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Kepone Toxicity to Estuarine Microorganisms

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Introduction

Microorganisms undoubtedly play a critical role in the metabolic transformation of many organic and inorganic compounds in nature. For many years an illusion of microbial infallibility persisted, which as summarized by Gale (25) stated, "It is probably not unscientific to suggest that somewhere or other some organism exists which can, under suitable conditions, oxidize any substance which is theoretically capable of being oxidized." Dagley (19) has emphasized the chemically dynamic state of the biosphere and the importance of microbial activity to the total carbon flux. As he points out, man-made compounds will be biodegradable as long as they are susceptible to attack by the enzymatic machinery microorganisms have evolved over time. Therefore man-made compounds containing structural features never encounted in natural products are less susceptible to biodegradation.

With the onslaught of the chemical revolution vast numbers of new organic compounds have been synthesized to meet the increasing demand of industry and agriculture. Few of these man-made chemicals have had as profound an effect on human life as the chlorinated hydrocarbons, particularly DDT, PCB's and most recently Kepone. They present a unique paradox of incredible effectiveness as an insecticide matched against the most devastating environmental problems yet encountered in our technological society. The fact that this class of compounds are known to

accumulate in humous soil and aquatic ecosystems, raises the question of whether they could potentially alter the basic processes of an ecosystem if the particular pesticide was notably toxic to microorganisms.

Simple shifts in delicate competition balances due to the killing of a naturally favored group of bacteria could significantly reduce transformation efficiency and even effect the extent of transformation. The possibility that pesticide residues may have deleterious effects on microorganisms and their activities has received a considerable amount of attention (3,21,53,56). Unfortunately most of these studies have involved soil microorganisms and relatively little effort has been applied to the effects on aquatic bacteria. Although the effects of pesticides on phytoplankton and photosynthesis has been well-documented (56) information is greatly lacking on the effects of organochlorine compounds on aquatic microorganisms and the processes they mediate.

In July of 1975, the discovery of Kepone poisoning in workers of a chemical plant, and the poisoning of the Hopewell, Virginia sewage treatment plant triggered a massive investigation to determine the fate of the insecticide in the James River Estuary.

Undetected for more than eight years, Kepone had seeped into the James River and Chesapeake Bay contaminating nearly every segment of the environment and spreading throughout the food chain. Surveys of the region revealed the presence of Kepone in finfish taken from areas beyond the confines of the James River and

Chesapeake Bay. Until recently, research on Kepone has concentrated on invertebrate toxicity and the effects on soil microorganisms (16,35,44). The purpose of this project was to determine the effects of Kepone on estuarine microbial populations and to determine the mode of toxicity to pure culture isolates obtained therein.

Literature Review

Natural Occurence of Organohalides

The halogens, because of their great reactivity never occur as free elements in nature. The bulk of these elements are found principally in the inorganic state either as dissolved salts or insoluble minerals. Additionally naturally occurring organohalides are known to occur in mammals, invertebrates, insects, plants, algae, fungi and bacteria: Approximately 200 such compounds have been identified (48). A majority of these products are of fungal or lichen origin, however, the chlorine containing antibiotics, chloramphenicol and chlorotetracycline are formed by species of Streptomyces. Most natural organobromine compounds are of marine origin, found predominantly in algae and sponges. In view of the natural abundance of bromine in marine water this is not surprising. Many structures of this group of compounds contain a bromine substituted benzene ring, i.e. Aeroplysinins-1 and -2 from sponges (24,46). Naturally occuring organochlorides can also occur as aliphatics, alicyclics and heterocyclic compounds. The natural occurrence of organofluorine compounds is confined to certain plants where it occurs as omega-fluoro-derivatives of acetic, oleic, palmixic, myristic and decanoic acids (14,43,55).

Industrial Sources

Since the end of World War II rapid developments in the fields of industrial and agricultural chemicals has resulted in the production of enormous quantities of organochlorine compounds. Between 1948 and 1961 chlorinated hydrocarbon production tripled, with 75% of all chlorine used in this country going into the production of chlorinated aromatic and aliphatic compounds (14). The degree of substitution in these chemicals can vary from the monochlorinated herbicide, 2-methyl-4-chlorophenoxyacetic acid, to the insecticide DDT, the highly substituted polychlorinated biphenyl and Kepone, and the completely substituted Mirex.

Recalitrance

With increasing chlorine substitution in such molecules the more persistant they become. They are not readily degraded by microorganisms, the main agents for returning organic compounds to the carbon cycle (19). The biodegradation of a halogenated compound can only be considered complete when its carbon skeleton has been converted to intermediary metabolites and its halide returned to the mineral state. Unfortunately the enzymes of the catabolic pathways available to bacteria for the degradation of unhalogenated compounds are rarely effective on halogenated substrate analogs. At best gratuitous metabolism occurs often resulting in the formation of a toxic and often more recalcitrant

intermediate. Gibson et al (26) has shown that 3-chloro-substituted catechols are formed by a toluene utilizing Pseudomonas putida when grown in the presence of chlorobenzene and 4-chlorotoluene. Additionally, organisms which utilize biphenyl as a sole carbon and energy source accumulated 4-chlorobenzoic acid from p-chlorobiphenyl (42,12). A phenol utilizing yeast accumulates 4-chlorocatechol from both 3-chlorophenol and 4-chlorophenol (54).

The cyclodiene insecticides; aldrin, dieldrin, chlordane, endrin and heptachlor are generally recognized as persistant pesticides in the environment. However, while transformation of several of these compounds has been demonstrated, the metabolites remain as persistant environmental contaminants. Tu et al (52) have demonstrated the epoxidation of aldrin to dieldrin with species of Trichoderma, Fusarium and Penicillium. Cultures of a Pseudomonas sp were found to transform dieldrin to a number of metabolites (41). The major metabolites were a ketone derived by rearrangement of an epoxide ring to a carbonyl function. It was assumed that dihydroxydihydroaldrin, an aldehyde and the acid were probably formed by loss of a carbon atom from the ring to which the epoxide function was formerly attached. Endrin is similarly metabolized to several ketones and aldehydes (40). From these above mentioned considerations it can be seen microorganisms may do little more than modify the structures of these persistant organochlorine compounds. There structures are

sufficiently different from the naturally occurring chlorinated products that degradation by specific or fortuitous enzyme systems is precluded.

There are, however, exceptions to this rule, and the research by Evans and Alexanders groups on the degradation of the chlorinated phenoxyacetic acid herbicides serves as a prime example. Their work reveals that a number of reactions involving chlorinated intermediates occur with the eventual elimination of halide (22,23,49,50). Evans et al (22) showed that two strains of Pseudomonas metabolized 2,4-dichlorophenoxyacetate (2,4-D) as a sole carbon source in mineral salts liquid media. With one of the Pseudomonas the appearance of 2-chlorophenol suggested a non-oxidative elimination of chlorine from 2,4-dichlorophenol or possibly 2,4-D itself, the exact mechanism of elimination still remaining to be elucidated. The accumulation of α-chloromuconate is reported as a further manifestation of this phenomenon since it is probably formed by the action of the ortho ring fission enzyme on 3-chlorocatechol. Subsequent metabolism of α-chloromuconate via an aldolase activity would presumably generate chloroacetate and succinate the later readily entering the TCA cycle. The second Pseudomonas (NCIB9340) metabolized 2,4-D via 2,4-dichlorophenol, 3,5-dichlorocatechol and two ring fission products tentatively identified as αδ-dichloromuconate and α-chloromaleylacetate. It is evident in this case that chloride

elimination is a function of lactonization of the ring fission product (23). Ando et al (5) claim that a soil Penicillium sp biosynthesizes 2,4-dichlorophenol. It is attractive to speculate that the ability of organisms to degrade 2,4-D may have evolved from their ability to grow with 2,4-dichlorophenol, a natural substrate.

Extensive degradation of the organochlorine insecticide lindane has been reported in moist soils (34). The dehydrohalogenation was attributed to the soil bacteria Clostridium sporogenes and Bacillus coli with trace amounts of benzene and monochlorobenzene produced by these organisms. MacRae et al (39) have demonstrated the production of CO_2 from lindane in submerged soils.

Kepone

Kepone (decachlorooctahydro-1,3,4-metheno-2H-cyclobuta(cd) pentalene-2-one) is a chlorinated hydrocarbon of special interest because of its extensive contamination of the James River System. It is commonly used for the control of banana root borer, tobacco wireworm, potatoe pests and has been effective against fire ants. Its introduction into the James River was due primarily to industrial effluents.

At this time there are no reports in the literature demonstrating any significant biologically mediated degradation of Kepone. Alley et al (4) have demonstrated photolysis of Kepone

in organic solvents to two major products; monohydro and dihydro-kepone. Hugget et al (36) has shown that Kepone levels in the James River sediment are 0.05-0.5mg/kg, particularly in those areas of high organic content. Since Kepone appears to be very recalcitrant and is only very slowly washing out of the James River sediment, it is possible that the residual Kepone could have an inhibitory effect on bacteria and the processes they mediate.

Methods of Analyzing Toxicity

Disc sensitivity assays were originally developed and widely used for determining the antibiotic sensitivity spectrum of strains of clinical bacterial isolates. Discs wetted with antibiotic solution are placed on the surface of an agar media spread with a lawn of bacteria. Zones of inhibition indicate that the drug is an active antimicrobial agent. The procedure has been widely adopted to determining antimicrobial activity of a number of pesticide formulations. Bourquin et al (8,9) demonstrated inhibition of estuarine bacteria by PCB formulations as did Brown et al (12,13) for mirex and mirex photoproducts. Analysis of this type provide little more than a rapid screening technique to test for inhibition by a compound on a large number of pure culture organisms over a range of concentrations.

Incorporation of a suspect toxic agent into petri dish

plate cultures for a viable count assay has several distinct advantages. First, it is the easiest method for assessing the toxic effects of a compound on a mixed population of bacteria. Second it allows one to determine a minimum inhibitory concentration while working at levels that would be a reflection of those seen under natural conditions. Third, individual isolates can be readily picked from these plates and used in subsequent experiments. Gray et al (31,32,33) employed such a procedure for the isolation and characterization of benzenehexachloride resistant bacteria from soil.

The use of physiological indices (sugar fermentation, enzyme activities, urea hydrolysis, hydrocarbon oxidation, etc.) as a possible tool to determine the toxic effects on specific physiological processes has not been extensively studied. Brown et al (13) employed this technique to demonstrate that Mirex showed no inhibition of growth, ammoniafication or hydrolyzing activity of gelatin, lipid or starch hydrolyzers. Gray (31,32,33) employed physiological indices in assessing the toxicity of benzenehexachloride (BHC) on autotrophic, heterotrophic and urea hydrolyzing bacteria. In these experiments BHC significantly repressed nitrification, thiosulfate oxidation and urea hydrolysis in solution media but had no effect on these processes when added to soils with organic matter. Additional experiments with heterotrophic bacteria (33) demonstrated inhibition within this

group, of oxidative and hydrolytic activities (i.e. Phenol oxidation, starch and cellulose hydrolysis, nitrate and sulfate reduction).

Growth studies by Gray and Rogers (30) demonstrated the growth only of Gram-negative organisms when mixed cultures from soils were incubated on nutrient agar with hexachloro cyclohexane. Similar experiments by Duda (20) showed that chlordane and hexachlorocyclohexane inhibited the growth of a number of Gram-positive organisms (B. subtilis, B. mycoides; S. griseus and Sarcina lutea) while the growth of Gram-negative isolates was unaffected. Growth studies with pure cultures of micro-organisms growing on synthetic media have yielded more consistent results on the nature of toxicity of organochlorine compounds. Results of Bourquin et al (8,9) and Brown et al (12,13) indicate marked reductions of bacterial growth in pure culture by increasing concentrations of PCB's and mirex respectively. Early experiments by Trudgill et al (51) showed similar effects on growth by technical chlordane. These results were correlated with inhibition studies of respiration rates which were determined by measuring oxygen consumption by whole cells with succinate as the substrate. The general pattern of increased sensitivity of Gram-positive isolates was further established by these experiments.

The inhibition by organochlorine insecticides of metabolic activities associated with respiration has been reported by a

number of workers. Wineley et al (58) reported inhibition of cytochrome C oxidase activity of Nitrobacter agilis extracts by 500 mg/l chlordane or heptachlor. Johnston (36) demonstrated the inhibition of rat heart succinooxidase by DDT at 35 mg/l even though electron flow from succinate to triphenyltetrazolium chloride was unperturbed. More recent studies by Widdus et al (57) showed technical chlordane at 20 mg/l to be a potent inhibitor of NADH oxidase activity and that disruption of the electron transport chain appeared to be general though more severe from NADH dehydrogenase to cytochrome C.

Materials and Methods

Reagents and Chemicals

Kepone (98% pure) was obtained from Chem. Services, Inc., West Chester, PA. N,N-dimethylforamide (DMF) was purchased from Aldrich Chemical Co. Microbiological media and substrates were obtained from Difco Laboratories, Detroit, MI.

Media

Zobell's 2216 marine medium (1) was prepared with Rila Marine Mix (Rila Products, Teaneck, NJ) to a final salinity of 15 $^{o}/_{oo}$ (Z-15). For solid medium, 2% Bacto-agar was added. Minimal salts basal (MSB) medium was prepared according to Stanier

et al., (47). Glucose (0.2%) was added as a growth substrate. Environmental isolates were also given 0.01% yeast extract (Difco) to compensate for some auxotrophic conditions of the cultures.

Kepone-Marine agar medium was prepared the same as Zobell's medium and after autoclaving Kepone was added in acetone to the desired final concentration in the medium prior to pouring the agar plates.

Physiological Indices

Media for determining physiological core characteristics (15) was supplemented with Rile Marine Mix to a final salinity of 15 $^{o}/_{oo}$. Starch hydrolysis plates were developed by flooding plates with iodine for 10 minutes. Clear zones indicated starch hydrolysis.

Bacterial Isolates

Bacterial isolates were obtained from Range Point Salt Marsh (Pensacola Beach). Escambia Bay surface water and the Sabine Island disposal pond. Water samples were appropriately diluted and plated on Zobell's 2216 marine medium supplemented with Kepone to 0.02, 0.20 and 2.0 mg/l plus a zero control. Plates were incubated at 30^{o}C for 7 days. At the end of the incubation period the dominant colony types from each sample at the respective Kepone concentrations were isolated. Stock

cultures of these isolates were maintained on Zobell's 2216 marine agar slants at 10°C. Gram reactions and cell morphology were determined for each isolate. Pure cultures used in the disc sensitivity assay's were part of a stock culture collection of bacteria and fungi maintained at the Gulf Breeze, EPA laboratory. They were originally isolated from batch culture enrichments of sea water using a large spectrum of substrates.

Disc Sensitivity Assays

Each pure culture was grown in Zobell's (Z-15) broth for two 18h growth cycles before spreading 0.1 ml of the culture fluid on Z-15 agar plates. Four paper discs (Schleicher & Schuell, 12.7 mm, No. 740-E, Keene, NH) designed for antibacterial substrate testing and treated with varying Kepone solutions were placed on the agar plate. Discs were prepared by wetting the discs in solutions of 0.1, 0.01, 1.0 mg Kepone per milliliter DMF or acetone. A central disc contained only DMF or acetone. The actual volume of Kepone solution per disc was calculated to be 0.11µl by determining the average increase in weight of DMF or acetone saturated discs.

Mixed Culture Inhibition Studies

Water and sediment samples retrieved from various estuarine and marine areas near Pensacola Beach, FL were diluted in water

blanks of the appropriate salinity and plated in replicates of 4, on Z-15 agar plates with and without Kepone. Water samples were incubated aerobically at 28°C and counted at 4 and 8 day intervals. Sediment samples were incubated aerobically and anaerobically in BBL Disposal Anaerobic Systems (BBL, Baltimore, Md.) at 28° for 8 days before counting. Dominant colony types were picked off individual plates with sterile toothpicks and spotted onto Z-15 agar plates. When colonies were grown, they were replicated onto various differential and selective media for physiological characterization.

Growth Studies

Growth studies were performed in Zobell's 2216 marine broth or 0.4% succinate mineral salts broth (MSB), supplemented 0.02, 0.20 and 2.0 mg/l Kepone plus a o control. Precultures were grown overnight (12 hours) in either of the above media, depending on the isolates being tested. Growth flasks were innoculated with a 5% innoculum and monitored turbidimetrically using a Klett-Summerson colorimeter equipped with a #42 blue filter (400-562 mμ.

Oxygen Uptake Studies

Pure cultures were selected from both control and experimental culture plates of the mixed culture inhibition studies. These

cultures were grown on 100 ml of Z-15 broth or MSB and glucose for 18 h prior to harvesting by centrifugation (10Xg, 10 min.). The cells were washed once with 0.05 M K_2HPO_4 buffer plus 1.5% NaCl pH 7.5. The washed cells were resuspended in 2 ml of buffer and kept in an ice bath until ready for use. Oxygen uptake was determined using a Gilson Oxygraph (Model K-ICT-C, Gilson Electronics, Middleton, Wisc.) equipped with a Clark electrode. The reaction vessel contained 1.7 - 1.8 ml phosphate buffer, 10 - 50µl cell suspension (100-300 µg protein), and 10 - 50 µl oxidizable substrate. When a baseline level of oxygen uptake was established, 10 µl of Kepone solution (0.19 - 19.0 µg Kepone/µl DMF) was injected to determine the effect on oxygen uptake. As a control inhibitor, 10 µl of a pentachlorophenol solution (20mM) was added to the same cultures. Protein determinations were performed according to the methods of Lowry et al (38).

Electron Transport Studies

Inhibition studies were performed with the electron transport particle (ETP) from isolate 32K. Cells were grown for 18 h in 1.0 liter of MSB/succinate and harvested by centrifugation at 10,000 x g for 10 minutes. Cells were washed twice in KH_2PO_4 buffer (0.05 M, pH 7.2) and resuspended in 5.0 ml of the same buffer. ETP was isolated by the method of Crane (17). Cells were subjected to sonic oscillation at 10 KHz with a Raytheon sonic oscillator (five 1.0 min

exposures). Cell debris was removed after centrifugation at 25,000 g for 15 minutes at 4°C. The supernate solution was removed and centrifuged at 150,000 x g for one hour at 2°C. The pellet obtained from the high speed centrifugation was resuspended in 5.0 ml of cold buffer and the procedure repeated. The final pellet was completely homogenized in 5.0 ml of the cold buffer by forcing the suspension through a 27G syringe needle. Succinooxidase activity in the ETP was assayed by the procedure of Crane et al. (18). Oxygen uptake was measured in a reaction mixture with a final volume of 2.0 ml. Succinooxidase activity was measured by adding 100 ul of cytochrome c solution (1.0%), 100 ul of 1.0 M sodium succinate, and 20 ul ETP preparation. A polarographic procedure was used similarly for NADH oxidase assays. The reaction mixture consisted of 20 ul of cytochrome c solution, 10 ul of ETP preparation (60 ug protein) and 80 ul NADH solution (0.4%) respectively. All rates were corrected for endogenous respiration.

Statistical Analysis

The experimental design of plate assay experiments predicated data interpretation by a three factor Model I analysis of variance (60). Factors compared were; Kepone concentration, aerobic versus anaerobic incubation, sampling dates, and an air treatment interaction component. In order to evaluate significant differences between population means a Student-Newman-Keuls (SNK) post hoc analysis (60) was performed. This test was used to contrast the

reduction in colony forming units (CFU's) affected by Kepone under aerobic and anerobic conditions. The critical values of the q distribution (q 0.05, ∞, p) were obtained from Table D.12 (60).

Results

Toxicity to Laboratory Cultures

The toxicity of Kepone to a variety of laboratory pure cultures was determined using the disc agar diffusion sensitivity method. The pure cultures were part of a stock culture collection of bacteria and fungi maintained at this laboratory. They were originally isolated from batch culture enrichments of sea water using a large spectrum of substrates. Table 1 gives a summation of the toxicity patterns. Of the 30 isolates tested, 33% were inhibited at the 3.65 µg/disc concentration and 47% were inhibited at the 14.6 µg/disc concentration. Higher concentrations of Kepone (20 µg/disc) inhibited all isolates. Chemical analysis of the Kepone has shown it to be 99% pure with no trace (above 0.5 ng/ml) of other chlorinated compounds. It is clear from the table that several of the isolates (Nos. 1,3,4,11,14,15,29) were particularly sensitive to Kepone; four of them were inhibited at the 1.46 µg/disc concentration.

Attempts to correlate their sensitivity at other physiological characteristics has proven negative in most cases. Kepone-sensitive cultures showed no significant difference from randomly selected stock cultures in terms of their morphology, aliphatic hydrocarbon utilization, pesticide tolerance (Aroclor 1242, 1016, methoxychlor, heptachlor, DDT, malathion, toxaphene, one or more)

lipolytic and proteolytic activity, nitrate reduction, sugar utilization and urea hydrolysis. Some of these results are summarized in Table 2a and 2b. However, many of the sensitive cultures (7/8) were gram positive whereas fewer of the Kepone-tolerant cultures (2/9) were gram positive. The same was true of amylolytic activity; 6/8 were positive for Kepone-sensitive cultures and 1/7 were positive for Kepone-tolerant cultures. There may also be some significance to the fact that greater percentage (50% versus 11%) of Kepone-sensitive bacteria metabolized one or more aromatic compounds (phenol, naphthalene, toluene, biphenyl and xylene tested). Only one of the Kepone-tolerant bacteria, in fact, grew on an aromatic hydrocarbon, benzene. All of the other aromatic utilizers were capable of growing on several of the aromatic compounds. Of the six fungal cultures tested, only one was poisoned by Kepone at the 14.6 µg/disc concentration. Two yeasts, Candida maltosa and Candida lipolytica, were both sensitive but at higher concentration than the bacteria tested.

Toxicity to Environmental Isolates

To determine the toxicity of Kepone to natural mixed populations of bacteria from a variety of marine habitats, standard total viable counts were performed using Zobell's seawater agar (Z-15) containing dissolved Kepone. Table 3 shows the results

of these assays. All results are reported from 8-day incubation periods and the percentage reductions in colony forming units (CFU) have been normalized against control plates which contained no Kepone. From these results, it is clear that Kepone as low as 20 µg/l has an inhibitory effect on the development of colonies on an agar plate. The sensitivity of these mixed populations was quite variable. Different degrees of inhibition were noted with samples taken from the same area at different times and in many cases it was apparent that concentrations below 20 µg/l were inhibitory.

Within each population, there are colony forming units which were quite resistant to Kepone. Tests at higher Kepone concentrations (10 and 100 mg/l) showed near 100% inhibition in many cases. However, in some of these assays colony forming units existed which were tolerant to these high concentrations.

A total of 17 colony forming units which grew in the presence of Kepone were selected (based on predominance) for further study. Examination of the cell type and enzymatic activities of these purified isolates showed significant correlation only with the amylolytic, lipolytic activities and the gram stain. When compared with 20 isolates randomly selected from Zobell's marine agar (no Kepone), 75% of the Kepone-tolerant isolates displayed amylolytic and lipolytic activity whereas only 55% of the non-tolerant isolates showed this activity. Ninety percent of these Kepone-

tolerant isolates were gram negative as compared to 55% of the isolates from Zobell's marine agar (no Kepone). There was no significant difference between the two groups of isolates when compared with the other characteristics tested.

Because Kepone is shown to be present in sediments and detritis in concentrations higher than that found in the water column, it was of interest to determine the toxicity of Kepone to bacteria isolated from sediment. Table 4 shows the results of a standard viable plate count on Zobell's marine agar containing Kepone and incubated under aerobic and anaerobic conditions. As can be seen, there was no significant difference in the number of colony forming units in the presence (0.2 mg/l) or absence of Kepone. Under anaerobic conditions, the high concentration of Kepone (2.0 mg/l) did show some reduction in the number of isolates on the agar plate under anaerobic conditions. However, the sensitivity of these anaerobic populations seem to be as variable as the aerobes from the water column. Sediment samples taken at different times showed different degrees of inhibition at 2.0 mg Kepone/l under anaerobic conditions (Table 4). The same samples when plated and incubated under aerobic conditions (Table 4) showed the same response as those cultures from the water column. The number of isolates growing on Kepone agar plates decreased with increasing Kepone concentration.

Statistical Analysis

The apparent decreased sensitivity to Kepone by organisms grown under anaerobic conditions and the variability of this response prompted further investigation into this phenomena. Data was collected from July 25, 1977 to November 14, 1977 for Range Point sediment plate assays incubated under aerobic and anaerobic conditions. The data, 150 observations and 4 variables (source) was pooled and subjected to a Model I Analysis of Variance. Figure 1 is an illustration of the mean number of colony forming units (CFU's) for aerobic and anaerobic incubations plotted against the Kepone concentrations tested. The mean response for sampling dates, aerobic vs. anaerobic treatments were all significantly different at a confidence interval of $\alpha = 0.05$ with a PR > F of 0.0001 (Table 5). A Student-Newmans-Kuels post hoc analysis was applied to the response means for aerobic and anaerobic incubations versus Kepone concentration in order to partition out any significant interactions between treatments (Table 6). Populations grown under anaerobic conditions were not significantly affected by Kepone at any of the concentration tested. However significant reductions in colony forming units were observed under aerobic conditions. Pair comparisons with $\alpha = 0.005$ revealed significant population differences between the control and 0.2 and 2.0 mg Kepone/l concentrations. Differences were also observed between the 2.0 mg

Kepone/l treatment and 0.02 and 0.20 mg Kepone/l treatments. This indicates then a minimum inhibitory concentration (MIC) of 0.2 mg Kepone/l.

Effects on Bacterial Growth

To determine the toxicity of Kepone to a variety of bacteria grown under different physiological conditions, growth studies were conducted with rich and defined media. The origin of the isolates are summarized in Table 7. Isolate 32K is a Gram-negative, rod-shaped bacterium. Growth of this organism was not inhibited by Kepone at any concentration tested when grown in the enriched Z-15 broth (Fig. 2). However, when grown in a defined medium such as MSB/Succinate, the growth rate decreased as Kepone concentration increased. The most notable effect was at 2.0 mg/l Kepone where the maximum optical density obtained was 3.5 fold lower than the control. When the inocula was increased to yield an initial optical density of 0.1 only a 2-fold reduction in the maximum growth yield was obtained with 2.0 mg/l Kepone.

Similar growth studies where performed on cultures of two Gram-positive cocci (49K and 19K) which grew, auxotrophically, only in Z-15 broth. Figure 3A shows the results obtained for isolate 49K, using 2 different innocula sizes corresponding to initial optical densities of 0.05 and 0.10. Only the 2.0 mg/l Kepone concentration was effective in inhibiting the maximum

obtainable optical densities. A 4-fold reduction observed for the higher innoculum and 20-fold for the lower innoculum. Apparently, lysis or clumping of cells was responsible for the decline in optical density with the lower innoculum culture. In comparison, 19K (Fig. 3B) under the same growth conditions had its maximum obtainable optical density depressed by only 28% and with a much smaller innoculum size. This would suggest that 19K was more tolerant to Kepone. This is not surprising considering that the organism was originally isolated from a toxic wastes holding pond which receives a considerable chlorinated hydrocarbon loading.

Oxygen Uptake Experiments

Initial Studies

In an attempt to further assess the sensitivity of bacteria to Kepone, oxygen uptake studies were conducted. In an initial screening several isolates were selected from environmental water samples. Log phase cells cultured in Zobell's marine broth were washed with 0.05M K_2HPO_4 buffer pH7.2 and then tested in an oxygraph to determine their oxygen uptake in the presence of Kepone with glucose (10mM) as substrate. As can be seen in Table 8, oxygen uptake did appear to be a sensitive indicator of Kepone toxicity. The isolates themselves showed varying degrees of

sensitivity. Most of them were inhibited at the 20 mg/l concentration, whereas relatively little inhibition occurred at the 2 mg/l concentration. This differs considerably with the plate count data where definite reduction in colony forming units occurred at the 0.2 mg/l concentration.

Of particular interest in the oxygen uptake studies was the fact that in many cases the inhibition at 20 mg/l was significantly greater than at the 200 mg/l (see isolates 32, 43 and 56 for example). This could indicate a threshold concentration of Kepone at which toxicity was maximal. It should also be noted that some isolates actually showed an increase in oxygen uptake in the presence of Kepone (see isolate 49 for example). This could be the result of an interference with the oxygen-reduction balance of the cells resulting in the production of hydrogen peroxide. The splitting of hydrogen peroxide by catalase could then account for the observed oxygen production.

Further polarographic experiments were conducted to obtain information on the possible mechanism of Kepone toxicity. Toxicity was measured as reduction in oxygen uptake in the presence of 8.5 mM succinate or 0.025% yeast extract and was related to Kepone concentration (0-20 mg/l). Analogous experiments were performed using 0-60 mg/l pentachlorophenol (PCP) as a reference toxicant. With all isolates tested, Kepone was 3 to 7 times more toxic than PCP. With isolate 32K, oxygen uptake on succinate was signifi-

cantly reduced by 25% at 0.1 mg Kepone/l and by 80% at 1.0 mg/l (Fig. 4A). In comparison 2.7 mg PCP/l reduced oxygen uptake by 55%. Isolate 28K, a Gram-negative rod isolated from the toxic wastes pond was far more tolerant to Kepone than was Isolate 32K: 5 mg Kepone/l reduced oxygen uptake by 34% and 20 mg/l by 75% (Fig. 4B).

Oxygen uptake by the Gram-positive coccus, Isolate 49K, was measured by using 250 mg/l yeast extract as the substrate. The complexity of the assay media required increased concentrations of Kepone to inhibit oxygen uptake, a condition paralleled in growth studies. Oxygen evolution occurred immediately after Kepone or PCP was added to the reaction mixture (Fig. 5), and was observed with each of the seven Gram-positive isolates tested.

Inhibition of Electron Transport Activity

Results thus far suggested that Kepone toxicity is related to the oxidative metabolism of microorganisms. Therefore, the effects of Kepone on electron transport activity were studied by measuring the inhibition of NADH oxidase and succinooxidase activities in the cellular particulate fraction or as commonly referred to in bacteria, the ETP (election transport particle). NADH oxidase activity, measured as oxygen uptake in the presence of ETP and cytochrome C, was inhibited 51% by 1.0 μM Kepone while succinooxidase activity (O_2 uptake by ETP with succinate) was inhibited by 25%

(Table 19). The maximum reduction in NADH oxidase activity (62%) was obtained with 2.0 μM Kepone while succinooxidase activity was inhibited by 50% in the presence of 10 μM Kepone. The three-fold lower activity of succinooxidase relative to NADH oxidase is characteristic of the electron transport particle (18).

Discussion

From the results presented in this thesis it is clear that Kepone is toxic to microorganisms. Kepone concentrations as low as 0.2 mg/l significantly reduced the number of colony forming units on Zobell's marine agar. Hugget (personal communications) has shown Kepone levels in James River sediment to be 0.05-0.5 mg/kg, particularly in those areas of high organic content. Since to date no one has demonstrated any significant chemical or biological degradation of Kepone and since Kepone appears to be only very slowly washing out of the James River system, it is possible that the residual Kepone could have an inhibitory effect on bacteria and the processes they mediate.

Kepone, however, was not universally toxic since both laboratory culture and environmental isolates could be shown to be quite resistant. The fact that many sensitive organisms were Gram-positive may be a partial explanation. Trudgill et al (51) has reported a similar sensitivity with Gram-positive bacteria exposed to chlordane and Bourquin et al (38) has also shown a correlation with PCB's. The increased tolerance of Gram-negative bacteria to Kepone may be a function of the higher lipid content in these bacterial cell walls. This influence is consistent with Widdus' et al (57) theory which proposed that chlordane has a non-specific but lethal swamping effect, that deforms the structural

integrity of the cell membrane. They proposed that chlordane would have had the same effect on E. coli cells as on B. subtilis but for the barrier action of the cell wall. In a series of elegant experiments they demonstrated the inhibition of succino-oxidase activity in E. coli sphaeroplasts thus supporting their theory. Grimes and Morris on (34) have implicated cellular lipids as having a major role in the bioconcentration of chlorinated hydrocarbons by microorganisms. Ware and Roan (56) also suggest that the microbial absorption of pesticides from water is a function of surface area and the lipid material within that surface.

Since not all types of bacteria are inhibited by Kepone at environmentally significant concentrations, it becomes important to know if tolerant species will replace the sensitive species thus maintaining the metabolic integrity of the ecosystem involved. In general, this replacement process is thought to occur in both soil (52) and aquatic (56) environments. But the investigations of the interactions of pesticides with aquatic populations of bacteria are not numerous particularly as they relate to overall activities such as total respiration and heterotrophic potential. My results indicate that Kepone is quite toxic (growth inhibition at 0.2 mg/l to certain types of aquatic bacteria). For example, it is ten to a hundred times more toxic than the PCB's, chlordane and heptachlor (37,38,49). Therefore, further effort should be

made to determine the overall population effects of Kepone on microorganisms. Unpublished results from the Gulf Breeze laboratory have shown that Kepone, at low concentrations decreases the rate at which the pesticide methyl parathion is degraded, thus implicating a possible interference with the metabolic integrity of an aquatic system.

Each of the three methods used in this study to determine toxicity, i.e. sensitivity disc, plate counts and oxygen uptake, superficially appears to give different results for toxicity levels. However, this is probably a reflection of the technique. Plate counts give the easiest method for assessing toxic effects on a mixed population of bacteria and the minimum inhibitory concentration (MIC) (0.02 mg/l) is probably a good reflection of that seen under natural conditions. Growth studies on individual isolates from the estuarine environment indicate a similar level for significant inhibition by Kepone. There seems to be a significant effect of inoculum size and growth media on the toxicity pattern of Kepone. This is probably due to the hydrophobic nature of Kepone. In a rich media Kepone may be complexed with organic substrates and thereby partitioned away from the cells. Increased inoculum sizes would have a similar effect. This time Kepone would be bound to a certain percentage of the cells in the inoculum allowing the remainder to grow at normal rates. One of the biggest problems in working with chlorinated hydrocarbons is knowing exactly what

concentrations the cells are seeing. Very little can be said about MIC from the agar-disc diffusion method except that very low levels of Kepone are inhibitory. Much higher levels of Kepone would appear to be necessary to inhibit oxygen uptake. However, these studies employed very high densities of cells. By back calculation (assuming 40% of a cell is protein and a cell weighs 10^{-12} gms) it can be shown that approximately 2 µg Kepone could give 100% inhibition of oxygen uptake by 2 x 10^6 cells/ml. Therefore, 2 µg Kepone/l could be inhibitory to approximately the same concentration of bacteria (10^3 - 10^4 cells/ml or gm) found in Escambia Bay waters or sediments. Thus these studies also reflect the very toxic nature of Kepone to bacterial metabolism.

The use of physiological indices (sugar fermentation, enzyme activities, urea hydrolysis, hydrocarbon oxidation, etc.) as a possible tool to determine the toxic effects on specific physiological processes has not been used extensively (41). In this study there is some correlation between these indices and Kepone toxicity: amylolytic activity seemed to be more prevalent in Kepone-tolerant organisms. Similar results were reported by Bourquin and Cassidy (38) for PCB toxicity. Exactly what relationship this activity could have to Kepone toxicity is not clear. It is apparently not enough of an effect to promote the enrichment of amylolytic-positive organisms in a mixed population since both static and flow through systems exposed to Kepone in our laboratory

did now show any enrichment.

The Model I ANOV used for the analysis of plate assay data is the required procedure in a factorial analysis of variance in which all factors are fixed effects. This model tests the null hypothesis of no difference between the levels of a factor by means of F = factor MS/error MS. In addition significant interactions can be tested by F = interaction MS/error MS. Since the analysis of variance rejected the multisample hypothesis of equal means it was desirable to perform a multicomparison test to determine the significant interactions. Multiple range testing is a highly debated area of statistical analysis, there being no agreement as to the best procedure to be used under a given set of situations. The SNK test appeared to be the most amenable to my data base allowing for stepwise mean comparisons; first between the largest mean and the smallest, then largest mean and next smallest. It is by way of the SNK test that the non-toxic effects of Kepone under anaerobic conditions was confirmed. While it is acknowledged that multiple range tests are more prone to Type II errors, not rejecting the null hypothesis when it is in fact false, the large number of data points should decrease its probability.

The non-toxic effects of Kepone in anaerobic plate assays and the marked inhibition of oxygen uptake with several isolates indicated a possible involvement with electron transport and

respiration. Widdus et al. (42) and Trudgill et al. (37) have shown that the inhibition of NADH oxidase activity in Bacillus subtilis by chlordane occurred indirectly through the apparent disruption of membrane mediated electron transport. Presumably then, if similar mechanisms are functioning during Kepone poisoning one would expect an effect from anaerobic versus aerobic conditions. Trudgill (1971) did not report on anaerobic growth but he did show that a Streptococcus species (which would have minimal cytochrome mediated electron transport activity) was significantly more resistant to chlordane. Based on these studies, Kepone is probably exerting a direct effect on bacterial membrane integrity and function. Studies of the membrane bound oxidases of the ETP from Isolate 32K show that indeed NADH oxidase and succinoxidase activity are inhibited by Kepone, indicating disruption of electron transport. These data and the oxygen evolution phenomenon observed with the Gram-positive isolates suggest that disruption of membrane mediated electron-transport may result in a transient imbalance in the oxidation-reduction potential of the cell. It is a well-known event of electron transport that NADH shuttles electrons to a flavoprotein dehydrogenase, generating a reduced flavoprotein. Gibson et al (27) and Ghisla et al (28,29) demonstrated that reduced flavins (free and as protein bound prosthetic groups) will activate molecular oxygen to produce a peroxy intermediate. If electron flow were blocked such that the flavoprotein could

not reoxidise through its normal sequence then one could expect the formation of hydrogen peroxide. In the presence of catalase, peroxide would be cleaved to yield water and molecular oxygen, thereby accounting for the oxygen evolution phenomenon observed. It should be emphasized here that the necessary stoichiometric experiments to support this theory were not within the technical capabilities of our lab and therefore this explanation is tentative at best.

The actual site of Kepone action was not determine. However, it has been observed in general that the Coenzyme Q factor in Gram-negative is an ubiquionone, while that of Gram-positive bacteria is a naphthoquinone, except in E. coli and Proteus which contain both (43). During aerobic growth of Proteus ruttgeri, ubiquione content exceeds that of naphthoquinone, while during anaerobic growth naphthoquinone is dominant (44). If these coenzymes serve as the site of Kepone toxicity, then investigations of the relationship between sensitivity to Kepone and physiological changes in these coenzymes may reflect the specific site of Kepone activity.

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TABLE 1. Sensitivity of Pure Cultures to Kepone

GBERL Isolate #	Acetone Control	Concentration Kepone (μg) per disc[a] 1.46	3.56	7.3	14.6
1	0	0	+ + +	+ + + +	+ +
3	±	+	+ +	+ + +	+ + +
4	0	±	+ + +	+ + + +	+ + +
5	±	±	±	±	+
8	±	±	±	±	+
11	±	+ +	+ + +	+ + +	+ + +
14	0	0	+	+ +	+ + +
15	0	0	+ +	+ + +	+ + +
29	0	+	+ +	+ + +	+ +
30	±	±	+	+	+
36	±	±	+	+	+
42	±	+	±	+ +	+ + +
47	0	0	±	±	+
54	±	±	+ +	+ +	+ +

[a]0, no zone; ±, 0 - 1 mm; +, 1 - 2 mm; + +, 2 - 3 mm; + + +, 3 - 4 mm; + + + + > 4 mm.

TABLE 2a. Distribution of Physiological Activities of Kepone Sensitive Microbes

Culture Number	Organism Type	Gram Stain	Alkane[a] Utilizer	Aromatic[a] Utilizer	Physiological Function			Pesticide Tolerance
					Lipolytic	Amylolytic	Proteolytic	
1	rod	pos	+	+	-	+	+	+
3	rod	pos	-	-	-	+	+	-
4	cocc-bac	neg	-	-	+	+	+	-
11	cocc-bac	pos	+	+	+	+	-	+
14	rod	pos	-	-	+	+	-	-
29	rod	pos	-	-	-	-	+	-
42	coccus	pos	-	-	+	-	-	-
54	rod	pos	-	+	-	+	+	-
8	yeast	-	-	+	+	+	+	-
15	fungus	-	+	+	N.D.[b]	N.D.	N.D.	-

[a]Hydrocarbons and pesticides listed in text

[b]N.D. - not determined

TABLE 2b. Range of Physiological Activities of Kepone Tolerant Microbes

Culture Number	Organism Type	Gram Stain	Alkane[a] Utilizer	Aromatic[a] Utilizer	Physiological Function			Pesticide Tolerance
					Lipolytic	Amylolytic	Proteolytic	
10	rod	neg	-	-	-	-	±	-
18	pleo	neg	+	-	+	-	-	-
20	rod	neg	+	-	N.D.[b]	N.D.	N.D.	-
23	rod	pos	+	+	N.D.	N.D.	N.D.	+
40	cocc-bac	pos	+	-	+	-	+	+
45	rod	neg	-	-	+	-	+	-
46	rod	neg	-	-	+	-	-	+
56	cocc-bac	neg	-	-	+	+	-	+
59	rod	neg	-	-	-	-	-	-
2	yeast	-	+	-	-	-	+	-
7	yeast	-	-	-	+	+	+	-
27	fungus	-	-	+	N.D.	N.D.	N.D.	+
44	fungus	-	-	-	N.D.	N.D.	N.D.	+

[a]Hydrocarbons and pesticides listed in text

[b]N.D. - not determined

TABLE 3. Effects of Kepone on Isolation of Aerobic Bacteria from Estuarine Water

Sample Source	Date[a]	Salinity (°/oo)	Temp. (C)	CFU's x 10^2 per ml water			
				Z-15	0.02[b]	0.20[b]	2.00[b]
Range Pt.	A	15	21	181.0	108.0(41)[c]	73.0(60)	35.0(81)
	B	15	24	86.5	65.0(25)	35.0(60)	33.0(61)
	C	20	23	17.7	12.0(28)	13.0(23)	13.0(25)
Escambia Bay	A	11	22	267.0	81.0(70)	233.0(13)	130.0(52)
	B	15	24	13.5	6.9(50)	10.0(26)	4.7(66)
	C	18	22	11.6	11.0(8)	9.0(23)	8.9(23)
Laboratory Pond	A	20	20	479.0[d]	448.9(7)[d]	409.0(14)[d]	396.0(18)[d]
	B	15	26	104.0	49.0(53)	-	19.0(82)
	C	20	27	544.0	501.0(8)	437.0(20)	309.0(43)
Gulf Mexico	A	33	18	270.0	62.0(78)	32.0(89)	46.0(83)
	B	33	22	13.3	10.5(21)	10.0(25)	8.0(40)
	D	33	24	15.0	6.1(59)	1.2(92)	.7(95)

[a]Sampling Dates: A = 4/1/77; B = 4/17/77; C = 4/29/77; D = 5/7/77.

[b]mg/l Kepone used in Zobell's Marine agar.

[c]( ) percentage reduction in CFU's normalized against growth on control plates of Zobell's marine agar containing no Kepone.

[d]CFU x 10^3/ml

TABLE 4. Colony-forming units in sediments[a]

Date	Air	Kepone (mg/l) 0	.02	0.2	2.0
8/2/77	Aerobic	207.50 ± 22.00	197.50 ± 41.00	13.75 ± 13.00	33.20 ± 16.00
	Anaerobic	34.00 ± 4.30	30.60 ± 10.00	41.00 ± 10.00	36.25 ± 3.10
8/9/77	Aerobic	235.00 ± 75.00	74.00 ± 17.00	53.70 ± 18.00	26.25 ± 10.00
	Anaerobic	76.75 ± 12.70	68.25 ± 20.30	67.50 ± 25.00	59.50 ± 23.00
8/31/77	Aerobic	215.75 ± 51.30	243.50 ± 98.60	207.50 ± 99.50	62.25 ± 56.00
	Anaerobic	26.25 ± 7.00	24.50 ± 3.40	18.00 ± 8.00	13.25 ± 1.50

[a]Cells x 10^2 per ml, average of 4 replicates

TABLE 5. Results of Model I ANOU procedure, Critical values of F obtained from Table D.11 by Zar (19).

Source	DF	Sum of Squares	F Value	PR F
Date	4	38002.33	11.96	0.0001
Air[a]	1	50006.87	62.97	0.0001
Treatment[b]	3	17498.36	7.34	0.0002
Air/Treatment	3	13628.83	5.72	0.0011

[a]Aerobic and anaerobic treatment

[b]Concentration Kepone tested (0, 0.02, 0.20, 2.0 mg/l)

TABLE 6. Sample means (calculated as a function of Kepone concentration versus aerobic/anaerobic treatments) used in the SNK test. $\nu = 138$, $\alpha = 0.05$ q value for pair comparisons from Zar Table D.12 ( ).

Air	Kepone Concentration	N[a]	Mean Responses
Aerobic	0.00	19	65.76
Aerobic	2.00	19	11.45
Aerobic	0.20	19	31.39
Aerobic	0.02	19	49.24
Anaerobic	0.00	20	3.48
Anaerobic	2.00	19	2.10
Anaerobic	0.20	21	2.82
Anaerobic	0.02	14	3.51

[a]N = the number of populations (plates) treated by concentrations of Kepone listed.

TABLE 7. Origin of isolates and media from which the organisms were isolated

Isolate	Origin	Media[a]
19K	Toxic Wastes Pond	0.02
28K	Toxic Wastes Pond	2.00
32K	Range Point	0.00
49K	Range Point	0.00

[a]Zobell's marine agar base supplemented with Kepone at the specified concentration (mg/l).

TABLE 8. Effect of Kepone on oxygen uptake for resting cells

Environmental Isolate[b]	Percentage Reduction in Oxygen Uptake[a] Kepone (mg/l)		
	2	20	200
EBŻ-49	+25	100	100
EB.02-51	0	100	100
EB.2-61	0	100	100
EB2-56	66	75	66
RPŻ-32	0	83	31
RP.02-35	11	100	100
RP2-43	-	64	33

[a] nM O_2/ml/min/µg protein

[b] EB - Escambia Bay, RP - Range Point, Ż-Zobell's, X,XX-Kepone concentration, - culture number.

TABLE 9. Inhibition of electron transport activity expressed as the percentage reduction in Specific Activity (nMO_2/min/mg protein) as a function of Kepone concentration. Kepone was added in 10 ul of acetone. All values are corrected for endogenous activity.

	NADH Oxidase		Succinooxidase	
Kepone (uM)	Specific Activity*	Reduction%	Specific Activity	Reduction %
0.0	223	0	67.60	0
0.5		4		0
1.0		51		25
1.5		57		25
2.0		62		--
4.0		62		40
10.0		--		50

Figure 1. Results of Model ANOV procedure.

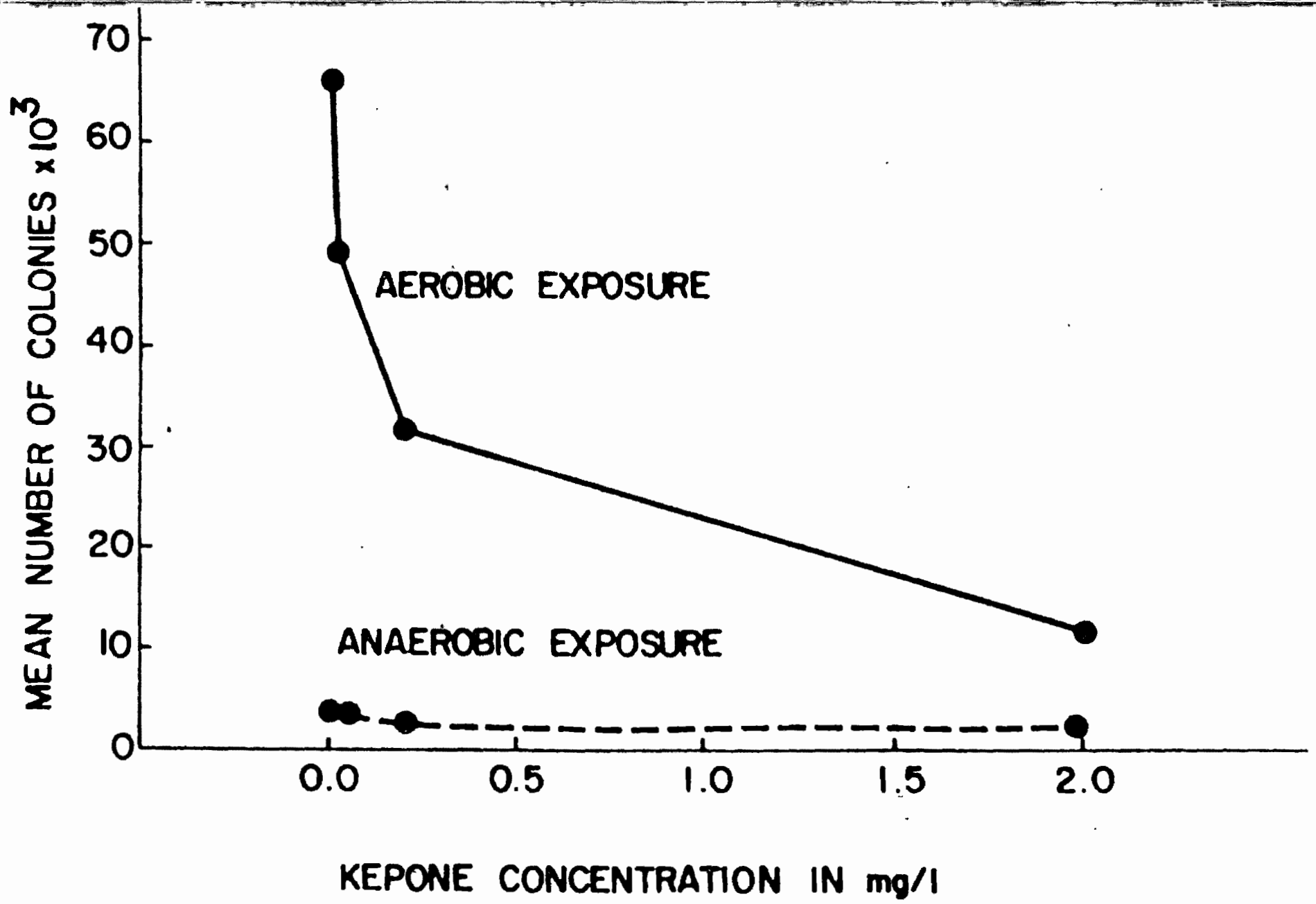

MEAN NUMBER OF COLONIES x 10^3
70
60
50
40
30
20
10
0
AEROBIC EXPOSURE
ANAEROBIC EXPOSURE
0.0
0.5
1.0
1.5
2.0
KEPONE CONCENTRATION IN mg/l

Figure 2. Growth response of isolate 32K (inoculum size?) in the presence of Kepone. Controls contain equivalent amount of acetone carrier as used in experimentals. (_____), MSB/0.4% succinate, (------), Zobell's marine broth (1.5%) NaCl.

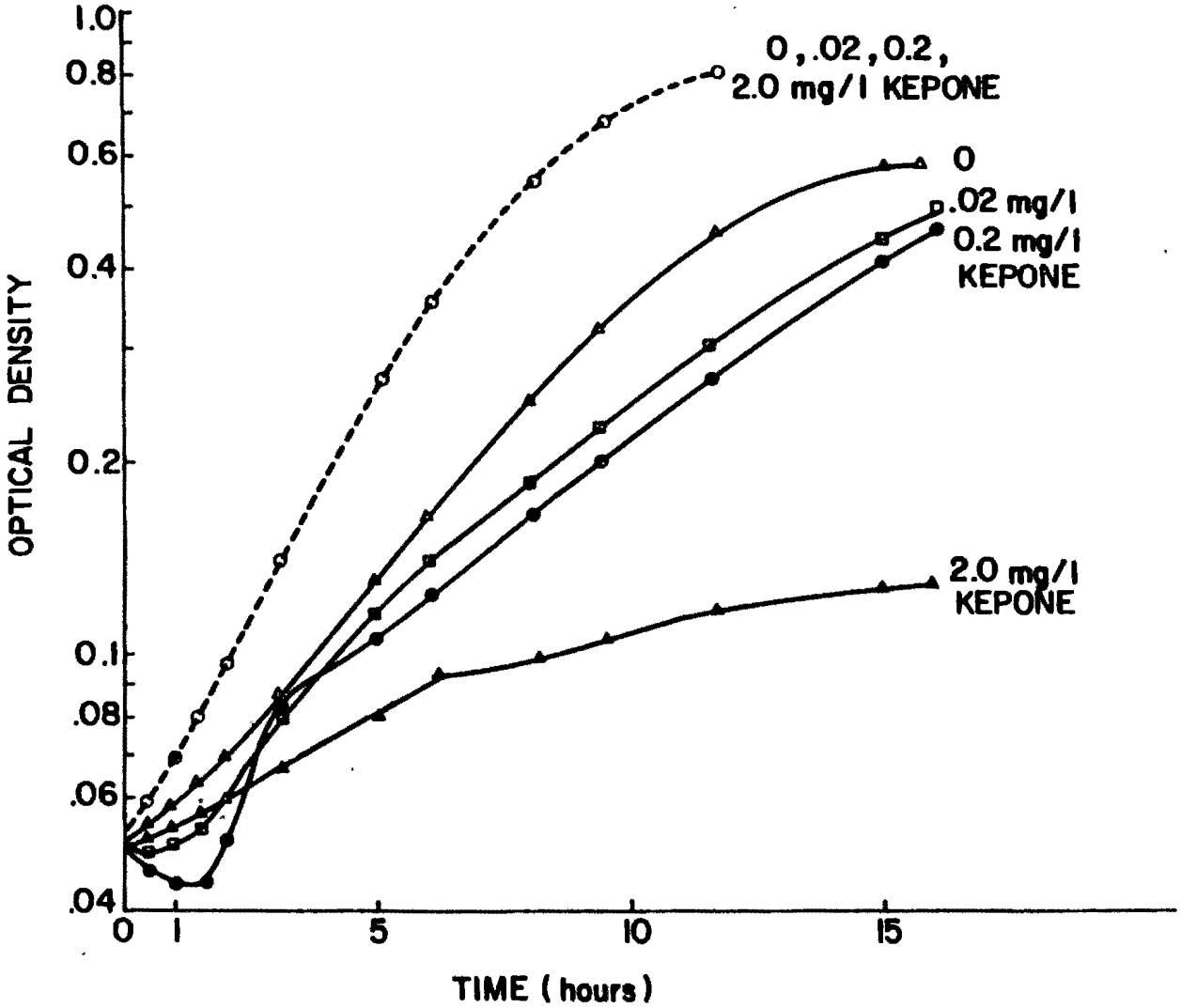
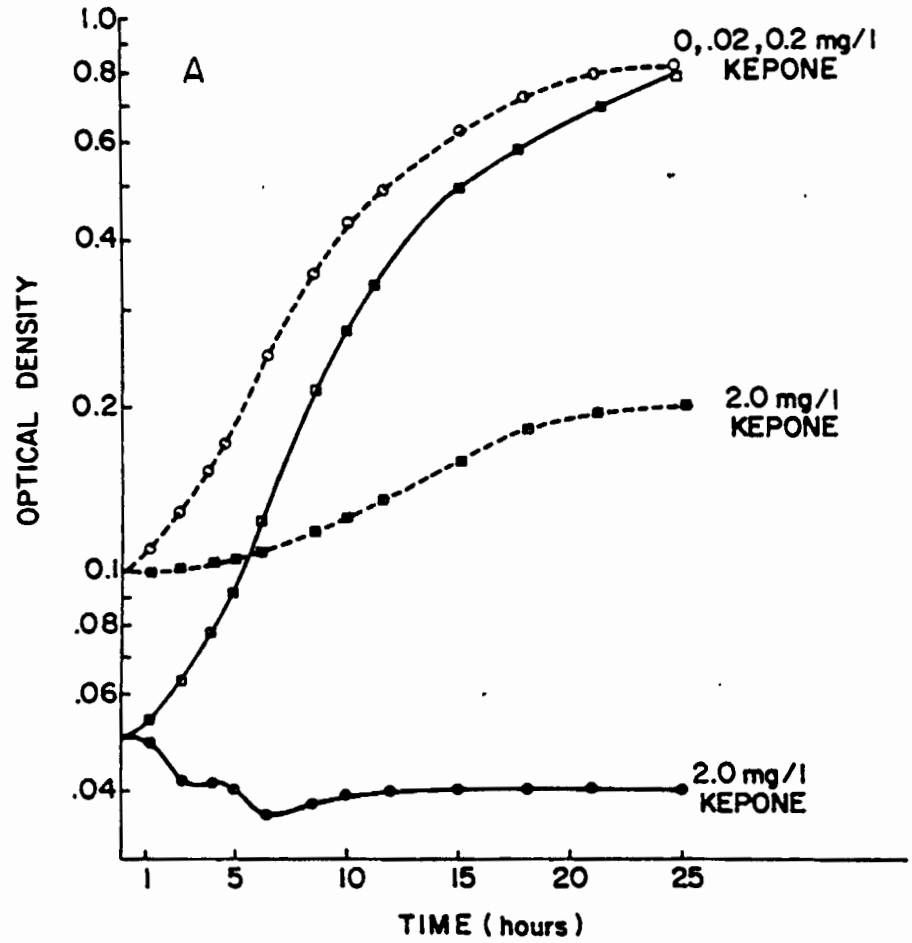

1.0
0.8
0.6
0.4
0.2
0.1
.08
.06
.04
OPTICAL DENSITY
0
1
5
10
15
TIME (hours)
0, .02, 0.2,
2.0 mg/l KEPONE
0
.02 mg/l
0.2 mg/l
KEPONE
2.0 mg/l
KEPONE

Figure 3. Effect of inoculum size on the growth of Isolates 49K and 19(K) in the presence of Kepone. (A) Isolate 49K: ( ); initial O.D. = 0.05, (-------); initial O.D. = 0.1. (B) Isolate 19K: ( ); initial O.D. = 0.02; (-------) initial O.D. = 0.1.


A
0, .02, 0.2 mg/l KEPONE
2.0 mg/l KEPONE
2.0 mg/l KEPONE
OPTICAL DENSITY
1.0
0.8
0.6
0.4
0.2
0.1
.08
.06
.04
1
5
10
15
20
25
TIME (hours)

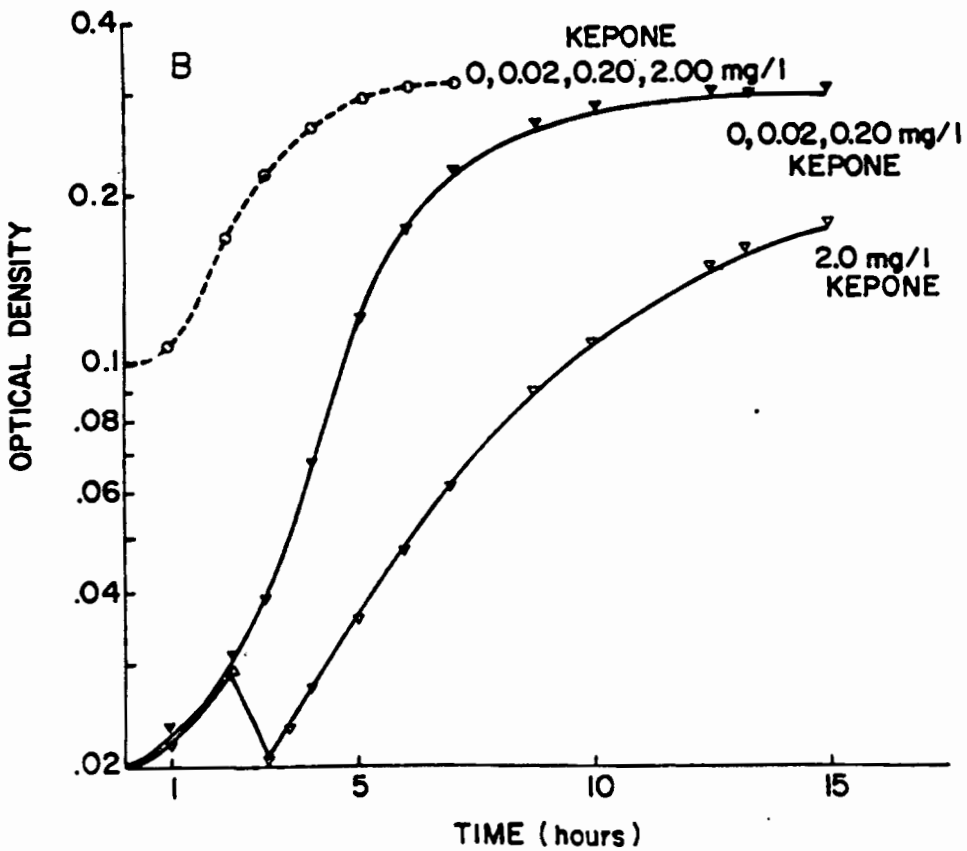
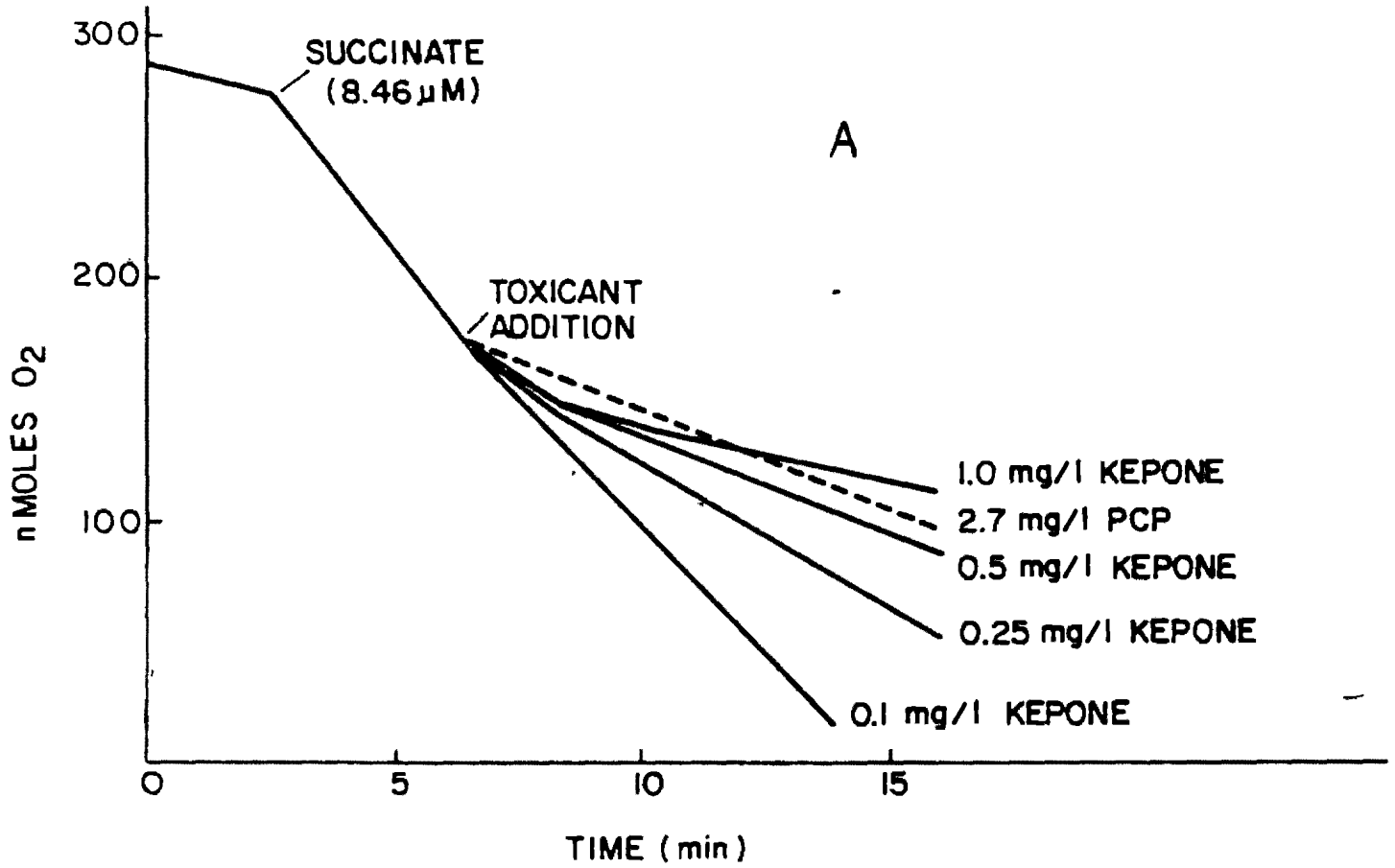

B
KEPONE
0, 0.02, 0.20, 2.00 mg/l
0, 0.02, 0.20 mg/l KEPONE
2.0 mg/l KEPONE
OPTICAL DENSITY
0.4
0.2
0.1
.08
.06
.04
.02
1
5
10
15
TIME (hours)

Figure 4. Oxygen uptake activity by whole cells: (A) Isolate 32K (140 µg protein). (B) Isolate 28K (180 µg protein). 10 µl of Kepone or PCP (reference toxicant) in dimethylformamide (DMF) carrier was added to each reaction mixture after a constant rate of O_2 uptake was observed.


300
200
100
nMOLES O2
SUCCINATE
(8.46 µM)
A
TOXICANT
ADDITION
1.0 mg/l KEPONE
2.7 mg/l PCP
0.5 mg/l KEPONE
0.25 mg/l KEPONE
0.1 mg/l KEPONE
0
5
10
15
TIME (min)

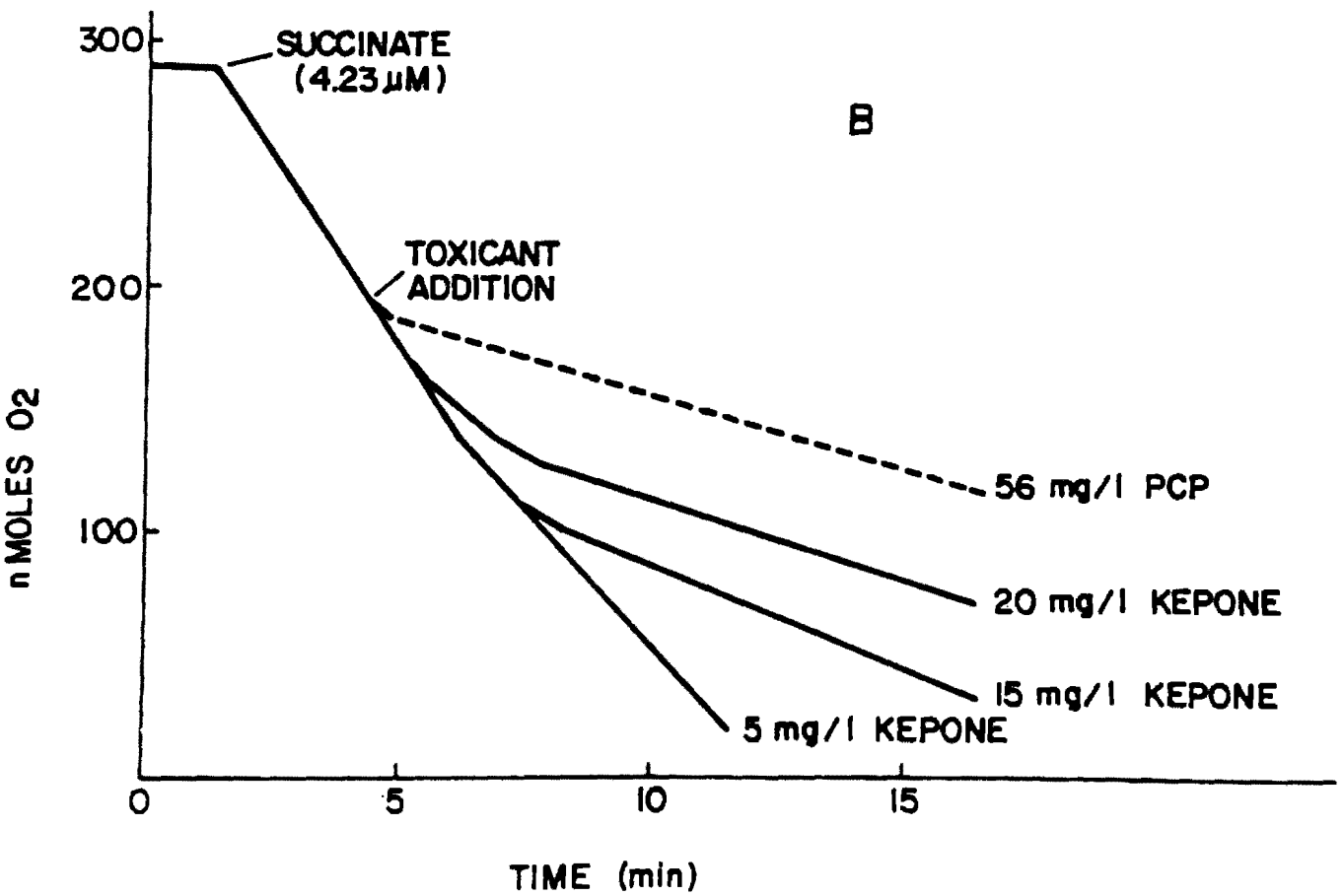
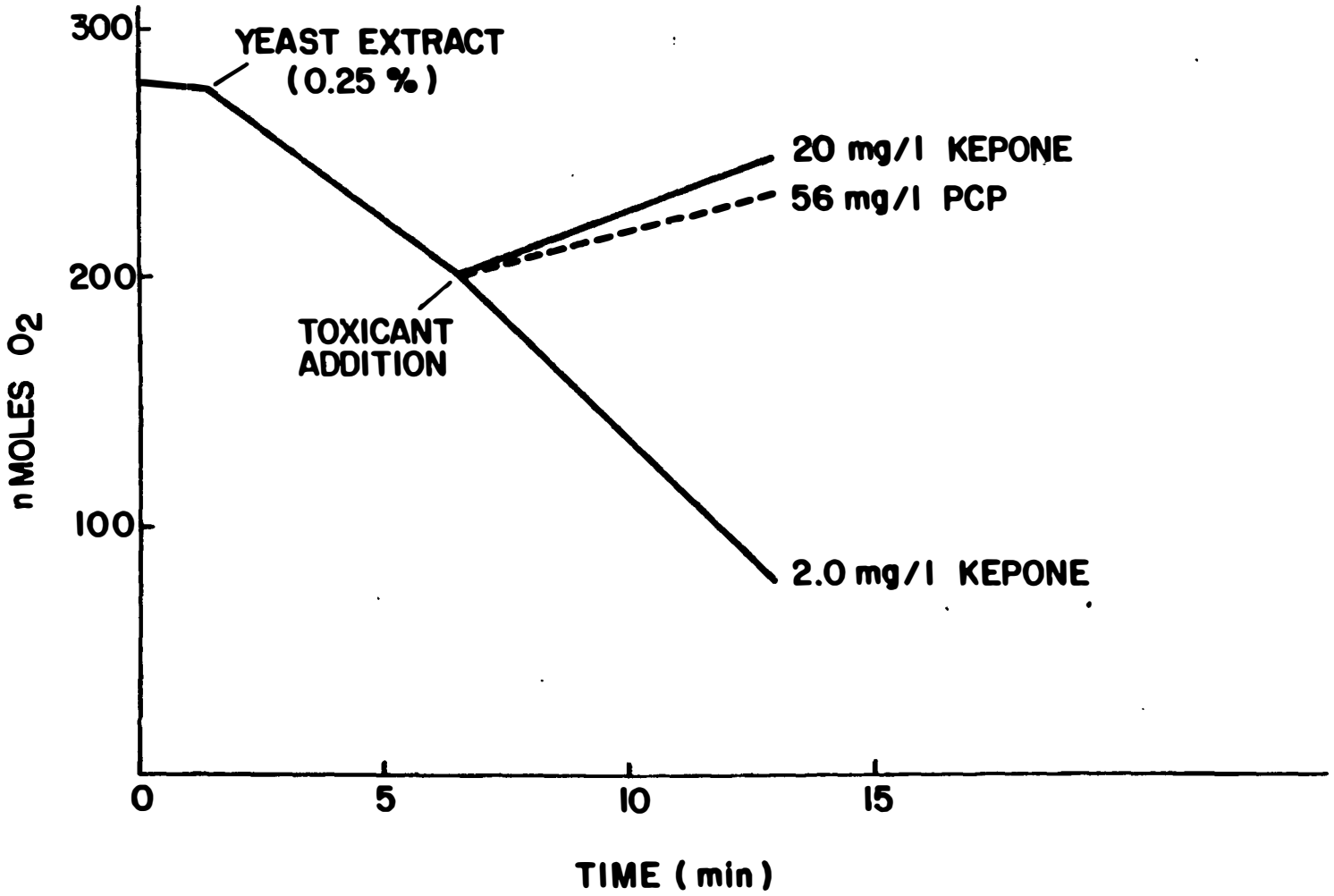

300
200
100
nMOLES O2
SUCCINATE
(4.23 µM)
B
TOXICANT
ADDITION
56 mg/l PCP
20 mg/l KEPONE
15 mg/l KEPONE
5 mg/l KEPONE
0
5
10
15
TIME (min)

Figure 5. Oxygen evolution by whole cells of Isolate 49 (200 µg protein) as affected by high concentrations of Kepone and PCP. 10 µl of toxicant in DMF carrier added to reaction mixture after a constant rate of O_2 uptake was observed.


300
200
100
nMOLES O2
YEAST EXTRACT
(0.25 %)
20 mg/l KEPONE
56 mg/l PCP
TOXICANT
ADDITION
2.0 mg/l KEPONE
0
5
10
15
TIME (min)