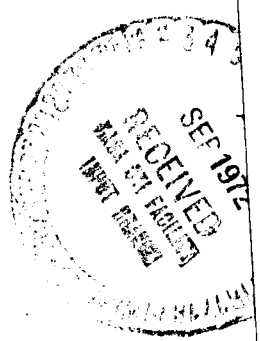
MICROBIAL DEGRADATION OF PARATHION

By

WILLIAM LEONARD GIBSON



A Dissertation
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
in the Department of Microbiology

State College, Mississippi

August 1972

MICROBIAL DEGRADATION OF PARATHION

By

WILLIAM LEONARD GIBSON

A Dissertation
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
in the Department of Microbiology

State College, Mississippi

August 1972

MICROBIAL DEGRADATION OF PARATHION

By

WILLIAM LEONARD GIBSON

Approved:

Dr. Lewis R. Brown
Assistant Dean of the College of Arts and Sciences and Professor of Microbiology and Director of Dissertation

Dr. Robert G. Tischer
Chairman, Department of Microbiology (Member of Graduate Committee)

Dr. John C. Mickelson
Professor of Microbiology (Member of Graduate Committee)

Dr. Howard W. Chambers
Associate Professor of Entomology (Member of Graduate Committee)

Dr. James R. Heitz
Assistant Professor of Biochemistry (Member of Graduate Committee)

Dr. J. Chester McKee
Dean of the Graduate School

August 1972

ACKNOWLEDGMENTS

Sincere appreciation is extended to Dr. Lewis R. Brown for his guidance and supervision in the direction of this research problem and for his constructive criticism regarding the preparation of this manuscript.

Gratitude is also extended to the members of my graduate committee, Dr. Robert G. Tischer, Dr. James R. Heitz, Dr. Howard W. Chambers, and Dr. John C. Mickelson for their advice and consultation.

My thanks is also extended to the staff and the graduate students of the Microbiology Department.

To my wife, Gayle, for her patience, understanding, and encouragement during the course of my graduate study, and for her clerical assistance in the preparation of this manuscript, I am especially indebted.

The author is grateful for the financial assistance awarded him by the Office of University Affairs, N.A.S.A. - Headquarters, Washington, D.C., Grant # NGL-25-001-040 entitled "A Multidisciplinary Research Program on the Environment."

W.L.G.

State College, Mississippi

August 1972

TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

TABLE OF CONTENTS (Continued)

TABLE OF CONTENTS (Continued)

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (Continued)

LIST OF FIGURES (Continued)

INTRODUCTION

Parathion is an organophosphorous insecticide still widely used in certain areas. It is marketed under various trade names primarily by two companies, American Cyanamid Corporation and Monsanto Chemical Company. Its popularity exists primarily due to its high insect mortality rate. However, when ingested or absorbed by wildlife, man or other nontarget organisms, parathion may exert a severe neurotoxic effect and is often fatal.

Parathion is effective in the control of aphids, mites, grasshoppers, moths, leafhoppers, wireworms, and many other insect pests (Monsanto, 1972). The degradability of parathion is of great practical importance since its toxicity as a cholinesterase inhibitor is rarely limited to the target organism but extends to nontarget species, including man. Properly formulated emulsifiable liquids, wettable powders, and dusts are not phytotoxic when applied at recommended dosage levels. Exposed or sprayed parathion is detoxified and decomposed by water or moisture, heat, microorganisms and earth, and partly by sunlight. However, little is known regarding the biological or chemical mechanics involved in parathion loss. It is interesting to note that parathion, which is very rapidly metabolized in tissues of higher

organisms and in the rumen, may still be present years after its introduction into soil (Ahmed, 1958).

As agriculture comes to rely more and more on pesticides, the importance of basic and applied research on the use, behavior, and toxicology of pesticides including parathion increases.

Despite an increasing concern with the persistence and residual effects of pesticides and the dominant position occupied by microbiological agencies in environmental detoxification, very little information is available on soil inhabitants. In addition, research in the area of microbial degradation may prove valuable in terms of a new system for waste disposal or perhaps as a new detection system for parathion pollution. Consequently, the objectives of this investigation were reported in the following manner:

(1) To selectively isolate by enrichment culture techniques one or more bacteria capable of readily metabolizing the parathion molecule as the sole carbon source,

(2) To study the physiological parameters of parathion utilization,

(3) To determine some of intermediates in the degradation of parathion by microorganisms.

REVIEW OF LITERATURE

History

The first esterification of alcohols and phosphoric acids is attributed to Lassaigne in 1820, and in the early 1900's numerous syntheses of phosphorus compounds containing nitrogen, fluorine, and various other substituents were reported (O'Brien, 1960). In all of the early investigations, however, there was no mention of the poisonous nature of these compounds, and it was not until the approach of World War II that the toxic properties of the organophosphates began to be realized. B.C. Saunders in England and Gerhard Schrader in Germany evaluated several organic phosphorus compounds for use as nerve gases. It was Schrader, however, who discovered their suitability as insecticides, originally as a substitute for nicotine against aphids (O'Brien, 1967).

It has been estimated that approximately 40,000 new potentially poisonous products enter the market each year. In less than 20 years, the use of synthetic chemical pesticides has increased from a level of a few million pounds a year to nearly one billion pounds annually. Helling, Kearney, and Alexander (1966) stated that today almost 60,000 different pesticide formulations are now registered in the United States and each of these contains one or more of the

approximately 600 different pesticide compounds. Chemical pesticides are produced at about two billion pounds per year at the present time. This is about 10 pounds per capita, or enough poison to kill every human being on earth.

Parathion Poisoning

In south Texas, the incidence of poisoning increased for several years, and was usually caused by parathion and methyl parathion. The leading cause of death among children is pesticides, resulting in approximately 5,000-6,000 cases per year according to Vercruysse (1964). When one considers that only about 10% of the true incidences of poisoning in the United States are reported and that the Poison Control Centers report over 100,000 cases per year, it is apparent that pesticide poisoning represents an important public health problem.

In Dade County, Florida, an estimated 250,000 pounds of organophosphate pesticides are used annually. During the past twelve years, over 100 persons have died as the result of pesticide poisoning. Since 1959, 56 deaths have resulted from parathion poisoning: 28 were accidental poisonings, 26 suicides, and 2 murders. Reich (1971) reported an estimated 2,000 nonfatal organophosphate poisonings occur yearly in the United States.

Chemistry of Parathion

The chemical name of parathion is O,O-diethyl O-p-nitrophenyl phosphorothioate. The structural configuration

of the parathion molecule is shown in Figure 1 in the Appendix. Parathion has a molecular weight of 291, and contains 41% carbon, 4.79% hydrogen, 10.95% phosphorus, 10.95% sulfur, 27.39% oxygen, and 4.79% nitrogen. It is a dark, brown liquid with a characteristic odor, melts at 6.0 C, and boils at 159-162 C. The specific gravity of parathion at 25 C is 1.26-1.28. Parathion is slightly soluble in water (24 parts per million at 25 C), and completely miscible with many organic solvents. Parathion is stable indefinitely in neutral and acid solutions at room temperature, but it becomes slightly hydrolyzed at 100 C. However, in solutions of pH 9 and higher, an appreciable increase in hydrolysis occurs as the temperature rises (Monsanto, Technical Bulletin, hereafter referred to as Monsanto, 1972).

Organophosphates are named as esters of phosphoric acid. The phosphorus contributes all of the electrons. Oxygen bonded to phosphorus draws electrons and creates an extremely positive phosphorus, resulting in a compound which is very soluble, reactive, and toxic (Lichtenstein and Schultz, 1967). Parathion is an organophosphate insecticide of the aromatic type. Parathion is readily oxidized to paraoxon, which is more toxic than the parent compound. As the oxygen analog, the phosphorus is more positive, soluble, and reactive. This probably accounts for the increased toxicity. The compound methyl parathion possesses methyl groups instead of ethyl. The reactions, utilization, and toxicity of this compound are quite similar to parathion.

Mode of Action

Pharmacological studies of parathion show that it has a dual effect: a stimulation of the parasympathetic nervous system and anticholinesterase activity. The systemic effects of parathion are qualitatively similar to those of other cholinergic agents, for example, muscarine, pilocarpine, etc. The effects of parathion result from inactivation of the cholinesterase enzyme, thus permitting accumulation of acetylcholine at the motor end plates of the parasympathetic and the voluntary nervous system, and in the ganglia of the sympathetics. The symptoms include giddiness, headache, nausea, vomiting, abdominal cramps, diarrhea, sweating, salivation, lacrimation, confusion, weakness, muscular fasciculations, and possibly coma and convulsions. Fatalities appear to result from respiratory failure.

Relative Toxicity of Parathion

Parathion and methyl parathion are up to 70 times as toxic as DDT; therefore, great care must be exercised in using formulations of parathion (Welling et al., 1970). The parathions are capable of producing severe toxic effects and death in animals and man. These effects can be produced after swallowing and breathing of mists, dusts or vapors or by absorption of parathion through the skin. The LC_{50} Oral Toxicity Tests on laboratory animals vary somewhat with the species (Reich, 1971). However, a lethal dose is in the range of 3-10 milligrams per kilograms of body weight for

parathion and 9-25 for methyl parathion. The LD_{50} values as tested against white albino rats are presented in Table 1 (Welling et al., 1970). The lethal dose for man is not known. It is impossible to interpret animal toxicity data directly in terms of man.

Monsanto (1972) reported that the organophosphates are 3 to 5 times as toxic as nicotine. Since the fatal dose of nicotine is estimated as 60 milligrams (1 grain), 12-20 mg of parathion may be considered as a poisonous quantity, potentially fatal. As little as 0.05 ml (1 small drop) of concentrated parathion splashed into the eye may be fatal. Fatal poisoning may result from contact with the skin by ingestion, or by inhalation of parathion or a formulation of parathion.

Applications

Large quantities of parathion are used to protect crop plant roots from soil insects, such as rootworms and wireworms. Parathion is widely recognized as the cheapest and most effective broad spectrum insecticide available. American Cyanamid (1972) reported that it can be used in formulations to control over 200 different types of insects on nearly 100 different crops. Parathion is principally formulated as an emulsifiable concentrate (2 to 8 pounds per gallon), wettable powder concentrates (15% and 25%), ready-to-use dry dust mixtures (1% to 10%), and granules (2% to 25%).

Ecological Effects

Pesticides found in the estuarine environment originate from three main sources: agricultural applications, applications to control noxious insects, and effluents from industries that formulate pesticides as a result of runoff and erosion. A pollutant may be defined as any substance added to the environment which has a measurable and generally detrimental effect upon the environment.

The chief hazard of pesticide residues in the aquatic environment lies in the biological magnification process in the food chain. Organophosphorus and organochlorine insecticides may be absorbed selectively by plankton which are later consumed by small fish and, in turn, the small fish are eaten by larger fish. The concentration of residue is magnified at each stage of the chain (Alexander, 1969).

Detection

The concentration of insecticides that will produce a perceptible odor in water has been determined for several pesticides according to Blackbourne (1970). An odor is imparted to water by concentrations of technical parathion as small as 3 parts per billion (ppb). This concentration is close to the commonly accepted limit of 1 ppb for phenol in water as shown in Table 2 (Frawley et al., 1958).

In occupationally exposed groups urinary excretion of para-nitrophenol (PNP), a metabolite of parathion has been used to monitor exposure. Acetyl-cholinesterase

activity has been employed to monitor exposure but the range of the norm for this test is broad and subject to considerable individual variation.

Phytotoxicity

Foster (1962) found that relatively heavy doses of parathion appeared to have a slight but temporarily depressing effect on germination and stand. Parathion had no apparent effect on the numbers of nitrifying, and denitrifying, cellulose-decomposing, manganese-oxidizing, or spore-forming bacteria according to Kasting and Woodward (1961).

Factors Affecting the Fate of Pesticides

There are approximately seven factors which are known to affect the fate and behavior of pesticides in the soil. These factors are as follows: (1) adsorption and desorption, (2) chemical decomposition, (3) photochemical decomposition, (4) microbial decomposition, (5) volatilization, (6) movement, and (7) plant and microbial uptake. Of the several mechanisms by which pesticides may be detoxified in soil, microbial degradation appears to be by far the most important. The presence of basic amino acids in soil organic matter catalyzes hydrolysis of organophosphorus esters (Alexander, 1965).

Photochemical Decomposition

Emulsions of parathion-aqueous salt solutions exposed to ultraviolet light (wavelength 1850-4000Å) develop anticholinesterase activity progressively according to Cook and Pugh (1957). The exposure of parathion to ultraviolet light gives rise to a mixture of compounds possessing greater *in vitro* anticholinesterase activity than parathion, but lower reactability to the Averill-Norris chemical method of parathion determination and lower toxicity to houseflies according to Frawley *et al.* (1958). Since similar conversion might occur on crops sprayed with parathion, the ability of the method to detect all toxic residues was in question. Samples of parathion and ultraviolet light-exposed parathion were fed subacutely to rats, and the *in vivo* anticholinesterase activity was found to parallel closely the loss in response to the Averill-Norris chemical method according to Cook *et al.* (1957). Oxidation of parathion at the P=S bond has been shown to occur under ultraviolet light; however, chemical oxidation of parathion in soils and waters is not prevalent.

Chemical Decomposition

The chemical structure of parathion is presented in Figure 1. Chemical hydrolysis of parathion may exist at the EtO-P and nitrophenyl, C-0-P ester linkages. Hydrolysis of organophosphates generally proceeds at the P-0-X bond. Hydrolysis at the nitro-phenyl C-0-P bond is the most likely chemical pathway for parathion degradation resulting in the

formation of p-nitrophenol and diethylthiophosphoric acid according to Menzer and Dauterman (1970). Chemical degradation of organophosphate insecticides involves hydrolysis of ester linkages. The hydrolysis may be either acid or alkali catalyzed.

Physical Factors of Decomposition

The action of oxygen in the air combined with the catalytic action of sunlight makes a very powerful oxidizing agent. Thus, insecticides that evaporate in the air are soon destroyed. The speed of the reaction is approximately doubled with each 10 degree rise in temperature. Therefore, the hot, sunny, summer days aid in the rapid oxidation of organic materials. Oxidation will also be rapid when deposits are exposed to the air, on the ground, and on leaves or solid surfaces (Fleck, 1965). On the other hand, insecticides that are mixed deep in the soil or in muddy river bottoms are shielded from this combination. The rate of parathion degradation was significantly influenced by soil temperature, moisture content, and acidity. Higher temperatures and soil moisture levels accelerated decomposition.

Persistence

It is interesting to note that parathion, which is very rapidly metabolized in tissues of higher organisms and in the rumen, may still be present years after its

introduction into soil (Alexander, 1967). For example, 100 ppm in the rumen persists for only one day according to Cook et al. (1957). Kasting et al. (1961) reported that 100 pounds/acre of formulated parathion applied to the soil persisted for 325 days.

Biodegradation of Pesticides

Soil and water have been considered almost universally as ideal receptacles for toxic natural or synthetic organic wastes. The use of pesticides in agriculture and nonfarm pest control operations has shown that, without question, the microorganisms of the soil and water are not the omnipotent detoxifiers that they were once believed to be (Alexander, 1969). Pesticides may be destroyed or detoxified by one or more of several different mechanisms. Nonbiological degradation or adsorption and inactivation by clay and other colloidal materials may contribute significantly to environmental decontamination (Alexander, 1969). Volatilization or photochemical degradation contribute to the removal of certain chemicals from a particular ecosystem and leaching may occasionally be responsible for the removal of pesticides from the surface zones of the soil. Leaching may lead to a detoxification of the treated soil, but the gain to man's aquatic environment may be very deleterious. These may pose major problems to the fish population or to the maintenance of an acceptable quality drinking water.

There is as yet no evidence that the fauna of the soil, either the protozoa or the higher subterranean animals, participate directly in pesticide destruction.

Biodegradation in general is considered to be a process of conversion by microorganisms of pesticides into one or more simpler non-toxic substances. On the basis of the available data, the biological detoxification of pesticides is thought to involve the following principle biochemical reactions: dealkylation, dehalogenation, hydrolysis, oxidation, reduction, ether fission, hydroxylation, ring cleavage, and cooxidation (Stojanovic, 1970).

In order for a pesticide to be subjected to biodegradation, five conditions must apply (Lichtenstein and Schultz, 1964): (1) an organism must exist in the soil or one must be capable of developing which has the capability of metabolizing the chemical; (2) the compound must be in a form suitable for degradation; (3) the chemical must reach the organism or the organism must find its potential substrate; (4) the compound must be capable of inducing the formation of the appropriate enzyme or enzymes concerned in the detoxification. Most enzymes concerned in these detoxification reactions require induction; few are constitutive. Low solubility or low concentration of the pesticide or permeability barriers at the surface of the organism may be associated with lack of induction; (5) environmental conditions must be suitable for the microorganisms to

proliferate and for the enzymes to operate. Should any of these conditions not be met, the pesticide will not be decomposed biologically, and it may persist for extended periods.

It may be observed that the carboxyl and hydroxyl groups favor decomposition and a notable retarding effect of the degradation when the molecule contains a sulfonate, nitro, or chlorine substituent (Alexander, 1965).

Helling et al. (1966) reported that the introduction of polar groups such as OH, NH_2, N-C(0), COO, NO_2 and others common to many pesticides often affords microbial systems a site of attack. Catalysts accelerating these reactions are induced or constitutive microbial enzymes. The ultimate objective of the organisms degrading pesticides is to obtain usable energy for other life processes. Only when the pesticide is fragmented to a compound that can be channeled into oxidative cycles, such as the Krebs cycle, does the organism derive any useful energy.

Biodegradation of Parathion

Activation

The organophosphorus insecticides are chiefly phosphate esters. The single ester organophosphorus compounds such as parathion are much more resistant than double ester compounds. Because the organic insecticides are less persistent than the inorganic ones, their use offers very real advantages. The organophosphorus insecticides contain

either the P=S (phosphorothioate) or the P=0 (phosphate) groupings. Frequently, the initial step in degradation is an activation of the molecule; such as, a phosphorothioate to a far more inhibitory derivative, a phosphate. Degradation of the phosphorothioates and phosphates may involve a hydrolase, phosphatase, dealkylase, or carboxyesterase.

Enzymatic transformation may involve a chemical change, such that all inhibiting properties are lost. This is termed "detoxification or inactivation," the conversion of active compounds to non-toxic derivatives through degradation. In the case of parathion, the material designated as the pesticide, parathion, is itself non-toxic, but it may be transformed microbiologically to the toxicant paraoxon, a process known as "activation." Erdos and Boggs (1961) reported that pure parathion is not at present considered to be an inhibitor of cholinesterase in vitro. It is probable that those who first reported to the contrary were using contaminated samples. It must first be activated to the p-nitrophenyl phosphate (paraoxon) form in order to possess anticholinesterase activity.

Cometabolism

In a cometabolic reaction sequence, the microorganism enzymatically transforms a compound which it cannot utilize as a source of energy (Alexander, 1968). An example of this is a bacterium which is not able to utilize p-nitrophenol

as a carbon and energy source, but oxidizes it to nitrate (Raymond and Alexander, 1967).

The detoxification mechanisms most likely to affect parathion are reduction, ring cleavage, cooxidation, oxidation, hydrolysis, and hydroxylation. Degradation of parathion has been demonstrated by either: (1) hydrolysis to p-nitrophenol and diethylthiophosphoric acid, or (2) reduction to its amino form depending on the population of soil microorganisms (Miyamoto et al., 1971). Yeasts were responsible for the reduction of parathion in soil to aminoparathion (Miyamoto et al., 1971).

Hydrolysis

Among the organophosphate insecticides, parathion is one of the most resistant to chemical hydrolysis (Graetz et al., 1971). Parathion was degraded by a strain of housefly to diethyl phosphate. The presence of this breakdown enzyme was the result of a mutation leading to an altered aliesterase that had lost most of its esteratic activity but acquired phosphatase activity. Paraoxon was hydrolyzed to diethylphosphate (Welling et al., 1970). Matsumura and Hogedijk (1964) and Dahm et al., (1950) showed that o,o-diethylphosphorothioic acid (DEPTA) is a major metabolite of both houseflies and rat microsomes, respectively. Methyl parathion was hydrolyzed to DEPTA by the supernatant fractions of rat liver and cockroach fat body homogenates under the influence of reduced glutathione according to Fukami and Shishido (1966).

Erdos and Boggs (1961) reported that paraoxon has been found to be hydrolyzed in mammalian blood by an aryl aromatic esterase. This enzyme occurs in the sera of various species. Among other properties, the arylesterase degrades to phenyl acetate, and it has been shown with this substrate that the enzyme requires the presence of calcium for activity. The arylesterase is activated by calcium and inhibited by sodium ethylenediaminetetracetate. Lineweaver-Burk plots showed a non-competitive inhibition. Mounter (1965) stated that Escherichia coli and Propionibacterium pentosaceum hydrolyze paraoxon.

Pseudomonas melophthora, an obligate, extracellular bacterial symbiote of the apple maggot, Ragoletis pomonella degrades parathion by way of the hydrolytic degradation through strong esterase activity (Matsumura and Hogedijk, 1964). Parathion is oxidized in vitro to paraoxon, which is the active anticholinesterase agent in poisoning. Dahm et al., (1950) concluded that p-nitrophenol, formed from hydrolysis of parathion in cattle was apparently reduced to aminophenol.

Reduction

The main microbial degradation pathway in soils and waters appears to be reduction of the nitro (NO_2) group to the amino analog (NH_2) with the formation of aminoparathion. Lichtenstein and Schultz (1967) investigated the persistence of several likely degradation products of parathion; namely,

aminoparathion, p-nitrophenol, and p-aminophenol. Complete degradation of p-nitrophenol occurred between 7 and 16 days after application. The major microbial degradation pathway inoculated with microorganisms obtained from a Lake Tomahawk sediment, was reduction of the NO_2 group to the NH_2 group to form aminoparathion (Miyamoto et al., 1971).

Parathion has been applied to polluted waters as a larvicide against Culex pipiens, one of the most common pest mosquito species almost all over the world and also the vector of Bancroftian filariasis in tropical areas. Parathion incubated with cultures of Rhizobium japonicum and R. meliloti was metabolized primarily by reduction of the nitro group (Mick and Dahm, 1971). Approximately 85% of the initial parathion was reduced to aminoparathion. About 10% of the parathion was hydrolyzed to o,o-diethylphosphorothioic acid (DEPTA). Both aminoparathion and DEPTA have negligible toxicity compared with parathion. The main reactions are conversion of P=S to P=0, cleavage of the diethyl phosphate or diethylphosphorothioate, and reduction of the nitro group.

The main cause of inactivation of fenitrothion, parathion, and methyl parathion by Bacillus subtilis was found to be due to the reduction of their nitro group to the amino group (Miyamoto et al., 1971). The major degradative pathway of methyl parathion (o,o-dimethyl o,p-nitrophenyl phosphorothioate) when incubated with the bacterium Bacillus subtilis was by reduction to the amino homologue of methyl parathion and hydrolysis to dimethyl-phosphorothioate.

Hirakose (1968) showed that methyl parathion was reduced to the amino homologue by bacteria found in polluted water.

In soils of low microbial populations (autoclaved soil) or of low microbial activity (dry soils) parathion persisted for a relatively long period of time. No aminoparathion was found in autoclaved soils. Yeasts were primarily responsible for the reduction of parathion in soil to aminoparathion, while bacteria apparently exhibited no effect on the reduction (Lichtenstein et al., 1968). Yasuno et al. (1965) observed similar results when applying various organophosphate insecticides to polluted waters. Parathion and methyl parathion showed almost complete inactivation of their insecticidal activity within several days after mixing with the polluted waters. When mixed with pure water, however, insecticidal activity persisted for a relatively long period of time. Among the organisms isolated from polluted samples, Bacillus subtilis was found to be highly effective for inactivating parathion and methyl parathion mixed in its culture media. Inactivation occurred as a result of reduction of NO_2 groups to NH_2 groups. According to Yasuno et al. (1965), insecticidal activity of parathion was diminished primarily through the reduction of NO_2 to NH_2 groups forming aminoparathion.

Ring Cleavage

Ring cleavage is a quite common mechanism of degradation of many pesticides by soil microorganisms.

Several phenoxyalkanoic acid herbicides are degraded to the corresponding catechols, which in turn are broken down to yield the corresponding muconic acids (Rogoff and Wender, 1959). Ring cleavage probably occurs only after hydroxylation. Complete ring cleavage, however, is considered to be an unlikely pathway.

When the organic carbon requirement of individual microorganisms was examined, some showed a high degree of versatility, whereas, others were extremely specialized. Certain bacteria of the pseudomonas group can use any one of over 90 different organic compounds as sole carbon and energy source, such as phenylacetate and phenol, as well as other hydrocarbons. Most (and probably all) organisms that depend on organic carbon sources also require carbon dioxide (CO_2) as a nutrient in very small amounts, because this compound is utilized in a few biosynthetic reactions (Rogoff, 1963). However, as CO_2 is normally produced in large quantities by organisms that use organic compounds, the biosynthetic requirement can be met through the metabolism of the organic carbon and energy source. The complete removal of CO_2 often either delays or prevents the growth of microorganisms in organic media, and a few bacteria and fungi require a relatively high concentration of CO_2 in the atmosphere (5 to 10 percent) for satisfactory growth in organic media.

Criteria for microbial utilization of pesticides are the disappearance or modification of the pesticide, the production of CO_2, acid formation, replication of

microorganisms or the consumption of oxygen in media consisting of mineral salts solutions enriched with pesticides as the only source of energy. The theoretical respiratory quotient for complete oxidation of a long chain paraffin hydrocarbon with the formula $C_nH_{2n} + 2$ is approximately 0.67 (Bennett, 1963). Carbon dioxide always results from the microbial assimilation of hydrocarbons. Crude oils, illuminating gases, petroleum ethers, gasolines, kerosenes, fuel oils, paraffin, or mineral oils, petrolatums, asphalts, waxes, and rubber, both natural and synthetic, have been shown to be oxidized by a great variety of microorganisms. Toluene, xylene, or phenol was oxidized readily by Bacterium benzoli (Bennett, 1963).

Evans and Simpson (1965) isolated a pseudomonad which grew in a mineral salt medium with 0.02% p-nitrophenol. They showed substitution of the nitro group by a hydroxyl group with elimination of the nitro group as nitrite ion. The p-nitro group may represent the point where enzymatic attack begins and the ring structure is broken. The oxidative metabolism of aromatic compounds by bacteria consists of manipulation of the ring systems in such a way that the products of degradation may enter the main terminal respiratory cycles of the cell at some point. Cleavage of the ring is preceded by hydroxylations necessary to provide two hydroxy groups on the aromatic rings. These are usually found in the ortho position (catechol, protocatechuic acid),

but in certain cases may appear in the para position (gentisic acid). When in the ortho position the ring is cleaved between the carbons bearing the hydroxyl groups; when they occur in the para position, there is ring cleavage across a bond adjacent to one of the carbon atoms which bears a hydroxyl group. The oxidases which cleave benzenoid rings catalyze reactions in which an atom of molecular oxygen enters directly into the oxidized product (Hayaishi et al., 1971). These enzymes also appear to have a group requirement for ferrous iron.

Phenol was oxidized faster under aerobic conditions; however, some attack of the compound can take place under anaerobic conditions as well. Investigators have found that a pH of 7.0 to 7.5 is optimum for oxidation of phenol by bacteria (Rogoff and Wender, 1959). A temperature of 37 to 55 C was optimum for oxidation of phenol by sewage bacteria; however, it may be oxidized at 4 C by these bacteria. Organic material may stimulate the oxidation of phenols by bacteria. Gunderson and Jensen (1956) isolated a strain of Corynebacterium simplex that oxidized p-nitrophenol, 2,4-dinitrophenol, and 2,4,6-trinitrophenol. Simpson and Evans (1953) isolated two species of pseudomonads that oxidized up to 100 ppm of o-nitrophenol and p-nitrophenol. Each strain utilized the appropriate nitrophenol but did not oxidize the other isomer. The maximum oxidation of 2,4-dinitrophenol and p-nitrophenol occurred at a pH near 8 and none of the compounds were oxidized at pH 7.0. Simpson et al. (1953)

observed that the maximum pH for the oxidation of o-nitrophenol was 7.0 and 7.5 to 8.0 for p-nitrophenol. Walker (1954) observed that soil bacteria were able to oxidize o-chlorophenol, p-chlorophenol, and 2,4-dichlorophenol. Bennett (1963) isolated pseudomonads which could oxidize phenol from metal-cutting fluids that had been treated with these inhibitors. In such instances, the addition of phenols may stimulate growth of the bacteria and deterioration of the product.

In general, bacterial enzymes cleave the aromatic ring by the insertion of oxygen between the atoms forming the C-H bonds of the two adjacent ring carbons, so that two hydroxyl groups are present on the ring in an ortho relationship; the carbon-carbon bond between the hydroxyls is subsequently split. Phenol is hydroxylated to catechol which is cleaved. Depending on the substitution in the original aromatic molecule undergoing oxidation, an alternate compound protocatechuic acid may be reached (Bennett, 1963). These key intermediates undergo ring cleavage and further transformation to form aliphatic compounds such as succinate and acetate that can enter the respiratory cycle. Catechol is split between the hydroxyl groups to form a cis-cis muconic acid. Hayaishi et al. (1971) tentatively identified cis-cis muconic acid as a product of the oxidation of catechol by a purified pseudomonas enzyme.

Gunner et al. (1966) reported a unique observation in the microbial degradation of the pyrimidyl ring of the

organophosphate insecticide diazinon by stretomyces and arthrobacter species. Approximately 66% of the hydrocarbon-oxidizing cultures were pseudomonas species. Hydrocarbon-oxidizing bacteria may be responsible for the fairly rapid disappearance of oils sprayed on various kinds of foliage as a carrier of insecticides. Zobell (1946) described many new species found in Bergey's Manual (Breed et al., 1957) which could utilize either naphthalene, cresol, or phenol, such as Pseudomonas rathonis or P. arvilla, both of which are very similar to the species of pseudomonas in question. The microbial assimilation of phenol in concentrations as high as 0.3% has been demonstrated.

The pseudomonads are usually obligately oxidative and can oxidize a wider range of simple organic compounds than any other known bacteria. Many pseudomonas species, such as P. fluorescens are devoid of accessory growth factor requirements, developing rapidly and abundantly in simple media with a mineral salt base and any one of many potentially oxidizable organic substrates (Fleck, 1965). The organic compounds whose oxidation has been studied thus far are simple substituted aromatic substances such as benzoic acid, phenol, and phenylacetic acid. The technique of simultaneous adaptation rests on two assumptions. One is the specificity of the adaptive response, and the other is that the cell must be permeable to all substrates tested. Kilby (1968) identified B-ketoadipic acid as an excretion product in cultures of vibrio enzymes capable of oxidizing either catechol or

protocatechuic acid to B-ketoadipic acid that can be isolated from dried cells adapted to aromatic precursors.

The development of gas chromatography and the electron capture detector has given researchers powerful tools for pesticide determinations. It is now possible to make qualitative determinations of pesticides in the range of 1-5 micrograms/liter (ppb) (Warnick and Gaufin, 1965).

With the use of thin layer chromatography, it was observed that both parathion and methyl parathion were hydrolyzed to p-nitrophenol, and diethyl and dimethyl-o-thiophosphoric acid, respectively (Stanley, 1964). The thin layer chromatographic procedure described by Graetz et al. (1971) demonstrated a small amount of parathion was present together with a compound having an Rf value of 0.23 relative to parathion. The thin layer chromatographic procedure employed by Graetz et al. (1971) was used in this dissertation.

As agriculture comes to rely more and more on chemistry, the importance of basic and applied research on the use, behavior, fate, and toxicology of these pesticides increase. For example, the disposal of empty metal cans and drums containing any potent pesticide, especially parathion, is a very serious problem. Hot water or caustic washing is not sufficient to rid the container of the pesticide (Stojanovic, 1970). Complete burning, crushing, or burial may be used to dispose of waste containers; however, none of these methods are completely effective.

MATERIALS AND METHODS

Cultures

Isolation and Screening of Microorganisms

Microorganisms indigenous to the Bay St. Louis area of the Gulf Coast, capable of degrading the parathion molecule, were sought by enrichment with parathion prior to the isolation of pure cultures. Isolation of microorganisms by selective enrichment is widely used in microbiology today and involves the stimulation of rapid growth of the desired organisms in a mixed culture by providing the proper nutrients and environmental conditions. Water samples collected from this area have been exposed to parathion as well as other pesticides via agricultural runoff, erosion, and flooding.

Enrichment cultures consisting of mineral salts, parathion (100 ppm), and a water sample were incubated at ambient temperature for various time intervals. Turbid cultures were selected for filter disc assay. Filter discs saturated with parathion in ethanol were placed on nutrient agar plates on which enrichment culture inocula was added. Filter disc assays were also conducted with mineral salts agar and parathion. Zones of growth surrounding parathion-saturated discs were considered evidence that the culture was a parathion utilizer. The purity of each culture was ascertained by streaking of a fresh nutrient agar plate.

Identification of Cultures

The culture was tested for purity by streaking on nutrient agar plates and observing colonial morphology. The cellular morphology was observed by staining the organism with Hucker's modification of the Gram stain. Capsular material was stained by the method of Tyler (1966). Bacterial flagella were stained according to the method of Ladner (1968). All stains were prepared in accordance with the Society of American Bacteriologist's Manual of Microbiological Methods (1957).

All physiological tests employed during the classification of this isolate were conducted in accordance with the Society of American Bacteriologist's Manual of Microbiological Methods (1957).

Chemicals

Parathion and By-Products

The parathion employed in this investigation was an analytical standard of 98.7% purity obtained through the courtesy of American Cyanamid Company, Agricultural Division. Reagent and analytical grade samples of reported parathion by-products were also supplied gratis by The Monsanto Company. These include p-nitrophenol, paraoxon, and p-aminophenol.

Emulsifiers

Atlas detergent 8-916P was incorporated in the mineral salts media as a dispersing agent. This detergent

had no effect on standard anticholinesterase assay according to Vardonis and Crawford (1964).

Hydrogen Concentration

The pH of water samples was determined in a 1:1 sample to distilled water suspension with the glass electrode using a Beckman Zeromatic II pH meter. The pH of the mineral salts medium was determined without dilution.

Aminoparathion Synthesis

Aminoparathion, a potential parathion by-product was not supplied; therefore, it was chemically prepared by reduction of parathion (2 ml of concentrated parathion to 100 ml of 5 N HCl and 5 grams of zinc dust) by the method according to Graetz et al. (1971). The reduction mixture was heated for 15 minutes and made alkaline with sodium hydroxide. The aminoparathion was extracted with 100 ml of benzene. The benezent extract of aminoparathion was employed in thin layer chromatographic analysis according to Graetz et al. (1971).

Cultural Procedures

Culture Techniques for Manometric Studies

All cells used in manometric experiments were grown on either nutrient broth or mineral salts plus parathion in 6 oz prescription bottles at ambient temperature. Fifty ml of the desired medium were added to prescription bottles, autoclaved, and inoculated with 0.5 ml of the culture. All

liquid cultures were incubated on a New Brunswick rotary shaker at approximately 180 rpms for a week. The pH of the medium was 7.0.

The mineral salts medium as described by Brown (1958) contained the following ingredients:

KNO_3	1.0 g/l
$MgSO_4$	0.2 g/l
K_2HPO4	0.4 g/l
$FeCl_3.6H_2O$	0.05 g/l
Distilled H_2O	1.0 liter
10% HCl	0.5 ml/l

The mineral salts medium was modified to buffer against an increased acidic medium by adding 50 g of dibasic potassium phosphate which is equivalent to 1 g/l of phosphate (PO_4) ions.

The pH of the medium was adjusted to pH 7.0 with either 10% (v/v) hydrochloric acid or 10% sodium hydroxide. A precipitate was formed in the resulting medium and the medium was allowed to settle for 24 hours before use. Only the resulting supernatant liquid was used unless otherwise noted.

The medium was dispensed in 50 ml quantities into 6 oz prescription bottles and sterilized. All of the media were autoclaved for 15 minutes at 121 C and 15 pounds per square inch (psi) in a Castle Automatic Autoclave.

Plate Count Procedure

Plate counts were conducted by the spread plate method. Nutrient agar plates were prepared in triplicate. The following dilutions were employed: 10^{-1}, 10^{-3}, 10^{-5}, 10^{-7}, and 10^{-9}. A 0.1 ml inoculum was added to each plate and spread confluently. Plate counts were incubated at 30 C unless otherwise noted.

Resting Cell Studies

Harvest and Preparation of Cells

Cell suspensions grown on nutrient broth, and on mineral salts-parathion medium were poured into 250 ml centrifuge bottles and centrifuged at approximately 8,000 X g for 20 min in a refrigerated International centrifuge model B-20. The cell sediment was washed from the centrifuge bottles with physiological saline (0.85% NaCl). The resulting cell suspension was then washed three times in physiological saline. The cells were suspended in physiological saline for subsequent manometric and Thunberg investigations.

The quantity of cells in the cell suspension was determined by comparing the light transmittance at 525 nanometer (nm) to a standard curve. Turbidometric measurements were accomplished in the following manner: a 1:20 dilution of the cell suspension was prepared and the light transmittance was determined. A standard curve was prepared from the undiluted cell suspension by preparing an array of serial dilutions. The light transmittance was determined

in a Bausch and Lomb Spectronic 20 colorimeter.

Manometric Studies

Manometric studies were conducted in accordance with standard manometric procedures as described by Umbreit et al. (1964). All investigations were carried out in either single or double sidearm flasks containing a total liquid volume of 3.2 ml on a Precision Scientific 18-place Warburg apparatus at 30 C. All ground glass joints were sealed with Dow Corning high vacuum silicone stopcock grease.

The center wells of the flasks contained 0.2 ml of either 10% (w/v) potassium hydroxide (KOH) or water (H_2O). Fluted filter paper was placed in the center well containing KOH. Each Warburg flask contained 1.5 ml of 0.067 M phosphate buffer at pH 7.0 and 1.0 ml of parathion solution at various concentrations in the body section of the flask. The sidearm contained 0.5 ml of the cell suspension. The flasks were placed on the manometers and transferred to the water bath. A ten min period was allowed for temperature equilibration. After this period the manometers were closed and an additional ten min period of equilibration was allowed. The contents of the sidearms were tipped into the main compartment and the initial readings were then taken. All experiments were conducted in duplicate. The consequent change in gas volume was measured at various intervals on the manometers.

Inhibitors

Irreversible inhibitors were used in manometric studies in an attempt to establish the nature of the enzyme system or systems involved in parathion utilization. Nutrient broth grown cells as well as parathion grown cells were inoculated into flasks containing the two substrates, nutrient broth and parathion (solubilized in 1.0% ethanol). Inhibitors of enzyme formation such as 2,4-dinitrophenol (an uncoupling agent) and chloramphenicol (an inhibitor of protein synthesis) were added to the sidearms of double sidearm Warburg flasks in 10 micromole (μM) concentrations. After the systems had equilibrated and a relatively consistent rate of oxygen consumption was noted, the inhibitors were tipped into their respective flasks. Additional control treatments were run in which no inhibitors were added.

Thunberg Techniques

Thunberg experiments were carried out in standard Thunberg tubes according to the procedure of Umbreit et al. (1964). The tubes contained 1 ml of 2.67×10^{-4}M methylene blue, 2 ml of the desired substrate (0.02M), 2 ml of phosphate buffer (0.067M), and 1 ml of cell suspension. The cell suspension was placed in the sidearm of the tubes. All joints were sealed with Dow Corning high vacuum grease. The tubes were evacuated with a Precision vacuum pump and flushed with nitrogen. A five-min period of temperature equilibration

was allowed and the contents were mixed. The reaction was followed on a Bausch and Lomb Spectronic 20 colorimeter at 660 mm. The light transmittance was recorded at periodic intervals for 30 min.

Aeration

The effects of increased carbon dioxide concentration and changes in the chemical composition of the mineral salts medium were determined by growth and pigment production of the culture. A cell suspension was added to six oz prescription bottles containing mineral salts and parathion. Following evacuation of each bottle, a positive pressure of 3 pounds per square inch (psi) of 80% air and 20% CO_2 was added. Growth was monitored by observation of turbidity and by pressure changes. It was impossible to compare turbidometric measurements due to differences in chemical compositions of the media. Seven different mineral salts media were compared under two different atmospheric conditions (air and 20% CO_2-enriched air). The mineral salts formulations (per liter) are presented as follows:

(1) KNO_3 ---------- 10 g
$MgSO_4.7H_2O$ ---------- 0.2 g

(2) Add Fe^{+++}
KNO_3 ---------- 10 g
$MgSO_4.7H_2O$ ---------- 0.2 g
$FeCl_3.6H_2O$ ---------- 0.05 g

(3) Add PO_4 + Fe^{+++}		
KNO_3	10.0	g
$MgSO_4.7H_2O$	0.2	g
K_2HPO_4	0.38	g
$FeCl_3.6H_2O$	0.5	g
(4) Add Fe^{++}		
KNO_3	10.0	g
$MgSO_4.7H_2O$	0.2	g
$FeCl_2.4H_2O$	0.05	g
(5) Add PO_4		
KNO_3	10.0	g
$MgSO_4.7H_2O$	0.2	g
K_2HPO_4	0.38	g
(6) Add PO_4 + Fe^{++}		
KNO_3	10.0	g
$MgSO_4.7H_2O$	0.2	g
$FeCl_2.4H_2O$	1.0	g
K_2HPO_4	0.38	g
(7) K_2HPO_4	0.38	g
$FeCl_3.6H_2O$	0.05	g

Chromatographic Determinations

Gas Chromatographic Analysis

Residual parathion from cultures containing mineral salts and parathion (10 ppm) was extracted with a 1:1 ratio of acetone and hexane, both pesticide quality reagents, and analyzed by electron capture gas chromatography as previously described by Walker (1970).

A Beckman GC-45 gas chromatograph equipped with an electron capture detector was used for the analysis of samples for parathion utilization. A glass column containing SE-30 Chromosorb WH, 80-mesh packing was employed. The column was designed specifically for the analysis of organophosphorus compounds. The carrier gas was helium and the flow rate was set at 40 ml/min. Samples were injected in

1.0 µl quantities. Electron capture helium flow rate was set at 80 ml/min. The carbon dioxide flow rate was peaked generally between 1.2 and 2.2 ml/min.

The electrometer settings were as follows: attenuation was set at 2, input at 1K x 2, suppression at 4, source current at 7, bias voltage at 0, and polarizing voltage at 760. The detector temperature was set at 30 C above column temperature which was usually operated at 200 C for parathion detection. Inlet temperature was adjusted at 50 C above column temperature. The retention time for analytical grade parathion standards was four min at the above mentioned settings. The results were expressed in ppm of parathion and plotted against time in days. Peak height reduction was converted to ppm by plotting a standard curve of peak heights of parathion standards at decreasing concentrations by serial dilution.

Thin Layer Chromatographic Analysis

Glass plates, 20 x 20 cm, precoated with silica gel G adsorbent (without fluorescent indicator) were obtained from Brinkman Instruments, Inc., EM Reagents Division, and used in all thin-layer chromatographic (TLC) analyses.

The pseudomonas species was grown on nutrient broth, centrifuged, and washed with physiological saline as previously described. The culture was inoculated as a 0.1 ml saline suspension at the rate of 2.0×10^8 cells/ml into 50 ml of sterile mineral salts medium containing 100 ug/ml

of parathion (5,000 ug total) and incubated for seven days. One control treatment received no bacterial cells. Another control treatment received no parathion. Following incubation, each sample was filtered, evaporated to dryness, extracted with acetone, and analyzed by thin layer chromatography according to the method of Graetz et al. (1971).

Ethanol solutions of analytical grade parathion and parathion by-products were applied in well-defined spots to thin-layer plates with five µl spotting pipettes. Ethanol and chloroform extracts from several mineral salts and parathion cultures were applied to TLC plates, and a comparison was made. Spotting was done along an imaginary line two cm from the bottom edge of the plate. A portable hair dryer enhanced solvent evaporation with a stream of hot air.

A Desaga Brinkman (DB) developing tank contained the TLC plates and 200 ml of 0.7 percent ethanol in chloroform for development (Graetz et al., 1971). Development was allowed to continue until the solvent front had traveled a distance of 16 cm from the origin. Following development, the thin layer plates were removed from the chamber and allowed to dry in a fume hood. Visual identification of parathion and parathion by-products was achieved by spraying with a palladium chloride solution (0.5 g $PdCl_2$ and 2 ml concentrated HCl in 98 ml distilled water). In order to permanently fix the visualized spots, the developed plates were then exposed to bromine fumes for approximately 30 sec

by placing in a DB developing tank containing 100 ml of bromine reagent (5% v/v bromine in carbon tetrachloride). A specially designed glass stand was employed to support the plate above the liquid bromine reagent. The plates were then allowed to stand in a fume hood for 2-3 min. Following drying, the plates were sprayed with fluorescein (0.25% w/v fluorescein in N-N dimethylformamide diluted 1:50 with ethanol). The plates were allowed to dry and sprayed with silver nitrate (1 ml of a 10% w/v aqueous $AgNO_3$ solution mixed with 10 ml of 2-phenoxy-ethanol and diluted to 200 ml with acetone), according to the method of Kadoum (1970). The plates were observed by exposure to ultraviolet light in a chromato-vue UV light cabinet (Ultra-Violet Products, Inc.). The spots representing parathion appeared brown to yellow against a brown background. Parathion by-products such as p-nitrophenol and phenol appeared white against a brown background.

Chemical Analyses

Pigment Extraction

Water insoluble pigments were extracted from the culture medium by a chloroform fraction method according to Graetz et al. (1971). Culture media (50 ml) containing the water-insoluble pigment was added to a 125 ml separatory funnel to which was added 25 ml of chloroform. After shaking vigorously the aqueous layer was eluted, leaving the pigment-chloroform fraction. The aqueous layer was

collected and employed in p-nitrophenol and phenol analyses.

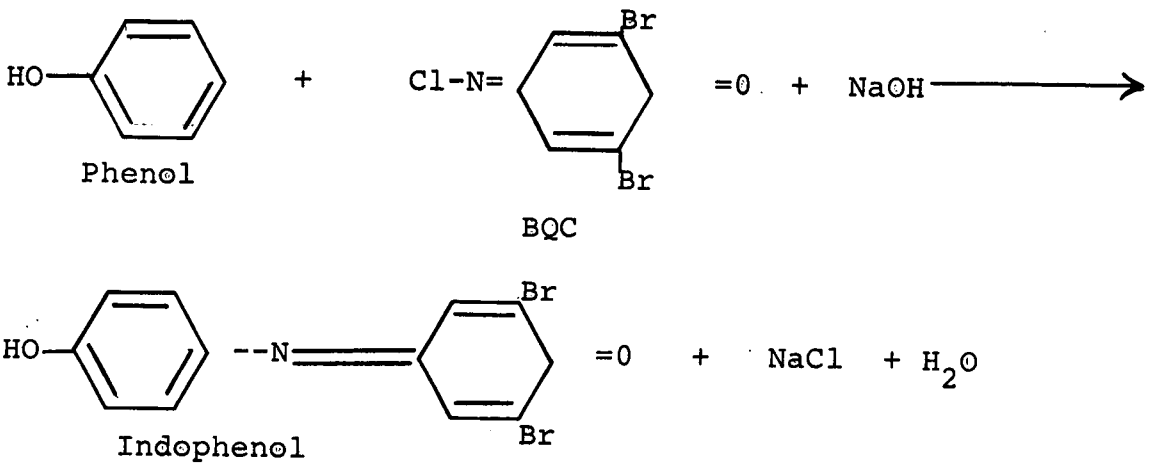
P-nitrophenol Analysis

P-nitrophenol analysis was conducted in accordance with the procedure described in the Monsanto Technical Bulletin (1972). A standard curve was prepared by weighing 0.1 gram of analytical grade p-nitrophenol and preparing a wide spectrum of dilutions. The sample was diluted with 1 N alcoholic potassium hydroxide and water. The color was measured at 400 nm wavelength and the results were plotted as micrograms of p-nitrophenol per ml versus optical density.

Free p-nitrophenol in the sample was determined by adding 1.0 ml of sample to a 100 ml volumetric flask. Alcoholic potassium hydroxide (50 ml) was added to the flask, stoppered and shaken for 30 min. The alcohol was diluted to the mark, mixed, and filtered. A 5 ml portion of the filtrate was added to a 250 ml beaker and evaporated with a gentle stream of air. The moist residue was redissolved in 25 ml of ether and transferred to a separatory funnel. The beaker was washed with two additional portions of ether and added to the funnel. The ether solution was extracted with four 20 ml portions of chilled 1% sodium carbonate solutions. The combined aqueous layers were collected into a 250 ml volumetric flask and 25 ml of 1 N alcoholic alkali solution was added. The solution was diluted to the mark with distilled water and the resulting color was measured.

Phenol Determination

Ten milliliters of the solution to be tested for free phenol were extracted by the chloroform fraction method mentioned previously. The extraction procedure was necessary to eliminate distorted readings on the colorimeter due to interference from the yellowish-green pigment secreted by the bacterium. The aqueous phenol layer was pipetted into a colorimeter tube. Five drops of 0.4 percent alcoholic solution of 2,6-dibromo-N-chloro-p-quinoneimine (BQC) were added, the tube mixed on a Vortex mixer, and then incubated for 15 min at 37 C to allow for maximum color development. The density of the blue color which developed was read with the colorimeter at 660 nm wavelength (Brown, 1958). The amount of free phenol present was calculated by comparing the reading thus obtained with a standard curve which had been prepared with known quantities of free phenol. The reaction involved is presented as follows:



A standard curve for phenol determination was prepared at pH 7.0, the pH at which the culture medium was buffered. One gram of phenol was weighed into a one hundred ml volumetric flask and made up to a volume with the buffered medium. This stock solution was diluted such that solutions containing 10 concentrations were obtained and 10 points were used to plot a standard curve. Phenol concentrations ranging from 0.002 mg/ml to 0.8 mg/ml of phenol were used. The contents of each flask were mixed with five drops of a 0.4 percent alcoholic solution of BQC in a colorimeter tube and incubated at 37 C for 15 min. The density of the blue color was then determined with a Bausch and Lomb Spectronic 20 colorimeter using the red filter and set at 660 nm. The readings obtained were plotted in optical density units against the phenol concentration used. The portion of the curve which was linear was used in determining the concentration of phenol in the test solutions.

Protein Determination

A modification of the Lowry method (Cook, 1965) was employed for the hydrolysis of bacterial cells. A sample (5 ml) of cell suspension was added to a test tube to which 5 ml of reagent A was added (40 g Na_2CO_3 dissolved in a liter of 2N NaOH). The cell suspension was heated in a boiling water bath for 10 minutes. Following removal, 5 ml of reagent B (0.2 g $CuSO_4$ in a 1 liter flask of water containing 0.4 g of sodium tartrate) was added. It was

allowed to stand at room temperature for 10 min after which 0.1 ml of Folin-Ciocalteau phenol reagent was added. After standing for 30 min at room temperature the hydrolyzed cell suspension was read at 655 nm.

A standard curve of bovine serum albumen was prepared employing the following concentration gradient: 40, 80, 120, 160, 200, 240, 280, 320, and 1000 mg/ml. The standard curve was expressed in terms of optical density versus micrograms of protein. Micrograms of protein in test samples were extrapolated from the linear portion of the standard curve.

Biochemical Determination of Capsule Composition

An attempt was made to obtain some estimation of the chemical composition of an ethanol-treated fraction of a pseudomonas cell suspension. The ultimate objective in this undertaking was to gain some insight into the chemical composition of the cell wall or capsular material in order to offer some satisfactory explanation to the permeability problem and the resultant solvent effects.

A series of qualitative biochemical tests were employed for the detection of simple and complex carbohydrates, lipids, and proteins in culture media containing the test organism. All of the biochemical tests were employed as described by Wilson (1969). The qualitative tests for the detection of simple carbohydrates were the Benedicts test, Seliwanoff's test, Benzidine reaction, and the Molisch

test. The analysis for complex carbohydrates was the Lugol's polysaccharide test. The analysis for the detection of fatty acids were the Fatty Acids and Glycerol test, the Unsaturation test, and the Acrolein test. The analysis for the detection of proteins, proteoses, and peptones was the modified Lowry (Cook, 1965) and the Biuret test.

Two groups of cell suspensions prepared from cells grown in mineral salts and parathion, were subjected to the qualitative tests. One group was treated by incubating in the presence of 1.0% ethanol under shake conditions at ambient temperature for five hours, whereas, the other group was not subjected to alcohol treatment. Both groups of cells were subsequently centrifuged for five minutes at 2500 X g. Cells and medium were tested from both groups of cells.

RESULTS AND DISCUSSION

The primary objectives of this dissertation are presented as follows: (1) to isolate, by enrichment techniques, one or more bacteria, capable of readily metabolizing the parathion molecules as the sole carbon source, (2) to study the physiological parameters of parathion utilization, (3) to determine some of the intermediates in the degradation of parathion by microorganisms.

Parathion Utilizing Microorganisms

Isolation

A bacterium was isolated from the Gulf Coast region near Bay St. Louis which exhibited the capability of metabolizing parathion according to enrichment culture data, filter disc assays, and manometric results previously described in the Methods section.

Preliminary Screening of Microorganisms for Parathion Utilization

Resting cell suspensions were prepared from cells grown on nutrient broth with parathion (10 ppm). A wide spectrum of water sample isolates was screened for the ability to consume oxygen in the presence of parathion as the substrate. Four organisms were selected which

demonstrated high activity in terms of oxygen consumption at 100 ppm concentration of parathion.

Parathion is only slightly miscible with water (24 ppm); therefore, a carrier solvent is required. Acetone and ethanol (1.0% concentration) were tested as potential parathion solvents. The results are shown in Figure 2. The organisms designated as PC and JP_2 were determined to be the same organism. Every organism tested demonstrated higher oxygen consumption with parathion solubilized in ethanol. Further, parathion solubilized in acetone exerted an inhibitory effect upon all of the organisms tested. It may also be noted that no oxygen consumption occurred with the ethanol control.

Morphological Characteristics

The organism selected for use in the subsequent experiments was a gram-negative, non-sporeforming, motile coccobacillus (0.5 micron by 1.0 micron). The bacterium was capsulated and monotrichously flagellated. At 18-24 hours cells were characterized as rod-shaped; however, after 30-36 hours they appeared to be coccobacilli. The cells, in general, exhibit no clustering and typically display single arrangement. The photographs presented in Figure 3 demonstrate the typical cellular morphology, presence of capsular material, and the presence of flagella, respectively.

Culture Characteristics

Turbidity was noted in cultures containing mineral salts, 10 ppm parathion, and the test organism after three days incubation under aerobic conditions (reciprocal shaking) at ambient temperature. Yellow-green pigmentation was produced only under the aforementioned conditions. Further, no pigmentation was noted when cells were grown in an enriched medium, such as nutrient broth. Cultures exhibited marked turbidity with a thick pellicle in stationary liquid cultures. Nutrient agar colonies were circular, convex, smooth, and thick with an irregular margin. The organism grows well at 25 - 42 C. However, the optimum temperature for growth is 30 C. The optimal pH for growth on mineral salts and parathion is 7.0-7.2. Further, acidic cultural conditions inhibit growth as well as parathion degradation.

Physiological Characteristics

The bacterium is motile, reduces nitrates to nitrites, hydrolyzes gelatin, and can utilize citrate as the sole carbon source. Indole was not produced and starch was not hydrolyzed. The litmus milk reaction was alkaline and hydrogen sulfide was not produced. No growth was noted on mineral salts alone. The following reactions were noted with the sugars during a 24-hour period of incubation.

Physiological Test	Result
glucose	Acid
sucrose	No acid
fructose	No acid
lactose	Acid*
maltose	No acid
mannitol	No acid

*Acid after 48 hours

Classification

On the basis of morphological and physiological characteristics, the bacterium has been classified as a species of the genus Pseudomonas in the family Pseudomonadaceae, according to Bergey's Manual of Determinative Bacteriology (Breed et al., 1957). The bacterium was tentatively identified as Pseudomonas aeruginosa. It may also be noted that according to Breed et al. (1957), this bacterium attacks naphthalene and other hydrocarbons.

Parathion Utilization

In an attempt to correlate manometric data with other parameters of parathion utilization, gas chromatographic analysis of incubated cultures was performed. The effect of light exposure during prolonged incubation (Cook et al., 1957) was also determined in order to establish if photochemical decomposition was taking place. Mineral salts cultures incubated in dark conditions were compared to those

exposed to normal room lighting conditions. A standard curve was prepared by injecting a series of parathion standards of decreasing concentrations. The values presented in Figure 4 are expressed in ppm parathion concentration as a function of time in days. A three-week period was monitored. Samples were collected daily and extracted for gas chromatographic analysis. According to the conditions of the test, normal room lighting conditions does not contribute significantly to parathion decomposition. It was also apparent that, following 21 days of incubation, approximately ninety percent of the original parathion had been degraded.

Effect of Solvents on Parathion Utilization

It may be observed in Figure 5 that a solvent was required in order for parathion to serve as a substrate for microbial attack. In Figure 2 it was noted that ethanol appeared to be a better solvent than acetone in terms of oxygen consumption. This same phenomenon was also apparent in the filter disc assays employed in the isolation of the bacterium. In order to provide some explanation why ethanol was a better solvent than acetone, an experiment was designed in which the cells were grown on nutrient broth, washed four times, and incubated in 1% ethanol for five hours under shake conditions at ambient temperature. The results are shown in Figure 6. Ethanol pre-incubated cells assayed on concentrated parathion demonstrated the highest activity in terms

of oxygen consumption, whereas, cells which were not incubated in ethanol did not exhibit this ability. Preincubated cells in ethanol utilized concentrated parathion and 1000 ppm parathion in ethanol at roughly the same rate. Preincubation in acetone resulted in a decrease in oxygen consumption. The experiment was repeated and the results were consistent with previous findings. It was apparent from Figure 6 that ethanol preincubation affects the permeability of the cells to parathion in some way. Perhaps, ethanol treatment results in denaturation of the cell wall or capsular component, or stripping off a layer that is susceptible to the effects of ethanol.

Additional experiments were conducted to describe further the mode of action of ethanol with reference to its effect upon the bacterial cell, especially the capsule. The first experiment was designed to determine the effect of varying ethanol concentrations on cells grown in the presence of parathion in nutrient broth. In the second experiment the cells were grown on parathion in mineral salts. The following concentrations of ethanol were added to two different substrates, parathion and nutrient broth: 0.1%, 1.0%, 2.0% and 3.0%. The results were shown in Figures 7 and 8.

The optimum concentration of ethanol was 1.0%, followed by 0.1, 2.0 and 3.0%, respectively, when the cells were assayed on parathion and grown on parathion. The optimum concentration of ethanol was 0.1% followed by 1.0%, 2.0%

and 3.0% when assayed on parathion and grown on nutrient broth.

The enzyme involved in parathion degradation appeared to be constitutive since the same response was noted on parathion grown cells and nutrient broth grown cells. The results given herein rule out the use of sequential induction studies according to Stanier (1950). In each experiment 2.0% and 3.0% ethanol concentrations were considered inhibitory. The experiment was repeated and the results were consistently the same.

It was hypothesized earlier that the pseudomonad capsule was stripped of some important layer, thus rendering the bacterial cell more permeable. However, the bacterial cell was still susceptible to denaturation or decomposition or at least, a loss of enzyme activity by 2.0% or 3.0% ethanol concentration which is characteristic of most bacteria reported in the literature (Villeneuve and Phillips, 1970).

The effect of the solvent, ethanol, on the permeability of the cell wall or capsule was investigated further.

A series of biochemical qualitative tests were employed in order to determine the composition of the layer affected by ethanol (Wilson, 1969). The results are presented in Table 3.

Reducing sugars, ketones, pentoses, or hexoses were detected in the ethanol-treated cells and medium which were not present in the untreated cells suspensions.

Polysaccharides were detected in both treated and untreated cells. No fatty acids were detected in either group. Proteins, proteoses, and peptones were prevalent in both groups of cells. The data presented in Table 3 suggest that ethanol affects the capsule or cell wall in some way.

Effects of Cultural Conditions on Growth

Effect of Temperature

The effect of temperature was measured as an expression of p-nitrophenol concentration. p-Nitrophenol production from parathion was measured at a series of temperatures in order to determine the optimum temperature for parathion utilization. The highest p-nitrophenol production in terms of parts per million was 30 C followed by 25 C, 35 C, 42 C, and 37 C as seen in Figure 9. p-Nitrophenol formation increased sharply on the fourth and fifth day of incubation which generally supports the data described in the next experiment. Cells grown at 42 C exhibited higher activity than expected and the shape of the curve was different from cells grown at lower temperatures. This phenomenon can be partially explained since some parathion hydrolysis to p-nitrophenol takes place above 40 C.

In order to verify further that parathion utilization takes place, the effect of temperature on cell population was determined by the plate count method. The results are illustrated in Figure 10. The increase in

cell concentration parallels the rate of p-nitrophenol production demonstrated in the previous experiment.

Effect of pH

An experiment was designed to compare the effects of pH on parathion utilization by Warburg respirometry. Manometric analysis was conducted on cell suspensions of samples collected daily. A 5.0 µm parathion concentration was employed. Following three hours incubation the highest activity occurred at pH 7.0 followed by 7.5, 8.0, 8.5, 6.5, and 6.0, respectively. The results are shown in Figure 11.

Daily samples were also collected, extracted and analyzed colorimetrically for p-nitrophenol concentrations. The results are presented in Figure 12. p-Nitrophenol concentration as a function of time in days is consistent with the findings demonstrated in Figure 11.

Aeration

In previous experiments it was discovered that maximum growth and color production was obtained when cultures were well aerated. In order to investigate the relationship of these findings to the amount of aeration, cultures were incubated at room temperature by three aeration schemes: static, shake, and static with a regulated air flow. A regulated air flow of 3,000 ml/min was allowed to bubble through mineral salts and parathion (10 ppm). The pH of the mineral salts medium was 7.0. Parathion was solubilized

in ethanol or suspended in an emulsifier. Growth and color was measured turbidometrically using a Bausch and Lomb Spectronic 20 set at 535 nm.

The emulsifier did not significantly affect the growth rate in terms of turbidity under the three test conditions. Ethanol was required in order for adequate growth to occur under the three test conditions. However, ethanol was not used as the substrate for growth since no turbidity was noted when ethanol was added as the sole carbon source. Static conditions with a regulated air flow stimulated maximum observable turbidity. It was not possible to measure color by the colorimetric method. However, visual observation was noted and the findings are consistent with turbidometric data.

It was apparent that enhanced aeration increased growth and pigment production. Therefore, attempts were made to determine if increased carbon dioxide concentration increased growth and pigment production. The effect of changes in the chemical composition of the mineral salts medium was also determined.

The results are illustrated in Table 4. It was apparent that the maximum growth in terms of observable turbidity was found in the cultures containing carbon dioxide-enriched atmospheres. No pressure changes were noted. The addition of dibasic potassium phosphate and ferric iron (Fe+++) enhanced growth in both atmospheres; air, and carbon dioxide-enriched air (80% air-20% CO_2).

The growth of the culture in terms of observable turbidity was negligible when no phosphate was added to the medium. These results coincide with earlier attempts to demonstrate growth with a poorly buffered medium containing parathion. In this instance the pH became very acidic (pH 3.0) and no growth or pigment production was noted.

Chemical Transformation during Growth Pigment Production

The yellow-green pigment was produced when the culture was incubated in mineral salts plus parathion. The presence of the pigment interfered with p-nitrophenol determinations. In order to distinguish between pigment production and p-nitrophenol concentration an extraction procedure described by Graetz et al. (1971) was employed. Yellow-green color production described previously was produced when the pseudomonas species was incubated in mineral salts and parathion for four days or more. Pseudomonas aeruginosa produces a pycocyanine pigment which is a yellow-green color. It is also pH sensitive in the same range as p-nitrophenol, a postulated parathion metabolite. In order to distinguish pycocyanine pigment production and p-nitrophenol production an extraction procedure employing chloroform was used. Pycocyanine separated out in the chloroform fraction, whereas p-nitrophenol was eluted in the aqueous layer. After pigment extraction, the aqueous layer of a culture

containing parathion was still yellow-green in color indicating that p-nitrophenol was present.

p-Nitrophenol Detection

The p-nitrophenol extraction procedure was described in the Methods section. p-Nitrophenol detection was expressed in ppm of p-nitrophenol concentration as a function of time in days. In order to convert p-nitrophenol concentration from optical density units to ppm values, a p-nitrophenol standard curve was necessary. p-Nitrophenol concentration in ppm was extrapolated from the linear portion of the standard curve. An increase in p-nitrophenol concentration appeared as a function of time in days. A sharp increase in p-nitrophenol concentration was noted from day four to day five of the six-day growth studies.

Manometric Studies

In an attempt to understand the completeness of parathion oxidation of this bacterium, the respiratory quotient (R.Q.) values of cells grown on nutrient broth and parathion (10 ppm) were determined. These results are illustrated in Figure 13. The R.Q. values illustrate the amount of carbon dioxide produced compared to the amount of oxygen consumed. The R.Q. values of approximately 0.60 were 80% of the theoretical R.Q. for parathion. R.Q. values of cells grown on mineral salts and parathion are presented in Figure 14. The R.Q. values herein are comparable to those

in which the cells were grown on nutrient broth and parathion.

A comparison of Q_{O_2} values was made of cells grown on mineral salts-parathion medium compared to nutrient broth-parathion medium. Several postulated metabolic intermediates were selected from a review of the literature to serve as substrates in a manometric study. Cells were grown on nutrient broth and parathion as well as mineral salts and parathion, and assayed on various test compounds. The results are expressed in terms of Q_{O_2} values. The Q_{O_2} values are an expression of the rate of oxygen uptake. In this case, it was µg of oxygen consumed per mg of protein per hour. Q_{O_2} values for cells grown on nutrient broth and parathion are illustrated in Table 5. Nutrient broth grown cells exhibited lower Q_{O_2} values than cells grown on nutrient broth and test compound. Further, no apparent difference in Q_{O_2} values of isomers of the test compounds was noted.

Cells were grown on mineral salts and test compounds and the results are presented in Table 6. The results in the two tables are comparable with the exception that the values were decreased for all test compounds when the cells were grown on mineral salts and parathion.

The results presented in Figure 15 demonstrate the optimum substrate concentration. Ethanol (1.0%) was employed to solubilize an array of parathion concentrations. The following concentrations of parathion were added as substrate in order to determine the optimum substrate

concentration: 1.0, 2.0, 3.0, and 5.0 μM/ml. It was observed that 5.0 μM/ml of parathion in 1.0% ehtanol exhibited the highest activity in terms of oxygen consumption. In additional experiments 10, 25, and 59 μM/ml concentrations were inhibitory. The theoretical R.Q. for parathion is 0.67 upon complete oxidation. In Figure 15 it was observed that the average R.Q. was 85% of the theoretical R.Q.

It was observed in Figure 16 the average R.Q. was again approximately 85% of the theoretical R.Q. for parathion. However, in this instance, resting cells were grown on nutrient broth and assayed on p-nitrophenol. It may also be noted that p-nitrophenol solubilized in water does not exhibit the high activity characteristic of its ethanol counterpart. On the other hand, phenol solubilized in water demonstrated the same type of curve as p-nitrophenol in ethanol, and the same approximate degree of oxygen consumption and CO_2 production. These results are illustrated in Figure 17. p-Nitrophenol is not utilized to the same degree with or without ethanol. These results were also supported by Thunberg data to be described later. An explanation for this phenomenon may be that the nitro (NO_2) group on parathion and p-nitrophenol affects the permeability of these compounds.

Thunberg Investigations

It has been stated previously by Graetz et al. (1971) that there are generally different enzyme systems involved in the metabolism of organophosphorus pesticides. Previous evidence has supported the fact that this is probably the case with this organism. The enzyme system involved in parathion degradation may be a mixed function oxidase system. In order to ascertain the general types of enzymes involved in parathion utilization, studies were conducted employing classical Thunberg techniques.

The results are presented in Table 7. The bacterial cell suspension was diluted until no reduction was noted within the time interval measured when tested in the absence of exogenous substrate. The Thunberg data corresponded very closely with the results of analyses previously described. However, it must be noted that, when no reduction occurs in Thunberg data it does not rule out the possibility that reduction could occur when different hydrogen accepters were employed. Pseudomonas cells in the presence of parathion, paraoxon, p-nitrophenol, and p-aminophenol were only reduced when ethanol was employed as the carrier solvent. However, only phenol was oxidized when no solvent was employed. The rate of oxidation was greater when ethanol was not added. No reduction occurred when ethanol and cells were allowed to react with methylene blue. Therefore, the solvent did not initiate the reduction reaction noted with the above mentioned substrates. Reduction occurred

when the emulsifier was added to a reaction mixture containing cells, parathion and ethanol. However, no reduction occurred when ethanol was deleted.

Oxidation occurred more rapidly with the additon of nutrient broth to a reaction mixture containing parathion solubilized in ethanol and cells. The reduction time was the same for reaction mixtures containing nutrient broth and cells with and without ethanol. The reduction time for phenol and cells was greater than the reduction time for any combination of reaction mixtures with the exception of nutrient broth and cells with and without ethanol. Assuming that the reduction time is a valid criterion for the rate of the reaction, then reduction occurs more quickly with cells assayed on phenol than other compounds tested. The results presented herein confirm earlier manometric data in which phenol demonstrated the same high activity as its ethanol-solubilized counterpart.

It was apparent that ethanol was required in order for parathion degradation to occur. The mode of action of ethanol in parathion utilization does not appear to be due solely to its role as a solvent. Various solvents have been employed such as acetone, ethyl acetate, and hexane in an attempt to duplicate the success of ethanol. A wide array of emulsifiers specifically formulated for parathion distribution have been employed. However, all attempts to date have failed. In view of this, it is postulated that ethanol denatures or strips some barrier affecting parathion,

p-aminophenol, and p-nitrophenol permeability. Phenol apparently was not affected by postulated impermeable layer since reduction readily occurred without the aid of ethanol. In addition, since the only difference in the proposed parathion metabolites is the presence of a nitro (NO_2) group, it is a logical deduction that the nitro group affects the permeability of these compounds. However, this phenomenon may be related to the presence of hydroxyl groups on ethanol and phenol molecules.

Inhibitors

Although resting cells of the organism grown in the absence of parathion could consume oxygen rapidly, it does not necessarily follow that the same metabolic pathway is being employed when the organism was grown without parathion as the sole carbon source. Therefore, studies were undertaken to establish the nature of the enzyme system with the use of irreversible inhibitors in manometric studies.

The results observed in Figures 18 and 19 substantiate the previous conclusion that the enzymes apparently were not adaptive. No difference was noted in the oxidative rates. It may also be noted that no enzyme inhibition was observed with chloramphenicol. Chloramphenicol is an inhibitor of protein synthesis; hence, the synthesis of new protein for enzyme formation would be disrupted. Constitutive enzymes are the product of the biosynthesis of normal cell constituents, and it is an inherent part of the cell

prior to exposure to the substrate for which the enzyme catalyzes a reaction. However, adaptive enzymes are synthesized only following exposure to its specific substrate. Therefore, chloramphenicol would inhibit the formation of inducible or adaptive enzymes. This proposed explanation is valid only when the assumption is made that oxygen consumption is a true expression of the rate of enzyme activity. Another assumption must be made regarding interpretation of the effect of chloramphenicol upon the metabolism of parathion. It must be assumed that permeability factors involving the capsule or cell wall are not influencing the inhibitor studies.

Figures 20 and 21 do not indicate any difference in the oxidative rate of the reactions. 2,4-dinitrophenol is an uncoupling agent for oxidative phosphorylation. Therefore, an inflection in the curve was expected. However, 2,4-dinitrophenol possesses a similar molecular configuration and, to some degree, similar chemical reactivity as p-nitrophenol which is a proposed metabolite in the postulated pathway of parathion degradation. It is not inconceivable that 2,4-dinitrophenol might be attacked by a nonspecific, constitutive enzyme system present in this culture.

Comprehensive Correlations of Results

In view of previous descriptions of experiments designed to determine the type and extent of parathion utilization, an attempt was made to relate all of the

findings described previously. Plate count data, p-nitrophenol analysis, phenol analysis, and parathion analysis were compared in order to determine the interrelationship between these parameters. The analyses also demonstrated the degree of correlation among the types of data.

Mineral salts and parathion (10 ppm) inoculated with the pseudomonas cell suspension was incubated under static conditions with a regulated flow of air at ambient temperature for eight days. Daily samples were withdrawn and each of the aforementioned analyses were performed. The results may be observed in Figure 22. All of the previously described parameters for monitoring parathion degradation exhibited the same trends and followed the same type of curve. This bacterium enters the logarithmic phase of growth after the fourth day of incubation. An inflection in the curve for p-nitrophenol, phenol, and gas chromatographic analysis paralleled the plate count growth curve on day four. The growth curve entered the stationary or death phase on day five with subsequent changes in the curve for the other parameters of parathion utilization. On the eighth day of incubation, the cell population declined, and p-nitrophenol and phenol equilibrated with a subsequent loss of 66% of the original parathion concentration. Data expressed in the four different types of analysis for parathion utilization compared very closely to a change in cell population and support the proposed pathway presented in Figure 28.

Physiological Age

Concurrent with the previously mentioned chemical analyses of parathion utilization by this culture, Warburg respirometric studies of the physiological activity as a function of the age of the culture was studied. Manometric studies were conducted according to the procedures outlined in the methods section. The substrates were parathion and p-nitrophenol in 5.0 μm concentration.

The results of the eight-day growth curve in terms of manometric data are presented in Figures 23 through 27. The peak activity as a function of oxygen consumption and carbon dioxide production is consistent with the data obtained by other methods of analysis. However, the decline in activity begins on day six, whereas, the decline was noted on day five by other methods of analysis. It may also be noted that an increase in activity was observed on day four which is consistent with other data collected for the four parameters of parathion utilization previously described. No withdrawals were made on day one or two since the cells were in the two-day lag phase in the growth curve.

The average R.Q. value for each substrate closely coincides with the trends noted in the growth curve data. In each instance, with the exception of day three, the average R.Q. values exhibited the same trend as the plate count data in the growth curve. Again, the decline in R.Q. values occurred after day six. In each case the average

R.Q. value for p-nitrophenol was higher than parathion. The same trend is reflected in the rate of oxygen consumption with the two substrates. Day three was the only exception to this trend. However, this exception may be due to the lag phase in the growth curve. Pseudomonas cells, young in terms of physiological age, may metabolize parathion more readily than p-nitrophenol; whereas, the opposite effects occur with older cell cultures. The data presented above further substantiate the proposed pathway presented in Figure 28.

Identification of Parathion Metabolites

Previous investigations mentioned in this dissertation have indirectly supported the proposed pathway presented in Figure 28. However, direct evidence for the pathway of parathion degradation was still uncertain. Therefore, thin layer chromatographic analyses were conducted in an effort to identify postulated intermediates in the microbial degradation of parathion.

Table 8 demonstrates the corresponding Rf values for samples as well as standards (parathion and its metabolites). A spot corresponding to the solvent (ethanol) was observed in all of the samples in which ethanol was employed as solvent. The spot was probably due to an impurity since ethanol is relatively volatile and would likely be lost before visualization. A standard sample of phenol

solubilized in ethanol produced two spots, one corresponding to ethanol and the other to phenol.

In the parathion-mineral salts medium inoculated with pseudomonad culture, five well-defined spots were present. Each of these spots correspond to one of the following standards: parathion and paraoxon, p-nitrophenol, p-aminophenol, phenol, and ethanol.

In the control with no cells two spots were found that correspond to ethanol and parathion. The control containing cells but no parathion did not show any evidence of a spot. Extracted cultures in which p-aminophenol was the sole substrate solubilized in ethanol, demonstrated four spots with Rf values corresponding to p-aminophenol, p-nitrophenol, phenol, and ethanol. When p-nitrophenol was employed as substrate, extracted samples exhibited the same four identical spots. It can be seen that the Rf value for the metabolite standards match the Rf values for the parathion degradation products. Spots corresponding to p-aminophenol, p-nitrophenol, and phenol were observed only in the presence of bacterial cells, and were regarded as true metabolites of parathion. Parathion and paraoxon seemingly occupy the same spot on the thin layer plate since they exhibit the same Rf values. The control containing no parathion was compared to the test treatment. No spots were detected on the thin-layer plate indicating that the four spots observed in the presence of cells and parathion represented

metabolites derived from the breakdown of parathion and not derived from compounds routinely produced by the bacterial cells alone.

It is possible that other metabolites exist which cannot be resolved by the system of development or visualization that was utilized here. The Rf values for parathion and metabolite standards presented in Table 8 represent analytical grade parathion, paraoxon, p-nitrophenol, p-aminophenol, and phenol.

The data presented in Table 8 strongly suggest that parathion is metabolized *in vitro* by the culture via the proposed pathway shown in Figure 28. The thin layer data support previous data suggesting that p-nitrophenol, phenol, and perhaps p-aminophenol lie in the pathway of parathion degradation.

SUMMARY AND CONCLUSIONS

A parathion-utilizing bacterium, previously isolated from a water sample, was characterized and classified tentatively as Pseudomonas aeruginosa. A pH of 7.0-7.5 and a temperature of 30 C were found to be optimum for the growth of bacteria and utilization of parathion. Resting cell suspensions demonstrated virtually no oxygen consumption when nonsolubilized parathion was employed. Parathion solubilized in ethanol, on the other hand, exhibited a high rate of oxygen consumption. The permeability problem was obviated by the use of ethanol as the solvent or preincubation of cells in ethanol.

Resting cell studies indicated that approximately 80% of the parathion consumed by resting cells was present terminally as carbon dioxide.

Results of qualitative biochemical tests on ethanol treated and untreated cell suspensions suggested that either cell wall or capsular material was stripped off by ethanol treatment. The optimum concentration of ethanol was 1.0% and enzyme activity was lost at 2.0% and 3.0%. The nature of the enzyme system was revealed to be constitutive. Increased aeration or increased carbon dioxide levels

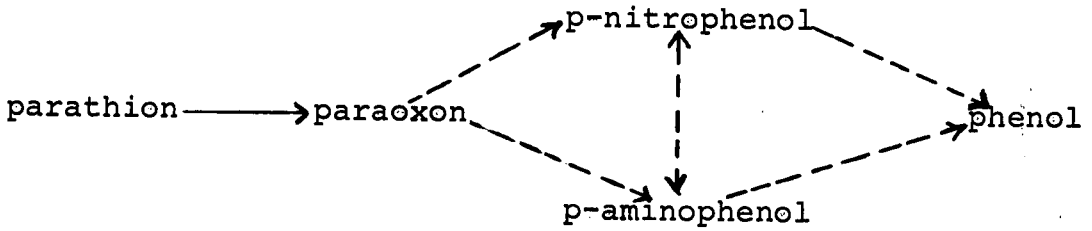
enhanced growth of parathion containing cultures in terms of turbidity and pigment production.

Gas chromatographic analysis revealed that ninety percent of the parathion residue in a culture containing parathion and mineral salts had disappeared after 21 days incubation. At the same time, chemical analysis for the detection of p-nitrophenol and phenol revealed that as parathion residue decreased, p-nitrophenol and phenol concentrations increased.

Turbidometric measurements indicated that the water sample isolate exhibited growth on parathion, paraoxon, p-nitrophenol, p-aminophenol, and phenol. Resting cell studies also indicate that these substrates were attacked by the Pseudomonas.

Thunberg investigations generally are in agreement with data obtained by other analytical methods. Thunberg studies revealed that an oxygenase was responsible for a portion of the attack on parathion and its metabolites by this pseudomonad. Further, the same permeability problems were encountered with the exception of phenol which was oxidized with or without the solvent, ethanol.

Plate counts, turbidometric measurements, chemical analyses, such as p-nitrophenol, phenol determination, and phosphate determinations, manometric studies, and Thunberg investigations suggest that the proposed pathway for parathion degradation is as follows:



Resting cell studies indicated that phenol was attacked terminally with the formation of carbon dioxide and water.

Parathion metabolites were tentatively identified by thin layer chromatography. Close agreement exists between the identified metabolites and the proposed scheme for parathion degradation.

ABSTRACT

William L. Gibson, Doctor of Philosophy, 1972

Major: Microbiology, Department of Microbiology

Title of Dissertation: Microbial Degradation of Parathion

Directed by: Dr. Lewis R. Brown, Professor of Microbiology

Pages in Dissertation: 113. Words in Abstract: 307.

ABSTRACT

An organism capable of utilizing parathion as the sole carbon and energy source was isolated by enrichment culture techniques. The bacterium was characterized and tentatively classified as Pseudomonas aeruginosa. A pH of 7.0 - 7.5 and temperature of 30 C were found to be optimum for the consumption of parathion. Virtually no oxygen utilization was observed with resting cell suspensions when nonsolubilized parathion was employed. The use of ethanol as solvent for parathion in resting cell studies or preincubation of cells in ethanol obviated this problem and rapid parathion oxidation was demonstrable. Approximately 80% of the parathion consumed by resting cells was present terminally as carbon dioxide.

Permeability of the cell to parathion or its metabolites was contingent upon the use of ethanol as either

solvent or denaturant. Interpretation of the biochemical analysis of ethanol-treated cells indicate that either cell wall or capsule components are stripped off as a result of ethanol treatment.

The nature of the enzyme system in parathion utilization was found to be constitutive. Enhanced parathion utilization was noted when grown in a carbon dioxide-enriched atmosphere. The isolate was capable of growth on parathion, paraoxon, p-nitrophenol, p-aminophenol, and phenol. Resting cell studies indicated that these substrates were attacked by this species of the genus pseudomonas. p-Nitrophenol and phenol determinations on extracted culture media containing parathion as the sole carbon source indicated that these metabolites lie in the proposed pathway of parathion degradation.

All of the analytical methods employed suggest that the proposed pathway for parathion degradation was via parathion--- paraoxon--- p-nitrophenol and/or p-aminophenol --- phenol. Resting cell studies indicated that phenol was attacked terminally with the formation of carbon dioxide and water.

Thunberg investigations indicated that an oxygenase was responsible for a portion of the attack on the parathion molecule by this pseudomonad.

Metabolites were tentatively identified by thin layer chromatography. Thin layer chromatographic data generally supported the growing evidence for the proposed pathway of parathion metabolism.

APPENDIX

Preceding page blank

Table 1. Relative Toxicities of Several Organophosphorus Insecticides.

Compound	LD_{50}*	Compound	LD_{50}*
Schradan	1.5	Azodrin	21
TEPP	2.0	Bidrin	22
Thimet	2.0	Trithion	30
Systox	2.5	Dursban	135
Parathion	3.0	Diazinon	150
Phosdrin	6.8	Naled	430
Methyl parathion	9.0	Ronnel	1250
Guthion	10.0	Malathion	1375

*In mg/kg as tested against white rats.

Table 2. Threshold Odor Concentrations of Pesticides in Water.

Compound Pesticides	Threshold Odor Concentration (ppm)
Parathion (technical grade)	0.003
Parathion (pure)	0.036
Endrin	0.009
Lindane	0.33

Table 3. Effect of Ethanol on the Composition of Cells of the Pseudomonas Isolate.

Test	Purpose	Cells	Medium	Ethanol Extracted Cells	Medium
Benedicts	Reducing sugars	-	-	-	+
Seliwanoff's	Ketones	-	-	+	+
Benzidine	Pentoses (glycosidic bonds)	-	-	-	+
Polysaccharide	Polysaccharides	+	-	+	+
Molisch	Pentoses and hexoses	-	+	+	+
Unsaturation	Unsaturated fatty acids	-	-	-	-
Acrolein	Acrolein	-	-	-	-
Biuret	Proteoses and peptones	pink	-	pink	pink
Modified Lowry	Proteins	+	-	+	+

+Positive reaction

-Negative reaction

pink-indicates the presence of proteoses and peptones.

Table 4. Effect of Carbon Dioxide and the Chemical Composition of the Mineral Salts Medium on Growth.

Medium	Growth	
	Air	80% air-20% CO_2
* (1) No additions	+	+
* (2) Added Fe^{+++}	-	-
* (3) Added PO_4 + Fe^{+++}	+++	+++
* (4) Added Fe^{++}	-	++
* (5) Added PO_4	+	++
* (6) Added PO_4 + Fe^{++}	+	++
(7) Added PO_4 + Fe^{+++}	+	++

-No turbidity

+Slight turbidity

++Increased turbidity

+++Heavy turbidity due to bacterial growth

*Contains KNO_3 and $MgSO_4.7H_20$.

Table 5. Q_{O_2} Values for Cells Grown on Nutrient Broth plus Parathion, and Nutrient Broth plus Other Test Compounds.

	Q_{O_2} Units					
	Test Compounds					
Growth Medium	Parathion	Paraoxon	P-Nitrophenol	P-Nitrophenol	P-Aminophenol	P-Aminophenol
NB + parathion	278	266	172	458	252	256
NB + paraoxon	358	458	420	458	492	420
NB + p-nitrophenol	402	439	400	648	457	395
NB + p-aminophenol	312	344	392	340	392	392
NB	60	80	80	0	82	34

Table 6. Q_{O_2} Values for Cells Grown on Mineral Salts plus Parathion and Mineral Salts Plus Other Test Compounds.

	Q_{O_2} Units				
	Test Compounds				
Growth Medium	Parathion	Paraoxon	p-Nitrophenol	p-Aminophenol	Methyl Parathion
MS + parathion	167	147	155	149	137
MS + paraoxon	183	244	156	210	173
MS + p-nitrophenol	145	130	176	198	168
MS + p-aminophenol	291	290	288	302	283
NB	60	80	0	36	54

Table 7. Thunberg Measurement of Reduction Potentials by Resting Cells of the *Pseudomonas* Species.

Sample	Time Required for Methylene Blue Reduction Time in Min
Cell control	*
Parathion + ethanol + cells	21
Parathion + ethanol	*
Parathion + cells	*
Ethanol + cells	*
Ethanol	*
Paraoxon + ethanol + cells	15
Paraoxon + ethanol	*
Paraoxon + cells	*
p-Nitrophenol + ethanol + cells	15
p-Nitrophenol + ethanol	*
p-Nitrophenol + cells	*
p-Aminophenol + ethanol + cells	15
p-Aminophenol + ethanol	*
p-Aminophenol + cells	*
Parathion + emulsifier + ethanol + cells	18
Parathion + emulsifier + cells	*
Emulsifier + ethanol + cells	*
Emulsifier + cells	*
Emulsifier	*
Nutrient broth + parathion (ethanol) + cells	9
Nutrient broth + ethanol + cells	12
Nutrient broth + cells	12
Nutrient broth	*
Phenol + ethanol + cells	24
Phenol + ethanol	*
Phenol + cells	12

*No reduction.

Table 8. Identification of Products Resulting from Microbial Degradation of Parathion.

Growth Medium	Rf Values: Ethanol Impurity 0.96	Phenol 0.46	p-Nitrophenol 0.24	p-Aminophenol 0.18	Paraoxon 0.32	Parathion 0.34	Amino Parathion 0.38
Phenol (ETOH)	0.96	0.46	*	*	*	*	*
p-Nitrophenol (ETOH) + cells	0.95	0.47	0.25	0.19	*	*	*
p-Aminophenol (ETOH) + cells	0.95	0.44	0.27	0.20	*	*	*
Paraoxon (ETOH) + cells	0.93	0.45	0.22	0.17	0.31	*	*
Parathion (ETOH) + cells	0.95	0.46	0.22	0.20	0.33	0.33	*
Ethanol + cells	0.96	*	*	*	*	*	*

*No observable spots.

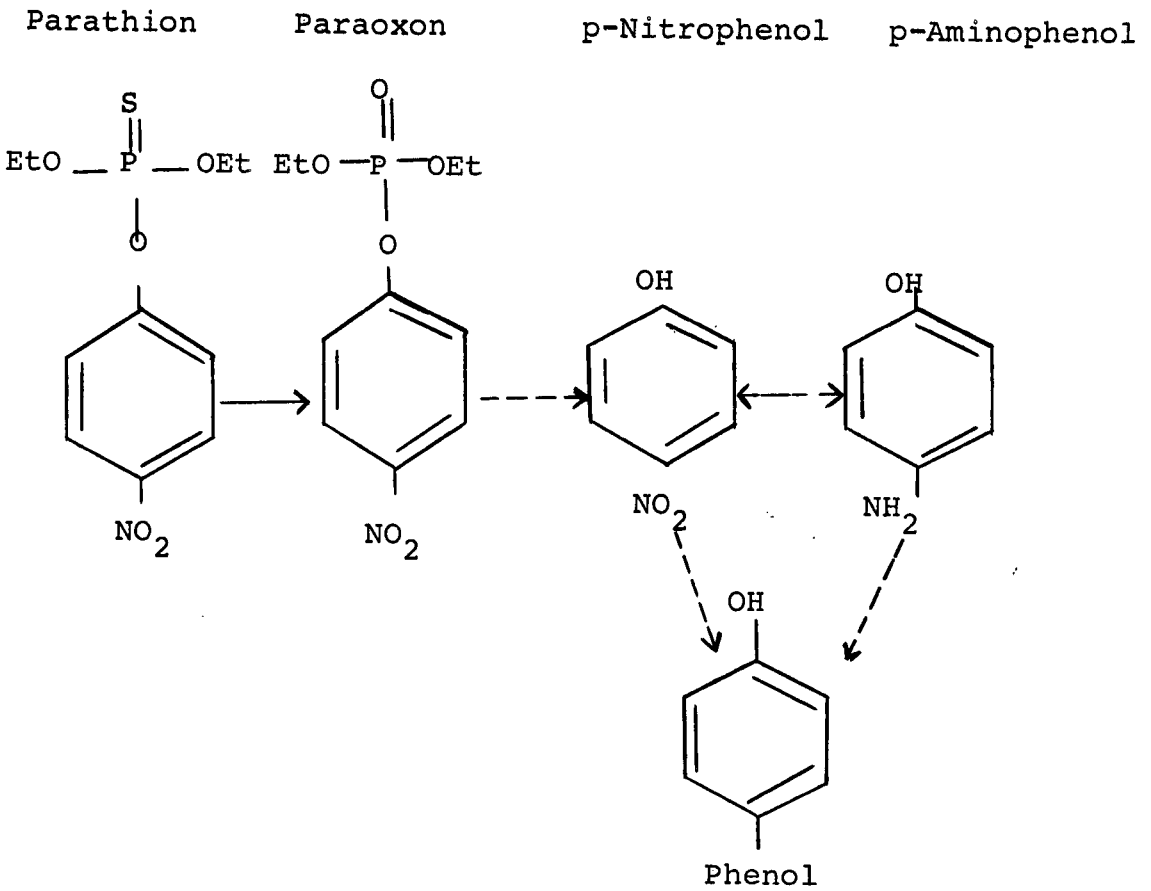


Figure 1. Structure of parathion and parathion metabolites.

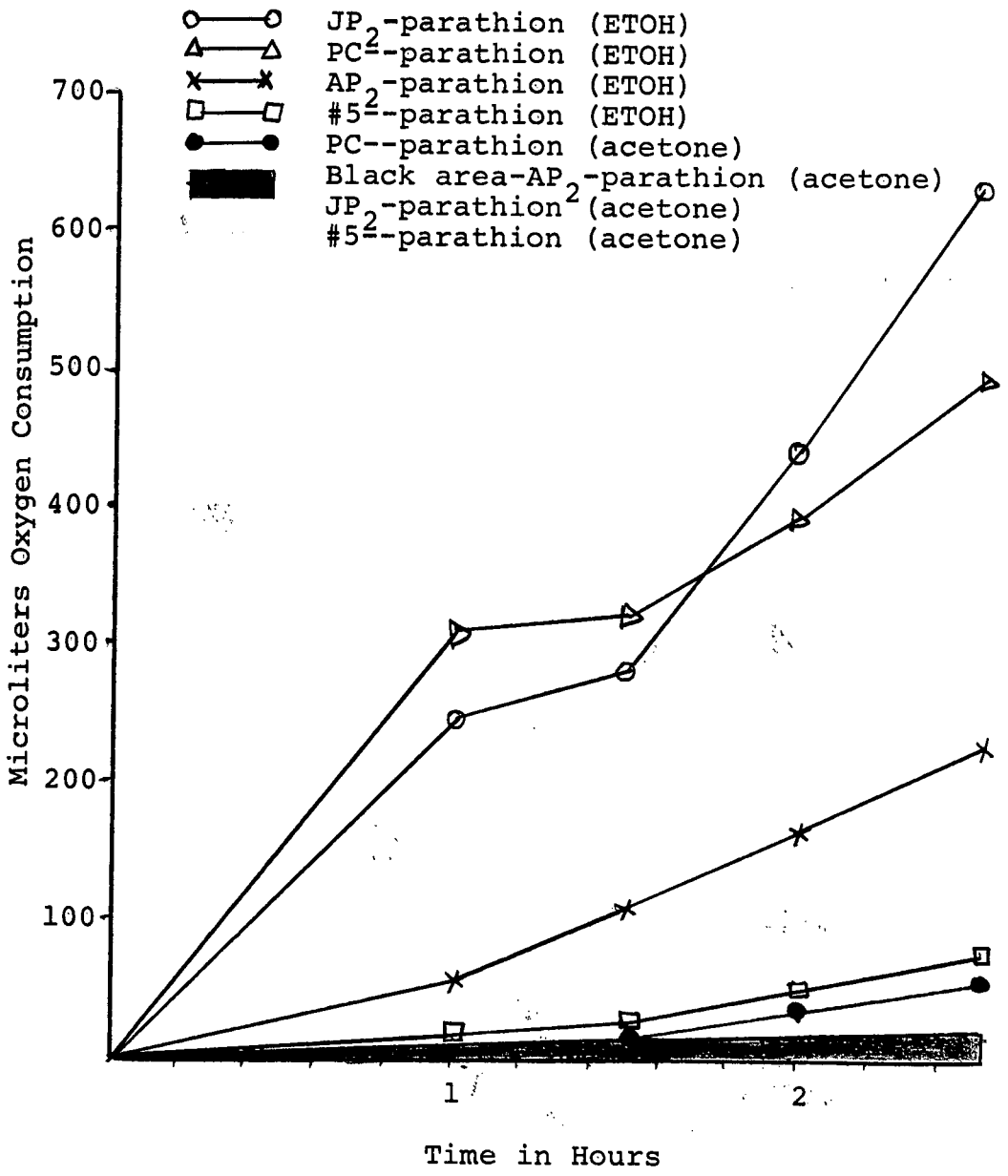
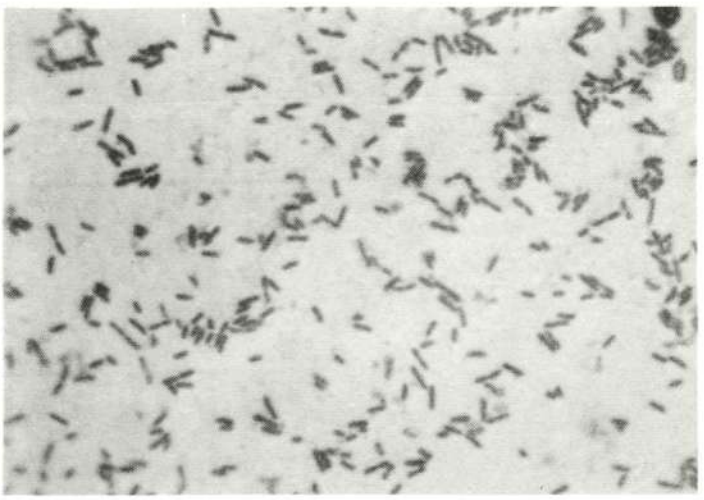
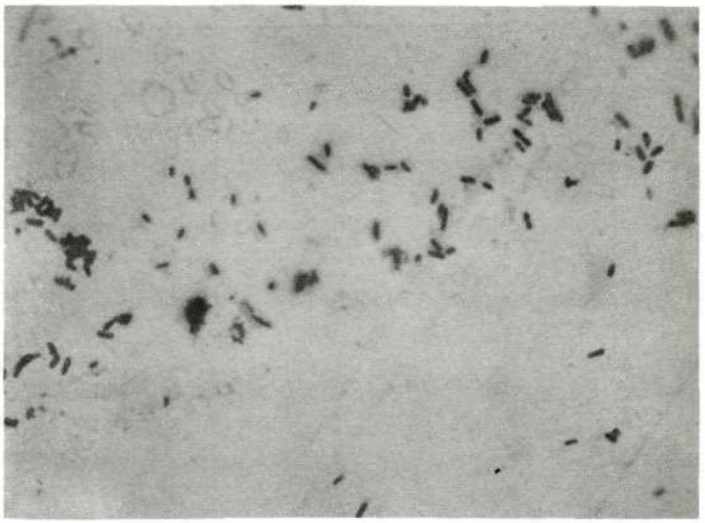


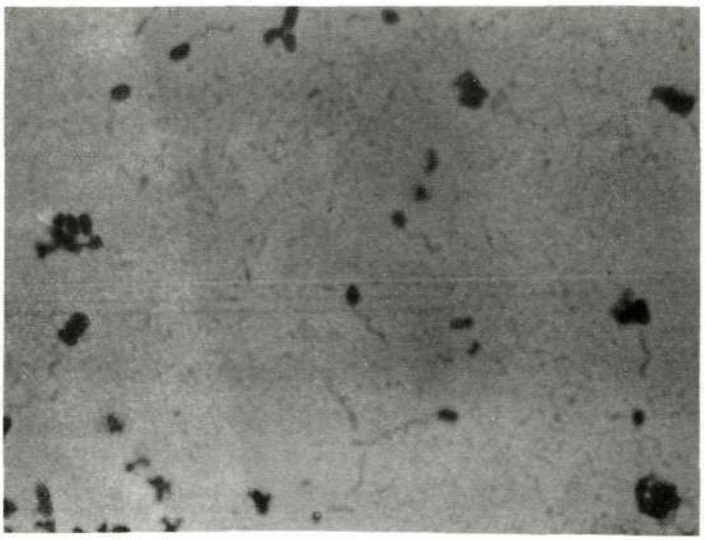
Figure 2. Preliminary screening of microorganisms for parathion utilization.



Typical cellular morphology(1500X) (crystal violet)



Pseudomonas capsule(1500X) (capsule stain)



Pseudomonas flagella(1500X) (flagella stain)

Figure 3. Photomicrographs of the cellular morphology of the Pseudomonas isolate.

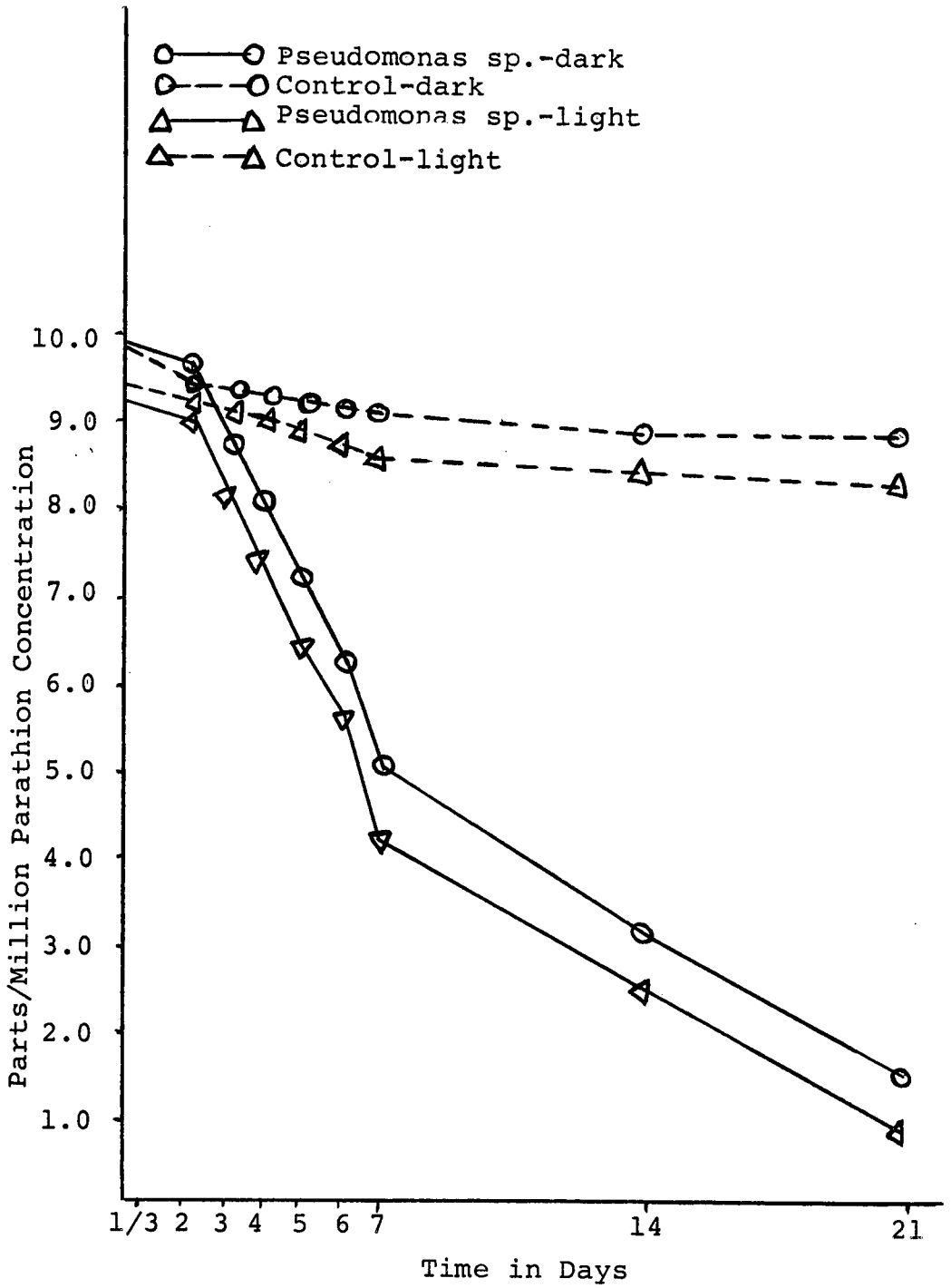


Figure 4. Changes in parathion concentration during the growth of the pseudomonad under both light and dark conditions.

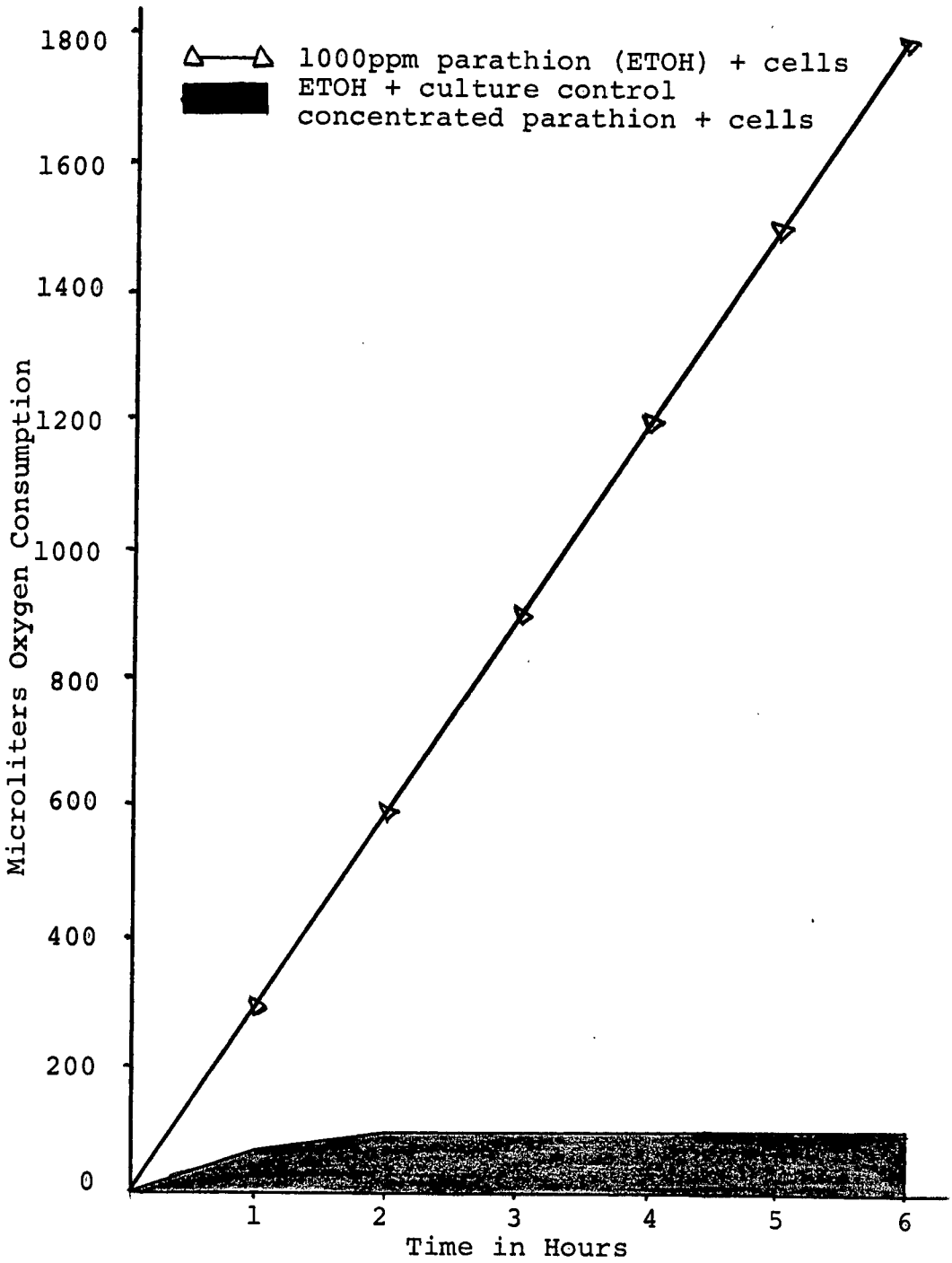


Figure 5. The effects of ethanol on oxygen consumption by the pseudomonad.

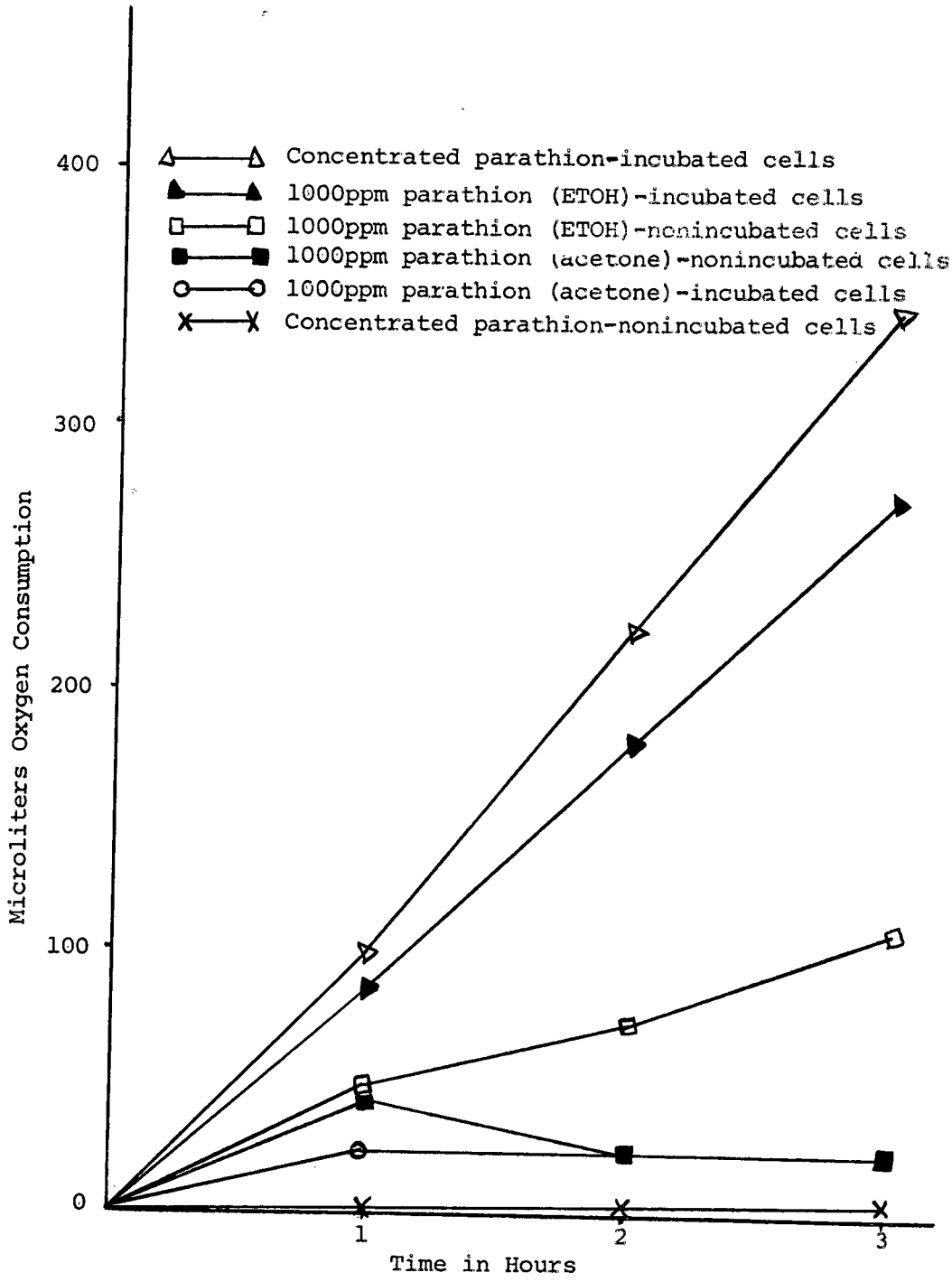


Figure 6. The effect of pre-incubation of cells in ethanol on oxygen consumption by the pseudomonad.

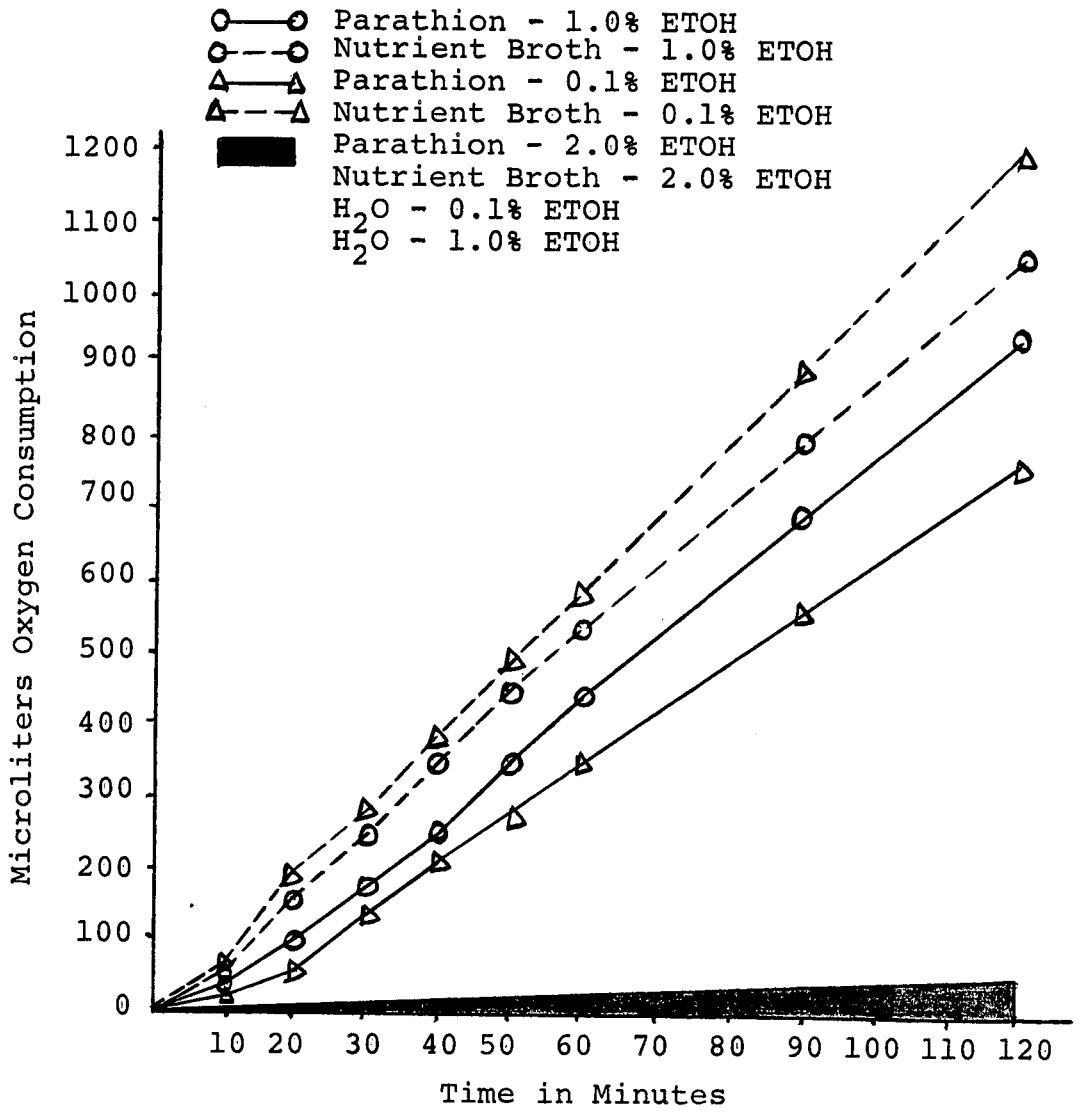


Figure 7. The effect of ethanol concentration on parathion utilization by resting cell suspensions grown on nutrient broth.

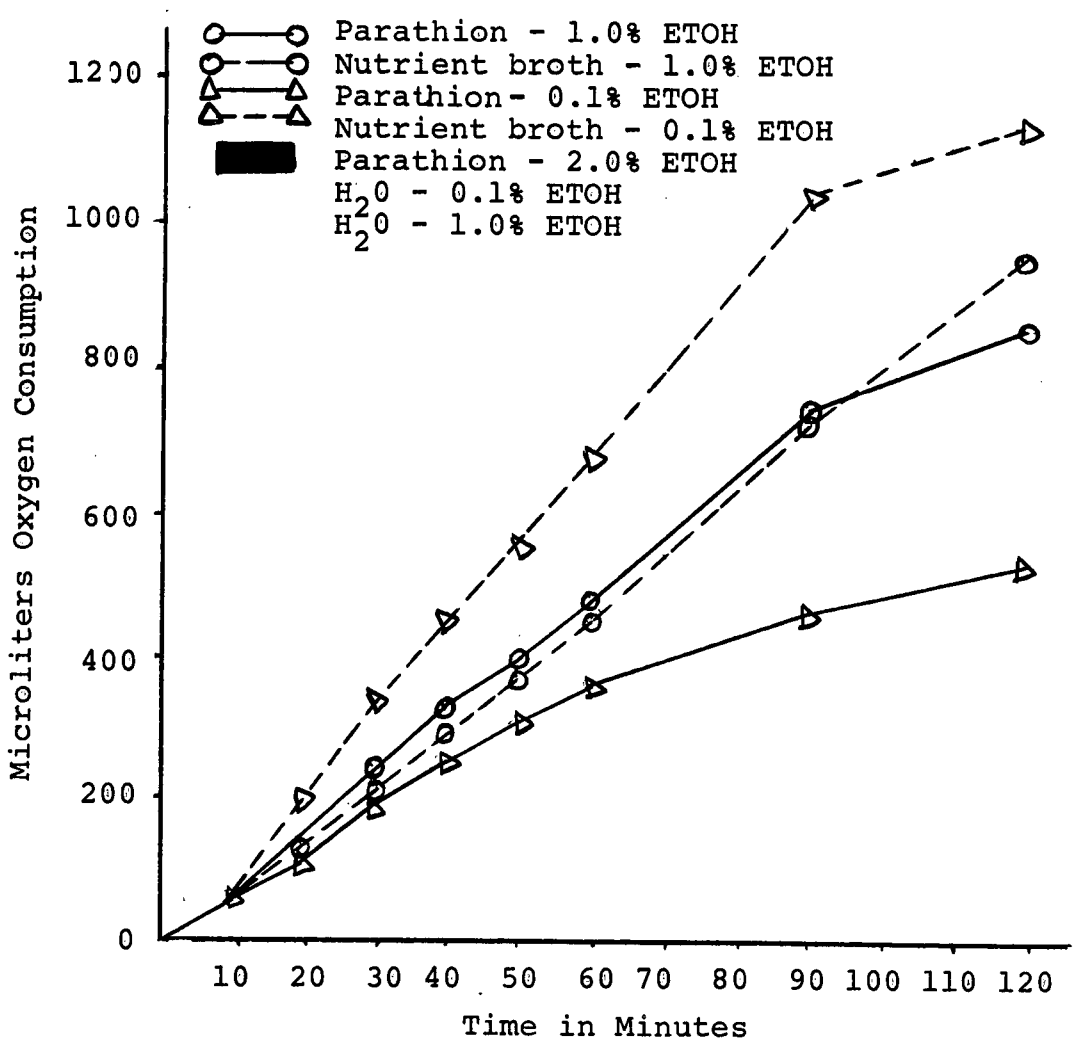


Figure 8. The effect of ethanol concentration on parathion utilization by resting cell suspensions grown on mineral salts and parathion.

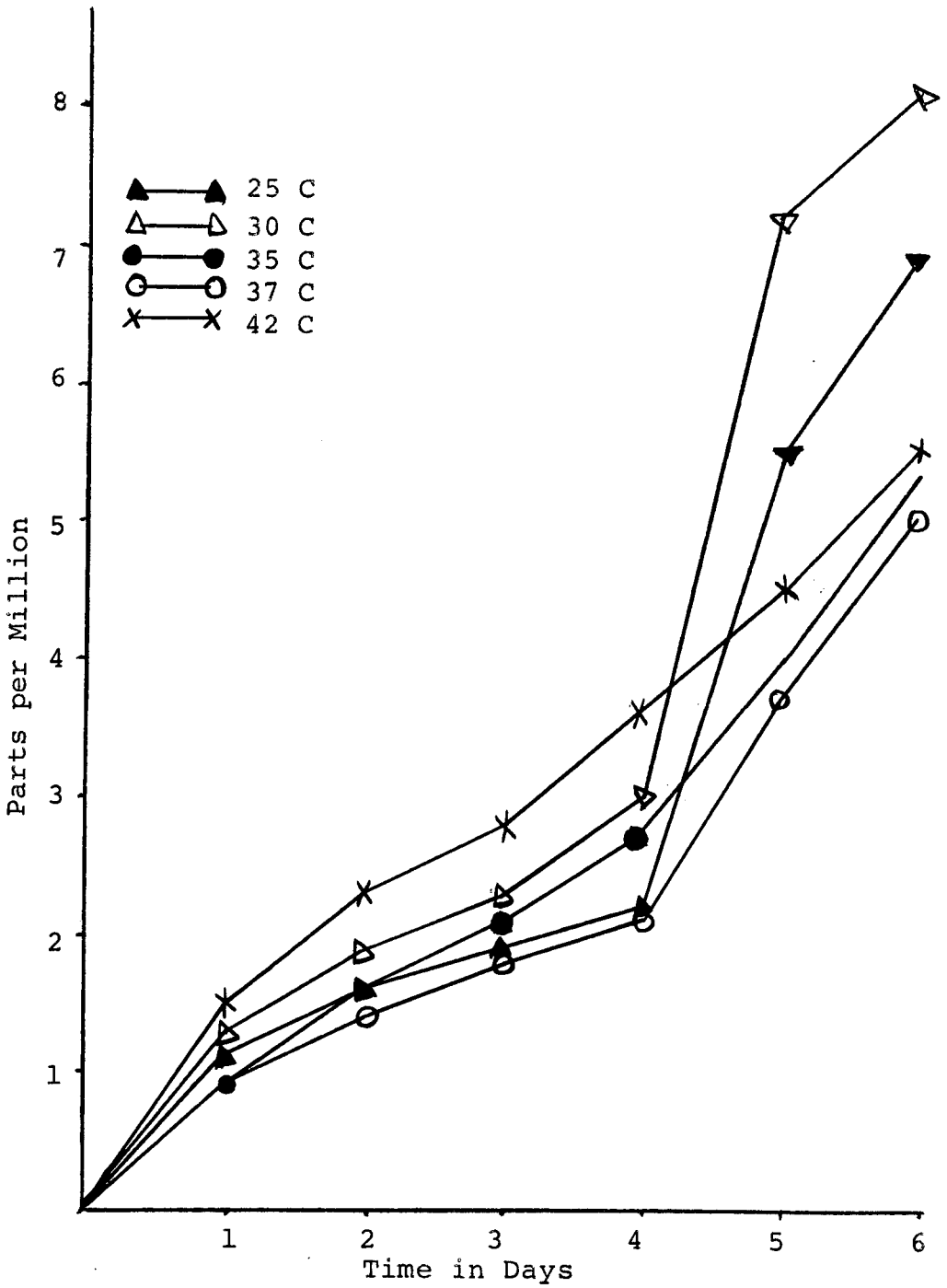


Figure 9. The effect of temperature on p-nitrophenol production on parathion utilization.

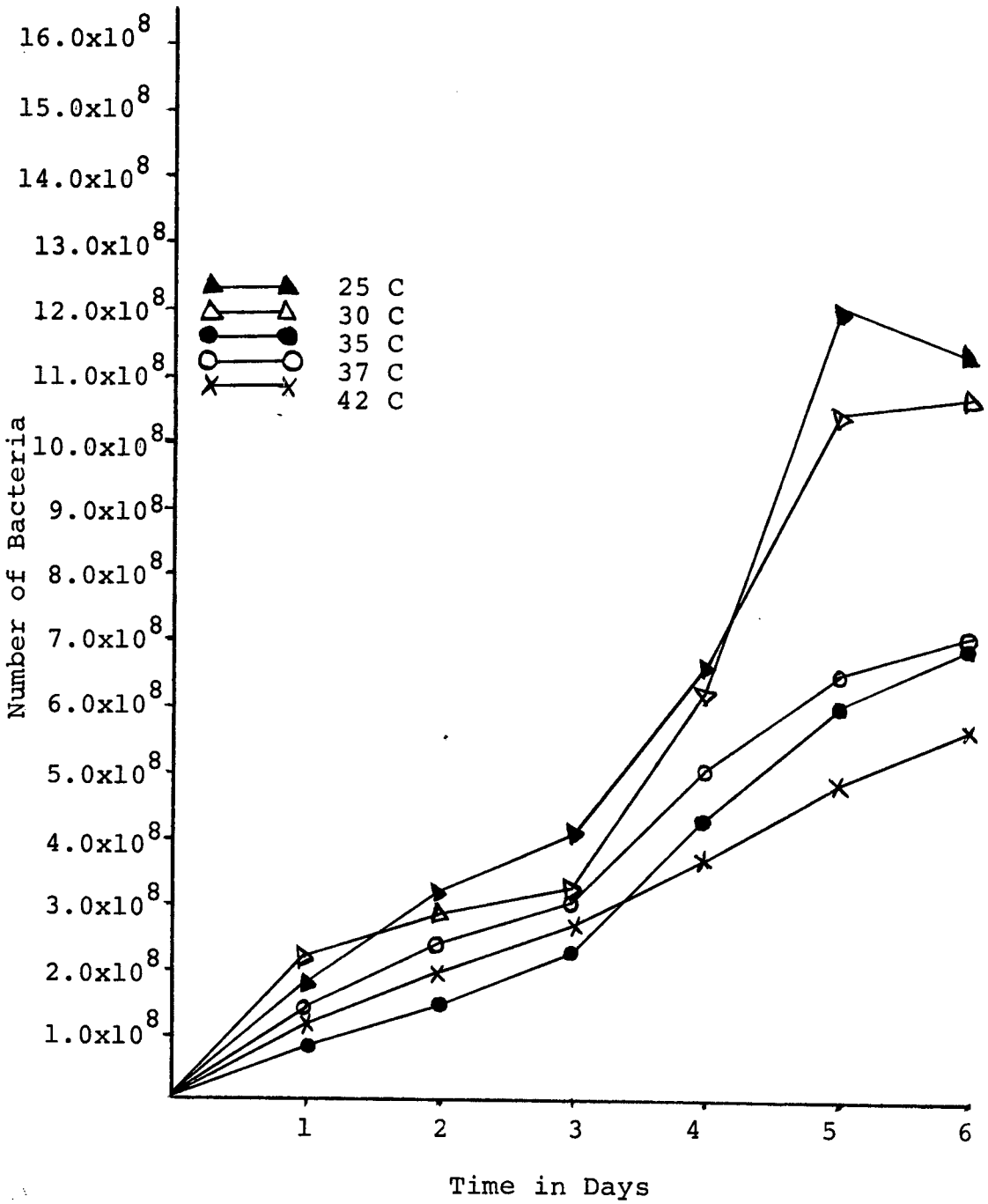


Figure 10. The effect of temperature on the growth of the pseudomonad in the presence of parathion as the carbon source.

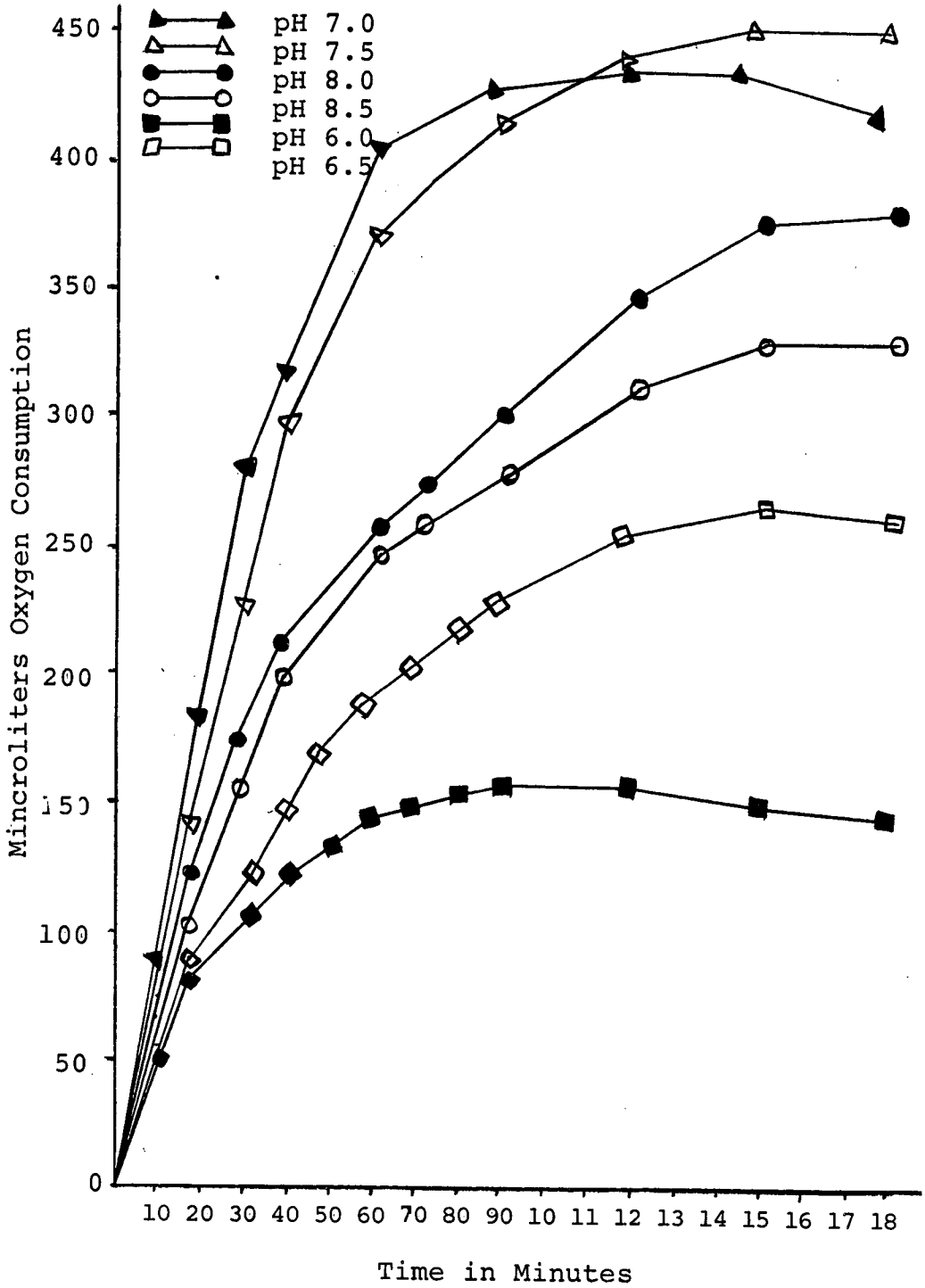


Figure 11. The effect of pH on the oxidation of parathion by resting cell suspensions.

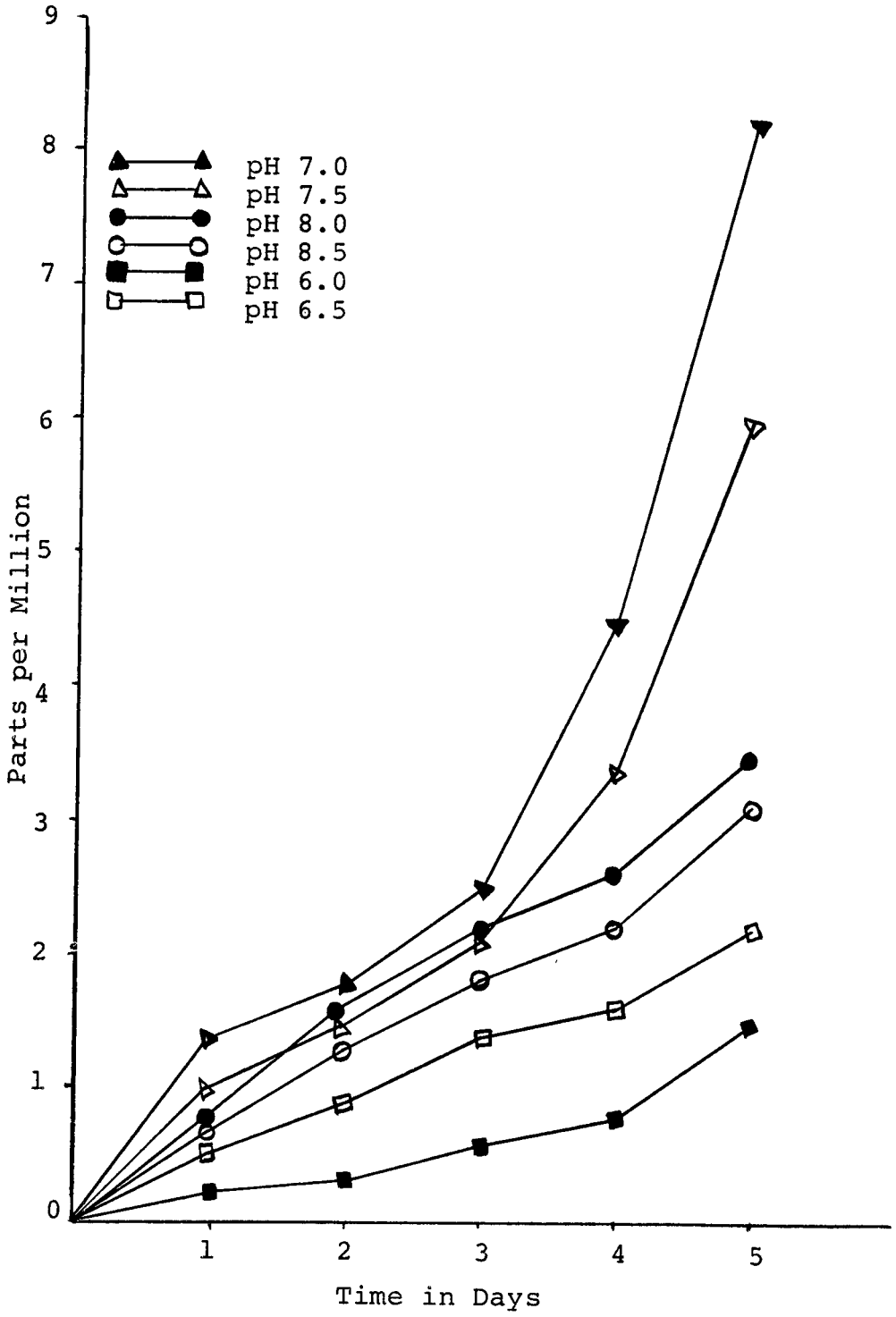


Figure 12. The effect of pH on the production of p-nitrophenol from parathion.

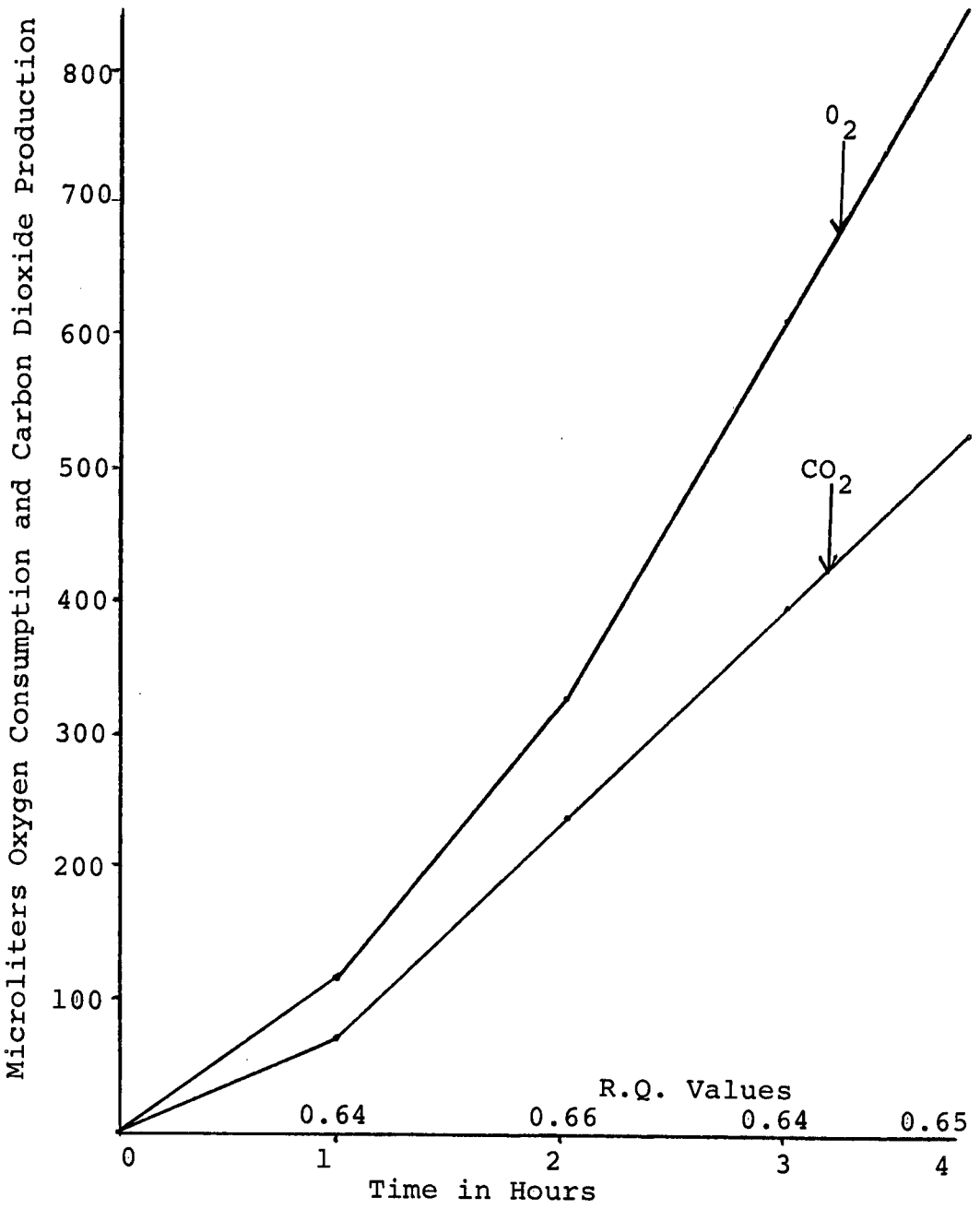


Figure 13. Oxygen consumption and respiratory quotients for resting cell suspensions grown on nutrient broth and parathion (10 ppm) and assayed on parathion.

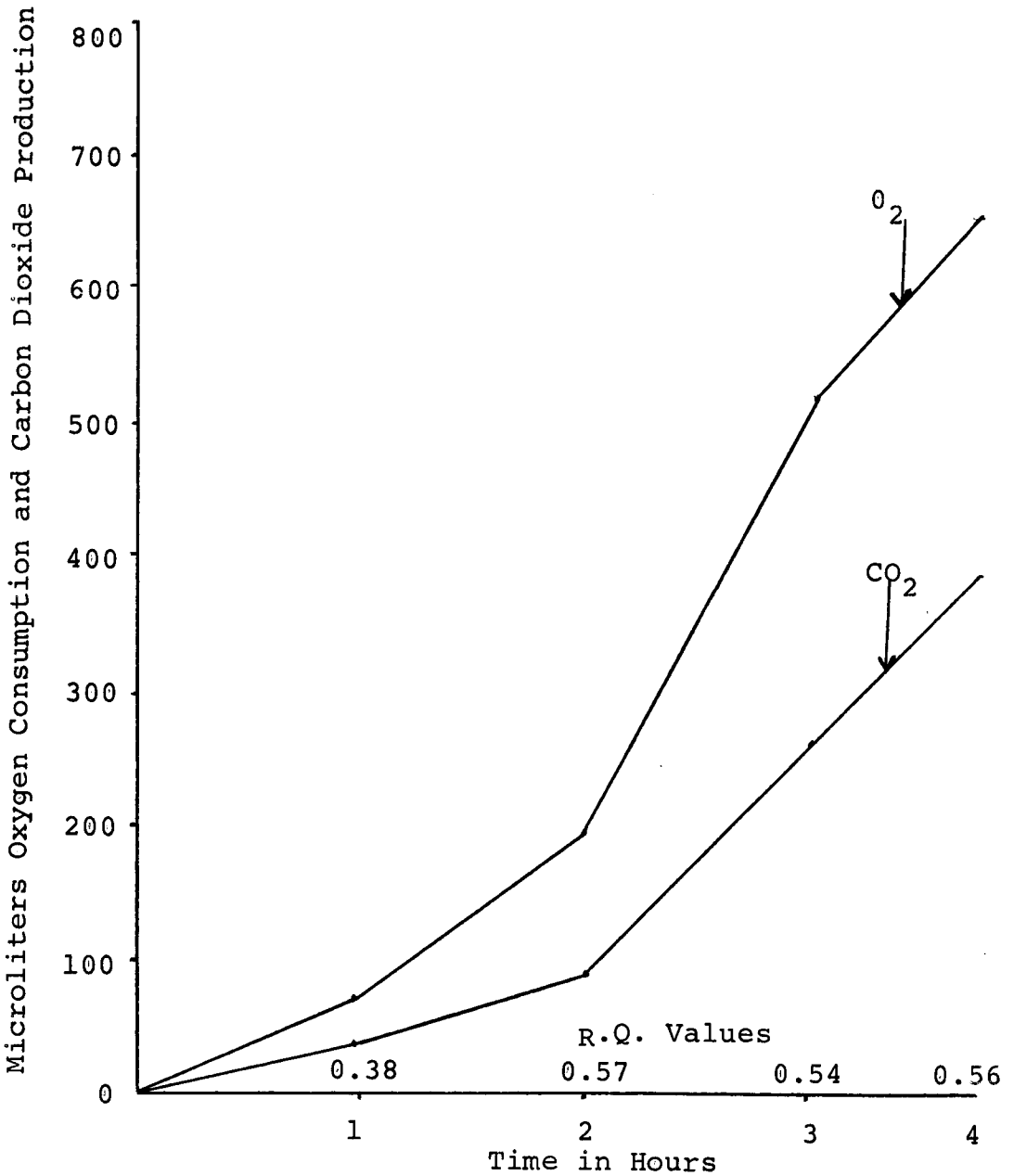


Figure 14. Oxygen consumption and respiratory quotients for resting cell suspensions grown on mineral salts and parathion (10 ppm) and assayed on parathion.

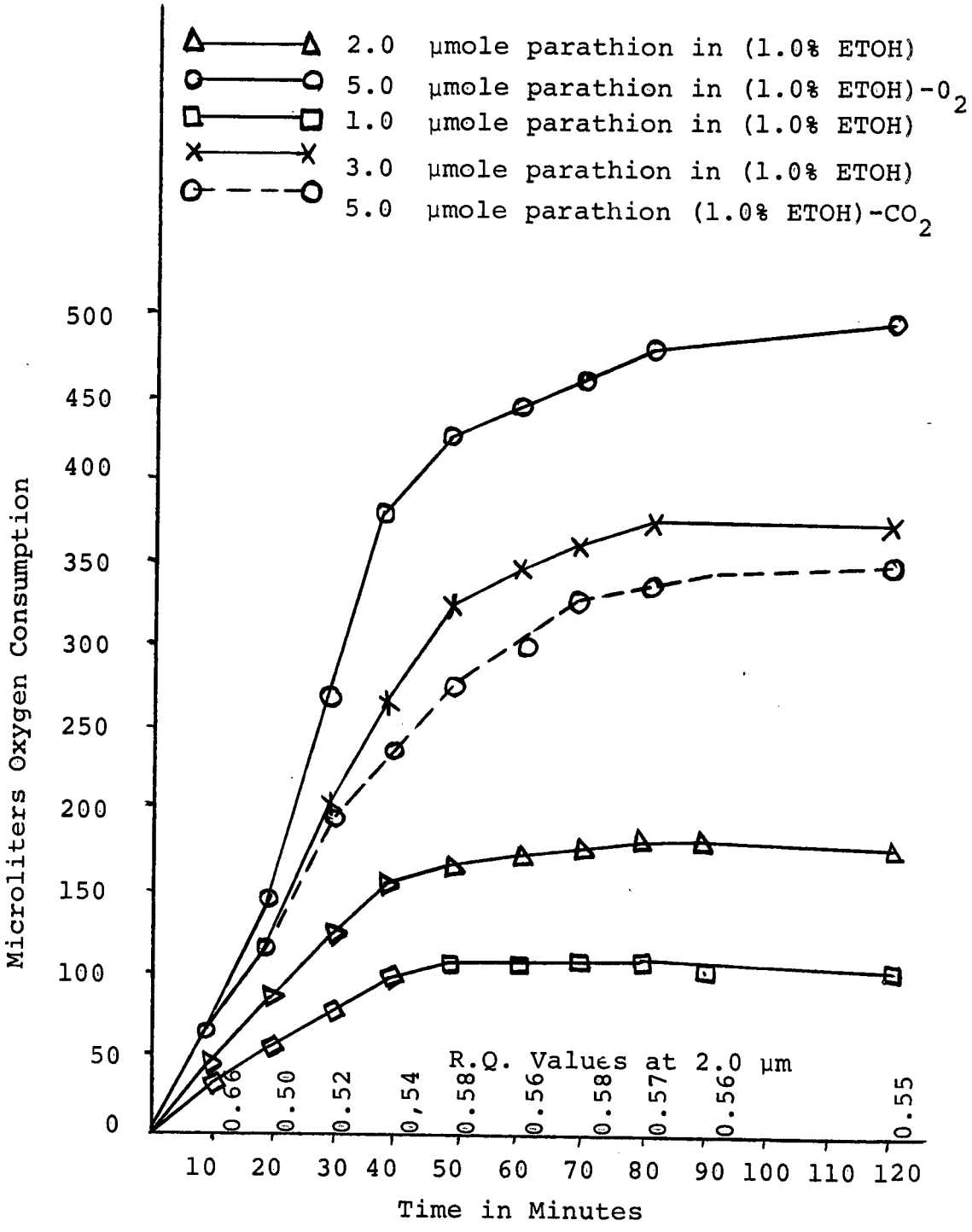


Figure 15. The effect of different parathion concentrations by resting cell suspensions.

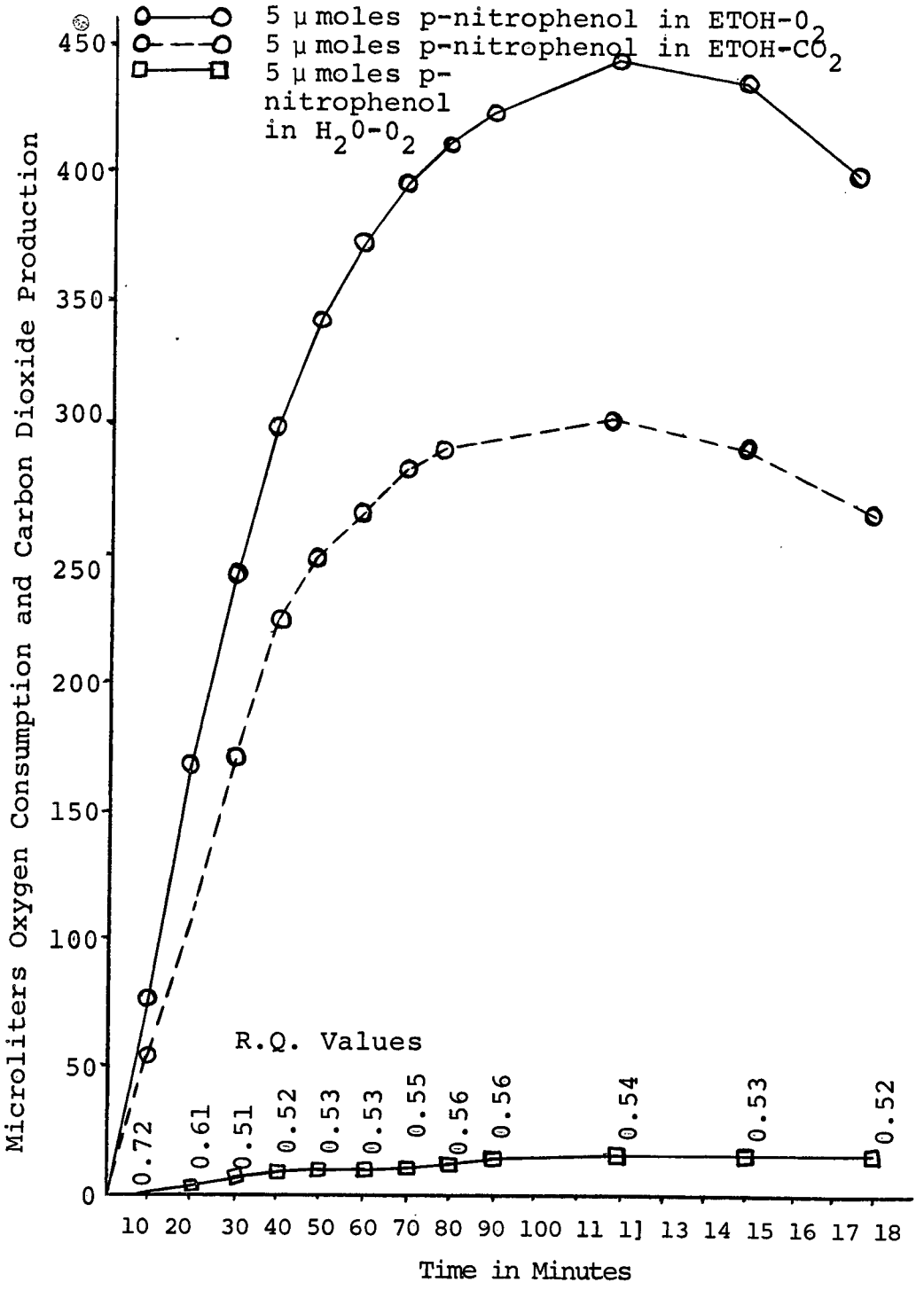


Figure 16. Oxygen consumption and respiratory quotients for resting cell suspensions grown on nutrient broth and assayed on p-nitrophenol.

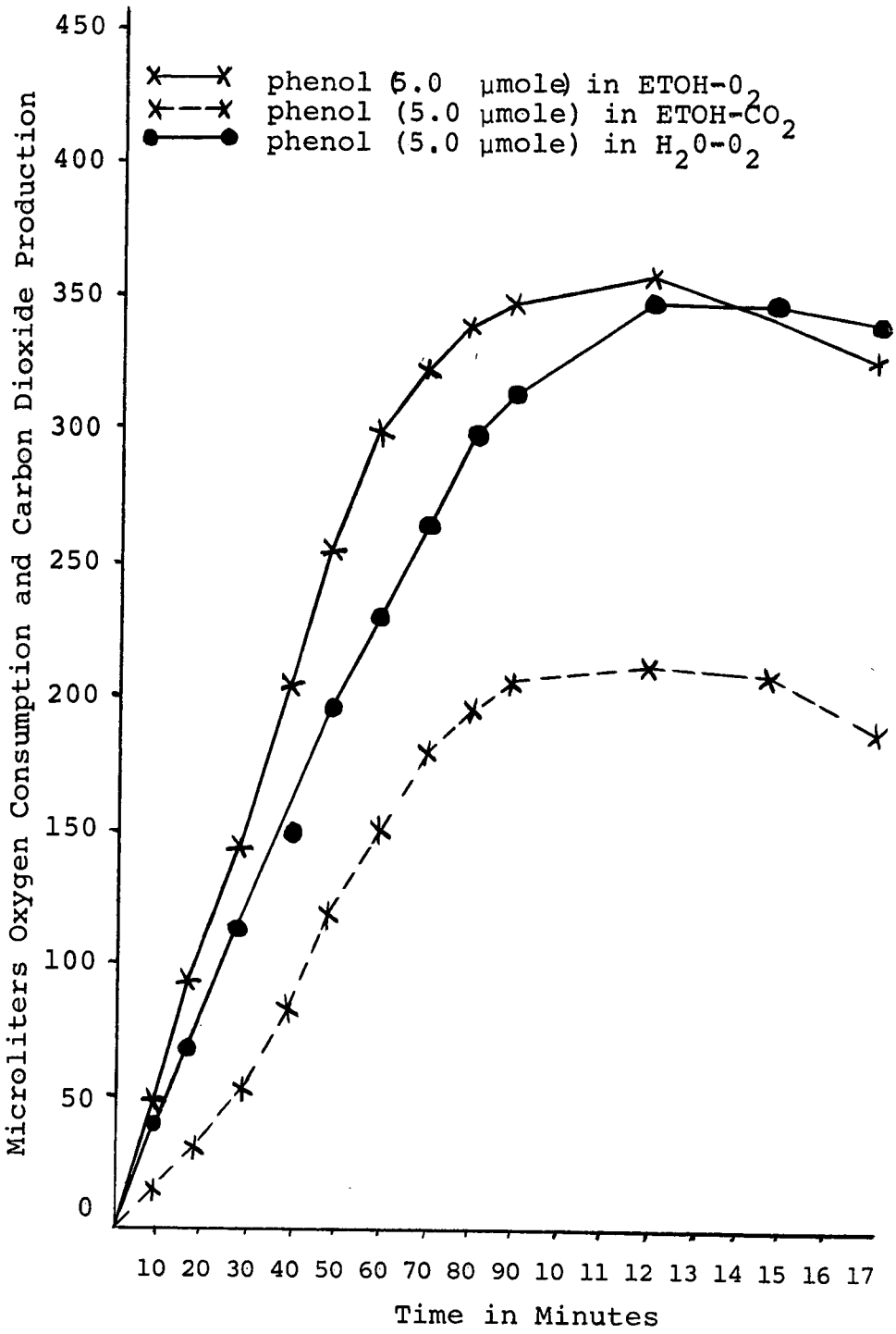


Figure 17. Oxygen consumption and respiratory quotients for resting cell suspensions grown on nutrient broth and assayed on phenol.

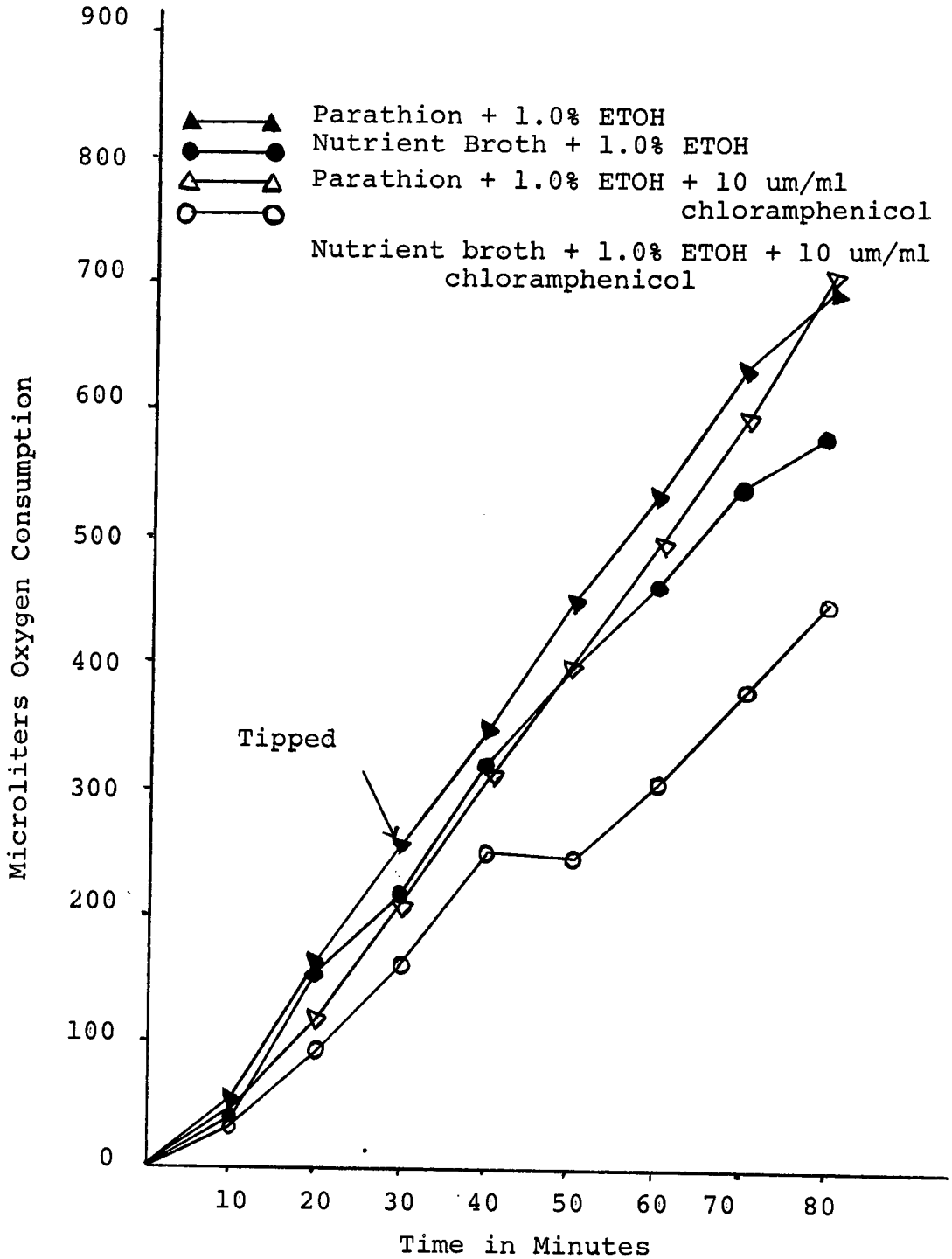


Figure 18. The effect of chloramphenicol on oxygen consumption by resting cells grown on parathion, and assayed on nutrient broth and on parathion.

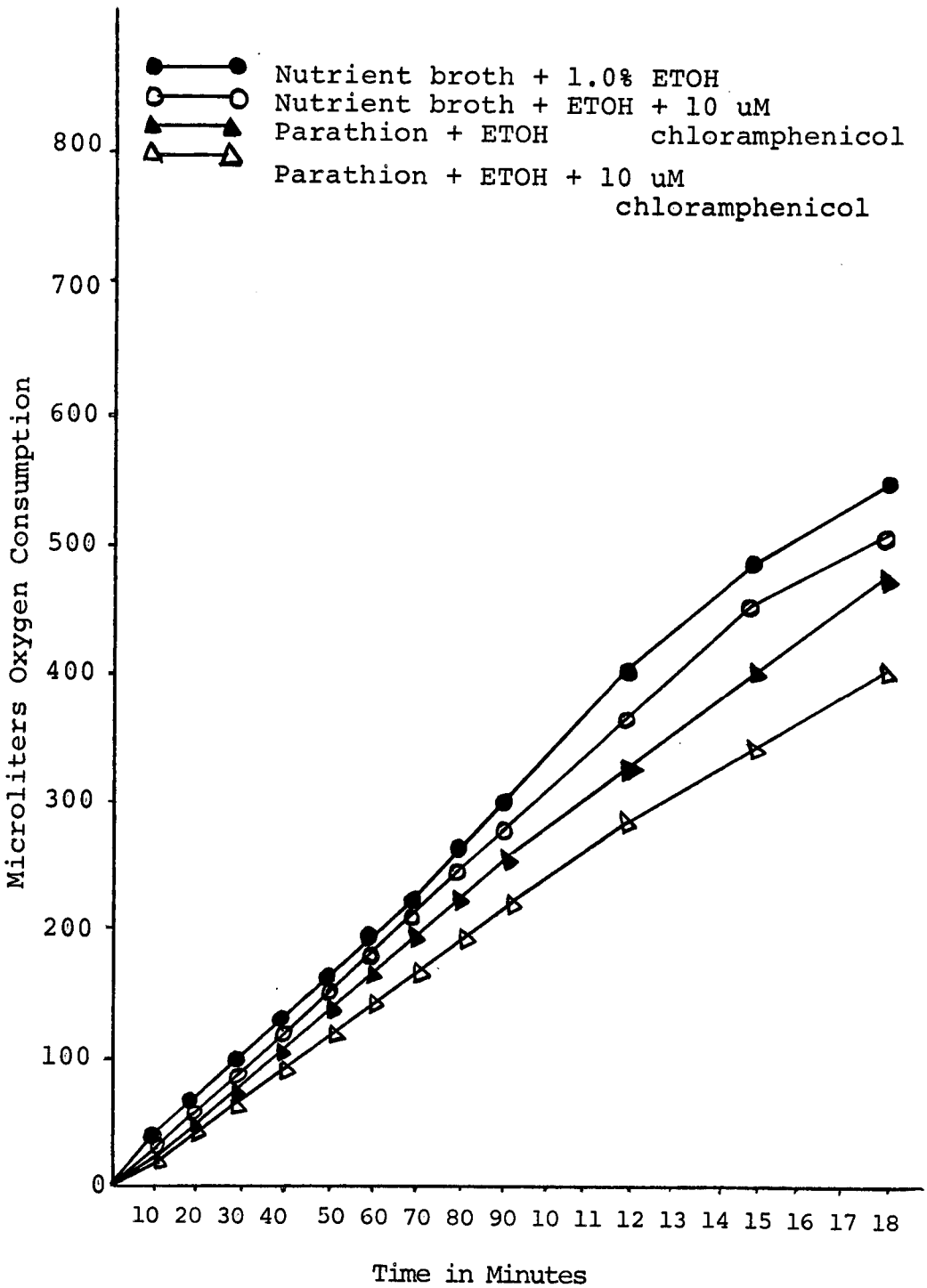


Figure 19. The effect of chloramphenicol on oxygen consumption by resting cells grown on nutrient broth, and assayed on nutrient broth and on parathion.

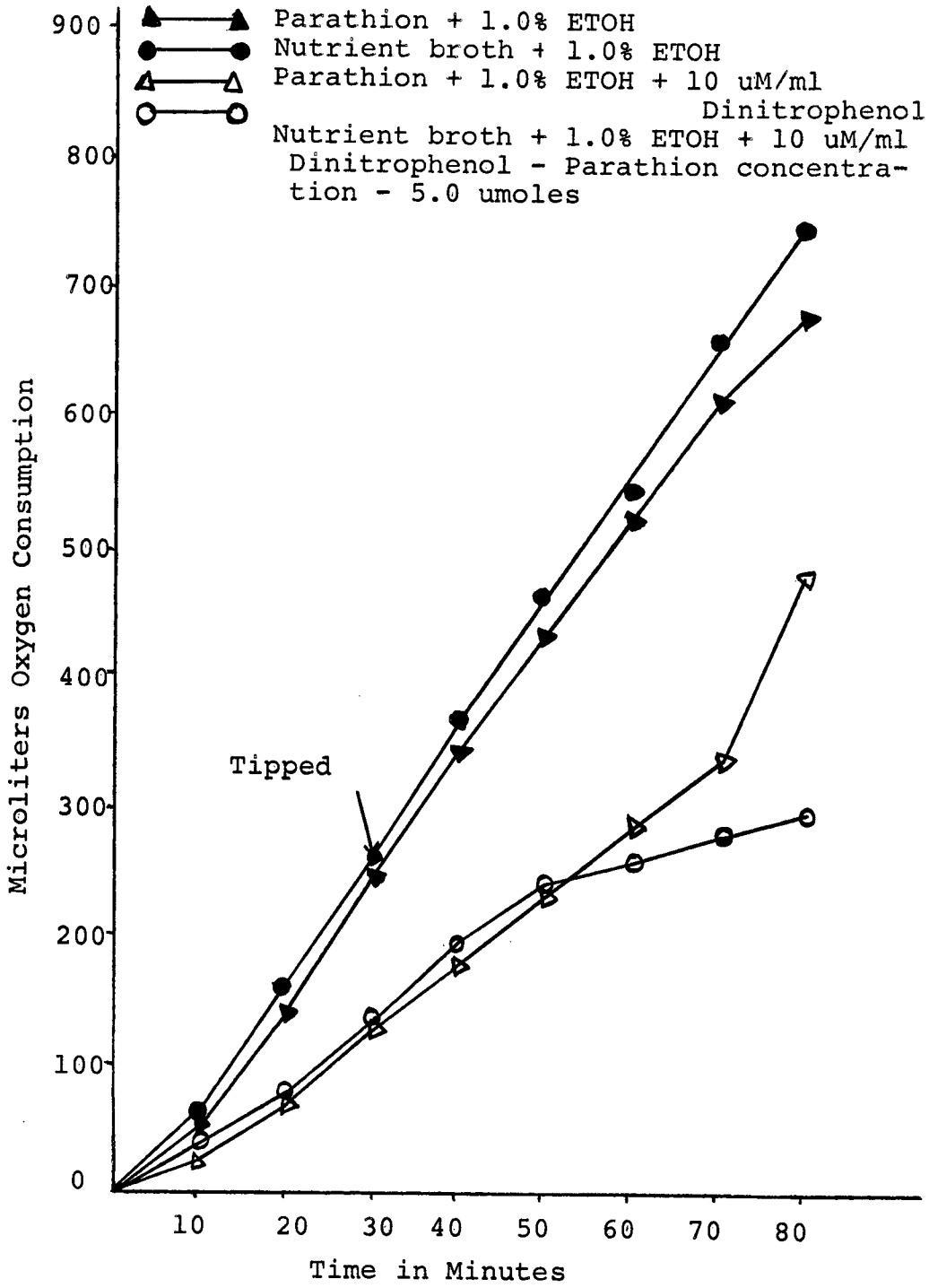


Figure 20. The effect of 2,4-dinitrophenol on oxygen consumption by resting cells grown on parathion, and assayed on nutrient broth and on parathion

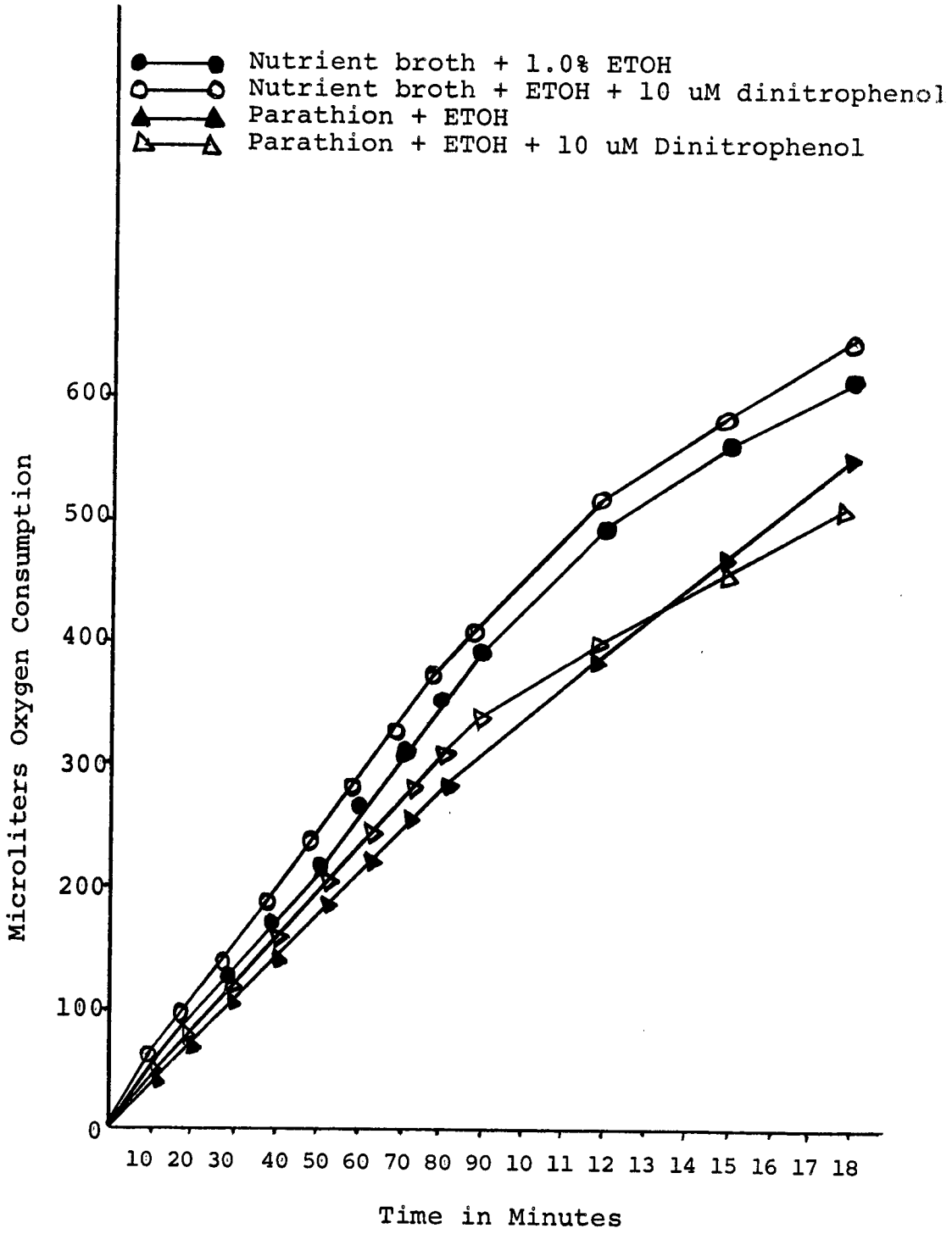


Figure 21. The effect of 2,4-dinitrophenol on oxygen consumption by resting cells grown on nutrient broth and assayed on nutrient broth and on parathion.

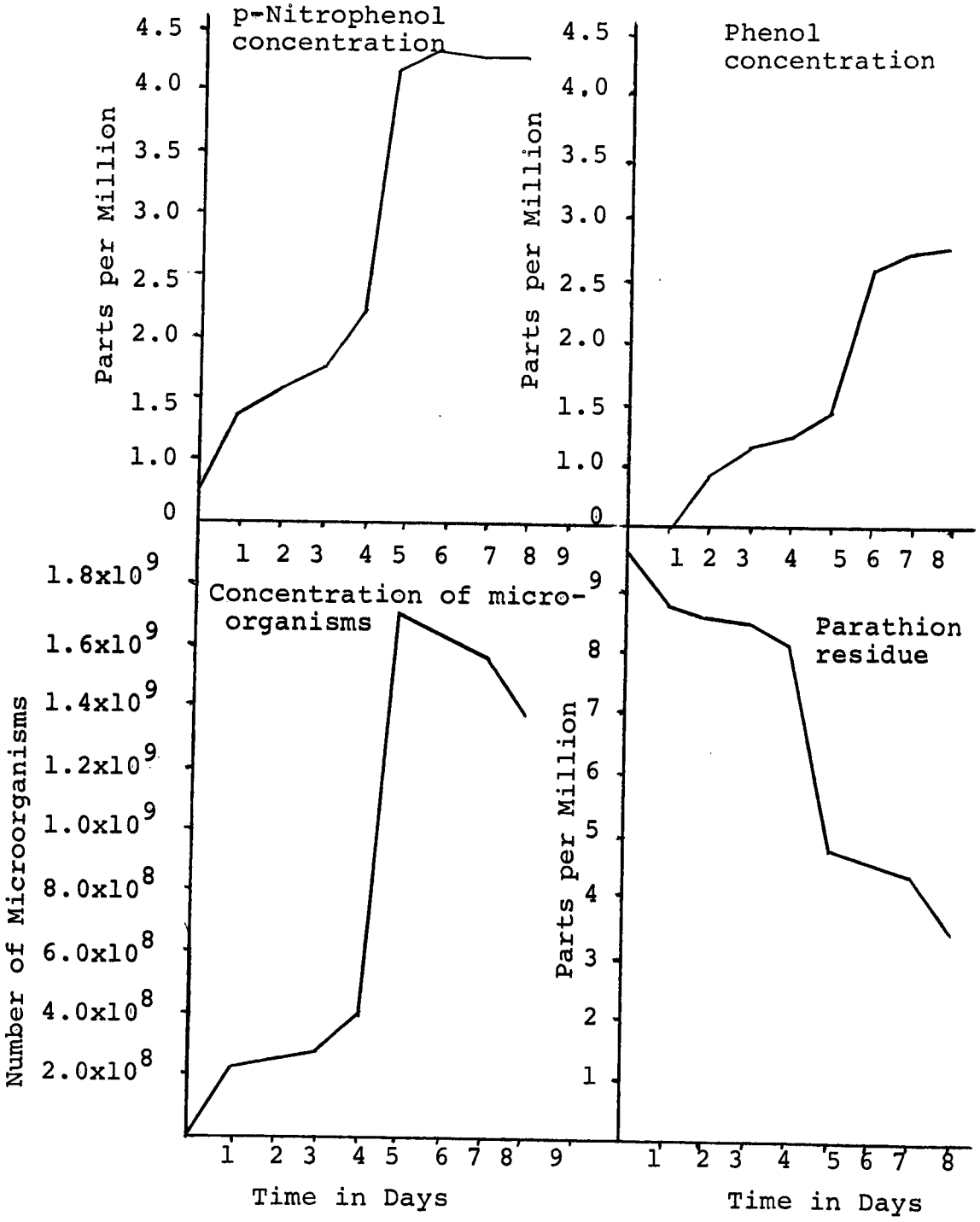


Figure 22. Cell populations, parathion residues, p-nitrophenol concentration, and phenol concentration of cells grown on mineral salts and parathion (10 ppm).

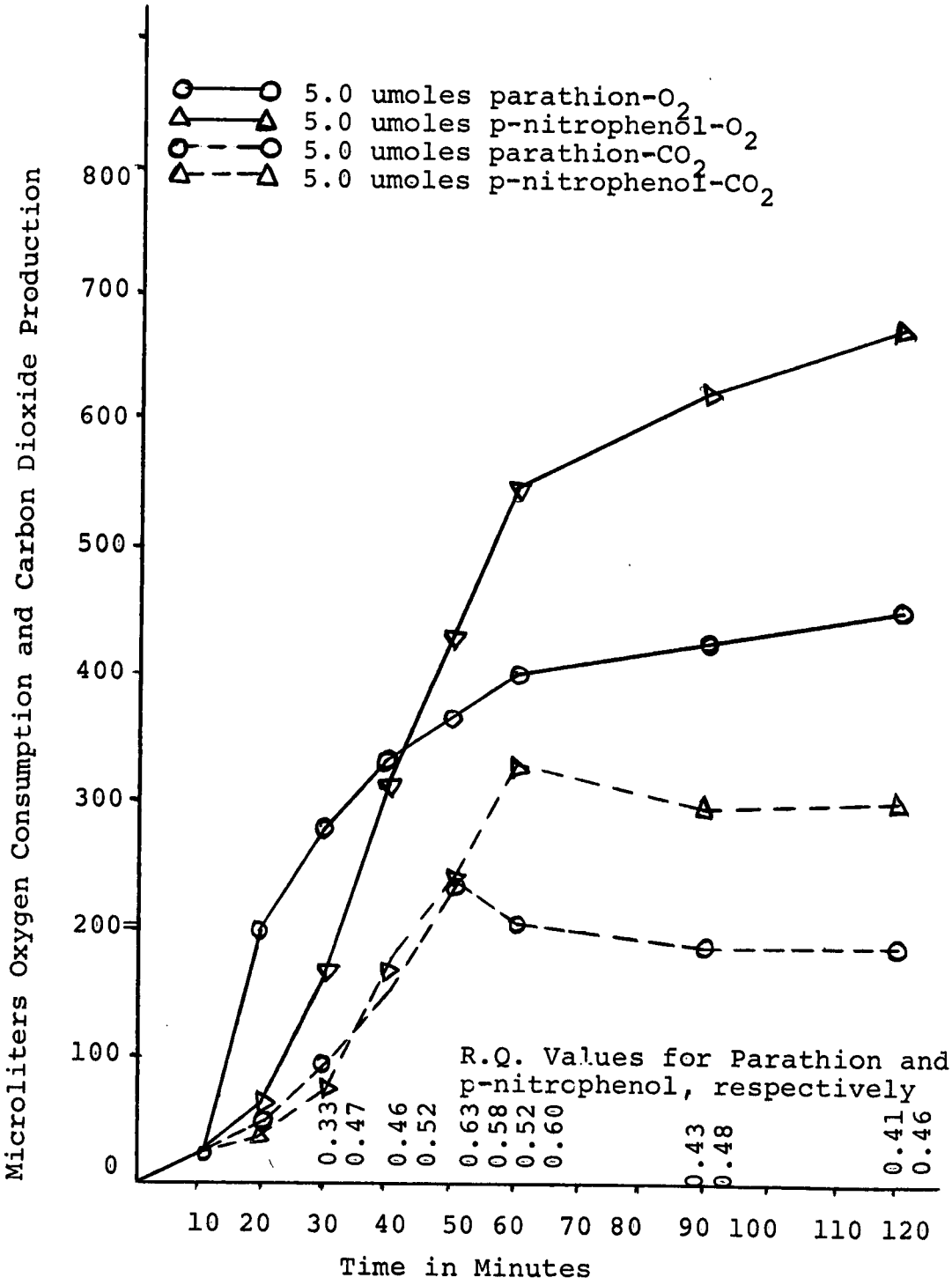


Figure 23. The effect of physiological age on parathion and p-nitrophenol utilization by resting cell cultures (Day 0).

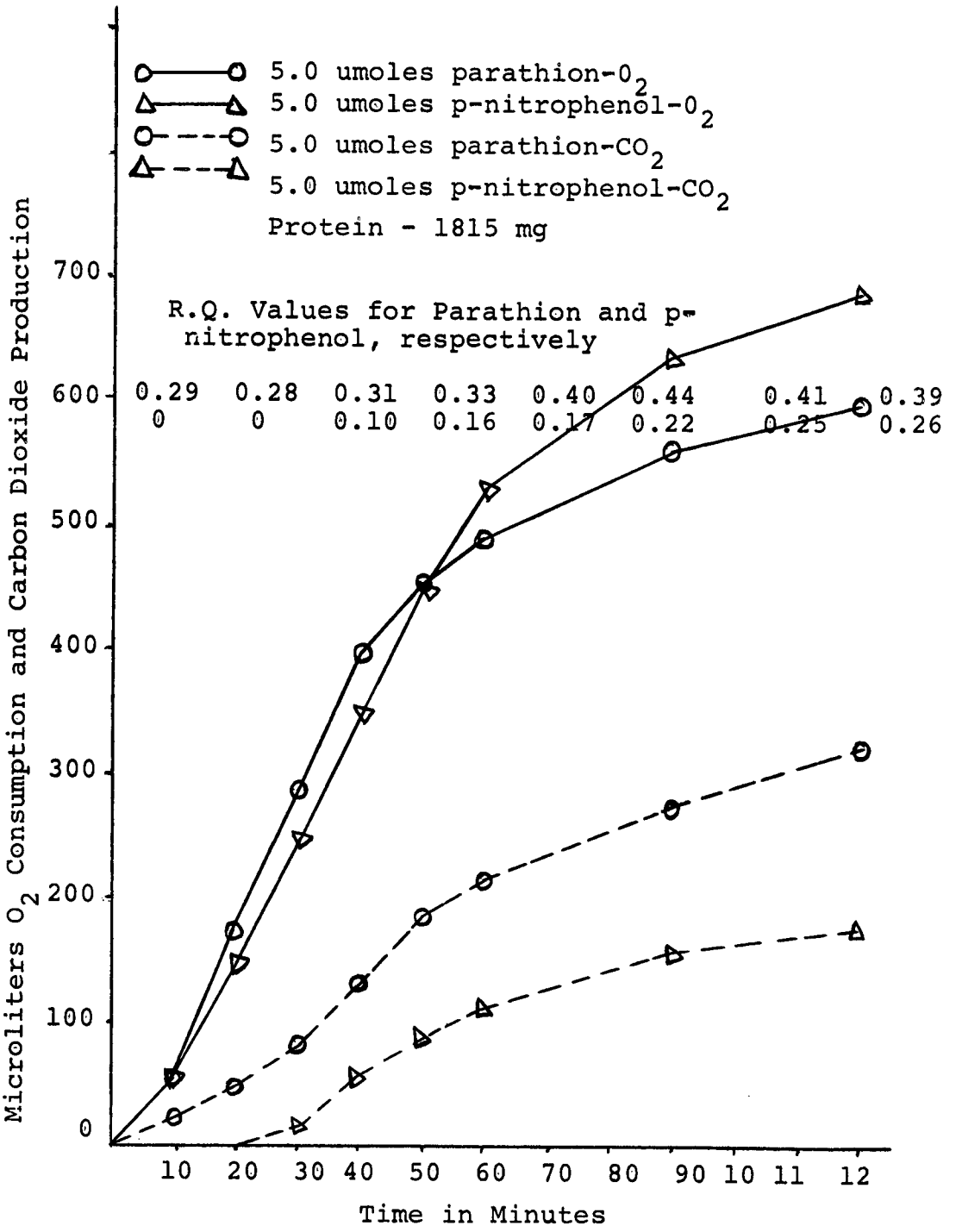


Figure 24. The effect of physiological age on parathion and p-nitrophenol utilization by resting cell cultures (Day 3).

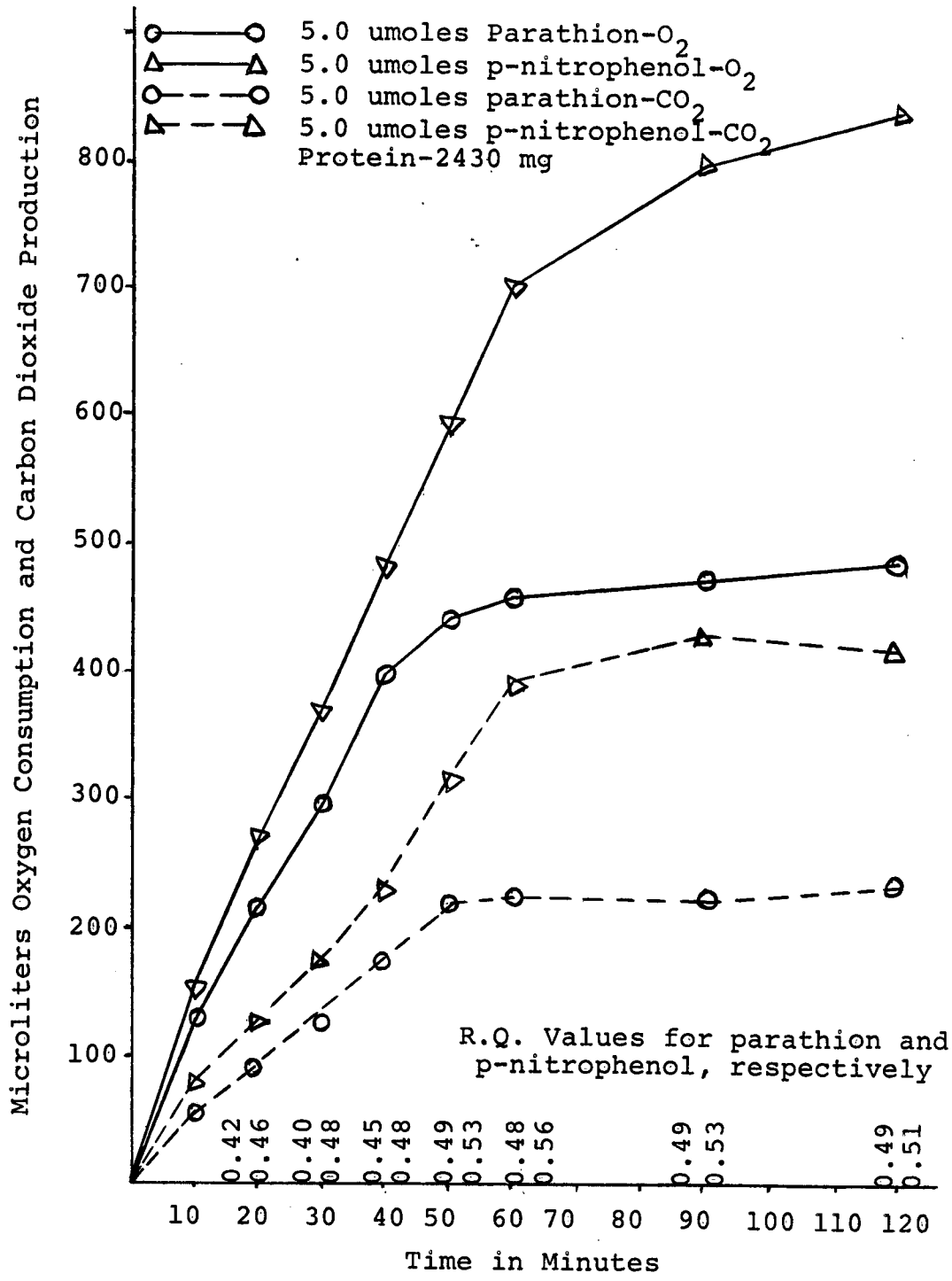


Figure 25. The effect of physiological age on parathion and p-nitrophenol utilization by resting cell cultures (Day 4).

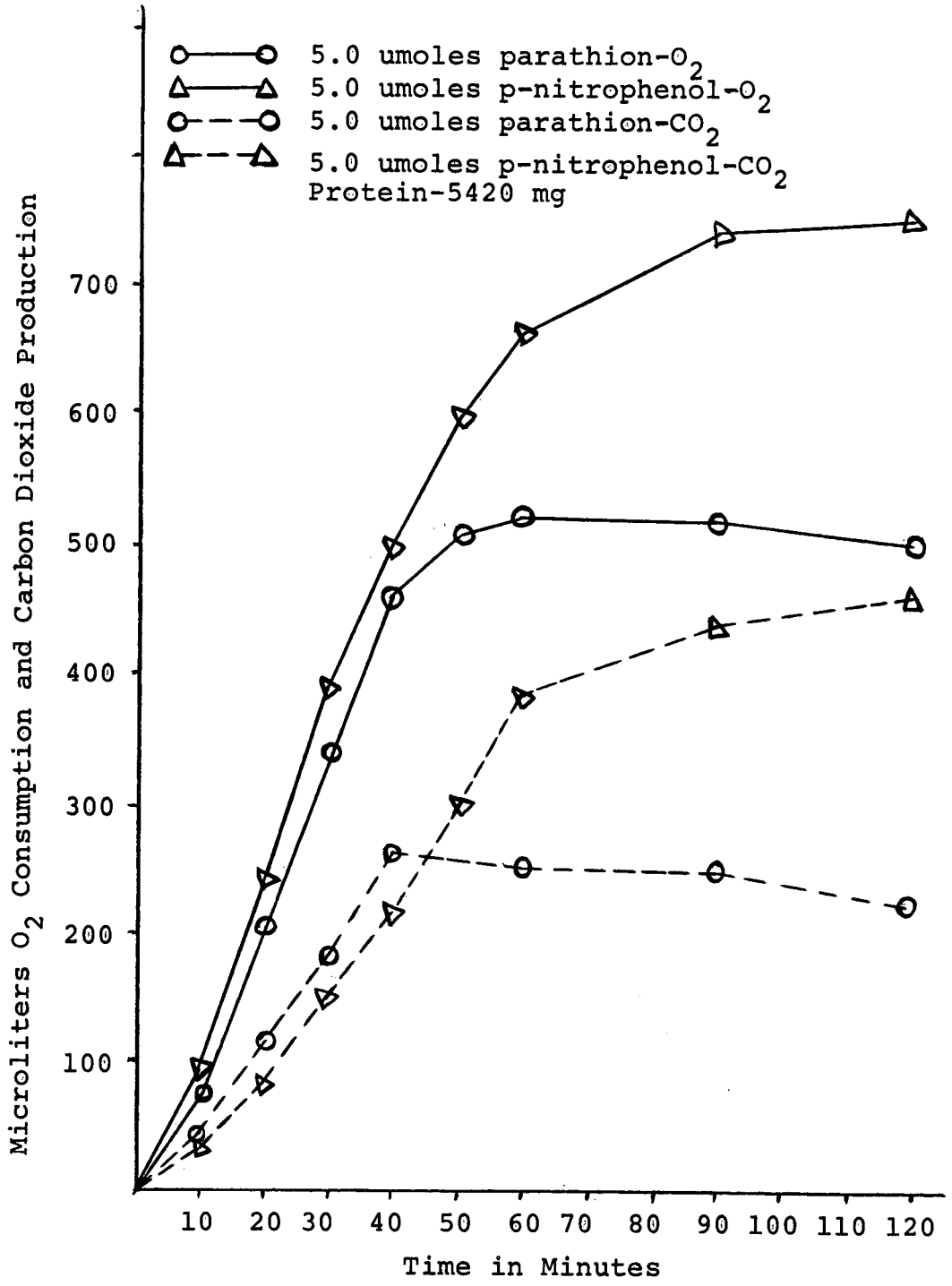


Figure 26. The effect of physiological age on parathion and p-nitrophenol utilization by resting cell cultures.

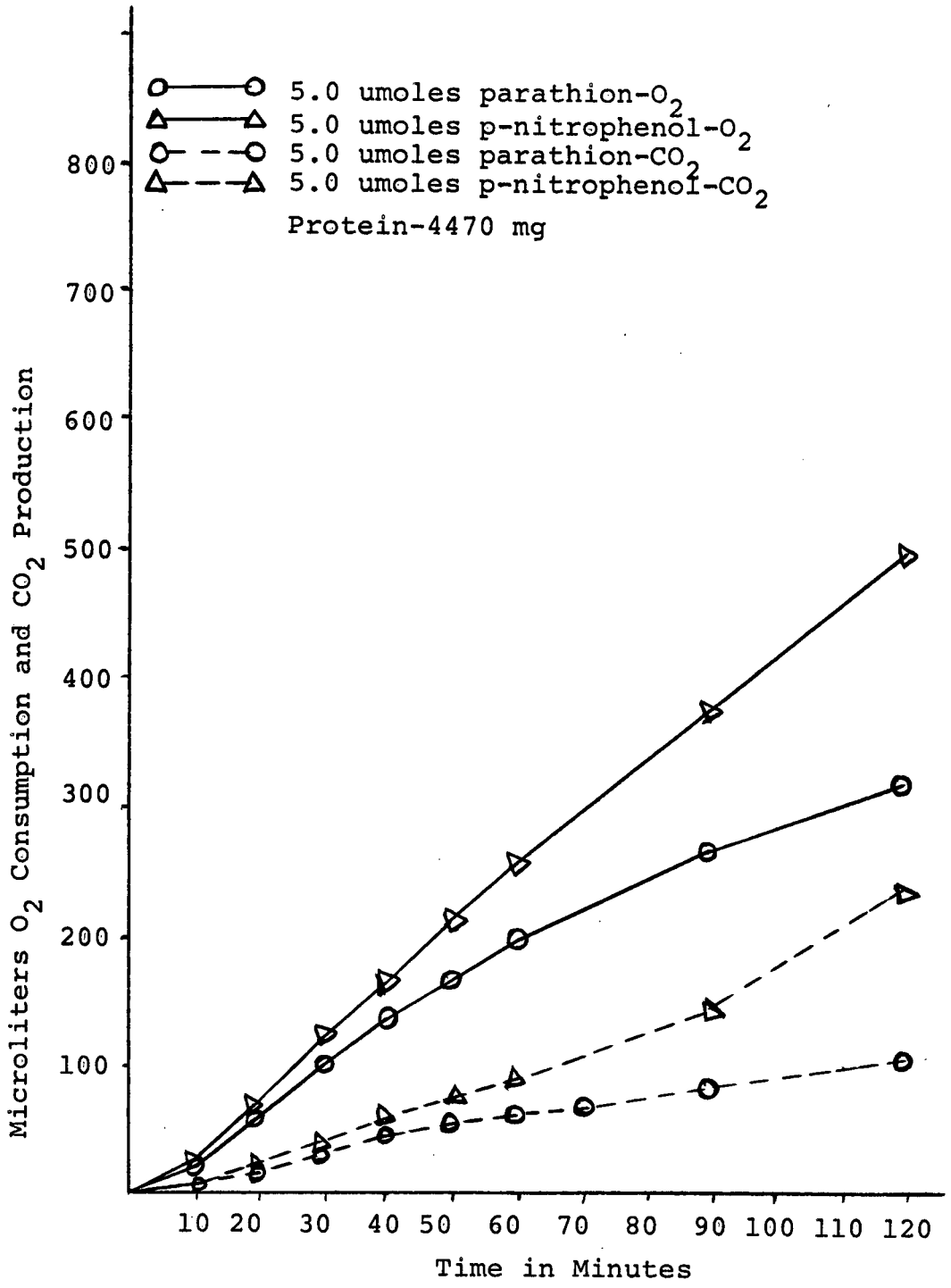


Figure 27. The effect of physiological age on parathion and p-nitrophenol utilization by resting cell cultures (Day 8).

C.3

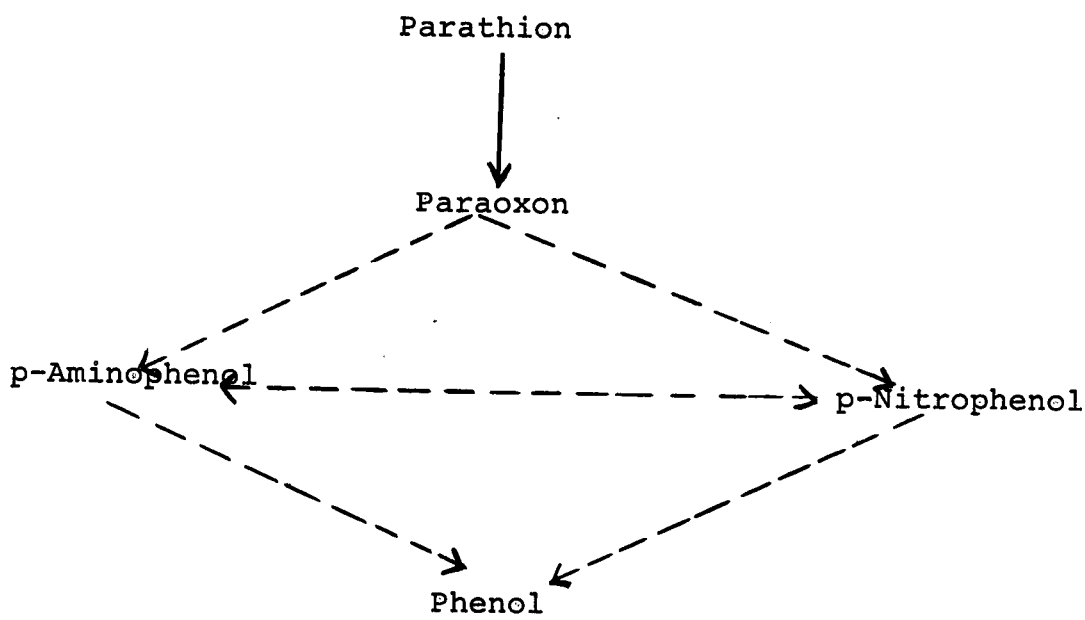


Figure 28. Proposed pathway for parathion degradation.

BIBLIOGRAPHY

Ahmed, M. K. and J. E. Casida. 1958. Metabolism of some organophosphorus insecticides by microorganisms. Agr. & Food Chem. 6: 10.

Alexander, M. 1965. Biodegradation of pesticides. Ann. Rev. Pesticides. 18: 6.

_____. 1967. The breakdown of pesticides in soil in "Agriculture and the Duality of our Environment." Am. Assoc. Adv. Sci. Washington, D.C.

_____. 1968. Microbial degradation and biological effects of pesticides in soil. J. Econ. Entomol. 51: 59-63.

American Cyanamid. 1972. Manual of Manufacturing Information for Thiophosparathion.

Bennett, E. O. 1963. Factors affecting the antimicrobial activity of phenols. J. Bacteriol. 70: 123-180.

Blackbourne, B. D. 1970. Pesticide poisoning. J. Biol. Chem. 209: 813-817.

Brown, L. R. 1958. Isolation, characterization and metabolism of methane oxidizing bacteria. Ph.D. Dissertation. Louisiana State University.

Breed, R. S., E. G. D. Murray and N. R. Smith. 1957. Bergey's Manual of Determinative Bacteriology. 7th ed. Williams & Wilkins Co.

Cook, D. W. 1965. Modified Lowry method. Ph.D. Dissertation. Mississippi State University.

Cook, J. W. and N. D. Pugh. 1957. A quantitative study of cholinesterase-inhibiting decomposition products of parathion formed by ultraviolet light. J. Assoc. Offic. Agr. Chem. 40: 277-281.

Dahm, P. A., F. C. Fountaine and J. E. Pankaskie. 1950. J. Dairy Sci. 33: 747.

Erdos, E. and L. Boggs. 1961. Hydrolysis of paraoxon in mammalian blood. Nature 190: 716.

Evans, W. C. and J. R. Simpson. 1965. Biochem. J. 62: 373-382.

Fleck, E. E. 1965. Chemistry of insecticides. J. Amer. Chem. Soc. 71: 1034-1036.

Foster, J. W. 1962. Bacterial oxidation of hydrocarbons. The oxygenases. O. Hayaishi, ed. Academic Press. New York.

Frawley, J. P., J. W. Cook, J. R. Blake and O. G. Fitzhugh. 1958. Effect of light on chemical and biological properties of parathion. J. Agr. & Food Chem. 6: 1.

Fukami, J. and C. Shishido. 1966. Glutathione-dependent enzyme system active in cleavage of methyl parathion to desmethyl parathion. J. Econ. Entomol. 59: 1338-1346.

Graetz, D. A., G. Chesters, T. C. Daniel, L. W. Newland and Lee Gerhard. 1971. Parathion degradation in lake sediments. J. WPCF 42: 2 Part 2.

Gunderson, K. and H. L. Jensen. 1956. Acta. Agr. Scand. 6: 100-114.

Gunner, H. B., R. E. Longley and B. M. Zuckerman. 1966. Microbial degradation of diazinon. Bacteriol. Proc. 66: 5.

Hayaiski, O., M. Hashimoto and J. Rothberg. 1971. J. Am. Chem. Soc. 77: 5450.

Helling, Charles S., Phillip C. Kearney and Martin Alexander. 1966. Behavior of pesticides in soils. Ann. Pestic. Rev. 17: 181-193.

Hirakoso, S. 1968. Inactivation of some insecticides by bacteria in mosquito-breeding polluted waters. Japan J. Expl. Med. 38: 327-334.

Kadoum, A. M. 1970. Thin-layer chromatographic separation and colorimetric detection of malathion and some of its metabolites from stored grains. J. Agr. & Food Chem. 18(3): 542-543.

Kasting, __ and __ Woodward. 1961. Insecticides and the soil. J. Agr. & Food Chem. 6(5): 127-134.

Kilby, B. A. 1968. The bacterial oxidation of phenol to B-ketoadipic. Biochem. J. Proc. Biochem. Soc. 43(5): 101-107.

Ladner, C. M. 1968. Flagella staining. Stain Technol. 43: 1.

Lichtenstein, E. P. and R. R. Schulz. 1964. The effects of moisture and microorganisms on the persistence and metabolism of some organophosphorus insecticides in soils with special emphasis on parathion. J. Econ. Entomol. 57: 618-627.

Lichtenstein, E. P., T. W. Fuhremann and R. R. Schulz. 1968. Effect of sterilizing agents on persistence of parathion and diazinon in soils and water. J. Agr. & Food Chem. 16(5): 871.

Lichtenstein, E. P. and R. R. Schulz. 1967. Persistence and metabolism of some organophosphorus insecticides in soils. J. Agr. & Food Chem. 14(5): 10-14.

Menzer, R. E. and W. C. Dauterman. 1970. Metabolism of some organophosphorus insecticides. J. Agr. & Food Chem. 18(6): 1031.

Matsumura, F. and C. J. Hogedijk. 1964. The enzymatic degradation of parathion in organophosphate susceptible and resistant houseflies. J. Agr. & Food Chem. 12: 447-453.

Mick, D. L. and P. A. Dahm. 1971. Metabolism of parathion by two species of rhizobium. J. Econ. Entomol. 63(4)

Miyamoto, J., K. Kitagawa and Sato Yoshishige. 1971. Metabolism of organophosphorus insecticides by Bacillus subtilis with special emphasis on Sumithion. Japan J. Expl. Med. 36(2): 211-225.

Monsanto Technical Bulletin. 1972. Parathion and Methyl parathion.

Mounter, L. A. 1965. Metabolism of organophosphates. J. Biol. Chem. 209: 813-817.

O'Brien, R. D. 1960. Toxic phosphorus esters. Academic Press, Inc. New York.

O'Brien, R. D. 1967. Insecticides, action and metabolism. Academic Press, Inc. New York.

Raymond, G. M. and M. Alexander. 1967. Microbial metabolism and co-metabolism of nitrophenols. Pesti. Biochem. Physiol. 1: 123-130.

Reich, G. A. 1971. The diagnosis and treatment of acute pesticide intoxication in man. EPA Pesticides and Public Health. Washington, D.C.

Rogoff, M. H. and J. Wender. 1959. J. Bacteriol. 77: 783-788.

Rogoff, M. H. 1963. Oxidation of aromatic compounds by bacteria. J. Bacteriol. 10: 1-23.

Simpson, J. R. and W. C. Evans. 1953. Biochem. J. 55: 24.

Society of American Bacteriologists. 1957. Manual of microbiological methods. McGraw-Hill Book Co., Inc.

Stanier, R. Y. 1950. Problems of bacterial oxidative metabolism. J. Bacteriol. 55: 477-494.

Stanley, C. W. 1964. Thin-layer chromatography of organophosphorus pesticides and acids on microchromatoplates. J. Chromatol. 16: 467-475.

Stojanovic, B. J. 1970. Pesticides in soils and crops. EPA Pesticides and Public Health. Washington, D.C.

Tyler, W. R. 1966. Procaryotic cell structure in: Fundamentals of Microbiology. W. B. Saunders. Philadelphia.

Umbreit, W. W., R. H. Burris and J. F. Stauffer. 1964. Manometric techniques. 4th ed. Burgess Publ. Co. New York.

Vardanis, A. and L. G. Crawford. 1964. Comparative metabolism of 0,0-dimethyl 0-p-nitrophenyl phosphorothioate (methyl parathion) and sumithion. J. Econ. Entomol. 57: 1.

Vercruysse, P. and P. Deslypere. 1964. Acute parathion poisonong. J. Forensic Med. 11: 3.

Villeneuve, D. C. and W. E. J. Phillips. 1970. The effect of acute ethanol administration of parathion toxicity. Bull. Environ. Contam. Toxicol. 5: 125-132.

Walker, N. 1954. Plant and Soils 5: 194-204.

Walker, W. W. 1970. Persistence of DDT, dieldrin and malathion in soil and their impact on the soil microflora. M.S. Thesis. Mississippi State University.

Warnick, Stephen L. and Arden R. Gaufin. 1965. Determination of pesticides by electron capture gas chromatography. J. AWWA. 1023: 1-6.

Welling, W. P., ~ Blaakmeer, G. J. Vink and S. Voerdman. 1970. In vitro hydrolysis of paraoxon by parathion resistant houseflies. Pest. Biochem. and Physiol. 1: 61-70.

Wilson, R. P. 1969. Laboratory Manual for Biochemistry Laboratory. Unpublished.

Yasuno, M., S. Hirakoso, M. Sasa and M. Uchida. 1965. Inactivation of some organophosphorus insecticides by bacteria in polluted water. Japan J. of Expl. Med. 35(6): 563.

Zobell, C. E. 1946. Action of microorganisms on hydrocarbons. Bacteriol. Rev. 10: 1.