Degradation of Alkyl Benzene Sulfonate by Pseudomonas Species

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Pseudomonas sp. HK-1 showed a direct relation between the concentration of alkyl benzene sulfonate (ABS) supplied and cell yields. Since growth on ABS alone did not occur, it was necessary to correlate the total energy obtained by the cells to the ABS concentration when glucose was supplied in a limiting concentration. Several types of metabolic attack in addition to the sulfonate removal were noted: (i) side-chain utilization as indicated by the production of tertiarybutyl alcohol and isopropanol and (ii) ring metabolism as indicated by the presence of phenol, catechol, mandelic acid, benzyl alcohol, and benzoic acid in spent growth media. Utilization of ABS was greatly enhanced by the presence of phenol. This enhancement suggests co-metabolism and that limited concentrations of phenolic products derived from ABS must be accumulated to get active metabolism of the ABS molecule.

Alkyl benzene sulfonate (ABS) is generally regarded as a biologically recalcitrant molecule. A steady increase in detergent use has been accompanied by problems such as foaming in sewage treatment plants and in the waters receiving the effluent from these plants (24). The accumulation of ABS in water is due primarily to its resistance to biodegradation, and several factors have been suggested which might affect the biodegradability. Swisher (31) stated that among the different ABS, the branched molecules are far more refractory in water and sewage than the straight-chain ABS. McKenna and Kalio (23) noted that the introduction of a single methyl branch imparts resistance to saturated hydrocarbons. This is especially true if the methyl branch is placed on the terminal carbon.

It was of biochemical interest to isolate an organism capable of degrading ABS (7). Although oxidation of the alkyl moiety of ABS would be expected to occur, attempts to isolate organisms capable of metabolizing this hydrophobic structure had led to a very limited success. This limited success did indicate, however, that organisms capable of utilizing this alkyl chain do exist in nature.

Oxidation of the benzene ring of ABS seemed to be a possibility, but this idea was not pursued initially. Kitagawa (21), studying toluene oxidation, showed the pathway to proceed through benzyl alcohol, benzaldehyde, benzoic acid, and catechol. Also, p-cresol was oxidized via the methyl group before attack on the ring (9).

House and Fries (20), while seeking information on the interaction of ABS and activated sludge, noted the production of significant amounts of inorganic 35S compounds from 35S-ABS. In 1952, Leclerc and Beauchelle reported on the production of hydrogen sulfide by bacteria metabolizing ABS (22). These observations, although they were not pursued further, tended to support the rationale that organisms capable of removing this sulfonate should occur in nature.

As previously reported, an organism was isolated from an enrichment culture, which was capable of cleaving the sulfonate moiety from alkyl benzene sulfonate and of using this sulfonate as its source of sulfur for growth (7). Approximately 70% ABS reduction was measured in 7 days in pure culture systems. The isolate, designated HK-1 and identified as a Pseudomonas sp., did not grow to a visible turbidity in a sulfur-poor glucose-salts medium but showed a good growth response to the addition of ABS to this medium. There appeared to be a direct relation between the ABS concentration and the energetics of the cell. Because the expected insoluble product, alkyl benzene,
could not be detected in these studies, investigations were initiated to ascertain the metabolic products derived from either the benzene ring or the alkane portion of the molecule.

**MATERIALS AND METHODS**

**Purification of ABS.** ABS as supplied by the Association of American Soap and Glycerine Products contained approximately 48% inorganic sulfate. This necessitated a purification procedure of ABS which was carried out as previously reported (7).

**Dry cell weight determination.** All dry weight determinations were done by measuring optical density and converting it to dry weight from a standard curve.

**Media and inocula.** The basal salts medium employed in these studies consisted of 1.0 g of NH₄Cl, 1.0 g of Na₂HPO₄, 0.5 g of KCl, and 0.1 g of MgCl₂ per liter of distilled water. ABS, glucose, inorganic sulfate, and various carbon and energy sources were added to this medium in various concentrations and combinations. A 1% inoculum was used in these studies and was taken from a 48-hr culture in basal medium plus 0.01% glucose and 0.1% ABS unless otherwise noted.

**Simultaneous adaptation growth responses.** Growth measurements for simultaneous adaptation growth responses were made by a Bonet, Maury, and Jouan biophotometer. Adapted cells were taken from a 9-day-old culture grown in ABS-glucose-salts medium. Nonadapted cells were taken from a 48-hr culture grown in glucose-salts medium. An inoculum containing 10⁷ bacteria/ml was used.

**Identification of metabolic intermediates.** Metabolic intermediates were identified by physical properties of the isolated compounds or of derivatives of the compounds. Amide derivatives were used for the identification of carboxylic acids, 3,5-dinitrobenzoate derivatives were used for identification of alcohols, and 2,4-dinitrophenylhydrazone derivatives and semicarbazone derivatives were used for the identification of aldehydes and ketones. All derivatives were prepared by procedures outlined by Shriner, Fuson, and Curtin (27). Molecular weight determinations were done by the freezing point depression method as described by Gattermann (13). Mixed melting point determinations using the isolated derivative and the same derivative of a standard compound corresponding to the suspected identity of the isolated compound were used to confirm tentative identifications whenever possible.

**Quantitative and qualitative procedures.** ABS was measured by the methylene blue procedure outlined in Standard Methods for the Examination of Water and Wastewater (3). Catechol was quantitated by the method of Arnow (4), and phenol determinations were accomplished by the 4-aminophenylpyrine method described by the United States Public Health Service (32). Benzaldehyde determinations were carried out by a modification of the diphenylamine procedure (5): 1 volume of the test sample was treated with 1 volume of a cold 20% trichloracetic acid solution and filtered. Benzaldehyde was quantitated as described by Ashwell (8). Qualitative periodic acid tests were used for the detection of epoxides and glycols according to Shriner, Fuson, and Curtin (27).

**RESULTS AND DISCUSSION.**

*Pseudomonas* sp. HK-1, the isolate employed in these studies, did not grow to visible turbidity in a sulfur-poor glucose-salts medium but showed a good growth response with the addition of ABS to this medium (7). This isolate was also capable of using inorganic sulfate as its sulfur source for growth, but, as seen in Table 1, the growth response to inorganic sulfur was not as great as the response exhibited when ABS was supplied as the sole sulfur source. Sulfur was not a limiting factor in these studies but glucose was limiting; therefore, it appeared that the greater growth response shown when ABS was added to the glucose-salts medium indicated that a greater amount of energy was available to the cell when ABS was present.

The tentative identification by ultraviolet spectrophotometry of a phenol-like compound and a catechol-like compound in spent media indicated that benzene ring oxidation was occurring in a manner similar to the oxidation of the aromatic ring structure studied in some detail by Stanier (28, 29, 30). This determination involved a counter-current distribution technique in conjunction with ultraviolet spectrophotometry. The phenol-like compound was identified in 18-day-old spent media, and the catechol-like compound, was identified in 30-day-old spent media.

Compounds isolated from spent growth media are shown in Table 2 with the melting points, derivative melting points, and molecular weight obtained.

To establish the origin of the isolated compounds as ABS, quantitation of benzaldehyde by the diphenylamine procedure and phenol

| Amt (mmoles) of ABS/liter | Amt (mmoles) of SO₄₂⁻/liter | Amt (mg)/liter (dry cell wt) |
|-------------------------|-----------------------------|-----------------------------|
| 2.870                   | 0.000                       | 378.00                      |
| 1.430                   | 0.000                       | 243.60                      |
| 0.715                   | 0.000                       | 151.20                      |
| 0.287                   | 0.000                       | 92.40                       |
| 0.000                   | 0.000                       | 50.40                       |
| 0.000                   | 0.287                       | 151.20                      |
| 0.000                   | 0.715                       | 193.20                      |
| 0.000                   | 1.430                       | 193.20                      |
| 0.000                   | 2.870                       | 193.20                      |
by the 4-aminoantipyrine procedure was carried out in ABS-glucose-salts media 3, 7, 10, and 18 days after inoculation and in glucose-salts media lacking ABS on days 3 and 18. The amount of ABS reduced was also determined by the methylene blue procedure. As seen in Table 3, from 76% (day 3) to 94% (day 18) of the reduced ABS could be accounted for as benzaldehyde-like and phenol-like compounds in the ABS-glucose-salts medium. No benzaldehyde-like or phenol-like compounds could be detected from the culture in the glucose-salts medium without ABS.

In view of the energy derived from oxidation of ring compounds by pseudomonads as studied by Stanier, it was of interest to determine the relation of degraded ABS to the energetics of the cell. Because an energy source such as glucose was required to allow growth, it was necessary to correlate the cell yield with the ABS utilized when the energy source, glucose, was present in limited concentration. By the method of calculation of Bauchop and Eldsen (6), the possible energy procedure from ABS was determined. It was calculated that 40.6 mg (dry weight) was formed for each millimole of ABS degraded. Energy calculations yielded a value of 4 mmoles of adenosine triphosphate (ATP) per mmole of ABS degraded.

Growth experiments and energy calculations were also employed to determine possible mechanisms for the reactions involved in the degradation of ABS (Table 4). Specific standards corresponding to compounds identified as intermediates of ABS degradation were used as the sole source of carbon and energy in a basal salts medium. The energy available to the cell from each standard compound utilized was calculated on the basis of maximum cell yield obtained. The amount of ATP produced by a growing culture was not measured directly. The ATP yields were approximated on the basis of total cell yield produced per millimole of substrate used. Measurement of ABS concentration, by the methylene blue procedure, before and after growth allowed calculation of ATP yields from ABS on the basis of the amount of ABS actually degraded. For all other substrates, the ATP yields calculated were related to the amount of substrate added to the medium.

The order of appearance of compounds involved in this sequence should follow Stanier's pathway for ring oxidation (28, 29, 30). Calculations based on bond energies were done to make correlations with the results obtained from growth studies. The reactions of benzyl alcohol to benzaldehyde and benzoic acid (Fig. 1) were consistent with reactions proposed for the metabolism of aromatic compounds by P. fluorescens. The conclusions based on cell yield data and bond energy calculations were the same as those based on respiratory data (28, 29). The formation of phenol by isolate HK-1 seems to be an important reaction in ABS degradation. The catabolism of the ABS molecule with glucose and the disappearance of ABS after phenol has been detected in the ABS-glucose medium suggest an active role for a phenolic compound in the degradation of ABS.

The proposed reaction for the oxidation of phenol to catechol is shown in Fig. 2. Since growth of isolate HK-1 in the absence of glucose occurred on phenol only in the presence of ABS, a coupled reaction could be postulated. This reaction could involve the desulfonation of ABS with a simultaneous oxidation of phenol to catechol. Energy calculations based on growth data indicated a net gain of 2.98 kcal per mmole of phenol oxidized by the isolate in the presence of ABS. However, the oxidation of phenol to catechol, in the absence of ABS, would be expected to release 31.7 kcal based on bond energy calculations. Apparently, the energy from this exergonic reaction is utilized to drive the endergonic desulfonation of ABS. Thus, bond energy calculations based on this proposed coupled reaction yield a value of 3.0 kcal of energy per mmole of ABS and phenol.

To obtain quantitative results in the determination of inorganic sulfate resulting from the degradation of ABS, it was necessary to oxidize inorganic sulfur products in spent
TABLE 3. Quantitation of benzaldehyde-like and phenol-like compounds and ABS recovered in spent growth media of Pseudomonas sp.

| Media* | Age (days) | ABS reduced (mM) | Phenol (mM) | ABS present as phenol (%) | Benzaldehyde (mM) | ABS present as benzaldehyde (%) | ABS present as benzaldehyde + phenol (%) |
|--------|------------|------------------|--------------|---------------------------|------------------|-------------------------------|---------------------------------|
| A      | 3          | 0.000            | 0.000        | 0.000                     | 0.000            | 0.000                         | 76.2                            |
| A      | 18         | 0.000            | 0.000        | 0.000                     | 0.000            | 0.000                         | 93.8                            |
| B      | 3          | 1.305            | 0.688        | 52.4                      | 0.311            | 23.8                          | 87.8                            |
| B      | 7          | 1.545            | 1.297        | 84.0                      | 0.151            | 9.8                           | 93.8                            |
| B      | 10         | 1.940            | 1.376        | 71.1                      | 0.245            | 12.6                          | 83.7                            |
| B      | 18         | 2.154            | 1.755        | 81.7                      | 0.074            | 3.4                           | 85.1                            |

* A = 0.01% glucose-salts; B = 0.1% ABS + 0.01% glucose-salts.

TABLE 4. Twenty-four-hour growth response of Pseudomonas sp. to the addition of compounds, which are possibly related to ABS degradation, to the basal salts medium*

| Medium supplement | Amt [mg (dry cell wt)] per mmole of substrate added | Amt (mmole) of ATP/ mmole of substrate added |
|-------------------|---------------------------------------------------|---------------------------------------------|
| Benzyl alcohol    | 2.01                                              | 0.201                                       |
| Benzoic acid      | 9.68                                              | 0.968                                       |
| Phenol            | 32.48                                             | 3.248                                       |
| ABS               | 0.00                                              | 0.000                                       |
| Phenol + ABS      | 0.00                                              | 0.000                                       |
| Glucose           | 80.80                                             | 8.080                                       |
| Glucose + ABS     | 121.40                                            | 12.140                                      |
| t-Butanol         | 18.87                                             | 1.887                                       |
|                   | 0.00                                              | 0.000                                       |

* Compounds were added at 2.4 mmoles/liter. Growth at the expense of glucose + ABS minus growth at expense of glucose = growth attributable to ABS. Values were 40.60 mg (dry cell weight) per mmole of substrate added and 4.060 mmole of ATP per mmole of substrate added.

media to sulfate by using hydrogen peroxide (7). This test required the generation of inorganic sulfur compounds either as sulfate or as those oxidizable to sulfate by addition of hydrogen peroxide. The sodium sulfite resulting from the proposed coupled reaction would explain the required oxidation by hydrogen peroxide to determine sulfate.

Cain and Farr (8) also reported the release of the sulfonate moiety of benzenesulfonate, p-toluene sulfonate, and benzene sulfonate detergent homologues as sulfite by P. aeruginosa growing at the expense of these compounds. However, phenol was not formed during growth of this organism on the benzene sulfonate substrates, and desulfonation appeared to

![Fig. 1. Proposed pathways for the oxidation of benzyl alcohol to benzaldehyde, benzaldehyde to benzoic acid, and benzoic acid to phenol by isolate HK-1.](image1)

![Fig. 2. Mechanism of desulfonation of an alkyl benzene sulfonate intermediate by isolate HK-1.](image2)
involving the incorporation of molecular oxygen into the aromatic nucleus to yield catechol.

Neither Focht and Williams (11) nor Ripin, Noon, and Cook (26) were able to detect any accumulation of sulfite during metabolism of p-toluene sulfonate by Pseudomonas sp. However, Ripin et al. (26) suggested that this might be due to the rapid enzymatic conversion of sulfite to sulfate. The direct production of sulfate as described in these reports (11, 26), without the intermediate formation of sulfite, is therefore questionable.

Phenol was not reported to be an intermediate product of p-toluene sulfonate degradation in either of the investigations (11, 26). However, Cain and Parr (8), Focht and Williams (11), and Ripin et al. (26) were concerned with the complete metabolism of aryl sulfonates by organisms capable of utilizing these compounds as sole sources of carbon and energy, rather than with co-metabolism of these aromatics in the presence of additional carbon and energy sources. Results obtained by Horvath (15, 17) and Horvath and Alexander (18) indicated that the co-metabolic attack of chloro-aromatic compounds differed from the complete metabolic attack (16) in that co-metabolism was not inhibited by meta-substitution whereas normal metabolism was inhibited (2). It is not surprising therefore, that the co-metabolism of aryl sulfonates would also differ from the mode of normal metabolic degradation of these substrates and than phenol was found to be an intermediate in the degradation of ABS by the process of co-metabolism.

Since benzyl alcohol, benzoaldehyde, and benzoic acid are present in the ABS-glucose-salts spent media, it was postulated that adaptation of the isolate to these compounds should occur in the growth medium. Simultaneous adaptation growth response experiments were employed to test this hypothesis. The adapted cells were grown in ABS-glucose-salts medium, and the nonadapted cells were grown in glucose-salts medium lacking ABS. Growth of the adapted strain began immediately at the expense of benzyl alcohol, benzoaldehyde, and benzoic acid. Significantly, the nonadapted strain exhibited a lag period of 45 to 75 min with these substrates, which was in agreement with the manometric results of Stanier, who reported a lag period of 30 to 80 min with nonadapted cultures (28, 29).

Accumulation of stoichiometric amounts of catechol in media containing ABS was not consistent with the ability of the isolate to utilize benzoic acid as a sole source of carbon and energy for growth in a medium not containing ABS. However, further metabolism of catechol appeared to be inhibited in the presence of the detergent. The Pseudomonas was capable of growth in a catechol-basal salts medium only in the absence of ABS. Growth was completely inhibited in the presence of ABS.

Initial accumulation of catechol could result from a lag in the appearance of an oxygenase required to further oxidize this compound, following the first sequential inductive steps as suggested by Hegeman (14). After 32 days, catechol was present at a 2.0 to 2.4 mM concentration, which equaled the amount of ABS degraded. With 2.0 mmole of catechol and only 0.25 mmole of ABS, growth would not occur at the expense of added glucose, indicating the presence of a toxic environment. The combination of accumulated catechol and nondegraded ABS seemed to produce a toxic environment, thus preventing further metabolism of the dihydroxylated benzene compound. This may also account for the fact that degradation of ABS never exceeded 75 to 80% of the initial ABS supplied.

The growth of isolate HK-1 in basal medium supplemented with isopropanol occurred to the extent of 18.9 mg (dry cell weight) per mmole of isopropanol in the absence of glucose. The reactions involved in the utilization of isopropanol appear to be those shown in Fig. 3. Allyl alcohol, compound no. 3 (melting point (mp) of 3,5-dinitrobenzoate derivative = 47°C; molecular weight = 58); glyceral, compound no. 4 (mp of 3,5-dinitrobenzoate derivative = 94°C; molecular weight = 74); and acetol, compound no. 5 (mp of semicarbazone derivative = 195, mp of 2,4-dinitrophenylhydrazone derivative = 129; molecular weight 73 to 75) were identified as products resulting from metabolism of isopropanol by isolate HK-1.

The formation of glyceral from allyl alcohol indicated that a monooxygenase might function in this system. A monooxygenase could also operate in the conversion of methylphenylcarbinol to phenylethylene glycol as well as in the formation of 8-phenyl-2, 4, 6, 8-tetramethyl-2-oc...
Fig. 3. Metabolism of isopropanol, an intermediate product in the degradation of ABS, by a Pseudomonas.

tanol is formed, t-butyl alcohol could result from hydrolysis of the bond between the α and β carbons to the first branch point. Hydrolysis of the bond between the α and β carbons to the next branch point would then explain the presence of isopropanol and 4-phenyl-2-pentanol. A repeat of the hydrolysis described above would yield a second mole of isopropanol and methylphenylcarbinol as shown in Fig. 5. Methylphenyl-carbinol could then undergo oxidative conversion to phenylethleneglycol. Formation of mandelaldehyde and mandelic acid would occur as does the formation of benzaldehyde and benzoic acid shown previously (Fig. 1). Bond-energy calculations yield an overall value of 4.04 mmoles of ATP per mmole of ABS for this reaction se-

Fig. 5. Proposed pathway for oxidation of the alkyl side chain and the aromatic ring of ABS by a Pseudomonas.
One possible means of desulphonation of the ABS molecule is by way of a coupled reaction with phenol (Fig. 2), and since we were unable to find any dodecyl benzene this must occur later in the degradation pathway.

The formation of significant amounts of phenol resulting from ABS degradation could occur directly by decarboxylation of benzoic acid or could result from a reduction of catechol formed from benzoate oxidation. Phenol could be detected in the supernatant fluids taken from the growth media 3 days after inoculation. Catechol concentrations did not reach significant levels until 30 days after inoculation. The order of appearance of these products would trend to support the pathway proposed for the formation of phenol from benzoic acid. The activation of growth in an ABS-salts medium by phenol, an apparent product of ABS metabolism, is in itself an interesting observation.

Results of this work and that reported by Horvath (15–17) and Horvath and Alexander (18, 19) indicate that the concept of molecular recalcitrance (1) may no longer be valid. Although those compounds previously considered to be nonbiodegradable may not support growth of microorganisms when supplied as sole sources of carbon and energy, they are subject to a co-metabolic attack, resulting in considerable alteration of the chemical and physical properties of the original molecule. The theory of molecular recalcitrance (1) should be reconsidered in terms of co-metabolism as well as complete mineralization.

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