Inhibition of RNA Synthesis in *Chlorella pyrenoidosa* and *Bacillus megaterium* by the Pine-blight Toxin, Dothistromin

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SUMMARY

Dothistromin, an anthraquinone derivative produced by the pine-blight fungus, *Dothistroma pini*, inhibits the growth of *Chlorella pyrenoidosa* and *Bacillus megaterium*. At growth inhibitory concentrations, dothistromin strongly inhibits incorporation of [³H]uridine into RNA of both species. With *B. megaterium*, marked inhibition of [³H]uridine incorporation is apparent within 5 min of addition of dothistromin, but only a slight inhibition of [³H]thymidine incorporation into the DNA-containing fraction or of [¹⁴C]leucine incorporation into protein is detectable after 10 min.

INTRODUCTION

Dothistroma pini Hulbary is a fungal pathogen causing the necrotic disease of pine foliage known as dothistromal blight (Gibson, 1972). Gadgil (1967), investigating the infection process on *Pinus radiata* needles, suggested that the disorganization and necrosis of the mesophyll tissue in advance of hyphal spread was due to an exotoxin or exoenzyme produced by the fungus. An orange-red pigment that inhibited the growth of *Chlorella pyrenoidosa* was subsequently isolated from the medium of *D. pini* cultures. This pigment, called dothistromin, has been isolated, purified and its molecular structure determined (Bassett *et al.*, 1970; Gallagher & Hodges, 1972). The structure was later confirmed and the absolute configuration established from X-ray diffraction studies (Bear, Waters & Waters, 1972). Dothistromin is a trihydroxyanthraquinone fused to a substituted tetrahydrobifuran ring system, and is structurally related to the mycotoxins, aflatoxin and sterigmatocystin.

Before a phytotoxic role can be assigned to dothistromin, it is necessary to investigate fully its biological activity and possible modes of action. *Chlorella pyrenoidosa* was selected as a test organism for a preliminary study of the effects of dothistromin on growth and biosynthetic processes. This organism had previously been used as the basis of an agar-plate bioassay during isolation of the toxin (Bassett *et al.*, 1970), and a survey of a wide range of micro-organisms showed that this species was highly sensitive to inhibition by the toxin. Since *C. pyrenoidosa* is a photosynthetic eukaryotic micro-organism, a study of the effects of dothistromin on its metabolism might throw light on the mode of action of the toxin on leaf cells of *Pinus* species. However, the usefulness of *C. pyrenoidosa* as a model system was limited by its low rates of incorporation of certain precursors. *Bacillus megaterium* KM was found to be equally sensitive to dothistromin and proved to be a suitable model system for this work.

METHODS

Organisms. *Dothistroma pini* and *Chlorella pyrenoidosa* were obtained from the Forest Research Institute, Rotorua, New Zealand. *Bacillus megaterium* KM (NRRL3694) was obtained from Dr W. C. Haynes, U.S. Department of Agriculture, Northern Regional Research Laboratories, Peoria, Illinois, U.S.A.

Growth of Dothistroma pini and isolation of dothistromin. *Dothistroma pini* was grown on a liquid medium containing, per litre: 100 g dried malt extract (A. Wander, Christchurch, New Zealand), 5 g dried yeast (Dominion Yeast Co., Auckland, New Zealand) and 20 mg cholesterol. The medium (pH 6.0 to 6.2) was sterilized at 121 °C for 15 min in 1 l flasks containing 500 ml medium. Cultures were grown on a gyratory shaker at 15 °C for 6 to 10 days. The total culture was then extracted twice with an equal volume of ethyl acetate: the solvent was added to the culture flasks which were shaken on a gyratory shaker for approximately 8 h and, after standing overnight, the ethyl acetate was removed and the extraction procedure repeated. The total ethyl acetate extract was washed several times with equal volumes of distilled water, and evaporated to dryness under vacuum at 40 °C. The resulting red deposit was purified by the procedure of Gallagher & Hodges (1972). *Dothistroma pini* cultures were maintained on malt agar of the same composition as the liquid growth medium plus 2.5 % (w/v) agar, and were subcultured at two-monthly intervals.

Growth of C. pyrenoidosa. This was maintained on an agar medium containing, per litre: 35 g Difco Chlorella agar and 5 g Davis agar (Davis Gelatin, New Zealand). Stocks were subcultured at intervals of 4 to 5 weeks. After inoculation, the slants were illuminated at 25 °C for 4 to 5 days and then stored at 4 °C. Cells for the inoculation of liquid cultures were taken from fresh slants (4 to 7 days old). For liquid cultures, *C. pyrenoidosa* was grown autotrophically in a modified Knop's solution containing, per litre: KH_2PO_4 , 1.25 g; KNO_3 , 1.25 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2.5 g; ferric ethylenediaminetetraacetate solution (Jacobson, 1951), 1 ml; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 1.81 mg; H_3BO_3 , 2.86 mg; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.22 mg; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.079 mg; $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$, 0.02 mg. The medium (pH 4.8 to 5.0) was sterilized at 121 °C for 15 min in 1 l flasks containing approximately 200 ml medium. Cultures were aerated with 5 % CO_2 in air at 25 °C and illuminated with two banks of four Atlas Super-Gro 20 W fluorescent tubes arranged to give an incident light intensity of 5000 lx at the surface of the culture vessel. For growth inhibition studies in batch cultures, growth was followed by measuring the extinction at 660 nm in a Bausch & Lomb Spectronic 20 spectrophotometer.

Synchronous culture of Chlorella. Cultures were grown as above except that instead of continuous illumination, three cycles of 16 h illumination followed by 8 h darkness were used. These three cycles were followed by 72 h incubation in the dark. The organisms were then harvested by centrifuging (13000 g; 8 min) in sterile 250 ml centrifuge tubes. The pellets were resuspended in cold sterile 0.02 M- KH_2PO_4 pH 4.5 and centrifuged at 100 g for 2 min; resuspension and centrifugation were repeated twice. After each centrifugation, the pellet containing the larger organisms was discarded. The small organisms, selected in this manner, were harvested by centrifuging (13000 g; 8 min), and resuspended in growth medium (pre-equilibrated at 25 °C and aerated with 5 % CO_2 in air) to give a density of 10^6 organisms ml^{-1} .

The synchronous cultures initiated in this way were grown with continuous illumination under the same conditions as for normal batch cultures. Samples were withdrawn at intervals throughout the growth cycle for estimation of the number of organisms and cell-

size distributions. Measurements were made of the sizes of 100 cells from each sample using a calibrated travelling eyepiece (Leitz).

Growth of B. megaterium. Cultures were grown at 30 °C in Erlenmeyer flasks on a gyratory shaker in a medium containing, per litre: KH_2PO_4 , 3 g; K_2HPO_4 , 7 g; sodium citrate dihydrate, 0.5 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g; $(\text{NH}_4)_2\text{SO}_4$, 1 g; glucose, 5 g; nicotinic acid, 0.5 mg; tryptone, 1 g. The medium (pH 6.8 to 7.0) was sterilized in 250 ml flasks containing 100 ml medium. Glucose and nicotinic acid were sterilized separately. Growth was followed by measuring the extinction at 540 nm (green filter) in a Klett–Summerson colorimeter.

Experimental procedures for precursor incorporation studies. Samples (5 ml) of suspensions of *C. pyrenoidosa* were taken from batch or synchronous cultures as indicated, and incubated with the appropriate radiolabelled precursor in 50 ml Erlenmeyer flasks suspended over a bank of fluorescent lights and aerated with 5 % CO_2 in air. Precursor uptake and metabolism were stopped at appropriate intervals by adding 20 ml boiling 80 % (v/v) ethanol to successive flasks. The ethanol-insoluble residues were collected by centrifuging and fractionated as described below.

Dothistromin was added to *C. pyrenoidosa* as a concentrated solution in ethyl acetate (usually 1 mg ml⁻¹). The concentration of ethyl acetate so added (up to 0.5 %, v/v) had little or no effect on growth or precursor incorporation; although concentrations greater than 0.5 % inhibited precursor uptake. Controls always contained an equivalent concentration of ethyl acetate.

For precursor incorporation experiments with *B. megaterium*, 10 ml samples of cultures were taken after 6 h growth, i.e. near the end of the exponential phase, and incubated with shaking at 30 °C with the appropriate radiolabelled precursor. Samples (1 ml) were taken at intervals during the next 11 min and added to 4 ml 95 % (v/v) ethanol at 85 to 90 °C. After 10 min in the boiling ethanol, the samples were cooled, and the ethanol-insoluble cell residues were collected by centrifuging and fractionated as described below.

Since precursor incorporation by *B. megaterium* was markedly inhibited by 0.5 % ethyl acetate, dothistromin and other antibiotics were added as concentrated stock solutions in dimethylsulphoxide (dothistromin and actinomycin D at 0.5 mg ml⁻¹; mitomycin C and chloramphenicol at 5 mg ml⁻¹). Dimethylsulphoxide had a minimal effect on the rate of precursor incorporation at the levels used (0.1 %, v/v). Controls always contained an equivalent concentration of dimethylsulphoxide.

Determination of precursor incorporation into cell constituents. [$\text{Me-}^3\text{H}$]Thymidine (23 Ci mmol⁻¹; final concentration 5 $\mu\text{Ci ml}^{-1}$) was used as the precursor for DNA labelling in *C. pyrenoidosa*; for *B. megaterium*, carrier thymidine to give a specific activity of 187 mCi mmol⁻¹ (final concentration 1.87 $\mu\text{Ci ml}^{-1}$) had to be added to get linear incorporation rates. [^3H]Uridine was used as the precursor for RNA labelling: 24 Ci mmol⁻¹ (2 $\mu\text{Ci ml}^{-1}$) for *C. pyrenoidosa*; and 2.5 Ci mmol⁻¹ (2.5 $\mu\text{Ci ml}^{-1}$) for *B. megaterium*. L-[U- ^{14}C]-Phenylalanine (513 mCi mmol⁻¹; 0.25 $\mu\text{Ci ml}^{-1}$) was used as the precursor for protein labelling in *C. pyrenoidosa*; and L-[U- ^{14}C]leucine (165 mCi mmol⁻¹; 0.125 $\mu\text{Ci ml}^{-1}$) for *B. megaterium*. To minimize errors from non-specific incorporation, the ethanol-insoluble residues were further fractionated according to the modified Schmidt–Thannhauser procedure of Buetow & Levedahl (1962). The residues from this extraction were resuspended in 0.5 ml 0.5 M-NaOH and heated (30 °C; 16 h) to hydrolyse RNA. The suspension was then neutralized with 0.5 M-HCl, 3 mg calf thymus DNA was added, and the DNA and protein were precipitated with 3 ml ice-cold 1 M-perchloric acid. The precipitate was separated by centrifuging and samples of the alkali-hydrolysed supernatant were taken to measure incorporation of isotope into the RNA-containing fraction. The precipitate was resuspended

Fig. 1. Effect of dothistromin on growth of *Chlorella pyrenoidosa*. (a) Dothistromin (1 mg ml⁻¹ in ethyl acetate) was added immediately after inoculation, at a cell density of 1.1×10^7 organisms ml⁻¹. ○, No dothistromin; ■, 0.5 µg dothistromin ml⁻¹; ●, 1.0 µg dothistromin ml⁻¹. (b) Dothistromin was added during the late-exponential phase of growth (2.4×10^8 organisms ml⁻¹). ○, No dothistromin; ●, 1.0 µg dothistromin ml⁻¹; △, 2.1 µg dothistromin ml⁻¹; ▲, 4.2 µg dothistromin ml⁻¹.

in 1 ml 1 M-perchloric acid, hydrolysed at 90 °C for 10 min, and then cooled in ice. The hot perchloric acid-insoluble material was separated by centrifuging, and samples of the supernatant were taken to measure incorporation of isotope into the DNA fraction.

The residue, the protein fraction, was redissolved in 0.5 M-NaOH, and used to determine [¹⁴C]phenylalanine incorporation into protein in *C. pyrenoidosa*: this incorporation was probably underestimated since not all of the protein was precipitated after RNA hydrolysis. With *B. megaterium*, the ethanol-insoluble residue after two initial 0.2 M-perchloric acid extractions was resuspended in cold 1 M-perchloric acid, and portions of this suspension were counted to determine the incorporation of [¹⁴C]leucine into the protein. An examination of the distribution of ³H among the different fractions from *B. megaterium* showed that over 90 % of the label from [³H]uridine was in the alkali-hydrolysed fraction and 80 to 90 % of the label from [³H]thymidine was in the hot perchloric acid-hydrolysed fraction.

Radioactivity was determined by liquid scintillation counting using Triton X 100-toluene scintillation fluid (Patterson & Greene, 1965). Samples (0.5 ml) were added to 7 ml scintillation fluid and counted in a Packard Tri-Carb liquid scintillation spectrometer.

Chemicals. All radiochemicals were obtained from The Radiochemical Centre, Amersham, Buckinghamshire. The antibiotics, chloramphenicol, mitomycin C and actinomycin D were from Sigma.

RESULTS

Effect of dothistromin on growth of C. pyrenoidosa

When dothistromin was added to *C. pyrenoidosa* cultures immediately after inoculation, it inhibited growth for a time which was dependent on the concentration of dothistromin used (Fig. 1a). Growth eventually recommenced, reaching a rate similar to that of the untreated control culture. Some evidence has been obtained from fluorometric estimation of dothistromin in agar plates (Harvey, unpublished results) that dothistromin is unstable in the light, which may account for the eventual onset of normal growth.

Fig. 2. Effect of dothistromin on the growth of a synchronous culture of *Chlorella pyrenoidosa*. A culture, synchronized as described in Methods, was continuously illuminated following addition of dothistromin [1 mg ml^{-1} in ethyl acetate; final ethyl acetate concentration, 0.07% (v/v)]. (a, c) Number of cells/ml; (b, d) size distributions of cells. (a, b) Samples taken from a control culture (lacking dothistromin, but containing ethyl acetate at the same concentration as the treated culture) during a single division cycle. (c, d) Samples taken from a culture treated with dothistromin ($0.7 \mu\text{g ml}^{-1}$).

Because of the low solubility of dothistromin in aqueous media, it was necessary to establish whether sufficiently high concentrations of it could be achieved to inhibit growth in the much denser cultures used for metabolic studies. Accordingly, the effect of addition of dothistromin to cultures of *C. pyrenoidosa* near the end of the exponential phase of growth was examined. At the population densities used (10^8 organisms ml^{-1}), a dothistromin concentration of $1 \mu\text{g ml}^{-1}$ ($2.7 \mu\text{M}$) was required to inhibit growth (Fig. 1*b*). This concentration could be achieved at well below the toxic level of the ethyl acetate solvent.

To determine whether dothistromin affected cell enlargement or division, the effect of the toxin on a synchronous culture was followed through one cell cycle. A toxin concentration of $0.7 \mu\text{g ml}^{-1}$ ($12.5 \mu\text{g}$ per 10^8 organisms) was used. Cell-size distribution histograms (Fig. 2) indicate that enlargement of, at least, a large proportion of the population was inhibited.

Effect of dothistromin on precursor incorporation by C. pyrenoidosa

Samples of a synchronously growing culture of *C. pyrenoidosa* were taken at the beginning of a cell cycle, and the incorporation of [^3H]uridine, [^3H]thymidine and [^{14}C]phenylalanine was followed over the first 1.5 h after cell division. Dothistromin ($1 \mu\text{g ml}^{-1}$) inhibited the rate of incorporation of [^3H]uridine by 81 % and [^{14}C]phenylalanine by 25 % (Fig. 3). The rate of incorporation of [^3H]thymidine by this organism was very low. In the experiment

Fig. 3. Effect of dothistromin on the incorporation of labelled precursors by *Chlorella pyrenoidosa*. Samples (5 ml) were taken from a synchronous culture at the beginning of the cell cycle; and dothistromin ($14 \mu\text{g}/10^8$ organisms) and radioactive precursor (at the concentrations shown) were added together. (a) $[^{14}\text{C}]$ Phenylalanine ($513 \text{ mCi mmol}^{-1}$; $0.25 \mu\text{Ci ml}^{-1}$). (b) $[^3\text{H}]$ Uridine (24 Ci mmol^{-1} ; $2 \mu\text{Ci ml}^{-1}$). (c) $[^3\text{H}]$ Thymidine (23 Ci mmol^{-1} ; $5 \mu\text{Ci ml}^{-1}$). ■, Control; □, with dothistromin ($1 \mu\text{g ml}^{-1}$).

Fig. 4. Effect of dothistromin concentration on the rate of $[^3\text{H}]$ uridine incorporation by *Chlorella pyrenoidosa*. A sample (5 ml; 6×10^6 organisms ml^{-1}) was taken from a synchronously growing culture 2 h after the start of the cell cycle and incubated with $[^3\text{H}]$ uridine (24 Ci mmol^{-1} ; $2 \mu\text{Ci ml}^{-1}$) and dothistromin. Samples were withdrawn after 15 min to determine ^3H incorporation into the RNA fraction.

described, the low rate of incorporation of thymidine might be attributed to the fact that very little DNA synthesis occurred during the first few hours of the cell cycle. However, in further experiments, samples were taken at various stages throughout the cell cycle. The results confirmed that the toxin inhibited $[^3\text{H}]$ uridine incorporation, and showed that incorporation of $[^3\text{H}]$ thymidine by *C. pyrenoidosa* was very low throughout the cell cycle. Other workers encountered the same difficulty in attempting to label *Chlorella* DNA with $[^3\text{H}]$ thymidine and attributed it to a lack of thymidine kinase in the organism (Wanka, Joosten & DeGrip, 1970).

Dothistromin inhibited $[^3\text{H}]$ uridine incorporation by synchronously growing cultures of *C. pyrenoidosa* near the beginning of the cell cycle (Fig. 4). After 15 min, $[^3\text{H}]$ uridine incorporation into the RNA (alkali-hydrolysable) fraction of *C. pyrenoidosa* was inhibited by 51 % with a dothistromin concentration of $1 \mu\text{g ml}^{-1}$ and by 85 % with $2 \mu\text{g ml}^{-1}$.

Fig. 5. Effect of dothistromin and various antibiotics on the incorporation of (a) $[^3\text{H}]$ juridine (2.5 Ci mmol^{-1} ; $2.5 \mu\text{Ci ml}^{-1}$), (b) $[^{14}\text{C}]$ leucine ($165 \text{ mCi mmol}^{-1}$; $0.125 \mu\text{Ci ml}^{-1}$) and (c) $[^3\text{H}]$ thymidine ($187 \text{ mCi mmol}^{-1}$; $1.87 \mu\text{Ci ml}^{-1}$), by late-exponential phase cells of *B. megaterium* (harvested after 6 h growth; Klett reading at 540 nm, 150). Dothistromin and other antibiotics were added as concentrated solutions in dimethylsulphoxide ($10 \mu\text{l}$) 2 min before the addition of labelled precursor. $\circ$, No added inhibitor; $\bullet$, dothistromin ($0.5 \mu\text{g ml}^{-1}$); $\square$, mitomycin C ($5 \mu\text{g ml}^{-1}$); $\blacksquare$, chloramphenicol ($5 \mu\text{g ml}^{-1}$); $\triangle$, actinomycin D ($0.5 \mu\text{g ml}^{-1}$).

Effect of dothistromin on growth of B. megaterium

In view of the relatively slow rates of precursor incorporation by *C. pyrenoidosa* and of the difficulties in studying DNA synthesis in this organism using $[^3\text{H}]$ thymidine as a precursor, other micro-organisms were investigated. An antibiotic-disc bioassay, in which filter-paper discs impregnated with $25 \mu\text{g}$ or $50 \mu\text{g}$ dothistromin were placed on the surface of an agar plate seeded with the test micro-organism, was used to determine the sensitivity of a number of bacterial species to the toxin. Of the species tested, *Bacillus megaterium* KM was very sensitive. *Bacillus megaterium* had also been found to be particularly sensitive to inhibition by aflatoxin B_1 (Burmeister & Hesseltine, 1966) and was selected as the basis for a microbiological assay for aflatoxin (Clement, 1968).

Cultures of *B. megaterium* KM in the late-exponential phase of growth (0.6×10^8 bacteria ml^{-1}) were treated with dothistromin, and subsequent growth was followed by measuring the extinction at 540 nm. Growth was markedly inhibited after 1 h by dothistromin at $0.2 \mu\text{g ml}^{-1}$ and completely inhibited after 30 min by $0.5 \mu\text{g ml}^{-1}$.

The effects of dothistromin and other antibiotics on precursor incorporation by B. megaterium

A study of the effects of dothistromin on the incorporation of [^3H]thymidine, [^3H]uridine and [^{14}C]leucine by *B. megaterium* was undertaken to investigate the specificity of the effect on RNA synthesis compared with that of DNA and protein. Antibiotics with established sites of action were included to ensure that precursor incorporation was a valid measure of particular biosynthetic activities. Chloramphenicol ($5\ \mu\text{g ml}^{-1}$) was selected as an inhibitor of protein synthesis, actinomycin D ($0.5\ \mu\text{g ml}^{-1}$) as an inhibitor of RNA synthesis and mitomycin C ($5\ \mu\text{g ml}^{-1}$) as an inhibitor of DNA synthesis. The concentrations used were the minimum required to give significant growth inhibition at the cell densities used in the incorporation studies.

Dothistromin ($0.5\ \mu\text{g ml}^{-1}$) inhibited RNA synthesis more than either thymidine incorporation into DNA or leucine incorporation into protein. The inhibition was similar to that of actinomycin D (Fig. 5*a*). Both leucine (Fig. 5*b*) and thymidine (Fig. 5*c*) incorporation by *B. megaterium* were slightly depressed by dothistromin. The reasons for this are not known, but the slight inhibition may represent some direct effect of dothistromin on the transport of these precursors into the cell. Chloramphenicol stimulated thymidine incorporation slightly (Fig. 5*c*) and uridine incorporation markedly; the rate of uridine incorporation in the presence of chloramphenicol was about 15 times that of the control (the data have been omitted from the graph). Stimulation of uridine uptake by chloramphenicol is well known and has been ascribed to the protection of ribosome-bound mRNA from degradation (Vasquez, 1966). Mitomycin C inhibited thymidine incorporation as expected (Fig. 5*c*); it also partially inhibited uridine incorporation although not as much as dothistromin and actinomycin D (Fig. 5*a*).

DISCUSSION

Dothistromin inhibited the growth of *Chlorella pyrenoidosa* and *Bacillus megaterium* at 1 to $2\ \mu\text{M}$, the actual concentration required depending on the ratio of dothistromin concentration to the number of micro-organisms/ml. At growth inhibitory concentrations, it inhibited the incorporation of [^3H]uridine into the RNA-containing fractions of both organisms. Although the RNA was not isolated, fractionation of the organisms by the Schmidt-Thannhauser procedure showed that 85 to 90 % of the [^3H]uridine was incorporated into the cold alkali-hydrolysable material of both organisms. Furthermore, the similarity between the effects of dothistromin and actinomycin D, which is known to inhibit the transcriptional process (Reich, Cerami & Ward, 1967), supports the view that this may be a primary site of action of the toxin in inhibiting microbial growth. However, further work with cell-free systems capable of RNA synthesis will be required to confirm this. It is also possible that dothistromin may inhibit the transport of exogenous precursor into the cell rather than its subsequent incorporation into RNA, although the toxin does not greatly inhibit thymidine or leucine uptake.

The inhibition of RNA synthesis by dothistromin is not surprising in view of its close structural similarity to aflatoxin. With techniques similar to those used in the present study, Clifford & Rees (1966) showed that aflatoxin B_1 inhibits RNA synthesis in liver cells, and more recent work has shown that aflatoxin binds to DNA and thereby impairs template function and RNA polymerase-mediated transcription (Neely, Landsden & McDuffie, 1970; Pong & Wogan, 1970). Although the effect of aflatoxin B_1 on precursor incorporation by *B. megaterium* has not yet been investigated, aflatoxin B_1 concentrations of 5 to $10\ \mu\text{g ml}^{-1}$ are required to inhibit growth of *B. megaterium* KM whereas comparable inhibition by

dothistromin required only 0.5 to 1 $\mu\text{g ml}^{-1}$ (C. D. Freke & A. M. Harvey, unpublished results).

The role of dothistromin in the pine-blight disease syndrome has yet to be established. The very low water solubility of the toxin might restrict its transport from the infected sites unless the toxin is normally present as a more soluble derivative.

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