*Biochemical Pharmacology*, Vol. 38, No. 18, pp. 3093–3102, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00
Pergamon Press plc

# SPECIFIC ASSOCIATION OF T-2 TOXIN WITH MAMMALIAN CELLS

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(*Received* 24 *May* 1988; *accepted* 25 *January* 1989)

**Abstract**—The binding of radiolabeled T-2 toxin to a mammalian cell line derived from a Chinese hamster ovary (CHO) was studied. The toxin bound to, or was taken up by, cells in a time-, temperature- and concentration-dependent manner. The binding was saturable, of high affinity ($K_d \sim 0.1$ to 1 nM), reversible at 37° (half-time ~2 hr), and specific. The kinetics of T-2–cell association and the rate of toxin-induced inhibition of protein synthesis closely paralleled one another. Likewise, the concentration–response for inhibition of protein synthesis and the toxin binding isotherm were similar. A synthetically derived epimer of T-2 bound less tightly to cells, but apparently to the same site as authentic T-2. The epimer was also less potent at inducing inhibition of protein synthesis. Two other trichothecene toxins, one more and one less toxic than T-2, blocked labeled T-2 binding to cells in a manner reflective of their protein synthesis inhibitory potencies. We conclude that the binding we defined is an accurate measure of the toxin responsible for inhibition of protein synthesis in CHO cells. The data also suggested that, at equilibrium, the interaction of T-2 with cells is not static, but is the sum of a continuous uptake and release process.

Trichothecenes are a group of structurally related sesquiterpenoids produced by several species of fungi. Depending on climate and storage conditions, growth of toxin-producing fungi on foodstuffs and fodder can present serious health problems for humans and livestock. Trichothecenes are demonstrably lethal to many animal species and have been implicated as the causative agents in human disease and death. Although the pathogenesis of toxicity in animals is complex and not well defined, it is clear that trichothecenes are potent inhibitors of eukaryotic protein synthesis [1]. Work from a number of laboratories has shown that trichothecenes block protein synthesis by binding to the 60S subunit of the eukaryotic ribosome [2, 3]. Although these toxins apparently bind to a common site [3, 4], mechanistically some appear to inhibit initiation whereas others block elongation or termination. Details on the molecular events involved in toxin–ribosome binding are sketchy, while information on other possible toxin–cell interactions, such as binding to a receptor, intracellular transport, binding to other organelles and excretion, is virtually nonexistent. In an attempt to fill this information gap, we have undertaken an in-depth study of the cellular and subcellular interactions of T-2 toxin.

T-2 is a trichothecene toxin produced by *Fusarium sporotrichioides*. *Fusarium tricinctum* and several other *Fusarium* species. T-2 inhibits protein synthesis at the level of initiation [1] and is toxic to yeast [5], HeLa [6], hepatocyte [7], fibroblast [8] or lymphocyte [9] cells. We have investigated the interactions of T-2 with a cultured cell line derived from a Chinese hamster ovary (CHO). This line appeared to be as sensitive to T-2 as are HeLa cells or reticulocytes. Using radiolabeled T-2, we examined association of the toxin with CHO cells as a function of time, temperature and concentration. T-2 cell association was specific, saturable, and of high affinity. The results of several other experiments suggested that the binding we studied is an accurate measure of that toxin responsible for inhibition of protein synthesis. However, comparison with results of similar binding experiments with isolated ribosomes [10] suggests that most cell-associated T-2 is bound intrecellularly to ribosomes.

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## MATERIALS AND METHODS

*Cells and cell culture*. Seed stock for the CHO (K1 subclone) line was obtained from the American Type Culture Collection (No. CCL-61). Cells were maintained in 75-cm$^2$ T-flasks (Costar No. 3075) with Earle's Minimal Essential Medium, 10% fetal bovine serum, and 50 μg/ml gentamycin.

*Media and sera*. All media, vitamins, antibiotics and amino acids were obtained from the Grand Island Biological Co. (Grand Island, NY). Fetal calf serum was obtained from Armour Pharmaceutical (Kankakee, IL). The serum was heat-inactivated for 30 min at 56° before use.

*Toxins*. T-2 was purchased from Calbiochem (La Jolla, CA), while the other trichothecene toxins were obtained from the Sigma Chemical Co. (St Louis, MO). T-2 was tritium labeled by New England Nuclear (Boston, MA) or Amersham/Searle (Arlington Heights, IL) using a previously published procedure [11]. The toxin preparations had specific activities from 9.0 to 14.0 Ci/mmol and were equipotent to unlabeled T-2 in a protein synthesis inhibition assay. All toxins were prepared in methanol and diluted so that the maximal alcohol concentration exposure to cells was 0.1%.

*Toxin–cell association assay*. Cells were seeded in

24-well tissue culture plates. On the day of experimentation (1–4 × $10^5$ cells/well), the growth medium was replaced with 0.5 ml Hanks' 199 supplemented with 10% fetal calf serum, 50 μg/ml gentamycin and 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) pH 7.4 (complete H-199). Further incubations and manipulations at 37° were carried out on top of a microscope slide warmer in a warm-box (both equilibrated to 37°). This arrangement allowed much better temperature control than did a standard $CO_2$ incubator. Radiolabeled T-2 was added to the cells in 50 μl volumes, and incubation was carried out under the conditions and for the times stipulated. To determine cell-associated toxin, cells were rinsed three times with Hanks' Balanced Salt Solution (HBSS) and solubilized in 1.0 ml of 0.1 M NaOH. A 0.5-ml aliquot was added to a scintillation vial, neutralized with 0.1 ml of 0.5 M HCl, 5 ml Aquasol (New England Nuclear) was added and the sample was counted in a liquid scintillation spectrophotometer.

*Protein synthesis assay.* Cells in complete H-199 were incubated with toxin for the times and under the conditions indicated. Protein synthesis was measured by the addition of 1 μCi/well of [$^3$H]leucine (New England Nuclear, 110–150 Ci/mmol) and incubation at 37°, usually for 30 min. The pulse was terminated by rinsing the cells twice with HBSS and adding 0.1 ml of 0.1 M NaOH. After 5–10 min at 37°, a prenumbered 11 mm disc (Schleicher & Schuell) was added to each well to absorb the solubilized cells. Each disc was then transferred to a bottle of 10% trichloroacetic acid, and the entire experiment was processed in mass as follows: two rinses with 5% trichloroacetic acid, two rinses with 50:50 ethanol: acetone, and one rinse with acetone. After drying, each disc was assayed for radioactivity in 2.0 ml Liquafluor:toluene (New England Nuclear).

## RESULTS

*Kinetics and concentration–response for toxin-induced inhibition of protein synthesis and toxin–cell association.* The kinetics of T-2-induced inhibition of CHO cell protein synthesis are shown in Fig. 1. At toxin concentrations of 0.05 to 0.1 μg/ml, protein synthesis inhibition was complete in ≤ 15 min. In the concentration range 0.005 to 0.05 μg/ml T-2, cellular protein synthesis decreased gradually and appeared to achieve a concentration-dependent, steady-state level rather than ultimately reaching zero. When plotted in a semi-logarithmic manner (data not shown), the initial rates of inactivation were linear, indicating apparent first-order kinetics.

The concentration–response relationship for T-2 inhibition of protein synthesis is shown in Fig. 2. Little or no toxic effect was seen below 0.002 μg/ml T-2. Between 0.002 and 0.01 μg/ml T-2, protein synthesis was increasingly inhibited; at concentrations above 0.02 μg/ml, inhibition of protein synthesis was complete. A striking feature of the concentration–response for T-2 inhibition of protein synthesis was the steep concentration dependence. For comparative purposes, the concentration–response relationship for cycloheximide inhibition of CHO cell protein synthesis is also shown in Fig. 2.

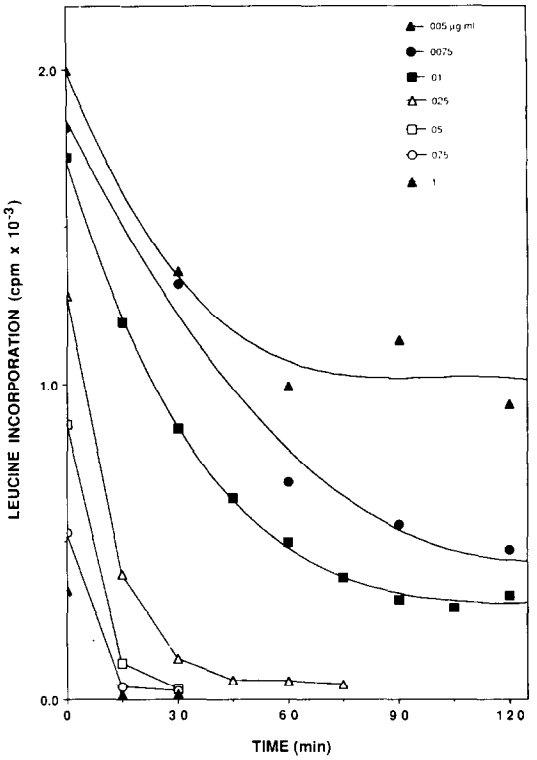


Fig. 1. Kinetics of T-2 inhibition of CHO cell protein synthesis. T-2 was added to CHO cells (37°) at the indicated concentrations (duplicates). After the time specified, 1 μCi of [$^3$H]leucine was added to each well and incubation continued for 15 min. Cells were then processed, as indicated in Materials and Methods, to measure leucine incorporation.

Note that an increase from 20 to 80% inhibition required a 3- to 4-fold increase in T-2 concentration, whereas a 75- to 100-fold increase in cycloheximide concentration was needed to bring about a similar inhibitory response.

The kinetics of radiolabeled T-2 association with CHO cells are shown in Fig. 3. The kinetics were studied using concentrations of labeled T-2 similar to those employed in the biological experiments depicted in Fig. 1. Earlier experiments (not shown) demonstrated that radiolabeled T-2 from both commercial sources was equipotent to unlabeled toxin at inhibiting protein synthesis. We observed both a time- and concentration-dependence in the association of T-2 with CHO cells. The association increased to an apparent plateau, which was maintained for the duration of the experiment. At the lower concentrations (Fig. 3A), both the initial rate and the final plateau level were proportional to the added toxin concentration. At higher concentrations (Fig. 3B), only the initial rates were concentration dependent. The plateau levels attained were approximately the same, suggesting a saturable process.

When a comparison was made between the degree of protein synthesis inhibition and the number of T-2 molecules associated with CHO cells, a direct correlation was observed (Fig. 4). The apparent level of saturation varied somewhat from experiment to experiment, but usually fell between 2 and 3 × $10^6$

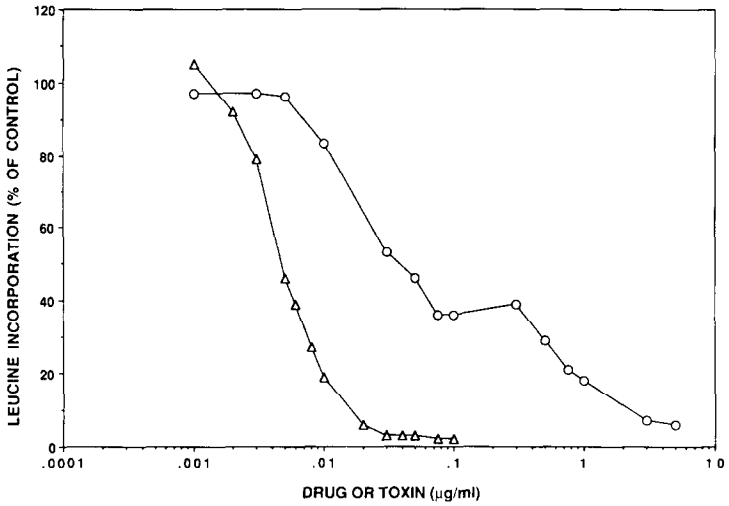


Fig. 2. Concentration–response of T-2 inhibition of CHO cell protein synthesis. T-2 (△) or cycloheximide (○) was added to CHO cells (37°) at the indicated concentrations (duplicates). After 2 hr at 37°, 1 μCi of [$^3$H]leucine was added to each well and incubation continued for 30 min. Cells were then processed, as indicated in Materials and Methods, to measure leucine incorporation. Controls on each plate were between 3000 and 3200 cpm.

T-2 molecules/cell. Both curves exhibit the very steep climb mentioned above, whereby a change in concentration of 2- to 3-fold resulted in an increase from a slight to a complete response (or binding). One subtle point which emerged from several experiments such as this was a threshold effect, where up to 600,000 T-2 molecules were cell-associated with no detectable inhibition of protein synthesis. This is evident in Fig. 4 at concentrations of $\leq 0.0075$ μg. In another part of this experiment, toxin-bound cells were washed, scraped off the culture plate, treated with trichloroacetic acid, and centrifuged. Essentially all (> 98%) of the T-2-derived radioisotope was found in the resulting supernatants fractions, indicating that [$^3$H]T-2 was not covalently bound to cellular proteins or nucleic acids (data not shown).

*Temperature dependence of T-2-CHO cell association and dissociation.* There was a marked difference in both the rate and overall pattern of T-2-CHO cell association at 4° compared to 37° (Fig. 5). In the cold, the association described a classical bimolecular reaction curve, increasing with time to a steady state at about 24–28 hr (Fig. 5). At 37°, T-2–CHO cell association was biphasic, rising to a plateau at 2–4 hr, followed by a more gradual, but continuous increase. Since the amount of labeled toxin employed for this experiment had no effect on protein synthesis, we believe the second, slower phase of binding simply reflects an increase in the number of cells during the course of the experiment and further binding of T-2 from the medium. This hypothesis is supported by the observations (i) that a second addition of [$^3$H]T-2 was followed by a rapid and approximate doubling of cell-associated toxin (Fig. 5, triangles) and (ii) that the slopes of the slow phases of T-2–cell association at 37° (triangles and circles, 6–12 hr) were the same as the cell growth rate.

In an attempt to understand the pattern of T-2–CHO cell association at 37°, we studied the dissociation kinetics. Cells were prebound with radiolabeled T-2 (3 hr), the monolayer was washed, and the level of cell-associated T-2 was determined at various time intervals. The amount of cell-associated toxin declined with time in an apparently exponential fashion. This suspicion was confirmed by a semilog plot (Fig. 6), from which we calculated an apparent first-order rate constant of 0.15 hr$^{-1}$, correlation coefficient 0.97 (half-time of 2.0 hr).

Another method we employed to assess T-2–cell dissociation was a "chase" experiment. Cells were preincubated with radiolabeled T-2 (3 hr), followed by addition of a 100-fold higher excess of unlabeled T-2; cell-associated radiolabeled toxin was assessed as above. The addition of a chase resulted in a decrease of prebound radiolabeled T-2 and a semilog plot of the data yielded a straight line (Fig. 6, correlation coefficient 0.96). A rate constant of 0.18 hr$^{-1}$ (half-time of 1.7 hr) was calculated, which is in excellent agreement with the value determined above. When similar experiments were performed at 4°, no dissociation was evident for up to 28 hr (data not shown).

*Biophysical parameters of T-2–CHO cell association.* Representative isotherms for T-2–CHO cell association at 4° and 37° are depicted in Fig. 7. Throughout the concentration range studied, the inclusion of a large excess of unlabeled T-2 substantially blocked the cell association of radiolabeled T-2, demonstrating a specificity to the interaction. At the very highest levels of toxin added, nonspecific binding was only 5–8%. Saturation was apparent at both temperatures, somewhere in the range of 0.03 to 0.1 μg/ml. Transforming the isotherm values by the method of Scatchard [12] produced data which fit well to a single, straight line at 4° (Fig. 7, inset). At 37°, the Scatchard transformation consistently produced data which fit a straight line in the higher

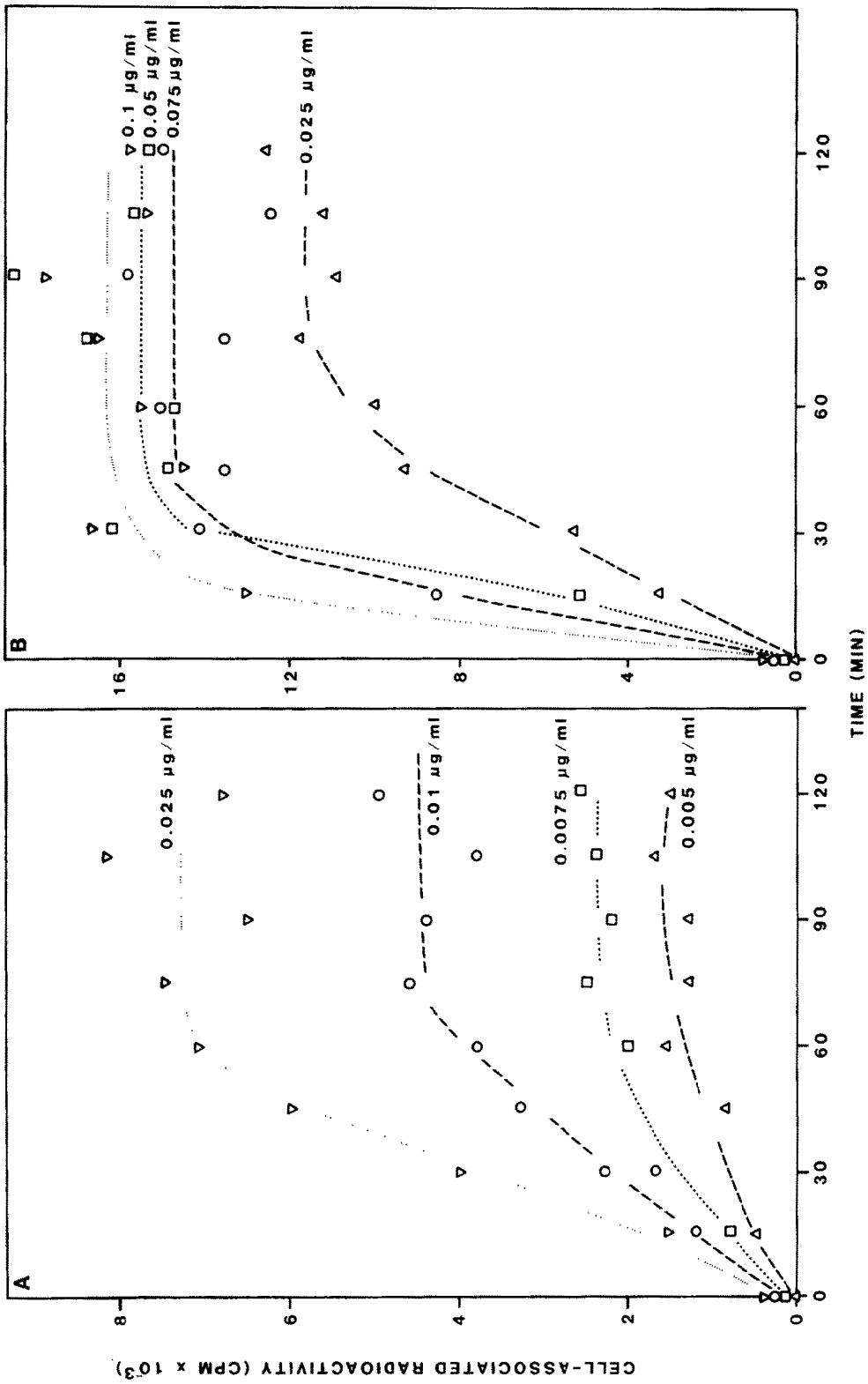


Fig. 3. Kinetics of [$^3$H]T-2 association with CHO cells at 37°. T-2 was added to cells (duplicates) at the concentrations indicated in the figure, and cell-associated radioactivity was determined at the indicated times, as described in Materials and Methods. Panels A and B represent two separate experiments over different ranges.

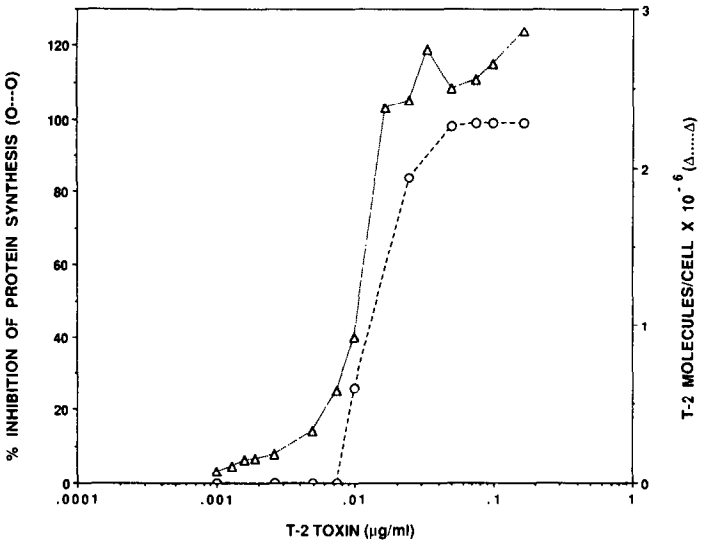


Fig. 4. Comparison of T-2 inhibition of protein synthesis with [$^3$H]T-2 association with cells. [$^3$H]T-2 was added to CHO cells at the indicated concentrations and incubation carried out at 37° for 2 hr. At that time, one set of cells (duplicates) was processed to determine the cell-associated T-2-derived radioactivity (△), as described in Materials and Methods. A second set of cells was processed as described in the text to determine the level of trichloroacetic acid-precipitable T-2-derived radioactivity (data not shown, < 60 cpm at all concentrations). [$^3$H]Leucine (1 μCi/well) was added to a third set of cells and incubation continued for 15 min at 37°. These cells were then processed, as described in Materials and Methods, to determine leucine incorporation (○) (control level *ca.* 5000 cpm).

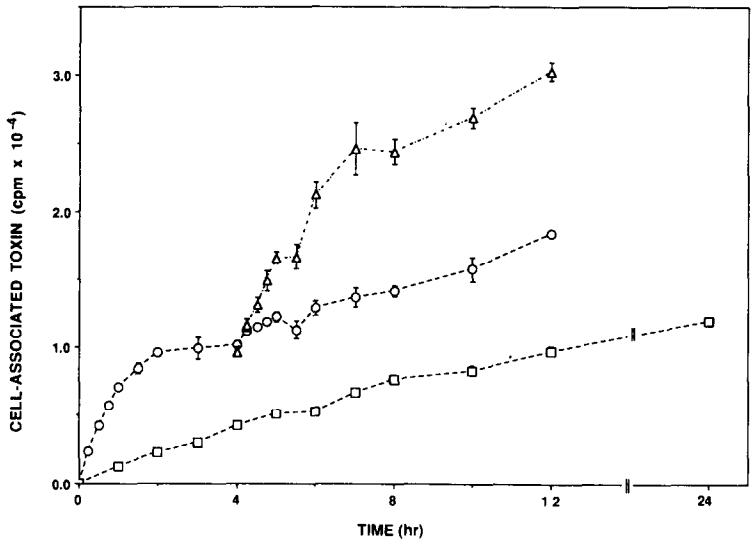


Fig. 5. Comparison of [$^3$H]T-2–CHO cell association at 4° and 37°. [$^3$H]T-2 (0.1 μCi/well) was added to CHO cells (triplicates), and incubation was carried out at 4° (□) or 37° (○). After 4 hr, a second addition of [$^3$H]T-2 (0.1 μCi/well) was added to a subgroup of cells being incubated at 37° (△). At the indicated times, the cells were processed, as specified in Materials and Methods, to determine the cell-associated, T-2-derived radioisotope. Error bars denote SE which, if not shown, were smaller than the symbol.

concentration ranges and a clustering about the ordinate. From this specific experiment, we calculated apparent dissociation constants of $3.6 \times 10^{-8}$ M and $7.5 \times 10^{-9}$ M for 4° and 37° respectively. Likewise, we obtained values of $1.8 \times 10^6$ and $2.9 \times 10^6$ as estimates for the number of binding sites/cell.

Another approach frequently employed to obtain estimates of dissociation constants is to measure the level of binding with a constant amount of labeled ligand while varying the concentration of unlabeled (competing) ligand. The level of unlabeled T-2 required to compete for 50% of the association is taken as an estimate of the dissociation constant. In experiments such as this, we consistently found that more unlabeled T-2 was required at 4° than 37° to attain the same degree of competition. At 37°, this

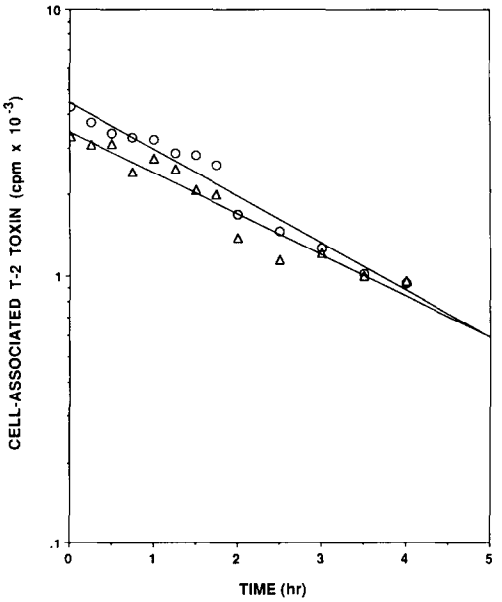


Fig. 6. Kinetics of [$^3$H]T-2–CHO cell dissociation at 37°. Cells were incubated with T-2 (0.05 μCi/well) for 3 hr at 37°. One-half of the wells were rinsed once and replenished with fresh medium (△). A 200-fold molar excess of unlabeled T-2 was added to the other wells (○). Incubation was continued at 37° and, at the times indicated, cells were rinsed and cell-associated radioactivity was determined (triplicates), as described in Materials and Methods. The data are presented as a semilog plot and the lines were fit by linear regression.

value was 0.005 μg/ml (~ $3 \times 10^{-8}$ M), whereas at 4° it was approximately 0.03 μg/ml.

*Stereospecificity of T-2–CHO cell association.* As synthesized by the organism, T-2 toxin is stereochemically pure. However, during the course of radiolabeling the toxin, epimerization occurs at the 3-position, resulting in the formation of stereoisomers; these epimers were purified and supplied to us separately. Tritiated T-2 with a configuration corresponding to the natural compound has been termed α-T-2, and that with the opposite configuration, β-T-2 [11]. As shown by the data in Fig. 8, the cell association of α- compared to β-T-2 was different. Both in the cold and at physiological temperature, CHO cells bound substantially more α-T-2 than β-T-2. A consistent finding was that the ratio of bound α-T-2 to β-T-2 was higher at 37° than 4°. Typically, at 37°, the α:β ratio was 13–17:1, whereas at 4° values were in the range of 3–5:1.

Binding isotherms for α- versus β-T-2 (Fig. 9) present a more complete picture of the concentration dependence of the toxin–cell interaction. Clearly more β-T-2 was required to attain the level of cell association achieved by a given concentration of α-T-2. However, the general shapes of the isotherms were similar, so that it was primarily the position on the graph which distinguished the binding of α- from β-T-2. The possibility that α- and β-T-2 bind to the same site(s), but with different affinities was tested by a competition experiment. The data (Fig. 10) demonstrated that α-T-2 blocked the association of both α- and β-T-2 at 4° and 37°. When normalized to a percentage of control association (no competitor), the curves at each temperature were very simi-

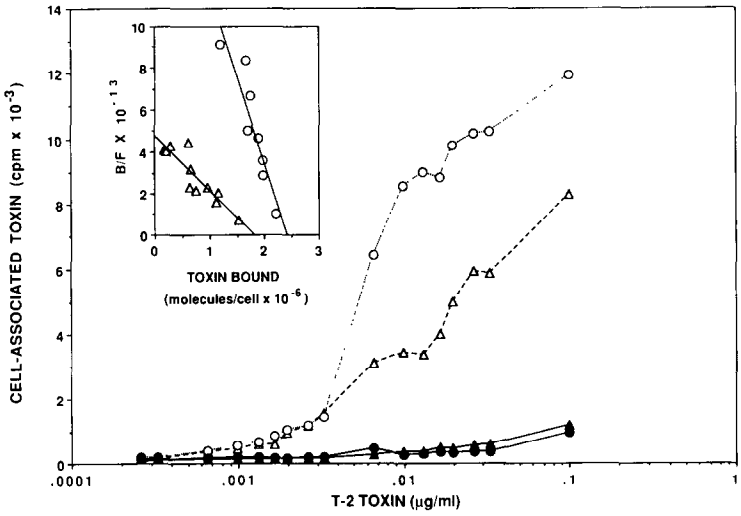


Fig. 7. [$^3$H]T-2–CHO cell association isotherms at 4° and 37°. Cells were incubated in duplicate with the indicated concentration of T-2 in the absence (open symbols) or presence (closed symbols) of a 200-fold molar excess of unlabeled T-2. After 2 hr at 37° (○, ●) or 20 hr at 4° (△, ▲), cell-associated radioactivity was determined, as described in Materials and Methods. Inset: Scatchard analysis of [$^3$H] T-2–CHO cell association. The specific cell-associated counts determined from the data shown in Fig. 7 were analyzed by the method of Scatchard [12], and the lines indicated fit by linear regression methods. Key: (○) 37°, and (△) 4°.

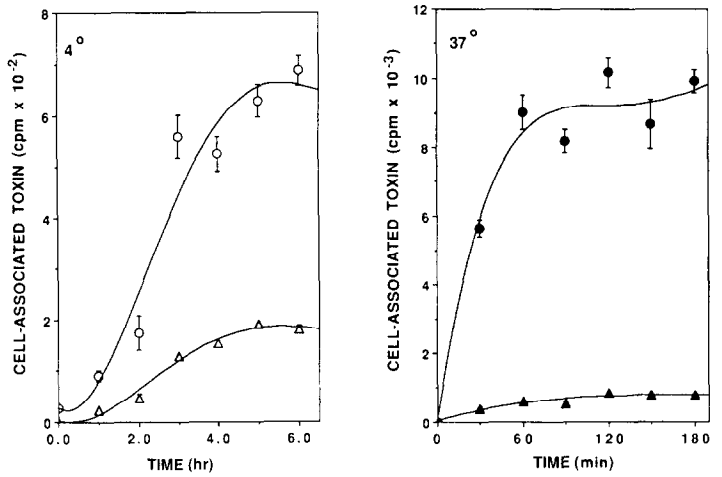


Fig. 8. Kinetics of $\alpha$- and $\beta$-T-2 association with CHO cells. $\alpha$- (○, ●) or $\beta$-T-2 (△, ▲) (0.3 $\mu$Ci/ml) was added to CHO cells (triplicates) and incubation carried out at 4° (open symbols) and 37° (filled symbols). At the times specified, cells were processed, as described in Materials and Methods, to determine cell-associated, T-2 derived radioactivity. Error bars denote SE which, if not shown, were smaller than the symbol.

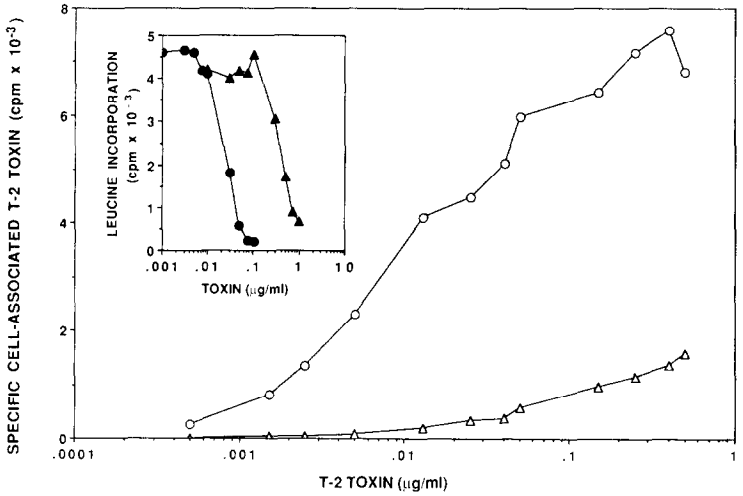


Fig. 9. Association isotherms for $\alpha$- and $\beta$-T-2 with CHO cells. [$^3$H]$\alpha$ (○) or [$^3$H]$\beta$-T-2 (△) (identical specific activities) was added to CHO cells at 4° and incubation carried out for 20 hr. Cells were then processed to determine cell-associated T-2, as described in Materials and Methods. Inset: Comparison of $\alpha$- and $\beta$-T-2 inhibition of CHO cell protein synthesis. $\alpha$- (●) or $\beta$-T-2 (▲) was added to CHO cells (duplicates) and incubation carried out at 37° for 30 min. [$^3$H]Leucine (1 $\mu$Ci/well) was added and incubation continued for an additional 30 min. The cells were then processed to determine leucine incorporation, as described in Materials and Methods.

lar. At both temperatures, the nonspecific association of $\beta$-T-2 was higher, a correction for which made the curves virtually identical. These data, along with the binding isotherms, suggested that $\beta$-T-2 would inhibit protein synthesis, but with a lower potency. The suggestion was confirmed by a bioassay (Fig. 9, inset), which demonstrated that $\beta$-T-2 inhibited protein synthesis in CHO cells with a potency about 15-fold less than $\alpha$-T-2.

A careful examination of chromatograms of our $\beta$-T-2 preparation revealed a low level ($\leq$ 2%) of a contaminant that migrated with the mobility of $\alpha$-T-2. To ascertain that the radiolabel-cell association and protein synthesis inhibitory activity we observed were due to the $\beta$-epimer, we incubated cells with that preparation, extracted and concentrated the cell-associated radioactivity, and chromatographed the sample in a standard system [13]. The results (not shown) clearly demonstrated that the predominant radioisotopically labeled material bound to cells migrated as $\beta$-T-2.

*Competition of other trichothecene toxins for T-2–CHO cell association.* When the efficacies of several trichothecene toxins were compared with their potential to compete for radiolabeled T-2–cell association, we observed a good correlation between the

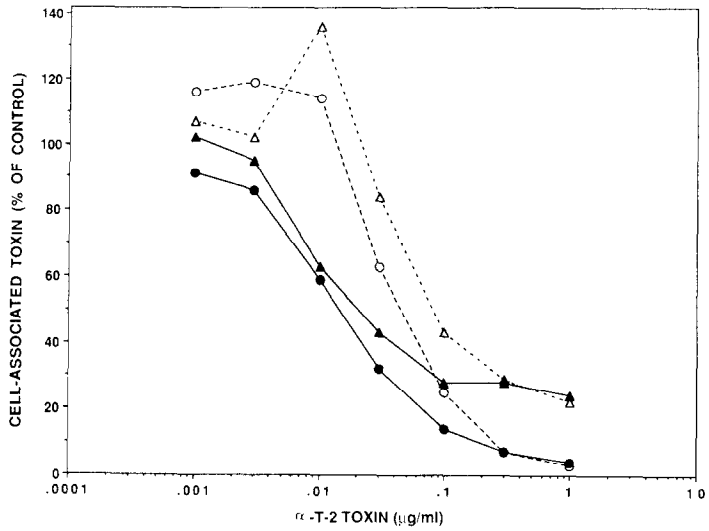


Fig. 10. Competition of α-T-2 for [$^3$H]α- and [$^3$H]β-T-2 association with CHO cells. Unlabeled α-T-2 was added to CHO cells (duplicates) to the indicated concentrations. [$^3$H]α- (○, ●) or [$^3$H]β-T-2 (△, ▲) (0.2 μCi/ml) was then added and incubation carried out for 1 hr at 37° (filled symbols) or for 16 hr at 4° (open symbols). Cells were then processed to determine cell-associated T-2-derived radioactivity, as described in Materials and Methods. Control association was as follows: α-T-2, 37°, 10,900 cpm; α-T-2, 4° 9600 cpm; β-T-2, 37°, 460 cpm; β-T-2, 4°, 800 cpm.

two effects (Fig. 11). Compared to T-2, verrucarin A was more potent at inhibiting protein synthesis (Fig. 11B). Correspondingly, verrucarin A was more effective at blocking radiolabeled T-2–cell association than was unlabeled T-2 (Fig. 11A).

In the other direction, HT-2 and, to a much greater degree, deoxynivalenol were less potent than T-2 at inhibiting protein synthesis. HT-2 was indistinguishable from T-2 in blocking radiolabeled T-2–cell association, while deoxynivalenol was clearly a much weaker blocker (Fig. 11, A and B).

## DISCUSSION

The interaction of trichothecene toxins with cells has been the subject of studies in several laboratories. Yeast, reticulocytes, spleen cells, thymic cells, and several established mammalian cell lines were used in the work [5–9, 14]. Most of the studies defined the effects of trichothecenes on various cell functions such as growth, macromolecular synthesis or ability to mount a mitogenic response. Some extended the work to the subcellular and molecular level, providing the basis for our present understanding of the pharmacology of the trichothecenes. Interesting though this work is, there remain large gaps in our knowledge of the action of trichothecenes on cells. In part, this results from the difficulty of comparing data from laboratory to laboratory, cell type to cell type, and toxin to toxin. In addition, most laboratories lacked radiolabeled trichothecenes with which to perform a number of obvious experiments. To fill these knowledge gaps and obtain a complete picture of the interaction of one trichothecene with one cell type, we carried out a comprehensive study with radiolabeled T-2 and the continuously cultured cell line, CHO. In this paper, we describe the results of experiments designed to measure the biophysical and biochemical interactions of T-2 with intact cells. In an accompanying paper [10], we present data on the interaction of T-2 with CHO cell-derived ribosomes.

There was considerable evidence that the cell-associated T-2 we measured was responsible for inhibition of protein synthesis. The rate of [$^3$H]T-2–cell association (Fig. 3) correlated well with the rate of protein synthesis inhibition (Fig. 1). It appeared that at concentrations around 0.025 to 0.075 μg/ml, a maximum in potential association was achieved (Fig. 3). This was also the concentration range which brought about a complete inhibition of protein synthesis (Fig. 1). While the experiments depicted in Fig. 1 and 3 were performed to compare the kinetics of toxin–cell association with the kinetics of biological response, the experiment shown in Fig. 4 was designed to directly compare the magnitude of response with the amount of toxin–cell association. Clearly, there was a close correlation between those two curves as well. It should be emphasized that the same solutions of [$^3$H]T-2 were used to measure binding and protein synthesis inhibition, so that correlation is highly reliable.

The measurement of the biophysical parameters for T-2–cell association at 4° appeared to be straightforward. We determined that there was no measurable metabolism of T-2 at that temperature (data not shown). The dissociation constant of about $3 \times 10^{-8}$ M was not extraordinarily strong, but was tight enough to be considered indicative of a specific interaction. The concept of specificity was further supported by competition for binding by unlabeled T-2 and other trichothecene toxins (Figs. 7, 10 and 11). The number of sites per cell was calculated to be approximately $2 \times 10^6$. Although we have not made a specific determination for CHO cells, this value is close to other estimates for the number of

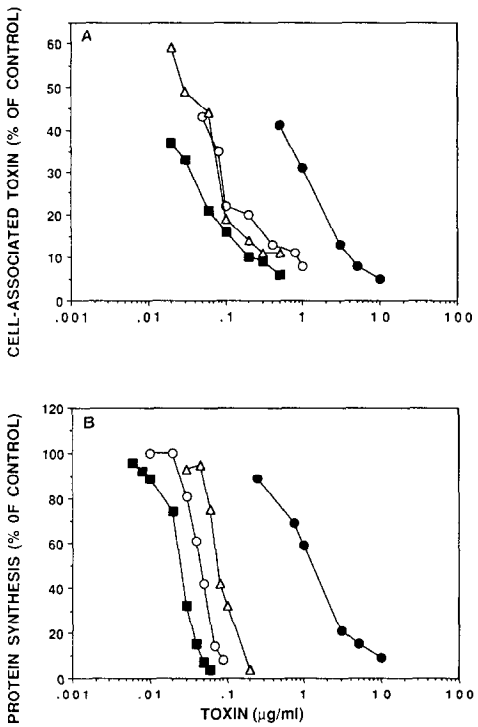


Fig. 11. Comparison of trichothecene inhibition of CHO cell protein synthesis with competition of [$^3$H]T-2–CHO cell association. (A): toxins were added to CHO cells (duplicates) to the indicated concentrations followed by [$^3$H]T-2 (0.2 $\mu$Ci/ml). After incubation at 4° for 20 hr, the cells were processed to determine cell-associated radioactivity, as described in Materials and Methods. Control association determined for each plate was *ca.* 2500–3000 cpm. Symbols: (○) T-2; (△) HT-2; (■) verrucarin A; and (●) deoxynivalenol. (B): toxins were added to CHO cells to the indicated concentrations and incubation was carried out at 37° for 30 min. [$^3$H]Leucine (1 $\mu$Ci/well) was added and incubation continued for 30 min. The cells were then processed to determine leucine incorporation, as described in Materials and Methods. Control incorporation measured on each plate was *ca.* 1800–2200 cpm.

ribosomes in a mammalian cell [15]. This, plus the fact that the estimated dissociation constant is similar to that determined for isolated CHO cell ribosomes [10], leads us to believe that cell-associated T-2 at 4° is predominantly T-2 bound to ribosomes.

The determination of biophysical parameters at 37° was more problematic. There is metabolism at this temperature [16], although not extensive during a 2-hr binding isotherm determination. At 37°, T-2 is converted to HT-2, then to T-2 tetraol by CHO cells. However, by 2 hr, no more than 10–20% is metabolized to HT-2 [16], a metabolite which is approximately equipotent to T-2 and also bound to CHO cells with essentially the same affinity (Fig. 11). The binding isotherm and Scatchard analysis should be evaluated cautiously. It is clear from the isotherm that more T-2 molecules associated with cells at 37° than at 4° (Fig. 7). Unlabeled T-2 competed for the association so, by that criterion, the increase at 37° compared to 4° was specific. A Scatchard plot of the 37° data was anomalous, defining two regions of behavior. The binding at high concentrations fit a straight line, while at lower concentrations presented as a cluster. The former may well represent binding to the ribosomes and, if so, would suggest both a higher affinity and more sites at 37° than 4°. It would certainly not be unusual to observe tighter binding at an elevated temperature, but the increase in sites remains to be explained. Since it has been determined that T-2 binds to free, but not bound, ribosomes [1], one possibility is that at 4° a fraction of ribosomes are in a protected state as polysomes, whereas at 37°, ribosomes continuously run off and are available for toxin binding. The conclusion of there being a higher affinity of toxin–cell association at 37° than at 4° was supported by the results of competition experiments, wherein less unlabeled T-2 was required at 37° to block [$^3$H]T-2–cell association by 50%.

Reversibility of toxin–cell association was demonstrated by two different experimental protocols: washing the cells or adding a chase. In theory, either approach should give a measure of dissociation and the two should agree. When run in parallel, we obtained similar curves (Fig. 6) and rate constants (text). The kinetics of dissociation were apparently first order with a half-time of approximately 2 hr. When compared to the kinetics of uptake (Fig. 3), it is obvious why T-2 is effectively concentrated from the medium by CHO cells. At higher concentrations, uptake was complete in 30 min, whereas elimination of 50% (starting at any point in time) took 2 hr. Furthermore, it is clear that cells were capable of taking up even more toxin when exposed to a second dose (Fig. 5).

Another aspect of the specificity of the T-2–CHO cell interaction is its stereospecificity. Epimerization at the 3-position markedly reduced the biological potency (Fig 9, inset) and the association with cells (Figs. 8 and 9). The reduction in association was more pronounced at 37° than at 4°. However, the fact that (on a percentage basis) unlabeled $\alpha$-T-2 competed equally well for the binding of either [$^3$H]$\alpha$- or [$^3$H]$\beta$-T-2 strongly suggests that both epimers bind to the same cell site(s). This would imply that the $\beta$-isomer has a lower affinity for these sites, at least one class of which we know to be ribosomal [10].

There is one published study wherein the binding of T-2 to spleen cells was examined in some detail [9]; the results were significantly different from ours. The number of toxin molecules taken up per cell, and the apparent binding affinity were one-tenth or less of those corresponding values determined for CHO cells. The rate of dissociation was faster and the specificity of binding lower than for CHO cells. No specific binding was observed at 0°, in marked contrast to the binding of T-2 exhibited by CHO cells. An explanation for these substantial differences in binding parameters is not readily obvious. However, one possibility is the heterogeneous nature of spleen cell populations. Thus, one could be measuring differential T-2 binding to subpopulations of cells and obtaining a weighted average of several interactions.

One interesting phenomenon brought to light by our data was the dynamic character of T-2–cell

association at physiological temperature. While the kinetics of this interaction described an elemental curve (Fig. 3), they were clearly a composite of at least two distinct processes: binding or uptake (Fig. 3) and out-transport or release (Fig. 6). The simplest model for these events would be to consider the cell membrane as a solvent barrier across which T-2 may cross with ease, to bind to (and dissociate from) the ribosomes. In that view, the kinetics of association and dissociation of T-2 with cells and ribosomes should be very similar. When that proposal was tested by comparing the data in this and the accompanying paper [10], it appeared that such is the case. In addition, with both cells and isolated ribosomes, lowering the temperature to 4° slowed the out-transport and dissociation, respectively, to essentially zero. Finally, the stereospecificity defined in several figures above was also observed in T-2–ribosome binding experiments [10].

A second possible model is to consider the cell membrane as a barrier to T-2. Presumably, T-2 would interact with a transport or receptor system to enter the cell where binding to the ribosomes would occur. The only evidence in this study which might support that proposal was the high threshold of T-2 molecules which must associate with cells before protein synthesis inhibition was evident (Fig. 4). It is possible that this threshold binding was T-2 bound to a receptor/transport system, but the bulk of our data would support a permeable membrane model.

*Acknowledgements*—The authors thank Lynn Trusal and Judith Pace for many helpful suggestions and Edward Hauer for computer assistance.

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