in rat and swine excreta obtained from T-2 and DAS-dosed animals. The *in vitro* system described above proves to be an excellent means for producing deepoxy compounds for analytical standards, toxicological evaluation and further metabolism studies. In the next year, we will attempt to produce 3'OH deepoxy HT-2 utilizing both liver microsomal incubations which produce 3'oxidation of T-2 and rumen incubation which yields the corresponding deepoxy compound.

TABLE 1. *In vitro* fermentation solution

Ingredients	Amount
Clarified rumen fluid	19.0 percent
Mineral 1	8.0 percent
Mineral 2	8.0 percent
Trypticase	0.1 percent
Starch	0.1 percent
Cellulose - chopped unscented tissue	0.1 percent
$NaHCO_3$	0.05 percent
Water	56.0 percent
Inoculum	19.0 percent

TABLE 2. Mineral solutions

Mineral 1	K_2HPO_4	0.6 percent
Mineral 2	KH_2PO_4	0.6 percent
	$(NH_4)_2SO_4$	0.6 percent
	NaCl	1.2 percent
	$MgSO_4\ 7H_2O$	0.2 percent
	$CaCl_2\ 2H_2O$	0.2 percent

IV. ANALYTICAL METHODS

A. A RAPID SCREENING PROCEDURE FOR THE DETECTION OF TRICHOTHECENES IN PLASMA AND URINE--Harold D. Rood, Jr., Steven P. Swanson, and William B. Buck

INTRODUCTION

Trichothecenes are a series of mycotoxins produced by several species of Fusarium fungi which commonly grow on agricultural products such as corn and wheat (1-5). These mycotoxins cause a number of adverse health effects associated with the consumption of Fusarium infected feedstuffs. Specific signs of toxicoses include emesis, diarrhea, lethargy, reduced weight gain and, in come cases, death (6-11).

The primary trichothecenes of concern in this paper are T-2 and diacetoxyscirpenol (DAS) along with their respective metabolites and deoxynivalenol (DON, vomitoxin). Many metabolites of T-2 and DAS have been found in the blood, urine, bile and tissues after exposure in a number of species including cattle, swine, chickens, rats and mice (12-24). These metabolites include HT-2, T-2 Triol, T-2 tetraol, 4-acetyl tetraol, 8-acetyl tetraol, 15-acetyl tetraol, neosolaniol, 4-deacetylneosolaniol, 3'OH T-2, 3'OH HT-2 in the T-2 group, (12-15,18,19,25) and monoacetyoxyscirpenol and scirpenetriol in the DAS group (22-24) (Figure 1). There have been relatively few metabolism studies with DON, therefore, metabolites of DON have not been identified with the exception of a novel deepoxide metabolite (26). Collectively, these compounds exhibit a wide range of chemical behavior due to the varied number and types of side groups. Current methods to analyze for T-2, DAS and their metabolites or DON can be lengthy, difficult or relatively costly. Standards for most of

the metabolites of T-2 toxin and DAS are not readily available, and if available, they are often expensive. This paper outlines a simple and rapid screening procedure that utilizes readily available standards and aides in the determination of DON, DAS and T-2 exposure.

EXPERIMENTAL

Materials

All solvents were HPLC grade, purchased from Fisher Scientific (Itasca, Illinois, USA) and tested in blank procedures.

Prep Sep silica cartridges were purchased from Fisher Scientific.

Clin Elut 1010 columns were purchased from Analytichem International (Harbor City, California, USA).

4-dimethyl aminopyridine (DMAP) and trifluoroacetic anhydride (TFAA) were purchased from Sigma Chemical Co. (St. Louis, Missouri, USA) and tested in blank procedures.

Deoxynivalenol was obtained from Myco Labs (Chesterfield, Missouri, USA).

T-2 tetraol and scirpenetriol were prepared in our lab by the alkaline hydrolysis of T-2 toxin and diacetoxyscirpenol, respectively (27).

All other reagents were analytical-reagent grade and tested in blank procedures.

Extraction

A 1 ml volume of saturated NaCl (aqueous) followed by 8 ml of centrifuged plasma or urine were added to a Clin Elut 1010 column. Two to three minutes after the sample had adsorbed onto the packing material, 2 x 40 mL of ethyl acetate was added to the column. The eluate was collected in a boiling flask and concentrated to dryness on a rotary evaporator. A quantitative aliquot was taken and saved for further confirmatory analysis, if necessary.

Hydrolysis

The residue was transferred to an 8 ml screw cap test tube and concentrated to dryness. A 200 µL volume of 0.25 N NaOH in 90 percent methanol was added, the test tube tightly capped then vortexed. The solution was heated for 5 minutes at 60°C then cooled at room temperature for 10 minutes. Immediately after the 10-minute cooling period, 200 µL of 0.25 N acetic acid in toluene was added to the test tube and vortexed. The solvent was concentrated to dryness under a stream of dry nitrogen and gentle heating in a 50°C water bath.

Silica Cartridge Cleanup

A silica cartridge was preconditioned with one column volume of (1:3) chloroform-acetone (v/v) followed by an equivalent volume of chloroform. The hydrolysis residue was transferred to the cartridge with 2 x 2 mL of (9:1) chloroform-acetone (v/v). After the rinse solvent had reached the top of the packing, an 8 mL screw cap test tube was placed beneath the cartridge. A 200 µl volume of absolute ethanol was added to the test tube originally containing the hydrolysis residue, vortexed, then followed with 1 mL of chloroform. The chloroform-ethanol mixture was transferred to the cartridge. To the test tube containing the original hydrolysis residue, 1 mL of (1:3) chloroform-acetone (v/v) was added, vortexed then added to the cartridge when the last portion of the chloroform-ethanol mixture had reached the top of the cartridge packing. Immediately after adding the first 1 mL of (1:3) chloroform-acetone, 4 mL of (1:3) chloroform-acetone (v/v) was added directly to the cartridge. All the eluate including the chloroform-ethanol mixture was collected in the same 8 mL screw cap test tube for a total volume of approximately 6 mL. The solvent was evaporated to dryness under a stream of dry nitrogen and gentle heating in a 50°C water bath.

Derivatization

To the residue, 1 mL of (9:1) toluene-acetonitrile (v/v) containing 2 mg/mL of DMAP was added then 50 µL of TFAA. The test tube was tightly capped and vortexed. The sample was heated for 20 minutes at 60°C then cooled at room temperature for approximately 5 minutes. After cooling, 1 mL of a five percent aqueous sodium bicarbonate solution was added. The sample was vortexed until the top layer was clear, a 100 µL aliquot of the top layer taken and diluted to 4 mL with hexane.

Gas Chromatography

A 2 µL volume was injected into a Hewlett Packard 5840A gas chromatograph equipped with an electron capture detector, a Hewlett Packard 7672A autosampler and a 1.8 m x 2 mm glass column packed with three percent OV-17 on 100/120 Supelcoport. The GLC conditions were as follows: oven temperature 165°C, injector temperature 275°C, detector temperature 300°C, flow rate 35 mL/minute.

RESULTS AND DISCUSSION

The method presented in this paper is a rapid and simple procedure to screen for trichothecenes in plasma and urine. About 20 samples can be easily prepared for GLC analysis in 8 hours. The method requires only three standards (deoxynivalenol, T-2 tetraol and scirpenetriol), which are commercially available or readily prepared by alkaline hydrolysis (27), to screen for at least 15 different trichothecene mycotoxins. Since the metabolites of T-2 and DAS are all converted to their respective parent alcohols, standards of the individual metabolites are not required for this screening procedure. This is advantageous since T-2 toxin and DAS are metabolized to a large variety of different metabolites by exposed animals.

T-2 and its metabolites (HT-2, T-2 triol, 4-acetyl tetraol, 8-acetyl tetraol, 15-acetyl tetraol, neosolaniol, 4-deacetylneosolaniol, 3'OH T-2, 3'OH HT-2) are all converted to T-2 tetraol by the hydrolysis of the acetyl and isovaleryl ester groups by the NaOH. DAS and its metabolite MAS undergo the same type of hydrolysis except the final product is scirpenetriol. T-2 tetraol and scirpenetriol, if present, undergo no change under the hydrolysis conditions used in this procedure. DON is not hydrolyzed; however, DON is susceptible to degradation under basic conditions that are slightly more severe than the hydrolysis conditions in this procedure. The hydrolysis conditions used in this procedure were optimized to achieve the complete conversion of T-2 to T-2 tetraol and DAS to scirpenetriol with minimal loss of DON. A reduced time and/or temperature parameter to insure the integrity of DON may not be adequate to insure the complete conversion to T-2 tetraol or scirpenetriol from their respective precursors. The time and temperature of hydrolysis are critical. Heating times beyond the optimized conditions lead to the loss of DON, scirpenetriol and T-2 tetraol (Figure 2). With pure standards of T-2, DAS and DON, heating for 5 minutes at 60°C (followed by immediate neutralization with acid) was sufficient for the complete conversion to T-2 tetraol and scirpenetriol with a minimal loss of DON. However, if residues from plasma or urine are present, a slightly longer incubation period was necessary for the total conversion to T-2 tetraol and scirpenetriol. An additional 10 minutes of incubation at room temperature immediately following 5 minutes of heating at 60°C is sufficient to achieve the full conversion with minimal loss of DON in the presence of sample residues. The percent of T-2 tetraol, scirpenetriol and DON remaining after 5 minutes at 60°C plus 10 minute room temperature incubation with real samples was the same as 5 minute heating at 60°C with

pure standards (90 percent T-2 tetraol, 95 percent scirpenetriol and 96 percent DON).

Chromatograms of control swine plasma and urine contained no interfering peaks (Figures 3 and 4). Baseline separation of T-2 tetraol, scirpenetriol and DON was achieved with a sensitivity level of approximately 25 ppb facilitating a multi-toxin analysis. Since both T-2 and DAS plus their respective metabolites are all converted to a single polar form (i.e., T-2 ---> T-2 tetraol, DAS ---> scirpenetriol), a cummulative effect results leading to a greater ability to detect trichothecene exposure. Since T-2 tetraol and scirpenetriol are the most sensitive members of their respective groups upon derivatization to their corresponding trifluoroacyl derivative, trichothecene exposure may be determined with greater sensitivity also. We have utilized this procedure to analyze urine samples from swine orally dosed with T-2. In previous examinations, T-2 toxin and its individual metabolites were not detected (detection limit 50 ppb). However, when several of these samples were rerun using this screening procedure, levels of 100-300 ppb of T-2 tetraol were found. Trichothecenes could be confirmed in these samples where the previous analysis gave no indication of exposure to T-2 toxin.

Recoveries were checked by spiking swine urine and plasma with DON, T-2, and DAS at several levels prior to extraction. The recoveries and standard errors are given in Tables 1 and 2. The recovery of T-2 tetraol directly off of a Clin Elut column is low (10 percent to 20 percent); however, current studies in cattle, swine, chickens, rats and mice have shown that T-2 tetraol is a minor metabolite in exposed animals (18,20,28). Plasma and urine extracts contain some residue upon concentration after hydrolysis. To achieve good recoveries, a total transfer of this residue to the silica cartridge is

necessary. The relatively nonpolar portion of the residue is transferred then eluted with the (9:1) chloroform-acetone. The 200 µL of absolute ethanol was necessary to transfer the more polar portion of the residue and the T-2 tetraol, scirpenetriol and DON which do not completely transfer with the (9:1) chloroform-acetone. The 1 mL of (1:3) chloroform-acetone was to insure a complete transfer of any remaining residue and trichothecene compounds. This multi-stage transfer was necessary to achieve good recoveries.

Kinetic studies have revealed that the half-life of T-2 and DAS to be approximately 10-20 minutes in the blood of pigs. At lethal IV doses, neither parent compound was found in the blood after 1-hour postdosing, and at lethal oral doses, no parent compound was found after 2 hours postdosing (29). For this reason, analysis of plasma and urine for T-2 or DAS after a prolonged period of time after exposure may be difficult and in many cases of minimal benefit. However, many metabolites of T-2 and DAS are found in the plasma and urine after the parent compounds are at low levels or no longer can be detected (12,14,15,18,29). The screening procedure outlined in this paper would be of benefit, especially if analysis was desired long after initial exposure to the source of trichothecenes--the parent compound would be undetectable, but its metabolites could still be present and available for hydrolysis to T-2 tetraol or scirpenetriol.

Analysis of plasma or urine by this method for the confirmation of trichothecene exposure in animals may be used as a complimentary procedure to the direct analysis of suspect feed. Analysis of feed suspected to be contaminated by trichothecenes may not always give an accurate account of the actual exposure levels for several reasons. The feed sample may not be available for analysis or may have been entirely consumed by the animal(s). Sample handling

techniques between exposure and subsequent analysis may have contributed to a decrease or increase of the toxin levels present especially if the feed samples were not dried properly. Also, the sample taken for analysis may not be a representive of the portion eaten. Analysis of blood and/or urine from the suspected animal for T-2 tetraol, scirpenetriol or DON would give a better indication of actual trichothecene exposure provided the urine and plasma samples were taken as soon as exposure was suspected.

This screening procedure provides a rapid and simple means of determining T-2, DAS and DON exposure by analysis of plasma and urine for trichothecene hydrolysis products. Also, high sensitivity and the availability of the necessary standards are added benefits.

ACKNOWLEDGEMENTS

This work was supported in part by contract DAMD17-82-C-2179 from the U. S. Army Medical Research and Development Command.

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TABLE 1. DAS, DON and T-2 recoveries in plasma after hydrolysis.

Level[1]	n	DAS (percent)	DON (percent)	T-2 (percent)
50 ppb	10	94 ± 4	93 ± 8	80 ± 6
250 ppb	10	96 ± 4	94 ± 3	80 ± 3
500 ppb	10	116 ± 7	114 ± 3	99 ± 5

[1]Molar percent.

[2]Mean ± standard error.

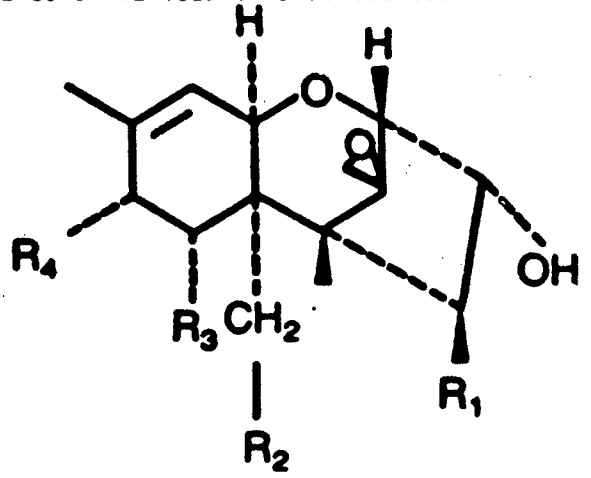
TABLE 2. DAS, DON and T-2 recoveries in urine after hydrolysis.

Level[1]	n	DAS (percent)	DON (percent)	T-2 (percent)
50 ppb	10	88 ± 5	78 ± 4	105 ± 10
250 ppb	4	119 ± 1	86 ± 1	97 ± 2
500 ppb	6	112 ± 3	95 ± 4	111 ± 4
1000 ppb	10	110 ± 4	93 ± 4	106 ± 9

[1]Molar percent.

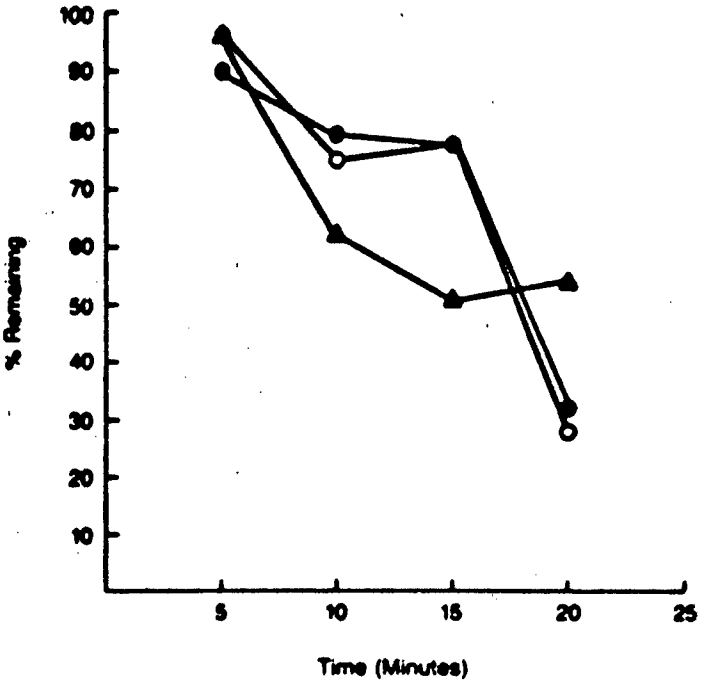
[2]Mean ± standard error.

FIGURE 1. Structures of various trichothecenes.



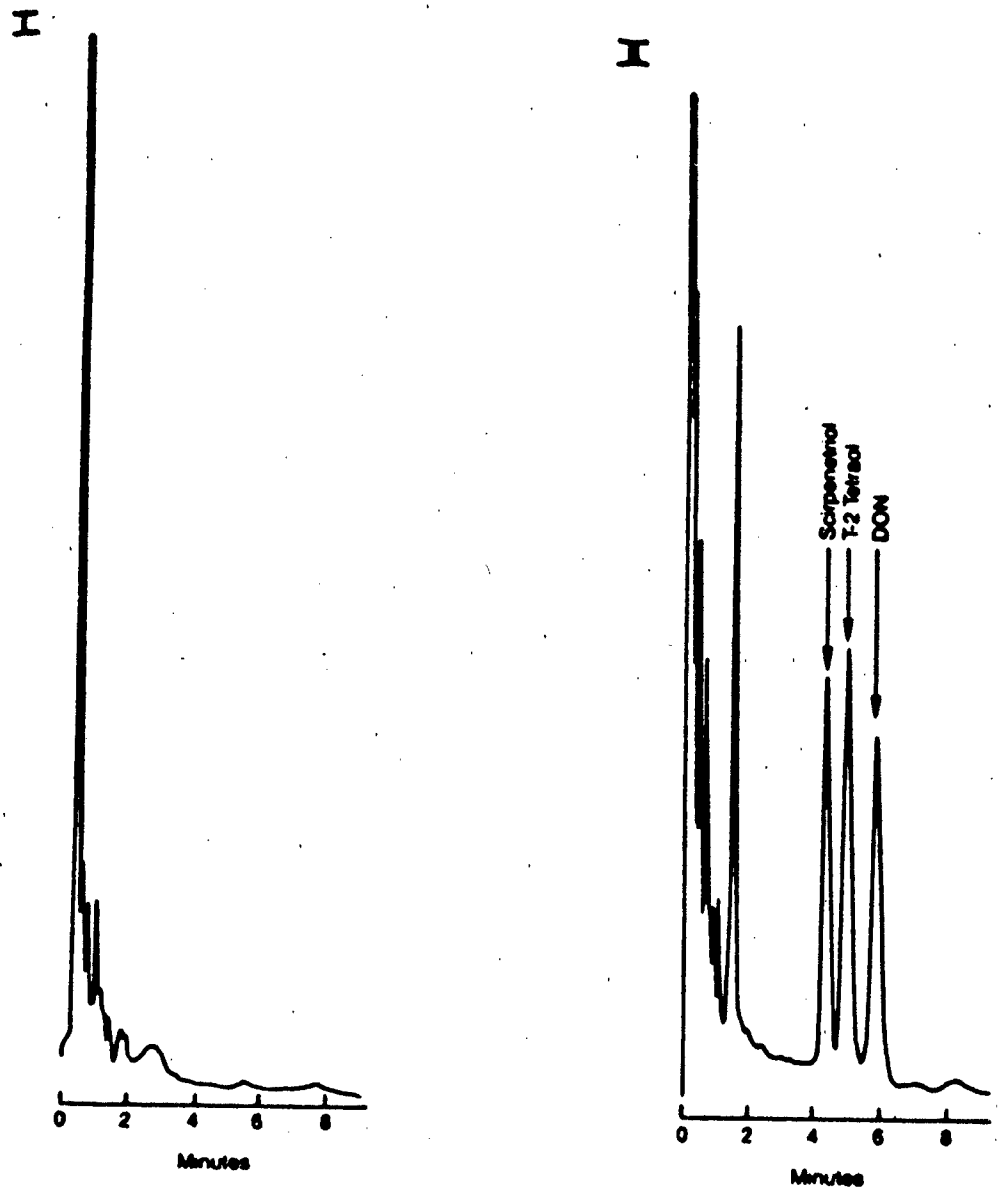
Compound	R_1	R_2	R_3	R_4
T-2 Group:				
T-2 toxin	OAc	OAc	H	$OCCH_2CH(CH_3)_2$ (O)
HT-2	OH	OAc	H	$OCCH_2CH(CH_3)_2$ (O)
T-2 triol	OH	OH	H	$OCCH_2CH(CH_3)_2$ (O)
T-2 tetraol	OH	OH	H	OH
Neosolaniol	OAc	OAc	H	OH
4-deacetyl neosolaniol	OH	OAc	H	OH
4-acetyl tetraol	OAc	OH	H	OH
8-acetyl tetraol	OH	OH	H	OAc
15-acetyl tetraol	OH	OAc	H	OH
3'OH T-2	OAc	OAc	H	$OCCH_2C(CH_3)_2$ (O, OH)
3'OH HT-2	OH	OAc	H	$OCCH_2C(CH_3)_2$ (O, OH)
DAS Group:				
diacetoxyscripenol	OAc	OAc	H	H
monoacetoxyscirpenol	OH	OAc	H	H
scirpenetriol	OH	OH	H	H
deoxynivalenol (DON)	H	OH	OH	O

FIGURE 2. Stability of T-2 tetraol, scirpenetriol and DON under hydrolysis conditions.



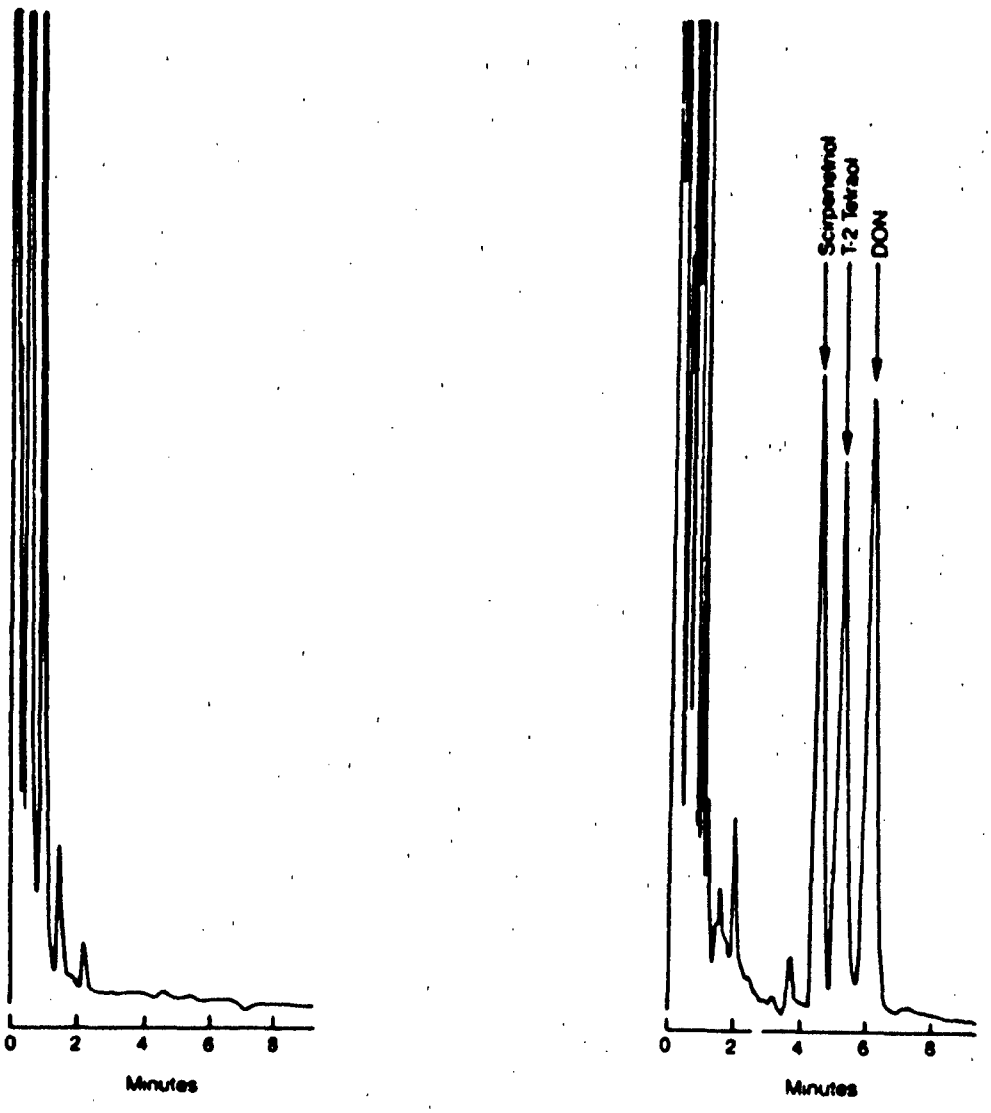
Percent of T-2 tetraol, scirpenetriol and DON remaining after incubation at 60°C of T-2, DAS and DON with 0.25 N NaOH in 90 percent methanol. ● denoted T-2 tetraol, ▲denoted scirpenetriol, O denoted DON.

FIGURE 3. Chromatograms of pig plasma.



GLC chromatograms of control pig plasma (I) and spiked pig plasma (II) at 250 ppb T-2, DAS and DON.

FIGURE 4. Chromatograms of pig urine.



GLC chromatograms of control pig urine (I) and spiked pig urine (II) at 500 ppb T-2, DAS and DON.

B. QUANTITATION OF DEOXYNIVALENOL AND ITS METABOLITE DOM-1 IN BOVINE URINE AND FECES BY GAS CHROMATOGRAPHY WITH ELECTRON CAPTURE DETECTION--A. M. Dahlem, S. P. Swanson, L-M. Cote, T. Yoshizawa, W. B. Buck

SUMMARY

Gas chromatographic procedures for quantitation of the mycotoxin deoxynivalenol (DON) and its metabolite DOM-1 in bovine urine and feces were developed to aid in diagnosis of DON exposure. Compounds in urine and feces were extracted from diatomaceous earth columns followed by silica cartridge clean-up. Trimethylsilyl (TMS) derivatives of the toxins were prepared and identified by gas chromatography with electron capture detection. Respective recoveries of DON and DOM-1 were 86.7 percent and 93.6 percent in urine, and 87.4 percent and 81.3 percent in feces.

Three dairy cows were fed mycotoxin-contaminated feed for 5 days, and their urine and feces were collected for analysis by these methods. DOM-1 was the major metabolite detected in both urine and feces. DON and its metabolite DOM-1 were quantitated in both urine and feces with a minimum detection limit of 50 ppb.

INTRODUCTION

Deoxynivalenol (DON, vomitoxin) is a naturally occurring trichothecene mycotoxin produced by several species of the fungus Fusarium, especially F. graminearum (1). Deoxynivalenol contaminated feed has been associated with numerous cases of sublethal toxicosis in animals resulting in feed refusal, reduced weight gain, emesis and diarrhea (2-4). Knowledge of residue transmission to humans through animal tissue remains incomplete.

DOM-1 is a metabolite of DON which is formed by the reduction of the epoxide group of DON leading to the loss of oxygen and formation of a carbon-carbon double bond (Figure 1). This metabolite was originally described in rats (5), it has been found in cattle (6) and it can be produced in vitro (7,8) by rumen microflora. The toxicity of DOM-1 remains unknown.

Diagnostic procedures for DON exposure in cattle have to date relied upon the analysis of feedstuffs, and several methods have been reported (9-11). This report presents procedures for rapid quantitative analysis of deoxynivalenol and its metabolite DOM-1 directly from bovine urine and feces. Gas chromatography (GC) with electron capture detection is used for the quantitative determination of these two mycotoxin compounds as trimethylsilyl ethers in both urine and feces.

EXPERIMENTAL

Chemicals

Deoxynivalenol [3 α, 7 α, 15-trihydroxy-12-13-epoxy trichothecene-9-en-8 one] was purchased from Myco Labs (Chesterfield, MO) for standard preparation. DOM-1, the de-epoxy metabolite of DON, was supplied by T. Yoshizawa.

Trimethylsilyl derivatizing reagent was prepared by mixing 5 parts trimethylsilylimidazole with one part trimethylchlorosilane purchased from Sigma Chemical Company (St. Louis, MO). Clin Elute columns were purchased from Analytichem International (Harbor City, CA). Prep-Sep, prepacked silica cartridges containing 300 mg silica were purchased from Fisher Scientific (Chicago, IL) and C-18 cartridges used in the initial feces extraction were Baker 500 mg (J. T. Baker, Phillipsburg, NJ). All solvents were distilled in glass.

Instrumentation

Gas chromatography was performed using a Hewlett Packard Model 5840A equipped with a ^{63}Ni electron capture dectector, a 1.8 m x 2 mm ID glass column packed with 3 percent OV-17 on 100 to 120 mesh supelcoport and a Hewlett Packard Model 7671A autosampler. Other conditions were as follows: carrier gas of argon-methane (95:5) at a flow rate of 35 mL/min, column oven temperature 220° C, injector temperature 275° C and detector temperature 325° C.

Standard Solutions

DON and DOM-1 were dissolved in absolute ethanol to give stock standard concentrations of 25 ng/μL. Spike recoveries were calculated by comparing DON-enriched samples of urine and feces with a standard calibration curve of 50, 100, 200, 400 and 800 pg of DON. Control samples of urine and feces were collected from experimental animals prior to addition of DON-contaminated feed to their ration.

ANALYTICAL PROCEDURES

Urine

One mL of saturated NaCl solution was introduced into a 10 mL Clin Elute column 3 minutes prior to addition of 9 mL urine. After 5 minutes, the mycotoxins were eluted with 100 mL of ethyl acetate into a boiling flask. The ethyl acetate was evaporated with a rotary evaporator and the residue transferred to a silica cartridge (preconditioned with $CHCl_3$). The flask was rinsed a third time with 200 μL of acetone followed by 1.8 mL of $CHCl_3$, then transferred to the silica cartridge and allowed to drain. The cartridge was then rinsed with an additional 3 mL of (9:1) $CHCl_3$-acetone. The toxins were eluted from the silica cartridge with 9 mL of (3:1) $CHCl_3$-acetone collected in a disposable test tube and the eluate was concentrated to dryness under an air stream.

Feces

Twelve grams whole feces or 4 grams lyophilized feces were extracted with 90 ml of 10 percent MeOH in water containing 1 percent NaCl. Samples were agitated for 1 hour on a wrist action shaker and filtered through Whatman No. 4 filter paper lined with glass wool. Twenty mL aliquots of sample extracts were partitioned twice with 20 mL of hexane and the hexane layers were discarded. The aqueous sample was added to a 20 mL Clin Elute column and left for 5 minutes to allow adsorption onto the diatomaceous earth packing. The toxins were eluted with 160 mL ethyl acetate into a boiling flask. The ethyl acetate was evaporated with a rotary evaporator, and the remaining residue was transferred to a silica cartridge and eluted by the same method described for urine.

Derivatization

The extracts were redissolved for derivatization in 25 µL of TMS derivatizing reagent and 100 µL ethyl acetate, vortexed and heated at 60° C for 5 minutes. The sample was then diluted with 9.9 mL of hexane and placed at room temperature overnight (18 hours). Two-hundred µL of diluted extract was then added to 800 µL of hexane in autosampler vials, and 2 µL was injected onto the gas chromatograph.

An alternate method for feces extraction was utilized prior to discovery of silica column effectiveness. Following evaporation of ethyl acetate from Clin Elute columns, the extract was transferred with 3 x 2 mL $CHCl_3$ to a Florisil column containing 2.5 g 60-100 mesh Florisil packed in $CHCl_3$. The Florisil columns were rinsed with 30 mL $MeCl_2$-acetone (95:5) and eluted with 50 mL $MeCl_2$-MeOH (9:1). The extract was evaporated, and the remaining residue was dissolved in 100 µL of MeOH followed by addition of 1 mL of water. The

sample in MeOH/water solution was then loaded onto Baker C-18 columns rinsed with 2.0 mL water and eluted with 2.0 mL of methanol-water (1+1). The C-18 extract was evaporated under air stream at 50° C and derivatized as previously described.

RESULTS AND DISCUSSION

Deoxynivalenol and its metabolite DOM-1 were quantitated in urine and feces by gas chromatography with a ^{63}Ni electron capture detector as the corresponding trimethylsilyl derivatives. The detection limits in both urine and feces for both trichothecenes were 50 ppb. The overall recoveries of urine and feces spikes (Table 1) were 86.7 percent and 87.4 percent for DON and 93.6 percent and 81.3 percent for DOM-1, respectively. The relatively low number of DOM-1 samples with respect to DON samples was due to a limited availability of standard.

Urine Analysis and Quantitation

Control cow urine was collected and frozen prior to analysis. Aliquots of 100 ml of urine were spiked with appropriate volumes of toxin 1 hour prior to analysis. Centrifugation of urine at 1,500 rpm prior to addition to Clin Elute columns aided recovery of the toxins probably due to a better adsorption of materials onto packing materials after removal of solid precipitate. Addition of saturated NaCl solution directly to the Clin Elute columns rather than to the sample prior to loading on column reduced the amount of salt residue after evaporation of the elution solvent and increased transfer efficiency. Disposable silica cartridges were an inexpensive and efficient method for purification of deoxynivalenol and DOM-1 in urine and use of chloroform and acetone mixtures as solvents allowed for quick solvent evaporation. Trimethylsilyl derivatization of extracts allowed short GC runs of

approximately 6 minutes at 220° C. No late eluting or interferring peaks were observed (Figure 2).

Feces Analysis and Quantitation

Cow feces were collected and determined to be trichothecene-free by G.C. prior to addition of DON and DOM-1 for recovery studies. Individual samples were spiked prior to extraction with appropriate volumes of mycotoxin standard solution. Partitioning of hexane soluble compounds from the aqueous extract aided the removal of extraneous GC peaks upon injection.

The use of a silica cartridge reduced the volume of solvent required and provided more rapid clean-up over the Florisil and C-18 method which was originally utilized. Recoveries of DON and DOM-1 were consistent between both methods so, due to the relative ease of use of silica cartridges, the silica cartridges were used.

Analysis of Urine and Feces From Orally Dosed Cow

We have utilized this method for analysis of urine and feces obtained from cows fed DON contaminated feed. DOM-1 was the major metabolite detected in both urine and feces and was present in most cases at 6 to 10 times the concentrations of DON. Maximal concentrations of DOM-1 in urine and feces were 18 ppm and 13 ppm, respectively. Urine was the specimen of choice for detection of DON exposure due to the relative ease of analysis and the high levels contained within, but feces provided a readily collectable and available specimen for analysis also.

Information on the metabolism and excretion of DON in dairy cattle fed DON-contaminated feed will be reported elsewhere.

ACKNOWLEDGEMENTS

This research was supported in part by a NC-129 grant from the University of Illinois Experiment Station and by contract DAMD17-82-C-2179 from the U.S. Army Medical Research and Development Command. The technical assistance of H. D. Rood, Jr., and A. B. Fidducia is gratefully appreciated.

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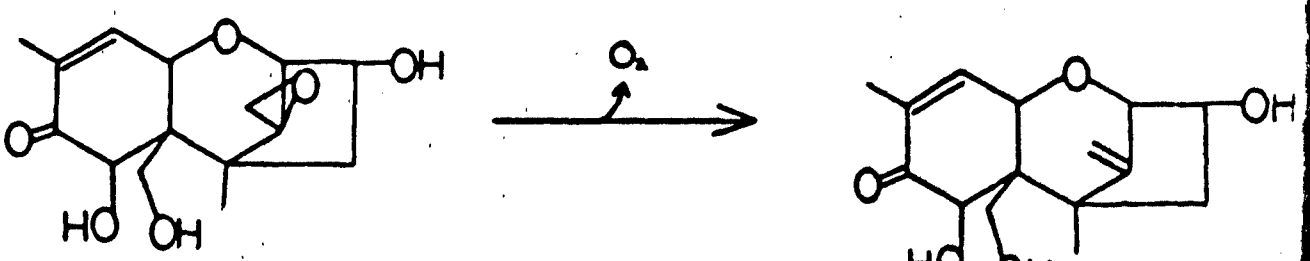
TABLE 1. Recovery of DON and DOM-1 from spiked urine and feces

Sample	Compound	ng/mL	n	Percent Recovered	S.E.
Urine	DON	200	15	97.8	3.9
		1,000	15	85.0	1.5
		5,000	15	77.3	1.3
Overall:			45	86.7	1.9
	DOM-1	200	3	93.6	2.9
Feces	DON	500	10	78.9	3.8
		1,000	10	88.4	3.8
		5,000	10	94.9	2.7
Overall:			30	87.4	2.3
	DOM-1	500	2	76.4	
		1,000	2	86.1	

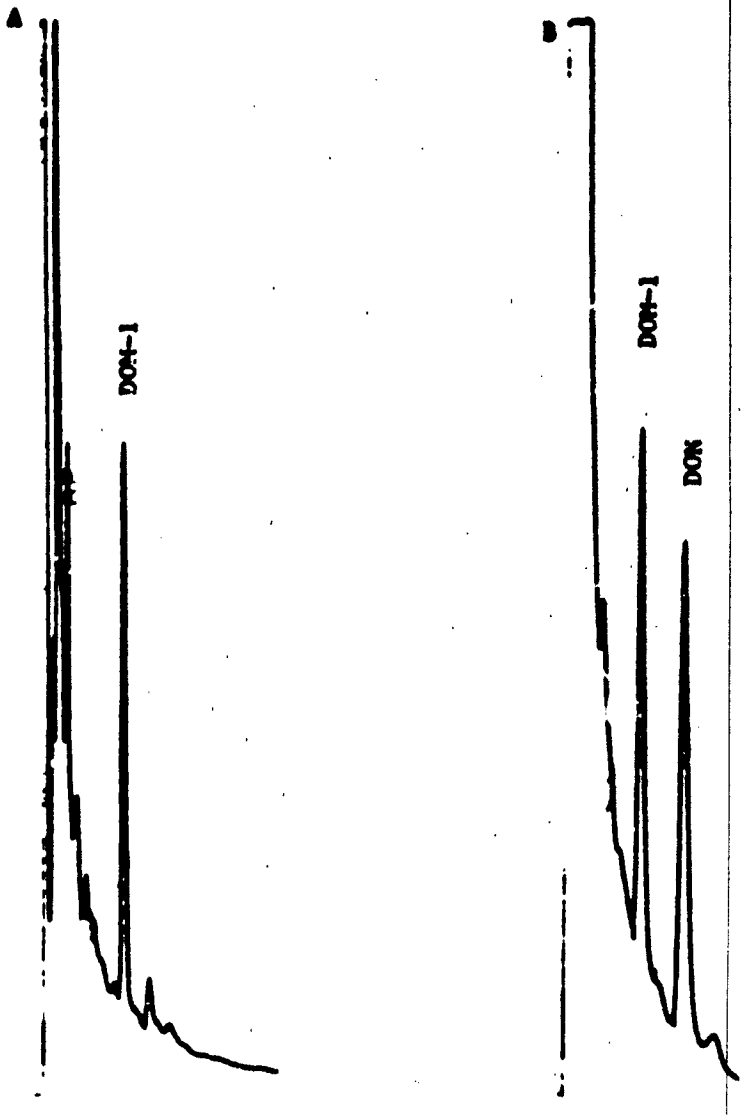
FIGURE 1. Reduction of deoxynivalenol to DOM-1.



DON

DOM-1

FIGURE 2. GLC chromatograms of derivatized urine extracts. A) Urine from cow fed DON-contaminated ration. B) DON / DOM-1 cow urine spike (200 ng / ml).



V. METABOLISM, IN VIVO

A. GLUCURONIDE CONJUGATES OF T-2 TOXIN AND METABOLITES IN SWINE BILE AND URINE--R. A. Corley, S. P. Swanson, W. B. Buck

ABSTRACT

Metabolite profiles in the bile and urine of two swine were determined by thin layer radiochromatography following the intravascular administration of 1.0 mCi of tritium-labeled T-2 toxin at a dose of 0.15 mg/kg body weight. A total of 13.11 percent and 1.29 percent of the administered dose was found in the gall bladders in addition to 17.93 percent and 42.46 percent in the urine of the two swine 4 hours after dosing. Free (unconjugated) metabolites represented less than 20 percent of the metabolite residues in bile and less than 31 percent in the urine. The parent compound, T-2 toxin, never exceeded 0.25 percent of the total metabolite residues present in bile or urine. The major free metabilites were 3'-OH HT-2 and T-2 triol. Glucuronide conjugates of T-2 toxin and its metabolites represented approximately 77 percent of the metabolite residues in bile and 63 percent in urine. The major conjugated metabolites in bile and urine were glucuronides of HT-2, 3'-OH T-2, 3'-OH HT-2 and T-2 toxin. Neosolaniol, 4-deacetylneosolaniol and T-2 tetraol in the free and glucuronide form were also identified in addition to three unknown metabolites designated PM-1 to PM-3.

INTRODUCTION

T-2 toxin (4ß, 15-diacetoxy-8α-(3-methylbutyryloxy)-3α-hydroxy-12,13,-epoxytrichothec-9-ene) is a toxic fungal metabolite produced by several species of Fusaria (Bamburg and Strong, 1971; Pathre and Mirocha, 1977). T-2 toxin has

been found in naturally contaminated corn, barley, and mixed feeds in the US and Canada at concentrations of 0.076-25 ppm (Vesonder, 1983). When present in the diets of livestock and poultry, T-2 toxin has been associated with feed refusal, infertility, diarrhea, intestinal irritation and possibly hemorrhage, perioral and pharyngeal irritation and lowered immunity (Hsu et al., 1972; Palyusik and Koplik-Kovacs, 1975; Speers et al., 1977; Weaver et al., 1977; Weaver et al., 1978a; Weaver et al., 1978b; Rafai and Tuboly, 1982; Hoerr et al., 1982). Trichothecene mycotoxins, including T-2 toxin, and their effects on humans have attracted considerable international attention because of their possible use in chemical warfare as the agent "Yellow Rain" (Rosen and Rosen, 1982; Mirocha et al., 1983; Watson et al., 1984).

Many procedures have been reported for the analysis of T-2 toxin in grains and mixed feeds (Scott, 1982). Analytical procedures designed to detect T-2 toxin alone in body fluids, excrement or tissues will probably fail to confirm exposure since several studies on the fate of T-2 toxin in laboratory animals, poultry, and livestock have demonstrated that the parent compound, T-2 toxin, is rapidly cleared from body fluids and tissues (Chi et al., 1978; Matsumoto et al., 1978; Robison et al., 1979; Yoshizawa et al., 1981). Toxicokinetic studies of T-2 toxin in growing gilts and heifers (Beasley, 1984) demonstrated that the disappearance of intravascularly administered T-2 toxin follows a two-compartment open model with mean plasma elimination phase half-lives of 13.8 minutes for swine and 17.4 minutes for cattle. In spite of administration of a lethal oral dose in swine (2.4 mg/kg) and a toxic oral dose in calves (3.6 mg/kg) no parent T-2 toxin was detected in plasma or urine at a detection limit of 25 ng/mL. These results indicate that the parent compound, T-2 toxin, is very rapidly eliminated in all species examined.

Studies of the in vivo metabolism of tritium-labeled T-2 toxin in laboratory animals, livestock, and poultry have demonstrated that hydroxylation at the 3'-carbon position and hydrolysis of ester linkages are important biochemical reactions for the metabolism of T-2 toxin (Yoshizawa et al., 1980; Yoshizawa et al., 1981; Yoshizawa et al., 1982). Although several metabolites were identified in these studies (Table 1) many were not structurally characterized. In a study of the metabolic fate of tritium-labeled T-2 toxin in a lactating cow, a significant fraction of the original radioactivity in urine (24-54 percent) was present as very polar metabolites, possibly conjugates (Yoshizawa et al., 1981). Kosuri et al., 1971, reported that the excretion of glucuronides in rats was increased following intraperitoneal administration of T-2 toxin at 2 mg/kg and increased further when rats were pretreated with phenobarbital, suggesting that glucuronide conjugation is a possible route of metabolism for T-2 toxin. Since swine are physiologically similar to the human (Pond and Houpt, 1975), and are an important agricultural commodity, this species was used to investigate the metabolism of tritium-labeled T-2 toxin. This paper reports the extent of glucuronide conjugation of T-2 toxin and its metabolites excreted in swine bile and urine.

EXPERIMENTAL SECTION

Reference Standards

Tritium-labeled T-2 toxin (radiopurity > 99 percent, specific activity 1.287 mCi/mg) was synthesized by the method of Wallace et al., 1977. Unlabeled standards of T-2 toxin, neosolaniol, HT-2, T-2 triol, 4-deacetylneosolaniol, and T-2 tetraol were produced from cultures of Fusarium tricinctum in our laboratory. Additional standards of 3'-OH T-2 and 3'-OH HT-2 were kindly provided by T. Yoshizawa (Yoshizawa et al., 1982).

Animal Treatment

Two 20 kg female crossbred swine (Yorkshire x Hampshire; Thrushwood Farms, Fairbury, IL) were injected with erysipelas bacterin (Rhusigen, Pitman-Moore, Inc., Washington Crossing, NJ) and acclimated to the large animal holding facility at the College of Veterinary Medicine, University of Illinois. All feeds offered to experimental swine were free from detectable concentrations of trichothecene mycotoxins and aflatoxins. Immediately prior to dosing, catheters were inserted into the urinary bladders of each swine. Tritium-labeled T-2 toxin was diluted with nonradioactive T-2 toxin in 1.5 mL of 50 percent ethanol such that each swine received 1 mCi of total radioactivity at a dosage of 0.15 mg/kg body weight intravascularly as a single bolus injection.

During the course of the experiment, the animals were restrained in a plastic-lined sling. Urine was collected hourly. The animals were euthanized 4 hours after dosing by administration of an anesthetic dose of pentobarbital followed by exsanguination. Bile was immediately collected from the gall bladders.

Determination of Total Radioactivity

The total radioactivity in urine was determined by adding 0.2 mL of urine directly to 5 mL of Aquasol-2 liquid scintillation cocktail (New England Nuclear Corp., Boston, MA). The total radioactivity in bile was determined by adding 0.02 mL of bile to 0.1 mL of 30 percent H_2O_2 followed by heating at 60°C for 1 hour. Aquasol-2 (5 mL) was added for scintillation counting. The counting of radioactivity was performed with a Packard Tri-Carb 300 M liquid scintillation counter (Packard Instrument Co., Inc., Downers Grove, IL). All data were corrected for background, dilution, quenching, and counting efficiency.

Extraction of Bile and Urine

A 0.2 mL volume of urine or 0.05 mL of bile was added in duplicate to 2 mL of 0.1 M acetate buffer (pH 3.8, Sigma Chemical Co., St. Louis, MO) and heated at 90°C for 30 min to inactivate enzyme inhibitors. After cooling, 1 mL of either 0.1 M acetate buffer or ß-glucuronidase (Sigma Chemical Co., type L-II from limpets; 4500 units/mL in 0.1 M acetate buffer) was added to duplicate samples and incubated with gentle mixing in a 38°C waterbath for 18 hours. A positive enzyme control utilizing phenolphthalein glucuronide (Sigma Chemical Co., 0.01 M, pH 7.0) and ß-glucuronidase in bile or urine was included with each set of samples. The ß-glucuronidase preparation used in this procedure also contained aryl sulfatase activity. Therefore, to confirm that metabolites liberated in this assay originated from conjugates of glucuronic acid a specific inhibitor of ß-glucuronidase, saccharic acid-1,4-lactone (10 mM final concentration; Sigma Chemical Co.), was added to replicate samples. Following incubation, samples were added to a 500 mg C18 cartridge (Baker-10 SPE, J. T. Baker Chemical Co., Phillipsburg, NJ; preconditioned with two column volumes of methanol followed by two column volumes of deionized water). The sample tubes were rinsed 2 x 1 mL with deionized water and added to the column. The aqueous eluates were combined and assayed for radioactivity to determine losses. Metabolites were eluted with 2 x 0.8 mL methanol and concentrated to 0.5 mL for TLC radiochromatography. The extraction efficiencies for all metabolites (total radioactivity) were (mean ± SE) 93.5 ± 1.6 percent (n = 40).

TLC Radiochromatography

Aliquots or each sample were spotted onto the outer channels of a precoated silica gel TLC plate (5 x 20 cm, 0.25 mm gel thickness, J. T. Baker; scored into three equal channels). Standard compounds were spotted in the middle

channel. The plates were developed in chloroform-methanol (9 + 1) and allowed to air dry. To obtain radiochromatographic profiles of each sample, 1-2 mm wide bands were scraped from the TLC plates directly into scintillation vials. A 0.1 mL volume of water was added to each vial followed by 0.25 mL of methanol. The samples were then counted in 5 mL of Aquasol-2. After scraping sample zones, the remainder of the plates were sprayed with 30 percent H_2SO_4 in methanol and heated at 120°C for 3-5 min. The standards were visualized under a long wave (365 nm) UV lamp.

Two-dimensional high performance thin layer chromatography was used to aid in the identification of metabolites. After co-spotting each sample with standards in the corner of a precoated silica gel HPTLC plate (10 x 10 cm, 0.2 mm gel thickness, Whatman HP-K), the plates were developed first in chloroform-methanol (9 + 1). After air-drying, the plates were developed the second direction in ethyl acetate-isooctane (3 + 1). The compounds were visualized with p-nitrobenzylpyridine (Takitani et al., 1979). Individual spots corresponding to known standards were scraped and analyzed for radioactivity as above to confirm the presence of metabolites.

RESULTS

Bile

A total of 13.11 percent and 1.29 percent of the administered dose was found in the gallbladders of swine S1 and S2, respectively, at necropsy. Since the bile was not sampled continuously over the course of the experiment, the total fraction of the dose eliminated by the liver into the bile could not be determined. Results are summarized in Table 2.

Several free metabolites, representing 9.93 percent (S1) and 19.41 percent (S2) of the metabolite residues in bile, were identified by thin layer radiochromatography (Table 4) with 3'-OH HT-2 and T-2 triol as the major metabolites. A significant fraction of the total radioactivity in bile (82 percent) was present at the origin of the TLC plates (see Figure 1). When aliquots of bile from both swine were incubated with ß-glucuronidase, the percentage of radioactivity at the origin decreased from 82 percent to an average of 8 percent of the total radioactivity. This decrease in radioactivity at the origin was matched by an increase in the concentration of several free metabolites with HT-2, 3'-OH HT-2 and the parent, T-2 toxin, as the major compounds identified. No significant differences ($p < 0.05$; t-test) were detected in metabolite profiles between samples incubated with ß-glucuronidase in the presence of 10 mM saccharic acid-1,4-lactone and those incubated in buffer alone (see Figure 1). These results confirm that T-2 toxin and its metabolites are conjugated with glucuronic acid. Glucuronide conjugates of known metabolites (listed in Table 1) represented 76.55 percent (S1) and 77.80 percent (S2) of the total radioactivity present in bile. Total known metabolites identified (free and glucuronide conjugates) accounted for 86.48 percent (S1) and 97.22 percent (S2) of the radioactivity in bile from both swine. An additional 2-8 percent of the total radioactivity was associated with metabolites whose structures are currently unknown (designated PM-1 to PM-3; see Figure 1).

Urine

A total of 17.93 percent and 42.47 percent of the administered dose was eliminated in the urine of swine S1 and S2, respectively, by 4 hours after dosing (Table 3). Concentrations of total metabolite residues peaked between

60 and 120 minutes in the urine from both swine and decreased rapidly by 4 hours.

Several free metabolites, representing 30.87 percent (S1) and 24.16 percent (S2) of the radioactivity in urine, were identified by thin layer radiochromatography (Table 5) with 3'-OH HT-2 and T-2 triol as the major metabolites. As with bile, a significant fraction of the radioactivity in urine (72 percent) remained at the origin of the TLC plate (see Figure 1). Following incubation with ß-glucuronidase, the percentage of radioactivity at the origin decreased from 72 percent to 2 percent while the concentration of several metabolites increased. No significant differences ($p < 0.05$; t-test) were detected in metabolite concentrations between urine incubated with ß-glucuronidase in the presence of 10 mM saccharic acid-1,4-lactone and urine incubated in buffer alone. Glucuronide conjugates of T-2 and identified metabolites (see Table 1) represented 59.21 percent (S1) and 67.76 percent (S2) of the radioactivity in urine. Total known metabolites identified (free and glucuronide conjugates) accounted for 90.14 percent (S1) and 91.90 percent (S2) of the radioactivity eliminated via the urine within 4 hours. As with bile, several unknown metabolites (PM-1 to PM-3) were detected; however, these metabolites accounted for less than 4 percent of the total radioactivity present in urine.

It should be noted that storage of urine longer than 1 year at -20°C resulted in a significant decrease in the ability of the ß-glucuronidase enzyme system to liberate metabolites of T-2 toxin. This was evidenced by an increase in the radioactivity located at the origin of the TLC plates following enzyme hydrolysis from an average of 2 percent in fresh urine to 36 percent in urine stored longer than 1 year. This phenomenon did not occur in bile stored under identical conditions.

DISCUSSION

Glucuronidation plays an important role in the metabolism of T-2 toxin in intravascularly dosed swine. Glucuronide conjugates of T-2 toxin and its metabolites listed in Table 1 represented approximately 77 percent of the metabolite residues in the bile and 63 percent in urine of these swine. The formation of glucuronide conjugates generally result in the elimination of pharmacological or toxicological activity of xenobiotics. If the glucuronides undergo hydrolysis by gut microflora, however, reabsorption of active compounds may occur.

As shown in Table 6, the parent compound never exceeded one-fourth of one percent of the total metabolite residues in bile or urine. Analytical procedures designed to detect only the parent compound in bile or urine will be very difficult and may fail to confirm exposure. The major free (unconjugated) metabolites in bile and urine were 3'-OH HT-2 and T-2 triol. These two metabolites, however, represent at most only 24 percent of the metabolite residues in urine and 15 percent in bile.

The major conjugated metabolites in bile and urine were glucuronides of HT-2, 3'-OH T-2, 3'-OH HT-2, and T-2 toxin. The glucuronide of T-2 toxin, however, represented a much higher percentage of the metabolite residues in bile (approximately 42 percent) than in urine (approximately 11 percent). If swine intestinal microflora are found to hydrolyze glucuronide conjugates of T-2 toxin and its metabolites, then a large amount of the parent compound in addition to several metabolites will be available for reabsorption and possibly enterohepatic recirculation.

Since the identification of glucuronide conjugates in this study was accomplished by analysis of metabolites following enzymatic hydrolysis, the

actual location(s) of the glucuronic acid moiety is (are) unknown. The parent compound, however, has only one hydroxyl group located at the C-3 position. Glucuronide conjugation is, therefore, likely to occur at this positon for T-2 toxin.

ACKNOWLEDGEMENT

The authors are greatly indebted to G. J. Gullo and L. Johnson for their technical assistance and to G. Lundeen, R. Poppenga, and V. Beasley for their surgical expertise.

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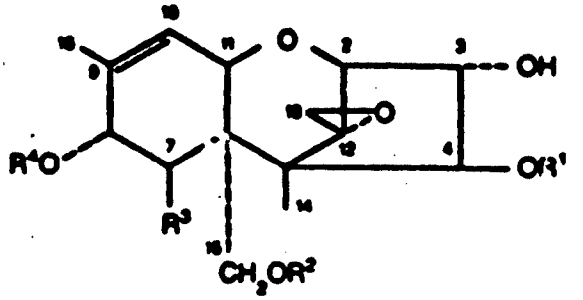
TABLE 1. Chemical structures of T-2 toxin and its metabolites.



Generic Name	Designations[a]	R^1	R^2	R^3	R^4
T-2 toxin[b-d]		Ac	Ac	H	$-COCH_2CH(CH_3)_2$
3'-OH T-2 toxin[c-e]	TC-1,(TB-1)	Ac	Ac	H	$-COCH_2C(OH)(CH_3)_2$
Neosolaniol (NEO)[b-d]		Ac	Ac	H	H
HT-2 toxin[b-d]	TC-2	H	Ac	H	$-COCH_2CH(CH_3)_2$
3'-OH HT-2 toxin[c-e]	TC-3,(TB-3)	H	Ac	H	$-COCH_2C(OH)(CH_3)_2$
T-2 triol[c]		H	H	H	$-COCH_2CH(CH_3)_2$
4-deacetylneosolaniol (4-DN)[c,d]	TMR-1, TC-4, TB-6	H	Ac	H	H
3'-OH-7-OH HT-2 toxin[d,f]	TC-6	H	Ac	OH	$-COCH_2C(OH)(CH_3)_2$
T-2 tetraol[c]		H	H	H	H

[a] Designations listed in parenthesis (TB-1 and TB-3) have not been confirmed.

[b] Matsumoto et al., 1978.

[c] Yoshizawa et al., 1980.

[d] Yoshizawa et al., 1981.

[e] Yoshizawa et al., 1982.

[f] Pawlosky and Mirocha, 1984.

TABLE 2. Total concentration and percent of administered dose Of T-2 toxin and its metabolites in bile collected from the gallbladders of two swine (S1 And S2) at time of euthanasia (4 hours).

Animal	Total Volume (mL)	Concentration (µg/mL)	Percent of Dose
S1	13.3	24.87 ± 0.51*	13.11
S2	3.9	10.02 ± 0.13	1.29

*x ± SE (n = 15), based upon specific radioactivity of administered tritium-labeled T-2 toxin.

TABLE 3. Total concentration and percent of administered dose of T-2 toxin and its metabolites in urine from two swine (S1 and S2).

Animal	Time After Dosing (min)	Total Volume (mL)	Concentration (μg/mL)	Percent of Dose	Cumulative Percent of Dose
S1	60	6.8	ND[a]	0	0
	120	9.4	26.89 ± 0.84[b]	10.02	10.02
	180	11.2	17.42 ± 0.76	7.73	17.75
	240	0.8	5.61 ± 0.13	0.18	17.93
S2	60	30.0	6.44 ± 0.05	6.38	6.38
	120	21.0	27.21 ± 0.41	18.87	25.25
	180	20.0	13.82 ± 0.30	9.13	34.38
	240	35.0	6.99 ± 0.12	8.08	42.46

[a]ND indicates none detected (detection limit of 0.1 ng/mL).

[b]x ± SE (n = 6) based upon specific radioactivity of administered tritium-labeled T-2 toxin.

TABLE 4. Concentration of T-2 toxin and its metabolites in bile collected from the gall bladders of two swine (S1 and S2) at time of euthanasia (4 hours)

	S1				S2			
	Concentration (ng/mL)			% of Dose	Concentration (ng/mL)			% of Dose
Metabolite	Free	Conjugated	Total	(Total)	Free	Conjugated	Total	(Total)
T-2 toxin	63 ± 22[a]	11,768[b]	11,831 ± 271	6.24	12 ± 5	3,622	3,634 ± 29	0.47
3'OH T-2	106 ± 30	1,096	1,202 ± 32	0.63	33 ± 2	399	432 ± 11	0.06
NEO	56 ± 7	177	232 ± 10	0.12	19 ± 6	74	93 ± 8	0.01
HT-2	236 ± 35	4,462	4,698 ± 24	2.48	87 ± 8	2,249	2,336 ± 55	0.30
3'-OH HT-2	924 ± 98	1,173	2,097 ± 40	1.11	696 ± 50	1,016	1,712 ± 32	0.22
T-2 triol	497 ± 52	304	801 ± 23	0.42	770 ± 68	238	1,008 ± 82	0.13
4-DN	289 ± 20	34	324 ± 10	0.17	119 ± 12	41	160 ± 12	0.02
T-2 tetraol	298 ± 63	24	322 ± 4	0.17	209 ± 14	157	366 ± 11	0.05
Total[c]	2,469	19,038	21,507	11.34	1,945	7,796	9,741	1.26

[a]X ± SE (n = 3), based upon the specific radioactivity of administered tritium-labeled T-2 toxin.
[b]Concentration of conjugated metabolites determined by subtracting free from total concentration.
[c]Total concentration of identified metabolites.

TABLE 5. Free and conjugated metabolites of T-2 toxin in total urine collected over 4 hours from two swine (S1 and S2) administered tritium-labeled T-2 toxin.

	Percent of Dose (S1)[a]			Percent of Dose (S2)		
Metabolite	Free	Conjugated	Total	Free	Conjugated	Total
T-2 toxin	ND[b]	2.27	2.27	0.03	4.20	4.22
3'-OH T-2	0.10	3.01	3.11	0.34	7.14	7.48
NEO	0.04	0.15	0.19	0.10	0.26	0.36
HT-2	0.14	3.37	3.51	0.60	8.94	9.54
3'-OH HT-2	3.31	1.54	4.85	5.51	7.51	13.02
T-2 triol	0.94	0.07	1.01	1.93	0.31	2.24
4-DN	0.33	0.08	0.42	1.03	0.02	1.05
T-2 tetraol	0.62	0.02	0.64	0.72	0.39	1.11
SUM[c]	5.48	10.51	16.00	10.26	28.77	39.02
PERCENT[d]	30.87	59.21	90.14	24.16	67.76	91.90

[a]The urine collected at 240 minutes was not included due to small sample size (0.8 mL).

[b]ND indicates non-detected.

[c]Total percent of administered dose represented by above metabolites.

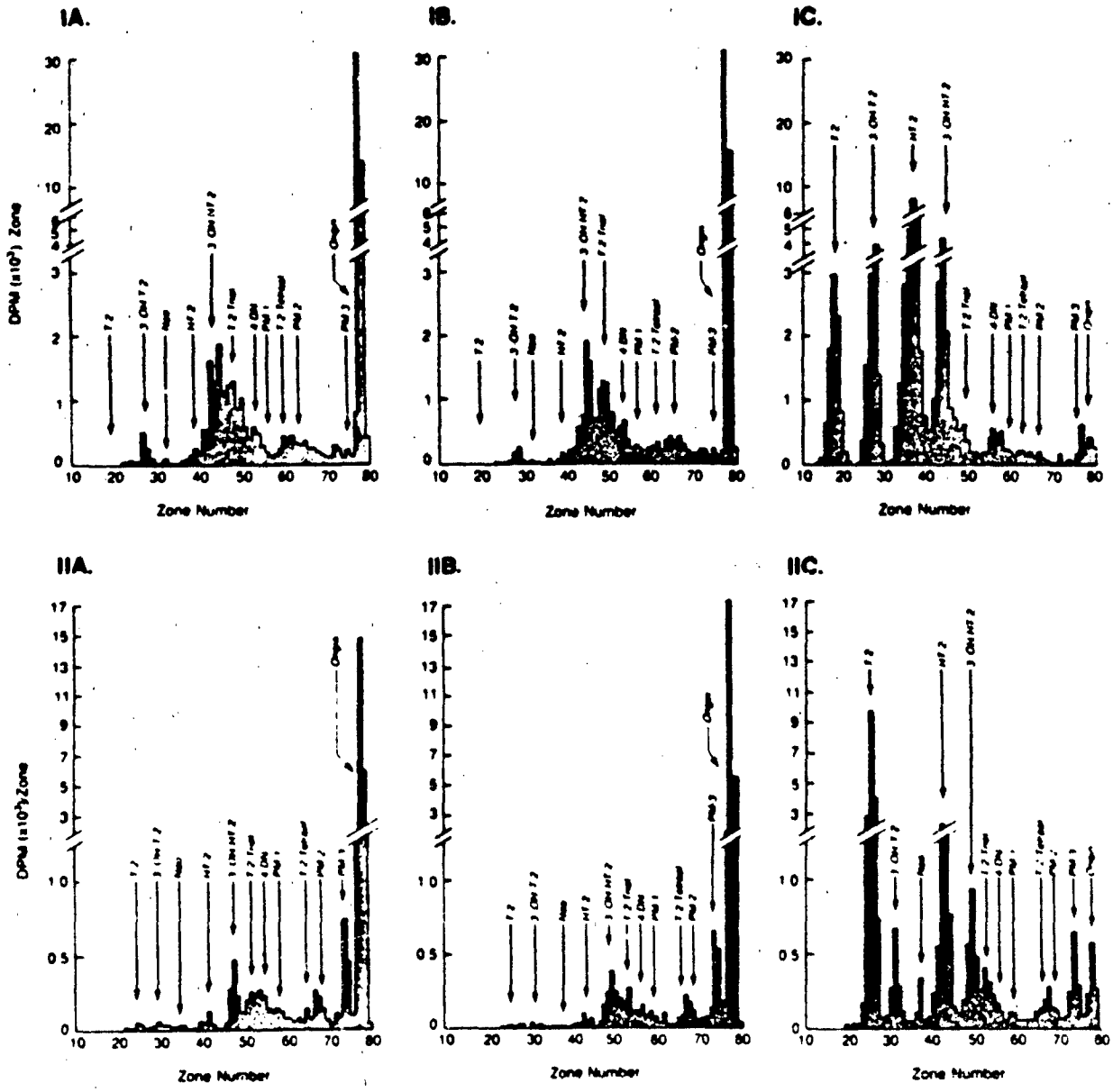
[d]Percent of total metabolite residues in urine represented by above metabolites.

TABLE 6. The relative abundance (%) of metabolites of T-2 toxin in urine[a] and bile[b] from two swine (S1 and S2) four hours following intravascular administration of 1.0 mCi tritium-labeled T-2 toxin at a dose of 0.15 mg/kg.

URINE				BILE			
S1		S2		S1		S2	
Metabolite	%[c]	Metabolite	%	Metabolite	%	Metabolite	%
HT-2-GLUC[d]	18.99	HT-2-GLUC	21.06	T-2-GLUC	47.33	T-2-GLUC	36.15
3'-OH HT-2	18.65	3'-OH HT-2-GLUC	17.69	HT-2-GLUC	17.95	HT-2-GLUC	22.49
3'-OH T-2-GLUC	16.96	3'-OH T-2-GLUC	16.82	3'-OH HT-2-GLUC	4.72	3'-OH HT-2-GLUC	10.14
T-2-GLUC	12.79	3'-OH HT-2	12.98	3'-OH T-2-GLUC	4.41	T-2 triol	7.68
3'-OH HT-2-GLUC	8.68	T-2-GLUC	9.89	3'-OH HT-2	3.72	3'-OH HT-2	6.95
T-2 triol	5.30	T-2 Triol	4.55	T-2 triol	2.00	3'-OH T-2-GLUC	3.99
T-2 tetraol	3.49	4-DN	2.43	T-2 triol-GLUC	1.22	T-2 triol-GLUC	2.38
4-DN	1.86	T-2 tetraol	1.70	T-2 tetraol	1.20	T-2 tetraol	2.08
NEO-GLUC	0.85	HT-2	1.41	4-DN	1.16	T-2 tetraol-GLUC	1.57
HT-2	0.79	T-2 tetraol-GLUC	0.92	HT-2	0.95	4-DN	1.19
3'-OH T-2	0.56	3'-OH T-2	0.80	NEO-GLUC	0.71	HT-2	0.86
4-DN-GLUC	0.45	T-2 triol-GLUC	0.73	3'-OH T-2	0.43	NEO-GLUC	0.74
T-2 triol-GLUC	0.39	NEO-GLUC	0.61	T-2	0.25	4-DN-GLUC	0.41
NEO	0.23	NEO	0.24	NEO	0.22	3'-OH T-2	0.33
T-2 tetraol-GLUC	0.11	T-2	0.07	4-DN-GLUC	0.14	NEO	0.19
T-2	ND[e]	4-DN-GLUC	0.05	T-2 tetraol-GLUC	0.10	T-2	0.11

[a]Total urine collected over 4 hours. The urine collected at 240 minutes from S1 was not included due to small sample size (0.8 ml).
[b]Bile taken from gall bladder at necropsy (4 hours).
[c]Percentage of total metabolite residues present in samples.
[d]GLUC abbreviation for glucuronide conjugate.
[e]ND indicates none detected.

FIGURE 1. Thin layer radiochromatograms of urine (Ia-c) and bile (IIa-c) following incubation with buffer alone (Ia,IIa), β-glucuronidase plus saccharic acid-1,4-lactone (Ib,IIb) and β-glucuronidase (Ic,IIc).



B. DISPOSITION OF T-2 TOXIN IN INTRAVASCULARLY-DOSED SWINE--R. A. Corley, S. P. Swanson, G. J. Gullo, L. Johnson, V. R. Beasley, W. B. Buck

INTRODUCTION

T-2 toxin (4ß, 15-diacetoxy-8α-(2-methylbutyryloxy) -3α-hydroxy-12, 13-epoxytrichothec-9-ene) is a toxic fungal metabolite produced by several species of Fusaria (Bamburg and Strong, 1971; Pathre and Mirocha, 1977). T-2 toxin has been found in naturally contaminated corn, barley, and mixed feeds in the U.S. and Canada at concentrations of 0.076-25 ppm (Vesonder, 1983). When present in the diets of livestock and poultry, T-2 toxin has been associated with feed refusal, infertility, diarrhea, intestinal irritation and possibly hemorrhage, perioral and pharyngeal irritation and lowered immunity (Hsu et al., 1972; Palyusik and Koplik-Kovacs, 1975; Speers et al., 1977; Weaver et al., 1977; Weaver et al., 1978a; Weaver et al., 1978b; Rafai and Tuboly, 1982; Hoerr et al., 1982).

Studies on the fate of T-2 toxin in laboratory animals, poultry, and livestock have demonstrated that the parent compound is rapidly cleared from body fluids and tissues. Tritium-labeled T-2 toxin and its metabolites rapidly distributed to tissues of orally dosed mice with maximum levels reached within 30 minutes declining to nondetectable levels by 72 hours (Matsumoto et al., 1978). Metabolites were eliminated in a feces to urine ratio of 3:1 over a 72 hour time period. Matsumoto et al. (1978) identified T-2 and HT-2 in rat feces at 2.7 and 7.5 percent of the administered dose, respectively, in feces in addition to two unknown compounds at 25.8 and 9.1 percent of the administered dose. No parent T-2 toxin was detected in the urine of rats, however, neosolaniol, HT-2 and three unknowns totaling less than 8 percent of

the administered dose were identified. Rats eliminated metabolites in a feces to urine ratio of 5:1 over a 24 hour period. Ueno (1977) reported similar results, finding HT-2 and neosolaniol in rat excreta.

T-2 and its metabolites are eliminated primarily through the bile into the gastrointestinal tract and excreta of orally dosed chickens (Chi et al., 1978). Maximum levels were reached by 4 hours in blood, plasma, abdominal fat, carcass, heart, kidneys, and liver and by 12 hours in muscle, skin, bile, and gall bladder, indicating a rapid distribution. Yoshizawa et al. (1980) identified several metabolites in the excreta of broiler chickens including: neosolaniol, HT-2, T-2 triol, 4-deacetylneosolaniol, and T-2 tetraol. Unknown compounds labeled TB-1 through TB-8 were found to be quantitatively more significant. Unknowns TB-1 and TB-2 were later identified as 3'-OH T-2; TB-3 (major metabolite) was identified as 3'-OH HT-2; TB-4 and TB-5 were identified as 8-acetoxy and 15-acetoxy T-2 tetraol in addition to another monoacetylated isomer of T-2 tetraol in the excreta of chickens following intraperitoneal injection of T-2 toxin (Visconti and Mirocha, 1985). Visconti and Mirocha (1985) also identified 3'-OH HT-2 (1370 ppb), HT-2 (233 ppb), T-2 triol (210 ppb) in addition to small amounts (ppb) of T-2 (4), 4-acetoxy T-2 tetraol (20), 15-acetoxy T-2 tetraol (22) and T-2 tetraol (18) in the liver of chickens 18 hours after administration of toxin.

Approximately 72 percent of orally administered trituim-labeled T-2 toxin was elminated in the feces and 29 percent in the urine of a lactating cow (Yoshizawa et al., 1981). Only 0.2 percent of the administered toxin was detected in the milk. Maximum concentrations of T-2 and its metabolites were reached by 8 hours in plasma (64 ppb); by 16 hours in urine (5.5 ppm) and milk 37 ppb); and by 44 hours in feces (9.2 ppm). Elimination phase half lives

were 12, 16, and 24 hours for urine, plasma and milk, respectively. In comparison, Beasley (1984) demonstrated a 17.4 minutes plasma elmination phase half life for the parent compound, T-2 toxin, alone following intravascular administration. Tissue levels were very low (< 20 ppb) in the lactating cow by 72 hours. Small amounts of HT-2, neosolaniol and 4-deacetylneosolaniol were detected in the urine along with significantly greater amounts of unknown compounds designated TC-1 to TC-8 with TC-1, TC-3, and TC-6 representing 40 percent of the total tritium residue 12 hours after toxin administration. The major metabolites found in feces were: TC-3, TC-6, and 4-deacetylneosolaniol. No T-2 or TC-1 was detected. In plasma, TC-1, TC-3, TC-6, and TC-8 were the major compounds identified. In milk, the major compounds were TC-1, TC-3, and TC-8. Very little unmetabolized T-2 (< 0.1 ppb) was detected by 36 hours. In contrast, unmetabolized T-2 toxin was found in milk at levels of 0-160 ppb by Robison et al., 1979a. Yoshizawa et al. (1982) later identified TC-1 as 3'-OH T-2 and TC-3 as 3'-OH HT-2. The metabolite TC-6 was tentatively identified as 3'-OH, 7-OH HT-2 by Pawlosky and Mirocha (1984).

The distribution of tritium-labeled T-2 toxin in swine was reported by Robison et al. (1979b). The distribution of radioactivity in the tissues of swine by 18 hours was very similar to that of chickens reported by Chi et al. (1978) with the exception that the kidneys of swine had a slightly higher level of radioactivity per g of tissue than liver, just the opposite of chickens, although the total radioactivity in the liver was greater than the total in the kidneys. Less that 50 percent of the administered radioactivity was accounted for with the remainder thought to be in the gastrointestinal tract. In a related study, unmetabolized T-2 toxin was found to be transmitted into the milk of a sow fed a diet containing 12 ppm T-2 toxin for 220 days

(Robison et al., 1979a). Only one sample, taken six days after parturition (day 190 of feeding study), was analyzed and found to contain 76 ppb T-2 toxin.

The metabolic fate of T-2 toxin in swine was unknown until Corley et al. (1985) reported on the glucuronide conjugates of T-2 toxin and metabolites in the bile and urine of swine following intravascular administration. Major free (unconjugated) metabolites identified were 3'-OH HT-2 and T-2 triol. These two metabolites, however, represented at most only 24 percent of the metabolite residues in urine and 15 percent in bile. Glucuronide conjugates accounted for 77 percent of the total metabolite residues in bile and 67 percent in urine 4 hours after toxin administration. The major conjugated metabolites were glucuronides of HT-2, 3'-OH T-2, 3'-OH HT-2 and T-2 with T-2-glucuronide accounting for a much greater percentage of metabolite residues in bile (approximately 42 percent) than in urine (approximately 11 percent). Total known free and conjugated metabolites accounted for approximately 92 percent of the metabolite residues in bile and urine 4 hours after dosing.

The purpose of this study is to determine the disposition of T-2 toxin in swine tissues, including the gastrointestinal tract and contents. An improved method for the chromatographic separation of tritium-labeled metabolites of T-2 toxin is also discussed.

EXPERIMENTAL SECTION

Reference Standards. Tritium-labeled T-2 toxin (radiopurity > 99 percent, specific activity 1.287 mCi/mg) was synthesized by the method of Wallace et al. (1977). Unlabeled standards of T-2 toxin, neosolaniol, HT-2, T-2 triol, 4-deacetylneosolaniol, and T-2 tetraol were produced from cultures of Fusarium tricinctum in our laboratory. Additional standards of 3'-OH T-2 and 3'-OH HT-2 were kindly provided by T. Yoshizawa (Yoshizawa et al., 1982).

Tritium-labeled T-2 toxin was used to prepare labeled standards of HT-2, T-2 triol and T-2 tetraol by alkaline hydrolysis (Wei et al., 1971) and 3'-OH T-2 by rat liver S-9 fractions (Wei and Chu, 1985). Tritium-labeled 3'-OH HT-2 was prepared from 3'OH T-2 by alkaline hydrolysis. De-epoxy derivatives of HT-2, T-2 triol and T-2 tetraol were prepared from tritum-labeled T-2 using bovine rumen microflora (manuscript in preparation).

Animal Treatment. Two 20 kg female crossbred swine (Yorkshire X Hampshire; Thrushwood Farms, Fairbury, IL) were injected with erysipelas bacterin (Rhusigen, Pitman-Moore, Inc., Washington Crossing, NJ) and acclimated to the large animal holding facility at the College of Veterinary Medicine, University of Illinois. All feeds offered to experimental swine were free from detectable concentrations of trichothecene mycotoxins and aflatoxins. Following preanesthetic administration of atropine sulfate, anesthesia was induced and maintained using halothane and oxygen. In-dwelling catheters for dosing and blood collection were surgically implanted in the aorta via the femoral artery and were tunneled subcutaneously anterior and dorsal to the pelvis. The swine were returned to holding pens and allowed to recover for at least 3 days following surgery.

Immediately prior to dosing, the subcutaneous catheters were exteriorized using Lidocaine HCl local anesthetic and Foley catheters were inserted into the urinary bladder. Tritium-labeled T-2 toxin was diluted with nonradioactive T-2 toxin in 1.5 mL of 50 percent ethanol such that each swine received 1 mCi of total radioactivity at a dosage of 0.15 mg/kg body weight intravascularly as a single bolus injection.

During the course of the experiment, the animals were restrained in a plastic-lined sling. Blood was collected in heparinized tubes on ice at 10,

20, 30, 45, 60, 90, 120, 150, 180, 210 and 240 minute intervals and centrifuged to obtain plasma. Urine and feces were collected hourly. The animals were killed 4 hours after dosing by administration of an anesthetic dose of pentobarbital followed by exsanguination. Bile was immediately collected from the gallbladders. The gastrointestinal tract was separated into stomach, duodenum, jejunum, ileum, and large intestine (including cecum). The contents were removed from each section, the lining scraped (residues added to contents) and rinsed with water. All tissues were weighed, homogenized, and flash frozen in dry ice-isopropanol.

Determination of Total Radioactivity. The total radioactivity in plasma was determined by adding a 0.2 mL aliquot directly to 5 mL of Aquasol-2® liquid scintillation cocktail (New England Nuclear Corp., Boston, MA). The total radioactivity in tissues, including the gastrointestinal tract and contents, and feces were determined by first homogenizing 5 g of sample in 15 mL of 0.1 M acetate buffer (pH 3.8). A 0.05 mL aliquot of the homogenate was incubated with Protosol® tissue solubilizer (New England Nuclear Corp., Boston, MA) for 2 hours at 60° C. The samples were decolorized by adding 0.2 mL 30 percent H_2O_2 followed by heating an additional 30 minutes and counted in 5 mL Aquasol-2®.

Extraction of Plasma. A 1 to 4 mL volume of plasma was diluted, in duplicate, to 6 mL with 0.1 M acetate buffer (pH 3.8). The pH was adjusted to 3.8 for larger volumes of plasma. All samples were heated at 90° C for 30 min to inactivate enzyme inhibitors. After cooling, 2 mL of either 0.1 M acetate buffer or ß-glucuronidase (Sigma Chemical Company, Type L-II from limpets; 400 units/ml in 0.1 M acetate buffer) were added and the samples incubated with gentle mixing in a 38° C water bath for 18 hours. A positive enzyme control

utilizing phenolpthalein glucuronide (Sigma Chemical Co., 0.01 M, pH 7.0) and ß-glucuronidase in control plasma was included with each set of samples.

Each sample was extracted four times with 20 mL of ethyl acetate after the addition of 10 mL saturated NaCl. Centrifugation was necessary between each partition. The extracts were combined and filtered through funnels containing 2 g $CuCO_3$ (Fisher Scientific, Itasca, IL; green, precipitated, basic) sandwiched between two layers of anhydrous sodium sulfate. The funnels were rinsed with an additional 20 mL of ethyl acetate and the extracts were concentrated. The residue was redissolved in 0.1 mL toluene-acetonitrile (95+5) for TLC radiochromatography according to the methods described previously (Corley et al., 1985).

Extraction of Tissues, Gastrointestinal Tract, and Contents. To the homogenates prepared for the determination of total radioactivity, 5 g of NaCl was added and the homogenates extracted three times with 20 mL of acetonitrile. A fourth extraction using acetonitrile-acetone (1+1) was necessary to improve recoveries of T-2 tetraol. Extracts were combined and 80 mL of methylene chloride was added to drive residual water out of solution. Enough anhydrous Na_2SO_4 (approximately 40 g) was added until the solution appeared clear. Cupric carbonate (2.5 g) was added and the sample filtered through Whatman No. 1 filter paper. The flask and filter were rinsed three times with 20 mL of methylene chloride-acetonitrile (1+1) and the samples concentrated. It was important that the extracts were clear prior to the addition of $CuCO_3$ since the presence of water in solution decreased the ability of the $CuCO_3$ to decolorize the extracts. We have also noted that different brands of basic $CuCO_3$ yield varying results. Fisher brand provided the best clean-up of these samples.

After the initial concentration, the residues were transfered to vials using methanol and concentrated. The samples were redissolved in 0.25 mL of methanol, 0.25 mL of water was added (solutions became very cloudy) and each sample filtered through a disposable membrane filter (Gelman Sciences, Ann Arbor, MI; ACRO LC13, 0.2 micron) for HPLC radiochromatographic analysis.

HPLC Radiochromatography. An HPLC system (Perkin Elmer Series 4, Norwalk, CT) equipped with a 15 cm x 4.6 mmid column packed with 5 micron C18 (Econosphere, Alltech Assoc., Deerfield, IL) and a 20 to 90 percent methanol in water linear gradient over 30 minutes at a flow rate of 1.0 mL/min was used to separate metabolites. Fractions (0.2 mL) were collected and assayed for radioactivity in Scinti Verse LC® liquid scintillation cocktail (Fisher Scientific Co., Itasca, IL). Resolution and reproducibility were greatly improved over thin layer radiochromatography.

RESULTS

Plasma. The total concentrations of T-2 and metabolites in plasma over time are given in Figure 1. The plasma elimination phase half-life was approximately 90 minutes for T-2 and metabolites. The major free (unconjugated) metabolites identified by one and two dimensional TLC were 3'-OH T-2, HT-2 and 3'-OH HT-2 in addition to the parent compound. These four compounds accounted for 60 percent of the total radioactivity in plasma samples taken 10 minutes after toxin administration but decreased to less than 10 percent by 4 hours, the lifetime of the swine. Other free metabolites detected at much lower concentrations (< 6 ppb) included: neosolaniol, T-2 triol, 4-deacetylneosolaniol and T-2 tetraol. When plasma samples were incubated with ß-glucuronidase, an average of 89.2 percent of the total extractable radioactivity (extraction efficiency: mean ± SE, 72.8 ± 3.1 percent, n = 11)

in plasma at all time periods sampled was associated with T-2, 3'-OH T-2, HT-2 and 3'-OH HT-2. The concentrations and percentages of these four compounds in the free and conjugated form are given in Figures 2 and 3. Although 3'-OH HT-2 was the predominant free metabolite in plasma, 3'-OH T-2 and 3'-OH HT-2 were nearly equal in concentration following enzyme hydrolysis.

Tissues, Gastrointestinal Tract and Contents. The distribution of T-2 and its metabolites (total radioactivity) in the tissues, gastrointestinal tract and contents is summarized in Table 1. The greatest amount of radioactivity was located in the contents of the gastrointestinal tract (10.08 and 15.97 percent of the dose for S1 and S2, respectively) followed by the gastrointestinal tract itself (5.37 and 8.14 percent), and remaining tissues sampled (5.19 and 4.69 percent).

Within the gastrointestinal tract and contents of both swine the ileum and its contents contained the greatest total amount of radioactivity (9.96 and 15.77 percent of the dose for S1 and S2, respectively) and the duodenum and its contents the least (0.07 and 0.23 percent of the dose). In the remaining tissues sampled, the muscle contained the greatest total amount of radioactivity (2.90 and 3.21 percent of the dose) followed by the liver (1.71 and 0.65 percent of the dose), kidneys (0.23 and 0.19 percent of the dose), pancreas (0.18 percent of the dose, S2 only), lungs (0.14 and 0.16 percent of the dose), heart (0.07 and 0.10 percent of the dose), mesenteric lymph nodes (0.05 and 0.10 percent of the dose), spleen (0.04 and 0.05 percent of the dose), bone marrow (0.03 and 0.03 percent of the dose), and brain (0.02 and 0.02 percent of the dose).

Although the total metabolite residues in many tissues of both swine 4 hours after dosing were present at very low concentrations (Table 1),

several metabolites were identified following HPLC separation. The concentrations of twenty-one metabolites, designated PM-I to PM-XXII (PM-XXI represents T-2) are given for each tissue and section of the gastrointestinal tract and contents in Tables 2 and 3. A representative radiochromatogram is shown in Figure 4. The total metabolite residues identified in all tissues represented 5.36 and 8.03 percent of the dose in swine S1 and S2, respectively. These free, extractable metabolites account for only 25.97 and 27.90 percent of the total radioactivity present in all tissues. Extraction efficiencies were consistent within a given tissue (coefficients of variation approximately 10 percent) yet, varied greatly between tissues, ranging from a low of 17 percent for brain (animal S2) to a high of 77 percent for large intestinal contents (animal S1). Recovery data is summarized in Table 4. The percent recovery for the HPLC separation step was 98.60 $\pm$ 0.66 percent ($\bar{X} \pm SE$, $n = 115$).

A total of 48.50 and 45.59 percent of the extracted radioactivity in tissues, gastrointestinal tract, and contents from animals S1 and S2, respectively had retention times identical to authentic tritium-labeled standards of T-2 (PM-XX), 3'-OH T-2 (PM-XVI), HT-2 (PM-XIX), 3'-OH HT-2 (PM-XIII), T-2 triol (PM-XVII) and T-2 tetraol (PM-I). The major metabolite, PM-XV, which represented 29.10 and 25.03 percent of the extracted residues in the tissues, gastrointestinal tract, and contents of animals S1 and S2, respectively, did not correspond to any standard.

DISCUSSION

The time course, dose and route of administration used in this study were selected to maximize the bioavailability of T-2 toxin, maintain adequate urine output and to facilitate tissue metabolite identification. Previous work demonstrated that swine are particularly sensitive to the emetic action of T-2

toxin at an intravascular dose of 0.3 mg/kg (Beasley, 1984) and at oral doses greater than 0.5 mg/kg (Robison et al., 1979b). In addition, a significant decrease in urine output (oliguria), a major route of elimination of T-2 and its metabolites in swine (Corley et al., 1985; Robison et al., 1979b), occurred following intravascular administration of a lethal dose (Beasley, 1984). A dose of 0.15 mg/kg was well below the threshold for emesis and oliguria. The swine were killed 4 hours after toxin administration since the parent compound, T-2, could not be detected after 2 hours in plasma or after 4 hours in tissues of swine administered 1.2 mg/kg intravascularly (Beasley, 1984).

A total of 52.62 and 73.98 percent of the administered radioactivity in the two swine have been accounted for (Table 5). The assumption that muscle and blood represent 25 and 6 percent of the total body weight, respectively, was used to calculate the total amount of radioactivity in these samples. The total mass of bone marrow and mesenteric lymph nodes could not be estimated. Therefore, the total radioactivity determined in these tissues was based on the amount collected at necropsy. The remainder is assumed to be in the carcass.

The distribution of the majority of radioactivity in both swine 4 hours after dosing was consistent with the gastrointestinal lesions reported in swine administered lethal doses of T-2 toxin intravascularly (Beasley, 1984; Weaver et al., 1978). Congestion and hemorrhage are reported to progress aborally in the small intestines with the duodenum appearing near normal. The distribution of radioactivity in the small intestines was the greatest in the ileum, followed by the jejunum and duodenum. Congestion and hemorrhage were also reported in the stomach (Beasley, 1984) and the large intestines (Beasley,

1984; Weaver et al., 1978). A large amount of radioactivity was also present in these tissues.

The source of the radioactivity in the gastrointestinal tract is assumed to be primarily from the bile, with some contribution from blood flow to these tissues. Since an average of 77 percent of the metabolites in the bile of these swine were present as glucuronides (Corley et al., 1985), no more than 23 percent of the total metabolite residues should have been extracted and purified for HPLC radiochromatography.

Extraction efficiencies were generally in agreement with this figure for the stomach and small intestines but increased in the large intestines to approximately 50 percent, indicating that gut microflora may be involved in the hydrolysis of glucuronides which may, therefore, result in enterohepatic recirculation and potentiation of the toxic effects of T-2 and its metabolites. The use of adsorbants, such as activated charcoal, in the management of T-2 toxicosis should be investigated for their potential in eliminating T-2 and its metabolites from the gastrointestinal tract.

Following HPLC separation, 21 metabolites were identified in tissues and the gastrointestinal tract at concentrations ranging from less than 0.01 to 67.56 ng/g. One of the major metabolites, identified thin layer radiochromatography in bile and urine (Corley et al., 1985), was found to be a minor metabolite by HPLC. A new major metabolite, PM-XV, identified by HPLC radiochromatography did not separate from T-2 triol and 3'-OH HT-2 by silica TLC. The other major metabolites in bile and urine, 3'-OH T-2, HT-2 and 3'-OH HT-2, were also found in plasma, tissues and gastrointestinal tract. The presence of the parent compound, T-2, as a major compound in the spleen, mesenteric lymph nodes, stomach (and contents) and ileum (and contents) is of

particular interest since these tissues in swine all became congested or hemorrhagic following intravascular administration of a lethal dose of T-2 toxin.

Several metabolites have been tentatively identified by comparing HPLC retention times of the unknowns with tritium-labeled standards. PM-I, PM-II, PM-XIII, PM-XVI, PM-XVII, PM-XVIII, PM-XIX and PM-XXI corresponded to T-2 tetraol, de-epoxy T-2 tetraol, 3'-OH HT-2, 3'-OH T-2, T-2 triol, de-epoxy T-2 triol, HT-2 and the parent compound, H-2, respectively. The major metabolites, 3'-OH HT-2, 3'-OH T-2 and HT-2, were present in urine in sufficient quantities to be confirmed by capillary gas chromatography with electron capture detection as the trifluoroacetic acid derivatives. Metabolites such as T-2 tetraol and T-2 triol were not available in sufficient quantities in these two swine to allow confirmation by gas chromatography but have been confirmed in other species. The de-epoxy derivatives of T-2 tetraol, T-2 triol and HT-2 have never been reported as in vivo metabolites were also not present in sufficient quantities for confirmation and their identification is, therefore, tentative. Work is currently in progress to structurally identify the major unknown metabolite, PM-XV, and to confirm the presence of de-epoxy metabolites of T-2 toxin in swine.

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TABLE 1. Total metabolite residues[a] in tissues, including the gastrointestinal tract and contents from two swine (S1 and S2) 4 hours following the intravascular administration of 1.0 mCi of tritium-labeled T-2 toxin at a dose of 0.15 mg/kg body weight.

	Concentration (ng/g)[b]		% of Admin. Dose[b]	
	S1	S2	S1	S2
Liver	106.5 ± 3.7	38.6 ± 2.8	1.71 ± 0.06	0.65 ± 0.05
Kidney	73.8 ± 1.5	68.1 ± 4.1	0.23 ± 0.01	0.19 ± 0.01
Spleen	29.0 ± 0.9	41.5 ± 1.4	0.04 ± 0.00	0.05 ± 0.00
MLN[c]	33.0 ± 1.2	45.8 ± 1.8	0.05 ± 0.00[d]	0.10 ± 0.00[d]
Muscle[e]	17.7 ± 0.5	19.3 ± 1.1	2.90 ± 0.08	3.21 ± 0.19
Lung	21.2 ± 1.1	23.7 ± 1.7	0.14 ± 0.01	0.16 ± 0.01
Heart	22.8 ± 0.7	26.1 ± 1.1	0.07 ± 0.00	0.10 ± 0.00
Brain	8.0 ± 0.7	12.8 ± 0.6	0.02 ± 0.00	0.02 ± 0.00
Pancreas	NA[f]	159.0 ± 4.5	NA[f]	0.18 ± 0.01
Bone Marrow	18.1 ± 0.9	24.9 ± 1.9	0.03 ± 0.00[d]	0.03 ± 0.00[d]
Stomach	90.7 ± 2.9	85.8 ± 3.6	0.43 ± 0.01	0.51 ± 0.02
St. Content	399.0 ± 7.9	79.4 ± 1.8	1.67 ± 0.03	1.10 ± 0.02
Duodenum	31.8 ± 1.0	101.3 ± 2.9	0.04 ± 0.00	0.16 ± 0.00
Duod. Contents	55.4 ± 1.2[g]	144.0 ± 1.7[h]	0.03 ± 0.00[g]	0.07 ± 0.00[h]
Jejunum	179.6 ± 2.5	149.1 ± 5.1	1.29 ± 0.02	1.39 ± 0.05
Jej. Contents	303.0 ± 6.2[g]	316.5 ± 7.1	1.07 ± 0.02[g]	1.47 ± 0.03
Ileum	554.2 ± 34.3	497.8 ± 15.8	3.27 ± 0.20	5.00 ± 0.16
Ileum Contents	1643.7 ± 112.4[g]	1405.7 ± 49.2	6.69 ± 0.45[g]	10.77 ± 0.38
Lg. Intestine	48.0 ± 3.9	142.1 ± 6.4	0.34 ± 0.03	1.08 ± 0.05
L.I. Contents	68.2 ± 2.2	181.1 ± 3.7	0.62 ± 0.02	2.56 ± 0.05

[a]All values based upon the specific activity of administered tritium-labeled T-2 toxin.
[b]X ± SE, n = 9
[c]MLN - mesenteric lymph nodes.
[d]Percent of administered dose based only on the amount of tissue collected.
[e]Total muscle mass estimated at 25 percent of body weight.
[f]NA- not analyzed.
[g]n = 6
[h]n = 3

TABLE 2. Concentration (ng/g) of the metabolites of T-2 toxin in swine tissues following HPLC radiochromatography.

Metabolite	Liver	Kidney	Spleen	MLN	Muscle	Lung	Heart	Brain	Pancreas	Bone Marrow
I	1.73,0.71[a]	0.26,TR[b]	0.05,0.04	ND[c],0.09	ND,ND	0.06,ND	0.04,0.04	ND,ND	--, 0.82	0.04,ND
II	1.61,0.33	0.88,0.69	0.29,0.34	TR,0.37	TR,ND	0.29,0.30	0.17,0.33	0.04,0.04	--, 1.83	0.30,0.36
III	1.72,1.66	4.30,4.03	0.92,1.52	1.51,1.05	0.98,1.13	1.03,1.28	0.88,1.38	0.28,0.32	--, 3.07	0.66,0.74
IV	TR,TR	TR,TR	0.16,TR	TR,0.14	TR,ND	TR,TR	0.14,TR	ND,0.09	--,ND	0.21,0.13
V	0.23,ND	0.20,ND	ND,ND	ND,ND	ND,ND	ND,ND	ND,ND	ND,ND	--,ND	ND,ND
VI	0.11,ND	ND,ND	ND,ND	ND,ND	ND,ND	ND,ND	ND,ND	ND,ND	--,ND	ND,ND
VII	ND,ND	0.19,ND	ND,ND	ND,ND	ND,ND	ND,ND	ND,ND	ND,ND	--, 0.56	ND,ND
VIII	0.54,0.30	0.17,0.17	ND,ND	ND,ND	ND,ND	ND,ND	ND,ND	ND,ND	--, 0.90	ND,ND
IX	0.38,0.15	ND,0.21	ND,ND	ND,ND	ND,ND	ND,ND	ND,ND	ND,ND	--, 0.74	ND,ND
X	1.31,0.37	0.34,0.24	ND,ND	ND,ND	ND,ND	ND,ND	ND,ND	ND,ND	--, 3.16	ND,ND
XI	1.03,0.30	0.31,0.25	ND,ND	ND,ND	ND,ND	ND,ND	ND,ND	ND,ND	--,ND	ND,ND
XII	TR,0.37	0.89,0.49	ND,ND	ND,0.09	ND,ND	ND,0.08	ND,0.07	ND,ND	--, 3.73	0.08,0.07
XIII	4.63,2.42	5.41,4.96	3.44,2.74	4.02,3.03	1.90,1.10	2.15,1.24	1.23,0.80	0.22,0.07	--,17.79	2.51,1.67
XIV	ND,ND	ND,ND	ND,ND	ND,ND	ND,0.14	ND,0.12	ND,0.24	ND,ND	--,ND	ND,0.15
XV	6.37,4.21	10.12,5.66	2.41,2.14	2.53,2.12	2.88,1.98	2.32,1.68	2.67,2.32	0.68,0.44	--, 5.67	1.84,1.12
XVI	0.67,0.78	0.81,0.47	0.54,1.78	0.63,1.69	0.48,1.07	0.27,0.87	0.19,0.58	0.07,0.10	--, 3.13	0.40,1.32
XVII	0.35,0.16	0.14,0.16	0.11,0.26	0.22,0.27	0.14,0.18	0.11,0.17	0.04,0.14	0.03,0.05	--, 0.65	0.07,0.15
XVIII	0.23,0.07	0.06,ND	0.03,ND	ND,ND	ND,ND	ND,ND	ND,ND	ND,ND	--,ND	ND,ND
XIX	2.66,1.47	1.08,1.45	2.16,2.25	5.21,3.76	2.06,1.69	0.85,1.69	0.48,0.68	0.10,0.13	--,11.68	1.16,1.35
XX	0.26,0.14	0.08,ND	ND,ND	ND,ND	ND,ND	0.13,ND	ND,ND	ND,ND	--,ND	ND,ND
XXI	0.71,0.27	0.05,0.08	2.73,1.04	1.81,1.37	0.64,0.85	0.13,0.85	0.06,0.32	0.02,0.05	--, 0.52	0.38,0.58
XXII	0.20,0.06	ND,ND	ND,ND	ND,ND	ND,ND	0.04,ND	ND,ND	ND,ND	--, 0.16	ND,ND

[a]Values expressed as the mean of three replicates for the two swine (S1, S2) based upon the specific radioactivity of administered tritium-labeled T-2 toxin.
[b]TR - Trace (less than 0.01 ng/g [^{3}H] T-2 equivalents).
[c]ND - None detected. Detection limit 0.005 ng/g [^{3}H] T-2 equivalents.

TABLE 3. Concentration (ng/g) of the metabolites of T-2 toxin in the gastrointestinal tract (lining and contents) of two swine following HPLC radiochromatography.

Metabolite	Stomach	Stomach Contents	Duodenum	Duodenum Contents	Jejunum	Jejunum Contents	Ileum	Ileum Contents	Large Intestine	Lg. Intestine Contents
I	2.09,TR[a,b]	11.86,0.11	0.19,0.69	0.13,0.09	3.78,2.67	7.52,3.37	13.36,4.29	41.75,13.66	ND[c],ND	ND,ND
II	0.57,1.04	TR,1.03	0.40,0.93	0.61,1.54	1.62,0.99	1.88,2.34	5.30,5.61	10.44,18.64	0.25,0.54	0.22,0.58
III	1.23,1.36	1.87,0.64	0.62,1.59	0.95,3.27	1.70,1.82	3.40,4.57	5.98,1.76	14.99,13.57	1.02,3.06	1.10,2.60
IV	TR,0.10	TR,0.12	0.10,TR	0.14,0.36	ND,ND	ND,0.69	TR,2.89	TR,ND	TR,TR	TR,1.93
V	ND,0.15	1.59,0.15	ND,ND	TR,ND	ND,ND	TR,ND	TR,ND	TR,ND	ND,0.74	0.73,1.48
VI	ND,ND	TR,ND	ND,ND	ND,ND	1.02,ND	1.27,0.87	4.71,ND	9.36,9.30	ND,ND	ND,ND
VII	ND,ND	1.43,ND	ND,0.33	ND,0.45	ND,ND	ND,ND	7.46,ND	21.13,TR	ND,ND	ND,ND
VIII	0.17,0.24	0.87,0.29	ND,0.16	ND,ND	1.66,1.19	2.58,2.37	6.27,2.38	5.25,14.39	ND,0.38	ND,0.50
IX	0.25,0.30	3.68,0.19	ND,0.65	ND,1.19	1.71,0.51	3.41,1.02	1.57,1.59	7.87,TR	ND,0.46	ND,0.62
X	0.94,0.89	4.87,0.90	ND,1.98	ND,2.44	TR,2.98	1.58,4.77	TR,6.97	TR,35.14	0.13,0.91	0.24,1.50
XI	0.40,0.67	1.39,0.23	ND,TR	ND,ND	1.38,TR	2.37,2.05	4.05,4.64	9.15,TR	0.21,1.11	0.36,1.23
XII	0.44,0.45	0.70,0.51	ND,0.81	ND,1.53	TR,3.28	0.60,3.76	1.13,9.77	2.78,26.12	1.54,2.23	1.54,5.48
XIII	3.45,2.53	8.68,1.47	2.87,5.83	4.08,7.76	7.36,7.38	12.23,15.85	29.27,24.83	66.70,67.56	3.56,13.99	7.02,13.49
XIV	0.68,2.35	ND,1.30	ND,ND	ND,ND	ND,1.50	ND,ND	ND,ND	ND,ND	0.42,ND	ND,ND
XV	5.76,5.17	13.56,6.16	2.04,6.04	3.01,8.61	5.45,7.52	11.52,14.97	24.16,16.33	45.92,40.40	10.25,23.23	38.04,45.44
XVI	1.11,2.09	2.77,1.62	0.51,1.42	0.55,2.10	0.99,1.71	0.64,2.42	1.41,3.02	6.64,5.43	1.45,3.65	ND,4.71
XVII	0.20,0.28	1.17,0.29	ND,0.40	0.13,0.33	0.39,0.36	0.40,0.54	0.67,0.86	6.24,3.63	0.33,1.38	0.55,2.17
XVIII	0.10,0.09	0.50,ND	ND,ND	ND,0.18	ND,ND	0.44,ND	0.75,0.55	ND,1.97	ND,ND	ND,0.76
XIX	3.21,2.86	2.80,0.76	1.80,2.84	1.76,3.75	3.54,3.58	4.22,4.25	7.09,9.75	6.77,17.69	1.50,7.20	1.32,9.91
XX	0.62,0.28	1.15,0.51	ND,ND	ND,ND	ND,0.40	ND,ND	ND,1.72	2.06,3.12	0.17,ND	0.31,ND
XXI	3.34,0.66	3.02,0.46	0.30,0.61	0.22,0.68	1.32,1.04	1.17,1.40	7.32,5.02	12.61,10.64	0.42,5.82	0.31,9.87
XXII	0.21,0.13	1.34,0.22	ND,ND	ND,ND	0.35,ND	0.39,ND	ND,ND	2.98,3.19	0.09,ND	0.17,ND

[a]Values expressed as the mean of three replicates for the two swine (S1, S2) based upon the specific radioactivity of administered tritium-labeled T-2 toxin.
[b]TR - Trace (less than 0.01 ng/g [^{3}H] T-2 equivalents).
[c]ND - None detected. Detection limit 0.005 ng/g [^{3}H] T-2 equivalents.

TABLE 4. Recovery (percent) of total metabolite residues from tissues, including the gastrointestinal tract and contents, of two swine (S1 and S2) prepared for HPLC radiochromatography.

Tissue	Extraction Efficiencies (% Recovery)[a] S1	S2
Liver	24.61 ± 2.83	38.43 ± 4.59
Kidney	33.13 ± 2.49	29.47 ± 4.21
Spleen	47.63 ± 2.86	31.20 ± 1.45
MLN[b]	52.46 ± 1.60	33.08 ± 4.68
Muscle	56.69 ± 0.53	48.38 ± 4.42
Lung	37.43 ± 1.05	35.35 ± 1.94
Heart	31.14 ± 5.67	29.30 ± 1.22
Brain	21.68 ± 4.29	16.86 ± 9.23
Pancreas	----	35.87 ± 0.66
Bone Marrow	40.42 ± 2.78	33.50 ± 7.55
Stomach	30.16 ± 3.00	27.05 ± 4.51
St. Contents	16.98 ± 0.15	22.99 ± 1.22
Duodenum	31.14 ± 1.80	26.29 ± 0.69
Duod. Contents	23.50[c]	26.22[d]
Jejunum	19.90 ± 0.32	28.02 ± 2.37
Jej. Contents	19.11 ± 1.45	22.31 ± 2.28
Ileum	23.21 ± 4.26	23.53 ± 1.22
Ileum Contents	18.92 ± 3.61	23.34 ± 4.25
Large Intestine	46.97 ± 5.64	46.40 ± 5.86
L.I. Contents	77.16 ± 1.70	48.49 ± 1.41

[a] X ± SE, n = 3
[b] MLN - mesenteric lymph nodes
[c] n = 2
[d] n = 1

TABLE 5. Distribution of radioactivity in two swine (S1 and S2) 4 hours after intravascular administration administration of 1.0 mCi of tritium-labeled T-2 toxin at a dose of 0.15 mg/kg body weight.

Sample	Percent of Administered Dose S1	S2
Blood[a]	0.94	1.43
Urine[b]	17.93	42.46
Bile[b]	13.11	1.29
Tissues	5.19	4.69
Liver	1.71	0.65
Kidney	0.23	0.19
Spleen	0.04	0.05
MLN[c,d]	0.05	0.10
Muscle[e]	2.90	3.21
Lung	0.14	0.16
Heart	0.07	0.10
Brain	0.02	0.02
Pancreas	NA	0.18
Bone Marrow[d]	0.03	0.03
Gastrointestinal Tract	15.45	24.11
Stomach	0.43	0.51
Stomach Contents	1.67	1.10
Duodenum	0.04	0.16
Duodenum Contents	0.03	0.07
Jejunum	1.29	1.39
Jejunum Contents	1.07	1.47
Ileum	3.27	5.00
Ileum Contents	6.69	10.77
Large Intestine	0.34	1.08
Large Intestine Contents	0.62	2.56
Feces	ND	ND
Total	52.62	73.98

[a]Total blood volume estimated at 6 percent of body weight. Concentration of radioactivity in whole blood was assumed to be equilavent to plasma.
[b]Corley et al. (1985)
[c]MLN = mesenteric lymph node.
[d]Percent of administered dose based only on the amount of tissue collected.
[e]Total muscle mass estimated at 25 percent of body weight.

FIGURE 1. The concentration (ng/ml) of T-2 and metabolites (total radioactivity) in plasma over time from 2 swine (S1 and S2) administered 1.0 mCi tritium-labeled T-2 toxin intravascularly at 0.15 mg/kg body weight.

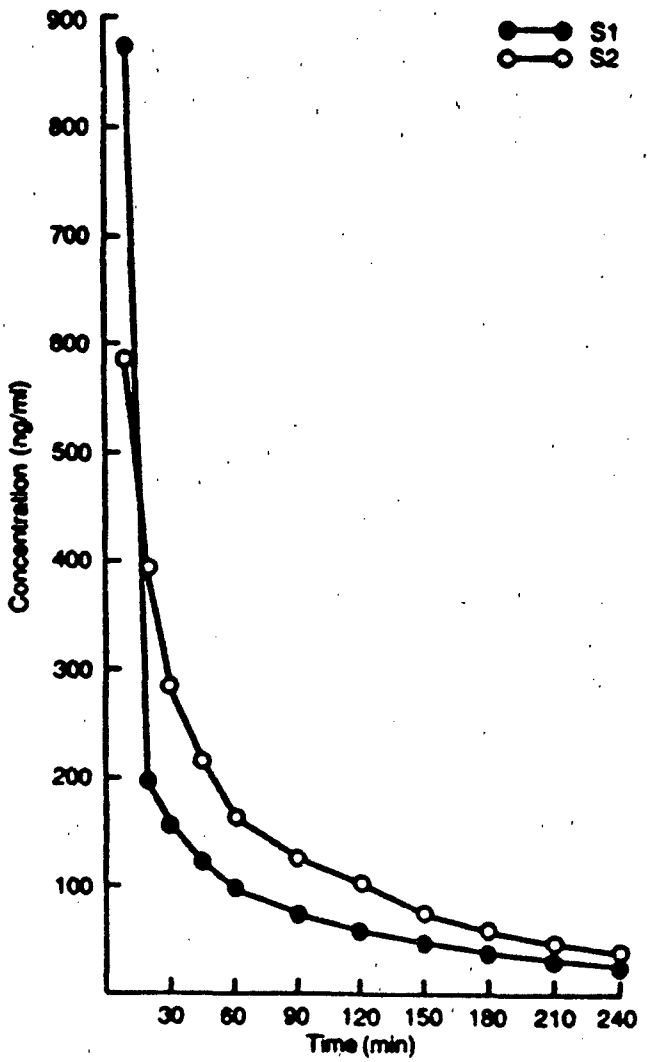


FIGURE 2. The concentration (ng/ml) of T-2 toxin, 3'-OH T-2, HT-2 and 3'-OH HT-2 in the free and conjugated form in plasma over time from a pig (S2 only) administered 1.0 mCi tritium-labeled T-2 toxin intravascularly at a dose of 0.15 mg/kg body weight.

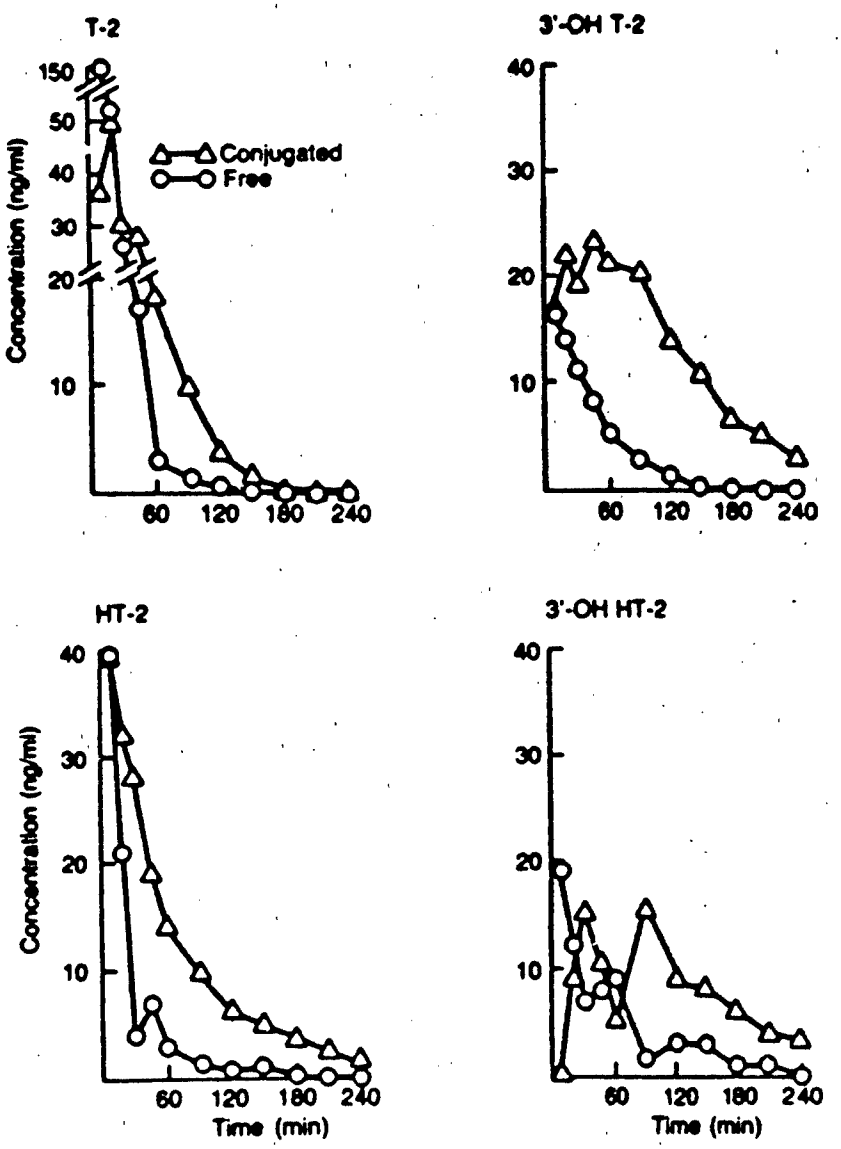


FIGURE 3. Percentages of T-2 toxin, 3'-OH T-2, HT-2 and 3'-OH HT-2 in the free and conjugated form in plasma over time from a pig (S2 only) administered 1.0 mCi tritium-labeled T-2 toxin intravascularly at a dose of 0.15 mg/kg body weight.

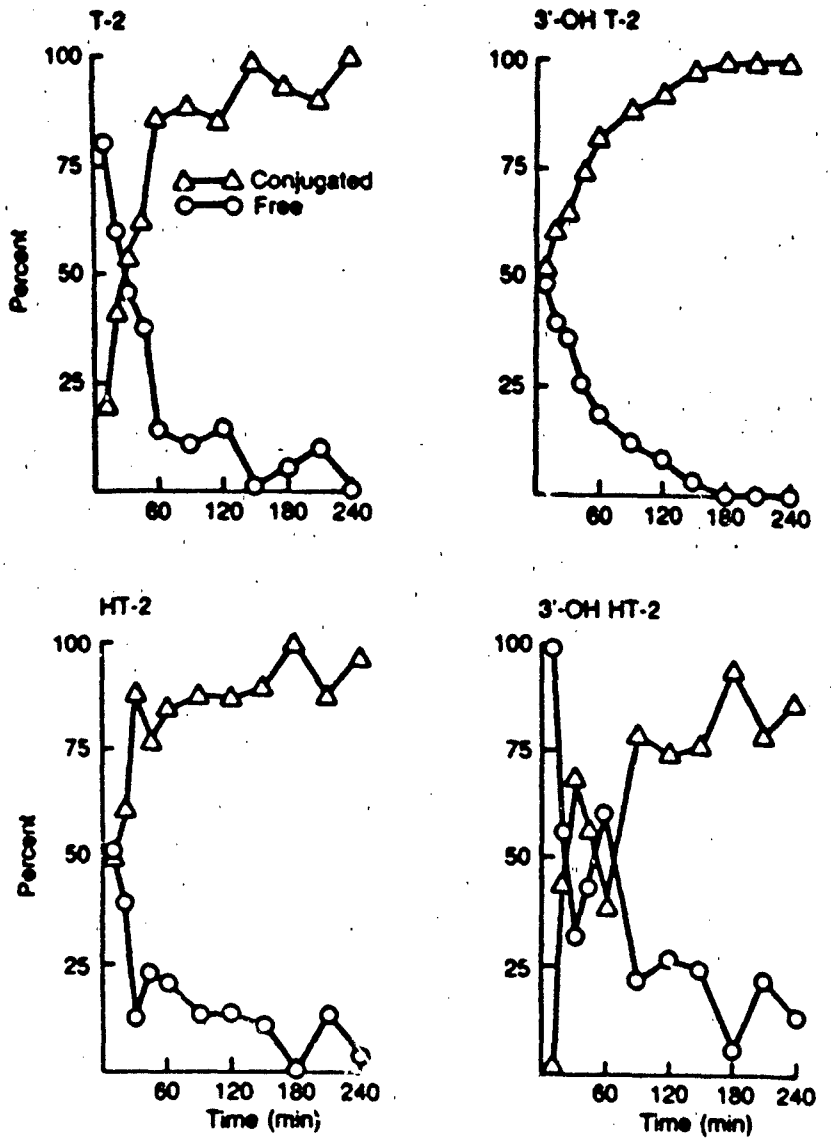
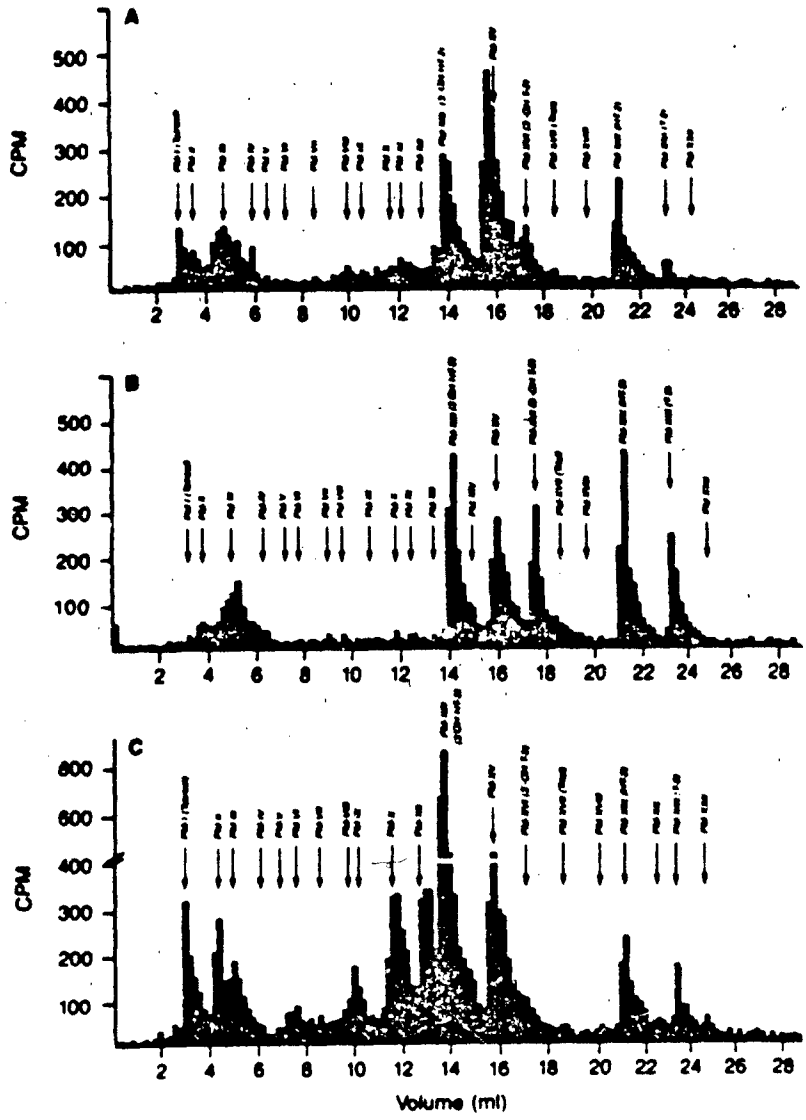


FIGURE 4. Reverse phase HPLC radiochromatograms of extracts from liver (A), spleen (B) and ileum contents (C) from a pig (S2 only) administered tritium-labeled T-2 toxin intravascularly at a dose of 0.15 mg/kg body weight.



C. STRUCTURES OF NEW METABOLITES OF DIACETOXYSCIRPENOL IN THE EXCRETA OF ORALLY ADMINISTERED RATS--Tae Sakamoto, Steven P. Swanson, Takumi Yoshizawa, and William B. Buck

ABSTRACT

Rats orally administered multiple doses of diacetoxyscirpenol (DAS, 2.8 mg/kg of body weight) eliminated 15-monoacetoxyscirpenol (15-MAS), scirpentriol (SCP), and two unknown metabolites named DRM-1 and DRM-2 in urine and feces. The parent compound was detected in neither urine nor feces. The metabolites, 15-MAS, SCP, DRM-1, and DRM-2 were detected in urine at 3.5 percent, 4.9 percent, 9.5 percent, and 7.2 percent of the administered dose, respectively, and the latter two metabolites were detected in feces at 9.5 percent and 18.9 percent, respectively. The new metabolites, DRM-1 and DRM-2 were identified as 15-acetoxy-3α,4ß-dihydroxytrichothec-9,12-diene, and 3a/4ß,15-trihydroxy-trichothec-9,12-diene, respectively on the basis of mass and nuclear magnetic resonance spectroscopy.

INTRODUCTION

Diacetoxyscirpenol (DAS, anguidine (17), 3α-hydroxy-4ß,15-diacetoxyl2,13-epoxytrichothec-9-ene) is one of the trichothecene mycotoxins produced by the species of Fusarium, frequently isolated from various agricultural commodities (3,11,13,18,20,24,25). This toxin has been found in barley, wheat, rice, oats, corn, safflower, and mixed feeds throughout the world (6,9,10,18,25,27) and the consumption of naturally contaminated feed has been associated with haemorrhagic bowel lesions in swine (18). Experimentally, DAS has been shown to have various biological activities including antifungal activity against Candida albicans (1), phytotoxicity (5), high acute mammalian toxicity

accompanied by "radiometic" injury of the tissues (14,23,30), teratogenicity to chicken embryos (29), and mutagenicity in onion cells (22), but not in the Ames test (31). Similarly, DAS was efficacious against colonic adenocarcinomas in mice (7,17). However, no remission of colonic adenocarcinomas was observed in human clinical trials (8).

To more fully understand the mode of action of DAS, it is important to clarify its metabolic fate in vivo. Currently, little information is available on the metabolism of DAS. In vitro metabolism studies utilizing liver microsomes of rats and rabbits demonstrated that 15-monoacetoxyscirpenol (15-MAS) was the only metabolite of DAS (19). In addition, 15-MAS was also reported as the sole metabolite when DAS was incubated with bovine rumen microorganisms (15). On the other hand, DAS, 15-MAS and scripentriol (SCP) were detected in the serum of pigs orally administered DAS (2). In this paper, we wish to describe the structural elucidation of the metabolites detected in the excreta of rats orally administered DAS.

MATERIALS AND METHODS

Mycotoxins. Diacetoxyscirpenol was purchased from Myco-Lab Co. (Chesterfield, MO); 15-MAS, SCP, and T-2 tetraol were prepared by alkaline hydrolysis of DAS and T-2 toxin respectively; and 4-Monoacetoxyscirpenol was prepared by acid hydrolysis of DAS (26). The purity of these toxins exceeded 96 percent as determined by gas-liquid chromatography (GLC) with a hydrogen flame ionization detector (FID).

Animal Treatment. Twenty male Wistar rats weighing 200 to 270 g were purchased from Holtman Co. (Madison, WI) and individually placed in metabolic cages (Nalgen, Rochester, NY) for seven days prior to toxin administration. Water and feed (Wayne rodent blox, Continental Grain Co., Chicago, IL) were

available ad libitum. Feed was analyzed and determined to be free from DAS, T-2 toxin, deoxynivalenol, and aflatoxin.

Diacetoxyscirpenol was dissolved in physiological saline containing 5 percent ethanol at a concentration of 1.4 mg/mL. Rats were orally administered DAS 2.8 mg/kg of body weight three times at seven day intervals. Urine and feces were collected daily for 21 days and stored at -20°C prior to analysis.

Extraction and Purification of DAS and Its Metabolites in Excreta. The urine and feces were separately pooled for the first 3 days after each toxin administration. After addition of 330 g sodium chloride, 1L of the urine was extracted with acetonitrile (3 x 0.5L) and acetonitrile:acetone (1:1 v/v, 3 x 0.5L) followed by centrifugation. The organic extracts were combined, concentrated, and redissolved in 10 mL water. The mixture was loaded onto five Sep-Pak C_{18} cartridges (Waters Associate Inc., Milford, MA), and the metabolites were eluted with 8 mL of 75 percent aqueous acetonitrile. The eluates were combined, evaporated to dryness, and redissolved in 12 mL methylene chloride:methanol (5:1 v/v). Anhydrous sodium sulfate (5 g) and cupric carbonate (2 g) were added, followed by filtration through Whatman No. 1 filter paper. The residue was rinsed twice with 5 mL methanol. The filtrate was evaporated, redissolved in 10 mL methylene chloride:methanol (9:1 v/v), added to a Florsil column (2 cm i.d., Florsil, 60 to 100 mesh, 20 g, Fisher Scientific, Fair Lawn, NJ), and the metabolites eluted with 200 mL of the same solvent.

Feces (1 kg) were extracted three times with 50 percent aqueous acetonitrile (1 x 2L, 2 x 1L) followed by centrifugation. The aqueous acetonitrile was defatted with n-hexane (2 x 1L) and the hexane was discarded. Sodium

chloride (1.2 kg) was added to the extracts to induce phase separation. The acetonitrile layer was collected and the remaining aqueous layer successively extracted with acetonitrile (2 x 1L) and acetonitrile:acetone (1:1 v/v, 3 x 1L) followed by centrifugation. The acetonitrile extracts were combined and purified as described above for urine.

Additional purification of metabolites for spectroscopic analysis was accomplished on silica gel TLC plates. Bands 5 mm wide with Rf values corresponding to 15-MAS and SCP were scraped and eluted with 5 mL acetone.

Quantitation of the metabolites was performed by extracting 135 to 165 mL of urine or 30 to 40 g of feces. Extraction and purification was accomplished as described above on a reduced scale. Florisil column eluates were concentrated, redissolved in 2 mL ethanol and aliquots were removed for GLC analysis.

Gas-Liquid Chromatography (GLC). Gas chromatography was performed on a Hewlett Packard Model 5790A gas chromatograph equipped with an electron capture detector (^{63}Ni-ECD) and a 12 m x 0.2 mm i.d., 200 μ film OV-1 bonded phase fused silica capillary column. The operating conditions were as follows: column temperature was programmed from 100°C [1] to 150°C at 25°C/min, from 150°C [4] to 230°C at 4°C/min and from 230°C to 270°C at 30°C/min (hold time in minutes is shown in brackets); injector temperature, 250°C; detector temperature, 325°C; helium carrier gas, 35 cm/s; make-up gas (argon:methane, 95:5) flow rate 45 mL/min. Quantitation was accomplished using T-2 tetraol as a GLC internal standard and 15-MAS and SCP as standards for quantitations of DRM-1 and DRM-2, respectively.

Derivatization of DAS and Its Metabolites. An aliquot of the sample extract was evaporated to dryness, redissolved in 500 μL of toluene: acetonitrile (95:5), 75 μL of N-methyl-bis (trifluoroacetamide) (MBTFA, Pierce

Chemical Co., Rockford, IL) was added and the mixture heated for 1 hour at 60°C. After cooling, the excess MBTFA was removed by partition- ing the organic phase with 1 mL of 5 percent aqueous sodium bicarbonate. The organic layer was diluted to 4 mL with n-hexane and the trifluoroacetyl (TFA) ester derivatives were analyzed by GLC-ECD. In addition, metabolites were derivatized to trimethylsilyl (TMS) ethers with TRI-SIL-TBT (Pierce Chemical Co.) for GC-MS analysis. Aliquots (ca. 0.5 g) of the isolated metabolites were hydrolyzed by reacting with 200 µL of 1 N KOH in 80 percent methanol for 30 minutes at room temperature to check for modification of the 12,13-epoxy ring according to the procedure described previously (35). After evaporation of the solvent, the hydrolized metabolites were analyzed by GLC-ECD as the TFA derivatives.

Thin-Layer Chromatography (TLC). Thin-layer chromatography was carried out on precoated silica gel plates (250 m gel thickness, 20 x 20 cm, J. T. Baker Chemical Co., Phillipsburg, NJ). Two TLC solvent systems were used; solvent A, chloroform:methanol (9:1 v/v) and solvent B, chloroform: acetone (3:2 v/v). Compounds were made visible under long wave (354 nm) ultraviolet light by heating the plates at 130°C for 5 min after treating with 30 percent H_2SO_4 in methanol. Additional TLC visualization was accomplished by treatment with 4-(a-nitrobenzyl)pyridine (28). Trichothecenes possessing a 12,13-epoxy ring yield blue colored spots with this reagent.

Spectroscopy. Gas chromatography-mass spectroscopy (GC-MS) was performed on a JEOL QH-100 mass spectrometer at 20 and 70 eV using both TFA and TMS derivatives. Proton and ^{13}C nuclear magnetic resonance (NMR) spectra were measured on a Hitachi R-90H Fourier transform NMR spectrometer (90 MHz) with Me_4Si as an internal standard.

RESULTS AND DISCUSSION

Quantitation of DAS and Its Metabolites in Rat Excreta. Diacetoxyscirpenol and its metabolites were quantified by GLC-ECD as TFA derivatives (Table 2). The parent compound was detected in neither urine nor feces eliminated within the first 3 days, indicating that DAS was significantly biotransformed *in vivo*. In urine, 15-MAS, SCP, and two unknown metabolites named DRM-1 and DRM-2 were detected at 3.5 percent, 4.9 percent, 9.5 percent, and 7.2 percent of the administered dose. On the other hand, only the metabolites DRM-1 and DRM-2 were found in feces at 9.5 percent and 18.9 percent of the dose, respectively.

Chemical Structures of the Metabolites. The metabolites 15-MAS and SCP detected in urine were analyzed by GC-MS as the corresponding TMS derivatives. The molecular ion of TMS-15-MAS was found at m/z 468 (calcd. for $C_{25}H_{40}O_5Si_2$, 468.2362). Other predominant fragment ions at m/z 378 (M^+ minus $(CH_3)_3SiOH$) and 159 (base peak) were also found in the authentic TMS-15-MAS. The mass spectrum of TMS-SCP was identical to authentic SCP displaying a molecular ion at m/z 498 (calcd. for $C_{24}H_{46}O_5Si_3$, 498.2652) and other fragment ions at m/z 483 (M^+ minus CH_3), 408 (M^+ minus $(CH_3)_3SiOH$) and 277 (base peak).

The molecular ion of DRM-1 TMS ether was found at m/z 452 (calcd. for $C_{23}H_{40}O_3Si_2$, 452.2413, M^+ of TMS-15-MAS minus an oxygen atom) with other fragment ions at m/z 437 (M^+ of TMS-DRM-1 minus CH_3), 362 (M^+ of TMS-DRM-1 minus $(CH_3)_3SiOH$), 289 and 261. All of these fragment ions were shifted down by 16 mass units compared with corresponding fragments in the TMS-15-MAS. Other predominant ions at m/z 175 and 159 (base peak) were observed in both DRM-1 and 15-MAS.

The molecular ion of DRM-2 TMS ether was found at m/z 482 (calcd. for $C_{24}H_{45}O_4Si_3$, 482.2702, M^+ of TMS-SCP minus an oxygen atom). Other fragment ions at m/z 467 (M^+ of TMS-DRM-2 minus CH_3), 392 (M^+ of TMS-DRM-2 minus $(CH_3)_3SiOH$), 379 and 261 were also shifted down by 16 mass units compared with corresponding fragments in the TMS-SCP. Other predominant ions including m/z 159 (base peak), and 147 were observed in both DRM-2 and SCP.

The proton NMR of DRM-2 dissolved in chloroform-d, dimethylsulfoxide-d_6 and D_2O was as follows: (90 MHz, ù) 1.04 (3H,s,C-14), 1.67 (3H,s,C-16), and 5.30 (1H, d, J = 5.0 Hz, C-10). Instead of a doublet resonance at ù2.59 and 2.80 (each 1 H, J = 4.2 Hz) due to methylene protons of the epoxy ring in SCP, singlet resonances were observed at Ü4.58 and 4.94 in DRM-2, which were assigned to terminal methylene protons at the C-13 position. The ^{13}C NMR of DRM-2 dissolved in acetone-d_6, chloroform-d and dimethylsulfoxide-d_6 was as follows: (ù), 11.31 (C-14), 20.12 (C-7), 23.11 (C-16), 27.90 (C-8), 44.12 (C-6), 52.32 (C-5), 60.95 (C-15), 67.35 (C-11), 78.63, 79.21, 79.88 (C-2,3,4), 119.61 (C-10), and 138.30 (C-9). Signals at Ü64.79 (C-12) and 45.91 (C-13) in SCP were shifted to ù152.91 and 105.25 in DRM-2, respectively. Chemical shifts of both carbons and protons at the C-12 and C-13 positions of DRM-2 were similar to those of verrucarin K (4), DOM-1 (32), and T-2 deepoxy metabolites (35) with a trichothec-9,12-diene skeleton.

In addition, DRM-1 did not give SCP, but rather DRM-2 as a parent alcohol after alkaline hydrolysis. DRM-2 remained unchanged indicating that the trichothecene nucleus of these compounds was modified. Furthermore, DRM-1 and DRM-2 were negative to the color reaction on a TLC plate after treating with 4-(à-nitrobenzyl)pyridine suggesting the absence of the 12,13-epoxy function in the metabolites. Based upon these data, chemical structures of DRM-1 and

DRM-2 were identified as 15-acetoxy-3α,4β-dihydroxytrichothec-9,12-diene, i.e., 15-acetoxy-deepoxy-scirpenol and 3α,4β, 15-trihydroxytrichothec-9,12-diene, i.e., deepoxy-scirpenol (Table 1).

Yoshizawa et al. (33,34), and Pawlosky et al. (21), identified hydroxylated T-2 toxin metabolites in the urine of a cow dosed orally with T-2 toxin (3'-OHT-2, 3'-OH HT-2,3'-OH-7-OH HT-2). In contrast, hydroxylated metabolites of DAS were not detected in the present study. Ohta et al. (19) reported that the microsomal nonspecific carboxyesterase from rat and rabbit liver hydrolyzed DAS to 15-MAS. These data indicate that the liver microsomal enzymes are involved in the in vivo hydrolysis pathway, i.e., DAS was hydrolyzed to SCP via 15-MAS. The only metabolites detected in feces in the present study were the deepoxy compounds, suggesting that the gastrointestinal microorganisms participate in the deepoxydation reaction of DAS in rats. In addition, King et al (16), reported that the deepoxydation of DON was observed in the incubation mixture with bovine rumen microorganisms. These data suggests that deepoxydation of 12,13-epoxy trichothecenes may be accomplished by either gastrointestinal or rumen microorganisms.

It is noteworthy that the deepoxy metabolites DRM-1 and DRM-2 were quantitatively more significant than DAS, 15-MAS, and SCP in rats. It was previously shown that the acid-catalyzed opening of the 12,13-epoxytrichothec-9-ene nucleus resulted in non-toxic products to culture cell, i.e., 10-13-cyclotrichothecane or apotrichothec-9-ene systems (12). Therefore, the deepoxy metabolites DRM-1 and DRM-2 identified in this study are also assumed to be detoxification products, although the toxicological significance of the deepoxydation in DAS metabolism remains to be established.

ACKNOWLEDGMENTS

This study was carried out under academic cooperation between the College of Veterinary Medicine, University of Illinois and the Faculty of Agriculture, Kagawa University.

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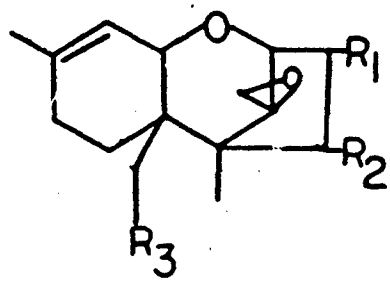
TABLE 1. Chemical structures and resolution of DAS and its metabolites by TLC and GLC.

Skeleton A



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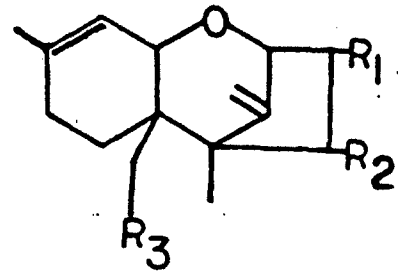

Compound[a]	Skeleton	R^1	R^2	R^3	TLC R_f[b] A	TLC R_f[b] B	GLC t_R[c] (min)
DAS	A	OH	OAc[d]	OAC	0.67	0.47	18.9
15-MAS	A	OH	OH	OAc	0.40	0.13	14.3
DRM-1	B	OH	OH	OAc	0.37	0.14	11.4
SCP	A	OH	OH	OH	0.17	0.03	10.4
DRM-2	B	OH	OH	OH	0.17	0.03	7.7

[a] Abbreviations: DAS, diacetoxyscirpenol; 15-MAS, 15-monoacetoxyscirpenol; DRM-1, 15-acetoxy-deepoxy-scirpenol; SCP, scipentriol; DRM-2, deepoxy-scirpenol.

[b] TLC on silica gel plates developed in solvent systems A and B (see text).

[c] Retention time of trifluoroacetate derivatives.

[d] OAc, acetate.

TABLE 2. Quantitation of DAS and its metabolites in excreta of rats orally administered DAS.

Excreta[a]	Recovery, percent of dose[b]					
	DAS	15-MAS	DRM-1	SCP	DRM-2	Total
Urine	[c]N.D.	3.5 ± 0.8	9.5 ± 1.8	4.9 ± 0.5	7.2 ± 0.3	25.0 ± 0.9
Feces	N.D.	N.D.	9.5 ± 1.0	N.D.	18.9 ± 1.2	28.5 ± 1.8
Total	0.0 ± 0.0	3.5 ± 0.8	19.1 ± 2.0	4.9 ± 0.5	26.1 ± 1.2	53.5 ± 1.4

[a]Urine or feces eliminated within the first 3 days after each dose was pooled and analyzed as TFA esters by capillary GLC-ECD.

[b]Molecular percent of the administered DAS. Values are the mean of three dosing replications ± standard deviation.

[c]N.D., none detected.

D. IN VIVO METABOLISM OF T-2 TOXIN IN RATS: EFFECT OF DOSE AND ROUTE--Richard Pfeiffer

INTRODUCTION

Two groups of male, Spraque Dawley rats (6 rats per group) weighing 250 grams each were dosed so that each treatment combination had been repeated two times. The routes of administration were oral, dermal and intravenous, and the dose levels were 0.60 mg/kg and 0.15 mg/kg. Urine and feces were collected at 24-hour intervals for a total of 7 days. Samples were stored at -20° C prior to analysis by HPLC.

DETERMINATION OF TOTAL RADIOACTIVITY IN URINE

A. Procedure. Aliquots of urine (0.2 mL) were placed in 7 mL glass scintillation vials. Water (0.4 mL) and Aquasol II (5 mL) were added, and the contents vortexed until clear. Samples were then counted on a Packard Tri-Carb Model 300 M liquid scintillation counter.

B. Results. After 7 days, approximately 15 to 22 percent of the administered dose was excreted in the urine. Table 1 gives a summary of the urinary excretion.

DETERMINATION OF TOTAL RADIOACTIVITY IN FECES

A. Procedure. One gram of feces is weighed into a 20 mL glass scintillation vial, and 10 mL acetate buffer (0.1 M and pH = 3.8) is added. Mix well using a Tekmar tissue homogenizer. Transfer a 50 µL aliquot to a 7 mL glass vial to which is then added 0.1 mL of 60 percent perchloric acid and 0.2 mL of 30 percent hydrogen peroxide. Vials are capped tightly and heated at 30° C in a oven for 24 to 36 hours until feces are completely digested. Vials are then removed and allowed to cool. Add 5 mL Aquasol

II scintillation cocktail before counting on a Packard Tri-Carb Model 300 M scintillation counter.

B. Results. Approximately 60 to 90 percent of the administered dose is excreted in feces after 7 days. A summary of the data is shown on Table 2.

PREPARATION OF METABOLITES (See Table 3 for HPLC Data)

A. Tritium labeled T-2 toxin was obtained from Amersham (500 mCi/mmol) with a radiochemical purity of > 96 percent.

B. The tritium labeled hydrolysis products of T-2 toxin; namely, HT-2, triol and tetraol were prepared using ^{3}H T-2 toxin and a mild alkaline hydrolysis (Wei et al., 1971). The identity of the products was confirmed by TLC and GLC/ECD.

C. The radiolabeled 3'-OH T-2 metabolite was prepared using a rat liver S-9 generating system and paroxon to inhibit esterase activity (Wilson et al., 1975 and Hansen et al., 1981). The details of the synthesis are described in the previous section entitled, "In Vitro Production of 3'-Hydroxy T-2 From T-2 Toxin By Rat Liver Homogenates." Confirmation of 3-'OH T-2 was by capillary GLC/ECD using the split peak TFA derivatives.

D. The preparation of 3'-OH HT-2 from radiolabeled 3'-OH T-2 was done using a mild alkaline hydrolysis (Wei et al., 1971). TLC was used to monitor the reaction with confirmation using GLC/ECD.

E. The tritium-labeled de-epoxy compounds of HT-2, triol and tetraol were produced using the conditions described by Leidle and Hespell (1980) with modifications described in the section entitled, "Metabolism of Three Trichothecenes, T-2, DAS, DON, By Bovine Rumen Microorganisms," substituting tritium-labeled T-2 toxin. Confirmation was done using TFA derivative and GLC/ECD.

HPLC ANALYSIS OF EXCRETA

A. Extraction Procedure.

1. Urine. An aliquot of urine (1-10 mL) is diluted to 15 mL with water and 15 mL acetonitrile is added. Following the addition of 5 g of NaCl, the sample is shaken vigorously and centrifuged to complete the phase separation. The top layer is transferred to an erlenmeyer flask. The above extraction is repeated three times with 10 mL acetonitrile and once using 10 mL acetonitrile/acetone (1+1), each time transferring the organic (upper) layer to the erlenmeyer flask. To the combined organic layer is added sodium sulfate (approximately 100 g) until a clear solution forms followed by 2 g cupric carbonate with swirling. The sample is filtered through analytical filter pulp and evaporated to dryness. The sample is redissolved in methanol/water (1+1) and filtered through a 0.2 micron filter disc before injecting a 100 µL aliquot for HPLC analysis.

2. Feces. An amount of feces (1 to 4 grams) is placed in a centrifuge tube followed by the addition of 10 mL acetonitrile and 10 mL 0.1 M acetate buffer (ph 3.6). Homogenize sample with a Tekmar tissue homogenizer, centrifuge and decant the fluid portion into another centrifuge tube. Repeat two times, each time decanting the acetonitrile/buffer with a centrifuge tube. To this extract, add 5 grams NaCl and proceed as with urine that was previously described.

B. HPLC Conditions. Alltech Econosphere 5 micron C-18 column, 150 mm x 4.6 mm. Gradient elution program of 10 percent MeOH/H_2O to 90 percent MeOH/H_2O. Collect 0.2 mL fractions (144) and add 5 mL Scintiverse LC cocktail for LSC.

C. Results. Figure 1 shows an HPLC radiochromatogram of ^{3}H T-2 and its alkaline hydrolysis products; namely, tritium-labeled HT-2, triol and tetraol. Figures 2 and 3 show a representative chromatogram of rat feces and urine, respectively. The HPLC analysis to compare the effects of dose and route on metabolism have just been started as have the confirmation procedures to positively identify the metabolites.

D. Discussion. The experiment comparing dosage and route is in progress. Representative HPLC chromatograms of feces and urine are given in Tables 2 and 3. Although the analyses are not complete, information on the major metabolites excreted are available. In urine the two major peaks detected were HT-2 and 3'OH HT-2, with smaller amounts of T-2, 3'OH T-2, T-2 triol, tetraol and several unknowns. In feces, 3'OH HT-2 was the major peak; however, significant quantities of HT-2, deepoxy HT-2, tetraol and several unknowns were detected. Preliminary evidence suggests the unknown eluting in fraction 82 is deepoxy 3'OH HT-2. Additional work is in progress on the identification and confirmation of these unknowns. As a result of those efforts, the compound was identified as 4'-Hydroxy T-2, 4β,15-diacetoxy-3α-hydroxy-8α-(3-methyl-4-hydroxybutyryloxy)-12,13-epoxytricho-thec-9-ene.

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TABLE 1. Percentage of the Administered Dose Excreted in Urine Over Seven Days*

Route	Dose (mg/kg)	Day 1	2	3	4	5	6	7	Total
IV	0.60	16.9	6.19	0.98	1.08	0.36	0.19	0.07	25.7
	0.15	20.2	2.49	0.62	0.27	0.22	0.12	0.10	24.0
Oral	0.60	18.2	2.26	0.79	0.29	0.19	0.13	0.11	21.9
	0.15	14.6	0.77	0.32	0.27	0.19	0.11	0.10	16.3
Dermal	0.60	7.20	3.95	2.42	1.45	1.13	0.92	0.64	17.1
	0.15	8.28	3.28	2.00	1.36	0.78	0.48	0.43	16.6

TABLE 2. Percentage of the Administered Dose Excreted in Feces Over Seven Days*

Route	Dose (mg/kg)	Day 1	2	3	4	5	6	7	Total
IV	0.60	42.4	9.45	2.00	6.67	1.27	1.10	--	62.9
	0.15	61.8	19.2	16.0	1.91	0.52	--	--	85.0
Oral	0.60	68.9	15.2	4.23	1.38	0.79	--	--	90.5
	0.15	79.3	3.87	0.25	0.63	0.49	--	--	84.5
Dermal	0.60	20.3	10.9	6.75	5.57	3.25	1.74	1.22	49.7
	0.15	31.9	14.3	8.05	6.06	3.59	1.45	0.52	65.8

*Average of two rats per treatment combination.

TABLE 3. HPLC Data for T-2 and Its Metabolites

Compound	Retention Time (Relation to T-2)	HPLC Fraction
T-2	1.00	116
de-epoxy HT-2	0.96	112
HT-2	0.91	105
de-epoxy triol	0.86	100
triol	0.79	92
3'-OH T-2	0.76	88
3'-OH HT-2	0.61	71
de-epoxy TOL	0.24	28
TOL	0.16	18

FIGURE 1. Representative Urine Chromatogram

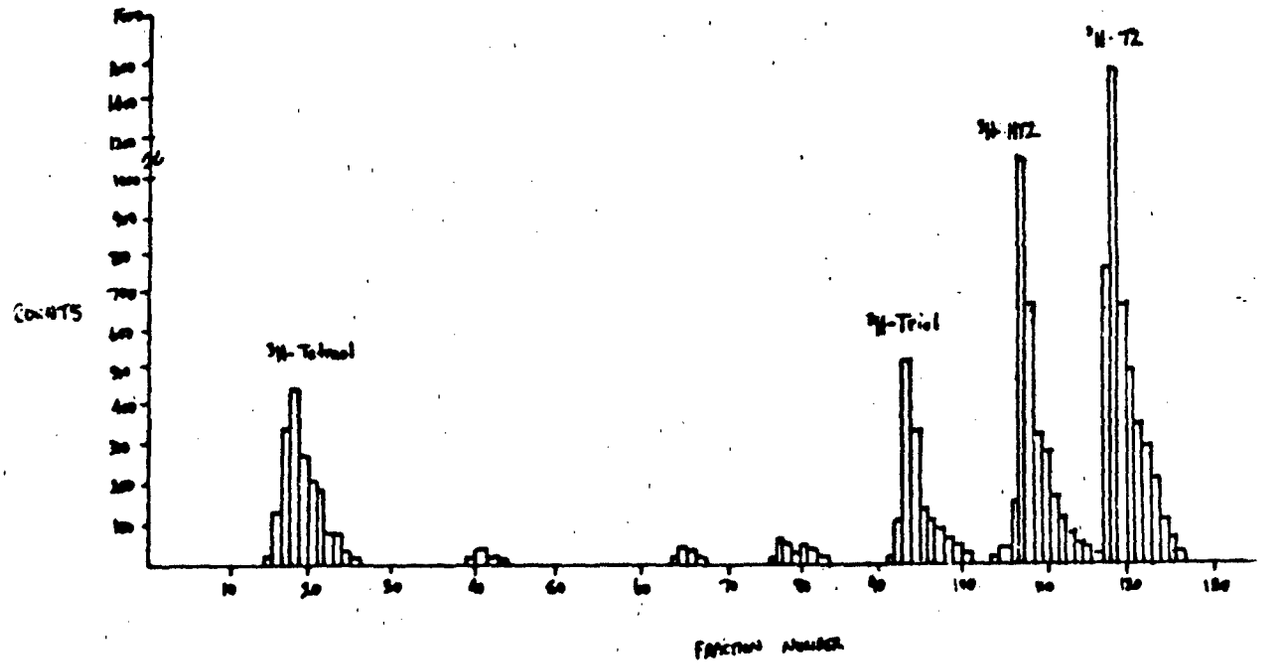


FIGURE 2. Representative Feces Chromatogram

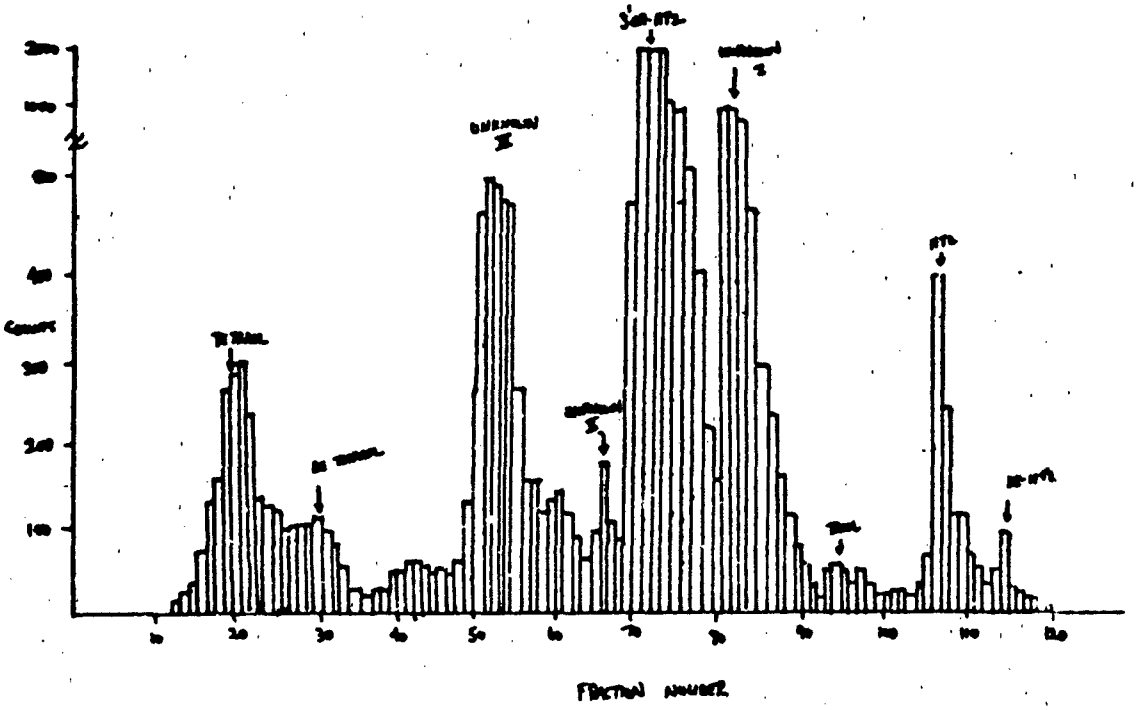
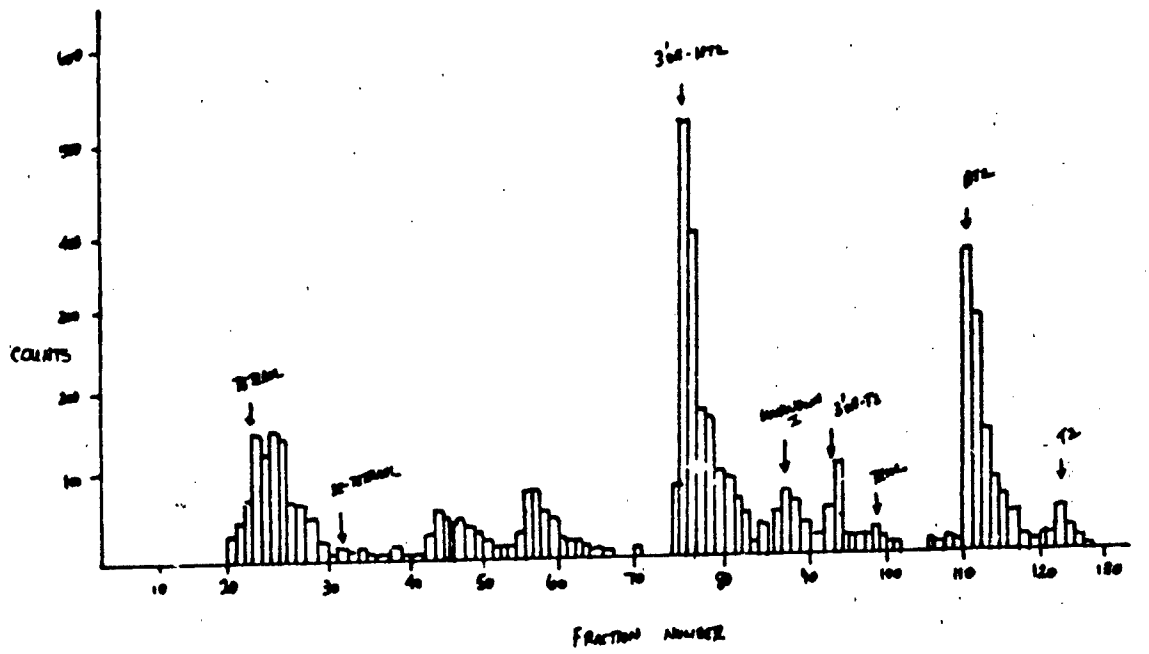


FIGURE 3. HPLC chromatogram of ^{3}H T-2 and its hydrolysis products



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VI. METABOLISM, IN VITRO

A. IN VITRO METABOLISM OF T-2 TOXIN BY RAT LIVER MICROSOMES--Cathy Knupp, Steven P. Swanson, and William B. Buck

ABSTRACT

Rat liver microsomes biotransform T-2 toxin to a variety of metabolites including: HT-2, Neosolaniol, 4-Deacetylneosolaniol, T-2 triol, 3'-OH T-2 and 3'-OH HT-2, in addition to two unidentified compounds. Metabolism resulting from esteratic cleavage of the parent T-2 toxin was more extensive than hydroxylation of the 3' carbon side chain by mixed function oxidases. Pretreatment of rats with phenobarbital enhanced oxidative hydroxylation of T-2 toxin at the 3' carbon position and the addition of paraoxon to the microsomes inhibited hydrolysis of the C3' oxidized product.

INTRODUCTION

T-2 toxin (4ß, 15-diacetoxy-8 -(3-methylbutyryloxy)-3 -hydroxy-12,13-epoxytrichothec-9-ene) is a trichothecene mycotoxin produced by species of Fusarium (Bamburg et al., 1971). Although detected only sporadically in nature compared with other trichothecene mycotoxins such as deoxynivalenol, T-2 has been implicated in several serious cases of human and animal toxicoses (Hsu et al., 1972; Joffe, 1971; Bamburg et al., 1971).

The distribution and metabolism of tritium labeled T-2 toxin was investigated after oral administration in chickens (Yoshizawa et al., 1980*), mice (Matsumoto et al., 1978) and a lactating cow (Yoshizawa et al., 1981) and after intravascular administration in swine (R. A. Corley, S. P. Swanson and W. B. Buck, 1985). In all species, T-2 was rapidly biotransformed to a

variety of metabolites. Minor metabolites in the cow and chicken were identified as simple hydrolysis products including HT-2, 4-deacetylneosolaniol and neosolaniol; however, the major metabolites remained unidentified.

Yoshizawa et al. (1983) characterized two of the main metabolites present in bovine urine, initially labeled TC1 and TC3, as 3'-OH T-2 and 3'-OH HT-2, respectively. Recently a third metabolite, TC6, was tentatively identified as 3'-hydroxy-7-hydroxy HT-2 (Pawlosky and Mirocha, 1984).

The compound HT-2 was reported as the only in vitro metabolite in human and bovine liver homogenates (Ellison and Kotsonis, 1974) and laboratory animals (Ohta et al., 1978). In addition to HT-2, 4-deacetylneosolaniol, T-2 tetraol, and neosolaniol were detected in rat liver homogenates and stomach strips incubated with T-2 toxin (Yoshizawa et al., 1980[b]). The hydroxylated products 3'-OH T-2 and 3'OH HT-2 were subsequently identified for the first time in vitro, using monkey and mice liver nomogenates or microsomal fractions supplemented with a NADPH generating system, cofactors necessary for mixed function oxidase activity.

The present study is concerned with the metabolism of T-2 by rat liver microsome fractions and the role of microsomal esterase and oxidase systems in the biotransformation of T-2 toxin in vitro.

EXPERIMENTAL SECTION

Microsomal Preparation. Male Sprague-Dawley rats weighing 400 to 500 grams were obtained from Harlan Co., St. Louis, MO. Liver microsome mixed function oxidase activity was induced by treating rats for three consecutive days with intraperitoneal injections of 75 mg/kg sodium phenobarbital (PB) dissolved in physiologic buffered saline. Following exsanguination, individual livers were perfused with cold homogenizing medium (0.25 M sucrose

containing 0.05 mM EDTA) through visible blood vessels, then homogenized with three volumes of the same medium. The homogenate was centrifuged at 10,000 x g for 10 minutes at 0°C and the supernatant filtered through glass wool. The filtrate was centrifuged at 100,000 x g for 60 minutes at 4°C and the supernatant decanted. The microsomal pellet was resuspended in the homogenizing medium to give 1.0 gram liver fresh weight/ml, flash frozen in liquid nitrogen and stored at -70°C. All microsome preparations were utilized within 2 weeks after preparation. For all assays, 1.0 mL of microsomal preparation was resuspended in 9.0 mL Tris buffer (0.05 M, pH 7.4) containing 0.15 M KCl. The final microsomal suspension averaged 22 mg protein/mL using the Hartree (1972) modification of the Lowry (1951) procedure.

Microsomal Assay. The reaction mixture consisted of 1.0 mL microsomal suspension from PB induced or noninduced control rats and 3.0 mL of the Tris-KCl buffer containing the following cofactors: 2.0 umol NADP+, 10.0 umol glucose-6-phosphate, 30 umol $MgCl_2$ and 1.33 units glucose-6-phosphate dehydrogenase. To each vial, 2.15 umol T-2 toxin (99+percent pure) dissolved in 0.05 mL ethanol was added and the vials incubated at 37°C in a water bath shaker. Additional microsomes from PB induced rats were also incubated with T-2 toxin in the presence of 50 nmol paraoxon. At the completion of each incubation period, enzyme activity was stopped by adding 0.5 mL of 1N HCl. Each incubation mixture was then applied to a 500 mg C18 cartridge (J. T. Baker, preconditioned with methanol and water) and the toxins eluted with 60 percent aqueous methanol (2 x 0.9 mL) followed by 100 percent methanol (2 x 0.9 mL) using an Analytichem Vac Elute system (Habor City, CA). The solvent was evaporated and the residue redissolved in ethanol. An aliquot was removed from each fraction for gas chromatographic analysis.

Derivatization and Gas Chromatographic Conditions. Sample extracts were dissolved in 1.0 mL toluene-acetonitrile (95 + 5) and derivatized with 0.05 mL trifluoroacetic anhydride at 60°C for 60 minutes. Samples were cooled to room temperature and vortexed with 1.0 mL of a 5 percent aqueous sodium bicarbonate solution. An aliquot was removed from the organic layer, diluted with iso-octane and 1 µL injected into the gas chromatograph. Gas chromatography was performed on a Hewlett Packard 5790 gas chromatograph equipped with a ^{63}Ni electron capture detector and a 12 meter x 0.2 mm id (.2 u film coating) fused silica capillary column. A multiramp column temperature program was used as follows: 90°C (hold 1 minute) to 170°C at 30 degrees per minute then from 170°C (hold 1 minute) to 245°C at 5.5 degrees per minute (hold 2 minutes). Other operating parameters were: injector 275°C, detector 340°C, hydrogen carrier gas at 45 cm/sec.

RESULTS AND DISCUSSION

The structures of T-2 metabolites and their gas chromatographic retention times are given in Table 1. The metabolic profiles from PB-treated and control rat liver microsomes are compared in Table 2. The major metabolite at all time periods and with both treatment groups was HT-2, demonstrating that enzymatic ester hydrolysis at the C-4 position occurs more readily than hydroxylation at C3' or hydrolysis at the C15 or C8 carbons. Six metabolites including: HT-2, 3'-OH T-2, 3'-OH HT-2, NEO, T-2 triol and 4-DN were detected within five minutes when incubated with microsomes from PB treated rats. Of the six metabolites detected above, only the compounds 4-DN and 3'-OH HT-2 were not detected at five minutes in microsomes from noninduced rats. The metabolite 3'OH HT-2 was not detected until 15 minutes and 4-DN was only detected at 60 minutes in noninduced microsomes.

Pretreatment of rats with phenobarbital induced both esterase and mixed function oxidase activity as indicated by the increase in HT-2 and the two 3'-hydroxylated metabolites at all time periods compared with noninduced controls. The induction of mixed function oxidase activity by PB, however was much more significant. The ratio of metabolite produced in PB induced compared to non-induced microsomes, averaged over all time intervals, was only 1.6 for HT-2 compared with a ratio of 4.8 for 3'-OH T-2 and 9.7 for 3'-OH HT-2.

At 60 minutes, 7 percent and 35 percent of the added T-2 remained unmetabolized in PB treated and noninduced microsomal preparations, respectively. All metabolites were still increasing in concentration at this time period.

The ratio of substrate to microsomal protein is critical in determining both extent and the pathway of metabolism. When the amount of T-2 toxin added to the PB induced microsomal incubation mixtures was reduced from 2150 nmoles (975 nmoles/mg protein) to 86 nmoles (39 nmoles/mg protein), only a trace amount of substrate remained unmetabolized by 60 minutes. In addition, the percentage of 3'-OH T-2 formed at the lower substrate to protein ratio increased threefold with no corresponding change in the HT-2 produced (data not shown).

Complete metabolism of T-2 toxin was reported in PB treated mice liver microsomes and a 60 minute incubation time with a substrate to microsomal protein ratio (nmoles/mg protein) of 154 (Yoshizawa et al., 1984). However, 3'-OH HT-2 was the major hydroxylated metabolite detected with mice microsomes compared with 3'-OH T-2 in the present study indicating significant differences between these rodent species.

The addition of 50 nmoles paraoxon to PB induced microsomes increased by six times the amount of 3'-OH T-2 formed as compared to untreated control microsomes. Paraoxon is a potent esterase (carboxyesterase, aliesterase) inhibitor (Stitzel et al., 1972). Therefore, it was expected that addition of paraoxon to the microsomal preparation would increase the percentage of the C3' hydroxylated product 3'OH T-2. Esterase inhibition not only increased the availability of added substrate (T-2) for oxidation but also decreased the rate of hydrolysis of the resulting oxidized product.

Two new compounds (presumed to be T-2 metabolites) were detected in the microsomal suspensions in addition to the six confirmed metabolites. These compounds were designated RLM-2 and RLM-3, and had GC retention times of 17.11 and 18.41 minutes. They were not found in either the T-2 plus buffer or microsomal blank incubations. Both compounds increased steadily over time and with phenobarbital induction. They also showed a 50 percent increase after the addition of paraoxon to the phenobarbital induced microsomes. These compounds were relatively minor metabolites, and did not exceed 3 percent of the administered T-2 even in the paraoxon plus PB microsomal system. Production of larger quantities of these compounds for structural identification is in progress.

ACKNOWLEDGEMENT

This work was funded by U.S. Department of Defense Grant No. DAMD17-82-C-2179.

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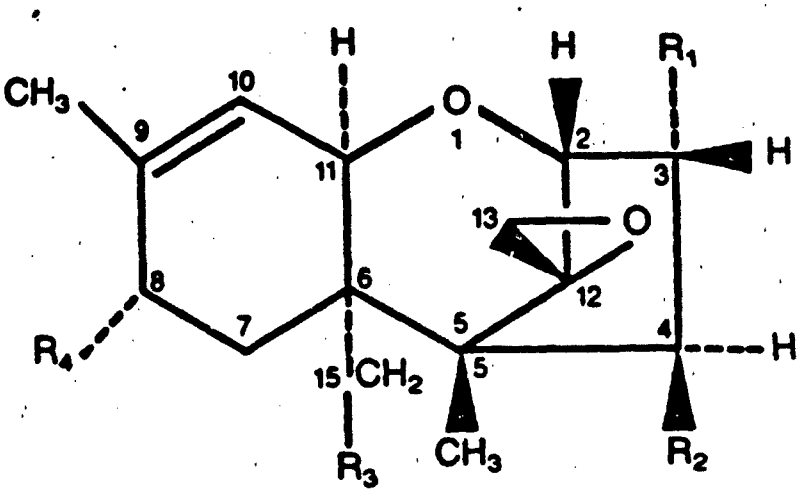

TABLE 1. Chemical structures and retention times of T-2 toxin and its metabolites by capillary gas chromatography.

Compound	R_1	R_2	R_3	R_4	GLC RT (min)[a]
1. T-2 toxin	OH	OAc	OAc	X1[b]	16.73
2. 3'-Hydroxy T-2	OH	OAc	OAc	X2	16.82, 17.87[c]
3. 3'-Hydroxy HT-2	OH	OH	OAc	X2	13.77, 14.80[c]
4. HT-2 toxin (HT-2)	OH	OH	OAc	X1	13.68
5. T-2 triol (Triol)	OH	OH	OH	X1	11.76
6. Neosolaniol (Neo)	OH	OAc	OAc	OH	11.59
7. 4-Deacetylneosolaniol (4-DN)	OH	OH	OAc	OH	8.79
8. T-2 Tetraol (TOL)	OH	OH	OH	OH	7.39

[a]Retention times of trifluoroacetate ester derivatives.
[b]X_1=$OCOCH_2CH(CH_3)_2$ X_2=$OCOCH_2C(OH)(CH_3)_2$
[c]The 3'-hydroxy metabolites form two isomers when derivatized with TFAA. (Pawlosky et al., 1984).

TABLE 2. In vitro metabolism of T-2 toxin by microsomal liver fractions of untreated rats and rats pretreated with phenobarbital.

	nmoles of Product[a]										
	5 min		10 min		15 min		30 min		60 min		60 min[e]
Compound	PB[b]	C[c]	PB	C	PB	C	PB	C	PB	C	PB
1. T-2	1240	1310	1130	1080	1140	1030	486	494	144	752	824
2. 3'OH T-2	51.2	9.3	69.7	16.0	95.6	18.5	153	32.7	193	44.4	279
3. 3'OH HT-2	1.2	ND[d]	2.2	ND	4.3	0.3	12.3	1.2	31.9	3.3	0.9
4. HT-2	302	168	491	265	567	332	912	658	1380	1060	6.7
5. Triol	2.2	1.2	2.7	2.2	3.6	3.1	3.8	3.1	8.7	4.5	ND
6. Neo	0.9	3.8	14.6	4.4	17.3	4.8	30.3	5.7	36.0	8.1	4.4
7. 4-DN	0.7	ND[d]	1.2	ND	1.5	ND	2.8	ND	6.2	0.8	ND

[a]To each vial 2150 nmoles of T-2 toxin was added. Values are averages of three replications.

[b]Animal pretreated with 75 mg/kg sodium phenobarbital intraperitoneally for three consecutive days prior to microsomal preparation.

[c]Noninduced control rats.

[d]ND = none detected.

B. METABOLISM OF THREE TRICHOTHECENE MYCOTOXINS, T-2 TOXIN, DIACETOXYSCIRPENOL AND DEOXYNIVALENOL BY BOVINE RUMEN MICROORGANISMS--Steven P. Swanson, Jean Nicolletti, Harold D. Rood, Jr. and W. B. Buck

ABSTRACT

The three trichothecene mycotoxins, T-2 toxin, diacetoxyscirpenol (DAS) and deoxynivalenol (DON), were incubated *in vitro* for 0, 12, 24 and 48 hours with rumen microorganisms obtained from a fistulated dairy cow. Gas chromatographic and GC/MS analysis of extracts indicated all 3 toxins were biotransformed to a variety of deepoxy and deacylated products. Deoxynivalenol was partially converted to a single product identified as deepoxy DON (DOM-1). Diacetoxyscirpenol was completely converted to 15-monoacetoxyscirpenol, scirpenetriol and two new products identified as 15-acetoxy-3α,4ß-dihydroxy-trichothec-9,12-diene (deepoxy MAS) and 3α,4ß,15-trihydroxytrichothec-9,12-diene (deepoxyscirpenetriol). T-2 toxin was also completely biotransformed to HT-2, T-2 triol and 2 new products identified as 15-acetoxy-3α,4ß-dihydroxy-8α-(3-methyl butyryl)-trichothec-9, 12-diene (deepoxy HT-2) and 3α-4ß,15-trihydroxy-8α-(3-methylbutyryloxy)- trichothec-9,12-diene (deepoxy T-2 triol).

MATERIALS AND METHODS

Inoculum

Fresh rumen fluid was obtained from a rumen fistulated dairy cow maintained on a timothy hay diet. Inoculum was prepared by filtering the rumen contents through cheese cloth and blending the filtrate for 1 minute in a waring blender under a carbon dioxide atmosphere.

Culture Conditions

The culture medium was prepared by adding to 125 mL erlenmeyer flasks 22.5 mL mineral solution I, 2.5 mL mineral solution II (Table 1), 0.125 mL of L-cysteine hydrochloride (2.5 percent w/v), 20 mL of water and 1.25 mg of deoxynivalenol, diacetoxyscirpenol or T-2 toxin dissolved in 0.5 mL of 10 percent aqueous ethanol. The mixture was saturated with oxygen-free carbon dioxide for 10 minutes, 25 mL of inoculum was then added and the pH adjusted to 6.8 with sodium bicarbonate. The media was bubbled with carbon dioxide for an additional 5 minutes, the flasks stoppered and then incubated on a water bath shaker at 38° C.

Bacterial Enumeration

At 0 and 48 hour, 1 mL aliquots were removed from selected flasks for bacterial enumeration, as outlined by Hungate (1950) and modified by Bryant (1972). Inoculation of roll tubes for enumeration was performed with 0.5 mL of appropriately diluted fermentation flask contents. Immediately after inoculation, each tube (medium 98-5, Bryant and Robinson, 1961) was rolled (30 to 40 seconds) using ice water applied to the tube with a sponge. The inoculated tubes were incubated for 5 days at 37° C after which the colonies were observed and counted.

Sample Analysis

At 0, 12, 24 and 48 hour, 10 mL aliquots of the incubation mixture were diluted with 5 mL water and centrifuged 10 minutes at 2000 rpm. A 10 mL aliquot of the supernatant was placed on a 12 x 1 cm column of Amberlite XAD-4. The column was rinsed with 100 mL water, the trichothecenes eluted with 100 mL acetone and the eluate concentrated on a rotary evaporator. The residue was transferred to a florisil column (2.5 g, 60-100 mesh, Fisher Sci)

with 3 x 2 mL of dichloromethane-methanol (9+1). The toxins were eluted with an additional 50 mL of the same solvent, the eluate concentrated and the residue redissolved in 1 mL of ethanol.

Gas Chromatography

A 0.2 mL aliquot of each extract was concentrated and redissolved in 1 mL of toluene-acetonitrile (95+5). Heptafluorobutyrylimidazole (HFBI, 0.05 mL, Pierce Chemical Co., Rockford, IL) was added and the mixture incubated at 60° C for 1 hour. After cooling, the excess HFBI was removed by mixing with 1 mL of 5 percent aqueous sodium bicarbonate. A 0.10 mL aliquot of the organic phase was removed and diluted to 5.0 mL with hexane. Two microliter aliquots were injected into a Hewlett Packard 5840A gas chromatograph equipped with an electron capture detector and a 1.8 m x 2 mm i.d. glass column packed with 3 percent OV-17 on 100 to 120 mesh Supelcoport. Other gas chromatographic conditions were as follows: injector, 275° C; detector, 325° C and argon-methane (95+5) carrier gas flow, 30 mL/min.

GC-MS Analysis

Mass spectra were obtained on a Hewlett Packard 5985 GC/MS and a VG 7070E GC/MS using both electron impact (70 eV) and methane positive chemical ionization. The metabolites were analyzed as trifluoroacetate (TFA) or trimethylsilyl (TMS) derivatives.

RESULTS

The concentration of anaerobic bacteria averaged 4.9×10^7 per mL immediately after inoculation. At 48 hours, the concentration of bacteria had declined to 2.8×10^6 per mL.

Gas chromatographic analysis of the incubation mixtures demonstrated a steady decline in added toxin concentration over time for all three trichothecenes, with a corresponding appearance of one or more new products.

Deoxynivalenol was partially biotransformed to a single metabolite with a GC retention time of 2.25 minutes (Table 2). The mass spectrum of this new product (TMS derivative) displayed a molecular ion at m/z 496 with major fragment ions at 481, 406, 391, 361 and 309. These fragment ions were 16 mass units less than the corresponding fragments for DON, and the spectrum was identical to authentic deepoxy DON (DOM-1) obtained from the urine of rats administered DON (Yoshizawa et al., 1983).

T-2 toxin was metabolized more rapidly and to a greater extent than DON. Only 3.6 percent of the parent T-2 was detected at 12 hours incubation, and none was detected at later time periods (Table 3). The hydrolysis products HT-2 and T-2 triol were detected as metabolites in addition to 2 new metabolites having GC retention times of 3.92 and 2.01 minutes, respectively. The chemical ionization mass spectra showed M+1 peaks at 601 and 655 for the trifluoroacetyl derivatives of the 2 unknowns consistent with the loss of oxygen from the epoxide group of HT-2 and T-2 triol to give deepoxy HT-2 and deepoxy T-2 triol, respectively. Alkaline hydrolysis (13) of both new metabolites yielded the same product, deepoxy T-2 tetraol, rather than T-2 tetraol. Additional evidence for the reduction of the epoxide groups were provided from methane chemical ionization mass spectra. Deepoxy HT-2 yielded a weak M+1 at m/z 601, with major fragment ions at 541, 499, 487, and 439. Deepoxy T-2 triol displayed chemical ionization mass spectrum with a M+1 at 655. These fragments were 16 mass units less than the corresponding fragments in HT-2 and T-2 triol, respectively. The structures of the two new metabolites were, therefore, proposed as 3α,4β-dihydroxy-15-acetoxy-8α (3-methylbutyryloxy)trichothec-9,12-diene (deepoxy HT-2) and 3α,4β-15- triacetoxy-8α-(3-methylbutyryloxy)trichothec-9,12-diene (deepoxy T-2 triol).

Diacetoxyscirpenol was also completely metabolized. No parent DAS was detected at any of the three time periods. Four products were detected including scirpenetriol, 15-monoacetoxyscirpenol and two new products with GC retention times of 2.14 and 0.93 minutes (Table 4). Chemical ionization mass spectra of the two new products yielded M+1 peaks at 555 and 501, respectively. All these fragments were shifted down 16 mass units compared with the corresponding fragments in 15-MAS and scirpenetriol. In addition, both new products did not react with 4-(p-nitrobenzyl)pyridine (9) on TLC plates suggesting the absence of the 12,13 epoxide group. Based upon the above evidence, the two new DAS metabolites were identified as 15-acetoxy-3α,4β-trihydroxytrichothec-9, 12-diene (deepoxy MAS) and 3α,4β,15-trihydroxytrichothec-9,12-diene (deepoxyscirpenetriol).

DISCUSSION

The ability of bovine rumen microorganisms to reduce the 12,13 epoxide group with the loss of oxygen to yield a carbon-carbon double bond was demonstrated for all three trichothecenes. King et al. (1985) previously reported the reduction of DON to the deepoxy product DOM-1 by rumen microorganisms. Our investigations confirmed their results. In addition, metabolites of T-2 toxin and DAS were reduced in a similar manner to yield deepoxy products. Deepoxy T-2 and deepoxy DAS were, however, not detected but rather their deaceylated products deepoxy HT-2 (DE HT-2), deepoxy T-2 triol (DE Triol) and deepoxy MS (DE MAS) and deepoxy scirpenetriol (DE Striol), respectively.

Kiessling et al. (1984) incubated both T-2 toxin and DAS with rumen microorganisms but detected only deaceylation products. The lack of deepoxidation reactions in that study may have been related to their relatively short

incubation times, 0.5 to 3 hours. In the present study, deepoxy biotransformation products were found only in small amounts, less than 8 percent of the added toxin, after 12 hours incubation, but increased steadily over time for the following 36 hours (see Tables 3 and 4) to approximately 1/3 of the total metabolites.

Although deepoxy metabolites of T-2 toxin (DE HT-2, DE T-2 triol) and DAS (DE MAS and DE Striol) were significant products at 48 hours after incubation, the simple hydrolysis products HT-2 and scirpenetriol were the predominant products, indicating deacylation of the C-4 acetyl group by microbial esterases occurred prior to the deepoxydation reactions. The pathway shown in Figure 1 is proposed for the in vitro metabolism of the trichothecene mycotoxins DON, DAS and T-2 toxin, which includes both enzymatic reduction and hydrolysis of the parent compounds.

Reduction of the 12,13 epoxide to yield a carbon-carbon double bond has been reported with several trichothecenes by fungi, animals and microorganisms. Verrucarin K, the deepoxy equivalent of verrucarin A was reported as a metabolite of Myrothecium verrucarin by Breitstein and Tamm (1977). Deepoxy DON (DOM-1) has been found in the urine and feces of rats orally administered DON (Yoshizawa et al., 1983) and recently in the urine, feces and milk of dairy cattle given DON-contaminated feed (Cote, Dahlem, Yoshizawa, Swanson and Buck, submitted for publication). Deepoxy metabolites of T-2 toxin including deepoxy T-2 tetraol and deepoxy-3'-hydroxy HT-2 have also been identified in rat excreta (Yoshizawa et al., in press). Recently, we have identified deepoxy MAS and deepoxyscirpenetriol as major metabolites in the urine and feces of rats orally administered DAS (Sakamoto, Swanson, Yoshizawa and Buck, 1986).

The identification of deepoxy metabolites in the urine and feces of animals administered trichothecenes suggests gastrointestinal microflora are capable of performing reductive deepoxidation reactions similar to the bovine rumen microorganisms. The toxicological significance of this reaction, however, is still unclear. Several authors have demonstrated alteration of the 12,13 epoxide group resulted in elimination of toxicity (4,5), although the reaction products examined were significantly altered in structure either by ring rearrangements or reductive cleavage of the epoxide to give a tertiary alcohol. Further work is needed to determine whether reduction of the epoxide to yield a carbon-carbon double bond as demonstrated by the rumen microorganisms in this study also eliminates toxicity.

ACKNOWLEDGEMENTS

This reseach was supported in part by a NC-129 grant from the University of Illinois Experiment Station and contract DAMD17-82-C-2179 from the U.S. Army Medical Research and Development Command.

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TABLE 1. Composition of incubation medium

Mineral Solution 1	Percent
K_2HPO_4	12 percent
Mineral Solution 2	
KH_2PO_4	0.6 percent
$(NH_4)_2SO_4$	0.6 percent
NaCl	1.2 percent
$MgSO_4 \cdot 7H_2O$	0.25 percent
$CaCl_2 \cdot 2H_2O$	0.16 percent

TABLE 2. Biotransformation of deoxynivalenol (DON) by bovine rumen microorganisms

Compound	RT (min) (a)	Molar Percent Recovered (b) 12	24	48
DON	3.43	88.0	62.6	41.2
DOM-1	2.25	7.4	14.2	18.7
Total	--	95.4	76.8	59.9

(a) Gas chromatographic retention time of the heptafluorobutyryl ester derivatives on OV-17 at 170° C.

(b) Each value is the average of two-three replications.

(c) Mean recovery of DON added to control rumen samples was 69 $\pm$ 4 percent, n = 3.

TABLE 3. Biotransformation of T-2 toxin by bovine rumen microorganisms.

Compound	RT (min) (a)	Molar Percent Recovered (b)		
		12	24	48
T-2 (c)	21.82	3.6	0.0	0.0
HT-2	6.14	60.4	44.8	30.9
DE HT-2	3.92	4.9	14.3	26.5
Triol	2.97	1.5	4.3	9.5
DE Triol	2.01	0.0	1.0	5.9
Total		70.4	64.4	72.8

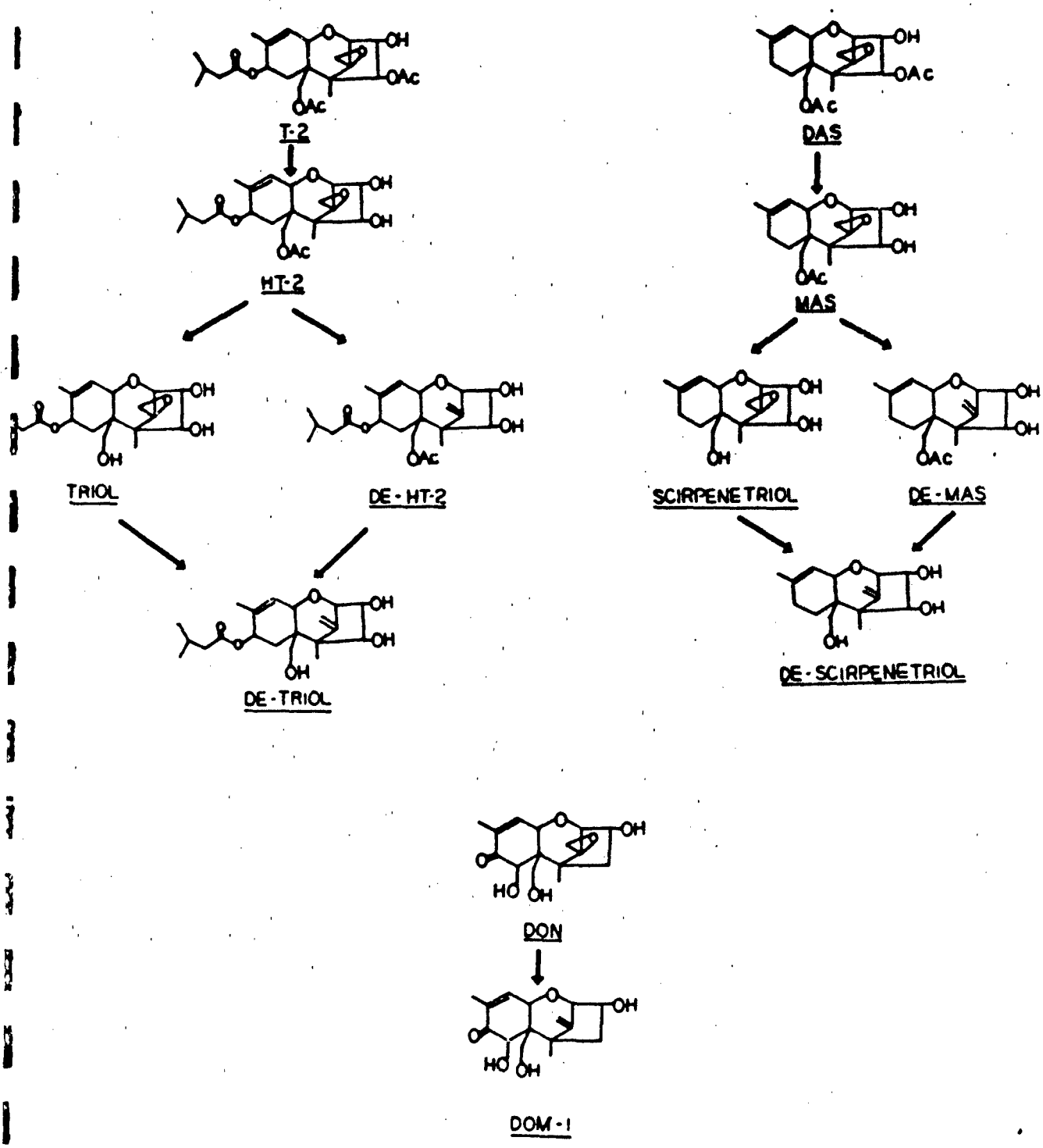
(a) Gas chromatographic retention time of the heptafluorobutyryl ester derivatives on OV-17 at a column temperature of 220° C.
(b) Each value is the average of three replications.
(c) Mean recovery of T-2 toxin added to control rumen samples was 86 ± 16 percent, n = 3.

TABLE 4. Biotransformation of diacetoxyscirpenol (DAS) by bovine rumen microorganisms.

Compound	RT (min) (a)	Molar Percent Recovered (b)		
		12	24	48
DAS	14.20	0.0	0.0	0.0
MAS	3.92	47.9	47.6	24.1
DE-MAS	2.14	5.3	12.2	21.4
Striol	1.48	13.2	25.0	25.7
DE-Striol	0.93	0.8	2.1	15.1
Total		67.2	86.9	86.3

(a) Gas chromatographic retention time of the heptafluorobutyryl ester derivatives on OV-17 at a column temperature of 190° C.
(b) Each value is the mean of three replications.
(c) Average recovery of DAS was 92 $\pm$ 8 percent, n = 3.

FIGURE 1. Proposed pathway for the metabolism of DON, DAS and T-2 toxin by bovine rumen microorganisms.



C. METABOLISM OF TRICHOTHECENE MYCOTOXINS BY BACTERIA

We have established in our laboratory that rumen microflora are capable of metabolizing DAS, DON, and T-2 to their deepoxide derivatives. It is important to find which pure strain(s) are able to metabolize the toxins.

There are many reasons why it would be advantageous to work with a pure culture. Rumen fluid is "dirty" to work with; it requires extensive clean-up with corresponding metabolite losses upon purification. Production of deepoxy trichothecenes by pure cultures grown on semidefined media would require little clean-up, the conversion would be more efficient, the reaction could be more controlled and kinetic studies could be done to obtain larger amounts of intermediate metabolites. Also, once we know which organisms are responsible for the toxin metabolism, we could examine the literature to find out what other animals, including humans, harbor that organism(s) as part of their endogenous intestinal microflora in attempts to predict which animal species may convert trichothecenes to the deepoxy metabolites. This is important as it is unlikely that humans will ever be dosed under controlled conditions.

Twenty rumen bacteria were selected from Dr. Marvin Bryant's culture collection (Table 1) (Rumen Microbiology, Department of Dairy Science, University of Illinois). Throughout this study, sterile anaerobic technques as outlined by Hungate (1950) and modified by Bryant (1972) were used.

Media

Bacterial media used was maintenance medium (Table 2) and toxin medium (Table 3). The test tubes were 18 x 100 mm. Eight ml of toxin media was added under an CO_2 atmosphere. Toxin medium was inoculated by transferring 0.2 mL of toxin medium to a 24 hour slant of inoculated maintenance medium.

After regassing the pipette with CO_2, 0.1 mL of toxin media/water of syneresis was transferred back to the toxin tube. At that time, the preferred substrate for each bacterium (Table 1) was added to the inoculated tube via a tuberculin syringe. The preferred sugars were kept in serum stopper bottles.

The inoculated toxin medium was incubated at 37° C for 5 days. Each bacterial strain was inoculated in duplicate, and uninoculated tubes were incubated as controls.

The incubation was terminated by adding all 8 mL of media into a Clin Elute 1010 column pretreated with 1 mL saturated NaCl solution. The column was eluted into a funnel, lined with 2 No. 1 Whatman filter papers, containing approximately 10 g $NaSO_4$ (arhys) and 3 g $CuCO_3$. Ethyl acetate was added (100 mL) to the column after the toxin medium. The filtrate was collected in a 250 mL round bottom flask. The sample was concentrated on a rotary evaporator. The extract was then transferred to a silica cartilage (Fischer) using chloroform. After rinsing the cartilage with chloroform, followed by toluene-acetone (95:5), the toxins were eluted with 6 mL chloroform-acetone (2:3). The eluate was concentrated and resuspended in 1 mL 100 percent ethanol.

At present, the samples have been extracted but not yet analyzed by gas chromatography. Analysis will be accomplished as follows. Aliquots will be removed, concentrated to dryness and redissolved in 1 mL (95+5) toluene-acetonitrile. Trifluoraacetic anhydride (TFAA) (0.05 mL) will be added and the mixture incubated 1 hour at 60° C. After cooling, the mixture is mixed with 5 percent bicarbonate, and a 0.1 mL aliquot of the organic phase is removed and diluted to 5 mL with hexane. A 2 µL aliquot is injected into GLC-ECD. GC conditions are as follows: 3 percent OV 17 on 100 to 120 mesh,

1.8 m x 2 mm id column, Det. 325° C, inj. 250° C, carrier rate 30 mL/min and column temperature 220° C.

TABLE 1. Microbial screening for toxin metabolism

Organism		Preferred Substrate
Ruminococcus albus	7	cellobiose
Ruminococcus flavefaciens	FD-1	cellobiose
Bacteroides succinogenes	S85	glucose
Butyrovibrio fibrosolvens	A38	glucose
Bacteroides ruminicola	118B	glucose
Selenomonas	HD-4	glucose
Treponema boyantii	B2-5	glucose
Streptococcus bovis	JB-1 Jim Russel	glucose
Lactobacillus vitulinus	GA-I	glucose
Eubacterium ruminantium	GA-195	glucose
Bacteroides ruminicola	23	glucose
Butyrovibrio fibrisolvens	49	glucose
Lachnospira multipares	D32	glucose
Selenomonas	GAr95	glucose
Megasphaera elsdenii	B159	glucose
Anaerovibrio lipolytica	7553	glycerol
B-385-like	B385-1	glucose
Bacteroides amylophilus	H-18	glucose
Ruminococcus bromii	6833	maltose/fructose
Succinivibrio dextrinosolvens	22-B	glucose

TABLE 2. Maintenance medium slants

	Percent
Resazurin solution	0.2 v/v
Rumen fluid, sterile	40.0 v/v
Mineral 1[a]	4.0 v/v
Mineral 2[a]	4.0 v/v
Glucose	0.1 w/v
Cellobiose	0.1 w/v
Soluble Starch	0.1 w/v
Agar	1.0 w/v
Water	46.0 v/v
8 percent $Na_2 CO_3$ solution (sterile, CO_2 equil.)[b]	5.0 v/v
2.5 percent cysteine solution (sterile, NO_2 equil.)[b]	1.0 v/v

[a]Table 3

[b]Add after autoclave

Tube in 13 x 100 mm sterile tubes. Maintain media under CO_2 atmosphere.

TABLE 3. Media For screening microbes for toxin metabolism

When Added	Ingredient	Percent
Prior to autoclaving	Water	47.0 v/v
	Trypticase	0.5 wt/v
	Rumen Fluid	30.0 v/v
	Hemin	
	Mineral 1	8.0 v/v
	Mineral 2	8.0 v/v
	Resazarin 0.1 percent w/v	0.1 v/v
After autoclaving	$NaHCO_3$ 15 percent w/v	5.0 v/v
	Cysteine HCl 2.5 percent w/v	2.0 v/v
	Toxin (0.5 percent ETOH w/ 0.094 percent w/v T_2)	10.0 v/v
After tubing	Preferred sugar 10 percent w/v (or add 0.16 ml stock/8 ml)	0.02 v/v

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15. Lorenzana, R. In vivo toxicity of trichothecenes. Invited speaker, FASEB Research Conference on Diagnosis, Toxicity, and Therapy of Tricothecene Mycotoxins, June 1984.

16. Poppenga, R. H., G. R. Lundeen. Therapy of acute T-2 toxicoses. FASEB Summer Research Conference on Trichothecene Mycotoxins, June 1984.

17. Lundeen, G. R., R. H. Poppenga, V. R. Beasley, W. B. Buck. Systemic distribution of blood flow in swine following intravascular administration of T-2 toxin. Poster presented at Gordon Research Conference on Trichothecene Mycotoxins, June 1985.

18. Beasley, V. R., R. Lorenzana, M. Manohar, C. Parks, V. Pang, W. Buck. Pathophysiology of acute T-2 toxicosis in swine. Poster presented at the Gordon Research Conference on Trichothecenes, June 1983.

19. Pang, V. F.,, W. M. Haschek, V. R. Beasley, P.J. Felsberg, S. P. Swanson, W. B. Buck. Morphologic and immunologic effects induced in swine by T-2 toxin following dermal exposure. Poster presented at Gordon Research Conference on Trichothecene Mycotoxins, June 1985.

20. Cote, L.-M., A. H. Dahlem, T. Yoshizawa, S. P. Swanson, W. B. Buck. Excretion of DON and its metabolite, DOM-1, in milk, urine, and feces of dairy cattle. Poster presented at Gordon Research Conference on Trichothecene Mycotoxins, June 1985.

21. Sakamoto, T., S. P. Swanson, T. Yoshizawa, W. B. Buck. In vivo metabolism of diacetoxyscirpenol in rats. Poster presented at Gordon Research Conference on Trichothecene Mycotoxins, June 1985.

22. Coppock, R. W. Toxicopathy and toxicokinetics of diacetylscirpenol and DON in swine. Poster presented at Gordon Research Conference on Trichothecene Mycotoxins, June 1983.

23. Corley, R. A. Metabolism of T-2 toxin in swine. Poster presented at FASEB Summer Conference on Trichothecene Mycotoxicosis, June 1984.

24. Cote, M.-L. Distribution, metabolism, and excretion of deoxynivalenol in swine, dairy cows, and rats. Poster presented at FASEB Summer Conference on Trichothecene Mycotoxicosis, June 1984.

25. Beasley, V. R. Ultrastructural and immunologic changes in acute T-2 toxicosis of swine. Presented at FASEB Summer Conference on Trichothecene Mycotoxicosis, June 1984.

26. Beasley, V. R., W. B. Buck, S. P. Swanson, J. R. Szabo, R. W. Coppock, H. R. Burmeister, R. F. Vesonder. Toxicokinetics of T-2 toxin in swine and cattle. Presented at Ann Meet AVMA, July 1981.

27. Beasley, V. R. An overview of acute T-2 toxicosis in swine. First American Symposium on Animal, Plant, and Microbial Toxins. Stillwater, OK, May 1984.

28. Pang, V. F., W. M. Haschek, V. R. Beasley, P. J. Felsberg, S. P. Swanson, W. B. Buck. Morphologic and immunologic effects induced in swine by T-2 toxin following inhalation exposure. Presented at Gordon Research Conference on Trichothecene Mycotoxins, June 1985.

INDIVIDUALS RECEIVING GRADUATE DEGREES WORKING ON TRICHOTHECENE PROJECTS

Name	Degree	Year
V. R. Beasley	PhD	1984
R. W. Coppock	PhD	1984
R.M. Lorenzana	PhD	1985
R. A. Corley	PhD	1985
V. F. Pang	PhD	1985
R. L. Pfeiffer	PhD	1986
G. R. Lundeen	PhD	1986
C. A. Knupp	MS	1986
P. M. Bratich	MS	1986
L. M. Cote	PhD	1986
R. H. Poppenga	PhD	1986

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