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Complete Mineralization of Methylparathion by *Pseudomonas* sp. A3

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Abstract

Organophosphorus insecticides are widely used in agriculture. Despite their biodegradable nature, some are highly toxic and their residues are found in the environment. Reports on the mineralization of a spectrum of these insecticides by a single potential strain are scarce. We have isolated a soil isolate, *Pseudomonas* sp. A3, through enrichment technique, able to degrade methylparathion (MP), malathion, monocrotophos, and Diazinon. The potential of this strain to mineralize MP as a carbon and/or phosphorus source has been evaluated. On hydrolysis of MP, the aromatic portion (*p*-nitrophenol) was used as a carbon and energy source whereas the alkyl moiety (dithiomethylphosphorothioate) was broken down for the phosphorus source. The results from the experiments involving [U-^{14}C]*p*-nitrophenol provided the evidence for incorporation of carbon into the cellular constituents and release of CO_2 from this insecticide. During the breakdown of MP, nitrite was released as a catabolic by-product.

Index Entries: Biodegradation; methylparathion; mineralization; organophosphates; *Pseudomonas* sp. A3.

Introduction

The continuous use of organophosphorus insecticides in large quantities for the control of a wide range of insects throughout the world and their potential neurotoxicity to the mammalian system have led to serious concern for the development of safe strategies to deal with its widespread dispersal in the ecosystem *(1)*. Methylparathion (*O*,*O*,dimethyl-*O*-*p*-nitrophenylphosphorothioate [MP]) is being increasingly used as a nonsystemic

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insecticide worldwide in the place of parathion *(2)*. Reports on microbes that grow on this compound as a sole source of both carbon and phosphorus appear to be rare *(3)*. Bacterial degradation of parathion has been studied more extensively *(4–6)*. The initial step in the breakdown of parathions was mediated through the hydrolysis of an organophosphate bond by the enzyme organophosphorus hydrolase (EC.3.1.8.2) *(7)*. Information on the genetic and enzymatic aspects involved in the complete mineralization of MP by bacterial strains that utilize MP as its sole source of carbon and phosphorus are yet to be studied in detail.

We have isolated a *Pseudomonas* sp. A3 that is able to degrade MP, malathion, Diazinon, edifenphos, and monocrotophos *(8)*. In this report, we propose a pathway for the complete breakdown of MP to CO_2 with the release of nitrite as a catabolic by-product. The efficiency of this isolate in utilizing MP as a sole source of carbon and/or phosphorus has been evaluated.

Materials and Methods

Chemicals

Technical grade methylparathion (95% purity) was supplied by Tropial Agrosystems, Madras, India. Spectrophotometric and analar grade *p*-nitrophenol (PNP) were purchased from Sigma, St. Louis, MO, and SRL Research, Bombay, India, respectively. Uniformly ^{14}C-ring–labeled PNP ([U-^{14}C]PNP) was supplied by Burkhard Schmidt, Aachen, Germany.

Culture and Cultural Conditions

Pseudomonas sp. A3 was isolated from rice field soils by enrichment technique *(8)* and identified using *Bergy's Manual of Determinative Bacteriology* based on its biochemical characteristics *(9)*. For biodegradation studies, basal salt medium (BSM) *(10)* was used. MP was added aseptically just before inoculation from a filter-sterilized stock solution. Appropriate modifications were made to BSM so that MP could serve as the sole source of carbon and/or phosphorus. When the efficiency of cells in utilizing MP as the sole source of phosphorus was assessed, filter-sterilized glucose was provided as the carbon source. In the flasks in which MP served as both carbon and phosphorus sources, BSM was devoid of glucose and inorganic phosphorus. The basal salt plates were prepared with 2% (w/v) agar. The isolate was maintained on basal salt slants amended with 3 m*M* MP and subcultured at regular intervals.

Biodegradation Studies

The efficacy of *Pseudomonas* sp. A3 in degrading MP was studied as follows. An 18- to 24-h culture grown in BSM with 0.2% (w/v) glucose was aseptically harvested, and the cells were washed thoroughly with sterile distilled water to remove the sugar. The cells were suspended in sterile BSM

to give an absorbance of 1.0 at 540 nm and was used as inoculum for all further degradation studies. In 250-mL Erlenmeyer flasks, 50 mL of BSM amended with different concentrations of MP was inoculated with 1 mL of inoculum and incubated on a rotary shaker (125 rpm/min) at room temperature. Uninoculated control flasks were maintained in all the experiments.

Estimation of Residual MP and Nitrite

Ten milliliters of the spent medium were withdrawn at regular intervals from the BSM–MP flasks inoculated with *Pseudomonas* sp. A3, and the cells were removed by centrifugation. The culture filtrate was extracted thrice with equal volumes of chloroform and diethyl ether; the solvent fractions were pooled and evaporated to dryness. The residue was dissolved in 500 µL of methanol and analyzed using thin-layer chromatography (TLC) for the estimation of residual MP as described by Siddharamappa et al. *(11)*. The amount of MP degraded during biodegradation was expressed as a percentage of MP in uninoculated control flasks. Nitrite released from MP into the medium was estimated calorimetrically using sulfanilamide and *N*-1-naphthylethylenediamine dihydrochloride *(12)*. All the experiments were repeated twice in duplicate.

Mineralization of [U-^{14}C]PNP

The efficiency of the strain A3 in utilizing PNP (hydrolytic product of MP) further as a carbon source was studied by utilizing [U-^{14}C]PNP. Mineralization of this labeled compound was studied as follows. Ten milliliters of BSM containing 0.5 m*M* unlabeled PNP as a sole carbon source was placed in a 40-mL Borosil-tube. [U-^{14}C]PNP was added to the medium and the medium was inoculated with overnight culture. The $^{14}CO_2$ evolved from [U-^{14}C]PNP as a final product of its degradation was trapped in 10 mL of a solution containing ethanolamine:methanol:Ready Gel™ Scintillation Cocktail (Beckman, Fullerton, CA) (4:1:5) as described by Kirk et al. *(13)* at 2-h intervals and assayed by liquid scintillation spectrometry. After 36 h, the cells were pelleted, and the aliquots of both the pelleted cells and culture filtrate were assayed in liquid scintillation spectrometry (LB1801, Beckman Instruments).

Analysis of Metabolites

The residue from the culture filtrate of MP-grown cells was resolved by TLC using a hexane:chloroform:methanol (7:2:1, v/v/v) system *(11)*. The compound from the prominent yellowish spot corresponding to the authentic PNP was eluted and given for spectral analysis. The culture filtrate of PNP-grown cells was acidified and extracted with chloroform: diethyl ether (1:1, v/v), and the residue was analyzed by TLC developed in chloroform:ethanol:0.1 *N* NaOH (100:5:1, v/v/v) *(14)*. The intermediates were located by observation under ultraviolet light. The residue from the culture filtrate of hydroquinone-grown cells was resolved by TLC using,

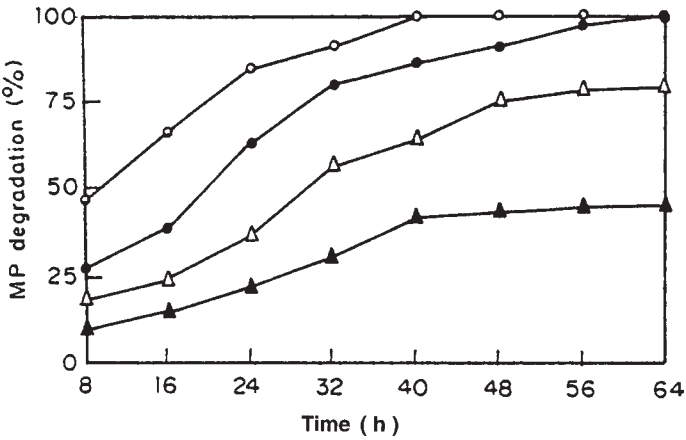

Fig. 1. Influence of pesticide concentration on MP degradation: MP was added at 250 (○), 500 (●), 750 (△), and 1000 (▲) mg/L.

hexane: dioxane:formic acid (85:15:1, v/v/v). The plates were sprayed with freshly prepared *p*-nitroaniline for identifying the spots. The compounds were carefully extracted from silica gel eluted from the TLC plates for spectral analysis. Proton-decoupled ^{13}C-NMR spectrum was recorded in a Bruker MSL 300 MHz nuclear magnetic resonance (NMR) spectrometer using dimethyl sulfoxide d6 as deuterated solvent and tetramethyl silane (TMS) as internal standard. Infrared (IR) spectrum was recorded by KBr pellet technique (sample/KBr:1/80) using a Nicolet Impact 400 Spectrometer keeping the resolution at 1 cm^{-1}. Mass spectrum was recorded in a Finnigan MAT 8230 GC mass spectrometer.

Results

Methylparathion Degradation

Pseudomonas sp. A3 readily utilized MP as a sole carbon and/or phosphorus source. In 40 h, the cells completely removed MP (100%) from the medium as a sole carbon source, whereas 93% of MP was degraded while it was provided as a phosphorus source. Over this period, only 65% of MP could be degraded, and complete disappearance of this compound was noted in 56 h, when the cells derived both carbon and phosphorus sources from this insecticide alone.

To study the effect of MP concentration on the rate of degradation, different concentrations, ranging between 250 and 1000 mg/L, were tested. An increase in the concentrations of MP gradually decreased the rate of degradation (Fig. 1). In flasks containing MP at 250 mg/L, complete removal of this compound was observed within 40 h. Over this period, the cells could utilize only 89.4% of MP added initially at 500 mg/L, and total disappearance of this insecticide was noted at 56 h. Only 79.3 and 45% of this compound was degraded when it was given at 750 and 1000 mg/L, respectively, in the medium.

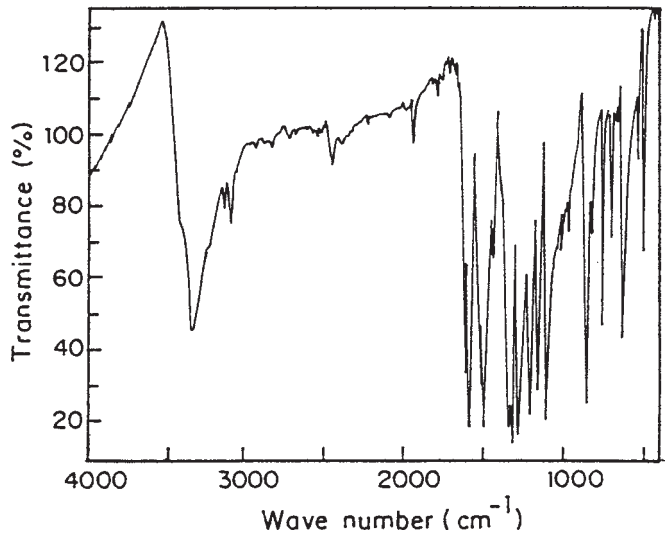

Fig. 2. IR spectrum of PNP.

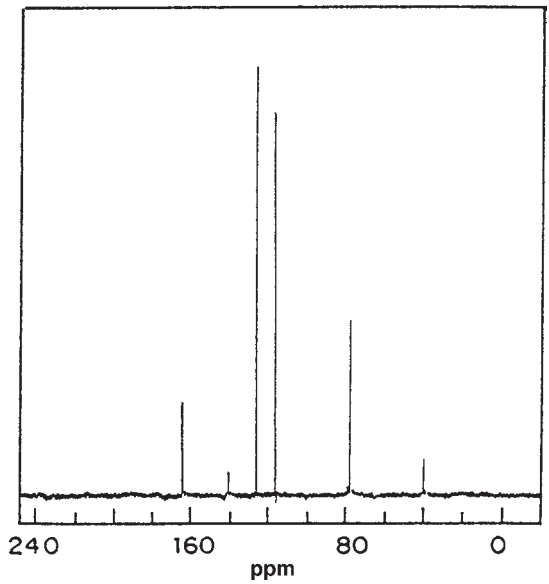

Fig. 3. ^{13}C-NMR spectrum of PNP.

Mineralization of [U-^{14}C]PNP

PNP was formed as the immediate hydrolytic product of MP during its breakdown. While the culture filtrate of MP-grown cells was resolved by TLC, the spot corresponding to PNP could be easily identified owing to its typical yellow color. The R_f value as well as the spectral analyses (Figs. 2–4) of PNP coincided with its authentic sample. Accumulation of PNP in the

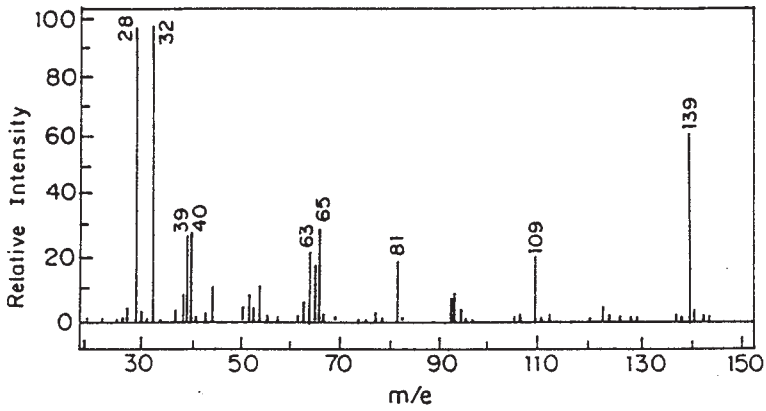

Fig. 4. Mass spectrum of PNP.

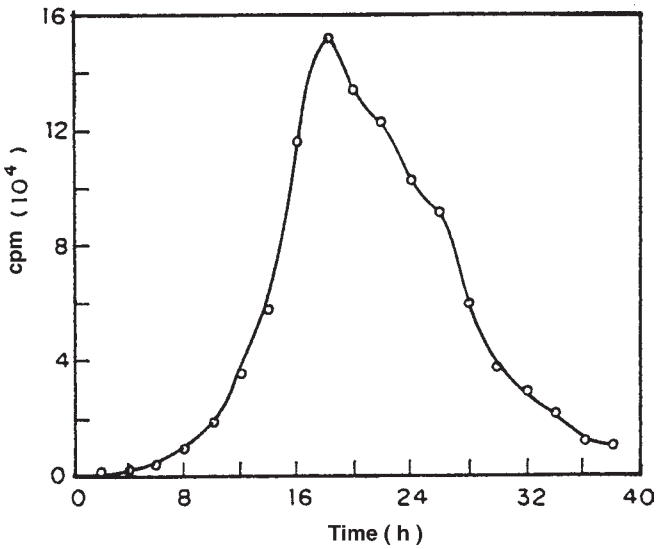

Fig. 5. Production of $^{14}CO_2$ (○) during growth on [U-^{14}C]PNP as a carbon source.

medium was not observed since the cells readily degraded this metabolite as and when it was released from MP. The ability of the bacterium to utilize PNP as a sole carbon and energy source was studied by utilizing [U-^{14}C]PNP. The strain A3 liberated $^{14}CO_2$ from the medium containing [U-^{14}C]PNP.

The release of $^{14}CO_2$ from the BSM-PNP medium inoculated with *Pseudomonas* sp. A3 was closely monitored by trapping $^{14}CO_2$ from the medium every 2 h. From 6 h onward, the release of radiolabeled CO_2 could be observed, and the highest amount of radioactivity was trapped at 18 h (Fig. 5). About 66% of the total radioactivity added initially was recovered as $^{14}CO_2$, 10% in the culture filtrate and 5% in the cell mass after 36 h of

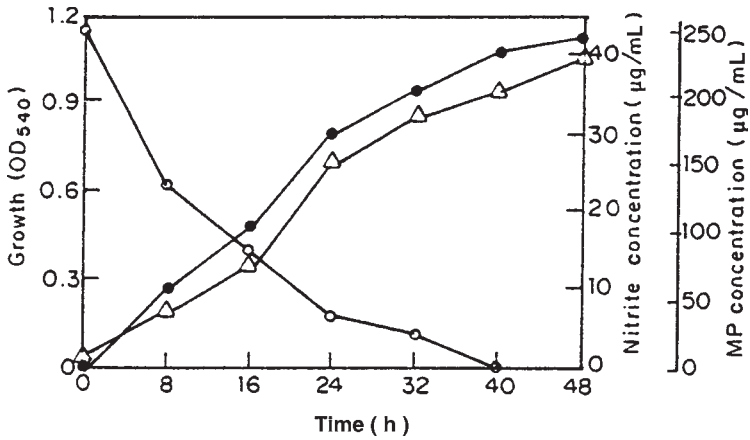

Fig. 6. MP degradation (○) and release of nitrite (●) during growth (△) on MP added at 250 mg/L.

incubation. In a control experiment, evolution of $^{14}CO_2$ was not observed from the uninoculated medium containing [U^{14}C]PNP.

Nitrite Release from MP

When *Pseudomonas* sp. A3 was grown on MP, nitrite was released in stoichiometric quantities. As the mineralization of this insecticide progressed, the level of nitrite in the medium rose (Fig. 6) and the amount of nitrite formed was proportional to the amount of MP that was degraded. After 48 h of incubation, when the cells completely metabolized the parent compound, the amount of nitrite accumulated in the medium was found to be 41.2 μg/mL, and it was almost equivalent to the theoretical value obtainable from the amount of MP added initially (250 μg/mL). In the uninoculated control flasks, nitrite was not detected.

Identification of Metabolites

The intermediates of the MP degradative pathway in *Pseudomonas* sp. A3 were identified. From the culture filtrate of PNP-grown cells, a brownish spot with an R_f value of 0.11 corresponding to authentic hydroquinone was noted. Spectral analyses of this compound confirmed its identity as hydroquinone (Figs. 7–9). A blackish spot with an R_f value of 0.42 corresponding to 1,2,4-benzenetriol was observed from the culture filtrate of the hydroquinone-grown cells, and the spectral analysis of this compound supported its identity as 1,2,4-benzenetriol (Fig. 10).

Discussion

Pseudomonas sp. A3, on initial hydrolysis, cleaved MP into dimethylphosphorothioate (DMPT) and PNP. The ring moiety served as a carbon source and was degraded to CO_2. Growth of this strain on MP as a phospho-

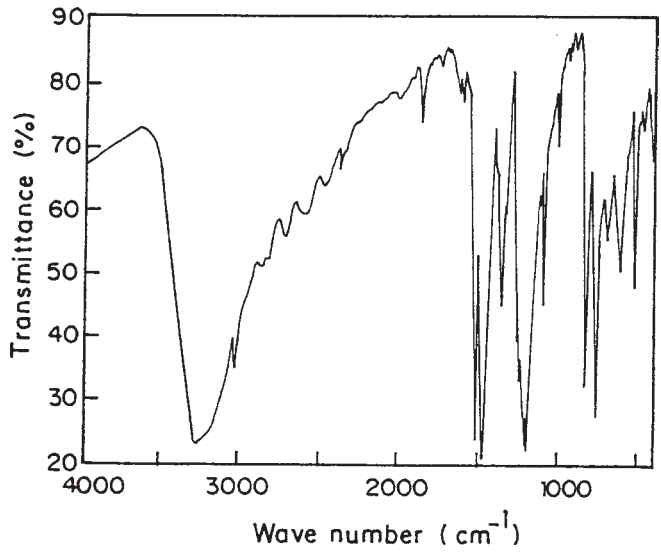

Fig. 7. IR spectrum of hydroquinone.

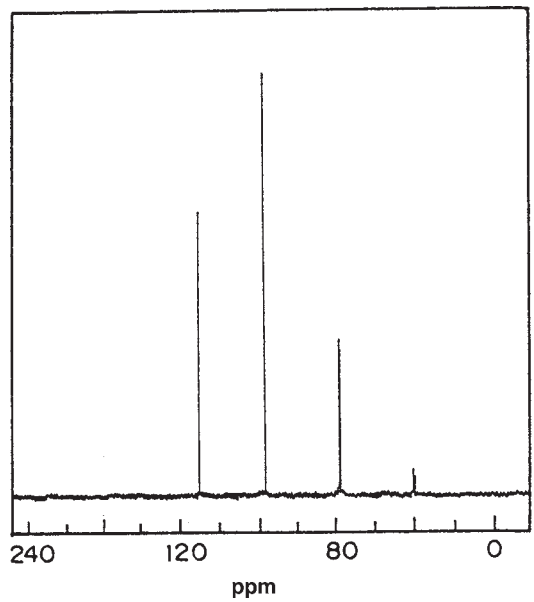

Fig. 8. ^{13}C-NMR spectrum of hydroquinone.

rus source indicates the breakdown of alkyl moiety prior to the release of phosphorus from DMPT. Utilization as a sole source of carbon and phosphorus, and degradation of this insecticide up to its final oxidation products such as CO_2 and H_2O with the release of nitrite, clearly revealed the potentiality of the bacterium to completely mineralize MP. From the studies on the degradation of [U-^{14}C]PNP, the results showed that most of the

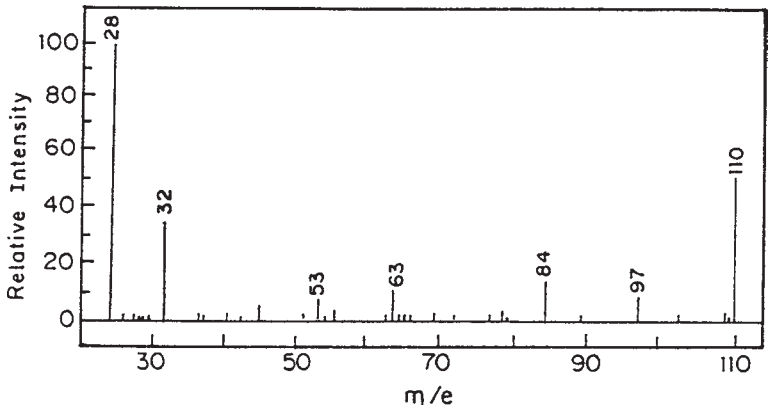

Fig. 9. Mass spectrum of hydroquinone.

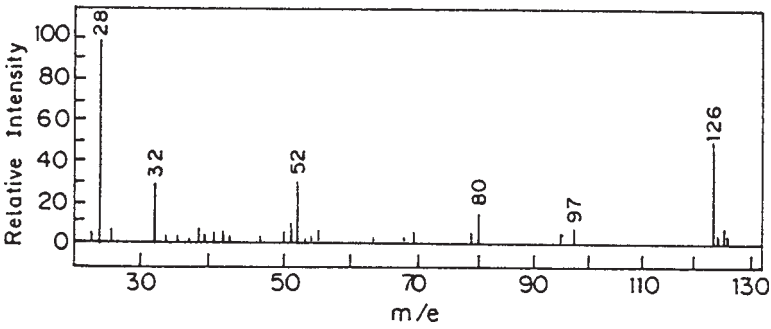

Fig. 10. Mass spectrum of 1,2,4-benzenetriol.

[U-^{14}C]PNP was degraded to $^{14}CO_2$. The incorporation of radioactivity in DNA and protein samples prepared (data not shown) from the cells grown on [^{14}C]PNP also confirmed the assimilation of carbon from the aromatic portion of MP into the cellular components. These results showed that PNP formed from MP was utilized as a carbon and energy source for its metabolism. After the PNP stage, hydroquinone, 1,2,4-benzenetriol, and maleylacetate were formed as the metabolites in the MP-degrading pathway (Fig. 11). Maleylacetate thus formed enters through the formation of β-ketoadipate into tricarboxylic acid (TCA) cycle and finally is transformed into gaseous CO_2 *(15)*.

In previous reports, microbial growth on MP as a sole carbon source was represented by two mixed cultures *(16,17)*. This pesticide was cometabolically dissimilated by *Pseudomonas* sp. *(16)* and *Bacillus* sp. *(17)* in the presence of additional carbon sources such as glucose and yeast extract, respectively. In these reports, the strains that could degrade diethylphosphorothioates failed to degrade dimethyl compounds and vice versa. In particular, an ethylparathion-degrading *Pseudomonas* sp. could grow on the diethyl compound Diazinon, but failed to hydrolyze the dimethyl ana-

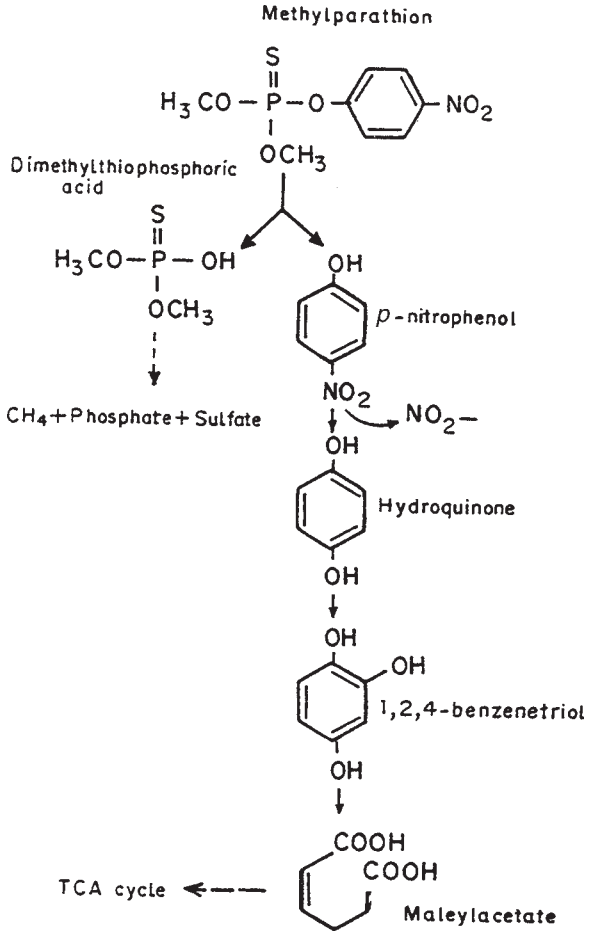

Fig. 11. Proposed pathway for MP degradation in *Pseudomonas* sp. A3.

log MP *(18)*. In another instance, an MP-degrading *Bacillus* sp. was unable to degrade the diethyl compound parathion *(2)*. The inability of bacteria to grow on both dimethyl- and diethylphosphorothioates suggested that the alkyl constituent attached to phosphorus is more important than the aromatic portion in determining the susceptibility of these compounds to bacterial degradation. Interestingly, *Pseudomonas* sp. A3 exhibited versatility in utilizing dimethyl compounds such as MP, malathion, and monocrotophos and diethyl compounds such as Diazinon and edifenphos as its carbon source *(8)*. It is noteworthy that the release of nitrite from MP by a strain that utilizes this insecticide as a carbon and/or phosphorus source is apparently the first report.

The detailed investigations in this field over the past decade have led to the overproduction of an organophosphate-hydrolyzing enzyme, phosphotriesterase in heterologous systems *(19)*, and its successful application in detoxifying coumaphos from a cattle-dip solution containing this

insecticide *(20)*. Primarily the enzyme-based strategies concern only the initial hydrolytic conversion of the organophosphates *(20–24)*. The soil isolates, such as *Pseudomonas* sp. A3, that ensure complete mineralization of these agrochemicals without any accumulation of toxic intermediates will be useful in developing whole cell-based remediation strategies. Our earlier work on the use of immobilized cells in detoxifying MP has offered promising results *(25)*. Extensive studies on the genetic and enzymatic components required for the mineralization of organophosphates will be useful in engineering pathways to widen the catabolic potential of a strain that is able to degrade a group of structurally related compounds. This approach may prove feasible for the development of simple and cost-effective biological disposal measures.

Acknowledgments

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