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NOTES

Toxicity of *Bacillus thuringiensis* var. *israelensis* Crystals to *Aedes aegypti* Larvae: Carbonate Reversal

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The toxicity of purified *Bacillus thuringiensis* var. *israelensis* crystals to larvae of *Aedes aegypti* could be reversed 100-fold by levels of K_2CO_3 as low as 0.15%.

The crystal-forming bacterium *Bacillus thuringiensis* var. *israelensis* is toxic to the larval stage of many mosquitoes and black flies. However, very little is known regarding the mode of action of the *B. thuringiensis* var. *israelensis* toxin. One approach to this question involves identification of physical or chemical factors which will counteract an observed toxicity. To this end, we screened 20 common inorganic salts at both 0.1 and 0.5% (wt/vol) to determine whether their presence reversed the toxicity of *B. thuringiensis* var. *israelensis* crystals purified on NaBr gradients (1). The crystal-containing salt solutions were bioassayed on *Aedes aegypti* larvae. The crystal concentrations at which 50% of the larvae were killed (LC_{50} values) were determined after 4 h as described previously (K. W. Nickerson and D. J. Schnell, J. Invertebr. Pathol., in press). Seventeen of the salts, $CaCl_2$, $Ca_3(PO_4)_2$, $FeNH_4(SO_4)_2 \cdot 12H_2O$, $K_2B_4O_7 \cdot 4H_2O$, KCl, $K_2HPO_4 \cdot 3H_2O$, KI, KNO_3, KSCN, $MgCl_2 \cdot 6H_2O$, $MgSO_4 \cdot 7H_2O$, $MnCl_2 \cdot 4H_2O$, NaBr, NaCl, NaF, $Na_2S_2O_3 \cdot 5H_2O$, and $(NH_4)_2SO_4$, did not reverse toxicity. The LC_{50} values were still $\leq$1 ng/ml. No attempt was made to detect enhanced toxicity.

In contrast, three salts, $BaCO_3$, K_2CO_3, and $MgCO_3$, did exhibit significant reversal. Of these, K_2CO_3 was chosen for further study because $BaCO_3$ and $MgCO_3$ are virtually insoluble in water. K_2CO_3 can undergo two ionization reactions (8) and, consequently, different carbonate species will be present depending on the pH chosen. We wanted to determine which of them is responsible for the observed reversal of toxicity. Figure 1 depicts the pH dependence of the *B. thuringiensis* var. *israelensis* LC_{50} values in the presence of 0.5% K_2CO_3. The LC_{50} values were strongly pH dependent; the carbonate reversal increased 30-fold as the pH was lowered from 8.0 to 6.0. Moreover, the pH dependence curve in Fig. 1 is identical in both shape and position to the demarcation line between H_2CO_3 and HCO_3^- in the carbonic acid equilibrium (8). Evidently it is the nonionized K_2CO_3 which accomplishes toxicity reversal.

Once the pH optimum for carbonate reversal had been determined (Fig. 1), it was then possible to construct a dose-response curve. The *B. thuringiensis* var. *israelensis* LC_{50} values at pH 5.5 in the presence of increasing levels of K_2CO_3 are presented in Fig. 2. As observed previously (Nickerson and Schnell, in press), the unsupplemented *B. thuringiensis* var. *israelensis* crystals gave an LC_{50} value of 1 ng/ml for *A. aegypti* larvae. However, this value increased rapidly with increasing K_2CO_3 until it reached a plateau of ca. 100 ng/ml at 0.14% K_2CO_3. Thus, a 100-fold reversal of toxicity is achieved with K_2CO_3.

However, four trivial explanations of carbonate reversal must be eliminated before it can be concluded that the phenomenon is actually operative in the larval gut and that it is related to the mode of action of the *B. thuringiensis* var. *israelensis* crystals. (i) It is not primarily a pH effect. pH 5.5 in the absence of carbonate did not achieve reversal in either a buffered (0.5% KH_2PO_4) or unbuffered (0.5% NH_4Cl or K_2SO_4) test solution. These solutions were monitored throughout the bioassay to ensure the maintenance of pH 5.5. (ii) It is not a crystal solubilization phenomenon. The *B. thuringiensis* var. *israelensis* crystals would, of course, be solubilized if exposed to the pH 10.5 to 11 of fresh K_2CO_3 solutions (3), and solubilized crystal preparations are generally found to be at least 1,000 times less toxic than intact crystals (3). However, the pH of the K_2CO_3 solutions employed was adjusted with HCl both before and after *B. thuringiensis* var. *israelensis* crystals were added. Additionally, the crystals could be harvested from 0.5% K_2CO_3 (pH 5.5 to 8.0) by centrifugation and suspended in distilled water

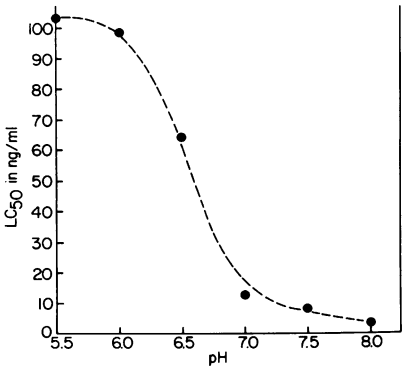

FIG. 1. Effect of pH on the toxicity of purified *B. thuringiensis* var. *israelensis* crystals to larvae of *A. aegypti* in the presence of 0.5% K_2CO_3.

with full retention of their toxicity; i.e., LC_{50} = 1 ng/ml. (iii) It is not due to protein carbamate formation. Alkaline carbonate buffers are known to convert the ϵ-NH_2 of lysine residues to the negatively charged carbamate (6). However, these protein carbamates are only formed under alkaline conditions, and they readily dissociate in mild acid (6). (iv) It is not a feeding inhibition phenomenon. Such a concern is reasonable since gaseous CO_2 is known to narcotize *A. aegypti* larvae (4). However, a quantitative particle consumption assay with radioactive cells of *Enterobacter cloacae* (Table 1) indicated that in the presence of K_2CO_3 at pH 5.5 the *A. aegypti* larvae actually experienced feeding stimulation rather than feeding inhibition.

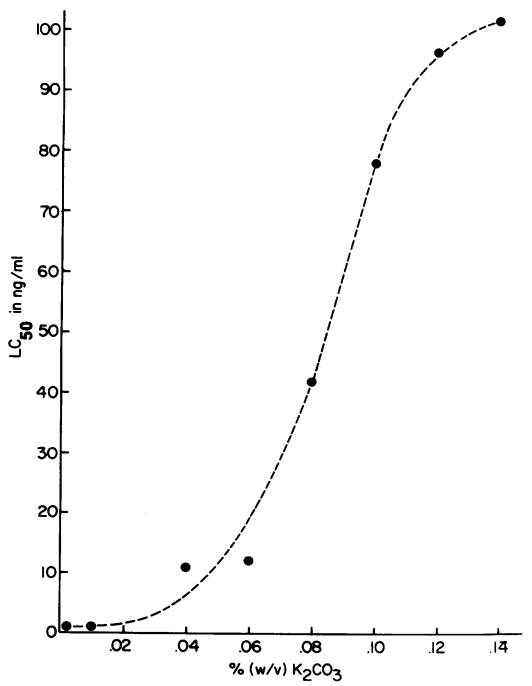

FIG. 2. Effect of K_2CO_3 concentration on the toxicity of purified *B. thuringiensis* var. *israelensis* crystals to larvae of *A. aegypti* at pH 5.5.

TABLE 1. Particle ingestion by larvae of *A. aegypti*[a]

Incubation conditions	Radioactivity ingested (cpm/larva)
pH 7, water	752
pH 5.5, 0.5% KH_2PO_4	793
pH 5.5, 0.5% K_2CO_3	1,131
pH 7, no bacteria	7
pH 7, no larvae	28[b]
pH 7, dead larvae	26

[a] Ingestion was measured by incubating seven to eight larvae at 23°C in 50 ml of a solution supplemented with 100 μl of a washed (two times) suspension of a capsule-free mutant of *E. cloacae* radiolabeled with L-[*U*-^{14}C]alanine. After 30 min the larvae were filtered through gauze, washed with 10 ml of 0.1% Triton X-100, crushed, and counted in 10 ml of a Triton X-100-containing liquid scintillation cocktail. The values reported are the average of 15 larvae, except for the controls with no bacteria and heat-killed larvae which employed 7 larvae.

[b] Counts per minute per filter.

Thus, we are left with the probability that carbonate exerts its toxicity reversal in the larval gut. This deduction has several implications with regard to the mode of action of the toxin. (i) Ca^{2+} ions did not induce toxicity reversal. It is well known (2) that external Ca^{2+} antagonizes insecticidal pyrethroid- and dichloro-diphenyl-trichloro-ethane-induced toxicity to nerves; consequently, it is unlikely that the *B. thuringiensis* var. *israelensis* toxin has a similar mode of action. (ii) External nonionized carbonate would undoubtedly shift the equilibrium of any carbonic acid preexisting in the larval gut. Such a shift could affect the overall larval gut pH, as well as influence the extent of protein carbamate formation on the toxin once it is ingested into the gut. (iii) A 100-fold toxicity reversal by carbonate is consistent with the suggestion (7; Nickerson and Schnell, in press) that both the *Lepidoptera*-active and mosquito-active toxins of *B. thuringiensis* act as ionophores, with the distinction that the *Lepidoptera*-active toxins influence cation transport whereas the mosquito-active toxins influence anion transport. More precise conclusions must await further data on the ionic composition of the larval gut in *A. aegypti* and the active mechanism (5) by which its highly alkaline pH is maintained.

Regardless of its ultimate mechanism, however, the mere existence of carbonate reversal

should have a profound influence on the reproducibility of data wherein the *B. thuringiensis* var. *israelensis* crystals were solubilized in carbonate buffers. Additionally, the possible presence of both soluble and insoluble carbonates must be considered in any further studies on the field efficacy and pH dependence of *B. thuringiensis* var. *israelensis*.

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