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Factors Influencing the Metabolism of Mexacarbate by Microorganisms

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A screening of microorganisms (bacteria, mold, and fungi) from soil, water, and stock cultures was undertaken to determine the extent and mode of degradation of mexacarbate (4-dimethylamino-3,5-xylyl methylcarbamate) in the environment. All organisms showed an ability to degrade mexacarbate and two organisms, HF-3, a bacteria, and *Trichoderma viride*, a fungus, were selected for more extensive study. By mass culturing and isolating the metabolic products it was possible to identify 4-dimethylamino-3,5-xylenol (DMAX) and 4-methylamino-3,5-xylyl methylcarbamate (MAZ) as the major metabolites of *T. viride* and HF-3, respectively. By studying the effects of various cofactors, inhibitors, and carbon sources it was found that *T. viride* could be induced to form N-desmethylation products (MAZ) where in standard media only decarbamylation (DMAX) occurs. Using these same factors HF-3 would only form N-desmethylation products (MAZ). In both cases mexacarbate was found to be metabolized fastest in the absence of an added carbon source.

As the use of the chlorinated hydrocarbon insecticides decreases, the importance of carbamate and phosphate insecticides as possible alternatives will increase. One carbamate insecticide which has shown good effectiveness and may be utilized extensively for control of forest insect pests is mexacarbate (4-dimethylamino-3,5-xylyl methylcarbamate). Numerous workers have studied the chemical (photooxidation) and enzymatic (plants and animals) degradation of mexacarbate. In animals and plants the primary site of attack is at the carbamyl end of the molecule (Williams *et al.*, 1964; Krishna and Casida, 1966); however, Roberts *et al.* (1969) showed that oxidation at the *N*-dimethyl end of the molecule can occur in the insect cuticle. Deamination of the molecule to form the hydroquinone can occur and conjugates of 4-dimethylamino-3,5-xylenol and 2,6-dimethyhydroquinone have been identified. Photooxidation of mexacarbate attacks the molecule at the dimethylamino group (Abdel-Wahab and Casida, 1967). Four products are normally formed; the toxic methylamino and amino analogs and the less toxic methylformamido and formamido are formed.

Although enzymatic and chemical degradations of mexacarbate have been studied extensively, the microbial aspects of degradation have not. The object of the work reported in this paper was to demonstrate the ability of microorganisms to degrade mexacarbate and to clarify the general pathway of this metabolism.

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EXPERIMENTAL SECTION

Screening. A total of 35 aerobic organisms were isolated from water and soils, some of which were collected from mexacarbate-treated forests, and from housefly gut, as described by Matsumura and Boush (1967). Pure cultures of these organisms were grown on 10 ml of yeast–mannitol culture media for 24 hr before amending with 10 μl of a 10^{-3} *M* ethanol solution of ring-labeled [^{14}C]mexacarbate (4 mCi/mmol obtained from Dow Chemical Co. and purified by tlc). Cultures were incubated for 2 weeks. Each tube was extracted with chloroform (Matsumura and Boush, 1967) and the radioactivity was counted in the chloroform and water fractions in order to determine recoveries (80–100%). The chloroform extracts were evaporated to 0.1 ml and a portion of the residue was spotted on a silica gel G tlc plate. The mobile phase employed for separating the metabolites was a mixture of ethyl ether, hexane, and ethanol (77:20:3). Chromatograms were developed to 15 cm and subsequently autoradiographed using Kodak No-Screen Medical X-ray film.

Two distinct types of metabolic patterns (Figures 1A and 1B) were observed in the screening study. Two microorganisms were therefore selected for further study: a bacteria isolated from housefly gut (HF-3) for the A-type pathway and a fungus, *Trichoderma viride* (culture No. 12, Matsumura and Boush, 1967), for the B-type pathway.

Mass Culture. For culturing HF-3, a modified culture media containing inositol (carbon source) and NADH (0.1 mg/ml) was incubated for 24 hr and then amended with 15 mg of unlabeled mexacarbate in 10 ml of ethyl acetate. After 2 weeks, at 30°, the mixture was extracted with

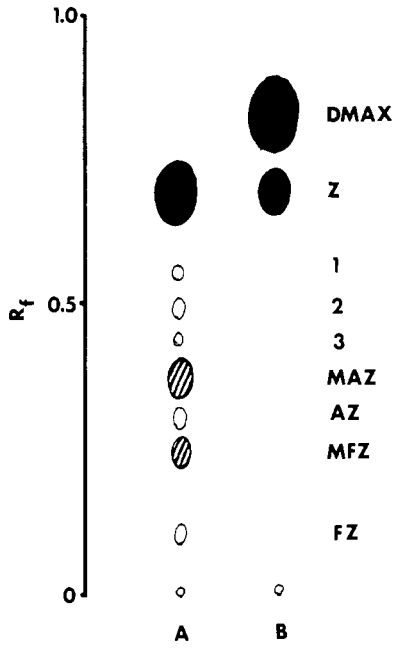

Figure 1. Representation of the autoradiograms of the two types of pathways of mexacarbate metabolism from tlc plates developed by using ethyl ether–hexane–ethanol (77:20:3): (A) metabolic pattern of the bacteria HF-3; (B) metabolic pattern of *Trichoderma viride;* DMAX, 4-dimethylamino-3,5-xylenol; Z, 4-dimethylamino-3,5-xylyl methylcarbamate (mexacarbate); MAZ, 4-methylamino-3,5-xylyl methylcarbamate; AZ, 4-amino-3,5-xylyl methylcarbamate; MFZ, 4-methylformamido-3,5-xylyl methylcarbamate; FZ, 4-formamido-3,5-xylyl methylcarbamate; 1, 2, and 3, unidentified metabolites.

chloroform. The chloroform was evaporated under reduced pressure and the residue dissolved in hexane. The metabolites were separated on tlc as previously described.

For the mass culture of *T. viride* 2 l. of standard media was amended with mexacarbate, incubated, extracted, and purified as described for HF-3.

Growth Factor Study. The two selected organisms were grown in the presence of [^{14}C]mexacarbate on standard yeast–mannitol medium with various cofactors and an inhibitor: NAD^+ (0.2 mg/ml), NADH (0.2), $NADP^+$ (0.2), NADPH (0.2), flavine adenine dinucleotide (FAD, 0.4), coenzyme A (CoA, 0.1), ATP (0.3), glutathione (GSH, 0.2), and mersalyl acid (0.18 μg/ml, a mercury inhibitor). The media were also modified by changing the carbon source to one of the following: potassium fumarate, sodium citrate, sodium succinate, inositol, or glucose. Extraction and autoradiographic techniques were the same as those described earlier. The quantity of each metabolite was determined by scraping the tlc plates where autoradiographic spots indicated metabolites were present. The silica gel was placed in scintillation fluid and the radioactivity measured using a scintillation counter.

Soil Study. A soil mixture was heated for 1 hr at 200°, washed with water and acetone, and a 20-g portion was placed in three 50-ml flasks. To each flask, 5 ml of nutrient solution containing 0.1 g of yeast extract, 2 mg of NaCl, 4 mg of $MgSO_4 + 7H_2O$, and 10 mg of K_2HPO_4 was added. To one of these flasks an additional 0.2 g of glucose was added. All the flasks were autoclaved at 15 lb pressure for 15 min. The flask containing glucose and one of the remaining flasks were inoculated with *T. viride,* and after 24 hr all three flasks were amended with 10 nmol of [^{14}C]mexacarbate. After 2 weeks the soil was extracted with a mixture of acetone and chloroform (1:1) and autoradiographed as previously described.

RESULTS

The metabolism of mexacarbate by a representative group of organisms is shown in Table I. Mexacarbate was degraded 70–100% during the 2-week test period and the major metabolite formed was 4-dimethylamino-3,5-xylenol (DMAX). Over this same period no degradation of mexacarbate occurred in sterile media. Only a few of the cultures of microorganisms did not exhibit the ability to decarbamylate the mexacarbate molecule. These organisms formed some of the same metabolites found by Abdel-Wahab and Casida (1967), namely methylamino mexacarbate (MAZ) and methylformamido mexacarbate (MFZ).

From the mass culture of *T. viride,* DMAX was confirmed as the major metabolite by comparing ir (Figure 2) and mass spectra (Figure 3) of the metabolite and chemically synthesized compound. Only one compound was isolated and identified from the mass culture of HF-3 which was incubated with mexacarbate. This compound was identified by ir (Figure 4) and mass spectra (Figure 5) and by comparison with a standard compound (obtained from Dow Chemical Co.) to be MAZ. Additional mexacarbate degradation products, including MFZ, amino mexacarbate (AZ), and formamido mexacarbate (FZ), were identified by tlc using standard compounds (Figure 1).

With *T. viride,* normally giving B-route metabolism, the addition of $NADP^-$, NADPH, and ATP gave the standard DMAX. The amount of DMAX decreased by 40% and MAZ increased by 6% in the presence of CoA, NAD^-, and NADH and when inositol and carbon-deficient media were used. Decreases in the amount of DMAX were from 50 to 100% and increases in the amount of MAZ were

Table I. Metabolic Products[a] of Mexacarbate Produced by a Group of Microorganisms as Detected by Autoradiography[b]

No.	Type of organism	DMAX	MAZ	MFZ	AZ	FZ	Met. 1	Met. 2	Met. 3	Origin (R_f 0)
12	*T. viride*	+++	−	−	−	−	−	−	−	−
HF-3	Short rod	−	+++	++	+	+	−	−	+	+
HF-1	Unidentified	+	++	+	+	+	+	+	+	+
33	*Pseudomonus*	++	−	−	−	−	−	−	−	−
71		++	−	−	−	−	±	±	−	±
204		+++	−	−	−	−	−	−	−	−
2601	Forest isolate	++	−	−	−	−	−	+	−	+
2602	Forest isolate	−	+++	+	+	+	−	++	±	+
2604	Forest isolate	+	+++	+	+	+	+	++	±	+
2608	Forest isolate	+++	+	−	−	−	−	−	±	+
2610	Forest isolate	−	+++	+	−	+	−	+	±	+
2614	Forest isolate	±	+++	+	−	+	−	+	±	+

[a] Metabolites detected by autoradiography of tlc plates: +++, very dark; ++, dark; +, light spot; ±, just detectable; −, no spot. [b] Abbreviations used are: DMAX, 4-dimethylamino-3,5-xylenol; MAZ, 4-methylamino-3,5-xylyl methylcarbamate; MFZ, 4-methylformamido-3,5-xylyl methylcarbamate; AZ, 4-amino-3,5-xylyl methylcarbamate; FZ, 4-formamido-3,5-xylyl methylcarbamate.

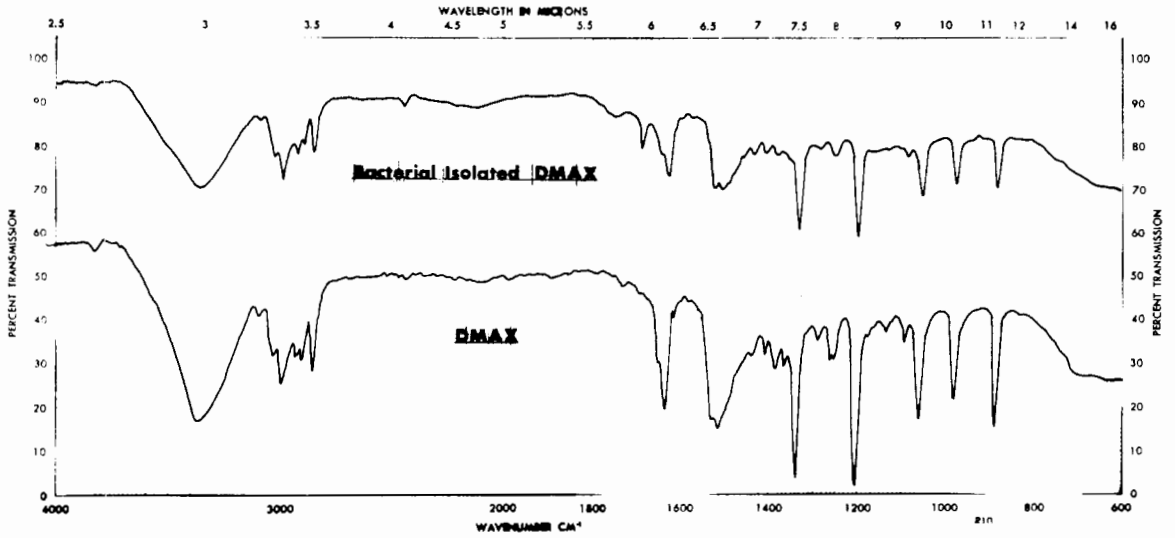

Figure 2. Infrared spectrum of standard 4-dimethylamino-3,5-xylenol (DMAX) and fungal metabolite DMAX.

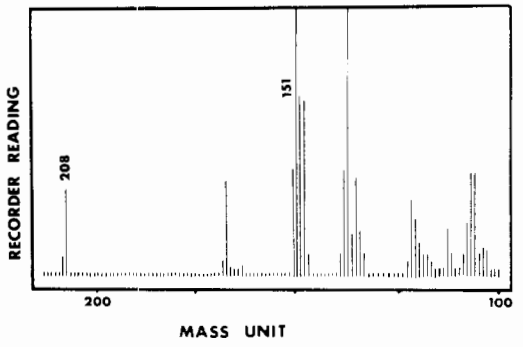

Figure 3. Mass spectrum of fungal metabolite DMAX. The two major mass peaks of pure DMAX are indicated on the spectrum by their mass number.

from 20 to 55% with citrate and glucose as carbon sources and in the presence of GSH and mersalyl acid. Some increase in other minor metabolites was found, particularly when succinate was used as the carbon source.

In the soil study, although recoveries were lower than the pure culture study (60% recovery for soil, 90% for pure culture), results were similar. Metabolism of mexacarbate in soil containing *T. viride* and glucose was 28% formation of MAZ, which was the same as untreated soil. This degradation was probably due to chemicals or microorganisms native to the soil used. In the low carbohydrate soil containing *T. viride*, 72% MAZ was formed, 44% higher than the control.

When HF-3 was studied, a maximum of 6% of DMAX was formed with any of the modified media. The major metabolite with most of the modified media was MAZ. In carbon-deficient and inositol media, and when mersalyl acid was used as an inhibitor, 50–86% of the total material extracted was MAZ. In the presence of NADH, ATP, and $NADP^+$, and when citrate was the carbon source, 20–38% of mexacarbate was converted to MAZ. All other modifications of the media resulted in the formation of less than 20% of this metabolite with the smallest amount of metabolism occurring in the presence of GSH, and when glucose was the carbon source. The amount of AZ, FZ, and MFZ increased up to 19% total in the presence of mersalyl acid, or when citrate, fumarate, succinate, or inositol was used as a carbon source. In the presence of FAD a maximum of 86% of these metabolites were formed: AZ, 20%; FZ, 9%; and MFZ, 37%. These metabolites were identified by comparing the R_f values of the metabolites with standard compounds.

DISCUSSION

T. viride, an example of a fungal organism which metabolizes mexacarbate principally through decarbamylation (route B, Figure 6), could be induced to produce metabo-

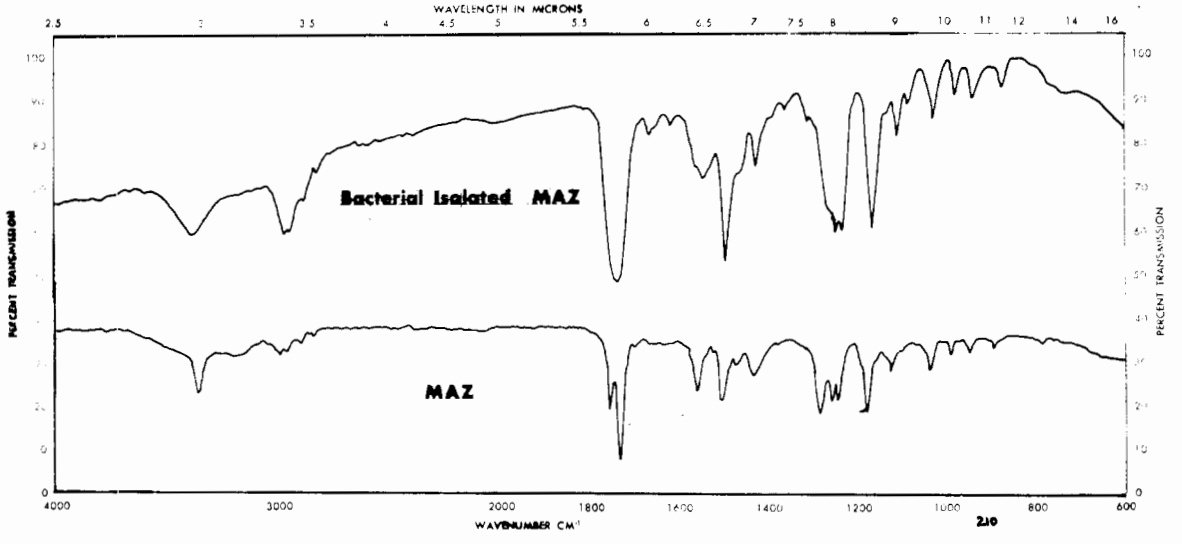

Figure 4. Infrared spectrum of standard 4-methylamino-3,5-xylyl methylcarbamate (MAZ) and bacterial metabolite MAZ.

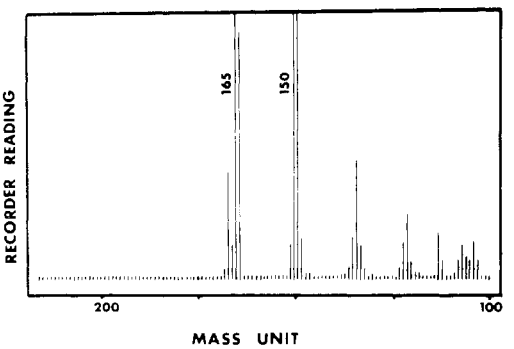

Figure 5. Mass spectrum of bacterial metabolite MAZ. The two major mass peaks of pure MAZ are indicated on the spectrum by their mass number.

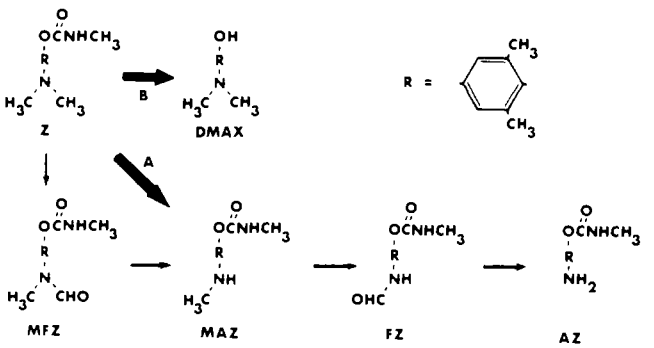

Figure 6. Proposed pathways for the metabolism of mexacarbate by two microorganisms: (A) N-desmethylation pathway that predominates for HF-3 bacteria; (B) decarbamylation pathway that predominates for the fungus, *T. viride*. A second less likely A pathway would be a methyltransferase system.

lites by desmethylation of the *N*-dimethyl group (route A, Figure 6), which includes 4-methylamino-3,5-xylyl methylcarbamate (MAZ), by altering the incubation and growth conditions. The decarbamylation system predominates in the presence of ATP, $NADP^+$, and NADPH, whereas the desmethylation process is facilitated by NAD^+ and FAD. The enzyme system responsible for decarbamylation is sensitive to mercury inhibition, indicating its nature to be an SH enzyme. In the presence of this inhibitor the amino desmethylation products predominate. There appear to be at least two different enzymes in *T. viride* causing the metabolism of mexacarbate. This is further substantiated by the fact that many of the growth factors favoring one system inhibit the other. It is interesting that mexacarbate seems to be an easily utilizable carbon source in that mexacarbate metabolism is higher in a carbon-deficient media and with sugars that are not readily utilized (inositol) than with easily utilized carbon sources (glucose).

The second type of microorganism, HF-3 as an example, does not form DMAX but metabolizes mexacarbate by desmethylation at the dimethylamine end of the molecule (Figure 6, route A). The enzyme system causing desmethylation seems to be resistant to inhibition by mersalyl acid. The amount of mexacarbate metabolism is increased in the presence of the inhibitor. Many of the respiratory enzymes are inhibited by mercury and, in organisms having the facilities, anaerobic and resistant systems would provide the energy requirements of the organism. The metabolism of mexacarbate increases under mercury inhibition and in carbon-deficient systems. In soil studies no enhanced microbial metabolism occurred in soil amended with glucose, whereas in low nutrient soil the formation of 44% MAZ could be attributed to enhanced microbial activity. This demonstrates the possible use of the methyl groups of mexacarbate as an energy source. Examples of such N-desmethylation processes can be found in kynurenineformamidase metabolism of tryptophan by microorganisms (Behrman, 1962). Further metabolism from MAZ to AZ occurs particularly in the presence of FAD.

The importance of the ability to metabolize mexacarbate by many microorganisms found in the environment is readily seen. Also important is the observation that some microorganisms can adjust their metabolic patterns according to the nutritional condition of their environment. If the above laboratory studies can be any indication of the actual field transformation activities, it is expected that the parent compound will rapidly be replaced by its metabolites, the N-desmethylation and/or the decarbamylation products depending on the nutritional conditions of the environment. Environmental studies are needed to clarify exactly which product will predominate under given conditions.

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