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Conversion of Fensulfothion by *Klebsiella pneumoniae* to Fensulfothion Sulfide and Its Accumulation

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Abstract

In a detailed study it was shown that washed cell suspensions of *K. pneumoniae* reduced the organophosphorus pesticide fensulfothion to fensulfothion sulfide. Temperature and pH optima for this conversion plus sensitivity to sulfydryl-reacting agents strongly suggested enzyme involvement. The reaction was also quite sensitive to molecular oxygen, only proceeding under conditions of low oxygen tension. Once formed, the fensulfothion sulfide was rapidly bound by living and heat-killed cells. A combination of lysozyme treatment and differential centrifugation showed 90% of the sulfide to be concentrated in the cell membrane fraction of exposed cells.

Introduction

Over the past 10–15 years a changeover from the use of traditional chlorinated hydrocarbon pesticides for crop protection to organophosphorus compounds has occurred. Knowledge of the fate of these newer pesticides in soil is incomplete. Fensulfothion, *O,O*-diethyl *O*-[4-(methysulfinyl)phenyl] phosphorothioate, is an organophosphorus pesticide that exhibits both insecticidal and nematicidal activity and is marketed under the trade names of Dasanit and Terracur-P. While its metabolism by plants and animals is well documented, its interaction with microorganisms is less well understood.

Metabolism of fensulfothion in plants (Everett and Gronberg 1967; Katague and Anderson 1967; Read 1971; Leuck and Bowman 1972) and animals (Everett 1968) is by oxidation to form the sulfone, oxygen analogue and the oxygen analogue sulfone. In addition to the oxidative pathways, rats possess the enzymes necessary to hydrolyse fensulfothion at the P–O–C bond (Everett 1968), leading to complete degradation of the molecule.

The first report of microbial interactions with fensulfothion was by Wood and MacRae in 1977. They demonstrated the reduction of fensulfothion to fensulfothion sulfide by washed cell suspensions of the bacterium, *Klebsiella pneumoniae*. Rosenberg and Alexander (1979) later isolated two pseudomonads able to utilize fensulfothion as a sole phosphorus source. Their data indicated that a major pathway for degradation in these bacteria was by hydrolytic attack by a phosphatase or phosphotriesterase to liberate diethyl phosphorothioate. However, these are the only reports to date on the interaction of microorganisms with the fensulfothion molecule.

In this paper the reduction of fensulfothion by *K. pneumoniae* is described and it is also shown that the product, fensulfothion sulfide, is bound to the cells rather than remaining in solution.

Materials and Methods

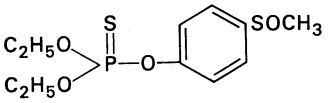
Microorganisms and Growth Conditions

Klebsiella pneumoniae (UQM 90) was obtained from the University of Queensland Culture Collection housed in the Department of Microbiology.

Sucrose mineral salts broth (Yoch and Pengra 1966) was used without added Tween 80. The 0·25 M phosphate buffer (pH 7·2) for washing and resuspending cells was the same as that used in the preparation of sucrose mineral salts broth.

Chemicals

Analytical grade fensulfothion (1), fensulfothion sulfide: *O,O*-diethyl *O*-[4-methylthiophenyl] phosphorothioate (2), Nemacur: *O*-ethyl *O*-[4-methylthio-m-tolyl]isopropyl phosphoroamidate (3), and HOL 4438: *O,O*-dimethyl *O*-[4-methylthiophenyl] phosphorothioate (4) were donated by the Farbenfabriken Bayer A.G., Leverkusen, Germany.



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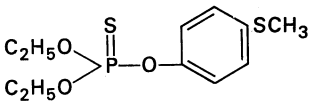

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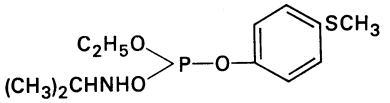

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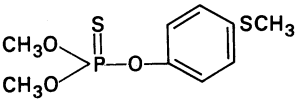

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Analysis by Gas Chromatography

Gas chromatographic analyses were performed with a dual-column Shimadzu model GC-4A instrument having dual flame thermionic detectors. Two stainless steel columns (0·4 and 2 m long, int. diam. 4 mm) packed with 10% (w/w) DC 200 silicone oil on 80/100 mesh Gas Chrom Q were used. The flow rate of oxygen-free nitrogen (carrier gas) was 40 ml/min while the temperatures of the column oven, detectors and injection ports were 208, 225 and 230°C respectively. Cultures and cell suspensions were extracted with nanograde hexane (1 : 1 v/v) containing diazinon (1·8 μg/ml) as internal standard. After phase separation, the hexane layer was dehydrated over anhydrous $MgSO_4$ and analysed by gas chromatography.

Reduction of Fensulfothion to Fensulfothion Sulfide

K. pneumoniae was grown at 36°C in 2-litre conical flasks containing 1 litre of growth medium sparged with sterile air at the rate of 350–400 ml/min. Cultures were harvested by centrifugation when they were in the logarithmic phase of growth and the cells resuspended in phosphate buffer to give washed cell suspensions with cell dry weights of 0·8–1·0 mg/ml. Replicate assay flasks were charged with 90 ml 0·25 M phosphate buffer (pH 7·2) containing fensulfothion (approx. 50 μg/ml) and 10 ml of washed cell suspensions. The flasks were incubated without shaking at 36°C and sampled at 0 and 3 h for analysis by gas chromatography.

Effect of Temperature, pH, Sulfydryl-reacting Agents and Oxygen on the Reduction of Fensulfothion by K. pneumoniae

The conversion of fensulfothion to fensulfothion sulfide during the first hour of the reaction was examined at five different temperatures: 23, 30, 36, 42 and 50°C. A single batch of washed cell suspensions was used and 10-ml lots added to 90 ml phosphate buffer. The starting concentration of fensulfothion was 50 μg/ml.

To examine the effect of pH, three buffer systems were used. Phosphate buffer (0·25 M) was used at pH 6·0 and 7·0, citrate phosphate buffer (0·1 M citric acid + 0·2 M Na_2HPO_4) at pH 4·0 and

5·0, and Tris-HCl buffer (0·2 M tris-(hydroxymethyl)aminomethane+0·2 M HCl) was used at pH 8·0 and 8·9. Washed cell suspensions were prepared at double strength in distillled water and 5-ml portions added to 95 ml of buffer solution at the appropriate pH. The pH values were checked after the mixing of cells and buffer and found not to change. All flasks were incubated at 36°C and duplicate 2-ml samples were taken at 0·25-h intervals for gas chromatographic analysis. Control flasks at each pH level contained distilled water in place of the washed cell suspensions.

The effect of sulfydryl-reacting agents on the reduction of fensulfothion to fensulfothion sulfide by washed cell suspensions of *K. pneumoniae* was tested using 100 mM, 1 mM or 0·01 mM *N*-ethylmaleimide, iodoacetic acid or *p*-hydroxymercuribenzoate respectively. The cell suspensions were first exposed to the sulfydryl-reacting agents for 30 min at 36°C. Fensulfothion was then added to each flask to give a final concentration of approximately 50 μg/ml and the flasks incubated for a further 60 min at 36°C. The rate of fensulfothion sulfide formation during this 1-h period was determined by gas chromatographic analysis of extracts of these mixtures.

The effect of oxygen on the reduction of fensulfothion was studied using growing cultures of *K. pneumoniae* containing fensulfothion at a concentration of 40–50 μg/ml. The level of dissolved oxygen in solution was adjusted by varying the flow rate of either nitrogen or oxygen gases. The dissolved oxygen level was monitored by means of a Beckman Model E2 oxygen analyser and electrode. In the first experiment the culture was held under anaerobic conditions and a level of 0% dissolved oxygen maintained for the first 4 h. The nitrogen gas was replaced with oxygen gas and the dissolved oxygen increased to 45–90% over the next 2 h. In the second experiment, dissolved oxygen was maintained at 100% for the first 4 h and 2–3% for the final 2 h. In both experiments duplicate samples were taken at hourly intervals for gas chromatographic analysis.

Effect of Exposing Cells of K. pneumoniae *to Fensulfothion on the Subsequent Rate of Reduction*

The effect of exposing *K. pneumoniae* to fensulfothion on the subsequent rate of reduction of the pesticide was studied using washed cell suspensions prepared from cultures grown for 24 h in sucrose mineral salts broth with and without the addition of fensulfothion. To 10 ml of each suspension of washed cells in the assay flasks was added 90 ml phosphate buffer containing fensulfothion at a concentration of approximately 50 μg/ml. Control flasks without fensulfothion were also included. Duplicate samples were taken at 0, 15, 30, 45, 60, 90 and 120 min during incubation at 36°C and extracted with n-hexane and analysed by gas chromatography for fensulfothion and fensulfothion sulfide.

Accumulation of Fensulfothion Sulfide and Structurally Related Compounds by Cells of K. pneumoniae

Washed cell suspensions containing from 8 to 10 mg dry weight of cells per millilitre were prepared and 5-ml lots added to replicate assay flasks containing fensulfothion sulfide (approx. concn 40 μg/ml) in 45 ml phosphate buffer. Control flasks without cells were included. The flasks were incubated without shaking at 36°C for 1 h and 5-ml samples taken and centrifuged at 1800 *g* for 15 min. The supernatant and cell pellet (resuspended in 5 ml phosphate buffer) were extracted with n-hexane and analysed by gas chromatography.

Flasks containing *K. pneumoniae* cells, autoclaved for 20 min at 121°C, were included to determine if killed cells would accumulate fensulfothion sulfide. Bioconcentration factors [i.e. ratio of total pesticide residue (ng/ml) in bacterial suspension to concentration of pesticide in water (ng/g)] were determined by the method of Johnson and Kennedy (1973).

To obtain information on the site of fensulfothion sulfide accumulation in bacterial cells *K. pneumoniae* cultures grown in the presence of fensulfothion for 13 h were harvested by centrifugation, washed and (i) disrupted by shaking with glass beads in a Braun homogenizer or (ii) treated with lysozyme at a concentration of 100 μg/ml plus 2 mM EDTA for 90 min at 37°C. These techniques were combined with differential centrifugation to produce the following corresponding cellular fractions: (*a*) cell wall and membrane, and intracellular material from the homogenized cells; (*b*) cell wall, cell membrane, and intracellular material from lysozyme-treated cells. Replicate samples of each fraction were extracted with n-hexane and the level of accumulated fensulfothion sulfide determined by gas chromatography.

To see if compounds structurally similar to fensulfothion sulfide could also be accumulated in bacterial cells, washed cell suspensions of *K. pneumoniae* were exposed to fensulfothion sulfide, Nemacur and HOL 4438 at concentrations of approximately 50 μg/ml for 1 h at 36°C. Duplicate

5-ml samples were centrifuged at 1800 *g* for 15 min. The supernatant and resuspended cell pellet fractions (5 ml phosphate buffer) were extracted with n-hexane, and analysed by gas chromatography.

Results

Reduction of Fensulfothion Sulfide by K. pneumoniae

Cell suspensions of *K. pneumoniae* reduced fensulfothion to fensulfothion sulfide in 3 h, initial and final concentrations of fensulfothion being 44·7 and 0·3 μg/ml, and of the sulfide 6·7 and 51·0 μg/ml, respectively, after this period. The authenticity of the metabolite was confirmed by comparison of retention times on two columns as well as by thin-layer chromatography with an authentic sample of the sulfide. Despite prolonged incubation (48 h) no further modifications of the fensulfothion sulfide molecule were detected.

Effect of Temperature, pH, Sulfydryl-reacting Agents and Oxygen on the Reduction of Fensulfothion by K. pneumoniae

The optimum temperature for the reduction of fensulfothion to the sulfide by *K. pneumoniae* was in the range 36–42°C. At 42°C, 23 μg fensulfothion were reduced per milligram cell dry weight per hour. Reducing activity declined sharply above this temperature and had almost completely disappeared at 50°C (1·8 μg per milligram cell dry weight per hour).

The pH optimum for the reaction was between 6·0 and 7·0 with hourly reduction rates of 20·0–22·3 μg/mg. Reducing activity was completely absent at pH 8·9 and, while markedly reduced, was still present at pH 4·0 (hourly reduction rate of 4·2 μg/mg). There was no change in the pH of the contents of any flask during the experiment and control flasks (without added cells) showed no conversion of fensulfothion to fensulfothion sulfide.

Other results (unpublished) showed that the buffer system had no effect on the reducing activity of the cells. At pH 7·2, all three buffer systems resulted in similar reduction rates.

At concentrations of 1 mM and higher all three sulfydryl-reacting agents (*N*-ethylmaleimide, iodoacetic acid, and *p*-hydroxymercuribenzoate) inhibited the reduction of fensulfothion after incubation for 1 h, as indicated in the following tabulation:

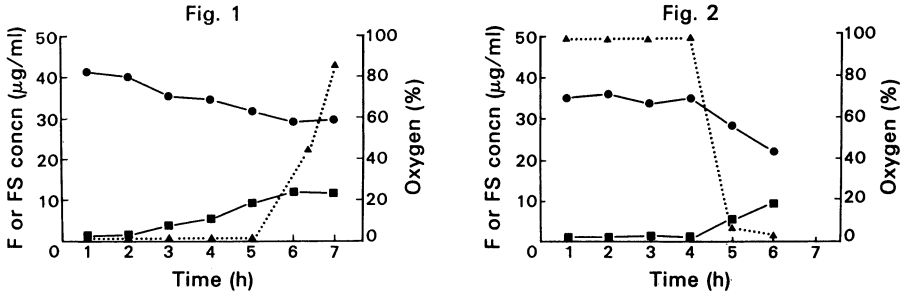
Sulfydryl-reacting agent	Fensulfothion sulfide formed (μg/mg)	Sulfydryl-reacting agent	Fensulfothion sulfide formed (μg/mg)
N-Ethylmaleimide (mM)		*p*-Hydroxymercuribenzoate (mM)	
100	0·1	1	0·4
1	0·2	0·01	3·8
0·01	4·0		
Iodoacetic acid(mM)		Control	4·0
100	0·0		
1	0·0		
0·01	4·2		

The conversion of fensulfothion to fensulfothion sulfide by *K. pneumoniae* occurred only under conditions of low oxygen tension. When cultures actively reducing fensulfothion were exposed to molecular oxygen, reducing activity rapidly ceased. Reducing

activity could, in effect, be switched off and on by the presence or absence of molecular oxygen (Figs 1 and 2). Once formed, the sulfide was not reoxidized to the sulfoxide but the presence of oxygen did prevent further reduction from occurring.

Effect of Exposing Cells of K. pneumoniae *to Fensulfothion on the Subsequent Rate of Reduction*

Exposing *K. pneumoniae* to fensulfothion did not alter reduction of the pesticide by washed cell suspensions. Hourly rates of reduction during the first 30 min were 70·5 μg per milligram cell dry weight for previously exposed cells.



Figs 1 and 2. Effect of molecular oxygen (▲) on the conversion of fensulfothion (F, ●) to fensulfothion sulfide (FS, ■) by *K. pneumoniae*. *1*, anaerobic for 4 h followed by increasing dissolved oxygen for the next 2 h; *2*, aerobic for 3 h followed by decreasing dissolved oxygen for the next 2 h.

Accumulation of Fensulfothion Sulfide and Structurally Related Compounds by Cells of K. pneumoniae

The accumulation of the sulfide by the cells was extremely rapid. Appreciable amounts were detected in the cells even after exposure for only 5 min at 36°C. After 1 h exposure at this temperature, accumulation of the sulfide had reached maximum values of 3·2 (3·3) and 32·2 (26·7) μg/mg in supernatant and cell pellets respectively, values in parenthesis being for heat-killed cells. Corresponding values for control flasks, which did not contain cells, were 37·4 and 0 μg/mg. Bioconcentration factors were calculated and showed that heat-killed cells of *K. pneumoniae* accumulated more than viable cells (factors of 10·1 and 9·1, respectively).

Lysozyme treatment of *K. pneumoniae* cells revealed that approximately 90% (9·4 μg/mg) of the fensulfothion sulfide formed by the cells (10·5 μg/mg) remained firmly bound to cell membrane components. Very little was associated with the cell wall and intracellular fraction (2·1 and 0·9 μg/mg respectively). Mechanical disruption methods confirmed these results with approximately 80% (8·1 μg/mg) of bound fensulfothion sulfide located in the cell wall and membrane component of exposed cells. Because of the vigorous nature of this method a higher level of sulfide was found in the intracellular fraction of mechanically disrupted cells (2·4 μg/mg) than with lysozyme-treated cells (0·9 μg/mg).

Fensulfothion sulfide and the closely related HOL 4438 were concentrated by *K. pneumoniae* cells (bioconcentration factors of 4·5 and 1·8 respectively) whereas almost all the Nemacur remained in solution (factor of 0·1). None of the compounds appeared to be metabolized during the incubation period.

Discussion

The conversion of fensulfothion to fensulfothion sulfide by *K. pneumoniae* had temperature and pH optima and was quite sensitive to molecular oxygen. Unless very low oxygen concentrations were used the reaction did not proceed. Consistent with these oxygen effects the reaction was quite sensitive to sulfydryl-reacting agents. These factors suggest that the reduction of fensulfothion by *K. pneumoniae* is enzyme-mediated.

Exposure of *K. pneumoniae* cells to fensulfothion did not enhance their subsequent reducing ability. This suggests that the enzyme involved is constitutive and probably serves other vital functions in the cell. Cells which were previously exposed to fensulfothion did, however, have a higher inital level of fensulfothion sulfide. This might be explained by our finding that once formed, the fensulfothion sulfide became firmly bound to the cells. Despite centrifugation and washing, the bound fensulfothion sulfide was retained by the cells. This bioconcentration effect occurred also with heat-killed cells. This suggests that the accumulation does not involve biological energy but is simply the result of a high affinity of fensulfothion sulfide for bacterial cell membrane components.

Our results suggest a possible sequence of events involved in the conversion of fensulfothion to fensulfothion sulfide by *K. pneumoniae*. An enzyme, which is extremely sensitive to molecular oxygen is present and continually being produced by *K. pneumoniae* cells. It results in the cleavage of oxygen from the fensulfothion molecule to produce fensulfothion sulfide. The presence of molecular oxygen at this stage does not reoxidize the sulfide but does prevent further reduction occurring. Once formed, the fensulfothion sulfide is rapidly bound by the cells. This bioconcentration is not enzyme-mediated and is not related to fensulfothion reducing ability since killed cells also concentrate the sulfide.

In our experiments, detectable amounts of fensulfothion were never found in hexane extracts of the cells of *K. pneumoniae* alone. Therefore, the experimental bioconcentration factor for fensulfothion would be close to zero. This may have been due to the fact that any fensulfothion which became bound to the cells was rapidly reduced to the sulfide. Our experimental bioconcentration factor for fensulfothion sulfide of 7·2–28·8, however, is about what is expected from water solubility data (Kenaga 1980). This accumulation effect was not unique to fensulfothion sulfide as the structurally similar compound HOL 4438 was also bound by *K. pneumoniae* cells. However, Nemacur showed little affinity for bacterial cells and this might be attributed to the methylation of the benzene ring and the large $(CH_3)_2$.CHNHO-group in the molecule.

Acknowledgments

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