Bacterial Metabolism of Arylsulfonates: Role of meta Cleavage in Benzene Sulfonate Oxidation by Pseudomonas testosteroni

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Pseudomonas testosteroni H-8 oxidizes certain lower alkylbenzene sulfonates at rates inversely related to the length of the alkyl group. Appreciable Q(O)₂ values were observed for benzene sulfonate (BS), toluene sulfonate (TS), and ethylbenzene sulfonate (EBS), but not for propylbenzene sulfonate (PS) and higher homologues. Catechol oxidation was catalyzed by a constitutive catechol-2,3-oxygenase (EC 1.99.2.1). Yellow meta cleavage products accumulated when BS-grown cells were exposed to catechol, 4-methylcatechol, 3-methylcatechol, EBS and PS, but not BS or TS. Traces of a yellow metabolite (probably 2-hydroxymuconic semialdehyde) were detectable during growth on BS. PS completely inhibited growth on BS, but not on L-leucine or nutrient broth. Also, PS antagonized respiration on BS and catechol, but not glutamate, the extent of inhibition being directly related to PS concentration. Formation of a meta cleavage product from PS, and inhibition of catechol oxidation by PS, suggested that the actual inhibitor may not be PS itself, but a metabolite.

Arylsulfonates are important intermediates and by-products in industrial chemical processes and frequently are present in industrial wastewaters. For this reason bacterial degradation of arylsulfonates may assume considerable significance in biological treatment of such wastewaters. It is known that benzene sulfonate (BS) and p-toluene sulfonate (TS) are readily oxidized by certain bacteria, and the overall reaction pathway in Pseudomonas has been outlined (2, 4, 6). A considerable body of information also exists on biodegradation of the related alkylbenzene sulfonate detergents (12). We have described a bacterium utilizing BS and TS as sole carbon source for growth and some characteristics of BS oxidation by this organism (11). This report presents additional information on oxidation of arylsulfonates by Pseudomonas testosteroni H-8.

MATERIALS AND METHODS

Organism and culture conditions. Mineral salts medium and conditions for growth of P. testosteroni H-8 in shaken flasks were as described previously (11). This organism has been deposited with the American Type Culture Collection, Rockville, Md., as ATCC 27911. For larger quantities of cells, 10-liter cultures in mineral salts medium with BS (1 g/liter) as carbon source were grown in a 14-liter Microform fermenter (New Brunswick Scientific Co.). The temperature was 30 C, with an impeller speed of 250 rpm and aeration at 4 liters/min.

Respiration of washed cell suspensions. Manometric measurements of oxygen uptake by washed cells were made as described previously (11).

Preparation of cell-free extract. Washed cells grown on BS were suspended at about 0.1 g (wet weight)/ml in distilled water containing 2-mercaptoethanol (10⁻⁴ M, final concentration) and disrupted by 5 min of sonic oscillation (Biosonic, Bronwill Scientific Co.). Cell debris was removed by centrifugation at 10,000 × g for 10 min in a Sorvall RC-2B refrigerated centrifuge, and the supernatant fluid was used as the crude extract (protein content, 3 mg/ml). Oxidation of diphenols by crude extract was measured manometrically at 30 C. Warburg vessels received substrate (10 μmol), extract (1.5 mg of protein), and pH 7.0 phosphate buffer (50 μmol) in a total volume of 2.8 ml (plus 0.2 ml of 40% KOH in center well).

Preparation of catechol-2,3-oxygenase. Cells were grown on BS and a partial purification of catechol-2,3-oxygenase was made by the method of Nozaki et al. (9). The preparation used corresponds to the dialyzed acetone precipitate fraction of Nozaki et al. (9).

Determination of protein. Protein was determined by the method of Lowry et al. (8) using bovine serum albumin fraction V as standard.

Preparation of yellow reaction products from catechols and certain arylsulfonates. meta Cleavage intermediates from catechol, 3-methylcatechol, 4-methylcatechol, ethylbenzene sulfonate (EBS) and propylbenzene sulfonate (PS) were isolated by a modification of the method of Feist and Hegeman (5).
Washed cells (3.3 mg, dry weight) were incubated with substrate (10 μmol) in 10 ml of pH 8.0 tris(hydroxymethyl)aminomethane hydrochloride buffer (0.003 M) at 30 C until maximum development of yellow color. Cells were removed by centrifugation at 10,000 × g for 10 min (Sorvall RC-2B), and the supernatant fluid was extracted three times with 25-ml portions of diethyl ether. The ether extract was discarded and the aqueous layer was adjusted to pH 2.0 with HCl before extracting again with three portions (25 ml each) of diethyl ether. This ether extract was recovered and immediately shaken with 10 ml of pH 7.6 tris(hydroxymethyl)aminomethane hydrochloride (0.2 M) to recover the product. The buffer solution was placed under vacuum to remove residual ether before determination of absorption spectra using a Beckman DB spectrophotometer.

Conversion of 2-hydroxymuconic semialdehyde to picolinic acid. A 10-ml sample of the yellow product prepared from catechol (presumably 2-hydroxymuconic semialdehyde) was treated with excess NH₄Cl (20 μmol). The mixture was adjusted to pH 5.0 and shaken for 18 h at 30 C to permit formation of picolinic acid (2, 3).

Chemicals. Sodium BS (BS, benzene sulfonic acid, sodium salt), sodium TS (TS, 4-methyl-benzene sulfonic acid, sodium salt) and sodium p-hydroxybenzoate were obtained from J. T. Baker Chemical Co., Phillipsburg, N.J. Catechol (1,2-dihydroxybenzene), sodium 2,4-dimethylbenzene sulfonate, sodium 2,5-dimethylbenzene sulfonate, and alpha-picolinic acid hydrochloride (pyridine-2-carboxylic acid hydrochloride) were from Eastman Organic Chemicals, Rochester, N.Y. 3-Methylcatechol (3-methyl-1,2-dihydroxybenzene) and 4-methyl catechol (4-methyl-1,2-dihydroxybenzene) were obtained from K and K Laboratories, Plainview, N.Y. Protocatechuic acid (3,4-dihydroxybenzoic acid), glutamic acid, and L-leucine were products of Sigma Chemical Co., St. Louis, Mo. EBS (EBS, 4-ethyl-benzene sulfonic acid), PS (PS, 4-propyl-benzene sulfonic acid, and butylbenzene sulfonate (butylbenzene sulfonate, 4-n-butyl-benzene sulfonic acid) were previously described (11) laboratory preparations made by treatment of the corresponding 1-phenyl alkane with fuming sulfuric acid. Under the conditions used the predominant reaction is a monosubstitution in the para position.

RESULTS

Oxidation of arylsulfonates by washed cell suspensions. P. testosteroni H-8 can oxidize BS and certain short-chain alkylbenzene sulfonates (11). Q(O)₂ values obtained for BS, TS, and EBS were relatively high, but oxidation rates were very slight for PS and two isomeric dimethylbenzene sulfonates (Table 1). An inverse relationship between respiratory rate and chain length of the alkyl substituent was evident.

Inhibition of BS oxidation by other arylsulfonates. Certain arylsulfonates were found to produce a significant inhibition of oxygen uptake with BS. Some 40 to 50% inhibition resulted when cells were incubated 10 min with 60 μmol of 2,4-dimethylbenzene sulfonate, 2,5-dimethylbenzene sulfonate, or PS before tipping in 20 μmol of BS. Inhibition with butylbenzene sulfonate was essentially complete (97%) and may have been due to partial lysis of the cells.

Oxidation of catechol by P. testosteroni H-8. Cells grown on BS were found to oxidize catechol rapidly and without lag (Fig. 1, curve A). This would be the expected result if H-8 metabolizes BS by the pathway described by Cain and Farr (2); i.e., if catechol serves as an obligatory intermediate in BS oxidation, growth on BS should yield cells fully induced for catechol oxidation. Cells of H-8 also were able to oxidize catechol (without lag) when grown on

Table 1. Oxidation of arylsulfonates by washed cells of P. testosteroni H-8 grown with BS as sole carbon source

| Substrate                  | Expt 1 (20 μmol/vessel) | Expt 2 (2 μmol/vessel) |
|----------------------------|------------------------|------------------------|
| BS                         | 89                     | 97                     |
| TS                         | 82                     | 85                     |
| EBS                        | 72                     | NT*                    |
| PS                         | 9                      | NT                     |
| 2,4-Dimethylbenzene sulphonate | NT                  | 4                      |
| 2,5-Dimethylbenzene sulphonate | NT                  | 10                     |

*NT, not tested.

![Figure 1](image-url)
other substrates, such as p-hydroxybenzoate (which presumably should be metabolized via protocatechuate, not catechol) and glutamate (Fig. 1, curves B and C). From this it was concluded that the catechol-oxidizing enzyme in H-8 is constitutive rather than inducible.

In conformity with its taxonomic assignment, P. testosteroni H-8 carries out a meta cleavage of diphenols. Formation of the yellow meta cleavage product from catechol (i.e., 2-hydroxymuconic semialdehyde) by a dilute cell suspension of H-8 is shown in Fig. 2. Much of the yellow product was extracellular and remained in solution after removal of the cells. Rupture of the cells by toluene treatment was not required for the reaction, but disappearance of the yellow product was more rapid in the toluenized cells.

![Figure 2](image)

**Fig. 2.** Formation of 2-hydroxymuconic semialdehyde from catechol by washed cells of P. testosteroni H-8 with (○) and without (O) toluene treatment. Reaction was begun by addition of catechol (0.2 μmol) to 74 μg (dry weight) of cells suspended in 3 ml of 0.016 M phosphate (pH 7.0).

A crude extract prepared from BS-grown cells was able to oxidize catechol and also other diphenols (Table 2). Oxidation rates for catechol, 3-methylcatechol, and 4-methylcatechol were about five to six times higher than for protocatechuate. It is not known whether the lower activity with protocatechuate truly reflects differences in amounts or activities of enzymes or simply greater inactivation during preparation of the extract.

The catechol oxygenase activity was partially purified by the method of Nozaki et al. (9), but proved unstable even when protected with acetone. This preparation catalyzed a rapid uptake of oxygen with catechol (approximately 28 μl of O₂/min per mg of enzyme protein), but not with protocatechuate, resorcinol, or pyrogallol. Oxygen consumption was proportional to amount of enzyme supplied over the range 100 to 500 μg of protein per vessel and was accompanied by formation of a yellow product with an absorption maximum at 377 nm. This activity required no added cofactors, was abolished by boiling, and was not inhibited by the following substances at 10⁻² M (final concentration): p-chloromercuriphenyl sulfonate, 2-mercaptopethanol, α,α-dipyridyl, and potassium cyanide. The yellow product from catechol was recovered by ether extraction of a larger reaction mixture and shown to have properties similar to 2-hydroxymuconic semialdehyde. At pH 7.6 it had an absorption maximum at approximately 377 nm, whereas at more acid pH values this peak was depressed and a new peak appeared at 320 nm (Fig. 3). It showed a melting point of 210 C and formed a 2,4-dinitrophenylhydrazone.

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**Table 2.** Oxidation of diphenols and glutamate by a crude cell-free extract prepared from BS-grown cells of P. testosteroni H-8

| Substrate          | Sp act (μl of O₂/min per mg of enzyme protein) |
|--------------------|-----------------------------------------------|
| Catechol           | 5.7                                           |
| 4-Methylcatechol   | 4.7                                           |
| 3-Methylcatechol   | 5.7                                           |
| Protocatechuate    | 1.0                                           |
| Glutamate          | 1.5                                           |

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when treated with 2,4-dinitrophenylhydrazine. It has been reported (2,3) that 2-hydroxyxymuconic semialdehyde is converted to picolinic acid by incubation with ammonium ion. When the yellow product was incubated with excess NH₄Cl under acid conditions, it was converted to a colorless substance with an absorption maximum at 266 nm. Authentic picolinic acid under these conditions also had an absorption peak at 266 nm. These properties suggest that the enzyme is a catechol-2,3-oxygenase (EC 1.99.2.a).

Ability of the crude extract to catalyze oxidation of protocatechuic acid could be interpreted as indicating induction of a protocatechuate oxygenase by BS. Presumably the enzyme involved is a protocatechuate-2,3-oxygenase because of the yellow color formation in the reaction vessels. Such activity was present, however, even in cells grown on a nonaromatic substrate. With washed cells grown on glutamate, the Q(O)₂ for protocatechuic acid was 21. This does not necessarily mean a complete absence of induction effects, because in another experiment cells grown on p-hydroxybenzoate showed a Q(O)₂ of 60 for protocatechuic acid.

Formation of yellow products during oxidation of EBS and PS. During manometric measurements it was observed that vessels receiving EBS and PS turned bright yellow, presumably because of accumulation of meta cleavage products. No such color developed with BS and TS. Formation of these yellow products could be monitored spectrophotometrically during incubation of EBS and PS with cell suspensions (Fig. 4). Appearance and disappearance of the yellow substances from the alkylbenzene sulfonates proved to be slower than the corresponding reactions with catechol.

The meta cleavage products from EBS, PS, catechol, 3-methylcatechol, and 4-methylcatechol were recovered by ether extraction of acidified reaction mixtures and the absorption spectra were compared (Table 3). On the basis of absorption maxima the products from EBS and PS seemed to resemble the product from 4-methylcatechol. This would be expected for operation of the pathway described by Cain and Farr (2).

Occurrence of meta cleavage product during growth on BS. No appreciable color was formed during oxidation of BS in Warburg vessels, but a slight yellowing of the medium often was noticeable during growth of the culture with BS. The absorption spectrum of clarified spent growth medium (16-h culture) showed an absorption peak in the neighborhood of 375 to 380 nm, similar to that for 2-hydroxyxymuconic semialdehyde. Also present were the characteristic triple peaks in the 240- to 280-nm region due to unused BS.

**Fig. 4. Formation and disappearance of yellow products from EBS (Δ), PS (○), and catechol (□) during incubation with P. testosteroni H-8. Mineral salts medium (100 ml) plus 0.2 µmol of substrate and 5.4 mg (dry weight) of cells was shaken in a 1-liter Erlenmeyer flask at 30°C. Samples (5 ml) were frozen in liquid nitrogen, thawed, and membrane filtered to remove cells.**

**PS as a growth inhibitor for P. testosteroni H-8.** The possibility that PS might act as an inhibitor of growth on BS was examined. It was found that PS does indeed strongly inhibit growth. When BS was supplied at 1 g/liter, a 38% inhibition of growth was produced by PS at a concentration of 0.02 g/liter, and a 96% inhibition by 0.04 g/liter.

The next question to be answered was whether PS acted as a general growth inhibitor for H-8, or whether it was limited to inhibition of growth on arylsulfonates. H-8 was inoculated into flasks of mineral salts medium containing selected growth substrates with and without added PS (0.25 g/liter). It was found that growth on BS was almost completely inhibited by PS, but there was no significant inhibition when the growth substrate was L-leucine or nutrient broth (Table 4). PS also produced a 42% inhibition of growth when p-hydroxybenzoate served as carbon source.

**PS as a respiration inhibitor.** Next, effects of PS concentration on inhibition of BS oxidation were examined. The cells were incubated with PS during the 10-min temperature equilibration period before tipping in BS. With 20 µmol of BS per vessel (i.e., 7.14 × 10⁻⁴ M), the extent of inhibition was a function of PS concentration (Fig. 5). It was noted that, as before, the contents of the vessels receiving PS became yellow, indicating some metabolism of PS.

It was of interest to rule out the possibility that PS was acting as a generalized respiratory inhibitor. The inhibitory effect of PS was found
TABLE 3. Absorption maxima of meta cleavage products from selected aromatic compounds

| Starting compound   | 48 h growth* |
|---------------------|--------------|
|                     | No PS | PS (0.25 g/liter) |
| **Growth substrate (1 g/liter)** |       |                  |
| BS                  | 0.63  | 0.03             |
| *p*-Hydroxybenzoate | 0.78  | 0.46             |
| L-Leucine           | 1.00  | 0.83             |
| Nutrient broth      | 1.10  | 1.30             |

* In pH 7.6 tris( hydroxymethyl) aminomethane buffer (0.2 M).

* Optical density at 600 nm.

to be selective (Table 5). With BS-grown cells, oxidation of catechol was strongly antagonized by PS, but oxidation of glutamate was unaffected.

**DISCUSSION**

There is a general impression that enzymes for aromatic metabolism in *Pseudomonas* are invariably inducible, being present in the cells only when the organism has been exposed to the particular inducer molecule or its metabolic precursors. This undoubtedly stems from the pioneer studies of Stanier (13, 14) on the simultaneous induction patterns for aromatic metabolism in the fluorescent pseudomonads. In the case of *P. testosteroni* H-8 the overall oxidation of arylsulfonates is inducible (11), but at least one key enzyme in the pathway, catechol-2,3-oxygenase, is constitutive. Although perhaps a minor point, it may be important for those attempting to trace sequences in aromatic pathways to be aware that these enzymes on occasion can be constitutive.

Another area of interest is the specificity of H-8 with respect to the alkylbenzene sulfonates that it will oxidize. We noted an inverse relationship between oxidation rates and size of the alkyl substituents. Because of the accumulation of meta cleavage products from EBS and PS, we are led to speculate that the rate-limiting reac-

![Fig. 5. Inhibition of BS oxidation by added PS. Cells were exposed to PS 10 min before substrate (BS, 20 μmol/vessel, or 7.14 × 10⁻³ M, final concentration) was tipped in.](image-url)

**TABLE 5. Effect of PS on oxidation of catechol and glutamate by *P. testosteroni* H-8 (grown on BS)**

| Substrate                          | Q(0)ₙ |
|------------------------------------|-------|
| Catechol (20 μmol)                  | 108   |
| PS (20 μmol)                        | <1    |
| Catechol (20 μmol) + PS (20 μmol)  | 34    |
| Catechol (20 μmol) + PS (60 μmol)  | 17    |
| Glutamate (20 μmol)                | 91    |
| Glutamate (20 μmol) + PS (60 μmol) | 97    |

| Substrate                          | Q(0)ₙ |
|------------------------------------|-------|
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be desulfonated to give the 4-alkylcatechol, which in turn would be cleaved yielding a 2-hydroxy-5-alkylmuconic semialdehyde. We suggest that increases in the size of the alkyl group render the latter molecule progressively less susceptible to the action of the 2-hydroxy
muconic semialdehyde hydrolase.

There are several possible explanations for the inhibitory effects of PS on growth and respiration. One possibility would be competition between substrates for entry into the cell. Competition between BS and PS for the same uptake site would seem plausible and would account for the lack of effect of PS on nonaromatic compounds. The partial inhibition by PS of growth on p-hydroxybenzoate and the strong inhibition of catechol oxidation would have to be explained by assuming either that the same uptake site was used for all of these molecules or else that PS was able to compete at more than one site. A trivial explanation would be that the observed effects might have been due to impurities or other reaction products present in the PS preparation. A third and more attractive explanation is that PS inhibits some step in aromatic metabolism inside the cell. Some metabolism of PS takes place, despite the low Q(O), values, because a meta cleavage product accumulates. Therefore, the actual inhibitor could be PS itself, the 4-propylcatechol resulting from desulfonation or the ring fission product, 2-hydroxy-5-propylmuconic semialdehyde. The inhibition of both BS and catechol oxidation suggests that the desulfonation reaction is probably not the one affected.

Regardless of the precise mechanism involved, this demonstration that one arylsulfonate can inhibit oxidation of another may have some practical significance in wastewater treatment. It may be that the inhibitory effects will prove to be less severe in complex microbial populations because of the variety of enzymes and pathways involved, but the possibility of such inhibition should be borne in mind when dealing with mixtures of arylsulfonates.

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