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THE ACTIVITY OF METRIFONATE AGAINST INFECTIVE LARVAE AND MAMMALIAN STAGES OF *BRUGIA PAHANGI*

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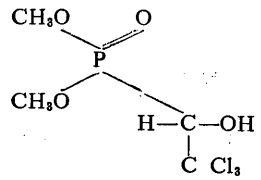
Shiu-Nan Chen (1982) The activity of metrifonate against infective larvae and mammalian stages of *Brugia pahangi*. *Bull. Inst. Zool., Academia Sinica* **21**(2): 113-119. Metrifonate, an organophosphorus cholinesterase inhibitor, was tested for efficacy against infective larvae and mammalian stage of *Brugia pahangi in vitro*.

In growth medium 199, levels equal to or above 10 mg/l concentration, the mortality of experimental worms was significantly higher than that of the control worms at 37±0.5°C. In 10 mg/l and 25 mg/l metrifonate solutions, the experimental worms were killed within 2 days *in vitro*. All the infective 3rd stage larvae were killed within 12 hours' incubation in a metrifonate concentration of 50 mg/l at 37± 0.5°C. There was no significant difference on the activity of metrifonate against infective larvae in growth medium 199 at pH values within a range of 6.3 to 7.6. In growth medium 199 with dog sarcoma cells, metrifonate at a concentration of 25 mg/l resulted in approximately 55% becoming inactive within 6 days' incubation at 37±0.5°C. At a concentration of 50 mg/l, the experimental larvae were killed after 24 hours' incubation.

In comparison, approximately 100-fold metrifonate concentration of suramin was used to killed the infective larvae with 12-24 hours' incubation in HBSS or growth medium 199 at 37±0.5°C.

Metrifonate, an organophosphorus cholinesterase inhibitor, has been used in the treatment of some animal and human helminthioses for several years[4,5,13]. The structural formula of this compound is shown below:



Originally this compound was marketed as Dipterex (Bayer) and used as general insecticide for crop protection. It was issued in pure form for human trials as Bayer 13/59, Bayer 2349, Dipterex® and Dylox® and the generic name of trichlorophon was accepted. This later changed to the international nonproprietary name of metrifonate. In 1962 Cerf *et al.*[4] first considered the potential of this compound for controlling invertebrates other than insects, including the helminths. Thereafter, considerable sucess was reported in the chemotherapy of human schistosomiasis by this compound[5].

Metrifonate was found to immobilise the microfilariae and adults of *Onchocerca volulus in vitro* and also demonstrated to be effective against adult worm of *O. volvulus* in human patients and followed by a decrease of microfilarial count[13]. In the experiments with *Brugia pahangi* infected cats Denham *et al.*[6] reported that the oral administration of metrifonate to the cat was followed by a decline in numbers of circulating microfilariae. They also indicated that metrifonate was active against

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both microfilariae and macrofilariae, but probably its greatest effect was on the macrofilarial stages.

The present study was an attempt to investigate the activity of metrifonate *in vitro* against macrofilariae including infective larvae early mammalian stages of *B. pahangi*. The effectiveness of metrifonate in the *in vitro* culture system was also compared with that of suramin and diethylcarbamazine (DEC).

MATERIALS AND METHODS

The procedures adopted for the preparation of the infective larvae and mammalian stages of *Brugia pahangi* were described previously[2]. Infective larvae and 2 and 3-day old juveniles of *B. pahangi* were used in this experiment. Prior to the experiments, larval worms were washed three times in sterile Hank's balanced salt solution (HBSS) with 1000 μg/ml of crystamycin (Glaxo Laboratories Ltd, Greenford, England) and then serilised as described previously[3]. Experiments were carried out in a series of 25 cm^2 culture flasks (Becton Dickson, UK Ltd) as described in the following sections.

The effects of metrifonate on the survival of infective larvae, and 2 and 3-day old juveniles of *Brugia pahangi*

Series of drug concentrations ranging from 50 mg/l to 1 mg/l were made by diluting 500 mg/l solution of metrifonate in tissue culture medium 199 with 400 μg/ml of crystamycin (GM 199) in Falcon flasks. 100 sterilised infected larvae or juvenile worms were then transferred from centrifuge tubes to flasks containing culture medium only or various concentration of metrifonate respectively. Both test and control experiments were performed at least in duplicate. The flasks were then incubated in a 37$\pm$0.5°C incubator and observed at 12 hours, 24 hours and then daily intervals, using a Zeiss Inverted Microscope and a Zeiss Dissecting Microscope. The mortality of the larvae and juvenile worms with times was recored.

The effects of pH on the efficiency of metrifonate against *Brugia pahangi* infective larvae

The infective larvae were tested to demonstrate the efficiency of metrifonate in media at different pH value. In this experiment 20 mmol HEPES (Flow Laboratories, Irvine, Scotland) buffered GM 199 was prepared and adjusted with 1N NaOH to give a pH of 6.3, 6.9 and 7.6 respectively. A drug concentration of 10 mg/l was made by diluting 100 mg/l solution of metrifonate in GM 199 with different pHs. Subsequently 100 sterilised infective larvae were added to the Falcon flasks containing 10 ml of culture alone or 10 mg/l metrifonate medium respectively. Each experiment was performed at least in duplicate. The larvae were incubated and observed as described above.

The effects of dog sarcoma cell line on the efficiency of metrifonate against *Brugia pahangi* infective larvae

Frior to the experiment, a series of Falcon flasks containing a good monolayer of dog sarcoma (DS) cell line was prepared as described previously[3]. Into the cell line, a volume of 10 ml fresh GM 199 containing a concentration 50 mg/l, 25 mg/l, 10 mg/l, 5 mg/l and 1 mg/l metrifonate was added respectively. Subsequently, 100 sterilised infective larvae were added to the medium. The infective larvae in 10 ml of GM 199 incorporated in the presence of DS cells, served as control. The larvae were incubated at 37$\pm$0.5°C and examined as described above.

The effects of diethylcarbamazine (DEC) and suramin on the survival of *Brugia pahangi* infective larvae

To compare the efficiency of DEC and suramin with metrifonate, the survival of infective larvae in media containing three different concentrations of suramin or DEC were tested, i. e. 100 mg/l, 500 mg/l and 1000 mg/l. Those various concentration of DEC were made by diluting a 2000 mg/l in GM 199. Whilst, the suramin was diluted in HBSS. The pH of DEC solution was adjusted to 7.0, using 4.4% $NaHCO_3$. 100 sterile infective larvae were added to each concentration of drug and the control. The experimental larvae were incubated and observed as described above.

RESULTS

Activity of metrifonate against infective larvae and early mammalian stages in GM 199

The effects of metrifonate in GM 199 on the mortality of infective larvae and 2 and 3-day old juveniles at 37±0.5°C are shown in Figs. 1-3. In 1 mg/l metrifonate solution the experimental larvae and juveniles showed approximately the same mortality rate as those obtained when the larval worms were incubated in 5 mg/l solution. At levels equal to or above 10 mg/l concentration, the survival of the experimental worms was significantly lower than that of the control worms. In 10 mg/l and 25 mg/l solutions, infective stage larvae and 2 or 3-day old juveniles were killed within 2 days *in vitro*. Metrifonate at concentration of 50 mg/l killed all experimental infective 3rd stage larvae within 12 hours' incubation at 37 ±0.5°C.

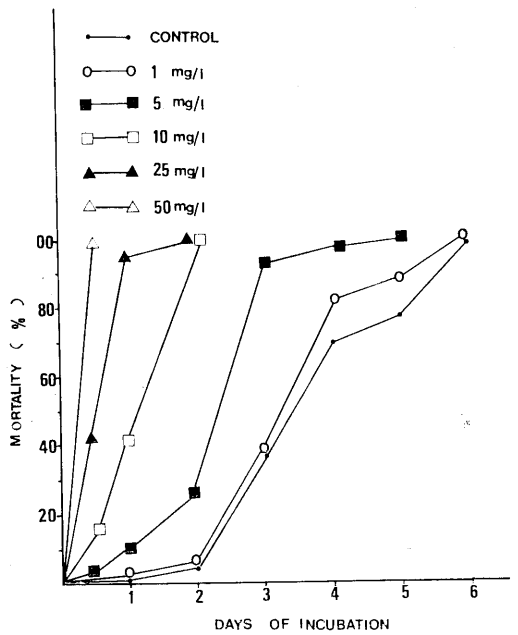

Fig. 1. Mortality of *Brugia pahangi* infective larvae from mosquito (*Aedes aegypti*) incubated in GM 199 containing various concentration of metrifonate at 37±0.5°C.

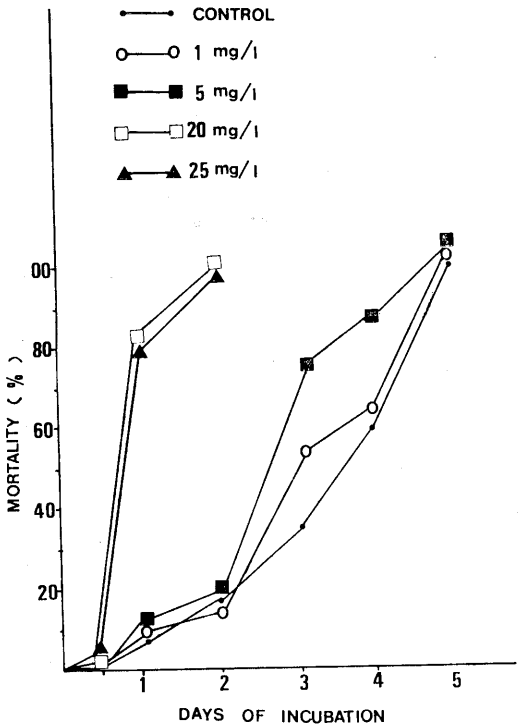

Fig. 2. Mortality of *Burgia pahangi* 2-day old juveniles from jird (*Meriours unguiculates*) incubated in GM 199 containing various concentrations of metrifonate at 37±0.5°C.

Activity of metrifonate against infective larvae in GM 199 with different pH value

The effect of incubating the infective stage larvae in 10 mg/l metrifonate solution with different pH value at 37±0.5°C was presented in Fig. 4. The result showed that there was no significant difference in the activity of metrifonate against infective larvae in GM 199 at pH values within a range of 6.3 to 7.6.

Activity of metrifonate against infective larvae in dog sarcoma (DS) cell culture

Fig. 5 shows the activity of metrifonate on infective larvae of *B. pahangi* in GM 199 with DS cells. Contral culture larvae remained active, with 17% mortality rate, throughout the 6 days of observation. Metrifonate at concentrations of 25 mg/l or lower resulted in

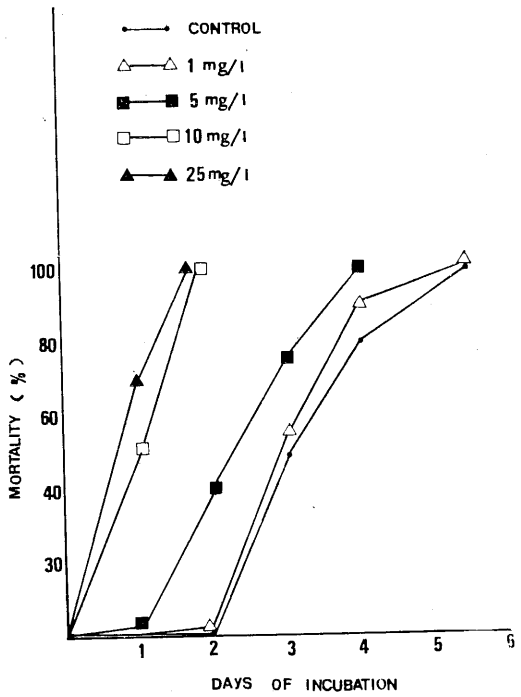

Fig. 3. Mortality of *Burgia pahangi* 3-day old juveniles from jird (*Meriones unguiculatus*) incubated in GM 199 containing various concentrations of metrifonate at 37±0.5°C.

up to 55% of larvae becoming inactive within 6 days' incubation. However, at concentrations of 50 mg/l 65% of the experimental larvae were killed within 12 hours' incubation. After 24 hours' incubation at this concentration, all the larvae were killed. This result constrasts markedly with that obtained in cell free culture system and illustrated in Fig. 2 and 5 demonstrated the decrease of efficiency metrifonate against infective larvae in GM 199-DS cell culture system when compare with GM 199 culture system.

Activity of DEC and suramin against infective larvae in GM 199 and HBSS

The activity of DEC on the infective larvae in GM 199 is presented in Fig. 6. The control culture larvae remained alive for 5 days in GM 199. In the larvae incubated in 100 mg/l concentration of DEC approximately the same mortality rate as that of the control culture was obtained throughout 5 days' incubation. However, all Infective larvae were killed by 1000 mg/l concentration with 6 hours and all larvae died within 24 hours in GM 199 containing 500 mg/l DEC.

Fig. 7 shows the activity of suramin against infective larvae of *B. pahangi* in HBSS. When the infective larvae were incubated in HBSS, they survived for approximately 48 hours. 100 mg/l showed no effect on the survival of 1000 and 500 mg/l suramin, the larvae were killed within 6 hours and 12 hours respectively.

DISCUSSION

The present results demonstrate that metri-

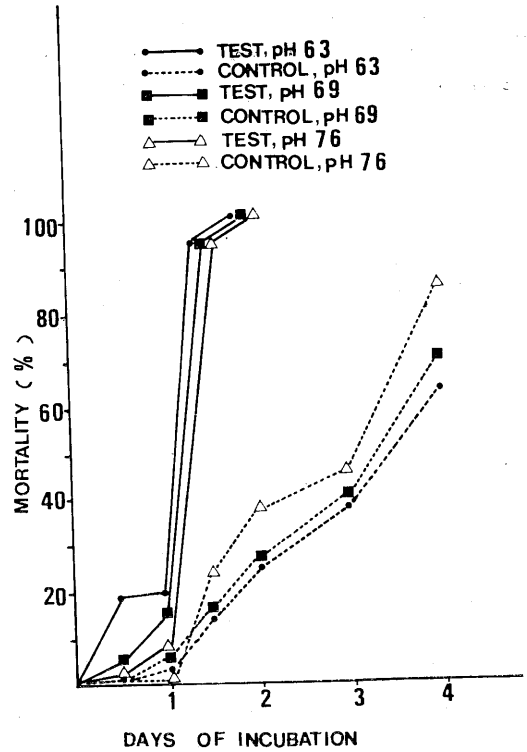

Fig. 4. Mortality of *Burgia pahangi* infective larvae from mosquito (*Aedes aegypti*) incubated in GM 199 with different pH value containing metrifonate of 1 mg/l at 37±0.5°C.

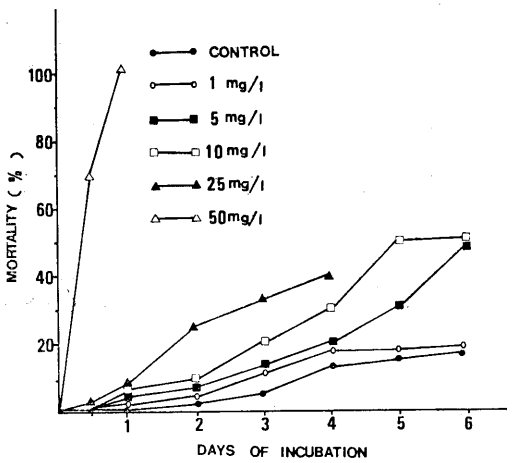

Fig. 5. Mortality of *Burgia pahangi* infective larvae from mosquito (*Aedes aegypti*) incubated in GM 199-DS cell culture containing various concentrations of metrifonate at 37±0.5°C.

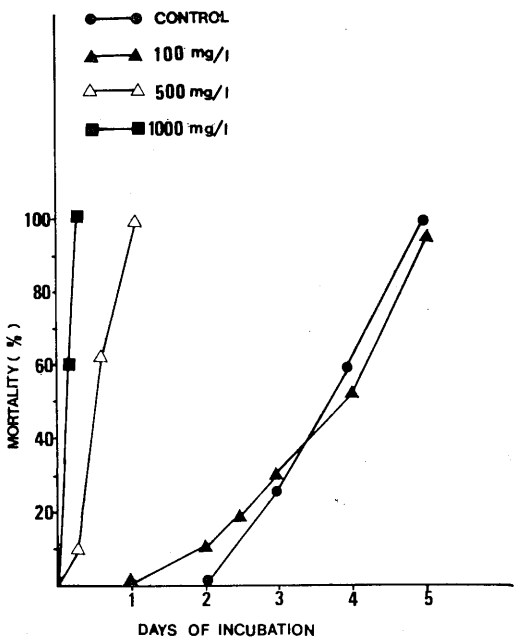

Fig. 6. Mortality of *Burgia pahangi* infective larvae mosquito (*Aedes aegypti*) incubated in GM 199 containing various concentrations of diethylcarbamazine (DEC) at 37±0.5°C.

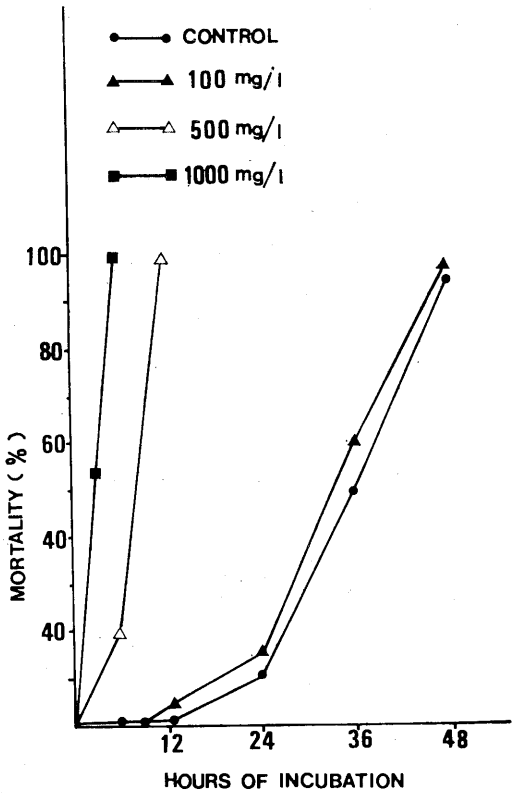

Fig. 7. Mortality of *Burgia pahangi* infective larvae from mosquite (*Aedes aegypti*) incubated in Hank's balanced salt solution (HBSS) containing various concentrations of suramin at 37±0.5°C.

fonate revealed filaricidal effects *in vitro* against the infective larvae and 2 or 3-day old juveniles at concentrations above 10 mg/l. *In vivo*, Denham *et al.*[6] reported that the dose used to kill the infective larvae in cat was at 25 mg/kg body weight/day. In comparison, in GM 199 and DS culture the concentration of drug required to kill the infective larvae within 24 hrs. was approximately 25-50 mg/l and 50 mg/l respectively. Those figures, therefore, indicate that the concentration of metrifonate required to kill the infective larvae *in vitro* is slightly higher than the concentration needed to kill the larvae *in vivo*.

Using levamisole Rogers and Denham[12] showed that *B. pahangi* infective larvae were

killed *in vitro* within 24 hours at approximately 0.05% concentration. This concentration was slightly higher than that needed to kill the infective larvae at the same period *in vitro* in this study.

No sign of effect of DEC against infective larvae was demonstrated by using 100 mg/l concentration of the compound. The infective larvae were killed in 24 hours *in vitro* in 500 mg/l DEC.

It is known that suramin is a drug which is effective against *Onchocerca volvulus*. However, the present results indicated that this compound possessed no filaricidal effect against *B. pahangi in vitro* at a concentration of 100 mg/l.

The mode of action of metrifonate on filarial worm is still not known. However, the insecticidal action of this compound depends on the inhibition of a specific esterase (cholinesterase) in ganglional synapses and neuromuscular junctions[5]. The presence of cholinesterase activity and reaction of chlinesterase with organophosphate in some nematodes were demonstrated by Lee and Hodsen[9], Kowles and Casida[8] and Hart and Lee[7]. The presence and distribution of this enzyme were also found in some filarias including adult worms of *Litomosoides carinii*[1], *Dipetalonema viteae*[10] and microfilariae of *Wuchereria bancrofti*[11]. Recently, Howells (unpublished results) demonstrated the presence and distribution of cholinesterase in the microfilariae and adult worms of *B. pahangi*. It is suggested that the activity of metrifonate against *B. pahangi* is probably also based on the inhibition of cholinesterase activity in the worms.

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REFERENCES

1. BUEDING, E. (1952) Acetycholinesterase activity of *Schistosoma mansnni*. *Brit. J. Pharmacol.* **7**: 563-566.
2. CHEN, S. N. and R. E. HOWELLS (1979*a*) The uptake *in vitro* of dyes, monosaccharides and amino acids by the filarial worms *Brugia pahangi*. *Parasitology* **78**: 343-354.
3. CHEN, S. N. and R. E. HOWELLS (1979*b*) The *in vitro* cultivation of the infective larvae and the early mammalian stages of the filarial worm, *Brugia pahangi*. *Ann. Trop. Med. Parasitol.* **73**: 473-486.
4. CERF, J. A. LEBRUN and J. DIERICKX (1962) A new approach to helminthiasis control: the use of an organosphorous compound. *Am. J. Trop. Med. Hyg.* **11**: 514-517.
5. DAIVS, A. and D. R. BAILEY (1969) Metrifonate in urinary schistosomiasis. *Bull. Wld. Hlth. Org.* **41**: 209-224.
6. DENHAM, D. A., T. POUNDURAI, G. S. NELSON, F. GUY and R. ROGERS (1971) The effect of metrifonate on *Brugia pahangi* infections in domastic cats. *Bull. Wld. Hlth. Org.* **45**: 423-429.
7. HART, R. J. and R. M. LEE (1966) Cholinesterase activies of various nematode parasites and their inhibition by the organophosphate anthelmintic haloxon. *Exp. Parasitol.* **18**: 332-337.
8. KNOWELS, C. O. and J. CASIDA (1966) Mode of action organophosphate anthelmintics cholinesterase inhibition in *Ascaris lumbricoides*. *J. Agric. Fd. Chem.* **14**: 566-572.
9. LEE, R. M. and M. R. HODSDEN (1963) Cholinesterase activity of *Haemonchus contortus* and its inhibition by organophosphorus. *Biochem. Pharmacol.* **12**: 1241-1252.
10. MCLAREN, D. J. (1972) Ultrastructural and cytochemical studies on the sensory organelles and nervous system of *Dipetalonema viteae* (Nematoda: Filarioida). *Parasitology* **65**: 507-524.
11. OMAR, M. S. and F. KUHLOW (1977) Cholinesterase activity in the microfilaria of *Wuchereria bancrofti* Cobbolt. *Tropenmed. Parasitol.* **28**: 267-470.
12. ROGERS, R. and D. A. DENHAN (1976) Studies with *Brugia pahangi* 12 the activity of levamisole. *J. Helminthol.* **50**: 21-28.
13. SALAZAR-MALLEN, M., D. GONZALEZ-BARRANCO and D. MARTINLEVY (1969) Trichlorphone treatment of onchocerciasis. *Ann. Trop. Med. Parasitol.* **65**: 393-398.

Metrifonate 對血絲蟲 (*Brugia pahangi*) 第三期幼蟲及青春期蟲體之影響

陳 秀 男

本實驗乃在探討二種合成藥物 Metrifonate 於試管內對血絲蟲，*Brugia pahangi* 之效用。

實驗結果顯示：在 G.M. 199 溶液內，於 37±0.5°C 之培育溫度下，當 Metrifonate 濃度在 10 mg/ℓ 以上時，則實驗蟲體之死亡率較控制組有顯着之增加。在藥之濃度 10 mg/ℓ 及 25 mg/ℓ 時則實驗蟲體於 2 天內死亡。若于 50 mg/ℓ 時則 12 小時內，蟲體會死亡。本實驗並證實在 G.M. 199 內，當溶液 pH 值於 6.3 至 7.6 時藥物效果並無顯着之不同，若將實驗於 G.M. 199-Dog Sarcoma 細胞內進行時，則實驗結果顯示：在 37±0.5°C 培育溫度下，Metrifonate 濃度 25 mg/ℓ 時經 6 天後，則 55%之蟲體會變得不活動。而於 50 mg/ℓ 之濃度下，實驗蟲體會於 24 小時內死亡。

另外，本實驗亦利用最常應用於血絲蟲病治療之藥物 DEC 及 Suramine 來比較 Metrifonate 之效果。實驗結果顯示：在 37±0.5°C 之培育溫度下，DEC 及 Suramine 必須 Metrifonate 100 倍濃度才能於 12～24 小時內殺死實驗蟲體。