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In Vivo Effects of T-2 Mycotoxin on Protein
and DNA Synthesis in Rat Tissues

W. L. Thompson and R. W. Wannemacher, Jr.

United States Army Medical Research Institute
of Infectious Diseases

Fort Detrick, Frederick, Maryland, USA 21701-5011

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mycotoxin.

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of laboratory Animal Care.

The views of the authors do not purport to reflect the positions of the Department of the Army or the Department of Defense.

Correspondence: William L. Thompson
Pathophysiology Division
US Army Medical Research Institute of
Infectious Diseases
Fort Detrick
Frederick, Maryland, 21701-5011
Phone: (301)663-7181

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ABSTRACT

In Vivo Effects of T-2 Mycotoxin on Protein and DNA Synthesis in Rat Tissues. Thompson, W. L. and Wannemacher, R. W., Jr. (). *Toxicol. Appl. Pharmacol.* __, ___-___. Rats were given an ip injection of T-2 mycotoxin (T-2); the T-2 metabolite, T-2 tetraol (tetraol); or cycloheximide. Serum, liver, heart, kidney, spleen, muscle, and intestine were collected at 3, 6 and 9 hr post injection after a 2-hr pulse at each time with ¹⁴C-leucine and ³H-thymidine. Protein and DNA synthesis levels in rats were determined by dual-label counting of the acid-precipitable fraction of tissue homogenates. With a lethal dose of T-2, tetraol, or cycloheximide, maximum levels of protein synthesis inhibition were observed in all the tissues at the earliest time period and continued for the duration of the study (9 hr). Additional rats given the same dose of any of the three treatments died between 14 and 20 hr. With sub-lethal doses of T-2 or tetraol, the same early decrease in protein synthesis was observed, but recovery in most of the tissues was seen with time. DNA synthesis in the T-2-treated rats was also suppressed, although to a lesser degree, in the six tissues studied. With sub-lethal doses, complete recovery of DNA synthesis took place in four of the six tissues by 9 hr after toxin exposure. The appearance of newly translated serum proteins did not occur in the animals treated with T-2 mycotoxin or cycloheximide, as evidenced by total and PCA-soluble serum levels of labeled leucine. An increase in tissue-pool levels of free leucine and thymidine in response to T-2 mycotoxin was also noted. T-2

mycotoxin caused a very rapid inhibition of protein and DNA synthesis in all of the tissues studied. Its effects in whole animals were the same as those of the T-2 metabolite, tetraol, and a known protein synthesis inhibitor, cycloheximide.

INTRODUCTION

T-2 mycotoxin (4 β ,15-diacetoxy-8 α -(3-methylbutyryloxy)-3 α -hydroxy 12,13-epoxytrichothec-9-ene) is one of the more toxic of the 12,13 epoxytrichothecene mycotoxins produced by various species of *Fusarium*. These toxins are responsible for illness and death in animals and man from ingestion of moldy agricultural products (Bamburg and Strong, 1971; Hayes, 1980). Extensive studies have been done on the pathology, distribution and metabolism of T-2 mycotoxin in various species of animals (Bunner et al.; Corley et al., 1985; Matsumoto et al., 1978; Pace et al., 1985; Robinson et al., 1979; Yoshizawa, 1982a). Numerous *in vitro* studies at the cellular and subcellular level have shown that T-2 mycotoxin is a potent protein synthesis inhibitor (Cundliffe and Davies, 1977; McLaughlin et al., 1977; Smith et al., 1975). However, in view of the rapid metabolism and clearance of the toxin, we questioned whether this same level of inhibition takes place in the tissues of the living animal. Other discrepancies also exist. The wide range of levels of *in vitro* protein synthesis inhibition caused by the various trichothecene mycotoxins does not correlate with their narrow

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range of lethality in animals (Sato and Ueno, 1977; Thompson and Wannemacher, 1986). Only a few limited studies of the effects of trichothecenes on *in vivo* protein synthesis have been done. These involved either sub-lethal doses of toxin at 8 and 24 hr (Rosenstein and Lafarge-Frayssenet, 1983), a single lethal dose studied during the terminal stage of intoxication (Robbana-Barnat et al., 1987), or feeding studies in which no significant effect on protein synthesis was observed (Suneja et al., 1983).

We studied the early time-course effects of various doses of T-2 mycotoxin, the metabolite, T-2 tetraol, and known protein synthesis inhibitor, cycloheximide, on *in vivo* protein and DNA synthesis in six different rat tissues and serum to see if a response similar to that seen *in vitro* could be demonstrated in whole animals.

MATERIALS AND METHODS

Animals. Male, Fisher-Dunning rats (F/344, FCRF, Frederick, MD) weighing 150-200 g were housed in a room maintained at 25-26°C and lighted from 0600 to 1800 hr. Sixteen hours prior to use, food was removed from the animals' cages.

Test agents. T-2 mycotoxin and T-2 tetraol (Myco Labs, Inc., Chesterfield, MO) were dissolved to make a concentration of 50 mg/ml in ethanol. The toxins were then diluted in ethanol: water:glycerol at a ratio of 2:5:3, respectively, to deliver an

ip dose in 0.1 ml of: for T-2 toxin, 2.0 mg/kg body weight (lethal dose) 0.75 mg/kg bw (sub-lethal dose), and 0.3 mg/kg bw (low dose); and for tetraol, 5.0 mg/kg bw (lethal dose), and 3.0 mg/kg bw (sub-lethal dose). Doses were based on an average weight of the rats used in the study. Cycloheximide (Sigma Chemical Company, St. Louis, MO) was made up similarly and diluted to deliver 10 mg/kg bw (lethal dose), in 0.1 ml to the rats. Control rats for all experiments were given 0.1 ml of the ethanol:water:glycerol mixture only.

Experimental procedure. For each experiment, 12 treated and 9 control animals were injected. At 1, 4 and 7 hr after T-2, tetraol, or cycloheximide injection, groups of three control and three treated rats were given an ip injection of 0.1 ml solution containing 10 μ Ci [14 C]leucine and 50 μ Ci [3 H]thymidine. Two hr later, the rats were anesthetized by CO₂ and killed by cervical dislocation. The posterior vena cava anterior to the diaphragm was severed, an aliquot of blood removed, and livers and kidneys quickly perfused *in situ* via the aorta with cold physiological saline. Liver, kidney, spleen, intestine, heart and muscle were removed and frozen in liquid nitrogen. Ten μ l aliquots of serum from the blood samples was added to Aquasol II (New England Nuclear, Boston, MA) and total circulating levels of [14 C]leucine and [3 H]thymidine determined with a liquid scintillation counter (Beckman LS5800) and a dual-label counting program for [3 H] and [14 C]. An additional 0.1 ml of serum was precipitated with 10.0 μ l of 2.0 N perchloric acid (PCA), centrifuged at 1300 x g for 5

min, and 10 μ l of the supernatant counted as above to determine circulating levels of unbound radiolabeled leucine and thymidine.

The three additional treated rats were observed for course of illness and time to death.

Sample processing. Tissues were thawed, weighed, and all but muscle, homogenized in 0.25M sucrose + 50mM hepes (pH 7.6), 75mM KCl and 5mM MgCl₂ (SHKM), to a final volume of 10 ml by using a motor-driven teflon pestle fitted to a Potter-Elvehjem homogenizer (Fisher Scientific, Columbia, MD). Muscle was homogenized in the same volume of SHKM with a Polytron homogenizer (Brinkman Instruments, Westbury, NY). All samples were kept on ice during the processing procedure. Samples were precipitated by adding 1 ml of 2.0N perchloric acid (PCA). They were centrifuged and the pellets washed twice with 0.2N PCA. Centrifugation speed for each tissue was variable (350 to 850 x g), based on sufficient g-force to recover precipitated protein quantitatively and still allow uniform resuspension of the pellets. Initial supernatants from each sample were saved and radioactivity of an aliquot determined by dual-label scintillation counting for tissue-pool levels of [³H]thymidine and [¹⁴C]leucine. After washing, the pellets were resuspended with a vortex mixer, 2 ml of 1N potassium hydroxide added and the samples hydrolyzed 1 hr in a water bath at 37°C. Fifty μ l of each sample was added to 4 ml of aquasol II and counted by liquid scintillation with a dual-label program.

Calculations and statistical analysis. Incorporation of both [³H]thymidine into DNA and [¹⁴C]leucine into protein and pool sizes in the tissue homogenates was determined. Initially, the protein content of each homogenate was measured (Lowry et al., 1951) in order to express the results in terms of dpm per g of tissue protein. However, there was no difference between the protein content of the control and of the toxin-treated animals during the course of the experiment, and results were just as accurately and more easily expressed in terms of tissue weight. Serum levels of labeled thymidine and leucine are expressed in terms of dpm per ml serum. Results from these studies represent the combination of two separate experiments (n=6 for each sample). Comparisons between control and toxin-treated animals in each tissue or serum sample, for each time period, were made by using analysis of variance with Stukey's studentized range test.

RESULTS

To establish baseline levels of protein and DNA synthesis in the various tissues, the combined levels of ¹⁴C-leucine incorporated into protein and ³H-thymidine incorporated into DNA per mg total protein from each tissue of the control animals tested in these studies was determined (Fig. 1). A significant, and consistent degree of variability existed in the relative rates of ¹⁴C-leucine and ³H-thymidine incorporation in the tissue of normal rats. Muscle and heart had the lowest level of leucine

incorporation (280 and 580 dpm per mg protein, respectively). Spleen, intestine, and kidney had intermediate levels, while liver had the highest level of protein synthesis (3600 dpm per mg protein), over 10 times that of the muscle. As would be expected, DNA synthesis levels were highest in the spleen and intestine (9600 and 11,500 dpm per mg protein). The other four tissues incorporated a 3- to 10-fold lower level of thymidine per mg protein. Because of the wide range of protein and DNA synthesis levels in the tissues studied, the results from these studies were plotted as a percent of their control level dpm per g tissue for each tissue, at each time period (Figs. 2-5). By presenting the data this way, we could compare the response to T-2, tetraol and cycloheximide in the various tissues on the same graph.

Clinical signs of T-2, tetraol and cycloheximide intoxication were similar. After a lethal dose, the rats were slightly lethargic at 3 hr and became increasingly immobile, showing signs of diarrhea by 6 hr. By 9 hr they were markedly ill, with reddened extremities and severe diarrhea; they appeared near death. Death occurred between 14 and 20 hr. With a sub-lethal dose, the most severe signs were similar to those seen at 6 hr after a lethal dose, while the low-dose rats exhibited only lethargy. All of the rats given less than a lethal dose of toxin appeared normal by 24 to 48 hr after exposure.

Protein synthesis levels were less than 25% of controls in all six tissues of rats 3 hr after exposure to a lethal dose of T-2 mycotoxin (Fig. 2A). Although there was some variability by 9 hr, the levels of protein synthesis remained significantly

inhibited, less than 50% of control levels in all the tissues tested. When given a sub-lethal (0.75 mg/kg, Fig. 2B) or lower dose (0.3 mg/kg, Fig. 2C) of T-2 mycotoxin, rats showed the same initial suppression of protein synthesis. This was followed by a general pattern of recovery in most of the tissues by 9 hr. Muscle and heart were the slowest to recover, still having significantly reduced protein synthesis levels at 9 hr. Kidney and intestine returned to above control levels by this time. A lethal dose of tetraol (5 mg/kg bw), which was approximately twice the lethal dose of T-2, had the same effect on protein synthesis as did the parent compound (Fig. 3A). Although still significant, protein synthesis in muscle and heart was not inhibited as much as in the other tissues. Recovery of protein synthesis from a sub-lethal dose of tetraol was also slowest in these two tissues (Fig. 3B), as was the case with T-2, while kidney and liver rebounded to above control levels by 9 hr. Rats given a lethal dose of cycloheximide also followed a very similar pattern of protein synthesis inhibition as rats treated with T-2 (Fig. 4A). Inhibition was greater than 70% of controls by 3 hr and remained significantly suppressed through 9 hr.

We observed a strong tendency toward overall inhibition of DNA synthesis in all tissues after T-2 treatment (high dose, Fig. 5A). However, there was more variability in DNA synthesis inhibition than inhibition of protein synthesis. Spleen and intestine, the two tissues with the highest inherent levels of DNA synthesis, showed the highest degree of inhibition at all three times. The pattern of recovery of DNA synthesis with lower

doses of T-2 mycotoxin (Fig 5B, sub-lethal;5C, low) was similar to that seen in protein synthesis with lower doses of the toxin. After an initial drop, complete recovery of DNA synthesis took place in the low-dose animals by 9 hr in all but spleen and intestine. After a lethal dose of cycloheximide, DNA synthesis was also inhibited in all but muscle (Fig. 4B). Muscle showed the most variability with both T-2 and cycloheximide treatment. This was probably due to the normally low levels of DNA synthesis in this tissue. As with T-2 mycotoxin treatment, spleen and intestine DNA synthesis was affected most by cycloheximide treatment, particularly at the later times.

A similar response in circulating levels of labeled leucine was seen in T-2 mycotoxin- and cycloheximide-treated animals (Fig. 5). The PCA-soluble radioactivity, representing the fraction of circulating ^{14}C -leucine which was not incorporated into protein, was 15-20% of the total serum levels in control animals. In toxin-treated animals, the PCA-soluble radioactivity was approximately twice that of control animals, but represented 90% or more of the total serum levels. This indicates that very little of the labeled leucine was being translated into proteins and excreted back into the serum. No significant change in circulating levels of labeled thymidine was seen between control and T-2- or cycloheximide-treated rats (data not shown).

To determine whether T-2 mycotoxin had an effect on pool size of labeled amino acids and DNA precursors in various tissues, the radioactivity of the PCA-soluble fraction from each tissue homogenate was determined. In all tissues except intes-

tine, pools of [^{14}C]leucine were significantly elevated after exposure to a lethal dose of T-2, when compared to control levels in the same tissue (Fig. 7). By 9 hr after toxin exposure, pools of labeled leucine had increased from over 2-fold in liver to a maximum of more than 8-fold in heart. The absence of increased pool size in the intestine may be due to the rapid turnover of cells in this tissue. A modest increase with scattered significance occurred in [^3H]thymidine pool levels in all of the tissues during the course of the experiment. Pool levels of [^3H]thymidine were significantly elevated above control in muscle, kidney and spleen.

DISCUSSION

Many *in vitro* studies have been done on the effects of T-2 mycotoxin in various systems in an effort to determine its mechanism of action. The general conclusion from these studies is that T-2 blocks cellular protein synthesis, by binding to the 80S subunit on the ribosome (Cannon et al., 1976) and interferes with peptidyl transferase activity at the transcription site (Wei and McLaughlin, 1974; Tate and Caskey, 1973). However, no complete studies have been done to try to relate *in vitro* results with whole-animal studies, which show a broad range of responses resulting in death of the animal.

These studies conclusively show that exposure of rats to T-2 mycotoxin results in rapid and extensive suppression of protein synthesis in a wide range of tissues. With a lethal dose of the toxin, protein synthesis remained suppressed through 9 hr after

exposure; death occurred after 14 hr. With lower doses, recovery of protein synthesis took place in the tissues of the rats which would survive. These results were supported by the lack of [¹⁴C]leucine incorporation into circulating proteins in the serum of toxin-treated rats and an increase in pools of labeled protein precursors in the various tissues after toxin exposure.

The response to T-2 mycotoxin was closely paralleled by that to another protein synthesis inhibitor, cycloheximide. Although cycloheximide inhibits protein synthesis by a different mechanism, the results, with respect to overall protein synthesis, DNA synthesis, serum effects, and time to death, were the same for both agents. Other toxins that have a well-defined mechanism of action involving only protein synthesis inhibition, such as diphtheria toxin (Middlebrook and Dorland, 1984; Eidels, et al., 1983), result in death with similar clinical signs.

In-vitro DNA synthesis inhibition by T-2 has been demonstrated (Oldham et al., 1980; Thompson and Wannemacher, 1984) but evidence exists that it is probably a secondary response to protein synthesis inhibition (Munsch and Muller, 1980; Cundliffe et al., 1974). Support for this conclusion was found in the reduced and more-delayed response in DNA synthesis inhibition as compared to protein synthesis inhibition in these *in vivo* studies.

Studies of the pathophysiology of T-2 intoxication in animals have shown widespread tissue and organ injury including haematological, hepatic, renal, pancreatic, muscular and cardiac effects (Bunner et al., 1985). The widespread effects of the

toxin parallels the generalized protein synthesis inhibition response seen in the tissues tested, and the response that might be expected from such an extensive interruption of this vital cellular function. More pronounced responses in tissues having a rapid turnover, resulting in effects such as immunosuppression in animals exposed to high doses of T-2, have been observed (Holt et al., 1988; Lafarge-Frayssinet et al., 1979; Tomar et al., 1988). These observations fit well with *in vitro* protein synthesis inhibition seen in lymphocytes exposed to T-2 (Thompson et al., 1986) and the general pattern from these studies of the most pronounced inhibition in tissues having the highest inherent levels of protein and DNA synthesis. Dyck et al. (1985) have shown that exposure of mice to T-2 blocks the acute-phase response, a phenomenon that results in release of mediators or acute phase reactants that contribute greatly towards the host defense against tissue damage. The authors concluded that this suppression is probably due to protein synthesis inhibition blocking the production of mediators or acute phase reactants.

Numerous *in vivo* studies have shown that T-2 is rapidly converted into more-polar, less-toxic metabolites, conjugated, and cleared by a many animal species. Metabolism and clearance may explain the discrepancy between the wide range of toxicities of T-2 and its derivatives in cell culture, shown by protein synthesis inhibition (Thompson et al., 1986), and the relatively narrow range of toxicity *in vivo*. The 2.5 log difference in *in vitro* protein synthesis inhibition levels of T-2 and tetraol, as compared to 2-fold difference seen in whole animals, is

reasonable when one considers that even during early intoxication, much of the injected T-2 is converted to less-toxic metabolites, conjugated, and/or cleared (Pace et al., 1985; Yoshizawa et al., 1980b), greatly reducing its toxicity. The fact that the ratio of protein synthesis inhibition levels of T-2 to tetraol in the rat are the same as the ratio of their lethality levels provides support for these observations.

The time course of illness in response to T-2, relative to protein synthesis inhibition, is of interest. Although protein synthesis inhibition was at a maximum at 3 hr (the earliest time measured), signs of illness were only beginning; the animals became progressively more ill until deaths began to occur around 14 hr. This time lapse shows that death in the animals was not a direct result of the early, prolonged, and widespread inhibition of protein synthesis. This conclusion is supported by the observation that T-2-intoxicated animals appear to die of a toxin-induced shock syndrome (Lorenzana et al., 1985; Bunner et al., 1985). Thus, we hypothesize that the early suppression of protein synthesis, especially in rapidly dividing tissues, results in extensive cellular damage. In the process of removing cellular debris, a cascade of events is set in place which results in shock and eventual death. We can not rule out all direct effects of T-2 as a cause of death, but it would be extremely difficult to further elucidate the mechanism of action without removal of the extensive protein synthesis inhibitory effects of this toxin.

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LEGENDS TO FIGURES

FIG. 1. Normal levels of protein synthesis (A) and DNA synthesis (B) in the six tissues used in these studies. Mean values \pm standard error bars are shown (number of independent measurements for each tissue = 53 to 62).







FIG. 2. Protein synthesis levels for each of the six tissues after 3, 6, and 9 hr exposure to 2.0 mg/kg T-2 mycotoxin (A), 0.75 mg/kg T-2 mycotoxin (B), and 0.3 mg/kg T-2 mycotoxin (C). Values are plotted as % of control level dpm [^{14}C]leucine/g tissue for each tissue at the same time period. Muscle  , Heart  , Kidney  , Liver  , Spleen  , Intestine  . (* = $p < 0.01$, + = $p < 0.05$, as compared to controls).







FIG. 3. Protein synthesis levels for each of the six tissues after 3, 6, and 9 hr exposure to 5.0 mg/kg T-2 tetraol (A), and 3.0 mg/kg T-2 tetraol (B). Values are plotted as % of control level dpm [^{14}C]leucine/g tissue for each tissue at the same time period. Muscle  , Heart  , Kidney  , Liver  , Spleen  , Intestine  . (* = $p < 0.01$, + = $p < 0.05$, as compared to controls).

FIG. 4. Protein (A) and DNA (B) synthesis levels for each of the six tissues after 3, 6, and 9 hr exposure to 10.0 mg/kg cycloheximide. Values are plotted as % of control level dpm







[¹⁴C]leucine/g tissue (protein synthesis) or dpm [³H]thymidine/g tissue (DNA synthesis) for each tissue at the same time period. Muscle  , Heart  , Kidney  , Liver  , Spleen  , Intestine . (* = p < 0.01, + = p < 0.05, as compared to controls).







FIG. 5. DNA synthesis levels for each of the six tissues at 3, 6, and 9 hr exposure to 2 mg/kg T-2 mycotoxin (A), 0.75 mg/kg T-2 mycotoxin (B), 0.3 mg/kg T-2 mycotoxin (C). Values are plotted as % of control level dpm [³H]thymidine/g tissue for each tissue at the same time. Muscle  , Heart  , Kidney  , Liver  , Spleen  , Intestine . (* = p < 0.01, + = p < 0.05, as compared to controls).











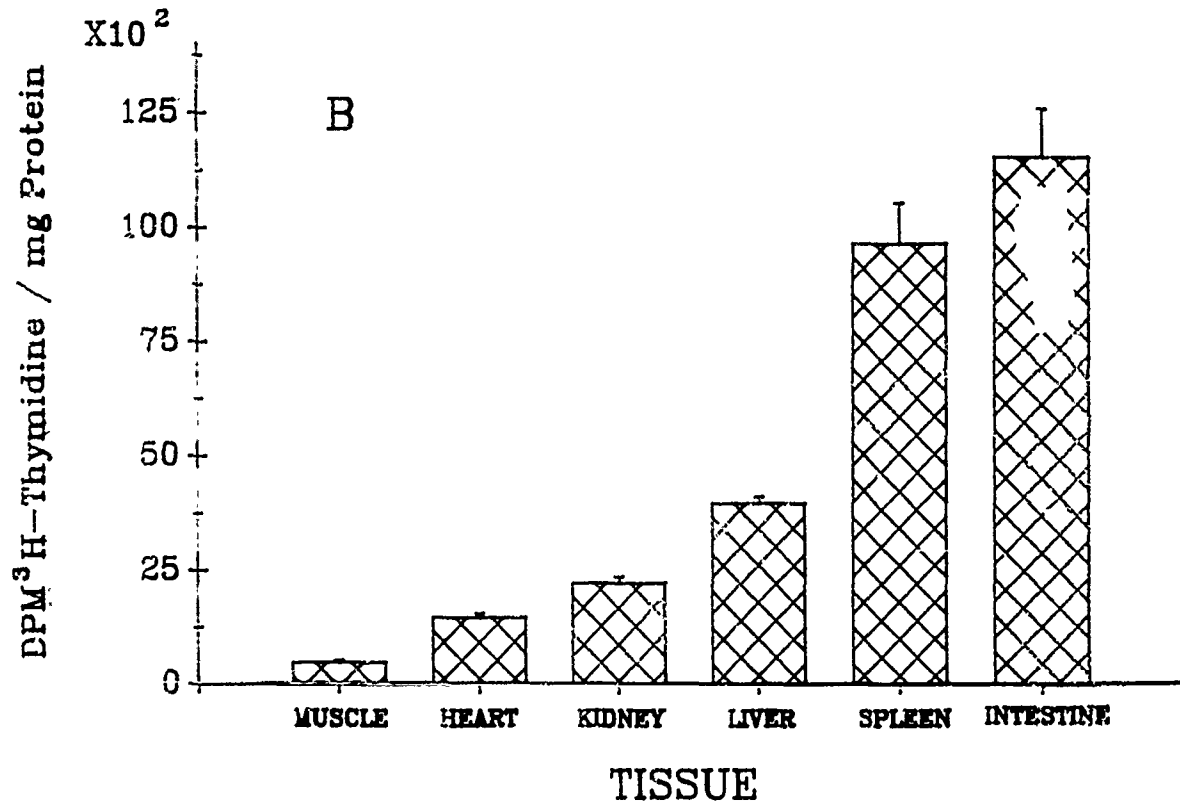
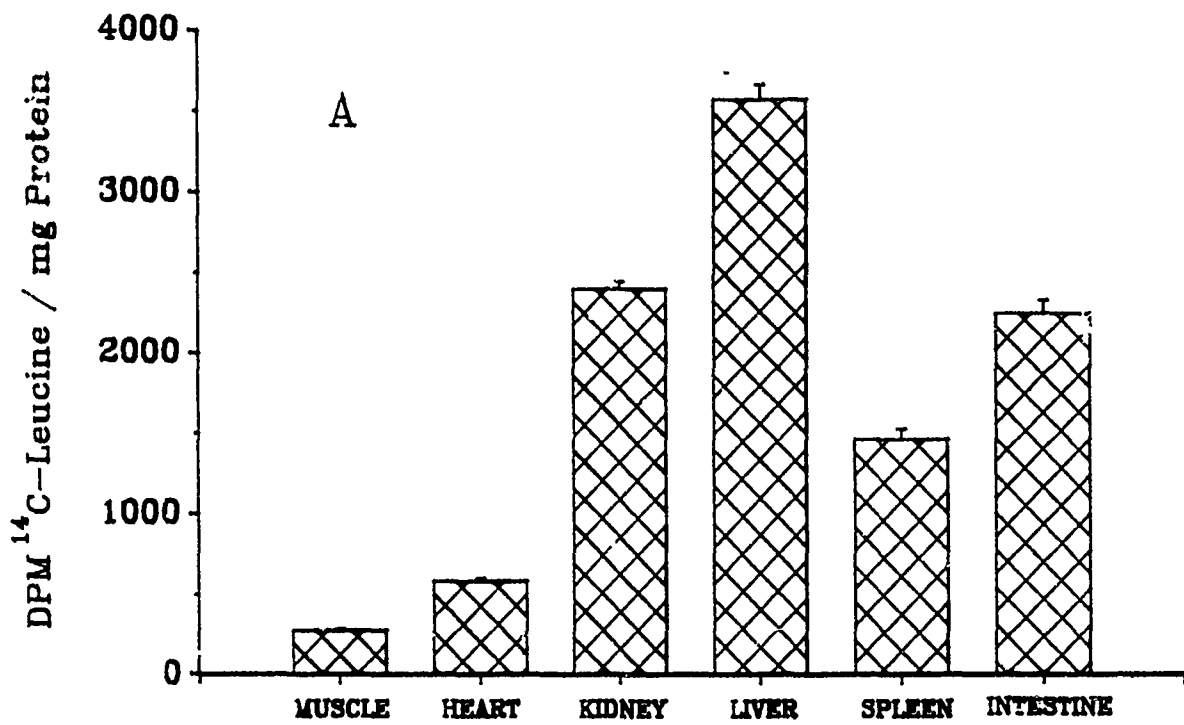
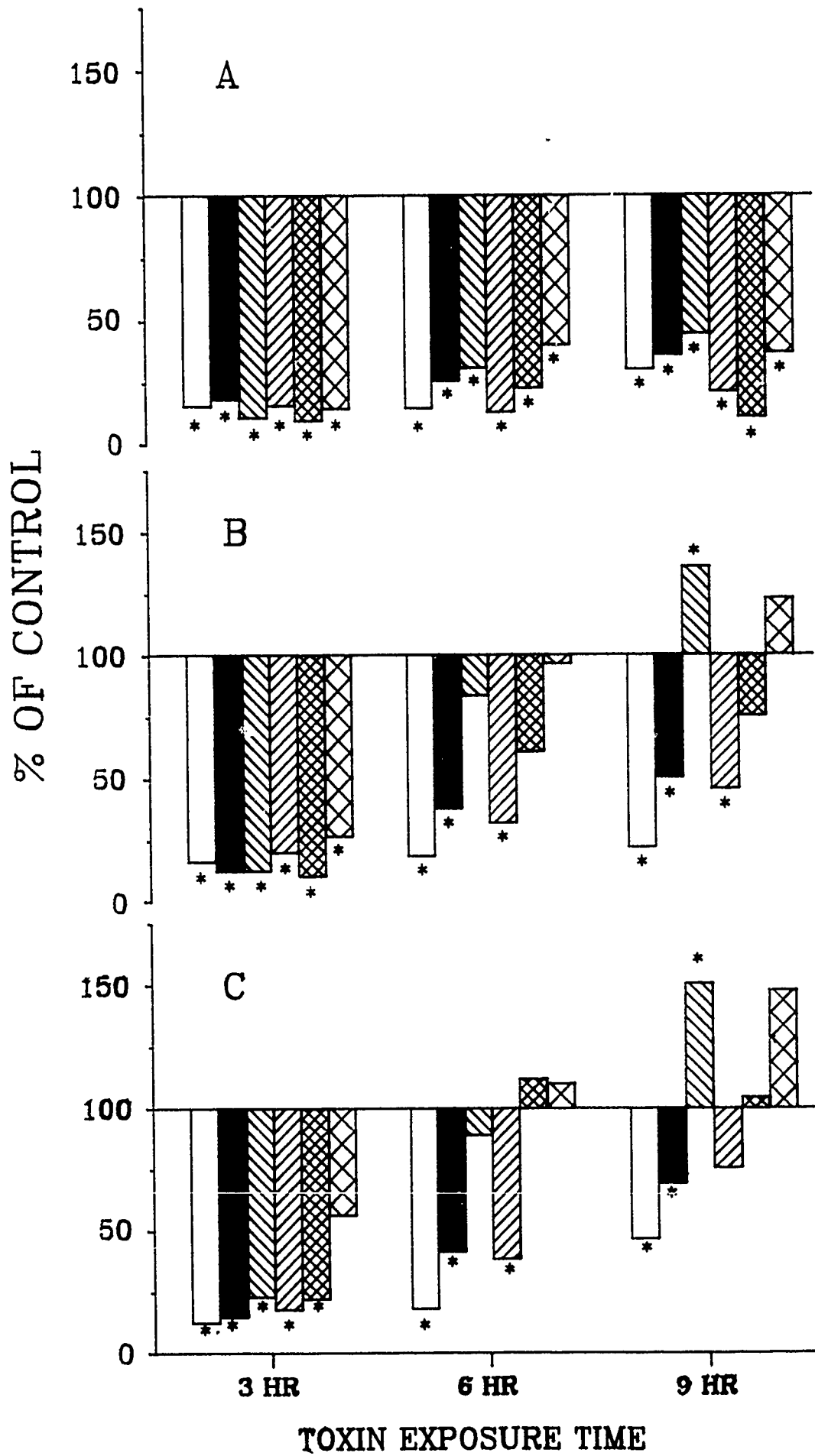
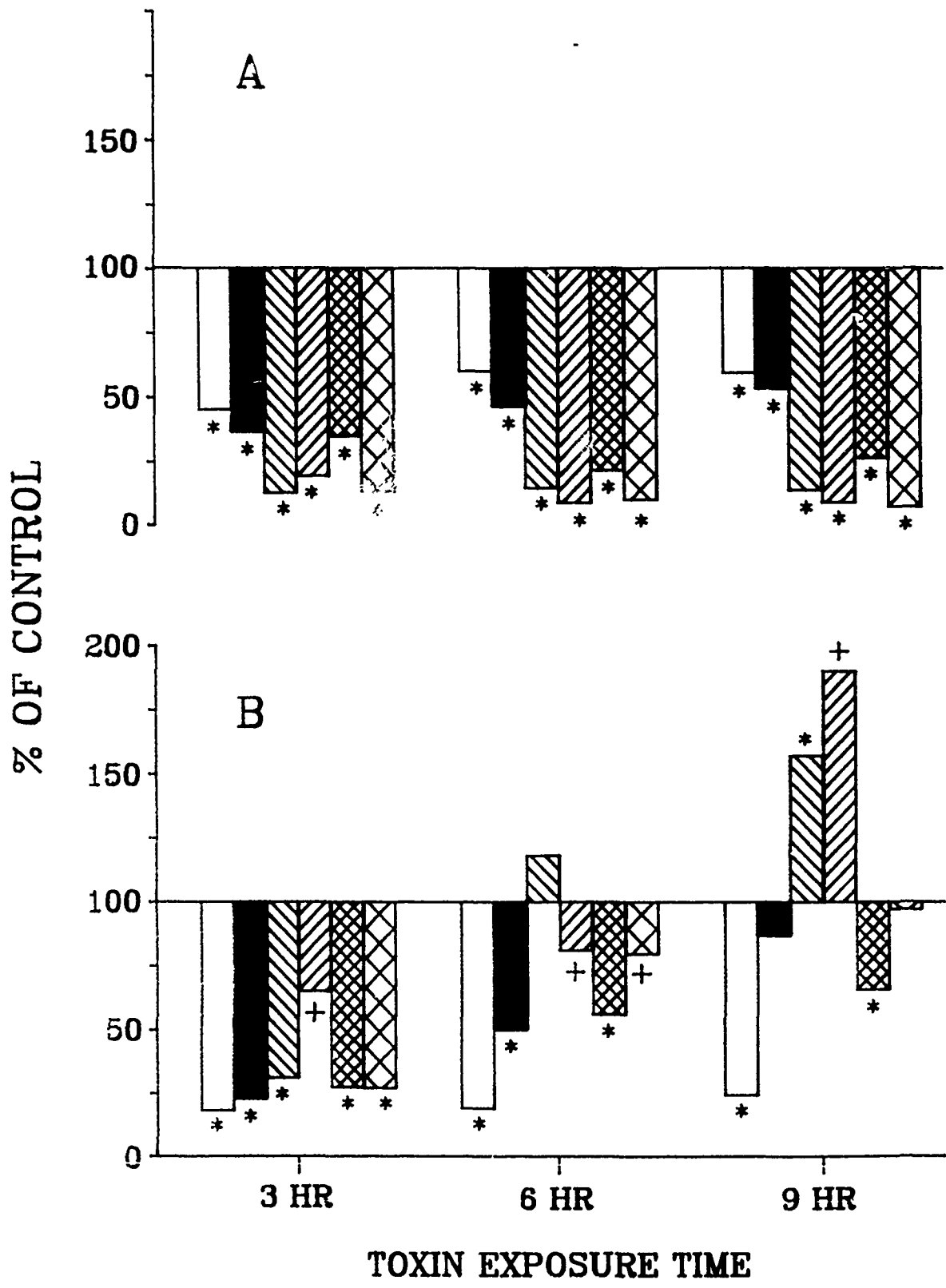
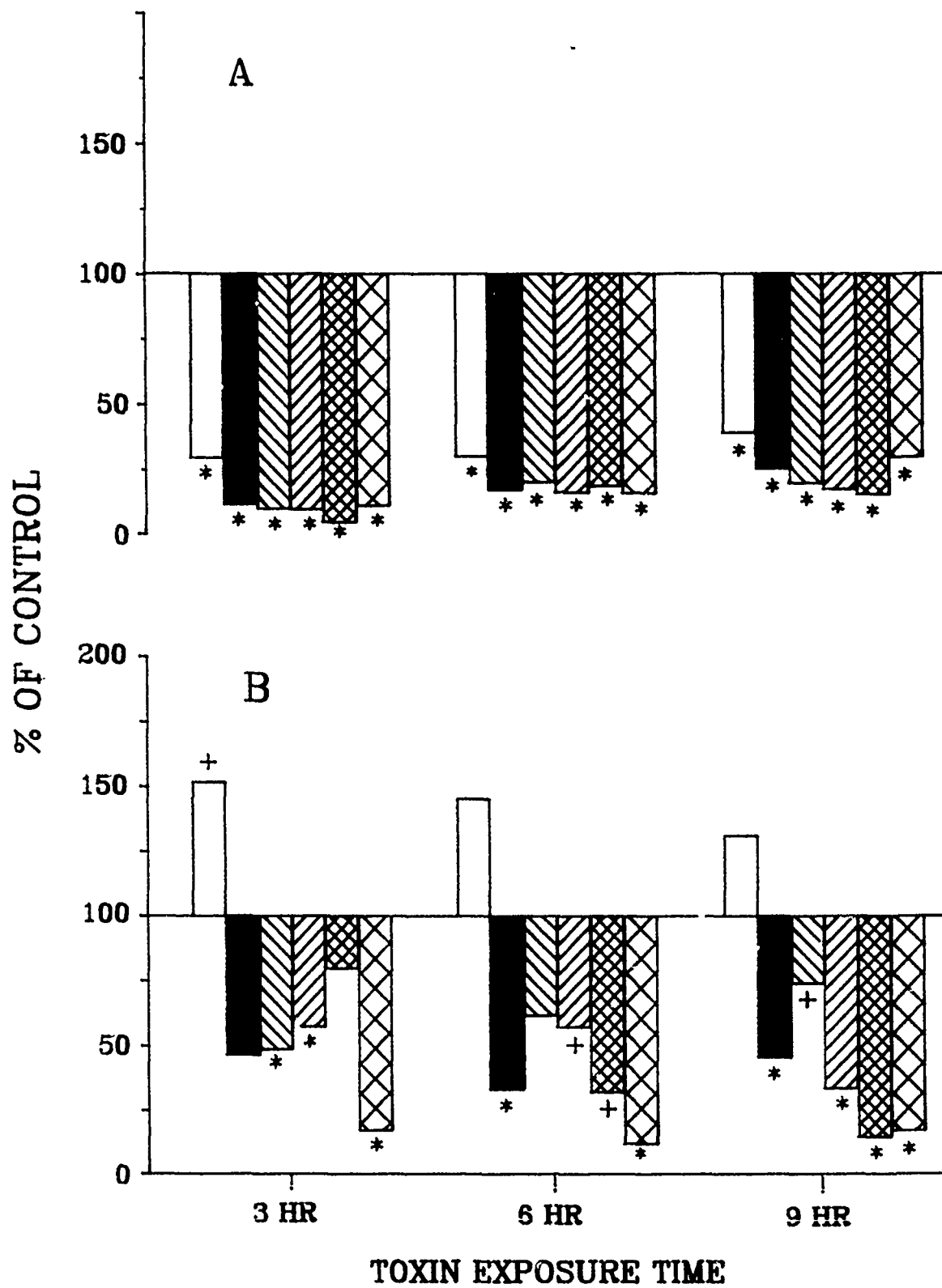
FIG. 6. Serum levels of radiolabeled leucine after a 3-hr exposure to 2 mg/kg T-2 mycotoxin (left) and 10 mg/kg cycloheximide (right). Control rats, total serum  ; treated rats, total serum  ; control rats, PCA-soluble fraction  ; treated rats, PCA-soluble fraction .

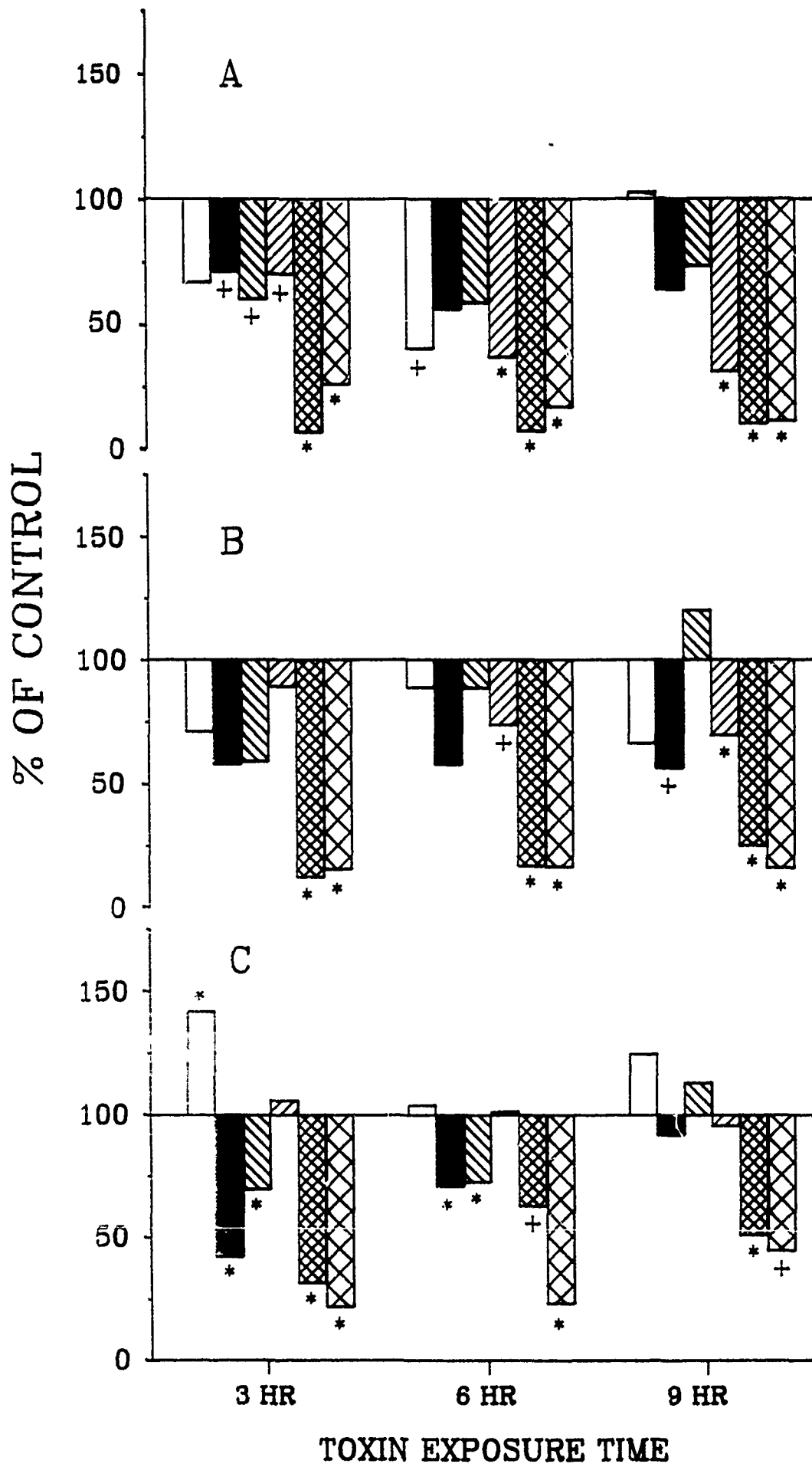
FIG. 7. Percent of control of labeled leucine (A) and labeled thymidine (B) in the PCA-soluble fraction of the six tissues studied after a 9-hr exposure to 2 mg/kg T-2 mycotoxin. Muscle  , Heart  , Kidney  , Liver  , Spleen  , Intestine . (** = p < 0.01, * = p < 0.05, as compared to controls).

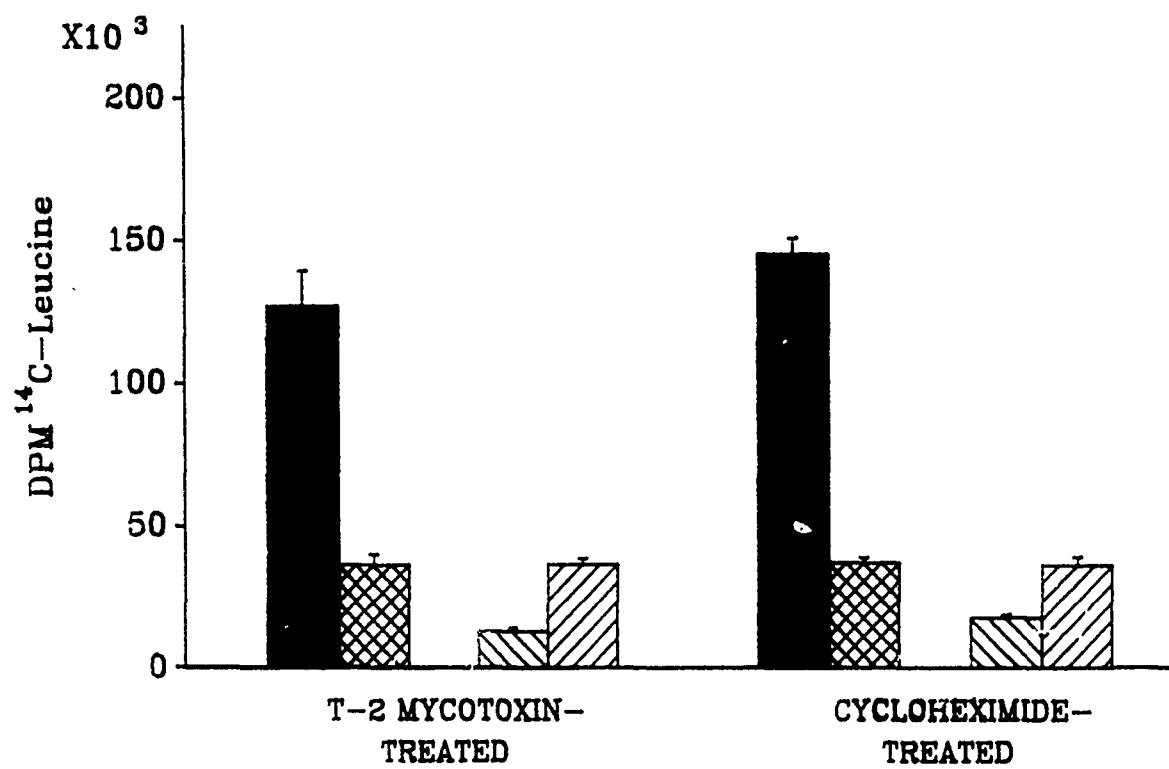


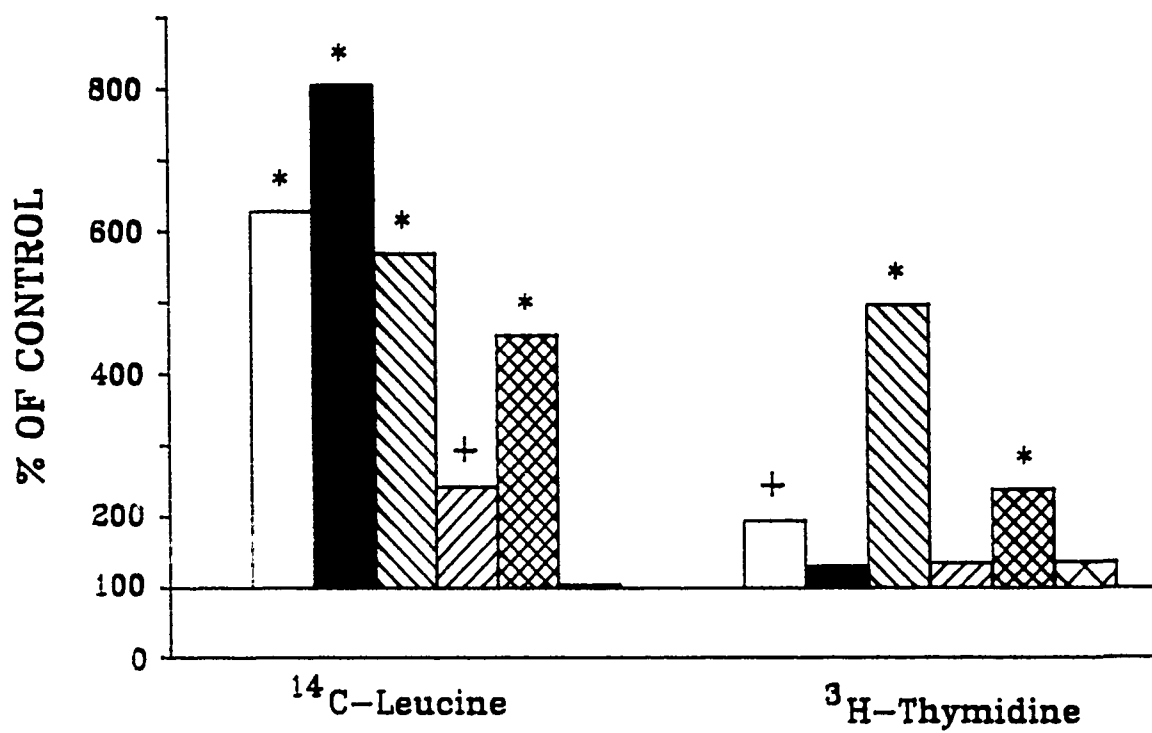












REPORT DOCUMENTATION PAGE

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19. ABSTRACT (Continue on reverse if necessary and identify by block number) <p><i>In Vivo Effects of T-2 Mycotoxin on Protein and DNA Synthesis in Rat Tissues.</i> Thompson, W. L. and Wannemacher, R. W., Jr. (). <i>Toxicol. Appl. Pharmacol.</i> __, __-__. Rats were given an ip injection of T-2 mycotoxin (T-2); the T-2 metabolite, T-2 tetraol (tetraol); or cycloheximide. Serum, liver, heart, kidney, spleen, muscle, and intestine were collected at 3, 6 and 9 hr post injection after a 2-hr pulse at each time with ¹⁴C-leucine and ³H-thymidine. Protein and DNA synthesis levels in rats were determined by dual-label counting of the acid-precipitable fraction of tissue homogenates. With a lethal dose of T-2, tetraol, or cycloheximide, maximum levels of protein synthesis inhibition were observed in all the tissues at the earliest time period and continued for the duration of the study (9 hr). Additional rats given the same dose of any of the three treatments died between 14 and 20 hr. With sub-lethal doses of T-2 or tetraol, the same early decrease in protein synthesis was observed, but recovery in most of the tissues was seen with time. DNA</p> <p style="text-align: right;">(Cont.)</p>				
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synthesis in the T-2-treated rats was also suppressed, although to a lesser degree, in the six tissues studied. With sub-lethal doses, complete recovery of DNA synthesis took place in four of the six tissues by 9 hr after toxin exposure. The appearance of newly translated serum proteins did not occur in the animals treated with T-2 mycotoxin or cycloheximide, as evidenced by total and PCA-soluble serum levels of labeled leucine. An increase in tissue-pool levels of free leucine and thymidine in response to T-2 mycotoxin was also noted. T-2 mycotoxin caused a very rapid inhibition of protein and DNA synthesis in all of the tissues studied. Its effects in whole animals were the same as those of the T-2 metabolite, tetraol, and a known protein synthesis inhibitor, cycloheximide. *Keywords:*

protein synthesis; DNA Synthesis, (KT) ←