GROWTH, RESPIRATION, AND ENERGETICS OF NITROBACTER AGILIS IN THE PRESENCE OF SELECTED PESTICIDES

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C. L. San Clemente
Major professor
C.L. San Clemente

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ABSTRACT

GROWTH, RESPIRATION, AND ENERGETICS OF *NITROBACTER AGILIS* IN THE PRESENCE OF SELECTED PESTICIDES

by Carl L. Winely

The purpose of this study was to determine the mode of action of several pesticides on both the growth of *Nitrobacter agilis* in aerated cultures and on the respiration of *N. agilis* cell suspensions in the Warburg respirometer. Those compounds which inhibited both growth and respiration were tested for an effect on several enzymatic activities to characterize the site of inhibition.

Two pesticides (aldrin and simazine) were not inhibitory to growth of *Nitrobacter*, but five compounds (CIPC, chlordane, DDD, heptachlor, and lindane) prevented growth when added to the medium at a concentration of 10 ug per ml.

Whereas CIPC (110 ug/ml) and eptam (275 ug/ml) prevented nitrite oxidation by cell suspensions, the addition of DDD (500 ug/ml) and lindane (500 ug/ml) resulted in only partial inhibition of the oxidation. Heptachlor (500 ug/ml) and chlordane (500 ug/ml) also caused only partial inhibition of oxidation with *Nitrobacter* cell suspensions, but were more toxic with cell-free extract nitrite oxidase.

None of the pesticides inhibited the nitrate reductase activity of cell-free extracts, but most of them, especially heptachlor, caused some repression of cytochrome c oxidase activity.

Two of the herbicides (CIPC and eptam) exerted an uncoupling effect on the oxidative phosphorylation linked to nitrite oxidation in Nitrobacter agilis. Although nitrite oxidation was inhibited, the more severe effect on phosphorylation caused decreased P/O ratios (umoles of ^{32}P incorporation per uatom of oxygen uptake). A 50% reduction in the P/O ratio occurred at approximately 2.3×10^{-4} M CIPC and at 8×10^{-4} M eptam.

A classical phosphorylation uncoupler, 2,4-dinitrophenol (DNP), affected the oxidative phosphorylation in N. agilis in a manner similar to that observed with the herbicides. Again, oxygen uptake was inhibited, but P/O ratios were lowered because of a greater effect on phosphate esterification. A 45% reduction in the P/O ratio occurred at 2.5×10^{-4} M DNP.

The maximum P/O ratio obtained was 0.36. This low P/O ratio was attributed to disruption of the internal integrity of the phosphorylating particle since an active ATPase was observed when the yeast hexokinase and glucose trapping system was omitted from the reaction system.

The actual modes of action of chlordane, DDD, heptachlor, and lindane on N. agilis were not determined, but were shown to be on biosynthetic processes of the cell rather than nitrite oxidation. In the case of the two herbicides, CIPC and eptam, uncoupling of oxidative phosphorylation in a manner analogous to 2,4 dinitrophenol is suggested as the mode of inhibition in Nitrobacter.

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By

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DEDICATION

This thesis is affectionately dedicated to my wife whose patience and encouragement made this achievement possible.

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INTRODUCTION

Biological nitrification, the oxidation of the ammonium ion to nitrate ion, is largely caused by the autotrophic soil bacteria of the genera *Nitrosomonas* and *Nitrobacter*.

Most of the experiments to study the effects of various pesticides on nitrification used field trials or determinations in the soil perfusion unit developed by Lees and Quastel (1946). Some (Hale *et al.*, 1957; Quastel and Scholefield, 1951; Farmer *et al.*, 1965; and others) have used both the perfusion apparatus and a manometric technique to measure the effect of pesticides on the nitrifers. When respiration studies were made, soil which had been percolated with an ammonium salt solution to increase the nitrifying population was usually added to the Warburg vessels and either the oxidation of ammonium or the appearance of nitrate was measured. Seldom were pesticides tested for an effect on respiration with concentrated cell suspensions of either *Nitrosomonas* or *Nitrobacter*. Likewise, aerated liquid cultures of these organisms were used infrequently as a test system for pesticides. In these studies the effect of the pesticides was reported only with regard to oxidation of the NH_4^+ or NO_2^- substrate; nothing was stated concerning effects upon biosynthetic processes of the organisms.

The purpose of this study was to determine the mode of action of several pesticides on both the growth of *Nitrobacter agilis* in aerated cultures and on the respiration of *N. agilis* cell suspensions in the Warburg respirometer. Characterization in terms of several enzymatic activities with respect to the site of inhibition was attempted with pesticides which inhibited both growth and respiration.

LITERATURE REVIEW

Media and Culture Techniques

Nitrobacter agilis is a chemoautotroph which derives its energy from the oxidation of nitrite to nitrate according to the following equation:

$$NO_2 + \frac{1}{2}O_2 \longrightarrow NO_3^- + 17.8 \text{ kcal}$$

Although *Nitrobacter* was first isolated in 1890 by Winogradsky, culturing difficulties have impeded knowledge concerning the metabolism of the organism. For instance, the first biochemical investigation was not carried out until 1957 (Lees and Simpson) and this was performed with impure cultures. One of the main problems confronting early investigators was particulate matter in the medium which was thought to be required for growth. Aleem and Alexander (1958) were the first to culture the organism successfully in particulate-free medium and they obtained relatively high cell yields. They established minimum concentrations of individual ions required for maximum growth. There were 5 ug per ml of phosphorus, 5 ug per ml of magnesium, and 7 ug per liter of iron.

The results that Aleem and Alexander obtained with nitrite illustrated the need for additions of the substrate during growth rather than the addition of a large initial nitrite concentration. As long as the original nitrite concentration did not exceed 40 ug of nitrite-nitrogen per ml, no lag phase occurred in growth, but at 130 ug of nitrite-nitrogen per ml, the lag phase was prolonged by two or three days. Additions of up to 500 ug NO_2^--N per ml could be made to logarithmic phase cultures without suppressing the growth and the logarithmic phase lasted until about 1500 ug NO_2-N per ml were oxidized.

Since one explanation for limited growth of Nitrobacter was the accumulation of nitrate during growth, Aleem and Alexander (1960) added various concentrations of nitrate to the medium. With an initial concentration of 1000 to 2000 ug NO_3^--N per ml the lag phase was prolonged and at 2000 ug NO_3^--N per ml no growth occurred. Gould and Lees (1960) observed a greater effect, however, as some inhibition of nitrite oxidation occurred after accumulation of 500 ug NO_3^--N per ml. Finstein and Delwiche (1965) observed decreased efficiency of growth due to nitrate accumulation, but only after a concentration in excess of about 0.1 M.

Other attempts at increasing yields included dialysis to remove nitrate (Gould and Lees, 1960; Boon and Laudelout, 1962) and growth in continuous culture (Gould and Lees, 1960). Only slight increases in yield were obtained by removing NO_3^-, but the continuous culture technique was an improvement as 1 g dry weight of cells could be obtained per week from a 5 l culture.

Inorganic Amendments To Medium

Molybdenum. Both inorganic and organic amendments to the inorganic medium (Aleem and Alexander, 1960) were sought which would increase cell yield. Perhaps the most noteworthy results were obtained on the addition of molybdenum. Using chemically purified medium, Finstein and Delwiche (1965) showed that Nitrobacter has an absolute requirement for molybdenum. In addition, molybdenum induced an 11 fold increase in both nitrite utilization and cell-mass development. With short term studies, concentrations as low as 10^{-9} M caused the maximal response and 10^{-6} M was usually added to large-scale cultures.

Aleem and Alexander (1958) reported the oxidation of 100 umoles nitrite in 9 days, Gould and Lees (1960) observed 140 umoles oxidized in 10 days while Finstein and Delwiche (1965) by adding molybdenum obtained oxidation of 250 umoles NO_2^- per ml in 10 days. However, since the presence of molybdenum decreased the efficiency of substrate utilization rapidly after oxidation of approximately 120 umoles of nitrite per ml, the cultures were usually harvested after 4 days of active growth. Finstein and Delwiche reported that a procedure was now available whereby large masses of Nitrobacter cells could be obtained with relative ease. Approximately 0.5 g (dry weight) of cells was obtained from 18 liters of culture.

Yeast extract and casamino acids. Various organic amendments to inorganic medium have also stimulated growth of the organism. Delwiche and Finstein (1965) found that addition of yeast extract (75 ug/ml) and casamino acids (200 ug per ml) to a medium adequate in minerals resulted in approximately 50% stimulation as judged by nitrite consumed per unit time, optical density, and plate count. Ashed yeast extract also stimulated growth if added to molybdenum-free medium, but a molybdenum concentration equivalent to the ash caused the same effect.

Acetate and formate. Delwiche and Finstein (1965) noted that formate and also acetate contributed some carbon to the cells. Since the formate contribution to cell carbon was small and Malavolta, Delwiche, and Burge (1962) had observed strong formate oxidation by *Nitrobacter* cell-free extracts, it was suggested that the formate was first reduced to carbon dioxide which in turn was fixed. In contrast to formate, acetate contributed strongly to cell carbon (Delwiche and Finstein, 1965) and under certain conditions stimulated growth. Neither formate nor acetate could substitute for CO_2 as the sole carbon source. Of the several amino acids which were tested by these workers, none stimulated growth but on the other hand, valine and threonine were inhibitory at 10^{-3} M.

Biotin. With four strains of *N. agilis* the addition of biotin (150 ug per l) to nitrite mineral medium increased nitrite oxidation by 2 to 4 fold and resulted in 100 to 1000 fold greater populations of cells (Krulwick and Funk, 1965).

Incubation of the four strains at 37 C imposed an absolute necessity for biotin requiring daily additions of 150 ug per 1 of medium. The stimulatory effects of the vitamin at 30 C suggested to these authors that in N. agilis, the synthesis of biotin was rate limiting for growth.

Obligate or Facultative Autotroph

Several investigators have attempted to find an organic substrate for Nitrobacter; these substances included acetate and formate primarily although certain other carbon and nitrogen compounds were studied. A significant report on acetate assimilation was made by Ida and Alexander (1965). They assumed that the inability of Nitrobacter to grow heterotrophically was caused by either: (i) impermeability of the cell to organic compounds; (ii) lack of appropriate enzymes to metabolize permeable organic molecules; or (iii) inability of the organism to derive energy from permeable organic compounds. Consequently, several compounds were tested for permeability and assimilation. Although none of the compounds tested could substitute for either CO_2 as a sole carbon source or nitrite as an energy source, the organism was permeable to acetate and some other simple carbon compounds. Other workers also found that acetate was converted to a number of products (Delwiche and Finstein, 1965). Since Nitrobacter was permeable to organic compounds and was also capable of metabolizing acetate, Ida and Alexander concluded that the obligate chemoautotrophic nature of Nitrobacter is probably associated with the inability of the organism to derive sufficient energy to sustain growth from the organic oxidations that it can perform.

Soon after these two reports of obligate autotrophy, formate was reported as an energy source for Nitrobacter (Van Gool and Laudelout, 1966_a). It had already been established that formate was oxidized by Nitrobacter (Silver, 1960; Malavolta, Delwiche, and Burge, 1962), but Van Gool and Laudelout found that, in addition, formate was also utilized as an energy source by Nitrobacter. The energy was used for CO_2 fixation and growth. The heterotrophic growth was much less than autotrophic growth as evidenced by a generation time of 144 hours with formate and 18 hrs with nitrite.

Another report of heterotrophic growth by Nitrobacter was given by Smith and Hoare (1968). Although acetate had no effect on the rate of nitrite oxidation or exponential growth, the compound was assimilated in the presence of nitrite. The assimilation of acetate was particularly marked since 33 to 39% of newly synthesized cell carbon was derived from acetate. Carbon from acetate was incorporated into all of the major cell constituents, including most of the amino acids of cell protein and poly-B-hydroxybutyrate. Cell suspensions were able to assimilate acetate in the absence of bicarbonate and even in the absence of nitrite. Contrary to previous reports, however, Nitrobacter was able to grow heterotrophically through seven transfers in a medium containing acetate and casein hydrolysate in the absence of nitrite and bicarbonate. With autotrophic and heterotrophic growth, the generation times were 20 hours and 90 hours, respectively. The authors concluded that *N. agilis* is a facultative autotroph rather than an obligate autotroph.

Nitrite Oxidase

Cytochromes involved. After improved cultural techniques allowed investigators to obtain high yields of the organisms (40 to 50 mg dry weight per liter), much attention was given to the physiology of the organism, particularly the nitrite oxidase system.

Using cell suspensions which were contaminated with a heterotrophic organism (approximately 2% by cell count), Lees and Simpson (1957) reported that a cytochrome with an absorption maximum at 551 appeared to be intimately associated with nitrite oxidation by Nitrobacter. Aleem and Alexander (1958) obtained cell-free extracts of Nitrobacter which oxidized nitrite to nitrate. When the extract was separated into particulate and soluble fractions by centrifugation at 144,000 x g, all of the nitrite oxidase (67% of the original) was associated with the particles. This particulate nitrite-oxidase was examined for cytochrome participation (Aleem and Nason, 1959). With the addition of nitrite, cell-free extracts showed absorption maxima representative of the alpha and beta peaks of a cytochrome of the c-type (550 and 520 nm) and representative of the alpha and gamma peaks of a cytochrome a-type (585 to 590 nm and 438 nm); therefore, both cytochrome c- and cytochrome a_1-like components appeared to be necessary for nitrite oxidase activity. Since a flavin did not seem to be involved, the transfer of electrons to oxygen via cytochrome c- and cytochrome a_1-like components was proposed as indicated in the following sequence:

$$NO_2^- \longrightarrow \text{cytochrome } c \longrightarrow \text{cytochrome } a_1 \longrightarrow O_2$$

Coupled oxidative phosphorylation. Some of the main problems needing resolution included the thermodynamics of a cytochrome linked oxidation of nitrite and the nature and succession of steps by which electrons are carried from nitrite to oxygen.

In 1960 Aleem and Nason reported that oxidative phosphorylation was linked to nitrite oxidation. The highest phosphate esterification to oxygen uptake (P/O) ratio obtained was 0.2. Attempts at uncoupling the phosphorylation from nitrite oxidation with 2,4 dinitrophenol, thyroxine, and dicumarol in various concentrations from 10^{-6} M to 5×10^{-5} M were unsuccessful. In fact, dinitrophenol at a final concentration of 5×10^{-4} M and thyroxine and dicumarol each at 5×10^{-5} M completely inhibited nitrite oxidation and accompaning phosphorylation.

In a study by Malavolta et al. (1960), the P/O ratio observed from the phosphorylation linked to nitrite oxidation was 0.5. The phosphorylation was uncoupled by 10^{-4} M dinitrophenol with no inhibition of nitrite oxidation. An ATPase was also reported to be active in the cell-free preparations.

Fischer and Laudelout (1965) obtained a maximum P/O ratio of 0.17 for nitrite oxidation by Nitrobacter winogradskyi. Attempts at purifying the particles by centrifugation depressed the ratio below 0.1. Since an active ATPase was present in the cell-free preparations, glucose and hexokinase were added to the reaction system to incorporate the esterified phosphate into glucose-6-phosphate.

Source of $NADH_2$. In 1965 Aleem reported that energy from nitrite oxidation was required for the reduction of nicotinamide-adenine dinucleotide (NAD). The requirement for ATP and $NADH_2$ in the reduction of CO_2 by cell-free extracts of Nitrobacter could be replaced by additions of ADP and NAD if O_2 and NO_2^- were present. The data thus indicated that nitrite oxidation resulted in the generation of not only ATP, but also NADH. It was concluded that some of the ATP generated by nitrite oxidation is required for the reduction of NAD.

The first seemingly satisfactory model for linking the nitrite oxidation to the generation of ATP and NADH was proposed by Kiesow (1964). Under anaerobic conditions, a reversal of the normal reaction of Nitrobacter occurred; nitrate was reduced to nitrite in the presence of $NADH_2$ as the electron donor. A coupled synthesis of ATP occurred with two moles of ATP being formed per mole of NO_3^- reduced.

Dinitrophenol (1.2 x 10^{-3} M) uncoupled this phosphorylation without inhibiting electron transport. Consequently, Kiesow proposed that in the normal reaction, nitrite oxidation, NAD is reduced at the cost of two units of ATP. Since $NADH_2$ was shown to be oxidized aerobically with the formation of three units of ATP per unit of $NADH_2$ oxidized, Kiesow proposed the following mechanism:

$$NADH_2 + \tfrac{1}{2}O_2 + 3ADP + 3P_i \longrightarrow NAD + H_2O + 3ATP$$

$$NO_2^- + 2ATP + NAD + H_2O \longrightarrow NO_3^- + 2ADP + NADH_2 + 2P_i$$

This sequence is essentially cyclic with the products of one reaction being the reactants of the other. The novel feature of this scheme is that the conversion of nitrite to nitrate reduces NAD; while the real source of metabolic energy is the oxidation of $NADH_2$ by an electron transport system in the usual way. The thermodynamic difficulty of this scheme is that the reduction of NAD by nitrite oxidation requires only two ATP units; however, the 750 mv difference in electron potential of the two systems ($NAD/NADH_2$ and NO_2^-/NO_3^-) represents an energy equivalent of 34,500 calories.

A mechanism which is consistent with the thermodynamic considerations of NAD reduction by nitrite oxidation was proposed by Aleem (1968). Contrary to earlier studies (Lees and Simpson, 1957; Aleem and Nason, 1960), Aleem reported that nitrite enters the nitrite oxidase system at cytochrome a_1 instead of cytochrome c. The coupled phosphorylation approached a P/O value of 1.0. These improved P/O ratios were attributed to the culturing techniques employed and the procedure used in the disruption of the intact cells. The ATP generated in the oxidation of nitrite was then shown to cause reduction of NAD by reverse electron flow (Sewell and Aleem, 1969). Since the stoichiometry of the reduction was 5 moles of ATP required per mole of NAD reduced, the results were in agreement with the calculated 35 kcal free energy difference.

Nitrate Reductase

Although much is now known about the nitrite oxidase system in *Nitrobacter*, almost nothing is known about the further metabolism of nitrate. Recently, Straat and Nason (1965) reported the isolation, solubilization, partial purification, and partial characterization of a particulate enzyme from *Nitrobacter* which catalyzes the reduction of nitrate to nitrite. The enzyme, designated as nitrate reductase, was shown to involve the transfer of electrons via the cytochrome pathway of the bacterium and consequently falls into the category of a respiratory-type nitrate reductase. This enzyme differs from most respiratory type reductases, however, because oxygen does not inhibit nitrate reduction. Nitrate reduction was shown not to be merely the reverse of nitrite oxidation in the organism.

Mode of Action of Pesticides

Insecticides. Aldrin, chlordane, and heptachlor are all cyclodienes. At present the specific mode of action of these compounds has not been established, but on the basis of symptomology and the effects of the compounds on isolated nerve endings, the cyclodienes appear to be neurotoxicants (O'Brien, 1967).

Lindane, a hexachlorocyclohexane, likewise appears to be a neuroactive agent as the gross symptoms of poisoning involve tremors, ataxia, convulsions, and prostration (O'Brien, 1967). Also, excessive electrical activity was seen in lindane poisoned axions (Lalonde and Brown, 1954).

No reports were found explaining the mode of action of the chlorinated hydrocarbon DDD. However, since this compound is the reductive dechlorinated analog of DDT, it seems reasonable to assume the modes of action of the two chemicals are similar. Contrary to DDD, DDT has been intensely studied. Although the specific mode of action of DDT has not yet been elucidated, all present evidence indicates that the compound is a neurotoxicant (O'Brien, 1967).

Herbicides. The herbicide CIPC was shown to inhibit protein synthesis in plants at points after the transport of amino acids across the cell membrane (Mann, et al., 1965). It was not determined whether there was inhibition on one of the steps directly involved in polypeptide formation or on the synthesis of one of the necessary cofactors such as ATP or messenger RNA. Mann et al. (1967) later concluded that CIPC inhibited the synthesis of an effector substance needed for germination; messenger RNA seemed likely. Another possibility for the mode of action of CIPC is uncoupling of oxidative phosphorylation. In cabbage mitochondria, CIPC at 10^{-3} M and 10^{-2} M severely inhibited the esterification of inorganic phosphate with a less severe inhibition of oxygen uptake (Lotlikar, et al., 1968).

Ashton (1963) attempted to determine the mode of action of eptam. Long exposure of plants to relatively high concentrations of eptam (100 ug per ml) inhibited $C^{14}O_2$ fixation, but did not alter the relative distribution of C^{14} in the various labeled compounds. When he measured phosphate esterification by cucumber mitochondria, Ashton observed a marked inhibition of both oxygen uptake and phosphorylation at 10^{-2} M eptam with a slight affect at 10^{-3} M. Since the reduction of phosphate esterification was greater than the depression of oxygen uptake, lower P/O ratios were obtained in the presence of the herbicide. Similar results were obtained when oxidative phosphorylation by cabbage mitochondria was measured in the presence of 10^{-3} M eptam (Lotlikar, 1968).

Simazine appears to affect the Hill reaction in susceptible plants. Moreland et al. (1959) found that the toxic effect of simazine for barley could be circumvented by supplying glucose through the leaf-tips of the simazine-treated barley plants. These results were confirmed by Allen and Pramer (1963) who noted that leaf-feeding glucose, sucrose, maltose and aspartate protected the simazine-treated barley. In a study of susceptible and resistant lines of corn, Eastin et al. (1964) also reported protection of the susceptible plants by leaf-feeding glucose and sucrose. This information along with the findings of Moreland et al. (1962) concerning the effect of simazine on isolated chloroplasts of barley and corn supports the theory that the s-triazines inhibit the Hill reaction and thereby stop photosynthetic production of carbohydrates.

Effect of Pesticides on Nitrification

Insecticides. Applications of 200 and 1000 ug of aldrin per gram of soil resulted in a slightly greater nitrite accumulation (Fletcher and Bollen, 1954). In most of the trials nitrate formation was not consistently decreased. Shaw and Robinson (1960) obtained similar results as no effect on nitrification was noted with applications of 10 to 100 lbs of aldrin per acre to a Cumberland silt loam soil. Likewise, Bartha et al. (1967) observed no inhibition of nitrification in laboratory trials at an application rate of 300 ug of aldrin per gram of soil.

Brown (1954) reported significant retardation of nitrification at 500 and 5000 ug chlordane per gram of soil, but Shaw and Robinson (1960) reported that applications of 30 to 300 ug of eptam per gram of soil had no effect on nitrification.

The chlorinated hydrocarbon, DDD, had no detrimental effect on nitrification in either field trials (Eno and

Everett, 1958) at application rates of 12.5, 50, and 100 ug per gram of soil or in laboratory studies (Bartha, et al., 1967) with applications of 300 ug per gram of soil.

In laboratory trials heptachlor did not affect nitrification with additions of the compound equivalent to 200 lb per acre (Shaw and Robinson, 1960), but it did cause a significant decrease in nitrate formation with additions of 5000 ug per gram of soil (Brown, 1954). With field applicants equivalent to 12.5, 50, and 100 ug of heptachlor per gram of soil, a severe decrease in nitrate production was noted at 1 month, but was no longer evident at 16 months (Eno and Everett, 1958).

Although the nitrate concentration was depressed in soil samples taken 1 month after field applications of lindane (100 ug per gram of soil), the nitrate concentration was normal after 16 months (Eno and Everett, 1958). In laboratory trials nitrification was significantly retarded by the addition of 5000 ug of lindane per gram of soil (Brown, 1954), but no effect was observed with 1000 ug of lindane per gram of soil (Bollen et al., 1954).

Herbicides. Using a soil perfusion technique, Quastel and Scholefield (1953) obtained a prolonged lag period (32 days) for nitrate formation by adding CIPC (3.3×10^{-3} M). This inhibition of nitrification by CIPC was confirmed by Bartha et al. (1967) since additions of 300 ug of both CIPC and eptam per gram of soil severely reduced nitrate formation in soil samples.

Conflicting results have been obtained for the effect of simazine on nitrification. Using a perfusion technique, Farmer _et al._ (1965) obtained inhibition of nitrite formation with 6 and 9 ug of simazine per ml of medium. In addition, 6 ug of simazine per ml of medium inhibited the oxidation of nitrite by _Nitrobacter_ in pure culture studies. Contrary to these results, Volk and Eno (1962) observed no significant decrease in nitrate formation with additions of simazine up to 1024 ug per gram of soil. Likewise, Shaw and Robinson (1960) and Bartha _et al._ (1967) have observed no inhibition of nitrification with additions of 100 lb per acre and 300 ug per gram of soil, respectively.

MATERIALS AND METHODS

Pestioides

Insecticides. The five insecticides investigated in this study were: 1, 2, 3, 4, 10, 10-hexachloro-1, 4, 4a, 5, 8, 8a-hexahydro-1, 4-*endo*-*exo* 5, 8-dimethanonaphthalene (aldrin); 1, 2, 3, 5, 6, 7, 8, 8-octachloro-2, 3, 3a, 4, 7, 7a-hexahydro-4, 7- methanoindene (chlordane); 1, 4, 5, 6, 7, 8, 8-heptachloro-3a, 4, 7, 7a-tetrahydro-4, 7-*endo*-methanoindene (heptachlor); gamma isomer of 1, 2, 3, 4, 5, 6-hexachlorocyclohexane (lindane); and 1, 1-dichloro-2, 2,-bis (*p*-chlorophenyl) ethane (DDD). Based on chemical configuration the three groups of insecticides represented are: (i) cyclodiene (aldrin, chlordane, and heptachlor), (ii) hexachlorocyclohexane (lindane); and (iii) chlorinated hydrocarbon (DDD) groups.

Herbicides. Isopropyl N-(3-chlorophenyl) carbamate (CIPC), S-ethyl di-N, N-propyl-thiocarbamate (eptam), and 2-chloro-4, 6-bis (ethyl-amino)-*s*-triazine (simazine) are the three herbicides which were investigated and they belong to the phenylcarbamate, thiolcarbamate, and s-triazine groups respectively.

Medium

Nitrobacter agilis (ATCC 14123), kindly supplied by David Pramer, Rutgers University, New Brunswick, N. J., was grown in the clear inorganic medium of Aleem and Alexander (1960) supplemented with $NaMoO_4 \cdot 2H_2O$ as suggested by Finstein and Delwiche (1965). The basal medium contained 0.3 g $NaNO_2$, 0.175 g K_2HPO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.1 g NaCl, 1.5 g $KHCO_3$, 35 ug $FeSO_4 \cdot 7H_2O$, and 25 ug $NaMoO_4 \cdot 2H_2O$ in 1.0 l of water.

For large-scale cultures, the medium was modified to include 1 mg $FeSO_4 \cdot 7H_2O$ and 10 mg $CaCl_2$ per l of medium. Solutions of potassium phosphate, potassium bicarbonate, and calcium chloride were each autoclaved separately and then added to the other constituents in order to obtain a clear medium.

Growth Effects

To determine the effect of the pesticides on growth of N. agilis, the chemicals were dissolved in acetone and 0.1 ml was added to 50 ml of sterile medium in a 250 ml Erlenmeyer flask. For simazine only, 12.5 mg of the dry chemical was added to the culture flasks. Control flasks received 0.1 ml of acetone. Inocula (1% v/v) were taken from log phase cultures which had oxidized 80 ug of nitrite-N per ml. The cultures were incubated at 30 C on a rotary shaker. Growth was determined every 12 hr by measuring the disappearance of nitrite by the method of Rider and Mellon

(1946). The range of pesticide concentrations tested was 1 to 250 ug added per ml and the duration of the experiments was 9 days.

Large-Scale Cultivation

Before acquisition of a MF-14 microferm fermentor (New Brunswick Scientific Co.) cell suspensions and cell-free extracts were obtained by cultivating N. *agilis* at room temperature in glass carboys containing 18 liters of medium. Air from the laboratory supply line was sterilized by passage through concentrated sulfuric acid. Inocula of 1% (v/v) were taken from 8-day stock cultures which had oxidized 200 ug nitrite-N/ml. Samples from the large cultures were assayed every 12 hours for nitrite by the method of Rider and Mellon (1946). Sodium nitrite was added as needed and 10 liters of culture (about 1 g wet weight of cells) were removed and replaced by sterile medium after 1500 ug NO_2-N/ml had been oxidized. Cells were harvested at 4 C by continuous-flow centrifugation (Sorvall Model SS-1 centrifuge equipped with the Szent-Gyorgyi and Blum continuous flow system). The sedimentated cells were washed three times with cold distilled water and then suspended in 0.1 M tris (hydroxymethyl) aminomethane hydrochloride (Tris) buffer (pH 8.0) at a concentration of 1 g (wet weight) per ml of buffer.

When the MF-14 microferm was used, 400 ml cultures which had oxidized 1 mg of nitrite-nitrogen per ml were used as inocula for 10 liters of medium. The fermentor was operated at 30 C with an aeration rate of 6 liters of air per min and a propeller speed of 200 r.p.m. Additional sodium nitrite (10 or 20 grams) was added to the cultures as needed. After oxidation of about 3.6 mg of nitrite-nitrogen per ml, cultures were harvested by continuous flow centrifugation. The usual yield obtained after 7 to 9 days of active growth was approximately 3 g wet weight of cells from the 10 liters of culture.

Preparation of Cell-Free Extracts

Cell pastes were suspended in either 0.1 M tris (hydroxy-methyl) amino methane (Tris)-hydrochloride buffer (pH 8.0) or the slightly modified isotonic sonication medium of Aleem (1968) at a concentration of 1 g wet weight of cells per ml of buffer. The isotonic medium contained 50 mM Tris-HCl buffer (pH 8.0), 250 mM sucrose, and 1 mM reduced glutathione.

Cell-free extracts were prepared by sonic disruption of the cells for 3 min (30 sec of sonic treatment alternated with 30 sec of cooling) with a 100-w ultrasonic disintegrator (Measuring and Scientific Equipment, Ltd., London, England). The debris was removed by centrifugation at either 15,000 x g for 15 min or 40,000 x g for 20 min in a Sorvall (model RC-2) refrigerated centrifuge (Ivan Sorvall, Inc., Norwalk, Conn.) and the supernatant fluid was stored at 4 C.

Purity of Cultures

Since *Nitrobacter* cannot grow on heterotrophic media, all cultures were routinely examined for heterotrophic contaminants by inoculating nutrient broth in quadruplicate and incubating one pair at 30 C and the other at 37 C for 7 days. In addition, each of the large-scale cultures was tested for purity by inoculation of (i) nutrient agar, (ii) nutrient agar with 1% yeast extract, (iii) Czapek Dox solution agar, (iv) basal salt medium (see page 20) with agar and 0.5% yeast extract, (v) basal salt medium with agar, nutrient broth (0.1%) and glucose (0.2%). Duplicate plates were incubated for 7 days at 30 C and another set was incubated at 37 C. Absence of growth was used as the criterion for purity and whenever a culture was found to be contaminated, it was discarded.

Oxidation of Nitrite

The effect of pesticides on nitrite oxidation was determined in the Warburg respirometer. The cell suspensions and cell-free extracts were diluted so that 50 to 60 ul of O_2 were consumed per hour. Usually, approximately 1.3 mg dry weight of cells and 1.0 to 2.5 mg of protein were required per Warburg vessel. The reaction system contained 0.1 ml of either diluted cell suspension or diluted cell-free extracts, 0.5 ml of 2×10^{-2} M $NaNO_2$, 0.3 ml of 1 M Tris buffer (pH 8.0), and 1.9 ml deionized, distilled water.

The center well contained 0.2 ml of 20% (w/v) KOH. Pesticides, dissolved in acetone, were normally added to the Warburg vessels in 10 ul or less. The control flasks received an equivalent volume of acetone. The reaction was started by tipping the nitrite from the side arm and the initial oxidation rates were determined after subtracting the endogenous oxidation. These initial activities obtained in the presence and absence of pesticides were compared.

Nitrate Reductase

The procedure of Straat and Nason (1965) was modified for determining the nitrate reductase activity. In a total of 1.0 ml the reaction mixture contained 0.5 ml of 0.1 M acetate buffer (pH 6.0), 0.2 ml of 0.1 M KNO_3, 0.1 ml of suitably diluted cell-free extract, 0.15 ml of 1% (v/v) mammalian cytochrome c (Sigma Chemical Co., St. Louis, Mo.) and 0.25 ml of 2×10^{-5} M sodium ascorbate. The reaction, maintained at 30 C, was started by addition of KNO_3; the nitrite formed was measured at the end of 10 min. Briefly, the nitrite was measured by first stopping the reaction with the addition of 0.5 ml of 1% (w/v) sulfanilic acid in 2 N HCl. Denatured protein was then removed by centrifugation and 0.5 ml of 0.12% (w/v) N-(naphthyl)ethylenediamine dihydrochloride was added. The volume was adjusted to 2.4 ml with distilled water and the absorbance at 540 nm was determined after 10 minutes. Amounts of nitrite ranging from 10^{-3} to 10^{-1} umoles contained in the final volume of 2.4 ml were used for preparation of a standard curve.

$NADH_2$ Oxidase

Spectrophotometric assay. Reduced nicotinamide adenine dinucleotide ($NADH_2$) oxidase was measured by the method of Smith and Hoare (1968). The assay solution contained 0.1 ml of $NADH_2$ (10^{-3} M), 0.1 ml of 0.5 M $MgCl_2$, 0.1 ml of 1 M Tris (pH 8.0), appropriate dilutions of enzyme, and distilled water to 1.0 ml. The decrease in absorbance of $NADH_2$ at 340 nm produced by the cell-free extract was either measured at 30-sec intervals for a 10-min period with a Beckman model DU spectrophotometer or measured by a Gilford-2000 multiple sample absorbance recorder attached to a Beckman DU spectrophotometer. The initial activities were calculated as the decrease in absorbance per min. Since the pesticides were added in acetone, the control cuvettes received an equal volume of acetone (usually 10 ul or less). Activities were corrected by subtracting the slight decrease in absorbance which occurred in the absence of cell-free extract.

Respirometer assay. Cell-free extracts were measured for $NADH_2$ oxidase activity and concomitant esterification of inorganic phosphate in the Warburg respirometer. Oxygen uptake was determined by the usual Warburg technique and esterification of inorganic phosphate was measured in the same reaction vessel. In a final volume of 2.3 ml the main compartment of the Warburg contained 50 umoles glucose, 15 umoles $MgCl_2$, 2 umoles ADP, 150 umoles Tris-hydrochloride buffer (pH 8.0), 20 umoles K_2HPO_4-^{32}P(0.3 ucurie), 1 mg hexokinase, and 4.4 mg N. agilis cell-free extract. Addition of 0.5 ml $NADH_2$ (20 umoles) from the side arm started the reaction. The center well contained 0.2 ml of 20% KOH (w/v).

Cytochrome C Oxidase

To measure cytochrome c oxidase activity, the following reaction system was used: 0.1 ml of 1.0 M Tris buffer (pH 8.0), 0.1 ml cell-free extract (0.36 mg protein), 0.3 ml of 1% (w/v) cytochrome c, 0.3 ml of 2×10^{-5} M sodium ascorbate, and 0.3 ml of deionized, distilled water. The reaction was started by addition of cell-free extract and the decrease in absorbance was measured at 550 nm with a Gilford-2000 multiple sample absorbance recorder attached to a Beckman model DU spectrophotometer. Initial activities were corrected by subtracting the decrease in absorbance which occurred in separate cuvettes without the enzyme. Because the pesticides were added in acetone, an equivalent amount of acetone was added to the control cuvettes.

Oxidative Phosphorylation Coupled To Nitrite Oxidation

Assay by K_2HPO_4-^{32}P incorporation. Oxidative phosphorylation coupled to nitrite oxidation by Nitrobacter agilis was tested by slight modifications of the method of Fischer and Laudelout (1965). Oxygen uptake was determined by the usual Warburg technique and esterification of inorganic phosphate was measured in the same reaction flask. The reaction system contained 50 umoles glucose, 15 umoles $MgCl_2$, 2 umoles ADP, 150 umoles tris(hydroxy-methyl) aminomethane (Tris)-hydrochloride buffer (pH 8.0), 20 umoles K_2HPO_4-^{32}P (0.3 ucurie), 1 mg hexokinase, and 1 to 2 mg N. agilis cell-free extract in 2.3 ml. The reaction was started by addition of 0.5 ml $NaNO_2$ (20 umoles) from the side arm.

After 60 minutes incubation at 30 C, the reaction was stopped by addition of 0.5 ml of 20% ice cold trichloroacetic acid. After removal of denatured protein by centrifugation, inorganic phosphate was separated from the organic phosphate by the method of Rose and Ochoa (1956). This separation procedure involved adding 0.5 ml of 10 N H_2SO_4 and 1.0 ml of 5% (w/v) ammonium molybdate to an aliquot of protein-free supernatant (0.4 to 0.8 ml). After the samples were diluted to 5 ml with distilled water, 5 ml of isobutanol were added. The samples were mixed well and allowed to stand for 15 min before discarding the upper isobutanol layer. The sides of the tubes were washed with 2.0 ml of isobutanol, and the upper layer was again removed. Extracted samples (1.0 ml) were taken to 15 ml final volume with the scintillation fluid described by Bray (1960) and were then assayed for radioactivity in a Model 6860 liquid scintillation system (Nuclear-Chicago). Since more quenching occurred in extracted samples than occurred in unextracted samples, the results were calculated as disintegrations per min by employing internal standards.

Glucose-6-phosphate dehydrogenase assay. In some instances, esterification of inorganic phosphate was determined by a glucose-6-phosphate dehydrogenase assay (Pinchot, 1953). The phosphorylation reactions were stopped by addition of 0.1 ml of 35% (w/v) $HClO_4$. After being stored in ice for 30 min, the solutions were neutralized by addition of 0.1 ml of 3.5 N KOH. Denatured protein was removed by centrifugation and the glucose-6-phosphate concentrations were determined by assaying samples (0.2 ml) in a reaction system containing 100 umoles Tris (pH 7.4), 10 umoles $MgCl_2$, 2 umoles EDTA, and 1 umole NADP. The reaction was started by addition of 0.5 units of glucose-6-phosphate dehydrogenase (Sigma Chemical Co.). The umoles of glucose-6-PO_4 present in the samples were determined from the increase in absorbance at 340 nm which occurred in 10 minutes. The molar extinction coefficient of NADPH at 340 nm is 6.22×10^3.

Analytical Methods

Hexokinase. Hexokinase (Sigma Chemical Co., St. Louis, Mo.) was assayed by the method of Darrow and Colowick (1962).

Protein. Protein concentrations were estimated by the method of Lowry *et al*. (1951). Crystallized, bovine fraction V albumin (Sigma Chemical Co.) was used as protein standard.

RESULTS

Growth Effects

Each pesticide was tested to determine its effect on the growth of *N. agilis* in aerated cultures. The range of pesticide concentrations tested was 1 to 250 ug/ml and the duration of the experiment was 9 days. The results of this experiment are illustrated in Table 1. Only two compounds, aldrin and simazine, were not inhibitory under the conditions of the experiment. The other six compounds: lindane, DDD, chlordane, heptachlor, CIPC, and eptam prevented nitrite oxidation for 9 days, while control flasks exhausted the nitrite within 3 days. Based on the concentration of pesticide required for 100% inhibition, the compounds were arranged in the following order: DDD (5 ug/ml), chlordane (5 ug/ml), CIPC (10 ug/ml), heptachlor (10 ug/ml), lindane (10 ug/ml), and eptam (75 ug/ml).

Oxidation Studies with Cell Suspensions and Cell Free Extracts

Cells grown in 18-liter carboys. The possible effect of growth inhibitors upon the nitrite oxidase activity of *N. agilis* was studied in the Warburg respirometer by measuring the nitrite oxidized by whole cell suspensions. When cells

TABLE 1. Effect of pesticides on growth of *Nitrobacter agilis* in aerated cultures.[a]

Pesticide	Concn (ug/ml)	NO_2-N remaining (ug/ml) after indicated days				Range of inhibition ug/ml[b]	
		3	5	7	9	Complete	Delayed
Aldrin	250	42	20	0	0		
Chlordane	10	42	42	42	42	5	
	5	42	42	42	42		
	1	27	0				
CIPC	25	42	42	42	42	10	5
	10	42	42	42	42		
	5	42	22	18	0		
DDD	10	42	42	42	42	5	1
	5	42	42	42	42		
	1	38	9	0	0		
Eptam	75	42	42	42	36	75	25-75
	50	42	42	31	20		
	25	42	42	17	17		
	10	42	20	0	0		

TABLE 1. continued

Pesticide	Concn (ug/ml)	NO_2-N remaining (ug/ml) after indicated days				Range of inhibition ug/ml[b]	
		3	5	7	9	Complete	Delayed
Heptachlor	25	42	42	42	42	10	5
	10	42	42	42	42		
	5	42	32	22	0		
Lindane	10	42	42	42	42		
	5	42	42	14	1	10	5
Simazine	250	32	10	0	0		
Control		25	0	0	0		

[a] Initial nitrite concentration in all flasks was 42 ug NO_2^--N per ml.

[b] This was the amount added per ml of medium. In most cases it was above the solubility level.

were grown in 18-liter carboys at room temperature, approximately 1 g wet weight of cells was obtained from 10 liters of medium after two to three weeks of growth. Upon the addition of aldrin, DDD, CIPC, eptam, heptachlor, and simazine to these cell suspensions, no effect on nitrite oxidation was observed with aldrin and simazine (250 ug per ml), but DDD, CIPC, eptam, and heptachlor were inhibitory (Fig. 1). CIPC at 90 ug/ml inhibited nitrite oxidation by 56%, and at 180 ug/ml caused 100% inhibition. Eptam caused 52% and 100% inhibition at 180 ug/ml and 270 ug/ml, respectively. The oxidation rate was reduced by 45% with heptachlor concentrations from 180 to 540 ug/ml, and only 58% at 720 ug/ml. The compound DDD was one of the most inhibitory pesticides in aerated cultures since no growth occurred at 5 ug/ml, but in the Warburg study only 54% inhibition resulted with 540 ug/ml.

The effects of pesticides on nitrite oxidation by cell-free extracts (Fig. 2) were similar to the results obtained with cell suspensions. CIPC completely inhibited nitrite oxidation at 180 ug/ml with 49% inhibition occurring at 90 ug/ml. DDD caused only 59% inhibition at 430 ug/ml. Heptachlor was slightly inhibitory with 33% inhibition at 535 ug/ml. Eptam was similar to CIPC as no oxidation was observed at 360 ug/ml and 33% inhibition occurred at 180 ug/ml.

Cells grown in MF-14 microferm fermentor. After acquiring an MF-14 microferm fermentor, we noted that the

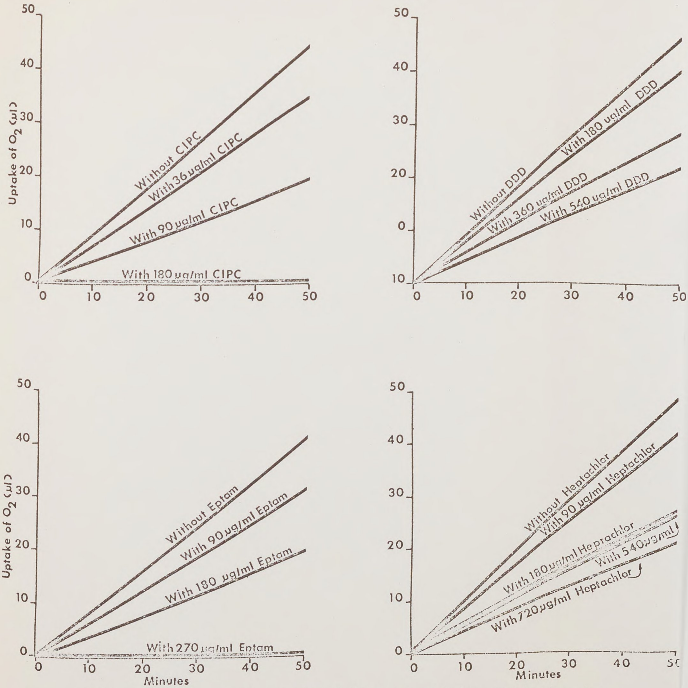

FIG. 1. The effect of pesticides on nitrite oxidation by <u>Nitrobacter</u> <u>agilis</u> cell suspensions.

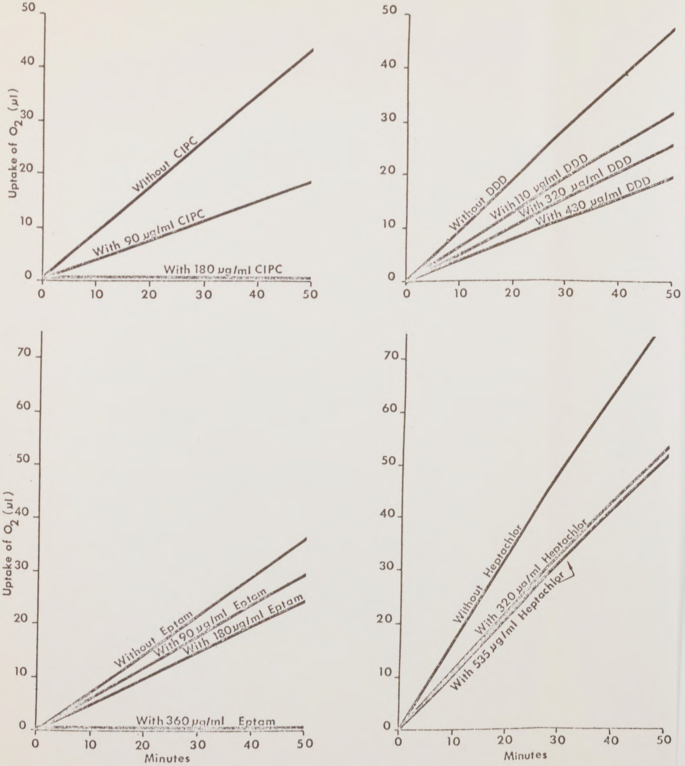

FIG. 2. The effect of pesticides on nitrite oxidation by <u>Nitrobacter agilis</u> cell-free extracts.

improved aeration and temperature control, as compared with the 18-liter carboys, resulted in a significant increase in Nitrobacter cell yields. About 3 g wet weight of cells were now obtained after 7 to 9 days growth. In addition, the stability of the cell suspensions was maintained by suspending the cells in the isotonic medium of Aleem (1968) in which the oxidase activity was stable for 7 days at 5 C and for over 2 weeks at -20 C. When the cells were suspended in 0.1 M Tris-hydrochloride buffer (pH 8.0), the nitrite oxidase activity normally decreased by 50% within 4 days at 5 C.

In order to determine whether the improved growth rate altered the effects of pesticides for cell suspensions and cell-free extracts, the earlier experiments were repeated (Table 2). Of the four pesticides previously tested, heptachlor was the only compound which affected both cell suspensions equally, whereas the other three chemicals were more inhibitory for the nitrite oxidase from the cells grown in the fermentor.

In spite of the increased inhibition in this latter experiment, high concentrations of the pesticides were still required for significant inhibition of the nitrite oxidation. Of the six pesticides which inhibited growth, only CIPC and eptam appreciably affected the nitrite oxidase activity of cell suspensions (Table 2). However, 110 ug/ml CIPC and 275 ug/ml eptam were required for total inhibition of oxidation,

TABLE 2. Effect of pesticides on nitrite oxidation by cell suspensions and cell-free extracts of *Nitrobacter agilis*.

Whole Cell Suspensions[a]						Cell-Free Extracts[b]					
		Oxygen Uptake		Nitrite Consumed				Oxygen Uptake		Nitrite Consumed	
Pesticide	µg/ml added	Q_{O_2}	Relative Activity	µmoles nitrite	Relative Activity	Pesticide	µg/ml added	Q_{O_2}	Relative Activity	µmoles nitrite	Relative Activity
			%		%				%		%
None		48.0	100	4.57	100	None		44.2	100	4.1	100
CIPC	110	0.0	0	0.0	0	CIPC	125	0.0	0	0.0	0
	100	8.5	18	0.23	5		100	0.0	0	0.35	9
	90	8.0	17	0.51	11		50	36.9	84	3.65	89
	75	12.8	27	0.83	18						
	50	25.4	53	2.43	53						
Chlordane	500	9.6	20	1.14	25	Chlordane	250	0.0	0	0.3	7
	250	15.8	33	2.1	46		200	5.4	12	0.4	10
	100	29.6	62	3.02	66		150	24.3	55	1.8	44
DDD	500	11.5	24	1.59	35	DDD	500	20.0	46	2.29	56
	375	17.6	37	1.44	31						

TABLE 2. continued.

Whole Cell Suspensions[a]						Cell-Free Extracts[b]					
		Oxygen Uptake		Nitrite Consumed				Oxygen Uptake		Nitrite Consumed	
Pesticide	µg/ml added	Q_{O_2}	Relative Activity	µmoles nitrite	Relative Activity	Pesticide	µg/ml added	Q_{O_2}	Relative Activity	µmoles nitrite	Relative Activity
			%		%				%		%
DDD (cont.)	250	20.1	42	1.8	39						
	125	33.8	70	2.66	58						
Eptam	275	0.0	0	0.0	0	Eptam	275	0.0	0	0.5	12
	250	9.6	2	0.57	13		250	12.1	27	0.47	12
	225	9.0	19	0.53	12		150	31.0	70	2.71	66
	200	10.2	21	1.37	30						
	150	20.6	43	2.17	47						
Heptachlor	500	21.0	44	2.47	54	Heptachlor	250	0.0	0	0.1	2
	250	30.0	63	3.0	66		200	0.0	0	0.3	7
	125	32.2	67	3.8	83		150	18.8	43	1.11	27
Lindane	500	22.4	47	1.78	39	Lindane	500	33.6	76	3.4	83
Simazine[c]	250	48.0	100	4.65	102						

TABLE 2. continued.

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[a] Oxidation rate, expressed as ul O_2 and as umoles nitrite consumed per mg dry weight of cells per hour.

[b] Oxidation rate, expressed as ul O_2 and as umoles nitrite consumed per mg protein per hour.

[a,b] Each vessel contained 0.1 ml of either cells (1.21 mg dry weight) or cell-free extract (1.06 mg protein), 0.5 ml of 2.0 x 10^{-2} M $NaNO_2$ (10 umoles), 0.3 ml of 1 M Tris buffer (pH 8.0) and 1.9 ml of distilled water. The pesticides were added in 10 ul acetone. Controls received 10 ul acetone and endogenous rates were determined in the presence of acetone (10 ul) and also with the pesticides.

[c] Simazine was added to Warburg vessels in 2 ml of methanol and evaporated to dryness at 50 C. Control vessels received an equivalent volume of methanol.

but only 10 ug of CIPC per ml and 75 mg of eptam per ml were required for cessation of growth (Table 1).

The nitrite oxidase activity of cell-free extracts was then tested in the presence of pesticides. Eptam, CIPC, DDD, and lindane affected both cell suspension and cell-free extract activities from fermentor grown cells similarly (Table 2), but heptachlor and chlordane were more inhibitory for the nitrite oxidase in the cell-free extracts. The extract activity was completely inhibited by the addition of 250 ug of chlordane per ml and 200 ug of heptachlor per ml, but the nitrite oxidase of cell suspensions was inhibited by only 50% and 75-80% with 500 ug of heptachlor and chlordane respectively. Neither DDD nor lindane at 500 ug/ml caused cessation of nitrite oxidation by either cell suspension or cell-free extracts.

When nitrite oxidase activity was measured by nitrite disappearance, the results were in close agreement with the oxygen consumption data (Table 2). Slight differences were noted, but these were expected since oxygen uptake was calculated as initial rate whereas the nitrite concentration was determined at the end of the 1-hr assay period. In some instances the initial oxidation rate was zero, but a small amount of oxygen was consumed before completion of the assay.

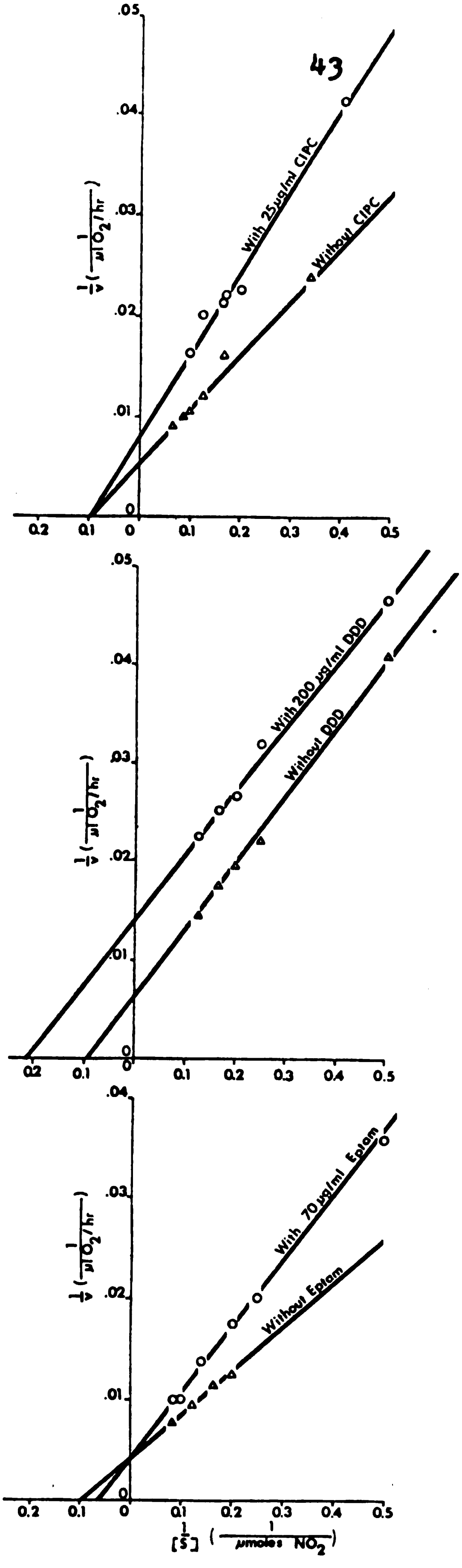
Kinetics of Oxidation

Using cell suspensions, three pesticides, CIPC, eptam, and DDD were selected for further study in the respirometer. The amount of the pesticides added were: 25 ug/ml of CIPC, 70 ug/ml of eptam, and 200 ug/ml of DDD. The concentrations of nitrite added to the Warburg flasks varied from 1.5 to 12 umoles. The types of inhibition which occurred (Fig. 3) were noncompetitive, competitive, and uncompetitive with CIPC, eptam, and DDD respectively. The Michaelis constant (K_m) for the nitrite oxidation without pesticides was 3.76 mM, while the Michaelis constants for the inhibited reactions were: CIPC, 3.76 mM; eptam, 5.49 mM; and DDD, 1.68 mM.

Nitrate Reductase

Although the metabolism of *Nitrobacter* depends on the oxidation of nitrite to nitrate, the microorganism contains an enzyme capable of reducing nitrate to nitrite (Straat and Nason, 1965). Since these authors have established that the reduction is not merely the reverse of nitrite oxidation, the enzyme could have physiological importance. When the nitrate reductase activity in cell-free extracts of *Nitrobacter* was measured with each of the pesticides which affected the nitrite oxidase activity, no deleterious effect occurred with any of the compounds up to 500 ug per ml. A representative value for the nitrite produced by the cell-free extracts during the 10 min reaction was 0.025 umoles per mg of protein.

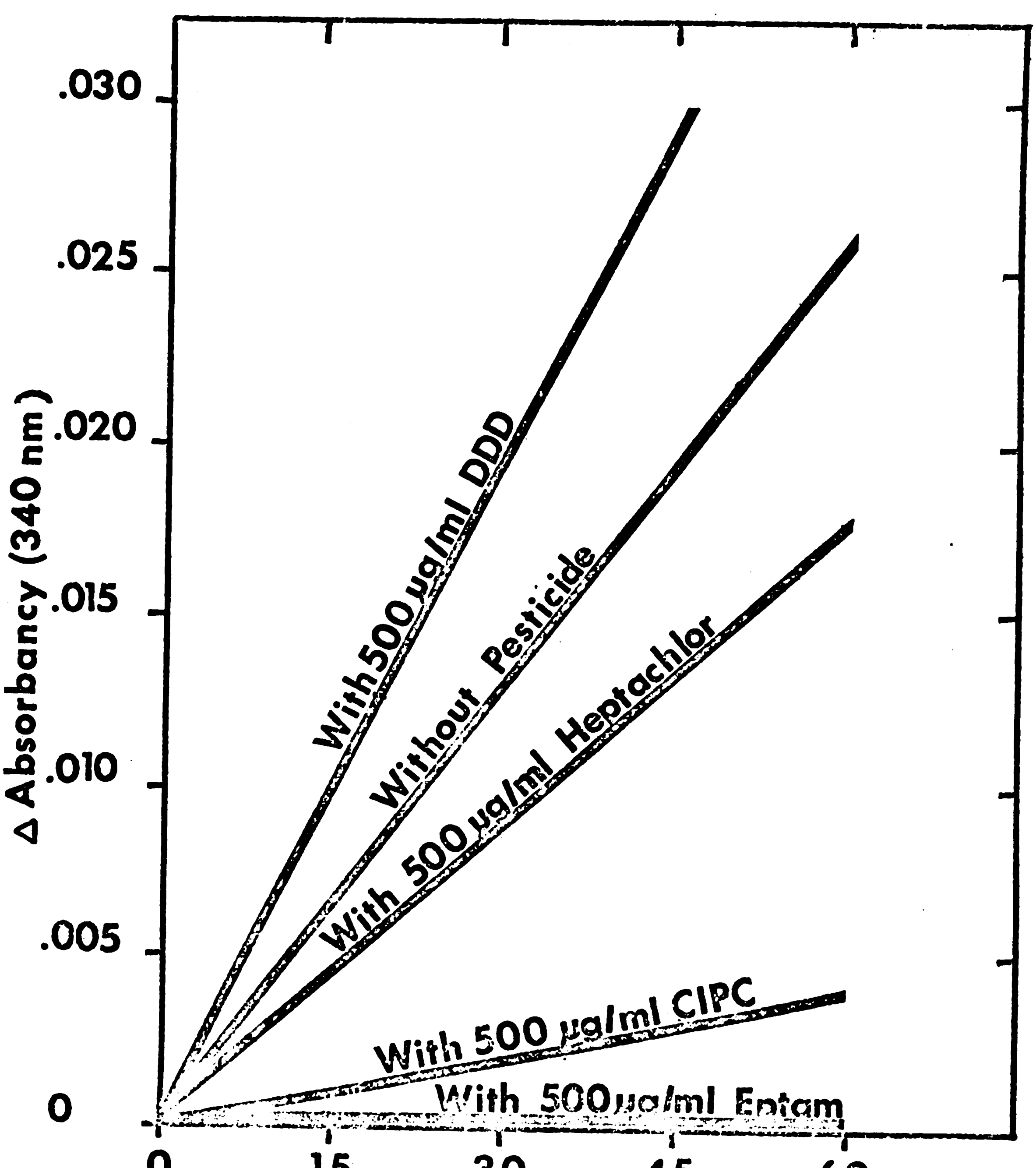
FIG. 3. The nature of the inhibition of nitrite oxidation by three different pesticides. Each Warburg flask contained 0.1 ml of *Nitrobacter agilis* cell suspensions (1.3 mg dry weight), 0.5 ml of $NaNO_2$ (2 to 12 umoles), and 0.3 ml of Tris buffer (pH 8.0). The center well contained 0.2 ml of 20% (w/v) KOH.


.05
.04
.03
.02
.01
0
$\frac{1}{v}\left(\frac{1}{\mu l\ O_2/hr}\right)$
With 25 µg/ml CIPC
Without CIPC
0.2
0.1
0
0.1
0.2
0.3
0.4
0.5
.05
.04
.03
.02
.01
0
$\frac{1}{v}\left(\frac{1}{\mu l\ O_2/hr}\right)$
With 200 µg/ml DDD
Without DDD
0.2
0.1
0
0.1
0.2
0.3
0.4
0.5
.04
.03
.02
.01
0
$\frac{1}{v}\left(\frac{1}{\mu l\ O_2/hr}\right)$
With 70 µg/ml Eptam
Without Eptam
0.2
0.1
0
0.1
0.2
0.3
0.4
0.5
$\frac{1}{[S]}\left(\frac{1}{\mu moles\ NO_2}\right)$

$NADH_2$ Oxidase

Although Smith and Hoare (1968) postulated that NADH oxidase is not involved in the autotrophic growth of *Nitrobacter*, Aleem (1968) showed that $NADH_2$ oxidation in *Nitrobacter* proceeds by an electron transport chain analogous to the mitochondrial respiratory chain reported by Chance *et al*. (1967). Consequently, the $NADH_2$ oxidase activity was measured in the presence of pesticides to obtain an indication of electron transport inhibition. The results of the preliminary experiment are shown in Fig. 4. At 500 ug pesticide per ml, heptachlor did not affect the reaction; DDD stimulated the oxidase activity; but CIPC and eptam were inhibitory (67% and 100% respectively). The experiment was repeated with various concentrations of eptam to determine the minimum concentration causing complete inhibition. As illustrated in Fig. 5 this phenomenon occurred with 375 ug eptam per ml. However, since the change in absorbance was only 0.05 per min per mg of protein, it was possible that NADH was reducing flavoproteins instead of the electron transport chain. To test this possibility, the reaction was repeated with another cell-free extract. A decrease in absorbance of 0.18 per mg of protein was noted at 10 min, but this activity was not affected by the addition of 10 umoles azide. A slightly increased rate (△ optical density of 0.25 per mg protein) was obtained by additions of either flavin mononucleotide (FMN) or flavin adenine dinucleotide (FAD)

FIG. 4. The effect of pesticides on $NADH_2$ oxidase activity of Nitrobacter agilis cell-free extracts. The reaction system contained 10^{-3} M $NADH_2$, 0.1 ml; 0.5 M $MgCl_2$, 0.1 ml; 1 M Tris buffer (pH 8.0), 0.1 ml; cell-free extract, 0.02 ml (0.49 mg protein); and water to 1.0 ml. Pesticides were added in 10 ul acetone. Decrease in absorbance of $NADH_2$ was measured at 340 nm.


.030
.025
.020
.015
.010
.005
0
Δ Absorbancy (340 nm)
0
15
30
45
60
Seconds
With 500 µg/ml DDD
Without Pesticide
With 500 µg/ml Heptachlor
With 500 µg/ml CIPC
With 500 µg/ml Eptam

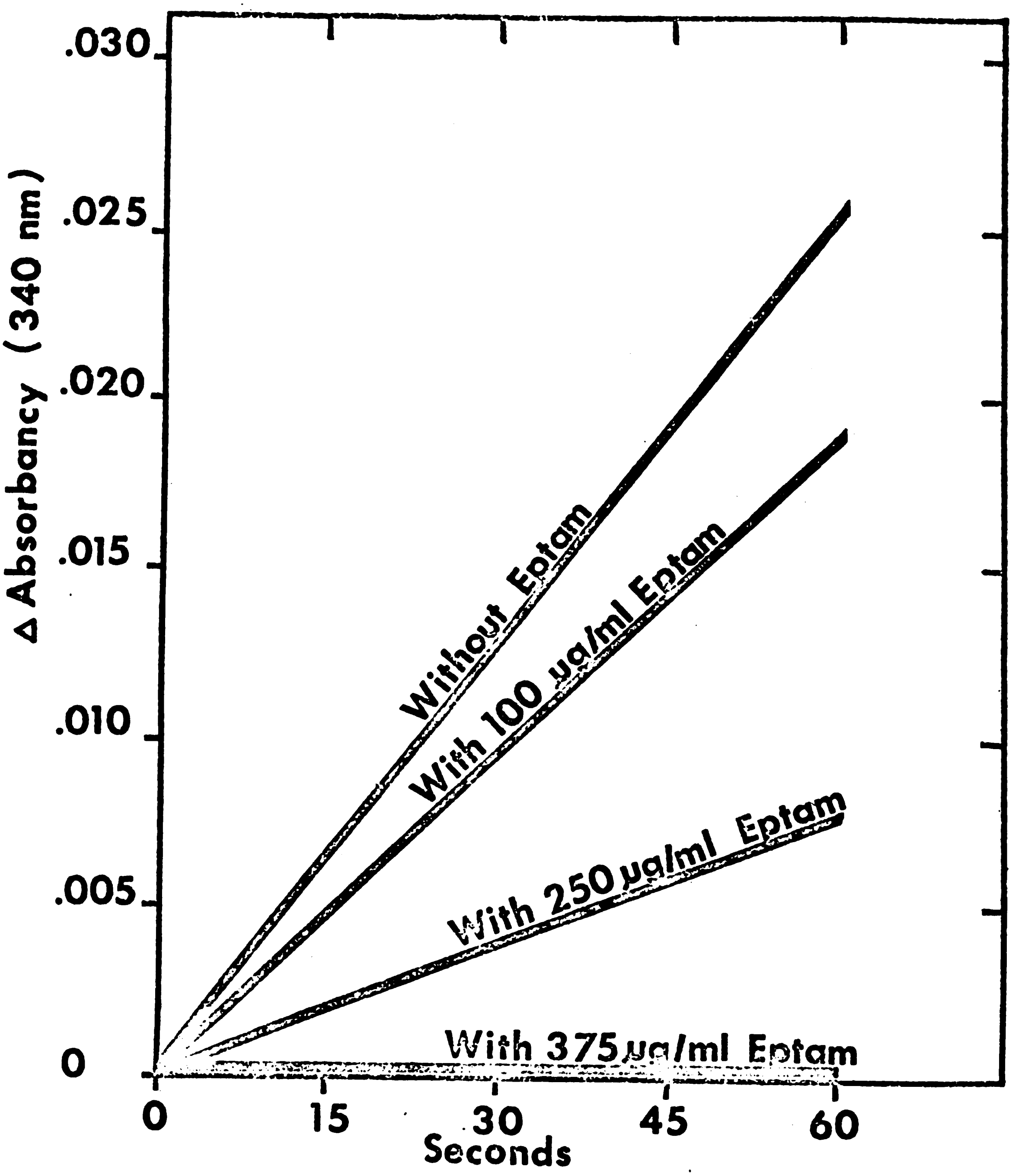


FIG. 5. The effect of eptam on $NADH_2$ oxidase activity of *Nitrobacter agilis* cell-free extracts. Conditions were the same as those described on Fig. 4.

at 2 x 10^{-4} M final concentration, but control cuvettes with azide (10 umoles) had equivalent rates. Likewise, using a phosphate buffer (0.05 M) at pH 7 and 8 resulted in no apparent $NADH_2$ oxidase activity; consequently, the absorbance decrease observed at 340 nm was probably caused by the reduction of flavoproteins.

In support of this conclusion was the observation of diaphorase activity in the cell-free extracts with 2,6-dichlorophenolindophenol (1.2 x 10^{-4} M) as the electron acceptor. A decrease in absorbance of 6.6 per min per mg of protein was noted at 600 nm.

Cytochrome C Oxidase

Pesticides which affected the nitrite oxidase activity of cell-free extracts were tested for an effect on cytochrome c oxidase. Most of the pesticides tested inhibited cytochrome c oxidase to some degree but none completely suppressed the activity (Table 3). Heptachlor, the most inhibitory, decreased cytochrome c oxidation by 76% at 500 ug/ml, but completely suppressed nitrite oxidase at 200 ug/ml (Table 2). At 500 ug/ml, DDD caused a similar effect on both oxidases as relative activities of 56% and 66% were obtained for nitrite oxidase and cytochrome c oxidase, respectively. Eptam was unique in this series of compounds because a slight, but reproducible, stimulation of cytochrome c oxidase was noted.

TABLE 3. Effect of pesticides on cytochrome c oxidase.[a]

Compound	Concentration (final)	Activity[b]	Relative Activity
	ug/ml		%
Control	---	0.139	100
Chlordane	500	0.081	58
CIPC	500	0.069	50
DDD	500	0.092	66
Eptam	500	0.167	120
Heptachlor	500	0.033	24

[a] Reaction mixture contained 0.1 ml of 1.0 M Tris buffer (pH 8.0), 0.1 ml cell-free extract (0.36 mg protein), 0.3 ml of 1% (w/v) cytochrome c, 0.3 ml of 2×10^{-5} M sodium ascorbate, and 0.3 ml distilled water. Sodium cyanide (10 umoles) completely inhibited the reaction.

[b] Activity expressed as decrease in absorbance at 550 nm per min per mg of protein.

Oxidative Phosphorylation Coupled To Nitrite Oxidation

Preparation of cell-free extracts. Aleem (1968) recently reported P/O ratios which approached 1.0 for the esterification of inorganic phosphate coupled to nitrite oxidation in *Nitrobacter agilis*. Aleem felt the high P/O ratios were caused by two important factors: (i) the cultural conditions of the organism and (ii) the procedures used for disruption of the cells. He stated that actively growing cultures must oxidize three additions of 20 mM nitrite per day for at least two days or the P/O ratios will be low. The cells obtained from such cultures were suspended in an isotonic sucrose-EDTA-GSH solution and disrupted by sonication for 3 min.

In accordance with these suggestions, only cultures which oxidized at least 60 mM nitrite per day for two or more days were used for phosphorylation studies. The turbidity (420 nm) and nitrite oxidation of such cultures during growth is illustrated in Fig. 6.

The generation time (27.6 hrs) was calculated from the time required for a doubling of absorbancy during the active phase of growth. When the turbidity (420 nm) was measured as a function of the nitrite oxidized (Fig. 7), the growth efficiency (ratio of absorbancy increase per unit of nitrite consumed) appears to decrease after oxidation of about 90 mmoles of nitrite per liter. The cells obtained from these cultures were suspended in the isotonic medium and sonicated for 3 mins.

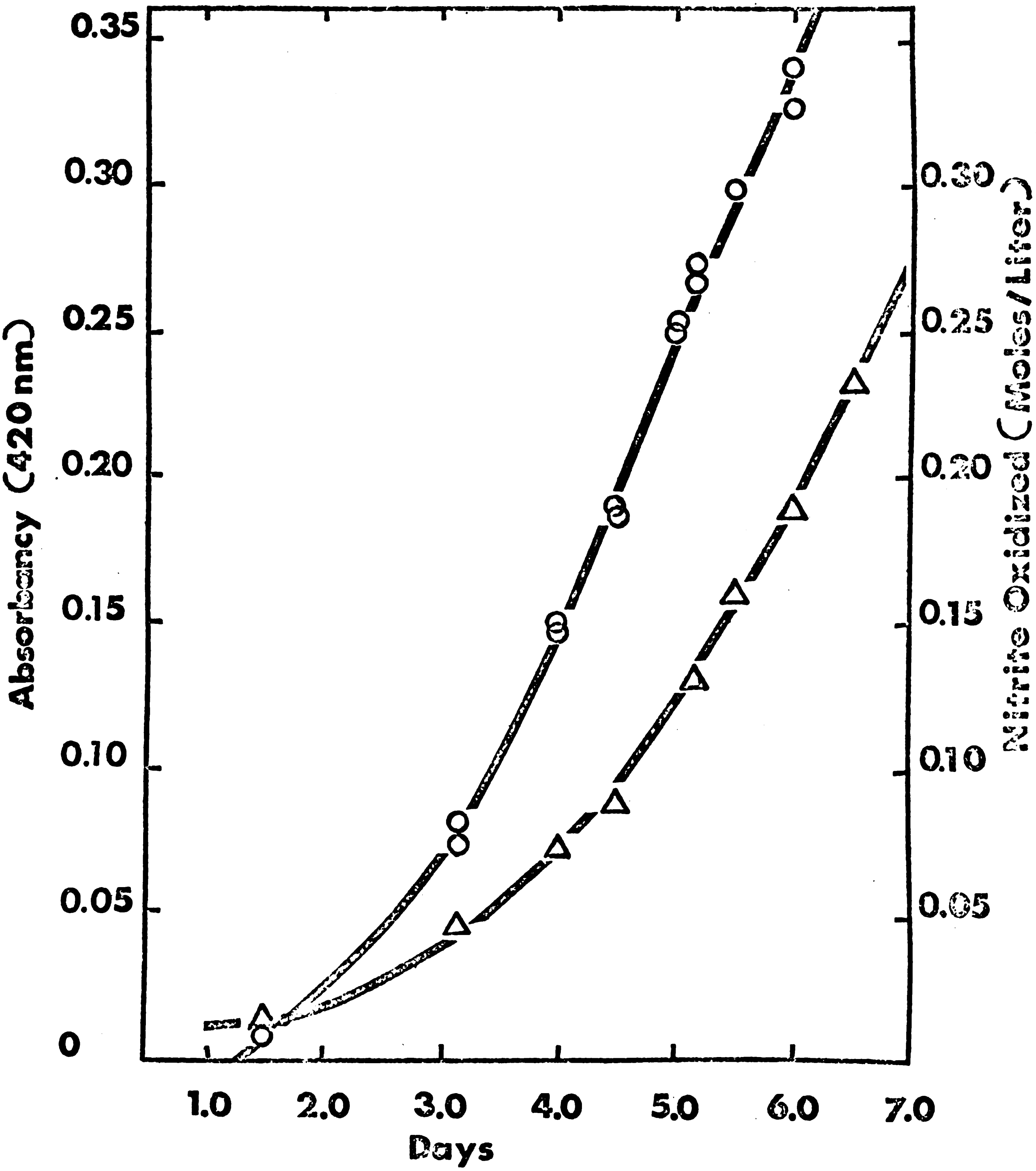


FIG. 6. Rate of nitrite oxidation (-Δ-Δ-) and turbidity (-o-o-) in large-scale cultures.

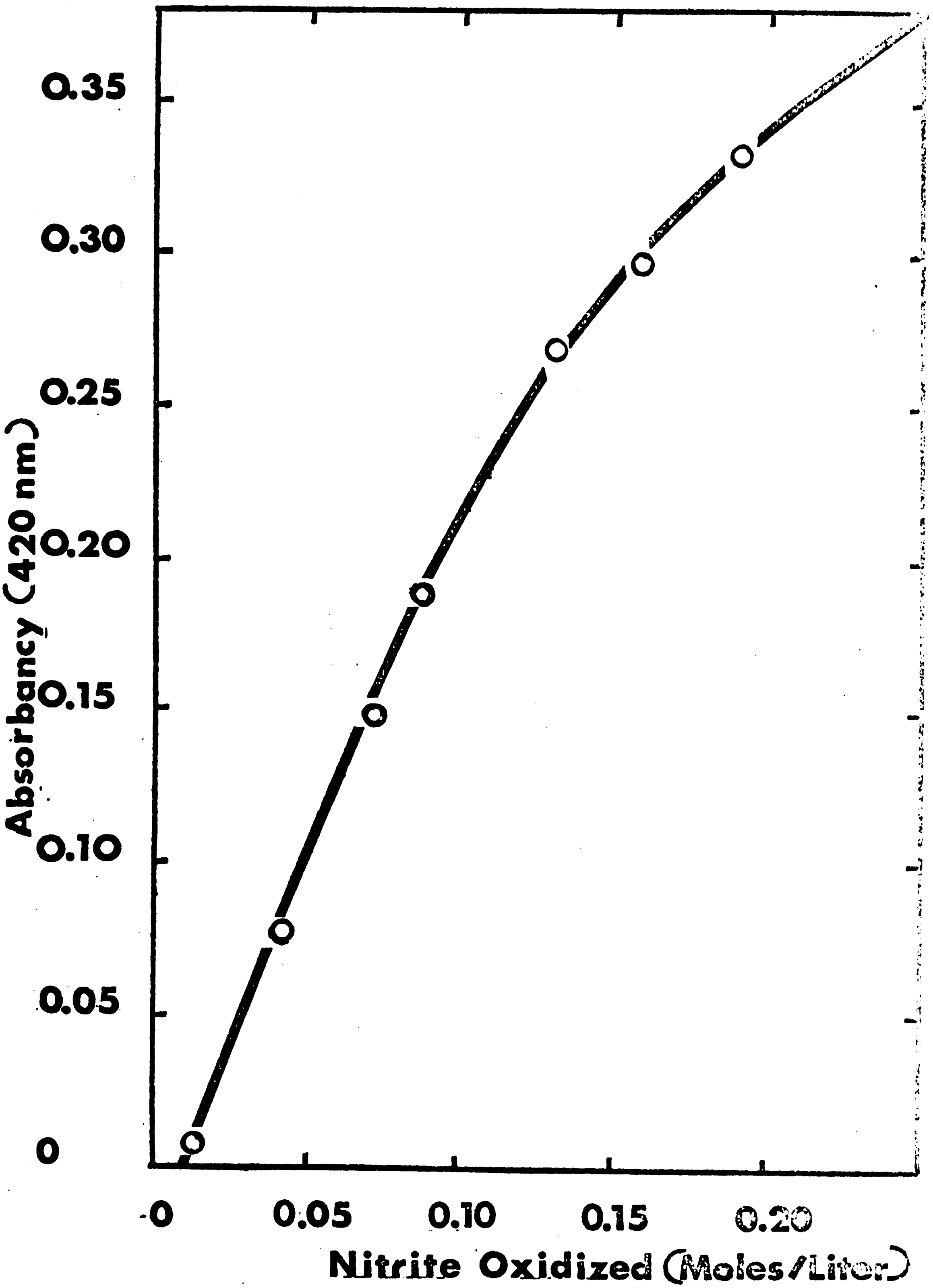


FIG. 7. Turbidity as a function of nitrite oxidation in large-scale cultures.

Effect of CIPC and eptam. CIPC and eptam, the two herbicides which inhibited the growth of *Nitrobacter* in flask cultures (Table 1), caused reduced P/O ratios with cabbage (Lotlikar, *et al.*, 1968) and cucumber mitochondria (Ashton, 1963). When nitrite oxidation and the concomitant esterification of inorganic phosphate were investigated in the presence of CIPC and eptam, both the nitrite oxidation and phosphate esterification were inhibited (Table 4). At 50 ug CIPC per ml, oxygen uptake was inhibited by 16%, but phosphorylation was reduced by 53%. With 75 ug of CIPC per ml, 51% inhibition of oxidation was accompanied by an 85% reduction in phosphorylation. With eptam (200 ug/ml) phosphorylation was inhibited by 93%, but oxidation was reduced by only 47%. Since reduced P/O ratios were obtained, both pesticides exerted an uncoupling effect on the oxidative phosphorylation linked to nitrite oxidation in *Nitrobacter agilis*.

Effect of 2,4-dinitrophenol. The inhibition of oxygen uptake in *Nitrobacter* by CIPC and eptam (Table 4) is not necessarily inconsistent with the observed uncoupling of phosphorylation since several workers (Butt and Lees, 1960; Aleem and Nason, 1960) have reported inhibition of nitrite oxidation in *Nitrobacter* by 2,4-dinitrophenol (DNP). When various concentrations of DNP were tested for an effect on oxygen uptake and phosphorylation by *Nitrobacter* cell-free extracts, oxygen uptake was decreased by 18% and 37% at 2.5×10^{-4} M and 1×10^{-3} M respectively (Table 5). However, since phosphorylation was inhibited by 47% (2.5×10^{-4} M) and 100% (10^{-3} M), the P/O ratios decreased with increased concentrations of DNP.

TABLE 4. Effect of CIPC and eptam on oxidative phosphorylation in *N. agilis* cell-free extracts.[a]

Pesticide	Concn.	O_2 uptake	Rel. Act.	Esterification	Rel. Act.	P/O[b]
	ug/ml	uatom	%	umole	%	
None	---	8.52	100	1.35	100	0.16
CIPC	25	8.4	98	1.09	81	0.13
	50	7.1	84	0.58	43	0.08
	75	4.2	49	0.20	15	0.05
	100	1.7	20	0.00	0	0.00
Eptam	50	7.9	92	1.26	93	0.16
	100	7.9	92	0.83	62	0.11
	150	6.5	77	0.34	25	0.05
	200	5.0	58	0.08	6	0.02
	300	2.4	29	0.00	0	0.00

[a] The reaction system in 2.8 ml contained 50 umoles glucose, 15 umoles $MgCl_2$, 2 umoles ADP, 150 umoles Tris (pH 8.0), 20 umoles K_2HPO_4-P^{32}, 20 umoles $NaNO_2$, 1 mg hexokinase, and 1.2 mg cell-free extract.

[b] Ratio of umoles of phosphate esterified to uatom of oxygen consumed.

TABLE 5. The effect of 2,4 dinitrophenol on oxidative phosphorylation in *N. agilis*.

Concn. of DNP	Oxygen uptake[b]	Rel. Act.	Esterification[b]	Rel. Act.	P/O[a]
mM	uatom	%	umole	%	
None	8.1	100	1.45	100	0.18
0.10	6.7	84	0.86	60	0.13
0.25	6.6	82	0.69	47	0.10
0.50	6.6	82	0.19	13	0.03
1.0	5.1	63	0.0	0	0.00
2.0	3.3	41	0.0	0	0.00

[a] Values corrected for endogenous rates. Reaction system was same as with Table 4. Protein concentration was 1.1 mg per Warburg vessel.

[b] Ratio of umoles esterified phosphate to uatom oxygen consumed.

Since the amounts of glucose-6-phosphate formed in these experiments were determined by assay with glucose-6-phosphate dehydrogenase, the dehydrogenase and hexokinase activities were tested in the presence of DNP to ascertain that the inhibitory effect observed with DNP was definitely on *Nitrobacter* phosphorylation. Neither hexokinase activity nor glucose-6-phosphate dehydrogenase activity was affected by DNP.

Necessity of trapping system. Since an ATPase has been reported in *Nitrobacter* cell-free extracts (Malavolta *et al*., 1960; Fischer and Laudelout, 1965), a hexokinase trapping system was included in our phosphorylation study. When hexokinase and glucose were excluded from the reaction system, the P/O ratios were reduced by approximately 40% (Table 6). Consequently, an ATPase was present in our cell-free extracts.

Efficiency of inorganic phosphate extraction. The P/O ratios obtained for *Nitrobacter* cell-free extracts without pesticides ranged from 0.16 to 0.33. Since these ratios were lower than the ratio recently reported by Aleem (1968), the procedure for extraction of the inorganic phosphate was tested to determine if any organic phosphate was lost in the inorganic phosphate fraction. Glucose-6-phosphate was synthesized in a reaction mixture containing 4 umoles of uniformly C^{14} labeled glucose, 20 umoles of ATP, 20 umoles of potassium phosphate, 10 umoles of $MgCl_2$, 150 umoles of Tris (pH 8.0), and 1 mg of hexokinase in 1.8 ml final volume.

TABLE 6. Necessity for trapping system for oxidative phosphorylation.

	System[a]	Oxygen uptake[b]	Inorganic phosphate[b] esterified	P/O
		uatom	umole	
I.	Complete	9.0	1.48	0.17
	Minus hexokinase	10.1	0.89	0.09
II.	Complete	12.1	4.30	0.36
	Minus hexokinase	14.7	3.54	0.24

[a] Complete system was the same as for Table 4. The system without hexokinase also contained no glucose, but contained 10 umoles ADP. The above results were obtained with two separate cell-free extract preparations (I and II). Protein concentration in both cases was 2.1 mg per Warburg vessel.

[b] Values were corrected by endogenous flasks with and without hexokinase.

After 30 minutes incubation, 0.2 ml of TCA (20%) was added to stop the reaction. The radioactivity present in extracted samples was then compared with the radioactivity in equivalent volumes of unextracted samples. After the results were corrected by the use of internal standards, 106% of the total radioactivity was obtained in the organic phosphate fraction.

Phosphorylation Coupled to the Oxidation of $NADH_2$

Phosphorylation coupled to the oxidation of reduced nicotinamide adenine dinucleotide ($NADH_2$) was tested to determine whether the pesticides were inhibiting electron transport in addition to phosphorylation. Earlier attempts to show the presence of $NADH_2$ oxidase activity by a spectrophotometric assay were unsuccessful, but when the oxygen uptake of cell-free extracts was measured in the Warburg respirometer with $NADH_2$ as the substrate, azide (10 umoles) inhibited the reaction by 42% (Table 7). Since the azide inhibition is a measure of the oxidation occurring via the electron transport chain, it was assumed that 2 umoles of NADH were oxidized by $NADH_2$ oxidase and 2.8 umoles were oxidized via flavoprotein reduction. Although CIPC (100 ug/ml) and eptam (300 ug/ml) inhibited the reaction by 50 and 64% respectively, the results are inconclusive for determining whether the pesticides are electron transport inhibitors. If all of the oxidation in the presence of the pesticides was by flavoprotein reduction, both compounds are potent inhibitors of $NADH_2$ oxidase; however, if only the flavoprotein

TABLE 7. $NADH_2$ oxidase activity of *N. agilis*.

Description	Oxygen Uptake[a]	Rel. Activity
	uatom	%
Complete system (CS)	4.8	100
CS with 100 ug/ml CIPC	2.4	50
CS with 300 ug/ml Eptam	1.7	36
CS with 10 umoles Azide	2.8	58

[a] Corrected for endogenous uptake. Reaction system essentially the same as with Table 4 with 10 umoles of $NADH_2$ used as substrate instead of $NaNO_2$. The protein concentration was 4.4 mg.

reduction was inhibited by the chemicals, $NADH_2$ oxidase was not affected. Consequently, the oxidation was measured in the presence of both the herbicide and azide. Since no phosphorylation was observed in the initial study, FMN (10^{-7} M), menadione (10^{-6} M) and cytochrome c (5×10^{-3}%) were included in the complete reaction system. Again no phosphorylation was observed, but the pesticides did not inhibit electron transport (Table 8). Since CIPC, eptam, and azide inhibited $NADH_2$ oxidation by 46%, 39%, and 23% respectively, the inhibitions caused by CIPC combined with azide and also eptam combined with azide should have been 69% and 61% if flavoprotein reduction rather than electron transport was affected. The actual inhibitions were 67% (CIPC with azide) and 69% (eptam with azide); consequently, CIPC and eptam did not inhibit electron transport.

TABLE 8. The effect of CIPC and eptam on $NADH_2$ oxidation.

Description	Oxygen Uptake[b]	Inhibition of O_2 Uptake
	uatom	%
Complete Reaction[a] System (CRS)	6.1	0
CRS with 10 umoles Azide	4.7	23
CRS with CIPC (100 ug/ml)	3.3	46
CRS with CIPC and Azide	2.0	67
CRS with Eptam (300 ug/ml)	3.7	39
CRS with Eptam and Azide	1.9	69

[a] Complete reaction system contained 10 umoles $NADH_2$, 10 umoles K_2HPO_4-^{32}P, 20 umoles glucose, 2 umoles ADP, 100 umoles Tris buffer (pH 8.0), 10 umoles $MgCl_2$, 3 umoles menadione, 0.3 umoles flavin mononucleotide, 0.15 mg cytochrome c, 2 mg yeast hexokinase, and 4.0 mg cell-free extract.

[b] Values corrected by endogenous uptake.

DISCUSSION

The eight pesticides considered in this report are currently recommended for agricultural uses. Field studies or laboratory investigations with soil systems have indicated no inhibition of nitrification by aldrin (Fletcher and Bollen, 1954; Shaw and Robinson, 1960; Bartha *et al.*, 1967), simazine (Bartha *et al.*, 1967), or DDD (Eno and Everett, 1960; Bartha *et al.*, 1967). The two chemicals (CIPC and eptam) which inhibited both the growth of *Nitrobacter* and nitrite oxidation were also inhibitory to nitrification in soil studies (Bartha *et al.*, 1967; Quastel and Scholefield, 1953). By applying 5000 ug of lindane and chlordane per gram of soil, Brown (1954) observed retardation of nitrification, but Shaw and Robinson (1960) observed no effect with chlordane at 300 ug per gram of soil and Bollen *et al.* (1954) reported lindane at 1000 ug per gram of soil did not inhibit nitrate formation.

We were particularly concerned in determining whether a correlation existed between oxidation studies with cell suspensions and the growth experiments. Aldrin and simazine neither affected growth in aerated cultures nor nitrite oxidation by cell suspensions or cell-free extracts. Among the six other pesticides tested which caused cessation of growth, eptam was the least inhibitory (75 ug/ml were required for complete inhibition), whereas only CIPC and eptam were completely inhibitory for the nitrite oxidase of cell suspensions. Although various degrees of nitrite oxidase inhibition were observed with 500 ug/ml of chlordane, DDD, heptachlor, and lindane, inhibition of nitrite oxidase activity was probably not the mode of action of these chemicals on Nitrobacter since 5 to 10 ug/ml stopped growth. Even with CIPC and eptam, inhibition of nitrite oxidase might not be the main mechanism of action since the concentrations of CIPC and eptam required for cessation of nitrite oxidase were approximately 10-fold and 4-fold greater, respectively, then the concentrations necessary for inhibiting growth.

It is noteworthy that heptachlor and chlordane were more inhibitory to oxidation by cell-free extract than by cell suspension. Complete inhibition of the nitrite oxidase activity in cell-free extracts was effected by 250 ug/ml chlordane and 200 ug/ml heptachlor, but additions of 500 ug/ml to cell suspensions resulted in 80% inhibition with chlordane and 50% inhibition with heptachlor. This difference in inhibition could be due to an impermeability of the cell suspensions to these two compounds or to binding of protein in the cell-free extracts since both chemicals are insoluble at the concentrations used. Likewise, sorption of cells or protein by insoluble pesticide particles could be responsible for the linear effect noted in the respiration experiments with increasing concentrations of the pesticides even though the solubility levels of the chemicals were exceeded.

Since marked inhibition of nitrite oxidase in cell-free extracts occurred with CIPC, chlordane, eptam, and heptachlor, the pesticides were tested for an effect on the cytochrome c oxidase activity in the cell-free extracts. Aleem and Nason (1960) and Lees and Simpson (1957) indicated that nitrite oxidation in Nitrobacter is mediated by cytochrome of c-like and a-like components, but Aleem (1968) recently showed that NO_2^- oxidation is mediated by cytochrome a and cytochrome oxidase components with the reduction of cytochrome c being energy dependent. None of the pesticides examined by us affected the cytochrome c oxidase activity sufficiently to account for the nitrite oxidase inhibition observed with cell suspensions and cell-free extracts. The percent relative cytochrome c oxidase activity obtained with 500 ug heptachlor per ml was similar to cell suspension respiration results, but heptachlor caused total inhibition of cell-free extract nitrite oxidase at 200 ug/ml. Thus, effects observed with heptachlor were probably only partially due to cytochrome c oxidase inhibition.

One complicating factor in comparing the nitrite oxidase and cytochrome c oxidase inhibitions is the relative insolubility of most of the compounds tested. However, CIPC is soluble at 100 ug/ml and eptam at 400 ug/ml. Since nitrite enters the cytochrome system at cytochrome a (Aleem, 1968), an inhibitor of nitrite oxidase should affect cytochrome oxidase unless the inhibition is competitive. As CIPC was shown to be a noncompetitive inhibitor of nitrite oxidase, inhibition of cytochrome oxidase was expected. Failure to obtain greater than 50% inhibition of cytochrome c oxidase by CIPC suggests the presence of more than one terminal oxidase in Nitrobacter. Straat and Nason (1965) likewise suggested that two terminal oxidases are present in the organism.

Another enzyme chosen for study was $NADH_2$ oxidase. Although it is probable that this oxidase is used for autotrophic growth only by the hydrogen bacteria (Smith et al., 1968), Aleem (1968) showed that $NADH_2$ oxidation in Nitrobacter proceeds by an electron transport chain analogous to the mitochondrial respiratory chain reported by Chance et al. (1967). Consequently, we were interested in studying the effects of pesticides on the oxidase as an indication of cytochrome inhibition. However, we were unable to detect $NADH_2$ oxidase activity in our preparations by spectrophotometric assay. A slight decrease in absorbance observed at 340 nm was presumed to be caused by reduction of flavoproteins.

No deleterious effect on nitrate reductase by the pesticides was noted in our system. Straat and Nason (1965) have postulated that the enzymatic reduction of nitrate in Nitrobacter might be the first step of a sequence for providing nutritional nitrogen, or it could be a means of recycling nitrite for further oxidation.

Though growth, nitrite oxidase, and enzyme studies were unsuccessful in characterizing the inhibitions caused by the pesticides studied, our results indicate that growth studies with low concentrations of pesticides were more suitable than the measurement of nitrite oxidation as indexes of the toxicity of pesticides for Nitrobacter agilis. Growth studies detect inhibition of biosynthetic reactions, whereas only inhibition of energy assimilation is detected by nitrite oxidation studies. The Nitrobacter growth studies indicated that chlordane, CIPC, DDD, eptam, heptachlor, and lindane would be expected to inhibit nitrification in the soil, whereas the nitrite oxidase studies suggested that only two of the pesticides, CIPC and eptam, would be inhibitory.

The oxidative phosphorylation coupled to nitrite oxidation was investigated as a possible site of action for two of the herbicides (CIPC and eptam). Both CIPC and eptam had previously been investigated for their mode of inhibition in plants. Ashton (1963) reported an uncoupling effect by eptam in cucumber mitochondria since the compound inhibited phosphate esterification more severely than oxygen uptake at 10^{-2} and 10^{-3} M. When added to cabbage mitochondria, 10^{-3} M eptam caused 77% inhibition of oxygen uptake and 100% inhibition of phosphate esterification, whereas 10^{-3} M CIPC reduced oxygen uptake and phosphate esterification by 85% and 91%, respectively (Lotlikar et al., 1968). Mann et al. (1965) obtained evidence for CIPC inhibiting protein synthesis at points after the transport of amino acids across cell membranes.

In microorganisms suggestive evidence for CIPC inhibition of oxidative phosphorylation was obtained by Bartha et al. (1967). The identical CO_2 production patterns obtained after applications of CIPC and DNP to soil samples caused Bartha *et al.* to propose that CIPC might be an uncoupler of oxidative phosphorylation in microorganisms.

Both CIPC and eptam exerted an uncoupling effect on oxidative phosphorylation by *Nitrobacter* at concentrations of the chemicals similar to those required for uncoupling cabbage mitochondria phosphorylation. At 2.3×10^{-4} M, CIPC inhibited *Nitrobacter* phosphate esterification by 53% and oxygen uptake by 16%. Complete inhibition of phosphorylation occurred at 4.6×10^{-4} M CIPC, but oxygen uptake was reduced by only 80%. Eptam at 10^{-3} M depressed oxygen uptake by 42% but reduced phosphate esterification by 97%.

It was of interest to determine whether 2,4-dinitrophenol, a classical phosphorylation uncoupler, affected oxidative phosphorylation in *Nitrobacter* in a manner similar to that observed with the two herbicides. An inhibition of nitrite oxidation in *Nitrobacter* has been reported previously with DNP at various concentrations. Aleem and Alexander (1958) reported 84% inhibition of oxygen uptake by *Nitrobacter* cell-free extracts at 10^{-2} M DNP. In 1960, Aleem and Nason were unable to demonstrate uncoupling of oxidative phosphorylation with DNP, but complete inhibition of nitrite oxidation occurred at 5×10^{-4} M DNP. Using *Nitrobacter* cell suspensions, Butt and Lees (1960) obtained 64% inhibition of nitrite oxidation at 2×10^{-3} M DNP. Van Gool and Laudelout (1966_b) reported 50% inhibition of nitrite oxidation at 5×10^{-4} M and 9×10^{-4} M DNP with *Nitrobacter* cell suspensions and cell-free extracts, respectively.

Nitrite oxidation by our cell-free extracts was reduced by 18% and 47% at 5×10^{-4} M and 10^{-3} M DNP, respectively. Since these oxygen uptake reductions were accompanied by inhibitions of phosphate esterification of 85% and 100% at 5×10^{-4} M DNP and 10^{-3} M DNP respectively, we concluded that the two herbicides and dinitrophenol had similar mechanisms of inhibition in *Nitrobacter*.

Because the phosphorylation occurring with nitrite oxidation is atypical in regard to inhibition of nitrite oxidation by an uncoupling agent, it is interesting to recall the scheme proposed by Kiesow (1964). Kiesow postulated that in nitrite oxidation the electrons from nitrite are utilized to reduce NAD with the consumption of two moles of ATP. The NADH is oxidized in the presence of O_2 and yields 3 moles of ATP with a net yield of one mole of ATP for the oxidation of nitrite to nitrate. In addition to explaining the inhibition of nitrite oxidation by uncoupling agents, this scheme also illustrates a need for NADH oxidase in autotrophic growth.

Various values have been reported for phosphorylation linked to NADH oxidation in *Nitrobacter*. In 1960, Aleem and Nason reported a P/O ratio of 0.05, but values approaching 2.0 (Aleem, 1968) and 3.0 (Kiesow, 1964) were obtained more recently. Because of these recent reports, the oxidation of NADH by *Nitrobacter* cell-free extracts was studied to determine the effects of the two herbicides on both electron transport and oxidative phosphorylation occurring in this reaction. Although we were unsuccessful in measuring $NADH_2$ oxidase activity by a spectrophotometric assay, increased protein concentrations in a respirometer assay indicated an $NADH_2$ oxidase activity of 0.3 to 0.5 umoles per hour per mg of protein. We observed no esterification of inorganic phosphate coupled to the oxidation. Although both pesticides did inhibit oxygen uptake with NADH as the substrate, a major portion of the oxidation proceeded via flavoprotein reduction. When each herbicide was used in combination with azide, the combined effect substantiated an inhibition of flavoprotein reduction and not an inhibition of electron transport by CIPC and eptam.

The modes of action of chlordane, DDD, heptachlor and lindane for Nitrobacter were not determined, but were shown to be on biosynthetic processes of the cell rather than nitrite oxidation. With CIPC and eptam, the results suggest an uncoupling of oxidative phosphorylation as the mode of action for both herbicides since phosphorylation was reduced by 57% with CIPC at 2.3×10^{-4} M and by 75% with eptam at 8×10^{-4} M. Nitrite oxidase was also affected by the chemicals, but uncoupling of oxidative phosphorylation with a less severe inhibition of nitrite oxidase was also observed with 2,4 dinitrophenol.

Although the P/O ratios of our cell-free extracts (0.16 to 0.36) were consistent with the maximum value of 0.2 obtained by Aleem and Nason (1960) and 0.17 reported by Fischer and Laudelout (1965) for Nitrobacter winogradskyi, they were lower than we expected. In 1968 Aleem listed two criteria for obtaining P/O ratios approaching 1.0 for the oxidative phosphorylation linked to nitrite oxidation in N. *agilis*: (i) cultures must oxidize at least three additions of 20 mM nitrite per day for two days and (ii) cells should be suspended in an isotonic medium and disrupted for only 3 min. Even though these criteria were met, we obtained a maximum value of 0.36. It was also noted that the nitrite oxidase activity was more stable than the coupled phosphorylation. After four days at 5 C no decrease was noted in the nitrite oxidase activity, but the P/O ratios usually decreased by about 50%.

To determine whether the low P/O ratios were caused by the loss of organic phosphate during removal of the inorganic phosphate, a reaction system containing uniformly labeled ^{14}C-glucose-6-phosphate was extracted in the usual manner. When the values were corrected by the use of internal standards, 106% of the radioactivity was obtained. Apparently the low P/O values were caused by destruction of the structural integrity of the phosphorylating particles during disruption of the cells. Proof of such destruction was the presence of an active ATPase.

Menadione (10^{-6} M), FMN (10^{-7} M), and cytochrome c (1.5×10^{-3}%) failed to increase the P/O values when added singly or in combination. However, no increase was expected since nitrite oxidation involves only cytochrome a- and a_1-like components (Aleem, 1968). One possibility for slight increases in the P/O values might be additions of flavin and cytochrome b inhibitors (i.e. rotenone and antimycin A) which would prevent the expenditure of ATP in reduction of the cytochrome c, cytochrome b, and flavin components of the cytochrome system.

In the large-scale cultures a cell yield of 2.86 gm wet weight was obtained from the oxidation of 2.61 moles of $NaNO_2$ (average of five determinations). Assuming the cells contained 80% water, the molar growth yield was 220 mg dry weight of cell material per mole of substrate. These values were in close agreement with the 231 mg dry weight per mole of substrate and 0.5 g dry weight of cells from 18 liters obtained by Finstein and Delwiche (1965). Using the P/O ratio of 0.36, the yield per mmole ATP (Y_{ATP}) was 0.22/0.36 or 0.6 mg per mmole ATP. Fischer and Laudelout with *Nitrobacter winogradskyi* obtained a Y_{ATP} value of 2 mg per mmole of ATP. For heterotrophs the average Y_{ATP} is 10.5 mg per mmole of ATP (Bauchop and Elsden, 1960).

The higher Y_{ATP} reported by Fischer and Laudelout can be attributed to a lower P/O ratio and a higher efficiency of growth with *N. winogradskyi* than we obtained with *N. agilis*. Since Fischer and Laudelout obtained 40 mg dry weight with the oxidation of 0.1 M nitrite, their cultures had a maximum efficiency of growth of 11.7% when calculated by the method of Baas-Becking and Parks (1927). In a study of the molybdenum requirements of *Nitrobacter*, Finstein and Delwiche (1965) reported essentially the same efficiency (11.2%) for cultures after 5 days of active growth. However, with 8 days of active growth the cultures had no increase in cell yield and an efficiency of only 6.4%. This decreased efficiency was apparently caused by nitrate accumulation in excess of

0.1 M. Although we did not measure cell weight at intervals during growth, turbidimetric measurements indicated only a slight decrease in efficiency after oxidation of 0.09 M nitrite; active growth continued even after oxidation of 0.2 M nitrite. After 5 days of active growth our cultures had an efficiency of only 6.2%.

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