Temperature-dependent activity of mosquito larvicidal factor(s) present in *Bacillus sphaericus* 1593-4 and 1691

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Summary. The larvicidal factor(s) of *Bacillus sphaericus* 1593-4 and 1691 was more active at high temperature (33-35 °C) as compared to low temperature (23-25 °C) for the larvae of *Culex fatigans* and *Anopheles subpictus.*

Certain strains of *Bacillus sphaericus* are found to be highly active against a variety of mosquito larvae[1-4]. The larvicidal factor(s) produced by these bacteria are attractive alternatives to chemical insecticides for the control of mosquitoes. Little attention has been given to the effect of the temperature of the larval environment on the action of *B. sphaericus* larvicidal factor(s). In view of the fact that the entry of certain toxins, like ricin, into mosquito cells is temperature dependent[5], it was thought to be worthwhile to study the effect of temperature on the toxin(s) (larvicidal factor(s)) activity as this information will help in the assessment of the suitability of using *B. sphaericus* for the control of mosquitoes in tropical countries.

Materials and methods. Bacillus sphaericus 1593-4 and 1691 were obtained from Dr L.A. Bulla of USDA, USA. Our studies have shown that strains 1593-4 and 1691 are more active against *Culex fatigans* of southern India than SSII-1 and 1404 strains (unpublished observations).

Heat-shocked spores of these strains were maintained in 50% glycerol at −20 °C. Cells were grown in a rotary shaker in nutrient broth medium at 30 °C. Under these conditions the culture with an inoculum of 10^7 spores/200 ml entered the stationary phase after 18 h and the sporulation phase after 24 h. The cells were harvested either after 12 h of growth or after 24 h of growth (sporulating cells). The extract of the sporulating cells was obtained by sonicating the cell suspension in saline at 0-2 °C for 2 min. The vegetative cells were heat killed at 80 °C for 15 min and then sonicated as above. (The larvicidal factor(s) is resistant to heat up to 80 °C.)

The cell extracts were added at suitable dilutions to 100 ml of tap water (source: from deep bore wells with low bacterial count) containing 10 3rd instar mosquito larvae. The temperature of the larval culture was brought to 23-25 °C or 33-35 °C, at least 2 h before the addition of the toxin(s). The species of mosquito tested were *C. fatigans* and *A. subpictus* (very common species on the Madurai Kamaraj University campus). Yeast tablets were used as food for the larvae. Each assay was set up in triplicate. Mortality counts were made after 24 h. The potency of the extract used in the assay is represented as the minimal concentration of the extract (expressed as µg protein) added to 100 ml of the larval culture that can cause 50% mortality (LD 50). LD 50 values were calculated graphically. Protein concentration in the extracts was estimated by the method of Lowry et al.[6].

Results and discussion. When the larval culture temperature was 33-35 °C *C. fatigans* required an extract of sporulating *B. sphaericus* 1593 cells containing 1.8 µg protein/100 ml to cause 50% mortality, whereas at 23-25 °C about 6 times more larvicide (extract) was needed to achieve this result (table).

A. subpictus was less susceptible to the larvicidal factor(s) than *C. fatigans.* Interestingly, in this case also the toxin(s) required for LD 50 at 23-25 °C was about 6 times more than that required for LD 50 at 33-35 °C (table).

Sporulating cell extract of *B. sphaericus* 1691 also showed a similar activity pattern. At low temperature (23-25 °C) the toxin(s) was about 5 times less active in comparison with its action at 33-35 °C against both *C. fatigans* and *A. subpictus.* Here again *C. fatigans* was more susceptible than *A. subpictus.*

The sporulating cells were several times more toxic than the vegetative cells to both *C. fatigans* and *A. subpictus* (table). Singer also observed that the most potent cells are those which have just finished vegetative growth and have entered the sporulation cycle[7].

The cadavers of the larvae obtained by the action of sporulating cell extract contained live bacteria. Infection, and multiplication of bacteria, could be faster at the higher temperature. To investigate this possible explanation for the increased mortality observed at high temperature, an extract from heat-killed vegetative cells of *B. sphaericus* 1593-4 was tested for larvicidal activity at 33-35 °C and 23-25 °C. Once again, the larvicidal action of the extract was approximately 6 times more at 33-35 °C than at 23-25 °C (table).

Culture as well as microscopic examination of the cadaver homogenate revealed the absence of any *B. sphaericus* cells, either alive or dead, in the case of treatment with heat-killed vegetative cells. Thus the present studies show that the action of the larvicidal factor(s) is dependent on temperature. Therefore the use of *B. sphaericus* 1593-4 and 1691 is particularly suitable for tropical countries provided other, useful, organisms are safe.

The larvicidal factor(s) has no effect on the pupae (non-feeding stage) when added in the medium. Our preliminary studies have shown that certain mid-gut protease(s) can release the toxin(s) from the cells (unpublished observations). Presumably, the released toxin(s) enters the gut epithelial cells.

In this connection it should be noted that ricin undergoes a conformational change after binding to its receptor present in the mosquito cell-surface which helps the entry of this toxin into the cell. Action of ricin is inhibited at low temperature[5]. It is quite likely that *B. sphaericus* toxin(s) also has a temperature-dependent entry mechanism into the gut epithelial cells.

Effect of temperature on the action of *B. sphaericus* 1593-4 and 1691 toxin(s) in mosquito larvae. Values are µg protein of the sonicated cells required for LD 50/100 ml larval culture containing 10 larvae

	Larval culture temperature of			
	Culex fatigans		*Anopheles subpictus*	
	33-35 °C	23-25 °C	33-35 °C	23-25 °C
Sporulating *B. sphaericus* 1593-4 cell extract	2.2 ± 0.24	12.7 ± 1.4	23.1 ± 2.3	154 ± 6.4
Sporulating *B. sphaericus* 1691 cell extract	3.1 ± 0.28	14.8 ± 1.6	24 ± 2.1	132 ± 5.2
Heat-killed vegetative *B. sphaericus* 1593-4 cell extract	101 ± 10.9	603 ± 49	-	-

Values at 33-35 °C are statistically different from values at 23-25 °C. $p < 0.001$ mean ± SE.

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The effects of naloxone on the analgesic activities of general anaesthetics

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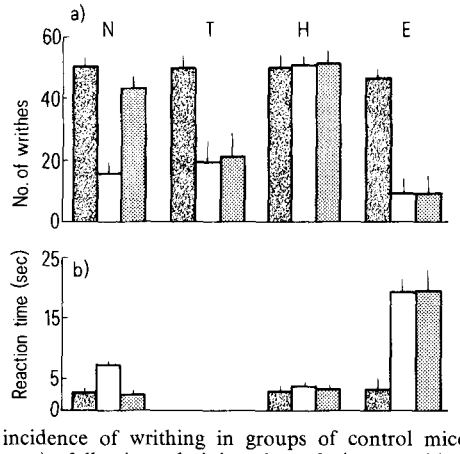
Summary. Inhaled concentrations of nitrous oxide (80%), halothane (0.5%), trichloroethylene (0.5%) and s.c. ethanol (1 ml/kg) caused similar degrees of excitation and ataxia in mice. Nitrous oxide, tricholoroethylene and ethanol caused analgesia (hot plate and writhing tests), but only that caused by nitrous oxide was antagonized by naloxone (20 mg/kg). Halothane lacked analgesic activity.

Anaesthetics differ in their analgesic potency at sub-anaesthetic concentrations. Whilst nitrous oxide and trichloroethylene are clinically useful analgesics, halothane has negligible acitivity[1], and barbiturates have been reported as being hyperalgesic[2]. The recent reports that the analgesic but not the anaesthetic action of nitrous oxide may be antagonised by naloxone implies that this drug releases endorphins[3,4]. The present experiments were designed to determine whether the analgesic actions of some other anaesthetics including trichloroethylene could be similarly explained.

Materials and methods. Groups of female mice (35–40 g) were used. Central depression was measured using an Animex meter set at 40 μA. Groups of 5 mice were placed on the counter in a Perspex equilibration chamber of floor area 580 cm^2. Activity was recorded at 1-min intervals for 15 min, then the gases were passed in at flow rates of 6 l/min (Rotameter). Activity was recorded for a further 35 min. Results are expressed as the difference from appropriate controls examined at the same time of day.

Mixtures of nitrous oxide in oxygen were delivered via Rotameters, those of halothane (Fluotec) and trichloroethylene (Tritec) using their respective vapourisers (Cyprane). Ethanol and naloxone were administered by s.c. injection.



a The incidence of writhing in groups of control mice (textured columns), following administration of nitrous oxide (80% N), trichloroethylene (0.5% T), halothane (0.5% H) and ethanol (1 ml/kg E) before (white columns) and after naloxone 20 mg/kg (shaded columns). *b.* Hot plate reaction times of groups of mice exposed to nitrous oxide, halothane and ethanol before and after naloxone (code as above). Limits are + SE.

Analgesia was assessed using 2 methods. Writhing was induced by the i.p. injection of 0.75% acetic acid (100 mg/kg). For the experiments using gases injection of acetic acid was immediately followed by naloxone or saline, and the mice placed in an equilibration chamber (9000 cm^3) through which gas passed at 6 l/min. The end point was taken as an extension of one or both hind legs accompanied by arching of the back and abdominal concavity. The number of such movements in the following 20 min was expressed as percentage inhibition from concurrent saline controls. The comparisons between naloxone and saline were performed 'blind'. The effect of naloxone was expressed as a percentage of the appropriate control. The hot plate was maintained electrically at 55 °C. Mice were placed in the same equilibration chamber through which passed the gas at a rate of 6 l/min. Following 10-min exposure a mouse was quickly transferred to an open-ended perspex box placed on the plate through which passed the same anaesthetic gas. The end point was taken as a sign of discomfort in a hind paw. A cut-off time of 45 sec was employed.

Each anaesthetic was examined using both tests except trichloroethylene which was not tested on the hot plate because of the drug's potential degradation on exposure to the heating element of the plate. Soda lime was used in the circuits in all experiments except those using trichloroethylene. Statistical comparisons were made using Student's t-test.

Results. The selection of doses of anaesthetics was made empirically based on the behavioural effects of a range of doses of anaesthetics. Nitrous oxide (100%), trichloroethylene and halothane (1%) and ethanol (2 ml/kg) caused rapid anaesthesia (< 3 min), followed by almost immediate recovery for the gases though ethanol had a longer duration of action.

The doses subsequently chosen were halothane and trichloroethylene (0.5%), nitrous oxide (80%) and ethanol (1 ml/kg). Doses of naloxone between 1 and 20 mg/kg were used. At these doses, halothane, trichloroethylene and ethanol all caused a similar increase in activity during the 15 min following administration. The increases (± SE) were respectively 257.7 (87), 258.1 (56.4) and 249.2 (145.7) Animex counts/15 min. These increases were significant ($p < 0.05$).