# Effect of Heptachlor and Related Compounds on Growth of *Staphylococcus Aureus*

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Previous work in this laboratory has shown that the growth of Staphylococcus aureus in broth media was affected by as little as 3 ug per milliliter of heptachlor (2,4). In addition, pesticides containing 72% heptachlor were found to be more inhibitory than those containing 99.8% heptachlor (4).

This study was made to determine the cause of the greater inhibition of 72% heptachlor and to obtain additional information on factors affecting the inhibition of S. aureus by heptachlor and related compounds.

## Methods and Materials

Pesticides. The following pesticides were used in this study: purified chlordane, 99.8% gamma chlordane, 99.5% nonachlor and 72, 73, 74, 99 and 99.8% heptachlor. Stock solutions of each pesticide were made to contain 3 mg per milliliter of absolute alcohol. The stock solutions were diluted with alcohol so that addition of 2 ml or less to 50 to 300 ml of sterile media would result in concentrations of 5 to 50 ug per ml of media. The pesticides were added to the sterile media prior to inoculation with S. aureus.

Organism and cultural procedure. A 15 to 18 hour trypticase soy broth (TSB) culture of S. aureus (Department of Animal Sciences, Food Science Section, University of Kentucky) was used to inoculate flasks containing the desired pesticide and either TSB or skimmilk. Control flasks contained media with and without alcohol and were treated the same as flasks containing pesticides. All flasks were incubated at 37 C either in a water bath or incubator. In one part of the study, the flasks were shaken at 140 oscillations per minute during incubation in a water bath.

Determination of growth. Determinations of numbers of bacteria were made every 2 hours during the initial 12 to 24 hours, and then daily for the duration of the incubation period. Numbers of S. aureus were determined by making standard plate counts on trypticase soy agar and incubating the plates at 37 C for 48 hours (1). In several trials, growth also was determined by following change in turbidity at 600 mu with a Klett-Summerson



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colorimeter. Plate counts were transformed to logarithms in order to facilitate comparison of the effect of pesticides on growth and to determine generation times. Generation times were determined according to Lamanna and Mallette (3).

## Results and Discussion

The effects of the pesticides studied on the growth of Staphylococcus aureus in trypticase soy broth are shown in Table 1. All pesticides were found to cause a fairly rapid decrease in the number of bacteria added to the broth (initial count) at 0 hour. They also caused increases in both the length of the lag period and the generation time.

Decreases in the initial count resulting from the pesticides, ranged from 8.3% (99% heptachlor) to 51.1% (99.8% gamma chlordane). Except for gamma chlordane, the largest decreases in the initial count were caused by pesticides containing 72 to 74% heptachlor. In general, pesticides containing at least 99% heptachlor had the least effect on the initial count. No detectable decrease in the initial count was caused by the absolute alcohol used as a solvent for the pesticides.

Depending on the pesticide, the number of bacteria continued to decrease for up to 24 hours before the start of their logarithmic growth phase. Thus, the lag period varied from less than 2 hours for control, absolute alcohol and nonachlor, to 24 hours for chlordane. Next to nonachlor, pesticides containing 99% or more heptachlor caused the shortest lag periods (4 to 8 hours).

The average generation time (32.5 min.) observed for S. aureus in TSB was increased by the pesticides as well as the alcohol. The increases observed ranged from 3 min. for alcohol to 324.5 min. for chlordane.

No relationship was found between percent decrease in the initial count and resulting generation time. Gamma chlordane, which caused the largest decrease in the initial count, caused the second smallest increase in generation time. Pesticides containing 72 to 74% heptachlor caused greater reductions in the initial count than those containing 99% or more heptachlor but, with one exception, had less effect on generation times.

Previous work has shown that growth of S. aureus in TSB was affected more by pesticides containing 72 to 74% heptachlor than by those containing 99% or more heptachlor (4). Results of this study suggest that the effect on viability as well as length of lag phase depends on the amount of chlordane and gamma chlordane rather than heptachlor contained in the pesticides. These compounds are common components of technical (72 - 74%) heptachlor. On the other hand, the generation time of S. aureus in TSB appears to depend on the amount of heptachlor

and possibly chlordane contained in the pesticide. Gamma chlordane appears to have the greatest initial effect on viability, but little effect on subsequent growth rate, whereas, both heptachlor and chlordane have little effect on initial viability, but considerable effect on subsequent growth rate.

TABLE 1

Effect of pesticides on the growth of Staphylococcus aureus in trypticase soy broth at 37 C

| Pesticide (10 ug/ml) | ($Log_{10}$)[a] | Minimum viable count ($Log_{10}$)[b] | (hr)[c] | Decrease in initial count (%) | Generation time[d] (Min) |
|---|---|---|---|---|---|
| Control | 5.00 | 5.00 | 1 | 0 | 32.5 |
| Absolute alcohol | 4.97 | 4.97 | 1 | 0 | 35.5 |
| Heptachlor - 72% | 4.98 | 2.88 | 12 | 42.2 | 106.0 |
| Heptachlor - 73% | 4.96 | 3.03 | 10 | 38.9 | 130.5 |
| Heptachlor - 74% | 4.94 | 2.85 | 12 | 42.3 | 190.6 |
| Heptachlor - 99% | 4.93 | 4.52 | 4 | 8.3 | 158.5 |
| Heptachlor - 99.8% | 4.96 | 4.33 | 8 | 12.7 | 210.7 |
| Chlordane - Purified | 5.26 | 3.38 | 24 | 31.7 | 357.0 |
| Gamma chlordane - 99.8% | 5.05 | 2.47 | 10 | 51.1 | 50.5 |
| Nonachlor - 99.5% | 4.91 | 4.32 | 2 | 12.0 | 42.8 |

[a]Number of bacteria added to the broth at 0 hr.
[b]Lowest count obtained during incubation.
[c]Time from 0 hr to reach minimum count and to enter logarithmic growth phase.
[d]Generation time determinations made only during logarithmic growth phase.

Effect of the size of the initial population. On the average, $9 \times 10^3$, $10 \times 10^4$, $8 \times 10^5$, and $10 \times 10^6$ cells per milliliter were added to TSB containing 5 ug/per milliliter of 73% heptachlor or chlordane and growth followed over a 12 hour period.

Since similar results were obtained for the three smaller inoculums, only the results obtained for the $10 \times 10^4$ inoculum (low initial population) and for the $10 \times 10^6$ inoculum (high initial population) are shown in Figure 1. Irrespective of the size of inoculum, numbers of S. aureus declined within the first two hours when exposed to heptachlor. The decrease continued until the 6th to 8th hour of incubation, at which time the logarithmic growth phase began. The decrease in the numbers of S. aureus was less as the size of the initial population was increased.

The decrease in numbers observed for chlordane was not as rapid or as great as that for heptachlor, but the decrease

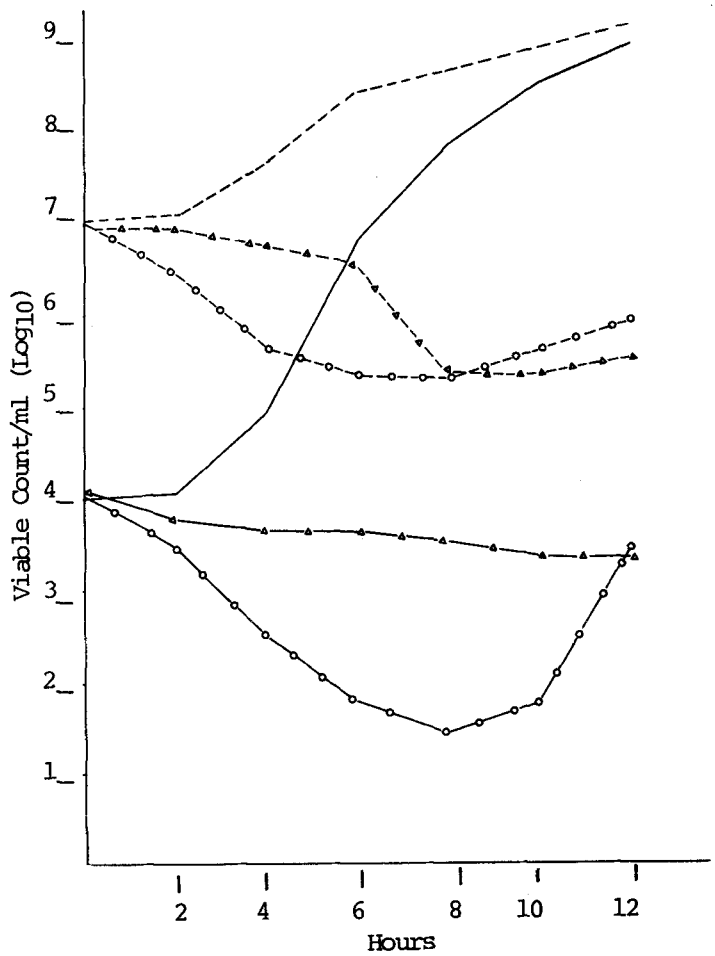


Fig. 1 Effect of initial population on the growth at 37 C of Staphylococcus aureus in trypticase soy broth containing 5 ug/ml of 73% technical heptachlor (TH) and chlordane (C). Symbols: low population control (———), TH (—o—) and C (—▲—); high population control (— — —), TH (--o--), and C (--▲--).

lasted for a longer period. Logarithmic growth phase was observed only for the high initial population ($10 \times 10^6$) and then only after the 10th hour. All other populations were still slowly decreasing after 12 hours. Further studies showed that logarithmic growth phase did not occur until the 24th hour.

Similar studies using the same size inoculums were made using skimmilk in place of TSB and increasing the level of 73% heptachlor from 5 to 50 ug per milliliter and chlordane from 5 to 10 ug per milliliter. Results obtained for growth of S. aureus at each of the initial populations in skimmilk plus the pesticides did not differ from those obtained for growth in the skimmilk controls. Thus the growth of S. aureus was not affected by heptachlor or chlordane when grown in skimmilk. Apparently, constituents of skimmilk prevent pesticides from affecting the growth of S. aureus (2,4).

The decreased effect of pesticides on S. aureus as the initial population is increased, is probably due to one or both of the following: a) more cells are able to survive the sublethal level of pesticide, b) better protective colloid (protein) effect due to increased numbers of cells (viable and nonviable). Casein has been shown to be able to protect S. aureus from being inhibited by up to 100 ug per milliliter of heptachlor (4). These previous results plus those obtained during this study suggest that bacterial protein also may help protect S. aureus from inhibition by pesticides. As the initial population is increased, there are proportionately more cells available to protect others from the pesticides. As a result, more cells are able to survive the toxic effect of the pesticide. Thus, the overall decrease in initial population is less and growth is able to begin sooner, and proceed at a more rapid rate.

Effect of static vs shaking incubation. Growth of S. aureus in skimmilk containing as much as 50 ug per milliliter of 73% heptachlor or 10 ug per milliliter of chlordane was not affected by the type of incubation nor by the size of the inoculum.

However, both the size of inoculum and type of incubation affect growth of S. aureus in TSB containing only 5 ug per milliliter of heptachlor or chlordane. The results obtained for the low initial population ($10 \times 10^4$) under both incubation conditions are shown in Figure 2. Growth, as measured by increase in turbidity in the pesticide samples, did not increase under shaking incubation during the 48 hr incubation period. On the other hand, under static incubation, the turbidity began to increase after 24 hours.

Unlike the results obtained for the low initial population, turbidity for the high initial population ($10 \times 10^6$) was greater in pesticide samples under shaking incubation (Figure 3). Under both incubation conditions, turbidity began to increase

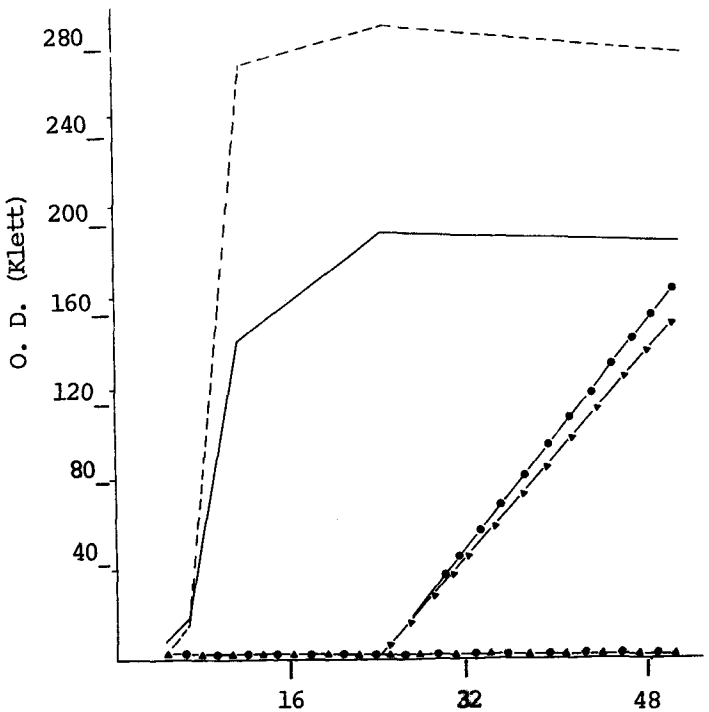


Fig. 2
Effect of static and shaking incubation at 37 C on growth of low initial population ($10^4$/ml) of Staphylococcus aureus in trypticase soy broth containing 5 ug/ml of 73% technical heptachlor (TH) and chlordane (C). Symbols: Static incubation control (——), TH (—●— ), and C (—▲—); shaking incubation control (— — —), TH and C (—▲— —).

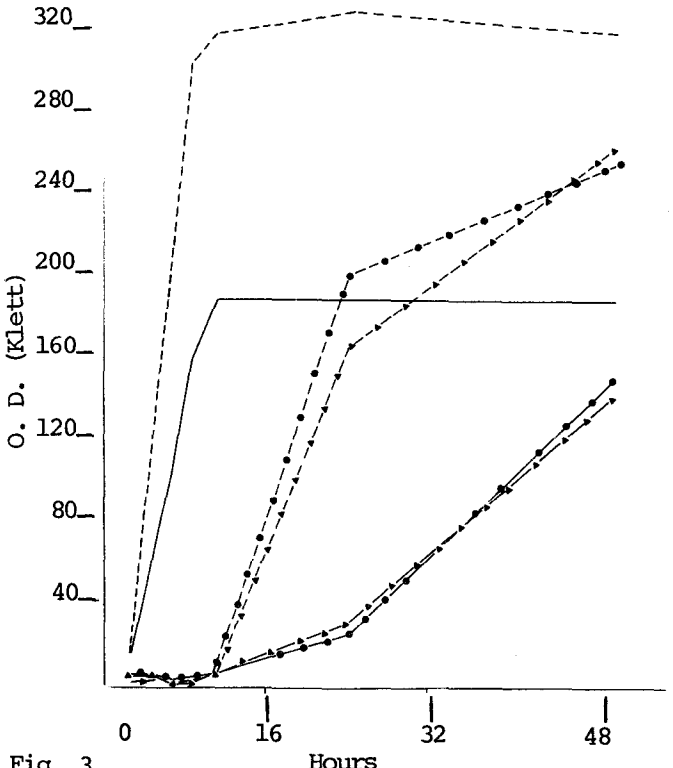


Fig. 3
Effect of static and shaking incubation at 37 C on growth of high initial population ($10^7$/ml) of Staphylococcus aureus in trypticase soy broth containing 5 ug/ml of 73% technical heptachlor (TH) and chlordane (C). Symbols: Static incubation (——), TH (—●—), and C (—▲—); shaking incubation control (— — —), TH (—●—) and C (—▲— —).

after 10 hours, but continued to increase at a much faster rate in samples under shaking conditions. Final turbidity was higher in the shaking pesticide samples than it was in the static controls.

Results show that the effect of incubation conditions on growth was related in part to initial population. When initial population was less than $10^5$/ml, growth was greater in the pesticide samples under static than under shaking incubation. The reverse was found when the initial population was over $10^6$/ml. Apparently at the lower population, shaking causes the pesticide to be in more direct contact with the limited number of cells and either prevents growth or causes a very long lag period. However, under static incubation, the poor solubility of the pesticides in TSB results in some settling and, as a result, the pesticides are in less contact with the cells. As a result, faster growth of S. aureus can occur.

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