RESISTANCE OF SOME SOIL BACTERIA TO
PENTACHLOROPHENOL AND SODIUM PENTACHLOROPHENATE

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The purpose of this study was to see if any soil bacteria were able to use pentachlorophenol or sodium pentachlorophenate either aerobically or anaerobically as a sole carbon source, to see if any soil bacteria could survive in high concentrations of sodium pentachlorophenate, to determine the maximum concentration of sodium pentachlorophenate which permitted the growth of some soil bacteria, to see the effects of varying concentrations of sodium pentachlorophenate on the growth curves of soil bacteria capable of growing in its presence, and to see if any soil bacteria could degrade sodium pentachlorophenate.

The thesis is divided into the five main divisions of Introduction, Materials and Methods, Results, Discussion, and Summary with an additional section of the Literature Cited. The data is that recorded from actual laboratory experiments.

The experiments revealed that no soil bacteria in the samples tested were able to use pentachlorophenol or sodium pentachlorophenate either aerobically or anaerobically as a sole carbon source. Two species of soil bacteria were isolated which were capable of surviving in concentrations of 180 parts per million sodium pentachlorophenate in a yeast
extract-basal medium. These bacteria were identified as members of the genera *Flavobacterium* and *Enterobacter*. The effects of different concentrations of sodium pentachlorophenate on the growth curves of these bacteria were revealed by an increased lag phase in the cultures and a decreased maximum optical density with an increased sodium pentachlorophenate concentration. Under the conditions employed, however, these soil bacteria which were capable of surviving in high concentrations of sodium pentachlorophenate were not able to degrade sodium pentachlorophenate.
RESISTANCE OF SOME SOIL BACTERIA TO
PENTACHLOROPHENOL AND SODIUM PENTACHLOROPHENATE

THESIS

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By

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INTRODUCTION

Pentachlorophenol is one of the most extensively used antibiotic substances today; that is, antibiotic in the sense of preventing, inhibiting, or destroying life. It is also effective in the salt forms of copper and sodium pentachlorophenate. It is used as a bactericide and fungicide in processing many products such as leather, wool, wood, plastics, and cellulose fibers. It is used to control mildew, slimes, and termite infestations, to mothproof fabrics, and to control many insects. It is also used as a preharvest dessicant, as a pre-emergent herbicide, as a general herbicide, and as a molluscicide.

Pentachlorophenol dissolved in fuel oil or other hydrocarbons is also one of the major preservatives for wood materials. It is a major constituent of creosote, which is used to preserve all railroad ties, utility poles, and heavy construction timbers. A survey by Hicock and Olson (14) showed that coal tar creosote which contained pentachlorophenol was effective as a wood preservative for periods up to fifteen years when impregnated in pine posts. In the same study it was shown that posts treated with pentachlorophenol only were still in excellent condition after a period of four years.

Pentachlorophenol is a white solid composed of needle-like crystals. Its solubility in water is only 14 to 19 parts
per million (ppm) although it is soluble in most organic compounds (3). The sodium salt of pentachlorophenol, sodium pentachlorophenate, is soluble in water to a concentration of 4,000 ppm at pH 8.

Goodnight (10) observed that 0.2-0.6 ppm sodium pentachlorophenate was sufficient to kill certain species of fish. The toxic effects probably followed penetration of sodium pentachlorophenate into the blood stream and tissues. The metabolic rate of the fish was changed and their blood pressure increased, causing capillary rupture and bleeding.

In man, poisoning symptoms occur when the concentration of pentachlorophenol reaches 0.4-0.8 ppm in the bloodstream (3). Fatal cases are preceded by high temperature, sweating, dehydration, rapid pulse, and early coma. Autopsy findings have shown that 28 to 96 ppm of pentachlorophenol have accumulated in urine while 20 to 140 ppm have been reported in tissues and blood. At least 97 deaths from pentachlorophenol have been verified during the period from 1900 to 1970 with probably many more going unrecognized or unreported. In man, pentachlorophenol is an environmental hazard in the leather, fiber, wood, and preservative industries. It has also caused deaths by inadvertent contamination of foods and drinking waters.

Weinbach (23) reported that in low concentrations in the tissues (0.01 to 1 ppm) the mode of action of pentachlorophenol is the uncoupling of oxidative phosphorylation. These results were later supplemented by Fujita, Ishikawa, and Yamashita (9).
who demonstrated that pentachlorophenol added to cells of *Micrococcus lysodeikticus* inhibited respiration immediately, and that this inhibition was removed by the addition of inorganic phosphate.

Weinbach (23) also reported that at higher concentrations of pentachlorophenol (1 ppm to 10 ppm) inhibition of mitochondrial and myosin ATPase occurred, while at concentrations of 10 ppm and greater there was inhibition of glycolytic phosphorylation, inactivation of respiratory enzymes, and gross physical damage to mitochondrial structures.

Because such a large variety of living organisms, including man and other higher vertebrates, are dependent for survival on oxidative phosphorylation, the interruption of this process by even small amounts of pentachlorophenol could be responsible for the wide-range effectiveness of this compound as an antibiotic substance.

Since pentachlorophenol is very toxic to such a large variety of living organisms and since it enjoys such widespread usage, its degradation in nature is very important. It must retain its toxic properties long enough to be used effectively and yet not be allowed to accumulate in natural systems lest it become hazardous to man and valuable animals. For this reason, experiments on residual toxicity of pentachlorophenol in nature have been numerous.

Harvey and Crafts (11) reported that in three California soils of different types, fine sandy loam, clay loam, and adobe
clay, there was no appreciable breakdown of pentachlorophenol in a warm, moist environment over a twelve month period of time. They also reported that pentachlorophenol was not fixed to the clay fraction of the soil and that it moved freely with water. Concentrations of 164 ppm pentachlorophenol increased oat crop yields in these same soils without crop damage, but at concentrations of 328 ppm crop yields were greatly reduced.

Dobrovohny and Haskins (7) found that the concentration of pentachlorophenol salts in solution was reduced in the presence of silts and clays. They believed that the principal process of disappearance was the absorption of the chemical by the fine particles of soil. They also reported that the greater the depth of mud in proportion to the depth of water, the more rapidly the pentachlorophenol disappeared from aqueous solutions. Soils with smaller quantities of organic matter showed decreased rates of pentachlorophenol disappearance. Soil that was rich in organic matter when agitated in water was most effective in removing pentachlorophenol from aqueous solution.

Loustalot (19) observed that the quantity of pentachlorophenol in soil decreased with time with the inactivation rate greatest in warmer temperatures. No appreciable sodium pentachlorophenate inactivation occurred in air-dry soil after a two-month period, while water-saturated soils reduced pentachlorophenol concentrations more rapidly than moderately moist soils.
Young and Carroll (24) believed that microbiological activity was in part responsible for pentachlorophenol degradation in soil. They found that disappearance of pentachlorophenol was greater in soils of high organic matter content where moisture and temperature were conducive to microbial growth and activity.

Appling, Buckman, and Meals (1) reported that Aspergillus niger could survive in the presence of 16 ppm pentachlorophenol. Later, Duncan and Deverall (8) grew the fungus Trichoderma sp. (P42) in sweetgum and sapwood blocks saturated with solutions of 0.1 to 1.3% pentachlorophenol dissolved in low boiling petroleum and toluene. They reported that this fungus had substantially depleted the pentachlorophenol from the treated wood blocks after an incubation period of 12 weeks.

Cserjesi (5) used several strains of Cephaloascus fragrans capable of growing in media containing 180 ppm sodium pentachlorophenate and adapted them to grow in media containing 280 ppm. This species, however, showed no apparent ability to degrade sodium pentachlorophenate in malt extract medium while strains of Trichoderma viride and T. virgatum which tolerated only 120 ppm sodium pentachlorophenate showed the ability to reduce the detectable concentration of sodium pentachlorophenate from over 1 ppm to virtually 0 ppm after 12 days of incubation.

Lyr (20) reported that chlorinated phenols including sodium pentachlorophenate could be detoxified through oxidation by the
enzymes laccase, tyrosinase, and peroxidase which are pro-
duced by many fungi. In a supplementary report (21), Lyr
showed that certain wood destroying fungi, one of which is
Trichoderma viride, can detoxify chlorinated phenols includ-
ing sodium pentachlorophenate by secreting laccase into the
culture medium.

Some researchers have challenged the concept of bio-
degradability of pentachlorophenol. Leutritz (18) found that
by his method of wood treatment, there was no different be-
tween pentachlorophenol losses in sterile and non-sterile
soil. He attributed losses of pentachlorophenol to such
factors as leeching, bleeding, and water displacement, or to
some combination of these.

Ingols and Stevenson (15) showed by ultraviolet absorp-
tion spectra and by chloride ion tests that sodium penta-
chlorophenate (at a concentration of 100 ppm) was the only
chlorinated phenol they studied that was resistant to bio-
degradation in conditions that were favorable to the growth
of microorganisms.

Biodegradation is not the only means of pentachlorophenol
disappearance that has been reported. Hiatt, Haskins, and
Oliver (13) reported the loss of sodium pentachlorophenate
from an aqueous solution by absorption of light in the wave-
length region of 290 to 330 millimicrons. Kuwahara, Natsuki,
and Munakata (16) reported that among the products of light
degradation were chloranilic acid and a yellow compound,
3, 4, 5-trichloro-6-(2'-hydroxy-3', 4', 5', 6'-tetrachlorophenoxy)-6-benzoquinone. Pentachlorophenol can also be decomposed by strong oxidizing agents (3).

Although biodegradation of pentachlorophenol by fungi has been studied to some extent, there is little or no work reported in the area of bacterial biodegradation of pentachlorophenol or sodium pentachlorophenate. This paper presents studies on the survival of some soil bacteria in aqueous solutions of pentachlorophenol and its sodium salt, sodium pentachlorophenate.
Soil Samples

Fifty-one soil samples were collected from various locations in Denton, Texas, in October, 1970. Samples #1 through #10 were obtained from soils adjacent to railroad ties; samples #11 through #41 were taken from the bases of utility poles; samples #I, III, IV, VIII, X, XI, XIV, XVI, and XVIII were taken from a creosoting plant yard in Denison, Texas; and sample #51 was taken from a flower bed on the NTSU campus. All vegetation and approximately one-fourth inch of soil were cleared away from each area to be sampled. The soils were then collected by use of a clean tablespoon from the next one-inch depth of soil and stored in screw cap test tubes in the refrigerator.

Preparation of Sodium Pentachlorophenate

A stock solution of sodium pentachlorophenate was prepared by the method of Hiatt, Haskins, and Oliver (13). Ten milligrams of pentachlorophenol (Eastman) were added to five milliliters of saturated aqueous solution of sodium bicarbonate. This mixture was heated on a steam bath under a hood until the crystalline pentachlorophenol had changed to the sodium salt and had gone into solution. The sodium pentachlorophenate solution thus prepared was allowed to cool to
room temperature, placed in an Erlenmeyer flask, protected from light, and stored at room temperature.

**Colorimetric Test for the Detection of Sodium Pentachlorophenate**

The procedure used is that originated by Haskins (12). Five milliliters of an aqueous solution containing five to fifty parts per million (ppm) sodium pentachlorophenate were placed in a 20 ml screw cap test tube. To this was added in sequence 5 ml of chloroform, 0.5 ml of saturated aqueous sodium bicarbonate, and 0.5 ml of a 0.02% aqueous safranin-0 (Allied Chemicals) solution. Each tube was capped and shaken vigorously for fifteen seconds and left standing for four minutes to allow the layers to separate and the color to develop. A 5 ml distilled water sample was treated in the same manner and used as a blank. Approximately 4 ml of the chloroform layer from each sample were transferred to a 10 ml cuvette and read in a spectrophotometer (Bausch and Lomb Spectronic 20) at a wavelength of 540 millimicrons after setting the instrument to zero with the blank.

**Procedure and Incubation Conditions**

Unless otherwise specified, all analytical, preparatory, and microbiological procedures were carried out under yellow light (Sylvania, 100 Watt) to prevent decomposition by light of a shorter wavelength. All cultures were incubated in the dark at room temperature under stationary conditions, unless otherwise stated.
**Basal Medium**

The basal medium used for culturing bacteria from soil inocula was a modification of L-salts (17) consisting of the following reagent grade chemicals in grams per liter of solution: \( \text{Na}_2\text{HPO}_4 \), 0.21; \( \text{NH}_4\text{NO}_3 \), 2.0; \( \text{MgSO}_4 \cdot 7\text{H}_2\text{O} \), 0.2; \( \text{FeSO}_4 \cdot 7\text{H}_2\text{O} \), 0.001; \( \text{K}_2\text{HPO}_4 \), 0.21; \( \text{KH}_2\text{PO}_4 \), 0.09; \( \text{KCl} \), 0.04; \( \text{CaCl}_2 \), 0.015; \( \text{CaSO}_4 \cdot 0.5\text{H}_2\text{O} \), 0.005; \( \text{H}_3\text{BO}_3 \), 0.01; \( \text{MnSO}_4 \cdot 5\text{H}_2\text{O} \), 0.01; \( \text{ZnSO}_4 \cdot 7\text{H}_2\text{O} \), 0.07; and \( \text{MoO}_3 \), 0.01. The basal medium was prepared at double strength concentration of salts (2X) and stored at room temperature. The reconstituted basal medium was sterilized before use and the final pH of the solution was adjusted to pH 7.5 after addition of nutrients or chemicals used in testing for growth of soil bacteria.

**Pentachlorophenol as a Sole Carbon Source**

Pentachlorophenol was tested as a possible sole carbon source for soil bacteria. Nine-tenths of a gram of pentachlorophenol were dissolved in 520 ml of diethyl ether (anesthesia grade). Ten milliliters of the diethyl ether-pentachlorophenol solution were added to each of 51 screw cap vials (32 ml). The vials were placed under the hood overnight in the dark to evaporate a large part of the ether. They were then transferred to a 55°C incubator to evaporate the residual organic solvent. Twenty milliliters of basal medium were added to the dried pentachlorophenol in each of the 51 screw cap vials prepared above. To each of these vials
was also added a pinch of soil. Each vial was labeled with the soil number, shaken vigorously and filled completely with basal medium to eliminate air. (Growth was checked by anaerobic incubation since pentachlorophenol acts as an uncoupler of oxidative phosphorylation.) These were incubated for 14 days and checked for growth by estimation of turbidity and by microscopic examination.

A second procedure was used to test pentachlorophenol as a sole carbon source. Two grams of pentachlorophenol were added to distilled water and warmed gently to prepare a saturated pentachlorophenol solution. This solution was filtered (Whatman #1 filter paper) and used as the aqueous phase to dissolve the chemicals. The medium thus prepared was basal medium saturated with pentachlorophenol. A pinch of soil was added to each of 51 screw cap vials (32 ml). These vials were completely filled with the basal medium-pentachlorophenol solution prepared above. Controls of basal medium without pentachlorophenol were prepared in the same way for each of the 51 soil samples. These were allowed to incubate for 14 days and were checked for growth by microscopic examination, by optical turbidity and by plating on nutrient agar plates.

Fifteen of the above primary soil cultures were transferred serially twice and incubated both aerobically and anaerobically along with the controls of basal medium only. These were checked for growth by plating on both nutrient agar plates and pentachlorophenol-basal medium plates that had been prepared
with the above solution and two per cent Bacto-Agar. The cultures which had been grown aerobically were checked for growth by incubating the plates aerobically, and the cultures which had been grown anaerobically were checked for growth anaerobically by incubation of the plates in a Gas-Pack Anaerobic Jar (Baltimore Biological Laboratories). Growth was checked after 96 hours of incubation.

Sodium Pentachlorophenate as a Sole Carbon Source

Sodium pentachlorophenate was also tested as a possible sole carbon source for soil bacteria. Of the above 51 soil samples, five were randomly selected for additional testing. One soil sample was taken from the edge of a railroad tie (#3), two were taken from the bases of utility poles (#18 and #23), one was taken from the Denison creosoting plant yard (#XVI), and one was taken from a flower bed on the NTSU campus (#51). These five soils were used in all of the remaining experiments.

A 50 ppm sodium pentachlorophenate-basal medium mixture was prepared by placing 2.5 ml of sodium pentachlorophenate solution, 50 ml of 2X basal medium, and 47.5 ml of an aqueous solution of saturated sodium bicarbonate in a dilution blank bottle. (The sodium bicarbonate was added to maintain a constant pH and to keep the sodium pentachlorophenate in solution.) The above solution was adjusted to pH 7.5 with concentrated HCl. Ten milliliters of this solution were added to each of five 32 ml vials for aerobic incubation and to each of five.
16 ml vials for anaerobic incubation in a Gas-Pack Anaerobic Jar. A pinch of each soil was added to both a 16 ml vial and a 32 ml vial. Basal medium was prepared and inoculated in the same way for controls. These vials were checked after eight days for growth by optical turbidity and by plating on nutrient agar and incubating both aerobically and anaerobically as described above.

The aerobic cultures of sodium pentachlorophenate-basal medium and basal medium only were transferred to their respective media as noted in the preceding paragraph and incubated. These were checked for growth at 8, 16, and 28 days of incubation by optical turbidity and by plating on nutrient agar and incubating both aerobically and anaerobically as previously described.

To a 250 ml Erlenmeyer flask were added 2.5 ml of sodium pentachlorophenate solution, 50 ml of 2X basal medium, and 47.5 ml of an aqueous solution of saturated sodium bicarbonate to make a final concentration of 50 ppm sodium pentachlorophenate. This was inoculated with approximately 1 g of a mixture of the five soil samples and incubated aerobically. This was checked for growth by optical turbidity and by plating on plain nutrient agar at 14 and 28 days of incubation.

**Growth of Soil Bacteria in the Presence of Sodium Pentachlorophenate**

The ability of soil bacteria to grow in the presence of high concentrations of sodium pentachlorophenate was determined.
Double strength concentrations (2X) of 50 ml of both Bacto-
Nutrient Broth and 0.5% Bacto-Yeast Extract in basal medium
were placed in dilution blank bottles. Sodium pentachloro-
phenate solution was added in amounts of 2.5, 3.75, 5.0, 12.5,
and 25 ml. An aqueous solution of saturated sodium bicar-
bonate was added to bring the total volume of each bottle
to 100 ml to make final concentrations of 50, 75, 100, 250,
and 500 ppm, respectively. Controls were prepared by adding
50 ml of an aqueous solution of saturated sodium bicarbonate
to both 50 ml of basal medium-yeast extract and to 50 ml of
nutrient broth.

Each of the above preparations was adjusted to pH 7.5
with concentrated HCl and divided into five 32 ml vials and
five 16 ml vials. To each preparation was added a pinch each
of soil samples #3, 18, 23, 51, and XVI. The 32 ml vials were
incubated aerobically and the 16 ml vials were incubated
anaerobically in a Gas-Pack Anaerobic Jar. After eight days
of incubation, growth was checked by plating on nutrient agar
and incubating the plates both aerobically and anaerobically
for 96 hours.

Identification of Soil Bacteria That Grow in the Presence of
High Concentrations of Sodium Pentachlorophenate

The bacteria isolated were tested by the following methods.
They were gram stained to check their morphology and gram
reaction. They were examined by wet mount preparations with a
phase contrast microscope to check motility and morphology. Motility was also tested by Bacto-SIM Medium, and the presence of flagella was ascertained by Leifson's flagella stain (21).

The indole reaction was determined by the Kovacs test (6) on Bacto-SIM Medium. Hydrogen sulfide production was tested on Bacto-SIM Medium and on Bacto-Triple Sugar Iron Agar. Starch hydrolysis was determined on starch agar by flooding the growth with Lugol's iodine. Bacto-Litmus Milk was used for determining milk protein hydrolysis. MR-VP Broth (Baltimore Biological Laboratories) was used to test acid production by the methyl red test (6) and acetyl-methylcarbinol formation by the Voges-Proskaur reaction (6).

Gelatin liquefication was checked by growth in Bacto-Nutrient Gelatin. Bacto-Nitrate Broth was used to determine the ability of the bacteria to reduce nitrate to nitrite and ammonia or nitrogen (2). The ability to use citrate as a sole carbon source was tested on Bacto-Simmons Citrate Agar.

Preliminary carbohydrate fermentation was tested on Bacto-Triple Sugar Iron Agar. The production of acid and gas from glucose was tested using Bacto-Phenol Red Broth Base and incubating aerobically and anaerobically in a Gas-Pack Anaerobic Jar. The production of acid and gas from glucose, lactose, sucrose, xylose, mannitol, and salicin was tested aerobically in Bacto-Purple Broth Base containing the respective sugars.

Tentative identification of the organisms isolated was made from the results of these tests by use of Bergey's Manual.
of Determinative Bacteriology (4) and Skerman's A Guide to the Identification of the Genera of Bacteria (21).

Maximum Concentration of Sodium Pentachlorophenate in which Resistant Soil Bacteria Can Grow

The maximum concentration of sodium pentachlorophenate which allowed the growth of resistant soil bacteria was determined. To fourteen dilution bottles was added 25 ml of nutrient broth at double strength concentration (2X); fourteen dilution bottles with 25 ml of yeast extract-basal medium were also prepared and both autoclaved. To these were added 0, 1.0, 2.0, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, and 8.5 ml of sodium pentachlorophenate, and the volume made up to 50 ml with an aqueous solution of saturated sodium bicarbonate to give final concentrations of 0, 40, 80, 120, 140, 160, 180, 200, 220, 240, 260, 280, 300, and 340 ppm sodium pentachlorophenate, respectively, in both media. Each preparation was adjusted to pH 7.5 with concentrated HCl, and each 50 ml was divided into two sterile 32 ml vials to make a final volume of 25 ml in each vial. To each set of vials was added 0.1 ml of a suspension of each of the bacteria previously isolated. These bacterial suspensions were prepared by adding 10 ml of sterile saline to each culture slant, shaking loose the cells, and pipetting from this suspension with a 1 ml pipette. These were incubated and examined daily for nine days for signs of growth by optical turbidity. At three days, growth was measured by taking optical
density readings of 4.5 ml of each culture on a Klett-Summerson Colorimeter using a blue filter. This experiment was repeated using concentrations of 120, 140, 160, 200, and 220 ppm sodium pentachlorophenate in both types of media with both bacterial isolates.

**Growth Curves of Isolated Bacteria in the Absence of Sodium Pentachlorophenate**

The growth curves of the two bacteria isolated when grown in the absence of sodium pentachlorophenate were determined. The medium was prepared in duplicate by placing 100 ml of a 2X concentration of basal medium containing 1.0 g yeast extract in each of two 1000 ml Erlenmeyer flasks and sterilizing. To each of these preparations was added 100 ml of a sterilized aqueous saturated solution of sodium bicarbonate. After the medium had cooled, it was adjusted to pH 7.5 with concentrated HCl.

To a nutrient agar slant of each organism were added 10 ml of sterile saline. This was shaken to loosen the cells, and 1 ml of each cell suspension was added to each of two flasks. A third flask of media was left uninoculated and it was used to zero the colorimeter.

At various time intervals, turbidity readings were taken on a 4.5 ml culture sample using a Klett-Summerson Colorimeter with a blue filter. Plate counts of each of the two cultures were also made in duplicate. These were prepared by the spread plate technique on nutrient agar using 0.1 ml of each culture to be
tested. The pH of the two cultures was also recorded at each time interval by use of a pH meter. This experiment was repeated as stated above.

**Growth Curves of Isolated Bacteria in the Presence of Varying Concentrations of Sodium Pentachlorophenate**

The effect of different concentrations of sodium pentachlorophenate on the two bacteria isolated was tested. Fifty milliliters of a 2X concentration of basal medium and 1.0 g of yeast extract were added to each of eleven side-arm flasks (Belco) and autoclaved. To each of these flasks were added 0, 1.0, 2.0, 4.0, and 8.0 ml sodium pentachlorophenate. The remainder of 100 ml was completed by adding a sterilized aqueous saturated solution of sodium bicarbonate to make final concentrations of 0, 20, 40, 80, and 160 ppm sodium pentachlorophenate, respectively, in a 0.5% yeast extract medium. Each culture flask was adjusted to pH 7.5 with concentrated HCl. One flask of media was left uninoculated and it was used to zero the colorimeter.

Cell suspensions of each bacterial culture were prepared by adding 10 ml of sterile saline to a nutrient agar slant and shaking loose the cells. To each set of flasks was added 0.5 ml of the suspensions of each of the isolated bacteria. These were incubated and read at various time intervals from 0 through 78 hours on a Klett-Summerson Colorimeter with a blue filter. The experiment was repeated as above and measurements were made during the interval from 0 through 48 hours.
Test for Biodegradation of Sodium Pentachlorophenate by Soil Bacteria

The ability of soil bacteria to degrade sodium pentachlorophenate was tested by the colorimetric test of Haskins (12). To six 250 ml Erlenmeyer flasks were added 50 ml 2X nutrient broth, 2.5 ml sodium pentachlorophenate, and 47.5 ml of an aqueous saturated solution of sodium bicarbonate. To four 250 ml Erlenmeyer flasks were added 50 ml 2X basal medium, 1.0 g of yeast extract, 2.5 ml sodium pentachlorophenate solution, and 47.5 ml of an aqueous solution of saturated sodium bicarbonate. To four 250 ml Erlenmeyer flasks were added 50 ml of 2X basal medium, 2.5 ml sodium pentachlorophenate solution, and 47.5 ml of an aqueous solution of sodium bicarbonate. One of each of these other types of media prepared above served as an uninoculated control, one of each of the three types of media was inoculated with a small amount of a mixture of the five soil samples, and two of each of the three types of media were inoculated with each of the two bacterial isolates. One flask of nutrient broth-sodium pentachlorophenate-sodium bicarbonate was left in regular laboratory light and the remaining flask of nutrient broth-sodium pentachlorophenate-sodium bicarbonate was inoculated with Escherichia coli, ATCC 11775, to serve as an additional control.

The colorimetric test was performed on each culture, using one part medium to nine parts of water. A reference curve of
absorbency of various known concentrations of sodium pentachlorophenate was performed with the same dilution of one part medium to nine parts water.

The above test was repeated with the exception of using 97.5 ml of medium to 2.5 ml of sodium pentachlorophenate with no excess of an aqueous solution of sodium bicarbonate. The same inoculations were used except that no culture was left in the light, no E. coli culture was tested, and neither of the bacterial isolates was tested in basal medium-sodium pentachlorophenate with no other carbon source.
RESULTS

The 51 vials containing basal medium and pentachlorophenol, which had been evaporated from diethyl ether, inoculated with the 51 different soil samples and incubated anaerobically, showed no growth as judged by either turbidity or microscopic examination after incubation for 14 days.

The saturated solution of pentachlorophenol supported trace amounts of growth from the original soil culture and the two serial transfers when tested by culturing aerobically on nutrient agar plates. However, the control of basal medium only showed trace amounts of growth of the same bacteria on aerobically incubated nutrient agar plates. Trace amounts of growth were also detectable from aerobic cultures by optical turbidity and microscopic examination.

On the second transfer, no growth was detected from cultures incubated anaerobically on either the pentachlorophenol transfer or the basal medium transfer. No growth was detected from any culture on the pentachlorophenol-basal medium plates.

The five soil samples added individually to 50 parts per million (ppm) sodium pentachlorophenate in basal medium and cultured aerobically showed trace amounts of growth after eight days incubation when tested by plating on nutrient agar, as did the basal medium control. Trace amounts of growth were also observed after 16 days of incubation. No growth in either
the basal medium control or the sodium pentachlorophenate-
basil medium was observed when incubated anaerobically.

Transfers of the aerobic five-soil-sample cultures
showed organisms growing from basal medium only when tested
on nutrient agar after eight days of incubation, but no or-
ganisms grew from the transfers of 50 ppm sodium pentachloro-
phenate. After these transfers had been incubated for 28 days,
they showed no growth from either basal medium only or 50 ppm
sodium pentachlorophenate when tested on nutrient agar.

The five-soil-sample mixture incubated aerobically was
checked for growth by plating on nutrient agar after 14 days
of incubation, at which time trace amounts of growth were
observed. After 28 days of incubation, no growth was observed
when tested by plating on either nutrient agar containing 100
ppm sodium pentachlorophenate or on plain nutrient agar.

The five soil samples cultured both aerobically and
anaerobically showed some bacterial growth in 50, 75, and 100
ppm sodium pentachlorophenate in both nutrient broth and yeast
extract-basal medium when tested both aerobically and anaero-
bically, respectively, on nutrient agar plates. No growth was
observed from either 250 or 500 ppm sodium pentachlorophenate
aerobically or anaerobically from any of the five soil samples.

A series of bacteria that formed white colonies on nutrient agar was isolated from all five soil samples in both yeast
extract-basal medium and nutrient broth at up to 100 ppm sodium
pentachlorophenate. A second species was isolated from sample
That formed yellow colonies on nutrient agar in up to 100 ppm sodium pentachlorophenate in both yeast extract-basal medium and nutrient broth.

Both isolates were subjected to various chemical tests and stains in order to determine their identity (Table I). From this information, the bacterium that formed white colonies was tentatively placed in the genus Enterobacter and the bacterium that formed yellow colonies was tentatively placed in the genus Flavobacterium.

The maximum concentration at which both Enterobacter sp. and Flavobacterium sp. could grow was 180 ppm sodium pentachlorophenate in yeast extract-basal medium and 160 ppm sodium pentachlorophenate in nutrient broth medium. After 24 hours of incubation, the highest concentration of sodium pentachlorophenate in which growth was visible by turbidity observation was recorded (Table II). Three out of four cultures had detectable growth in the highest concentration in which the organism could grow after 48 hours of incubation, and the fourth culture had detectable growth in the highest concentration in which the organism could grow after 96 hours of incubation.

Klett readings were taken after three days of incubation of both bacterial species in both types of media with concentrations of 0 to 220 ppm sodium pentachlorophenate (Fig. 1). These values were recorded as an average of two consecutive tests. The cultures containing no sodium pentachlorophenate
# Table I

Tests for the identification of soil bacteria isolated from the presence of high concentrations of sodium pentachlorophenate

<table>
<thead>
<tr>
<th>Test</th>
<th>Reaction</th>
<th>White Colony Bacterium</th>
<th>Yellow Colony Bacterium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram Stain</td>
<td></td>
<td>Gram -</td>
<td>Gram -</td>
</tr>
<tr>
<td>Morphology</td>
<td></td>
<td>Small Rod</td>
<td>Small Rod</td>
</tr>
<tr>
<td>Motility</td>
<td></td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Flagella Stain</td>
<td></td>
<td>Peritrichous</td>
<td>None</td>
</tr>
<tr>
<td>Litmus Milk</td>
<td></td>
<td>No Change</td>
<td>No Change</td>
</tr>
<tr>
<td>Methyl Red</td>
<td></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Acetyl-Methyl Carbinol</td>
<td></td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Starch Hydrolysis</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Indole Production</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Nitrate Reduction</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Citrate Utilization</td>
<td></td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sugars</th>
<th>Acid</th>
<th>Gas</th>
<th>Acid</th>
<th>Gas</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Glucose (Anaerobic)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Lactose</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sucrose</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Xylose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Mannitol</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Salicin</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>
TABLE II
HIGHEST CONCENTRATION OF SODIUM PENTACHLOROPHENATE 
IN PARTS PER MILLION ALLOWING GROWTH

<table>
<thead>
<tr>
<th>Time (Days)</th>
<th>Yeast Extract-Na PCP*</th>
<th>Nutrient Broth-Na PCP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Entero-bacter</td>
<td>Flavo-bacterium</td>
</tr>
<tr>
<td>1</td>
<td>160</td>
<td>140</td>
</tr>
<tr>
<td>2</td>
<td>180</td>
<td>180</td>
</tr>
<tr>
<td>3</td>
<td>180</td>
<td>180</td>
</tr>
<tr>
<td>4</td>
<td>180</td>
<td>180</td>
</tr>
<tr>
<td>6</td>
<td>180</td>
<td>180</td>
</tr>
<tr>
<td>9</td>
<td>180</td>
<td>180</td>
</tr>
</tbody>
</table>

*Na PCP = Sodium Pentachlorophenate

were more turbid than those containing a given concentration 
of sodium pentachlorophenate. There was little difference in 
turbidity from concentrations of 40 ppm to 140 ppm sodium 
pentachlorophenate in yeast extract-basal medium. Peaks 
appeared at 160 ppm sodium pentachlorophenate in yeast extract-
basal medium followed by a large decrease in turbidity at 180 
ppm sodium pentachlorophenate. The nutrient broth-sodium 
pentachlorophenate culture appeared to have less growth when 
measured by turbidity at all concentrations than yeast extract-
basal medium-sodium pentachlorophenate cultures. At three days, 
the readings of the nutrient broth cultures showed fairly
Fig. 1—Turbidity readings of various concentrations of sodium pentachlorophenate at three days incubation.
constant turbidity up to concentrations of 160 ppm but no growth at 180 ppm sodium pentachlorophenate.

The viable cell counts and turbidity readings of Enterobacter sp. in the absence of sodium pentachlorophenate were recorded as an average of two tests of duplicate readings (Fig. 2). There was a lag phase of approximately eight hours with maximum growth at 20 hours of incubation. Maximum growth was approximately \(10^9\) cells per milliliter as determined by viable cell counts. The stationary phase of the culture tested by viable cell counts was relatively short with a rapid decrease in viable cell numbers after 24 hours of incubation. Turbidity readings showed a more gradual decline. The pH increased rapidly during the first eight hours of incubation after which the culture became more alkaline at a slower rate (Table III). After reaching a pH of 8.4, the culture entered the death phase.

The viable cell counts and turbidity readings of the Flavobacterium sp. were also recorded as an average of two tests of duplicate readings (Fig. 3). There was a lag phase of approximately eight hours and a maximum viable cell count of \(10^9\) viable organisms per milliliter at 20 hours of incubation. This culture remained in the stationary phase for some 48 hours. The turbidity readings, however, continued to increase up to 48 hours of incubation, after which they also began to decrease. The pH of the Flavobacterium sp. also increased during the first eight hours of incubation after which the culture became more
Fig. 2—Growth of *Enterobacter* sp. in the absence of sodium pentachlorophenate.
Fig. 3—Growth of *Flavobacterium* sp. in the absence of sodium pentachlorophenate.
TABLE III

THE PH READINGS OF ENTEROBACTER SP. AND FLAVOBACTERIUM SP.
GROWN IN THE ABSENCE OF SODIUM PENTACHLOROPHENATE

<table>
<thead>
<tr>
<th>Time (Hours)</th>
<th>Enterobacter sp.</th>
<th>Flavobacterium sp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>7.5</td>
<td>7.5</td>
</tr>
<tr>
<td>8</td>
<td>8.1</td>
<td>8.0</td>
</tr>
<tr>
<td>12</td>
<td>8.3</td>
<td>8.2</td>
</tr>
<tr>
<td>16</td>
<td>8.3</td>
<td>8.4</td>
</tr>
<tr>
<td>20</td>
<td>8.4</td>
<td>8.5</td>
</tr>
<tr>
<td>24</td>
<td>8.5</td>
<td>8.6</td>
</tr>
<tr>
<td>32</td>
<td>8.7</td>
<td>8.7</td>
</tr>
<tr>
<td>40</td>
<td>8.8</td>
<td>8.8</td>
</tr>
<tr>
<td>48</td>
<td>8.9</td>
<td>8.9</td>
</tr>
<tr>
<td>60</td>
<td>9.0</td>
<td>9.0</td>
</tr>
<tr>
<td>84</td>
<td>9.1</td>
<td>9.1</td>
</tr>
</tbody>
</table>

alkaline at a slower rate (Table III). However, the culture did not enter the death phase until it had reached a pH of 8.9.

Turbidity readings of Enterobacter sp. (Fig. 4) and Flavobacterium sp. (Fig. 5) at 0, 20, 40, and 80 ppm sodium pentachlorophenate, recorded as an average of two consecutive tests, revealed (1) an increased lag phase at each higher concentration of sodium pentachlorophenate, and (2) a decreased maximum optical density at each higher concentration. No growth was observed with either bacterium at 160 ppm sodium pentachlorophenate.
Fig. 4—Growth of Enterobacter sp. at different concentrations of sodium pentachlorophenate.
Fig. 5--Growth of *Flavobacterium* sp. at different concentrations of sodium pentachlorophenate.
Although some variation occurred in the colorimetric test for pentachlorophenol detection (12), which was used to test for the biodegradation of sodium pentachlorophenate by soil bacteria, this variation was not significant. Neither the cultures of media-sodium pentachlorophenate-sodium bicarbonate (Table IV) nor the cultures of media-sodium pentachlorophenate (Table V) showed any significant difference between different media or between different inocula. The curve relating absorbency to parts per million of sodium pentachlorophenate (Fig. 6) shows that absorbency would need to drop to approximately .6 for a drop to 40 ppm sodium pentachlorophenate, and such a drop was never observed.
<table>
<thead>
<tr>
<th>Medium</th>
<th>Time (Days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>YE-BS-NaPCP - Control</td>
<td>.72</td>
</tr>
<tr>
<td>YE-BS-NaPCP - Soil</td>
<td>.74</td>
</tr>
<tr>
<td>YE-BS-NaPCP - Ent.</td>
<td>.74</td>
</tr>
<tr>
<td>YE-BS-NaPCP - Flav.</td>
<td>.73</td>
</tr>
<tr>
<td>NaPCP-BS - Control</td>
<td>.72</td>
</tr>
<tr>
<td>NaPCP-BS - Soil</td>
<td>.71</td>
</tr>
<tr>
<td>NaPCP-BS - Ent.</td>
<td>.71</td>
</tr>
<tr>
<td>NaPCP-BS - Flav.</td>
<td>.73</td>
</tr>
<tr>
<td>NB-NaPCP - Light - Control</td>
<td>.70</td>
</tr>
<tr>
<td>NB-NaPCP - Dark - Control</td>
<td>.72</td>
</tr>
<tr>
<td>NB-NaPCP - E. coli</td>
<td>.73</td>
</tr>
<tr>
<td>NB-NaPCP - Soil</td>
<td>.74</td>
</tr>
<tr>
<td>NB-NaPCP - Ent.</td>
<td>.70</td>
</tr>
<tr>
<td>NB-NaPCP - Flav.</td>
<td>.72</td>
</tr>
</tbody>
</table>

NB = Nutrient Broth
YE = Yeast Extract
BS = Basal Salts
NaPCP = Sodium Pentachlorophenate
Ent. = Enterobacter sp.
Flav. = Flavobacterium sp.
### TABLE V

RESULTS OF THE COLORIMETRIC TEST FOR DETECTING
THE CONCENTRATION OF SODIUM PENTACHLOROPHENATE
WITH NO EXCESS OF SODIUM BICARBONATE
RECORDED AS ABSORBENCY

<table>
<thead>
<tr>
<th>Medium</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaPCP-BS - Control</td>
<td>.70</td>
<td>.75</td>
<td>.70</td>
<td>.70</td>
<td>.71</td>
<td>.72</td>
</tr>
<tr>
<td>NaPCP-BS - Soil</td>
<td>.71</td>
<td>.73</td>
<td>.70</td>
<td>.70</td>
<td>.71</td>
<td>.72</td>
</tr>
<tr>
<td>YE-BS-NaPCP - Control</td>
<td>.71</td>
<td>.74</td>
<td>.72</td>
<td>.72</td>
<td>.74</td>
<td>.73</td>
</tr>
<tr>
<td>YE-BS-NaPCP - Soil</td>
<td>.71</td>
<td>.74</td>
<td>.72</td>
<td>.75</td>
<td>.72</td>
<td>.72</td>
</tr>
<tr>
<td>YE-BS-NaPCP - Ent.</td>
<td>.71</td>
<td>.75</td>
<td>.72</td>
<td>.72</td>
<td>.75</td>
<td>.74</td>
</tr>
<tr>
<td>YE-BS-NaPCP - Flav.</td>
<td>.73</td>
<td>.75</td>
<td>.72</td>
<td>.73</td>
<td>.75</td>
<td>.74</td>
</tr>
<tr>
<td>NB-NaPCP - Control</td>
<td>.73</td>
<td>.75</td>
<td>.73</td>
<td>.73</td>
<td>.75</td>
<td>.74</td>
</tr>
<tr>
<td>NB-NaPCP - Soil</td>
<td>.73</td>
<td>.74</td>
<td>.74</td>
<td>.73</td>
<td>.75</td>
<td>.74</td>
</tr>
<tr>
<td>NB-NaPCP - Ent.</td>
<td>.73</td>
<td>.75</td>
<td>.75</td>
<td>.75</td>
<td>.75</td>
<td>.75</td>
</tr>
<tr>
<td>NB-NaPCP - Flav.</td>
<td>.73</td>
<td>.75</td>
<td>.71</td>
<td>.73</td>
<td>.75</td>
<td>.74</td>
</tr>
</tbody>
</table>

NB = Nutrient Broth
YE = Yeast Extract
BS = Basal Salts
NaPCP = Sodium Pentachlorophenate
Ent. = Enterobacter sp.
Flav. = Flavobacterium sp.
Fig. 6--Reference Curve showing the correlation between different concentrations of Sodium Pentachlorophenate and absorbance at 540 millimicron wavelength.
DISCUSSION

From the data presented from testing pentachlorophenol and sodium pentachlorophenate as possible sole carbon sources for soil bacteria, it appears that there were no bacteria in the soil samples tested able to use either of these compounds as their sole carbon source. The first 51 vials showed no bacterial growth when incubated anaerobically. Throughout the experiment no growth of anaerobes was observed when pentachlorophenol and its sodium salt were included as a possible sole carbon source.

Although some bacterial growth was observed from aerobic incubation of the same soil sample cultures in sodium pentachlorophenate and in pentachlorophenol, growth of the same bacteria was also observed in basal medium with no carbon source. These results indicated that either there were trace contaminants in the basal medium preparations or the organisms lived on carbon in the soil or from the air or a combination of these. The lack of growth of bacteria on pentachlorophenol-basal medium plates and the lack of growth after a prolonged incubation of 28 days help to support this hypothesis.

Although Cserjesi (5) has reported the adaptation of certain fungi to concentrations as high as 280 ppm sodium pentachlorophenate in malt extract medium, few fungi can be adapted to withstand even 120 ppm sodium pentachlorophenate. No bacteria
have been reported to be capable of survival in the presence of sodium pentachlorophenate. The ability of the two bacterial species isolated in the presence of sodium pentachlorophenate to withstand 180 ppm sodium pentachlorophenate in yeast extract-basal medium and 160 ppm sodium pentachlorophenate in nutrient broth is, therefore, noteworthy. It is also noteworthy that one of the bacteria was found in all five soils that were tested extensively, suggesting the widespread occurrence of this organism.

The two bacteria isolated were tentatively placed in the genera Enterobacter and Flavobacterium, although it was not possible to assign them to known species. The reactions of the bacterium that formed white colonies were very similar to the reactions described in Bergey's Manual (4) for Aerobacter aerogenes, now placed in the genus Enterobacter. The main difference was in the nitrate reduction test which was negative on the isolated organism and positive according to the manual.

The bacterium that formed yellow colonies on nutrient agar was more reluctantly placed in the genus Flavobacterium. This classification was based largely on its gram negative reaction, its yellow colonial pigmentation, and its lack of gas production in the sugars tested. It did have a greater ability to produce acid from sugars than most Flavobacterium species, although some species of the genus Flavobacterium have been reported in Bergey's Manual (4) to form acid from
various sugars. Its ability to grow under anaerobic conditions and to ferment glucose anaerobically cast some doubt on this classification; however, at least one species of *Flavobacterium* has been reported in Bergey's Manual (4) to have this activity.

The turbidity readings taken on the Klett-Summerson Colorimeter after three days of incubation (Fig. 1) seem to indicate that any concentration of sodium pentachlorophenate in a given medium which allows growth will allow almost the same amount of growth (except at the concentration of 160 ppm sodium pentachlorophenate in yeast extract-basal medium). However, the growth curves recorded at varying concentrations of sodium pentachlorophenate show that this is not exactly correct (Fig. 5 and Fig. 6). The graphs of these last two figures show that increased concentration does have a definite effect on the growth curve by increasing the lag phase time and decreasing the maximum turbidity. In these two experiments different amounts of inocula and media were used in different types of containers (screw-cap vials in the first experiment and 250 ml Belco side-arm flasks in the second experiment) which would effect the amount of oxygen present. Also, in each experiment different diameter tubes and different amounts of media were measured in the colorimeter. Therefore, no definite proof can be obtained from the latter tests with respect to the former tests. However, it is probable that in the first graph (Fig. 1), the growth curves had leveled off during the culture
death phase. Since no growth was reported in the second tests from 160 ppm sodium pentachlorophenate, it is difficult to say much about the peaks recorded at 160 ppm sodium pentachlorophenate in yeast extract-basal medium. However, since the lag phase is increased in the cultures in Figure 5 and Figure 6, it is possible that in Figure 1 the cultures at 160 ppm sodium pentachlorophenate in yeast extract-basal medium are still in the stationary phase of growth while the other cultures in Figure 1 are probably in late death phase.

The growth curves of turbidity and viable cell counts and the pH readings in the absence of sodium pentachlorophenate give a better idea of some of the characteristics of the bacteria isolated. The Enterobacter sp. undergoes lysis shortly after it reaches its maximum growth as determined by turbidity readings. The turbidity readings correspond well with the viable cell counts. The increase in turbidity of Flavobacterium sp. after the stationary phase has been reached in the viable cell counts is probably due to pigment production.

Biodegradation of sodium pentachlorophenate and pentachlorophenol is still questionable. While several organisms have been shown to tolerate sodium pentachlorophenate, the only microorganisms which have been shown by any researcher to reduce sodium pentachlorophenate are two species of the fungus Trichoderma (5). It is not unusual, therefore, that the bacteria isolated and the soil inocula were unable to show a decrease from a concentration of 50 ppm sodium pentachlorophenate.
Although no degradation was detected at this concentration or under these circumstances, it is possible that degradation of sodium pentachlorophenate can occur under other conditions or at other concentrations. The slight variations of the readings of the colorimetric test are within the range of technical error (e.g. variations due to pipetting technique and in the time that a culture was exposed to light before readings were taken).
The purpose of this study was to see if any soil bacteria were able to use pentachlorophenol or sodium pentachlorophenate either aerobically or anaerobically as a sole carbon source, to see if any soil bacteria could survive in high concentrations of sodium pentachlorophenate, to determine the maximum concentration of sodium pentachlorophenate which permitted the growth of some soil bacteria, to see the effects of varying concentrations of sodium pentachlorophenate on the growth curves of soil bacteria capable of growing in its presence, and to see if any soil bacteria could degrade sodium pentachlorophenate.

The study showed that none of the soil samples collected contained bacteria that could use sodium pentachlorophenate or pentachlorophenol as a sole carbon source. Two species of bacteria, one of the genus Enterobacter and the other of the genus Flavobacterium, were isolated that could survive up to 180 ppm sodium pentachlorophenate in yeast extract-basal medium. It was shown that an increased concentration of sodium pentachlorophenate affected the growth curves of both of these bacteria by increasing the lag phase time and decreasing the maximum turbidity. These bacteria, however, were unable to degrade sodium pentachlorophenate in concentrations of 50 ppm as tested by the colorimetric test of Haskins (12).
LITERATURE CITED


