# Biochemistry and Mode of Action of the *Bacillus sphaericus* Toxins

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Bacillus sphaericus *produces at least two toxins which are highly toxic to mosquito larvae. The binary toxin, which is comprised of proteins of 51.4 and 41.9 kDa, is present in all highly insecticidal strains. The 100 kDa SSII-1 toxin is present in most highly insecticidal as well as the weakly insecticidal strains. The current status of studies on biochemistry and mode of action of these toxins is reviewed.*

Key words: biochemistry - *Bacillus sphaericus* - toxins

*Bacillus sphaericus* truly fits the description of "pathogen", with both mechanisms for killing the host (the toxins) and the ability to multiply and produce a stable, transmissible form (the spore) within the dead host. *Bacillus sphaericus* as a species is unable to utilize most carbohydrates as carbon sources, utilizing instead fatty acids, carboxylic acid intermediates, and amino acids (Priest et al. 1988). Therefore in nature insecticidal. *B. sphaericus* probably relies almost exclusively upon dead insect hosts for multiplication, since with the exception of certain man-made situations (e.g. sewage), nitrogen-containing compounds are generally very limited in accessibility. This organism derives obvious benefits from production of highly specific insecticidal toxins, and from restricting its ability to germinate and reproduce to those insects which are susceptible to its toxins. These characteristics also make *B. sphaericus* a very useful biological control agent.

The insecticidal strains of *B. sphaericus* have been placed into nine groups based on flagellar antigen serotypes and eight groups based on sensitivity to lytic phages (Yousten 1984, de Barjac et al. 1985). Among these strains, insecticidal activity ranges from slightly to highly toxic. The best strains require only ca. 100 spores/ml to kill susceptible mosquito larvae. Phage type, serotype and insecticidal activity are closely linked in *B. sphaericus* (unlike *B. thuringiensis*), suggesting that genes for the insecticidal toxins are chromosomal, along with the genes responsible for phage and serotype. Four highly insecticidal strains have received the most study: 1593 and 2362 (serotype 5a5b, phage type 3), 2297 (serotype 25, phage type 4), and IAB59 (serotype 6, phage type 3). Further discussion of the taxonomy of *B. sphaericus* insecticidal strains will appear elsewhere in this volume (Aquino de Muro, Zahner, Priest).

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## SYNTHESIS AND STRUCTURE OF THE *BACILLUS SPHAERICUS* TOXINS

Highly insecticidal *B. sphaericus* strains produce at least two different types of toxins, the binary toxin and the "SSII-1" toxin. The binary toxin is present in all highly insecticidal strains, but not in weakly toxic strains (Baumann et al. 1987). The binary toxin is comprised of two proteins, 41.9 and 51.4 kDa, respectively, both of which are required for a high level of insecticidal activity, and both of which are expressed from a single 3.5-kb *Hind*III fragment following a single promoter (Baumann et al. 1987, 1991, Porter et al. 1993). The SSII-1 toxin, ca. 100 kDa in molecular weight, was first cloned from the weakly insecticidal strain SSII-1 but is present in both highly insecticidal and weakly insecticidal strains (Thanabalu et al. 1991). The binary and SSII-1 toxins bear no sequence homology to each other, nor to any known insecticidal toxin, including those of *B. thuringiensis*. The SSII-1 toxin does, however, bear some similarity to ADP-ribosylating toxins produced by certain mammalian pathogens (below).

The binary toxin of *B. sphaericus* is produced as a rectangular or parallelepiped crystalline inclusion which forms alongside the spore, first appearing at stage III (forespore engulfment) of sporulation (Yousten & Davidson 1982, Payne & Davidson 1984). Toxicity to larvae appears at the same time as the crystals, and increases as sporulation progresses (Myers et al. 1979, Yousten & Davidson 1982, Broadwell & Baumann 1986). The crystal bears regular internal striations of 6.3 nm, and generally remains attached to the spore by an exosporium (Yousten & Davidson 1982). The binary proteins are produced in relatively

equal concentrations. After sporulation or during alkaline extraction both proteins may be enzymatically processed to similar size, ca. 39-43 kDa (Baumann et al. 1991). The binary proteins also have a strong affinity for one another, making purification of one protein in the presence of the other difficult. This led to considerable confusion over the role of these proteins in early studies. Only after each protein was cloned and expressed separately was it recognised that insecticidal activity depends on the presence of both proteins (Broadwell et al. 1990). Using cloned protein and protein extracted from *B. sphaericus*, it was shown that preparations previously thought to be pure protein of ca. 43 kDa were probably contaminated with small amounts of the ca. 41 kDa degradation product of the 51.4 kDa protein, leading to the insecticidal activity of the protein complex (Davidson et al. 1990).

The binary toxin proteins have been cloned and expressed together and separately in *Escherichia coli*, *B. thuringiensis*, *B. subtilis*, and nontoxic or low toxicity *B. sphaericus*. Deletion analysis, site direcited mutagenesis, and protein sequencing studies have delineated approximate minimal regions required for insecticidal activity. The minimal active fragment of the 51.4 kDa protein is ca. 41 kDa, resulting from the deletion of ca. 35 amino acids from the N-terminus and ca. 50 amino acids from the C-terminus. The minimal active fragment of the 41.9 kDa protein is ca. 39 kDa, resulting from deletion of up to 16 amino acids from the N-terminus and 16 or 17 from the C-terminus. Fusion proteins formed by deleting the noncoding region between the toxin protein genes were toxic in one study but required addition of the 51.4 kDa protein in another study (reviewed by Baumann et al. 1991, Porter et al. 1993). The binary toxin proteins share four regions of substantial sequence homology. Three of these, designated Regions B, C and D by Baumann et al. (1988), were found to be necessary for activity; interestingly, these regions are separated from each other by similar numbers of amino acids in each protein (B to C = 59 or 60 amino acids; C to D = 45 amino acids). Each protein contains three cysteine residues, one of which resides in Homology Region B in each protein. Results in our laboratory (EW Davidson & AL Bieber, unpublished) have shown that incubation of binary toxin with the reducing agent dithiothreitol leads to 100- to 1000-fold reduction in larvicidal activity, and subsequent dialysis of these preparations against buffer restores some of the activity. When binary toxin is unfolded by treating with urea or heating without reduction, a band with mobility of ca. 90 kDa appears on electrophoresis. Reduction of this band and subsequent electrophoresis yields a band of ca. 41 kDa. These results suggest that disulfide bridges are important for toxic activity, and that under nonreducing conditions two or more toxin molecules may be associated by disulfide bridging. The nature and significance of this association is not yet known.

While it is true that both binary toxin proteins are required for maximum activity toward mosquito larvae, the 41.9kDa protein alone is sufficient to lyse cultured *Culex quinquefasciatus* cells (Baumann & Baumann 1991), and in large excess this protein alone will kill *C. quinquefasciatus* larvae (Nicolas et al. 1993). However even in very large excess, the 51.4 kDa protein alone is not insecticidal. Maximum insecticidal activity is produced by a mixture of approximately equal quantities of 41.9 kDa and 51.4 kDa proteins. An excess of 41.9 kDa protein does not enhance activity, whereas an excess of 51.4 kDa protein reduces activity of the toxin mixture (Davidson et al. 1990). These results suggest that the *B. sphaericus* binary toxin may follow the well-known "A (active)-plus-B (binding)" scenario which is exhibited by toxins produced by mammalian pathogens such as cholera. However each binary toxin protein will bind to the larval gut in the absence of the other (Davidson et al. 1990). Recently Oei et al. (1992) have shown that highly regionalized binding and internalization of the binary toxin proteins in the gastric caecum and posterior midgut of *C. quinquefasciatus* larvae occur only in the presence of both proteins. N-terminal amino acids 39 to 45 of the 51.4 kDa protein are required for localized binding, and amino acids in the C-terminus of the 51.4 kDa protein are required to direct binding of the 41.9 kDa protein or internalization of the complex. Regionalization of binding is therefore a function of the 51.4 kDa protein. This protein apparently acts as a chaperone to the 41.9 kDa protein or facilitates its binding in specific regions of the gut, however both proteins are required for internalization of the toxin. These early steps in the pathology will be discussed in further detail below.

The *B. sphaericus* binary toxin exhibits a highly specific host range. Mosquito larvae are the only organisms killed by this toxin. *C. quinquefasciatus* larvae are most susceptible ($LC_{50}$ = ca. 50-100 ng toxin protein/ml), members of the genus *Anopheles* are intermediate in susceptibility ($LC_{50}$ = ca. 360-5000 ng/ml) and *Aedes aegypti* is the least susceptible ($LC_{50}$ = 42,000 ng/ml) (Davidson 1989a). The three highly toxic serotypes differ somewhat in their host range. Strains 1593 and 2362 (serotype

H5a5b), 2297 (serotype H25) and IAB59 (serotype H6) are all approximately the same in activity toward *C. quinquefasciatus* after 48 hr, although the larvae die more quickly when ingesting 2362 toxins than when ingesting toxins from the other serotypes (Berry et al. 1993). In addition, strains 2297 and IAB59 are not toxic to *Ae. aegypti*, whereas strains 1593 and 2362 are somewhat toxic to these larvae (Thiery & de Barjac 1989). When the binary toxin genes from these strains were cloned, it was learned that the toxins from strains in serotype H5a5b (1593 and 2362) are identical, but toxins from the other two serotypes differ from the H5a5b toxin and from each other in amino acids in both the 41.9 and 51.4 kDa proteins (Baumann et al. 1988, Berry et al. 1989). Using site-directed mutagenesis, the difference in host range and time to death were shown to be due to amino acids centered around amino acid 100 in the 41.9 kDa protein (Berry et al. 1993).

Strain SSII-1 (serotype 2a2b, phage type 2) was the first *B. sphaericus* strain discovered with a potentially useful level of insecticidal activity (Singer 1973), although SSII-1 is far less toxic than strains such as 1593, 2362 and 2297 which were isolated later. Toxicity in SSII-1 is unstable and does not increase at sporulation. The binary toxin genes are missing from strain SSII-1, therefore another toxin must be present. Thanabalu et al. (1991) cloned a gene coding for a protein of 100 kDa from SSII-1, and found that it was also present in high as well as low toxicity strains. As in SSII-1 itself, the 100 kDa toxin was found to be unstable when expressed in *E. coli* although subcloning produced a more stable toxin. When purified from an *E. coli* expression system, the 100 kDa protein is equal in activity to the binary toxin. The weak insecticidal activity of *B. sphaericus* SSII-1 is apparently due to low expression and/or instability of the toxin in this strain. The 97 kDa protein formed by the toxic subclone is cleaved by trypsin or mosquito gut enzymes to give peptides of 27 and 70 kDa. The SSII-1 toxin bears one sequence with homology to the S1 subunit of pertussis toxin and the A (active) subunits of cholera and *E. coli* heat-labile toxins, as well as another short region with homology to *Pseudomonas aeruginosa* exotoxin and the A subunit of diphtheria toxin (Thanabalu et al. 1993). Drawing on the knowledge that these toxins exert their actions by ADP-ribosylation, Thanabalu et al. (1993) have shown that a nontoxic 57 kDa peptide derived from the SSII-1 toxin can ADP-ribosylate *C. quinquefasciatus* cell membrane proteins. However the 57 kDa peptide cannot induce changes in cultured *C. quinquefasciatus* cells, whereas the 97 kDa intact toxin and its 27 kDa derivative can induce these changes. This toxin, therefore, appears to have at least two functional domains, a C-terminal 70 kDa peptide and an N-terminal peptide, both of which are required for toxicity.

## MODES OF ACTION OF THE *BACILLUS SPHAERICUS* TOXINS

When spores and crystals of *B. sphaericus* are ingested by a sensitive *C. quinquefasciatus* mosquito larva, the crystals are dissolved very quickly in the highly alkaline anterior midgut region (Yousten & Davidson 1982, Charles 1987). The binary toxin is subsequently activated by gut proteases. The 51.4 kDa protein is converted into a stable 43 kDa protein while the 41.9 kDa protein is converted to ca. 39 kDa (Broadwell & Baumann 1987). The activated proteins, either separately or perhaps in association, traverse the peritrophic membrane and bind to cell membranes in specific regions of the gastric caecum and posterior midgut. Binary toxin binds to the brush border of *C. quinquefasciatus* midgut cells, and eventually enters the midgut cells by receptor mediated endocytosis (Davidson 1988). The interactions of the 51.4 and 41.9 kDa protein in this process are discussed above. Ultrastructural studies have demonstrated swelling of mitochondria during the first hour after feeding, followed by the appearance of large cytolysosomes or vacuoles in cells of the gastric caecum and posterior midgut ca. 4 hr following ingestion of spores and crystals (Davidson 1981, Charles 1987). Midgut cells separate from one another at their bases as the midgut swells, eventually lying against the body wall. The peritrophic membrane with its food contents falls into zig-zag folds. Spores of 1593 or 2297 germinate within a few hours of ingestion, multiply within the peritrophic membrane of the dying larva, and eventually colonize the cadaver. Approximately $10^5$ fresh spores are produced in each larval cadaver over the following 48 hr (Davidson et al. 1984). The relationship between intoxication of the larva and spore germination is not yet understood.

The ultrastructural effects of ingesting strain SSII-1 vegetatives, known to contain only the 100 kDa toxin, are similar in several aspects to those observed in larvae fed strain 1593 or 2297 spores and crystals, which contain both the 100 kDa toxin and the binary toxin. SSII-1 intoxication proceeds more slowly than in strains containing the binary toxin, however large cytolysosomes and mitochondrial damage are characteristic of both. SSII-1 intoxication leads to loss of microvilli and sloughing of posterior midgut cells, which is uncommon in pathology induced by the more toxic strains (Davidson 1979).

Toxin binding and ultrastructural effects are somewhat different in larvae which are less sensitive than *C. quinquefasciatus*. Fluorescent-labelled binary toxin does not bind to midgut cells of relatively insensitive *Ae. aegypti* larvae, and binds weakly, without being internalized, to midgut cells of *Anopheles* spp., which are intermediate in sensitivity (Davidson 1989a, Oei et al. 1992). *A. stephensi* larvae develop large areas of low electron-density instead of large cytolysosomes or vacules in midgut cells early in the intoxication, along with abnormal mitochondria. *Ae. aegypti* larvae fed a very large dose of strain 2297 spores and crystals exhibit alteration of mitochondria and endoplasmic reticulum, but no further symptoms until death (Charles 1987).

The binary toxin has rapid effects on cultured *C. quinquefasciatus* cells. These cells round up, become phase-dark, detach from the support, and eventually lyse (Davidson 1986). The toxicity of the binary toxin to cultured cells is enhanced over 50-fold by enzymatic activation before application to the cells (Broadwell & Baumann 1986, 1987, Davidson et al. 1987, Baumann & Baumann 1991). Activity of the toxin toward cultured insect cells of various species parallels the activity of the toxin toward the larvae themselves. When cloned separately and overexpressed in *B. subtilis*, the 39 kDa product of the 41.9 kDa protein was found to produce full toxicity to cultured *C. quinquefasciatus* cells without the presence of the 51.4 kDa protein (Baumann & Baumann 1991). Toxin binds strongly to sensitive *C. quinquefasciatus* cells, but much less strongly to insensitive cell lines. In *C. quinquefasciatus* cells, the toxin is internalized by receptor mediated endocytosis as seen in the larval midgut (Davidson et al. 1987). A toxin-resistant cell line was selected by increasing toxin pressure, and this cell line also bound and internalized toxin, demonstrating that binding and internalization alone are not sufficient for toxicity (Schroeder et al. 1989). Ultrastructural changes induced in cultured *C. quinquefasciatus* cells include swelling of the mitochondria and endoplasmic reticulum as well as loss in integrity of the cell membrane (Davidson & Titus 1987). Colloid osmotic lysis is the probably cause of death of these cells, since sugars of molecular radius above 0.5-0.6 nm were capable of blocking lysis (Davidson 1989b).

The SSII-1 toxin also has dramatic effects on cultured *C. quinquefasciatus* cells. Within 10 min cells lose their shape, and eventually fuse with one another to form large clusters. This effect is very different from that seen with the binary toxin, but bears interesting similarities to the action of certain ADP-ribosylating *Clostridium* toxins which alter the cytoskeleton (Thanabalu et al. 1993). A 70 kDa peptide derived from the 97 kDa insecticidal fragment of the SSII-1 toxin was sufficient to induce these changes in cultured cells. The 70 kDa fragment was not toxic to larvae when fed alone, but was toxic in the presence of another nontoxic, 57 kDa fragment. This scenario is similar to the situation with the binary toxin, in which a derivative of the 41.9 kDa protein will lyse cultured cells, but both are required to kill larvae. It is not yet known whether the SSII-1 toxin binds to the insect cell membrane.

Binding of the binary toxin to midgut cells is almost certainly the step which determines host range. Relatively insensitive *Ae. aegypti* larvae maintain the highly alkaline gut pH required for toxin dissolution, and possess enzymes capable of activating the toxin, but do not bind the toxin on the midgut cells. *Anopheles* larvae also solubilize and activate the toxin, but bind the toxin in a weak manner without internalization, suggesting that weaker binding may be responsible for the intermediate sensitivity of these larvae (Davidson 1989a). As discussed above, several lines of evidence strongly suggest that the initial step in the interaction of the binary toxin with the cell is association of the 51.4 kDa protein with a component of the cell membrane. Treatment of cultured cells with trypsin leads to temporary loss of sensitivity to toxin, and toxicity is not inhibited by various lipids, suggesting that the receptor is a protein or glycoprotein (Davidson et al. 1987).

An *in vitro* binding assay has demonstrated the presence of saturable, specific receptors on membranes from midgut cells dissected from *C. quinquefasciatus* larvae. A single class of receptors with high affinity was found on *C. quinquefasciatus* cells, whereas *Ae. aegypti* cell membranes did not exhibit any significant binding. Both binary toxin components were found to bind to these membranes in approximately equal concentrations (Nielsen-Leroux & Charles 1992).

Labelled toxin was used to detect binding of toxin on Western blots of cell membrane proteins extracted from *C. quinquefasciatus* midgut or cultured cells. Proteins of 30-32, 28 and 15 kDa were bound, and affinity chromatography using biotinylated toxin and avidin columns retrieved proteins of similar size as well as a larger protein of ca. 150 kDa (Fig. 1). Antibody to the 30-32 kDa membrane protein inhibited binding and activity of the toxin to cultured cells (Fig. 2). The receptor for the *B. sphaericus* toxin may therefore be comprised of a complex of several relatively small cell membrane proteins (E Davidson, T Grounds, A Bieber and M Meyer, unpublished).

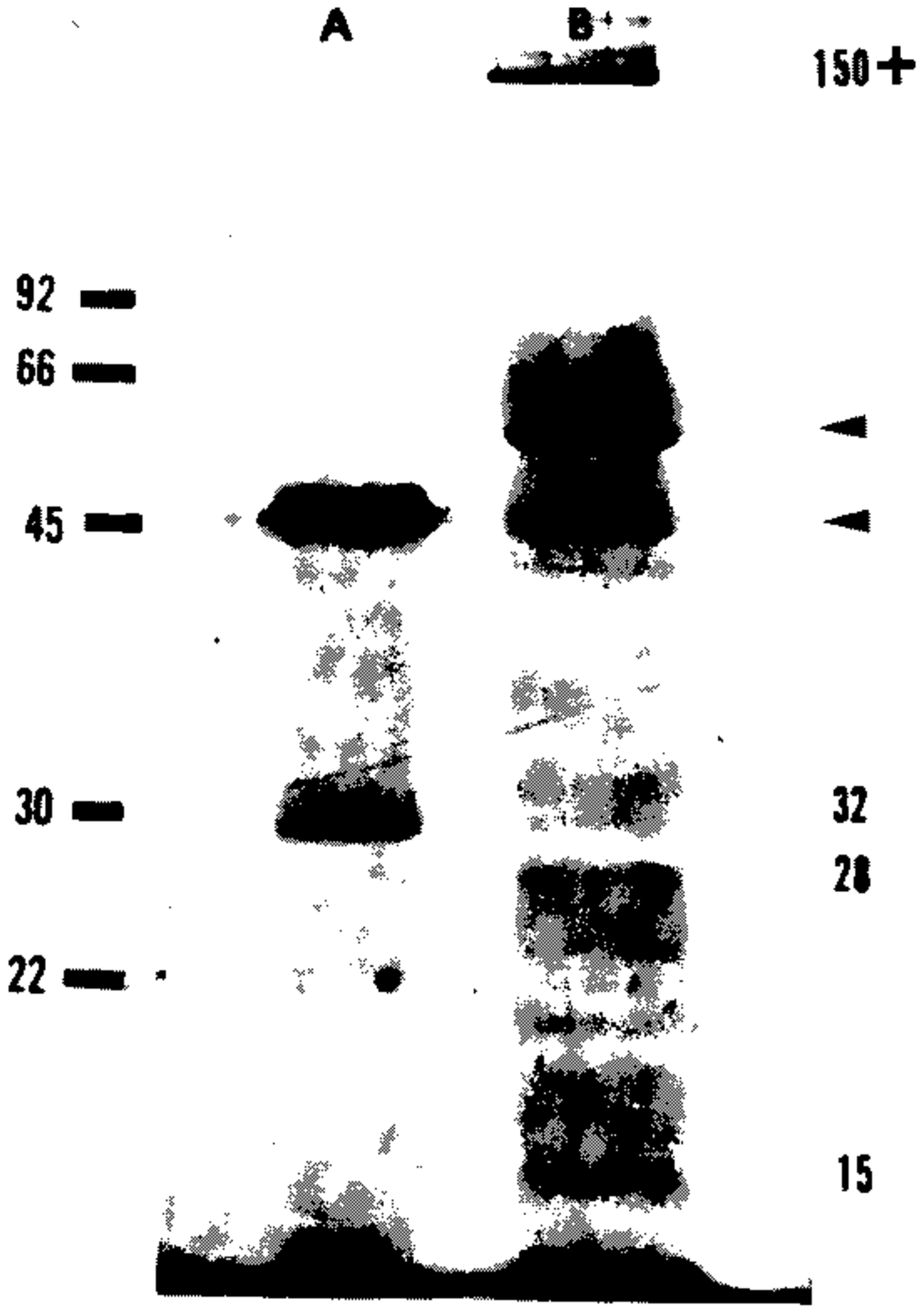


Fig. 1: proteins retrieved by affinity chromatography on avidin column from a mixture of biotinylated native toxin incubated with *Culex quinquefasciatus* midgut cell extracts. Separated on 12% SDS-PAGE, silver stained gel. A: DTT-sample buffer fraction; B: first PBS fraction following DTT. Molecular weight standards as Fig. 2. Mr of major toxin-binding proteins indicated on the right. Arrows- biotinylated toxin.

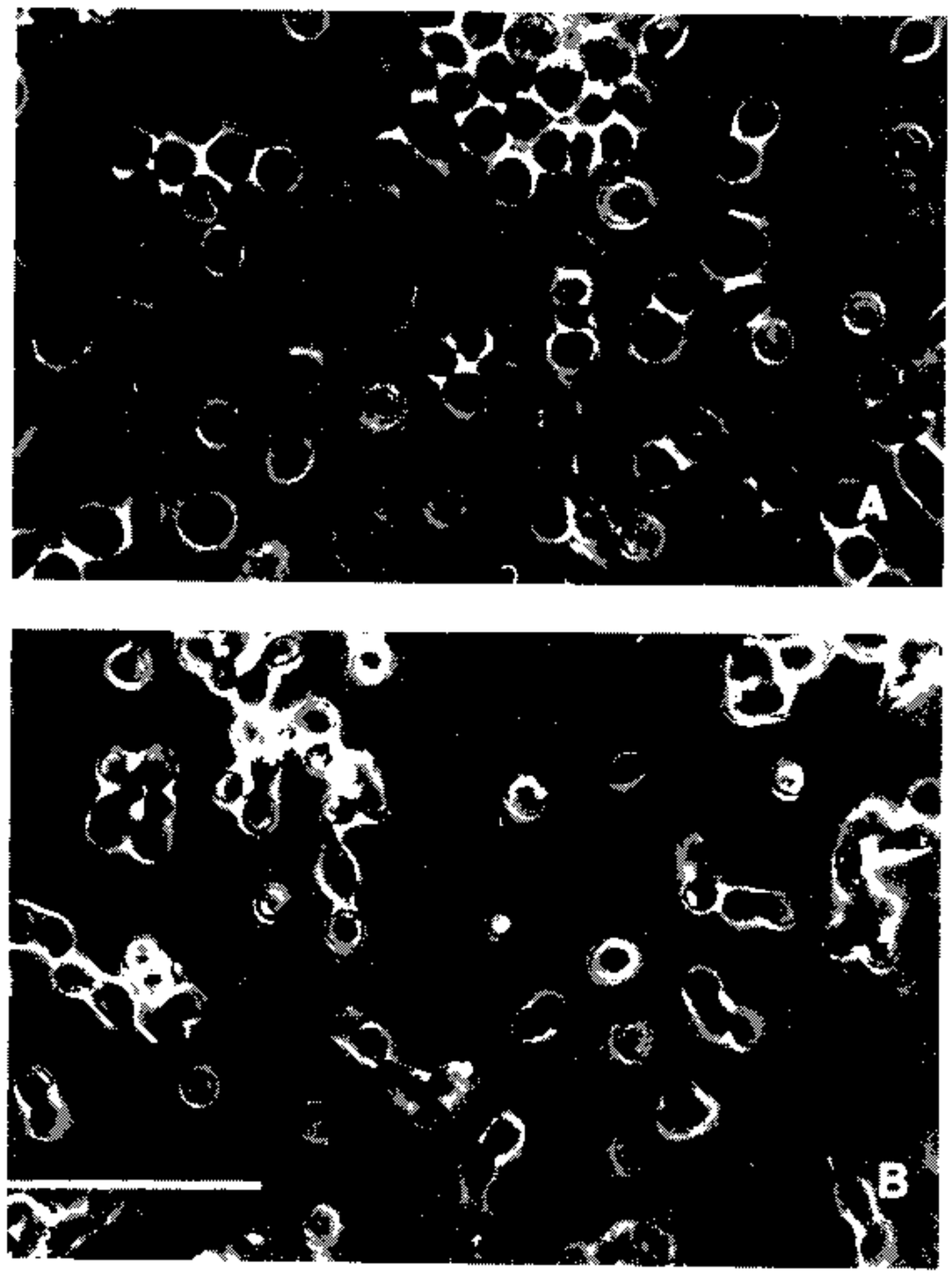


Fig. 2: toxin-sensitive cultured *Culex quinquefasciatus* cells. A: incubated with *Bacillus sphaericus* toxin: B: incubated with toxin in the presence of antibody raised to the 30-32 kDa *C. quinquefasciatus* midgut membrane protein. Bar = 50 µm.

Although the first steps in the pathology of *B. sphaericus* binary toxin, binding and internalization by receptor mediated endycytosis, have been elucidated, it is not yet known what further steps lead to death of the larva. We have therefore only begun to understand the mode of action of this toxin.

For further details on this subject, the reader is encouraged to consult three recent reviews: Aronson et al. (1986), Baumann et al. (1991) and Porter et al. (1993).

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