ANAEROBIC TRANSFORMATIONS OF KEPONE BY DENITRIFYING BACTERIA

by

George Cornelius Allen, II

Thesis submitted to the Faculty of the

Virginia Polytechnic Institute and State University

in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

in

Microbiology

APPROVED:

R.E. Benoit, Chairman

R.W. Young

J.L. Neal

L.V. Holdeman

J-S. Chen

December, 1983

Blacksburg, Virginia

ANAEROBIC TRANSFORMATIONS OF KEPONE BY DENITRIFYING BACTERIA

by

George Cornelius Allen, II

(ABSTRACT)

Anaerobic bacteria isolated from Kepone-contaminated sediment were screened for their ability to degrade Kepone under anaerobic conditions. The most extensive degradation was produced by denitrifying bacteria grown on benzoate-nitrate medium. In enrichment cultures, Kepone was transformed more extensively than by pure cultures isolated from the enrichments. A gram negative-faculative rod, called K bacterium, transformed 8.8% of the Kepone in a benzoate-nitrate medium in the presence of 2000 ug/ml potassium nitrate under anaerobic conditions. Kepone transformation by K bacterium increased to 21.1% when the potassium nitrate concentration in this medium was 500 ug/ml of medium. Although monohydro-Kepone and dihydro-Kepone were produced as products of the transformation, less than 20% of the transformed Kepone was recovered as these products. Both K bacterium and the enrichment culture transformed [^{14}C]Kepone. No $^{14}CO_2$, or new radioactive insoluble or soluble products were detected in spent media. Products which were more polar than Kepone, but could not be identified, were observed in GLC chromatograms. K bacterium attached to the Kepone crystals and was pleomorphic during Kepone transformation. These data support the hypothesis that Kepone is transformed when it is used by certain anaerobes as an alternate electron acceptor.

ACKNOWLEDGEMENTS

My thesis is dedicated to Dr. Robert E. Benoit. May he continue to do courageous, difficult experiments that lead to important new ideas. I thank the members of my committe, which consisted of talented people from diverse backgrounds. Dr. Neal, who introduced me to the world of nitrogen fixation. Maybe someday these excellent ideas will be considered fundable by the powers that be. Dr. Holdeman, whose course gave me the first taste of what graduate school in microbiology was all about. Dr. Chen, an expert in the physiology of anaerobes, who gave me insight into hydrogenase and nitrogenase with a depth of knowledge unsurpassed. Finally, Mr. Roddy Young, who saw this research important enough to support it despite the lack of external funds. Simply said, this research would not have been possible at V.P.I. without his help and kindness.

My work in Roddy's lab had a joyful byproduct. I met several unique people who were both friends and co-workers who have helped me through these turbulent years. Their dedication to this project made it possible. Joy Burroughs and Jean Dickinson, who spent many extra hours injecting samples on the gas chromatograph. Finally, Sarah Palmer and Patty Dickinson, the people responsible for the most tedious part of the analysis - extraction and purification of the many samples that I brought over.

Due to the probable controversy regarding this work, several methods of confirmation were necessary. I am indebted to Kim Harich of the V.P.I. Biochemistry Department for his mass spectra analysis. A

key piece of evidence was supplied by Mr. Joel Jessee of Bethesda Research Laboratories, who tirelessly analyzed our samples by thin layer chromatography and confirmed our results.

TABLE OF CONTENTS

INTRODUCTION

Kepone is one of the most recalcitrant compounds ever synthesized (6, 26, 39). The remarkable stability of Kepone was one of the reasons it was suggested for use in 55 commercial products, which ranged from a pesticide to a fire retardant. Kepone has an affinity for lipids (14, 26, 40) and, therefore, it has a bioaccumulative character which affects many species in the food chain from microorganisms (7, 31, 32) to humans (10, 20, 43). The question, "Can microorganisms degrade Kepone?", has important environmental ramifications of indefinite duration because Kepone has accumulated in some detritus sinks such as James River sediment in Virginia (13). There was speculation that Kepone cannot be biologically degraded, because no microorganism that can use Kepone as a carbon or energy source has been isolated. Furthermore, no evidence of [^{14}C]Kepone degradation was observed with mixed cultures in anaerobic or aerobic hydrosoils (26) or anaerobic sediment (39). However, several claims for Kepone degradation, and closely related compounds, have been published. For example Mirex and Kepone were degraded by sewage sludge organisms (3, 5). Schrauzer and Katz described the complex chemistry of Kepone and the theoretical degradation intermediates (37) and demonstrated that Kepone could be degraded in a highly reduced, anaerobic, abiotic system, if vitamin B_{12s} was available. Orndorff and Colwell were the first to demonstrate that Kepone can be transformed by pure cultures of bacteria (33). In that study, a strain of Pseudomonas aeruginosa isolated from the Kepone-contaminated sludge lagoon at Hopewell, Va., transformed

14.2 and 15.6% of the Kepone to monohydro-Kepone and dihydro-Kepone after 7 days of aerobic incubation in a proteose peptone-yeast extract-salts medium. Monohydro-Kepone and dihydro-Kepone have been detected in Kepone-contaminated portions of the James River (6, 11, 21); these products may indicate either in situ microbial degradation, or photolytic products.

Since the initial dehalogenation of organochlorine compounds is favored by reducing conditions (27), our objective was to determine if bacteria could transform Kepone under anaerobic conditions. We assumed that the reductive dechlorination reactions necessary to make Kepone susceptible to chemical attack would occur during anaerobic respiration. We hypothesized that a suboptimum concentration of an essential electron acceptor such as nitrate might divert the electrons of a respiring microorganism into an electron-poor organochlorine compound such as Kepone. Since Kepone is lipophilic, the concentration of Kepone at the bacterial membrane will exceed the concentration in the polar aqueous environment and be available to the electron transport system. Mahaffey et al. (31) demonstrated that Kepone did not reduce the number of colony forming units of estuarine microorganisms during anaerobic growth, although gram-positive bacteria were more inhibited by Kepone than gram negative bacteria (7, 31, 32). We report here the isolation of a Kepone-transforming bacterium (K bacterium) from an anaerobic enrichment culture which was grown in a defined medium containing benzoate, Kepone, nitrate and salts. Kepone resistant cultures were subsequently screened for the capacity to degrade Kepone. Preliminary reports of this work have been presented

(Allen, G.C., R.W. Young and R.E. Benoit, Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, Q85, p. 214 and 1983, Q 138, p. 283).

Chemical Structures

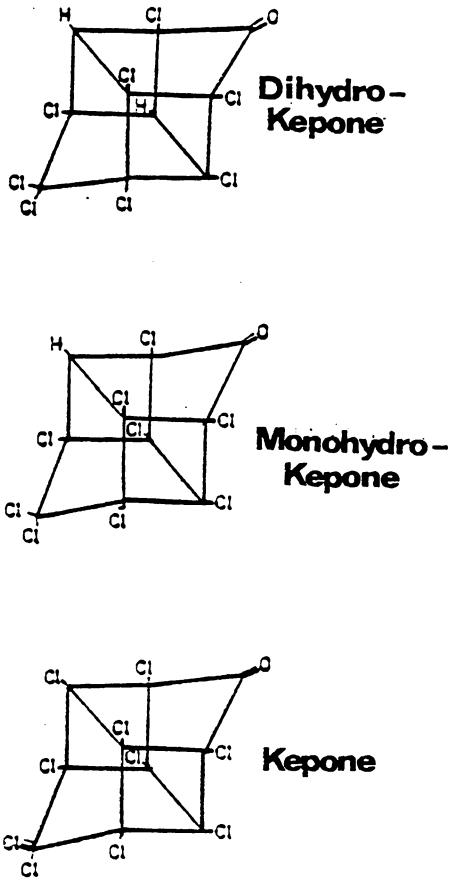

FIG. 1. Chemical structures of Kepone and the reduction products monohydro-Kepone and dihydro-Kepone.

MATERIALS AND METHODS

Chemicals. Technical-grade Kepone (88.6%) was obtained from Allied Chemical Corp., Baltimore, Md. Pure monohydro-Kepone [1a,3,3,4,5,5a-5b,6-nonachloro-octahydro-1,3,5-methano-2H-cyclo-buta-(cd) pentalen-2-one] and dihydro-Kepone [1,3,-4,5,5,5a,5b,6-octachloro-1,3,4,metheno-2H-cyclobuta (cd) pentalen-2-one] standards were supplied courtesy of the U.S. Environmental Protection Agency, Research Triangle, N.C. [^{14}C]Kepone (sp act = 3.2 mCi/mmole) was obtained by the courtesy of Mr. R.D. Zehr, U.S. Environmental Protection Agency, Research Triangle, N.C. Organic solvents used in the study were pesticide grade (Burdick and Jackson, Muskegon, Mich. and Mallinckrodt, St. Louis, Mo.). Other chemicals were reagent grade (Fisher Scientific, Raleigh, N.C.).

Sediment sample collection. Samples were collected in 1-liter polypropylene bottles from the Kepone-contaminated leachate-outfall at Hopewell, Va. municipal sewage treatment plant in March, 1980. The samples were stored at 5°C for 1 mo prior to initiation of the study.

Media. All anaerobic culture media were prepared according to the V.P.I. and S.U. Anaerobe Laboratory Manual (22). One mg of technical-grade Kepone was added to each culture tube in an acetone carrier, which was evaporated prior to medium addition. All media were sterilized at 121°C for 15 min. Standard anaerobic culture tubes (Bellco Glass, Inc.) containing 10 ml of medium per tube were used throughout the study, unless otherwise indicated. The media tested for

enrichment were as follows: Brain Heart Infusion broth (22), Lactate-Sulfate broth (35), Pyruvate-Sulfur broth (34), Modified Acetate broth (30), and Benzoate-Nitrate broth (BN medium) (48). The BN medium, which was extensively used, contained (g/l): Na-benzoate, 0.72; Na_2HPO_4, 3.12; KH_2PO_4, 1.5; NH_4Cl, 0.3; KNO_3, 2; $MgSO_4 \cdot 7H_2O$, 0.1; and 5 ml of trace element solution. The trace element solution contained (g/l): disodium EDTA, 50.0; $ZnSO_4 \cdot 7 H_2O$, 22.0; $CaCl_2$, 2.27; $MnCl_2 \cdot H_2O$, 5.06; $FeSO_4 \cdot 7 H_2O$, 4.99; $(NH_4)_6 Mo_7O_{24} \cdot 4 H_2O$, 1.1; $CuSO_4 \cdot 5 H_2O$, 1.57; and $CoCl_2 \cdot 6 H_2O$, 1.61.

Enrichment and isolation. Sediment (150 g wet wt) was added to 500 ml of the respective anaerobic media in 1-liter Erlenmeyer flasks, each containing 50 mg of technical-grade Kepone. The flasks were incubated in the dark at 23°C 18 d prior to transferring one ml into culture tubes containing 10 ml of the same medium. Each enrichment culture was transferred weekly in duplicate. One culture and a corresponding uninoculated control were frozen until they were analyzed for residual Kepone. Pure culture isolates were obtained from the third transfer from BN medium, Lactate Sulfate medium, and brain heart infusion medium enrichments which contained 10 ml of the appropriate medium. Anaerobic roll tubes (22), supplemented with 1 mg of technical-grade Kepone, were used in all isolations. Isolated colonies were streaked until pure cultures were obtained. Stock cultures were maintained on anaerobic slants containing the respective medium with Kepone. The BN medium isolates were screened for the potential to degrade Kepone by inoculating a loopful of surface growth into 10 ml of

BN-Kepone broth, incubating for 23 d at 30°C, and analyzing for the residual Kepone.

Kepone and metabolite extraction and purification. A modification of the technical procedures described by the Division of Consolidated Laboratory Services (4) was used to extract residual Kepone from the media. The spent culture medium (10 ml) was added to a 250-ml separatory funnel. The culture tubes were rinsed with 25 ml of acetonitrile and the mixture combined and shaken vigorously for 5 min. on an automatic wrist shaker. Fifteen ml of 1:1 (v/v) ethyl ether-petroleum ether was added, and the shaking was continued for 1 min. Two ml of NaCl-saturated water was then added, and the mixture was shaken for 30 sec. The water-acetonitrile layer was drained into a second 250-ml separatory flask and extracted, as above, with 1:1 (v/v) ethyl ether-petroleum ether. The ether extracts were combined in the first separatory flask and washed twice with 1 ml of NaCl-saturated water and 20 ml of distilled water. The combined ether extract was then drained through a 2 in. column of anhydrous Na_2SO_4 into a 100-ml beaker and evaporated to dryness in a 38°C water bath. The evaporated material was suspended into 10 ml of 2% methanol-4% benzene- and 94% hexane (v/v) and transferred to a graduated centrifuge tube.

Activated florisil (1.6 g) was topped by anhydrous Na_2SO_4 (1.6 g) in a 12 mm internal diameter microflorisil column. The initial 7 ml eluate contained no Kepone and was discarded. The Kepone-containing sample was eluted from the column with 40 ml of 1% methanol-4% benzene-2% acetonitrile, and 93% hexane (v/v) and collected in a 100-ml beaker. The eluate was evaporated in a 38°C water bath to

dryness, and transferred to a graduated centrifuge tube with 10 ml of 1% methanol in benzene. Extracts were stored at 5°C in the dark until analyzed. Recovery from samples containing 1 mg of Kepone averaged 98%.

Gas liquid chromatography (GLC). The sample extracts were analyzed with a Microtek 220 gas-liquid chromatograph equipped with a ^{63}Ni electron-capture detector. The column (6 ft. length X 4 mm) was packed with Chromosorb WHP 80/100 mesh with OV-17/QF-1 liquid phase (Supelco, Inc., Bellefonte, Pa.). Nitrogen (Industrial Gas, Radford, Va.) was the carrier gas; flow rate was 70 ml/min. flow rate. The temperature parameters were as follows: injector, 225°C; column, 205°C; and detector, 350°C.

Kepone concentrations were determined by injecting 1 ul of a 1/1000 dilution of the 1% methanol in benzene sample extract and comparison of peak areas with those from 0.1 ng analytical Kepone standards. Changes in Kepone concentrations were determined by comparing inoculated and abiotic samples. In order to determine monohydro-Kepone and dihydro-Kepone, a 10 ml sample of the 1% methanol in benzene extract was concentrated to 1 ml, and 0.1 ul of concentrated sample was chromatographed. Results were compared with the response of the GLC to 0.1 ng of analytical standards. Samples were run in duplicate and standards were injected after every third sample.

Mass spectrometry (MS). Mass spectra were obtained by using a Varian Mat 112 gas-liquid chromatograph-mass spectrometer in the electron impact mode. Experimental conditions were: source temperature, 190°C; column temperature, 180°C; separator

temperature, 280°C; GLC line temperature, 280°C; helium carrier gas, 20 ml/min. flow rate; electron energy, 70 eV; and emission current, 0.7 mA. A 6 ft. glass column containing 1.5% OV/ 1.95% QF1 liquid phase with Chromosorb WHP 80/100 mesh was used. The sample was scanned in the range from 50-600 m/e and the data were digitized for subsequent retrieval and analysis with a Varian Mat Spectrosystem 100 MS. Following the GLC analysis, selected 1% methanol in benzene samples were concentrated to 20 ul under nitrogen and 7 ul were injected for MS analysis.

The Kepone in the spent media that was observed in given peak of the GLC analysis was confirmed by MS analysis of that peak (45). These major ion clusters were identical to the analytical Kepone standard with C_5Cl_6, m/e 270; C_5Cl_5, m/e 235; and C_5OCl_4, m/e 216. Monohydro-Kepone was confirmed in some samples and was identical to the results of Orndorff and Colwell (33) with major ion clusters of C_5HOCl_3, m/e 182; C_5HCl_4, m/e 201; C_5OCl_4, m/e 216; C_5Cl_5, m/e 235; C_5HCl_5, m/e 236; and C_5Cl_6, m/e 270. It was not possible to prove the presence of dihydro-Kepone in any of the spent culture media because the concentrations formed were below the minimum detection limit. We were not able to identify other peaks in the chromatograms of the BN enrichment (Fig. 3), possibly because they were unstable in our storage conditions, or the concentration of the products may have been too low for mass spectrometry.

Inocula preparation. Two types of inocula were prepared according to the following procedures: (i) One loopful of K bacterium grown anaerobically on an agar slant was inoculated into 500 ml of

anaerobically prepared BN broth containing 50 mg of technical grade Kepone and (ii) 5 ml of the BN enrichment culture that had been transferred weekly 30 times was inoculated into 500 ml of anaerobically prepared BN broth containing 50 mg of technical-grade Kepone. Both cultures were incubated 16 days at 23°C. An additional inoculum type of a dense cell suspension of K bacterium was prepared for the [^{14}C]Kepone transformation experiment as follows: One loopful of K bacterium was inoculated into 500 ml of aerobically prepared trypticase soy broth (Difco) containing 50 mg of technical-grade Kepone. The culture was incubated for 12 d at 23°C prior to centrifugation at 11,500 RPM for 20 min at 5°C in an International Centrifuge Model IEC B-20A. The resulting pellet was resuspended in 120 ml of 0.8% NaCl and incubated further for 24 h at 23°C.

[^{14}C]Kepone transformation. To increase the dispersion of the insoluble Kepone, 6.8 g of 60-80 mesh glass beads were added to each 60-ml serum vial (Wheaton) to be used. Two uCi in 3 mg of analytical Kepone (99%) in an acetone carrier were then added to the serum vials. After the acetone was evaporated as previously described, thirty ml of anaerobically prepared BN broth were added to each vial. Three ml of each of the two previously described inocula was then added by syringe to each of ten serum vials. Three ml of 1% (w/v) $HgCl_2$ (final concentration = 0.083% w/v) were added to 5 serum vials for each inoculum set. To compare the [^{14}C]Kepone data with the GLC results, the same inocula were used to initiate growth in identical subsets of BN medium containing 3 mg of unlabelled Kepone in serum vials. The culture in vials with unlabelled Kepone was extracted and analyzed by

GLC as previously described. All cultures were incubated statically in the dark at 30°C for 80 days.

The spent media containing [^{14}C]Kepone were flushed for 12 h with oxygen-free nitrogen into two phenylethylamine (Fisher) traps and analyzed for $^{14}CO_2$(Fig. 2). The contents of each serum vial were then extracted twice with 10 ml of 1% methanol in benzene and shaken for 1 min. Following each extraction, the methanol-benzene layer was pipetted into 20 ml scintillation vials (Fisher) and evaporated to dryness by oxygen-free nitrogen . The remaining aqueous phase was then extracted twice with 10 ml hexane and combined with the methanol-benzene extract (insoluble product extract) and evaporated. The final insoluble product extract was then taken up in 15 ml Scinti Verse (Fisher) and the appropriate dilutions were made for counting. Five replicates of each sample were counted. The organic solvent layer that remained on the surface of the aqueous phase was evaporated under nitrogen. Two ml of the aqueous phase (Soluble products) were pipetted into each of five scintillation vials which contained 15 ml Scinti Verse. ^{14}Carbon was measured with a Beckman Model LS 3150T Scintillation Counter equipped with a gamma source external standard. All samples were corrected for quenching with an internal standard.

Thin layer chromatography. Cellulose MN-300 Uniplates (10 X 20 cm) from Analtech (Newark, Del.) were dried at 80°C for 10 min. prior to sample application. One ul samples of a 1/10 dilution of the insoluble fraction [^{14}C]Kepone extract was applied and developed using water-saturated CCl_4 (Fisher Scientific, Raleigh, N.C.). The chromatograms were exposed for 72 h on Kodak XR-5 X-ray film. Spots were detected

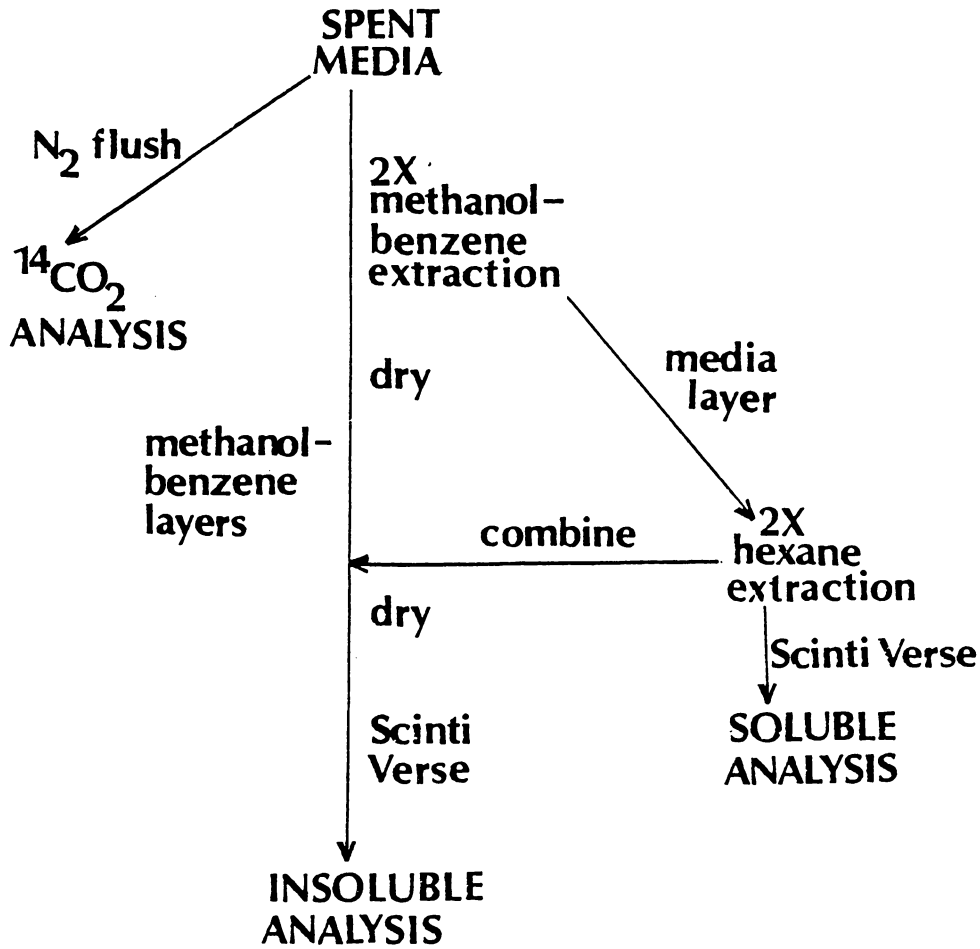

FIG. 2. Flow diagram for the extraction and fractionation of [^{14}C]Kepone and [^{14}C]Kepone products.

and analyzed by a Camag TLC scanner (Applied Analytical).

Effect of varying nitrate concentrations on Kepone degradation. Ten ml of anaerobically prepared BN broth (1 mg of Kepone/10 ml medium) containing 0, 0.1, 0.5, and 1.0 (g/l) KNO_3, respectively, was added to culture tubes. One ml of the appropriate inoculum was added to 5 replicate culture tubes of each nitrate concentration. One ml of 1% $HgCl_2$ (final concentration = 0.083% w/v $HgCl_2$) was added to an identical subset of the inoculated culture media. This treatment was used as a killed control to measure absorption losses or abiotic transformation of Kepone. All cultures were incubated for 80 days at 30°C prior to analysis for residual Kepone and Kepone transformation products. Kepone contained in the inocula were determined by analyzing ten one-ml inoculum samples. Protein content in each inoculum was determined by the method of Lowry et al. (29) following alkaline digestion. Bovine serum albumin (Sigma) was used as the standard. Qualitative tests for nitrate and nitrite were performed according to the Manual of Methods for General Bacteriology (41).

Effects of Kepone on bacterial morphology. Technical grade Kepone in an acetone carrier was added to 10 ml of trypticase soy broth (Difco) to give final concentrations of 1, 5, 10, 50, and 100 ug/ml. Controls contained the acetone carrier without Kepone. The 5 test organisms (K bacterium, Escherichia coli ATCC 12435, Bacillus polymyxa ATCC 842, Staphylococcus aureus ATCC 12600, and Pseudomonas flourescens ATCC 13525) were grown to mid-log phase in trypticase soy broth (minus Kepone). One loopful of these broth cultures were inoculated into media containing the different Kepone concentrations. The cells were

grown aerobically at 30°C and examined with a phase contrast microscope (1000 X) every 24 h to determine if Kepone affected the morphology of the cells.

RESULTS

Kepone Degradation in Enrichment Culture. Kepone transformation was defined in this study as the percent of Kepone decrease in culture media relative to uninoculated media. During the initial 5 serial transfers of the enrichment cultures, the average Kepone transformation in the modified acetate and pyruvate media was less than 2%. The average decrease in the brain heart infusion and lactate-sulfate enrichment media was 10.3 and 9.5% respectively in the same experiment. Whether the media were incubated 1 or 2 weeks, the quantity of Kepone transformed did not change. The brain heart infusion and lactate-sulfate enrichment media became very turbid and these bacteria formed copius quantities of capsule. Orndorff and Colwell (33) also noted that Pseudomonas aeruginosa formed extracellular material when grown in a peptone-Kepone medium. In the brain heart infusion and lactate-sulfate enrichments, the Kepone transforming capacity was reduced after each successive transfer. For example, Kepone transformation in the brain heart infusion was reduced from 17.7 to 2.8% between the first and fifth transfer. The reason for this loss of activity is not known, but the high concentration of Kepone (100 ug/ml) may have selected for Kepone tolerant organisms instead of Kepone degraders.

There was little Kepone transformation during the first two enrichment transfers in the benzoate-nitrate medium, but on the third transfer 62.4% of the Kepone was transformed. This was the maximum Kepone transformation observed throughout this study. The long lag may be related to long "adaptation" times reported for benzoate-dependent CH_4 production in sediment (24). Several different morphological

types of bacteria were observed in the enrichment. The mean of the Kepone transformation in the spent media of the 18 weekly transfers was 7.5 ug/ml (Fig. 3). If the organisms in the enrichment culture had different capacities to transform Kepone and the quantities of these organisms varied in different transfers, this might explain in part why the BN enrichment culture had an unpredictable pattern of transformation in the different transfers. But in any given transfer, consistent results were observed between replicates. Kepone transformation was not enhanced by long incubations times which favors the hypothesis that Kepone degradation occurs during the most active phases of the growth cycle in this enrichment. In order to test this hypothesis, pure cultures were isolated from the BN enrichment. A small, gram negative, motile rod, designated K bacterium, was chosen for further study because it transformed more Kepone than other members of the enrichment.

Transformation of Kepone by K Bacterium. Kepone transformation was measured in the initial experiments by GLC analysis (Fig. 3) and confirmed by [^{14}C]Kepone fraction analysis in this experiment (Table 1). The Kepone was adsorbed to glass beads in the medium in an attempt to increase the surface area of the Kepone relative to the medium and thereby increase the potential for Kepone degradation. There was significant Kepone transformation in all inoculated flasks, and there was general agreement between the GLC and [^{14}C]Kepone techniques. There was more Kepone transformed in the enrichment culture system than in the K bacterium cultures. K bacterium, unlike the enrichment culture, consistently transformed a certain quantity of Kepone in all

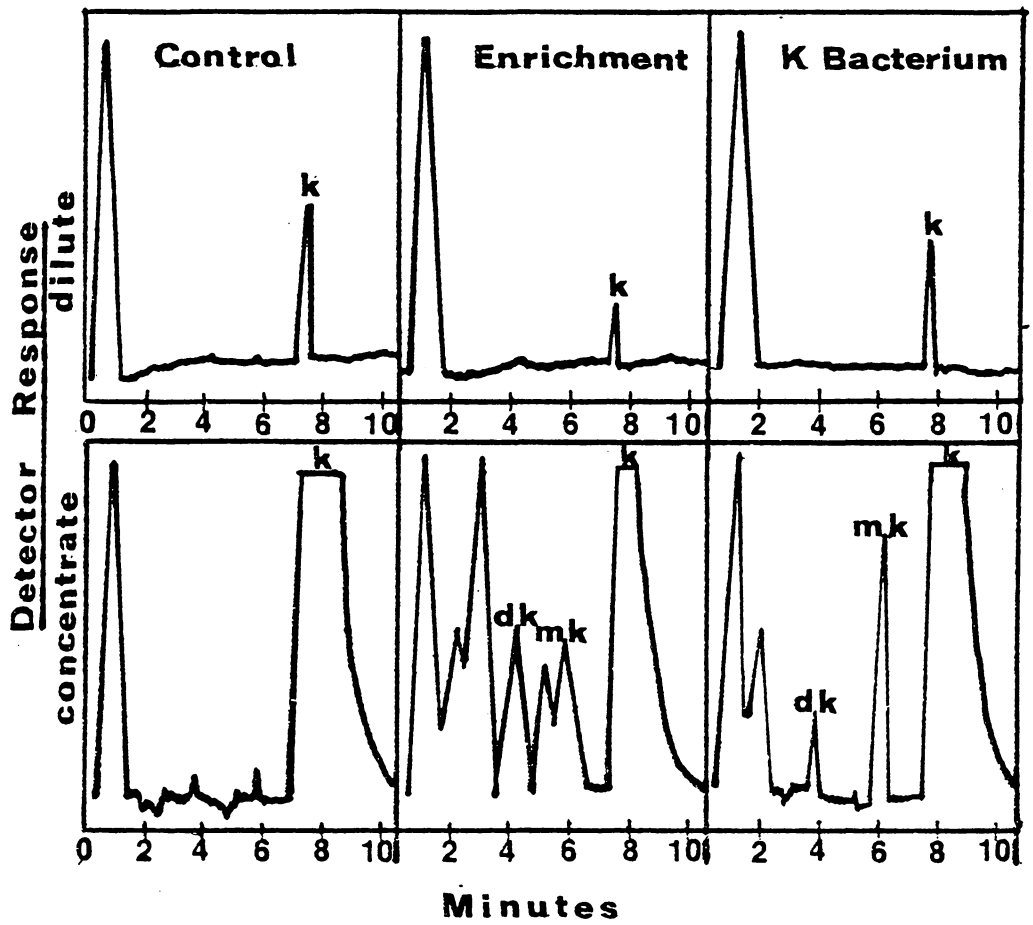

FIG. 3. Kepone transformation in BN medium by enrichment and K bacterium cultures after 10 days incubation at 30°C. The samples were diluted to measure residual Kepone (K) shown in the top row and were concentrated to measure monohydro-Kepone (mK) and dihydro-Kepone (dK) shown in the bottom row.

TABLE 1. Biotransformation of Kepone by enrichment culture and different inoculum quantities of K bacterium on BN medium after 80 days of incubation at 30°C.

Sample treatment	Kepone[a] (ug/ml)	monohydro-Kepone (ug/ml)	dihydro-Kepone (ug/ml)	Percent of ^{14}C in insoluble fraction	Percent of ^{14}C in soluble fraction
Uninoculated	96.7 (±2.1)[b]	0.3	0.0	100 (±2.3)	2.8 (±0.1)
Killed K bacterium	97.2 (±2.2)	0.2	0.0	100 (±2.3)	2.8 (±0.1)
K bacterium (4.8 mg protein)[c]	91.0 (±3.2)	0.2	0.0	73.6 (±1.2)	3.3 (±0.0)
K bacterium (41 ug protein)[c]	87.9 (±3.3)	0.3	0.0	84.4 (±3.2)	2.9 (±0.2)
Enrichment (156 ug protein)[c]	79.1 (±4.0)	0.1	0.0	63.1 (±2.2)	2.3 (±0.0)

[a]GLC analysis

[b]Mean of five replications (± SEM)

[c]Protein per ml was used to measure quantity of culture per ml in the inoculum.

experiments. There was no significant difference between results obtained with different inocula when the GLC technique was used to measure Kepone transformation by K bacterium. However, the high cell inoculum transformed more Kepone than the low cell inoculum when both were compared by ^{14}C-analysis. No $^{14}CO_2$ was observed as an endproduct in any treatment. There were no significant differences between the amounts of [^{14}C]Soluble material in the spent medium or the controls. The GLC method used to detect monohydro- and dihydro-Kepone could detect small changes in the quantities of these intermediates, but the concentration of these intermediates in the spent medium was not significantly different than the controls. In the many preliminary experiments that were run with the enrichment culture and K bacterium, we did not detect significant accumulations of monohydro- and dihydro-Kepone in the spent medium. Products which were more polar than Kepone were detected in GLC chromatograms (Fig. 3), but it was not possible to isolate these products. A variety of standard thin layer chromatography techniques failed to detect any [^{14}C]product other than [^{14}C]Kepone in the nonsoluble fraction of the spent medium of enrichment and K bacterium cultures. The use of glass beads in the BN medium did not affect Kepone transformation by K bacterium.

Kepone transformations under sub-optimum nitrate conditions in BN medium. In order to test the hypothesis that Kepone can be transformed if it serves as an alternate electron acceptor in a competitive system when the quantity of the "normal" external electron acceptor, such as nitrate, is limited, varying quantities of nitrate were added to the BN

medium. K bacterium transformed small amounts of Kepone in the BN medium that contained no added nitrate. The inoculum may have contained small amounts of nitrate which enabled the organism to achieve a limited growth yield. Maximum Kepone transformation was observed when the initial KNO_3 concentration in the BN medium was between 100 and 500 ug/ml (Fig. 4). When the KNO_3 concentration was greater than 500 ug/ml in the BN medium, Kepone transformation by K bacterium was reduced. The quantity of Kepone transformed by K bacterium was greater in this experiment than previous experiments where the KNO_3 concentration was 2000 ug/ml. The quantity of nitrate in the BN medium did not affect the quantity of mono- or dihydro-Kepone produced in the spent medium. Small amounts of mono- and dihydro-Kepone were usually found in the spent BN medium of K bacterium, whereas, there were only traces of these products in the uninoculated control medium. Throughout our entire study, a maximum of only 20% of the Kepone transformed by K bacterium could be recovered as mono- or dihydro-Kepone.

The enrichment culture transformed as much Kepone as K bacterium when 100 ug/ml of KNO_3 was present in the medium. Unlike K bacterium, the enrichment culture did not show a reduced potential to transform Kepone at the 1000 ug/ml KNO_3 concentration. However, based upon previous experiments which used BN medium with quantities of KNO_3 greater than 1000 ug/ml, it can be inferred that Kepone transformation by the enrichment culture can be reduced by providing higher quantities of nitrate. In most of the experiments in our study, the enrichment culture was able to transform more Kepone than K

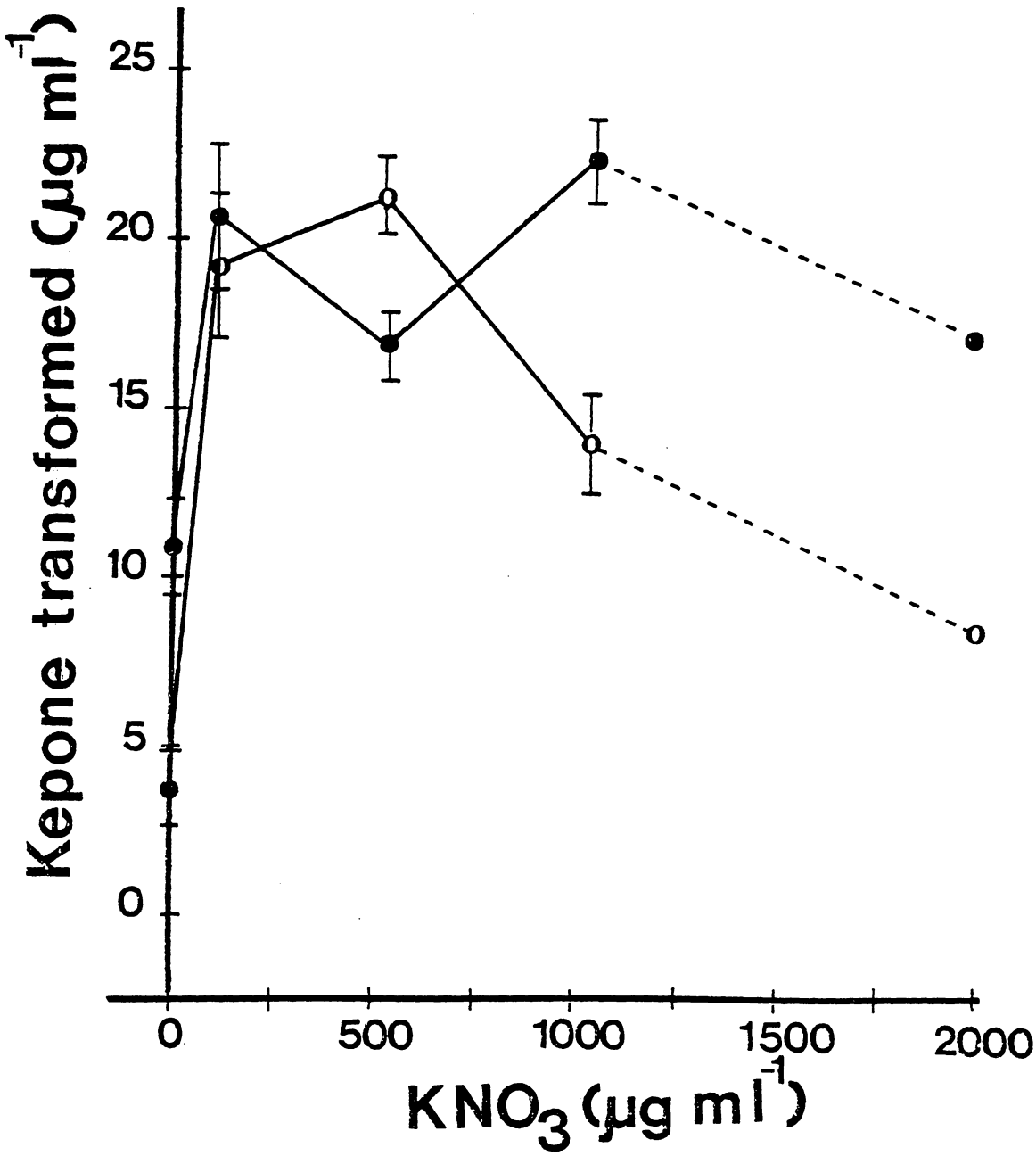

FIG. 4. Decrease in residual Kepone from enrichment (closed symbols) and K bacterium (open symbols) cultures at different nitrate concentrations. Values are the mean of five replicates (± SEM). Cultures were grown 80 days in anaerobic BN medium at 30°C in the presence of 100 ug/ml Kepone. Residual Kepone was measured by GLC and Kepone transformation was measured as the difference between spent media, and uninoculated and killed controls. The 2000 ug/ml KNO_3 values (dashed lines) represent an average of two experiments.

bacterium, but in some cases the amount of Kepone the enrichment transformed was equal to, or less than that transformed by K bacterium. The amount of Kepone transformed by K bacterium in different experiments was more consistent than was observed with the enrichment culture. These differences in Kepone transformation between the enrichment culture and K bacterium are consistent with the hypothesis that Kepone can be biologically transformed when certain biological, chemical, and physical conditions are operating in concert. Small quantities of monohydro- and dihydro-Kepone were detected in the spent media of the enrichment culture, but only a small portion of the transformed Kepone was recovered as these products.

Effect of Kepone on Cell Morphology. It was our hypothesis that the morphogenesis of K bacterium might be an important factor in the transformation of Kepone, especially if direct contact of the cell and Kepone particles is one of the variables. The growth and morphology of some gram negative bacteria, such as Escherichia coli K-12, was not affected by the presence of Kepone in aerobic trypticase soy medium at any of the concentrations tested. High concentrations (100 ug/ml) of Kepone increased the length of the lag growth phase of Pseudomonas flourescens. During the early log phase many of the P. flourescens cells were pleomorphic, and the motility of some cells was uncoordinated. During the late log and stationary growth phases, P. flourescens regained a typical rod shape. Neither E. coli or P. flourescens had an affinity for the Kepone particles. In contrast, K bacterium had a high affinity for the bundles of Kepone crystals, especially during the early log growth phase. K bacterium had a

typical rod shape during growth in aerobic trypticase soy broth in the absence of Kepone, but in the presence of Kepone the cells were pleomorphic in the log growth phase. The bundles of Kepone crystals that frequently floated on the surface of the medium became dispersed after K bacterium attached to them. Furthermore, the large Kepone bundles separated into single crystals and became smaller during the growth of K bacterium. During the stationary growth phase, K bacterium had a typical rod shape and only a small portion of the cells remained attached to the Kepone crystals. Based upon these observations it is plausible that optimum cell-Kepone contact in certain phases of the growth cycle is one of the variables affecting Kepone transformation. Failure to arrange the ideal geometry between the medium, Kepone and certain bacteria may reduce the rate of Kepone transformation. For example, the acetone carrier used to add Kepone to the culture tubes occasionally caused the Kepone to float in the culture medium or to stick to the bottom of the culture tube. Therefore, the Kepone may not have been in the optimum position in the culture tube to permit transformation by bacteria during the phase of life cycle when metabolic conditions are most advantageous for a cometabolic process.

Several different laboratories have reported that Kepone is toxic to certain gram positive bacteria (7, 31, 32). Similarly, we observed that the addition of 1 ug/ml of Kepone to trypticase soy medium was toxic to both Staphylococcus aureus and Bacillus polymyxa.

DISCUSSION

Cometabolism is one of the best documented mechanisms for the degradation of organochlorine insecticides (25, 28). Reductive dechlorination of these compounds is one of the less studied, but important alternate mechanisms, for microbial transformation of recalcitrant compounds such as Kepone. Some of the compounds that can be degraded by anaerobic microorganisms include phenol (16), benzoate (24, 42), and Atrazine (27). Bouwer and McCarty (8, 9) recently have shown that halogenated aliphatic compounds, such as carbon tetrachloride, can be transformed either by batch denitrification systems or methanogenic systems. Suflita et al. (42) have shown that microorganisms of lake sediment can metabolize halobenzoates anaerobically. They speculated that these microorganisms contain a novel pathway which may be important in the removal of some chlorinated xenobiotics from the environment.

The novel structure and high degree of chlorination are major factors in Kepone's stability. Any biological reaction which affects Kepone probably involves an enzyme, or enzyme complex, of wide substrate specificity. We have demonstrated that Kepone can be biologically transformed if certain conditions are met. Our data confirm the conclusion of Orndorff and Colwell (33) that Kepone can be degraded by microorganisms. There are, however, some important differences between these two studies since different microorganisms, oxygen concentrations, and media were used.

Unless novel modifications of the traditional enrichment culture technique are utilized, it is likely that Kepone-tolerant bacteria, and

not Kepone-transforming bacteria, will be the dominant population in the enrichment system if large quantities of readily available energy sources are added in addition to the Kepone. The enrichments used in this study demonstrated that a variety of anaerobic bacteria have the potential to transform Kepone and those bacteria with anaerobic respiration systems may be the best candidates. Nitrate respiration results in the degradation of phthalic acids (1, 2), and chloro-, bromo-, and iodobenzoates can be dehalogenated by methane producing freshwater sediment (24, 42).

K bacterium was selected for study rather than some of the cultures which were isolated because it consistently transformed Kepone in replicate treatments. The enrichment culture from which K bacterium was isolated usually transformed more Kepone than K bacterium alone, but in some cases, little or no Kepone was transformed by the enrichment. This loss of activity apparently was not due to mutation, because when a culture that failed to transform Kepone in one experiment was transferred to new medium, Kepone transformation usually was observed. The reason why Kepone transformation by the enrichment culture was variable is not known, but the following hypotheses are consistent with observations made during this study: (i) The quantity of an activator or inhibitor of Kepone transformation may have varied in the different experiments. Holmstead (23) observed that Mirex, a compound closely related to Kepone, was catalytically transformed by reduced hematin if Tween 80 was present in the reaction mixture; otherwise, the reaction was not homogeneous. We observed that when Kepone was transformed by K bacterium, the Kepone became more

hydrophilic as the growth cycle proceeded. If different amounts of detergent-like compounds were produced in different experiments by K bacterium or the enrichment culture, this may have affected the extent of Kepone transformation. (ii) The metabolic system responsible for Kepone transformation may be subject to regulation. Therefore, inhibitors, inducers, and the energy status of the bacteria may vary in the different enrichments. Ebel (15) has shown that Kepone induces cytochrome P-450, which is responsible for oxidative metabolism of a wide variety of chemicals including pesticides, in the hepatic microsomal fraction in rats. K bacterium may have a "cytochrome P-450 like system" because Kepone is toxic to aerobic respiratory systems. (iii) Kepone transformation may be influenced by the geometry of the bacteria, the Kepone particles, and the chemicals in the medium. The quantitative technique used to add Kepone to the medium resulted in the Kepone either floating on the medium in some cases, or adhering to the glass walls in other cases. Since the inoculum was added to the top of the medium, the bacteria may have attached to the Kepone particles early in the growth cycle, and therefore, achieved more Kepone transformation in this position than was possible in culture tubes when most of the Kepone was adhered to the glass. The pleomorphism observed in K bacterium when attached to Kepone may have helped establish a micronitch where the concentration of nitrate or benzoate was reduced and Kepone was in the ideal position to serve as an electron acceptor during reductive dechlorination. Other similar cyclodiene insecticides, such as chlordane, exert their effects by deformation of membrane integrity (47). The primary result is then a

cessation of membrane associated aspects of cell division and biosynthesis while an effect upon energy metabolism may be a closely associated secondary phenomena. Such deformation of the membrane may be explained by changes in fatty acid and phospholipid composition in the presence the organochlorine insecticide as has been previously observed in E. coli (36). Sethunathan, et al. (38) reported that the conversion of benzene hexachloride to gamma-pentachlorocyclohex-1-ene by a Clostridium sp. was inhibited by KNO_3, and Chu and Kirsch (12) noted that pentachlorophenol degradation was enhanced by keeping the concentration of substrate low, relative to cell concentration. Other variables may also affect the Kepone transformation by changing membrane composition. For example, Tomlins et al. (44) observed that benzoate elevated the saturated /unsaturated lipid profile of Salmonella typhimurium and had a marked effect on the composition of phospholipids and neutral lipids.

Some of the other bacteria in the BN enrichment culture besides K bacterium had the capacity to transform Kepone. Therefore, the other members of the enrichment culture may have transformed Kepone in concert with K bacterium and thereby increased total activity. These bacteria also may have increased the growth of K bacterium by providing growth factors, or by utilizing the endproducts of K bacterium in their metabolism. If members of the enrichment culture that were not primary transformers of Kepone established early dominance in the BN-Kepone medium, this may have reduced the growth of K bacterium and other Kepone-transforming bacteria so that the total Kepone transformation for that experiment was low.

K bacterium and the P. aeruginosa strain of Orndorff and Colwell transformed similar quantities of Kepone in their respective media. The transformation of Kepone by K bacterium was confirmed by GLC, [^{14}C]Kepone product analysis, and [^{14}C]Kepone thin layer chromatography techniques. Monohydro-Kepone and dihydro-Kepone were the primary products of Kepone transformation by P. aeruginosa, whereas these compounds were minor products in the spent medium of K bacterium. The product differences in the spent medium of K bacterium were always determined by difference with uninoculated controls and mercury-killed inoculated controls; therefore, the amounts of monohydro-Kepone and dihydro-Kepone represent products of biological activity. K bacterium did not produce any $^{14}CO_2$, [^{14}C]soluble or [^{14}C]insoluble products. No product with R_f values different than the original [^{14}C]Kepone was found by thin layer chromatography techniques. GLC analysis of spent medium of K bacterium frequently showed several unusual peaks, but with the exception of monohydro-Kepone and dihydro-Kepone, it was not possible to determine if these peaks represented degradation products. A suggested model for the anaerobic transformation has the following steps: (i) In the presence of suboptimum quantities of a suitable electron acceptor, such as nitrate, Kepone is transformed to monohydro-Kepone and dihydro-Kepone but these intermediates may not accumulate in the medium. Orndorff and Colwell used a static culture system where oxygen and nitrate were both available; therefore, denitrification may have occurred. A variety of anaerobic bacteria may have a certain potential to transform Kepone, but those bacteria capable of anaerobic respiration may present the

best models. (ii) The hydrogenated dihomocubane cage structure of Kepone may be transformed to small molecular weight chlorinated compounds. This step is consistent with the model proposed by Schrauzer and Katz (37). They observed reductive dechlorination of Kepone in a chemical system with catalytic amounts of vitamin B_{12s}, and an organocobalamin having a $C_3Cl_3H_2$ residue was formed. Holmstead (23) also used a chemical system to show that reduced hematin reacts with Mirex to yield products formed from reductive dechlorination which included mono-, di-, tri-, and tetrahydro derivatives as well as more polar products. In our experiment using [^{14}C]Kepone, the small molecular weight chlorinated products may have been present, but lost during our procedure used to trap CO_2. (iii) The small molecular weight chlorinated compounds may be transformed to CO_2 or CH_4, or polymerized intermediate products. The possibility of polymer formation was discussed by Schrauzer and Katz. No CO_2 was observed from [^{14}C]Kepone transformation in this study, but if we had used an aerobic incubation after the anaerobic incubation of K bacterium and the BN enrichment culture, the possibility of CO_2 formation might have been enhanced. Fogel et al. (18) noted that anaerobic or denitrification conditions were necessary for biodegradation of Methoxychlor in soil at a concentration similar to the concentration of Kepone used in this study. Methoxychlor degradation was enhanced when an aerobic incubation followed an anaerobic incubation.

The amount of Kepone transformed by K bacterium in this study and by the P. aeruginosa of Orndorff and Colwell (33) was in the range of 21 to 28% of the total Kepone present in the medium. When the

incubation time of K bacterium was extended from 7 to 80 days at 30°C Kepone transfromation was not enhanced. Kepone transformation may be limited in pure culture systems to an early portion of the culture growth cycle, or inhibitors which prevent further transformation may accumulate during cell growth.

If Kepone was added to the BN medium at concentrations equal to the maximum solubility of the pesticide (2 ppm), K bacterium was able to transform only a portion of that Kepone. Whereas, when Kepone was added to the BN medium at a concentration of 100 ppm, approximately 20 ppm of the Kepone was transformed. French and Hoopingarner (19) showed that reductive dechlorination of DDT by cell-free preparations of E. coli occurred only under anaerobic conditions in the presence of FADH and the membrane fraction. One of the factors which determine the capacity of K bacterium to transform Kepone may be the surface area of the organism which is in direct contact with Kepone.

If K bacterium is representative of bacteria found in Kepone-contaminated environments, then Kepone transformation may be a function of the presence a Kepone-transforming microbial population, readily available energy sources, sub-optimum concentrations of external electron acceptors, and an optimum geometry between the microorganisms and the Kepone. One could speculate that the model used to describe how thiobacilli metabolize an insoluble compound such as elemental sulfur (46) may have some physiological parallels when K bacterium attacks Kepone particles. Since Kepone can be transformed by chemical reactions which fall in the range of physiological processes, and Kepone has been transformed by humans and rats (17), it is likely,

given the diversity of the microbial world, that Kepone can be transformed by microorganisms if there is a juxtaposition of the necessary environmental conditions.

REFERENCES

1. Aftring, R.P., B.E. Chalker, and B.F. Taylor. 1981. Degradation of phthalic acids by denitrifying, mixed cultures of bacteria. Appl. Environ. Microbiol. 41:1177-1183.

2. Aftring, R.P. and B.F. Taylor. 1981. Aerobic and anaerobic catabolism of phthalic acid by a nitrate-respiring bacterium. Arch. Microbiol. 130:101-104.

3. Andrade, P.S.L. and W.B. Wheeler. 1974. Biodegradation of Mirex by sewage sludge organisms. Bull. Environ. Contam. Toxicol. 11: 415-416.

4. Anonymous, Commonwealth of Virginia, Division of Consolidated Laboratory Services. 1979. Chlordecone (Kepone) Mirex metabolites in fish and shellfish. Procedure 2-126 DCLS, Richmond, Va.

5. Bell, B.A., F.C. Whitmore, and R.R. Cardenas, Jr. 1978. Anaerobic biodegradation of Kepone in sewage sludge. Proc. of the 1978 Natl. Conf. on Control of Hazardous Material Spills. Miami, Fl. pp. 240-243.

6. Borsetti, A.P., and J.A.G. Roach. 1978. Identification of Kepone alteration products in soil and mullet. Bull. Environ. Contam. Toxicol. 20:241-247.

7. Bourquin, A.W., P.H. Pritchard, and W.R. Mahaffey. 1978. Effects of Kepone on estuarine microorganisms. Dev. Ind. Microbiol. 19:489-497.

8. Bouwer, E.J. and P.L. McCarty. 1983. Transformations of 1- and 2-carbon halogenated aliphatic organic compounds under methanogenic conditions. Appl. Environ. Microbiol. 45:1286-1294.

9. Bouwer, E.J. and P.L. McCarty. 1983. Transformations of halogenated organic compounds under denitrification conditions. Appl. Environ. Microbiol. 45:1295-1299.

10. Cannon, S.B., J.M. Veazey, R.S. Jackson, V.W. Burse, C. Hayes, W.E. Straub, P.J. Landrigan, and J.A. Liddle. 1978. Epidemic Kepone poisoning in chemical workers. Am. J. Epidemiol. 107: 529-537.

11. Carver, R.A., and F.D. Griffith, Jr. 1979. Determination of Kepone dechlorination products in finfish, oysters, and crustaceans. J. Agric. Fd. Chem. 27:1035-1037.

12. Chu, J. and E.J. Kirsch. 1973. Utilization of halophenols by a pentachlorophenol metabolizing bacterium. Dev. Ind. Microbiol. 14:264-273.

13. Cutshall, N.H., I.L. Larson, and M.M. Nichols. 1981. Man-made radionucleotides confirm rapid burial of Kepone in James River sediments. Science 213:440-442.

14. Desaiah, D. 1981. Interaction of Chlordecone with biological membranes. J. Toxicol. Environ. Health 8:719-730.

15. Ebel, R.E. 1982. Alterations in microsomal cytochrome P-450-catalyzed reactions as a function of Chlordecone (Kepone) induction. Pest. Biochem. Physiol. 18:113-121.

16. Evans, W.C. 1977. Biochemistry of the bacterial catabolism of aromatic compounds in anaerobic environments. Nature (London) 270:17-22.

17. Fariss, M.W., R.V. Blanke, J.J. Saady, and P.S. Guzelian. 1980. Demonstration of major metabolic pathways for Chlordecone (Kepone) in humans. Drug Met. Disposition 8:434-438.

18. Fogel, S., R.L. Lancione, and A.E. Sewall. 1982. Enhanced biodegradation of methoxychlor in soil under sequential environmental conditions. Appl. Environ. Microbiol. 44: 113-120.

19. French, A.L. and R.A. Hoopingarner. 1970. Dechlorination of DDT by membranes isolated from Escherichia coli. J. Econ. Ent. 63:756-759.

20. Guzelian, P.S., G.Vranian, J.J. Boylan, W.J. Cohn, and R.V. Blanke. 1980. Liver structure and function in patients poisoned with chlordecone (Kepone). Gastroenterology 78:206-213.

21. Harless, R.L., D.E. Harriss, G.W. Sovocool, R.D. Zehr, N.K. Wilson, and E.O. Oswald. 1978. Mass spectrometric analyses and characterization of Kepone in environmental and human samples. Biomed. Mass Spectrometry 5:232-237.

22. Holdeman, L.V., E.P. Cato, and W.E.C. Moore (ed.). 1977. Anaerobe Laboratory Manual, 4th ed., Virginia Polytechnic Institute and State University, Blacksburg, Va.

23. Holmstead, R.L. 1976. Studies of the degradation of Mirex with an iron(II) porphyrin model system. J. Agric. Fd. Chem. 24: 620-624.

24. Horowitz, A., J.M. Suflita, and J.M. Tiedje. 1983. Reductive dehalogenations of halobenzoates by anaerobic lake sediment microorganisms. Appl. Environ. Microbiol. 45:1459-1465.

25. Horvath, R.S. 1972. Microbial co-metabolism and degradation of organic compounds in nature. Bacteriol. Rev. 36:146-155.

26. Huckins, J.N., D.L. Stalling, J.D. Petty, D.R. Buckler, and B.T. Johnson. 1982. Fate of Kepone and Mirex in the aquatic environment. J. Agric. Fd. Chem. 30:1020-1027.

27. Jessee, J.A., R.E. Benoit, A.C. Hendricks, G.C. Allen, and J.L. Neal. 1983. Anaerobic degradation of cyanuric acid, cysteine, and Atrazine by a facultative anaerobic bacterium. Appl. Environ. Microbiol. 45:97-102.

28. Lal, R., and D.M. Saxena. 1982. Accumulation, metabolism, and effects of organochlorine insecticides on microorganisms. Microbiol. Rev. 46:95-127.

29. Lowry, O.H., N.J. Rosebrough, A.L. Farr, and R.J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.

30. Mah, R.A., M.R. Smith, and L. Baresi. 1978. Studies on an acetate-fermenting strain of Methanosarcina. Appl. Environ. Microbiol. 35:1174-1184.

31. Mahaffey, W.R., P.H. Pritchard, and A.W. Bourquin. 1982. Effects of Kepone on growth and respiration of several estuarine bacteria. Appl. Environ. Microbiol. 43:1419-1424.

32. Orndorff, S.A., and R.R. Colwell. 1979. Distribution and degradation potential of Kepone resistant bacteria in the James River and Upper Chesapeake Bay, p. 396-407. In A.W. Bourquin and P.H. Pritchard (ed.) Workshop: Microbial Degradation of Pollutants in Marine Environments, EPA-600 / 9-79-012. EPA Environmental Research Information Center, Cincinnati, Ohio.

33. Orndorff, S.A., and R.R. Colwell. 1980. Microbial transformation of Kepone. Appl. Environ. Microbiol. 39:398-406.

34. Pfennig, N. and H. Biebl. 1976. Desulfuromonas acetooxidans gen. nov. and sp. nov., a new anaerobic sulfur-reducing, acetate-oxidizing bacterium. Arch. Microbiol. 110:3-12.

35. Postgate, J.R. Postgate's Medium C. In "The Sulfate-Reducing Bacteria," Cambridge University Press, 1979.

36. Rosas, S.B., M. del Carmen Secco, and N.E. Ghittoni. 1980. Effects of pesticides on the fatty acid and phospholipid composition of Escherichia coli. Appl. Environ. Microbiol. 40: 231-234.

37. Schrauzer, G.N., and R.N. Katz. 1978. Reductive dechlorination and degradation of Mirex and Kepone with Vitamin B_{12S}. Bioinorg. Chem. 9:123-143.

38. Sethunathan, H., E.M. Bautista, and T. Yoshida. 1969. Degradation of benzene hexachloride by a soil bacterium. Can. J. Microbiol. 15:1349-1354.

39. Skaar, D.R., B.T. Johnson, J.R. Jones, and J.N. Huckins. 1981. Fate of Kepone and Mirex in a model aquatic environment: sediment, fish, and diet. Can. J. Fish. Aquatic Sci. 38:931-938.

40. Skalsky, H.L., M.W. Fariss, R.V. Blanke, and P.S. Guzelian. 1979. The role of plasma proteins in the transport and distribution of chlordecone (Kepone) and other polyhalogenated hydrocarbons. Ann. N.Y. Acad. Sci. 320:231-237.

41. Smibert, R.M. and N.R. Krieg. 1981. General characterization. In Manual of Methods for General Bacteriology. P. Gerhardt (ed.) American Society for Microbiology, Washington, D.C.

42. Suflita, J.M., A. Horowitz, D.R. Shelton, and J.M. Tiedje. 1982. Dehalogenation: A novel pathway for the anaerobic biodegradation of haloaromatic compounds. Science 218:1115-1117.

43. Taylor, J.R., J.B. Selhorst, S.A. Houff, A.J. Martinez. 1978. Chlordecone intoxication in man. Neurology 28:626-630.

44. Tomlins, R.I., T.R. Watkins, and R.J.H. Gray. 1982. Membrane lipid alterations and thermal stress in Salmonella typhimurium 7136. Appl. Environ. Microbiol. 44:1110-1117.

45. Uk, S., C.M. Himel, and T.F. Dirks. 1972. Mass spectral pattern of Mirex (dodecchlorooctahydro-1,3,4-metheno-2H-cycolbuta [cd] pentalene) and of Kepone (decachlorooctahydro-1,3,4-metheno-2H-cyclobuta [cd] -pentalen-2-one) and its application in residue analysis. Bull. Environ. Contam. Toxicol. 7:207-215.

46. Weiss, R.L. 1973. Attachment of bacteria to sulfur in extreme environments. J. Gen. Microbiol. 77:501-507.

47. Widdus, R., P.W. Trudgill, and D.C. Turnell. 1971. Effects of technical chlordane on growth and energy metabolism of Streptococcus faecalis and Mycobacterium phleii: a comparison with Bacillus subtilis. J. Gen. Microbiol. 69:23-31.

48. Williams, R.J. and W.C. Evans. 1975. The metabolism of benzoate by Moraxella species through anaerobic nitrate respiration. Biochem. J. 148:1-10.

The three page vita has been removed from the scanned document. Page 1 of 3

The three page vita has been removed from the scanned document. Page 2 of 3

The three page vita has been removed from the scanned document. Page 3 of 3