Conversion of Dexon (p-Dimethylaminobenzenediazo Sodium Sulfonate) to N,N-Dimethyl-p-Phenylenediamine by Pseudomonas fragi Bk9

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The metabolism of the fungicide Dexon (p-dimethylaminobenzenediazo sodium sulfonate) by a soil bacterium is reported for the first time. The organism which is capable of using Dexon only by a co-metabolic process was obtained by enrichment culture and was identified as Pseudomonas fragi. The first metabolic product of Dexon was identified as N,N-dimethyl-p-phenylenediamine. The presence of an enzyme, p-dimethylaminobenzenediazo sodium sulfonate reductase, capable of reducing Dexon to N,N-dimethyl-p-phenylenediamine has been demonstrated in the cell-free extracts of the organism. The enzyme is found to be in the soluble fraction and requires dithiothreitol as a reductant.

One of the important ways by which pesticides are removed from the environment is by microbial metabolism. Depending upon their basic chemical structure they are susceptible to varying degrees to microbial attack. Thus, different groups of pesticides like organo-phosphates, organo-chlorines, mercurials, carba-mates, triazines, etc. are known to be degraded by microbes to different products (4, 5). However, not much information is available on the breakdown of pesticides having a diazo group. The fungicide Dexon (p-dimethylaminobenzenediazo sodium sulfonate, DABDS), for example, is widely used to protect different crops against “damping-off” diseases caused by phycomycetes (13). Several workers have shown the disappearance of this compound in soil, a process which is rather fast under tropical conditions but somewhat slow in other regions (1, 9). However, the actual mode of degradation of Dexon was not established. The purpose of this communication is to report studies on the metabolism of Dexon by a soil pseudomonad.

MATERIALS AND METHODS

Materials. N,N-dimethyl-p-phenylenediamine (DMPDA) was purchased from K and K Laboratories, Hollywood, Calif. Analytical grade Dexon was a gift from Farbenfabriken Bayer, A.G., Germany. All the other chemicals were of A.R. grade available commercially.

Isolation of the organisms. Enrichment cultures were set up using basal salt medium (11) containing 0.01% Dexon as carbon or nitrogen, or carbon and nitrogen source, and were inoculated with soil previously exposed to Dexon. Repeated attempts to isolate organisms using Dexon as a sole source of carbon or nitrogen, or both, failed. Dexon was then added to a complete medium containing glucose (0.1%), and ammonium nitrate (0.1%) and fresh enrichments were started. After several transfers the cultures were streaked on a similar medium solidified with 2% agar. The colonies that developed were picked and purified by the usual methods. The isolates were again checked for growth on the Dexon-containing medium and those strains that caused a distinct fall in Dexon concentration were retained for further study. To follow Dexon disappearance, suitable samples were taken and centrifuged to remove the cells. To 1 ml of the culture filtrate, 50 ml of 1% sodium sulfite was added and Dexon was estimated by the method of Anderson and Adams (2). The bacterial isolates were characterized by following Skerman’s key (15) and Bergey’s Manual (3). One of the strains (Bk9) was identified as Pseudomonas fragi and was used in these investigations.

Analysis of culture filtrate and replacement medium. The organism was grown in the mineral medium described by Seubert (14) containing 0.5% glucose supplemented with 0.01% Dexon. After 18 h of growth on a rotary shaker at 30 C, the cells were harvested, and washed twice with 0.1 M sodium phosphate buffer (pH 7.0). The cells were used for replacement studies. The culture filtrate was then adjusted to pH 8.0 with 1 N NaOH and extracted twice with equal volumes of peroxide-free ether. The aqueous layer was discarded and the ether extract was evaporated under a vacuum. The residue was dissolved in 1 ml of ethyl acetate, and a 0.5-ml sample was used for the quantitative determination of N,N-dimethyl-p-phenylenediamine as detailed later; the
other 0.5 ml was used for paper chromatography. Suitable samples were spotted on Whatman no. 3 paper and subjected to ascending paper chromatography by using the organic phase of benzene: acetic acid: water (4:4:1, vol/vol/vol) as the solvent system. The chromatogram was sprayed with Ehrlich’s reagent (2% p-dimethylaminobenzaldehyde in ethanolic hydrochloride, prepared by mixing 80 ml of 95% ethanol with 20 ml of 6 N HCl).

The washed cells (5.0 g) were resuspended in 100 ml of 0.1 M sodium phosphate buffer (pH 7.0) containing 80 μmol of Dexon (henceforth referred to as the replacement medium). The culture was incubated at 30 C for 24 h with gentle agitation. Samples (10 ml each) from the replacement medium were removed aseptically at different time intervals. The samples were treated as above to isolate the metabolic products of Dexon.

Preparation of cell-free extract. Eighteen-hour-old cultures were harvested, washed twice with 0.1 M sodium phosphate buffer (pH 7.0), suspended in four volumes of 0.025 M sodium phosphate buffer (pH 7.0), and disrupted for 5 min in a Branson sonifier. The temperature of sonic treatment was less than 5 C and the current strength was held at 5 A. The lysate was then centrifuged at 10,000 × g for 10 min. The supernatant fluid was designated as the crude enzyme (16 to 18 mg of protein per ml). The crude enzyme was centrifuged at 100,000 × g for 1 h in a Beckman model L-3-50 preparative ultracentrifuge. The pellet was resuspended in 0.025 M sodium phosphate buffer (pH 7.0) and again centrifuged at 100,000 × g for 1 h. The pellet was uniformly dispersed in 0.025 M sodium phosphate buffer (pH 7.0) with the help of a fine hypodermic syringe. This preparation is referred to as the particulate fraction and the earlier 100,000 × g supernatant fluid is referred to as the supernatant fraction.

Assay of DABDS reductase. DABDS reductase activity was measured by incubating, for 30 min at 30 C, a reaction mixture (1.5 ml) containing sodium phosphate buffer, pH 7.0, (70 μmol), Dexon (2.5 μmol), and the required enzyme preparation (3 mg of protein). The reaction was stopped by adding 0.1 ml of 30% trichloroacetic acid. The pH of the reaction mixture was then adjusted to 8 by adding 0.2 ml of 10% NaOH. The contents of each tube were extracted with 4 ml of distilled ethyl acetate. To 1.0-ml samples of the organic phase was added 0.5 ml of Ehrlich’s reagent followed by 4.0 ml of 95% ethanol. The intensity of the yellow color developed was determined in a Klett-Summerson photoelectric colorimeter against a reagent blank, by using a no. 42 filter (400 to 465 nm). The response to increasing concentration of DMPDA was linear up to 0.1 μmol.

Isolation and identification of the enzyme product. Dexon (80 μmol) was incubated with the crude enzyme (30 ml) under the conditions of the assay procedure. After 2 h of incubation the pH of the reaction mixture was adjusted to 8.0 with 10% NaOH and the solution was extracted thrice with peroxide-free ether. The ether extracts were pooled, dried over anhydrous sodium sulfate, and evaporated to dryness under vacuum. The residue was dissolved in a small volume of ethyl acetate and chromatographed on Whatman no. 3 paper by using benzene:acetic acid: water (4:4:1, vol/vol/vol), organic phase) as the solvent system. A sample strip from the paper was cut out and the position of DMPDA was located by spraying with Ehrlich’s reagent. The band on the paper corresponding to the yellow spot of DMPDA was cut out and eluted with ether and dried. The chromatographic behavior of the product was studied in the different ways listed below:

(i) Ascending paper chromatography on Whatman no. 3 paper using the following solvent systems: (a) benzene:acetic acid:water (4:4:1, vol/vol/vol), (b) benzene:methanol:acetic acid (45:8:4, vol/vol/vol), (c) n-butanol:pyridine:water (140:30:30, vol/vol/vol).

(ii) Thin-layer chromatography on Silica gel G plates (0.25 mm thickness) using the following solvent systems: (d) n-butanol:acetic acid:water (40:10:50, vol/vol/vol), (e) isopropanol:ammonia:water (20:1:2, vol/vol).

The ultraviolet (UV) and infrared (IR) absorption spectra were also recorded. UV spectra were taken in ethyl acetate using the Unicam SP-700A recording spectrophotometer. The IR spectra were recorded in a Carl Zeiss, Jena UR 10 spectrophotometer, the material being taken as a film.

Since Dexon is known to be decomposed by light, all studies were conducted under yellow-green light (7).

RESULTS AND DISCUSSION

The analysis of the culture filtrate and replacement medium on paper chromatograms gave two minor and one major Ehrlich-positive spots. Of the two minor spots, the one which moved with the solvent might be the same unidentified compound as observed by Hills (6). The second spot, with an Rf value of 0.12, corresponded well with authentic DMPDA. In the replacement medium, this compound could be detected within 2 h and continued to accumulate until 16 h, after which its concentration decreased (Fig. 1). When a Dexon solution decomposed by exposure to light (7) was incubated with the cells, DMPDA could not be detected in the medium. This shows that DMPDA is formed from Dexon and not from any of its light-decomposition products.

The crude enzyme, DABDS reductase, had maximum activity at pH 7.0, and the activity was proportional to the amount of protein added up to 6 mg. The reaction was linear for 150 min and the crude enzyme showed no requirement for any cofactor. Also, the light-decomposition product(s) was not found to be the substrate for DABDS reductase.

The distribution of DABDS reductase activity in different fractions is given in Table 1.
Table 1. Intracellular distribution of DABDS reductase from P. fragi Bk9

| Fraction                  | DMPDA formed (nanomoles) |
|---------------------------|--------------------------|
| Crude                     | 388                      |
| Particulate               | 0                        |
| Supernatant               | 0                        |
| Supernatant + particulate | 354                      |
| Supernatant + DTT\*       | 378                      |
| Particulate + DTT         | 34                       |

* Dithiothreitol, 2 × 10⁻¹ M.

The 100,000 × g supernatant did not show any activity when tested without cofactors. As shown in Table 2, when various cofactors and thiol compounds that are generally required for reductases were tried, it was found that addition of dithiothreitol (DTT) resulted in the recovery of more than 95% of the activity. Other reagents allowed only partial recovery (20 to 26%).

The activity could also be restored in the 100,000 × g supernatant when a suspension of the 100,000 × g pellet was added, suggesting the presence of a membrane-bound factor which is functionally similar to DTT and which may be responsible for in vivo activity. (Attempts are being made to reveal the possible nature and properties of this factor.)

The enzyme product was identified as DMPDA by comparing its properties with an authentic sample. Both authentic DMPDA and the isolated product showed the same $R_s$ values on thin-layer chromatographic plates as well as on paper chromatograms in different solvent systems (Table 3). Co-chromatography of the product with an authentic sample in any of the solvent systems gave a single spot. The UV spectrum of the enzymic product showed a broad peak at 258 nm in ethyl acetate and was indistinguishable from that of authentic DMPDA. Also, the IR spectra of both the enzymic product and the authentic sample were identical as may be judged from Fig. 2. The results indicate that DMPDA is formed by the reductive cleavage of the diazoate-Dexon (Fig. 3). The other product of the reaction is yet to be identified. Attempts are underway to purify DABDS reductase.

It is interesting to note that DMPDA is also the first product formed during the metabolism of azo compounds such as $p$-dimethylamino-azobenzene (butter yellow) in mammalian liver. In these systems DMPDA is known to be metabolized further (12). It has been recently

Table 2. Effect of cofactors and thiol compounds on DABDS reductase activity in the supernatant fluid of cell-free extracts from P. fragi Bk9

| Additions   | DMPDA formed (nanomoles) |
|-------------|---------------------------|
| Nil         | 0                         |
| NADH        | 0                         |
| NADPH       | 0                         |
| FAD + NADH  | 93                        |
| FAD + NADPH | 97                        |
| β-mercaptoethanol | 85                      |
| Reduced glutathione | 0                       |
| Sodium metabisulfite | 70                       |
| DTT         | 375                       |
| DTT + FAD   | 371                       |

* The concentrations used were 2 × 10⁻¹ M for thiol compounds and 10⁻¹ M for cofactors. The activity was assayed as described under Materials and Methods.

Table 3. $R_s$ values of authentic DMPDA and enzymic product in different chromatographic systems

| Chromatograms | Solvent* system | Authentic | Enzyme product |
|---------------|-----------------|-----------|----------------|
| Paper         | a               | 0.12      | 0.13           |
| Paper         | b               | 0.31      | 0.32           |
| Paper         | c               | 0.82      | 0.82           |
| Thin layer    | d               | 0.43      | 0.43           |
| Thin layer    | e               | 0.72      | 0.72           |

* Details in the text.
reported that the administration of DMPDA itself affects the levels and activities of several oxido-reductases in animal tissues (8). The present observation that DMPDA is the first product formed during microbial co-metabolism of Dexon further emphasizes the need for the detection of major products in addition to the parent compounds when doing residue analysis.

The inability of the organism to use Dexon as a sole source of carbon or to metabolize it to a stage where it can assimilate the carbon demonstrates that Dexon might be disposed of only by a co-metabolic process. In this connection it is of interest that the addition of Dexon to soil without any other amendments resulted in a significant fall in the organic carbon content of the soil (10). We could also recover DMPDA from soils treated with Dexon. DMPDA is as toxic as Dexon to *Pythium aphanidermatum* (unpublished data), which is interesting from the point of view of microbial associations and activation of pesticides in the environment.

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