Effect of Heptachlor on the Growth, Viability and Respiration of *Staphylococcus aureus*

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Researchers have begun only recently to elucidate mechanisms which can be involved in the different interactions possible between organochlorine pesticides and microorganisms. Most published information deals with general effects that various pesticides have on population of soil microorganisms (BOLLEN 1961; MARTH 1965; MARTIN et al 1959; MARTIN 1963; SHAW and ROBINSON 1960) and on degradation of pesticides (CHACKO 1966; LICHENSTEIN and SCHULZ 1960; LICHENSTEIN et al 1963; MACRAE et al 1969; MATSUMURA and BOUSCH 1961; MATSUMURA et al 1968; MILES et al 1969; RAGHU and MACRAE 1966). Little information is available concerning the mechanisms by which certain halogenated pesticides affect the viability and metabolism of bacteria (TRUDGILL et al 1971).

PFISTER (1972) has reviewed interactions which can occur between organochlorine pesticides and microorganisms. Heptachlor is believed to be degraded in four ways through chemical and microbial decomposition: a) volatilization, b) microbial epoxidation, c) chemical hydrolysis followed by microbial epoxidation and d) bacterial dechlorination followed by epoxidation (BOVARD et al 1971 and FERGUSON 1964).

Previous research in this laboratory showed that 3 to 5 ppm of technical grade heptachlor (72%) inhibits the growth of Staphylococcus aureus in broth media for up to 24 h (COLLINS and LANGLOIS 1968; LANGLOIS and COLLINS 1970; LANGLOIS and SIDES 1972). The effect caused by heptachlor appears to be one of inhibition as evident by increase in lag phase of growth when heptachlor concentration exceeds 3 ppm. Inhibition remained until some mechanism(s) permit bacteria to overcome the effect of the pesticide or its metabolite and then proceed to grow exponentially.

This study was conducted to obtain additional information concerning the inhibition of S. aureus by heptachlor and to determine the mechanisms involved in the inhibition.

Materials and Methods

Organism and cultural procedure. Species and cultural procedure used in this study have been previously described (LANGLOIS and SIDES 1972). The inoculum used to inoculate the flasks was prepared by diluting an actively growing culture in sterile phosphate buffered distilled water. This permitted 10^4

Published with the approval of the Director of the Kentucky Agricultural Experiment Station as journal article no. 75-5-143.

Bulletin of Environmental Contamination & Toxicology,
Vol. 16, No. 3 © 1976 by Springer-Verlag New York Inc.

to 10^6 cells per ml to be added when 1 ml of diluent was added to 500 to 700 ml media.

Pesticides. Stock solutions of 72% heptachlor were made so final concentrations of 3 or 5 ppm could be obtained by adding 1 ml to 500 to 700 ml media. Stock solutions were made up in absolute alcohol, except that acetone was used for making the solutions used in the respiration studies. For studying effect on morphology and leakage, the pesticide solutions and alcohol were added when cells of S. aureus were just entering their exponential phase of growth. The acetone-pesticide solutions were added to Warburg flasks and allowed to evaporate before cells of S. aureus suspended in 1 ml of phosphate buffer were added.

Determination of growth. Methods used to determine growth were similar to those previously described by LANGLOIS and SIDES (1972).

Morphology. To broth cultures of S. aureus in their exponential phase of growth, 1 ml of alcohol or pesticide solution was added. A 0.1 ml sample was removed aseptically from flasks 0, 1, 1.5, and 2 h after addition of solutions and gram stained. Stains were examined under oil immersion magnification using a Zeiss Photomicroscope and photographs were taken of several areas within each field. A 0.1 to 0.01 mm stage micrometer was used as a standard reference to determine cell size as observed by the photographs. Mean diameter of cells was determined using a Zeiss Particle Size Analyzer TGZ-3.

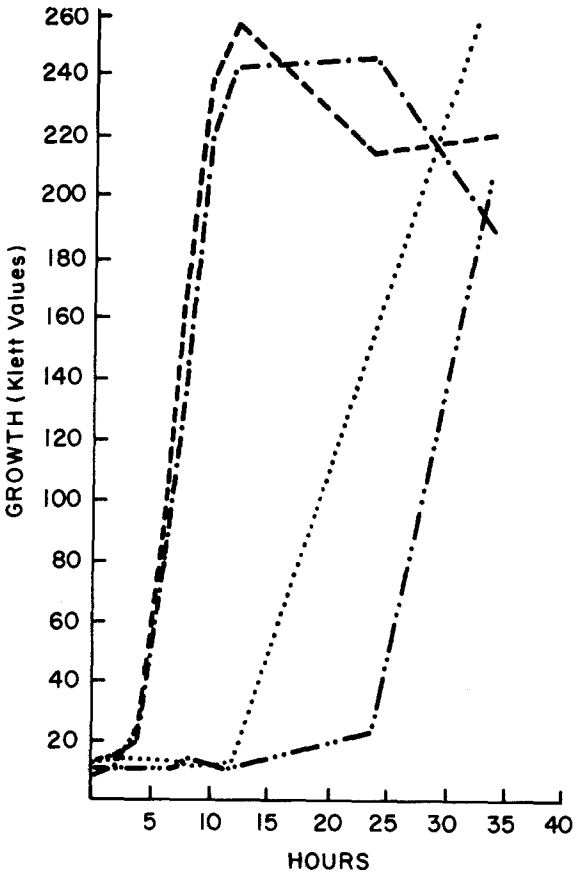
Determination of Dry Matter. A modification of the procedure of WHITE and FREEMAN (1967) was used to determine dry matter. Aliquots were removed from cultures growing in Trypticase Soy Broth (TSB) control, TSB plus 1 ml alcohol and TSB plus 1 ml alcoholic pesticide solution, at 2 h intervals beginning during lag phase and ending after the exponential phase. Samples were centrifuged in a Lloyd Model Betafuge at 4,000 x G for 20 min, the pellet was washed twice with distilled water and dried to a constant weight at 40C in a vacuum oven (30" Hg). Optical densities (OD) and viable counts were obtained on each TSB sample at the time each aliquot was removed for determination of dry matter in order to permit construction of standard curve. This curve showed correlation between dry matter and OD or viable count at a given time.

Determination of leakage. Alcohol or alcoholic pesticide solutions (1 ml) were added to broth cultures of S. aureus during their exponential growth phase. Aliquots of 10 ml were removed aseptically at 0, 1, and 2 h and centrifuged at 3,000 x G for 30 min. The supernatant was filtered through a 0.45 micron filter and the OD was determined at 265 mu using a Beckman Model DB Spectrophotometer. Leakage was determined by comparing UV absorbing material in control with that in pesticide containing material.

Determination of Respiration. Oxygen uptake was measured with a Warburg Respirometer using standard technique of UMBREIT et al (1972). The main compartment of the single sidearm Warburg flask contained residue remaining from evaporation of either 1.5 ml acetone or 1.5 ml pesticide acetone solution and 0.7 mg dry weight of S. aureus cells suspended in 1 ml phosphate buffer. The center well contained 0.2 ml 20% KOH (W/W) and the side arm contained 0.5 ml 2% glucose (W/W).

Results and Discussion

FIGURE 1

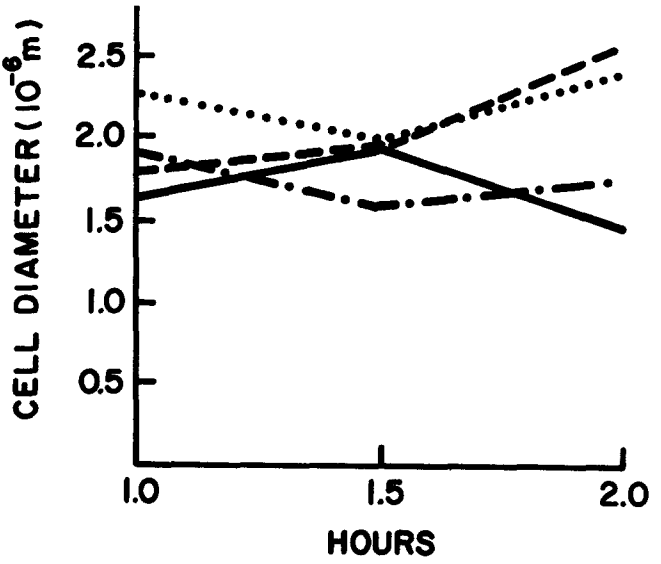


Growth of Staphylococcus aureus in Trypticase Soy Broth (TSB) containing heptachlor. Symbols: TSB control (-·-); TSB + alcohol (---); TSB + 3 ppm heptachlor (....); TSB + 5 ppm heptachlor (-···-).

Growth curved for Staphylococcus aureus in TSB, TSB plus alcohol and TSB plus 3 and 5 ppm heptachlor are shown in Figure 1. Lag phase of S. aureus in TSB increased from 12 to 24 h when the concentration of heptachlor was increased from 3 to 5 ppm. The lag phase in controls and controls plus alcohol was 3 to 4 h. The results of the growth phase of the study agree with the findings of COLLINS and LANGLOIS (1968); LANGLOIS and COLLINS (1970); LANGLOIS and SIDES (1972); SANDFORD (1974).

The size of cells of S. aureus growing in TSB, TSB plus alcohol and TSB plus 3 and 5 ppm heptachlor is shown in Figure 2. Problems were encountered with preparation of gram stains when 10^4 to 10^6 cells per ml were added to broth before the addition of pesticide. As a result, the effect of heptachlor on morphology was determined by permitting the cultures to reach exponential growth phase before the pesticide was added.

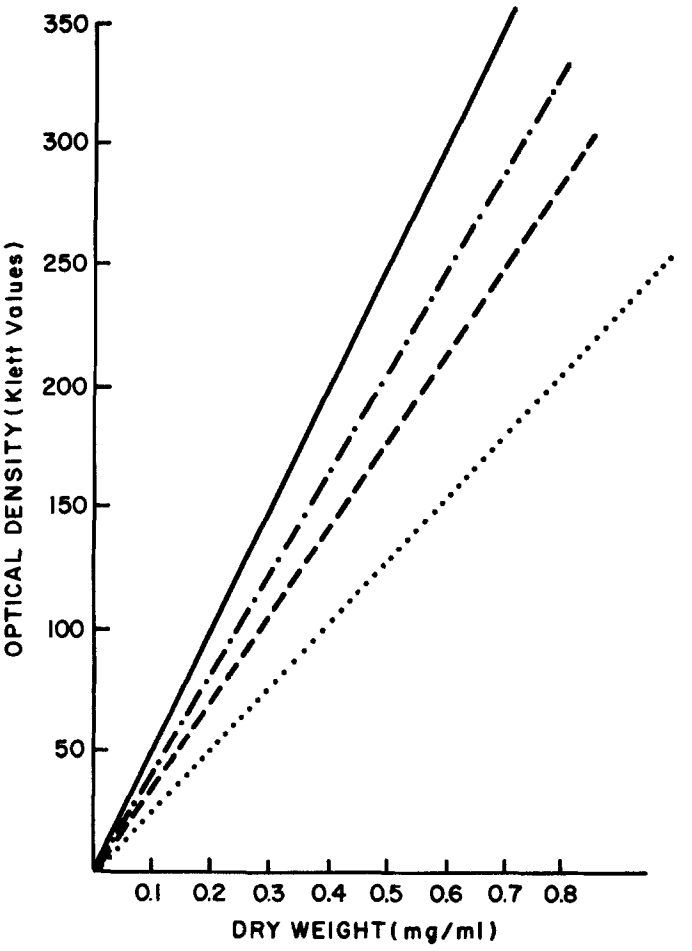
FIGURE 2



Effect of heptachlor on the size of Staphylococcus aureus cells in Trypticase Soy Broth (TSB). Symbols: TSB control (———); TSB + alcohol (-·-); TSB + 3 ppm heptachlor (---); TSB + 5 ppm heptachlor (....).

Compared with controls, cell size increased approximately 20 and 28% when 3 and 5 ppm heptachlor were added, respectively. This increase indicates an expansion of the cell wall, owing to either a thickening of cellular material comprising the wall or a stretching condition resulting from a weakening of the wall. The increased size resulting from dividing of the cells prior to inhibition of

FIGURE 3

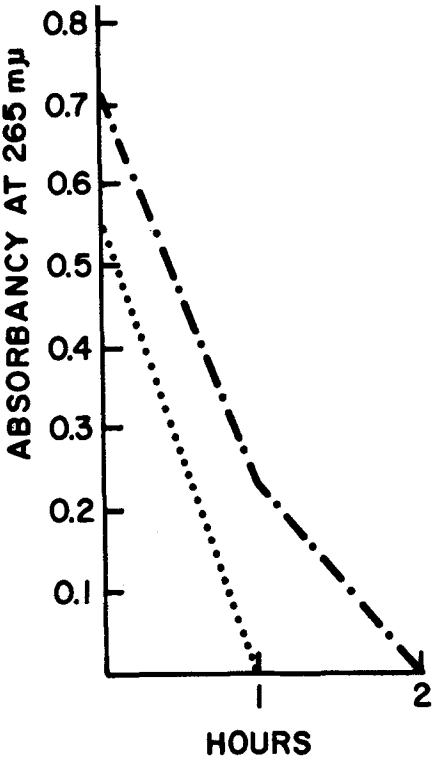


Effect of heptachlor on the dry matter content of Staphylococcus aureus cells in Trypticase Soy Broth. Symbols: TSB control (———); TSB + alcohol (-·-); TSB + 3 ppm heptachlor (---); TSB + 5 ppm heptachlor (....).

division also could be a reason for the increased size of the cells. The greatest increase in cell size occurred 2 h after addition of pesticide to the growing culture. Clustering of S. aureus cells did not appear to be affected by heptachlor at the concentrations used.

Figure 3 compares the dry matter of S. aureus cells grown in TSB and TSB plus alcohol or heptachlor. Dry matter of the cells increased with an increase in size of the cells, which was related to an increase in concentration of heptachlor. The results obtained for dry matter tend to support the data shown in Figure 2,

FIGURE 4



Effect of heptachlor on leakage of cellular material from cells of Staphylococcus aureus grown in Trypticase Soy Broth (TSB). Symbols: TSB + 3 ppm heptachlor (-·-); TSB + 5 ppm heptachlor (....).

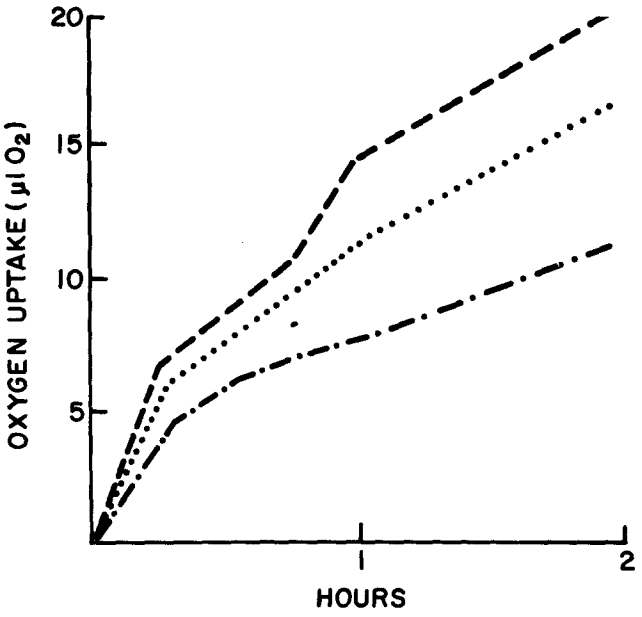
which show that as cell size increased an increase in dry matter also occurred. Staphylococcus aureus grown in TSB plus 5 ppm heptachlor had cells which were larger and contained more dry matter than cells from cultures grown in TSB plus 3 ppm heptachlor. Growth of S. aureus in presence of heptachlor produced cells which were larger and contained more dry matter than cells from controls or TSB plus alcohol. Studies involving changes in composition of cell wall or binding of heptachlor molecules to cell wall components need to be done in order to determine cause(s) for the increase cell size and dry matter content.

Initial exposure of S. aureus cells to heptachlor in TSB resulted in leakage of some ultraviolet light absorbing cellular materials (265 mu) into the growth medium. More leakage was observed in TSB plus 3 ppm heptachlor than in TSB plus 5 ppm heptachlor (Figure 4). Leakage appeared to cease after incubation for an hour in 5 ppm heptachlor but continued for an additional hour in 3 ppm heptachlor. The slight leakage observed may have been due to disruption of cell wall of some of the peripheral cells in a cluster, but cells within the cluster were not affected. As a result, disruption of cell wall did not occur for all cells in a culture which would have caused total lethality.

The cumulative oxygen consumption of S. aureus based on 0.7 mg/ml dry weight in 2% glucose at 37C is shown in Figure 5. Heptachlor caused an increase in rate of respiration during growth when compared with growth in control. The greater rate of oxygen consumption supports other data obtained in this study, especially the effect on cell size and dry matter. A greater rate of oxygen consumption occurred for cells growing in the lower concentration of heptachlor. During the initial 15 min, the rate of respiration was similar for S. aureus in 2% glucose control and in glucose plus pesticide. After 30 min, rate of respiration was less in control than for cells in the presence of pesticide. The difference in respiration rate became greater with increase in time until it leveled off at 3 h.

Inhibitory levels of heptachlor appeared to shift the metabolism of S. aureus from a phase of normal maintenance and multiplication to a phase of increased cell size, dry matter content, and rate of respiration and an inability to multiply.

FIGURE 5



Uptake of oxygen by cells of Staphylococcus aureus during growth in 2% glucose containing heptachlor. Symbols: control (-·-); 3 ppm heptachlor (---); 5 ppm heptachlor (....).

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