METABOLISM OF 4-HYDROXYISOPHTHALIC ACID BY A *PSEUDOMONAS* SPECIES

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ABSTRACT

A bacterium capable of utilizing 4-hydroxyisophthalic acid as a sole carbon source was isolated from tap water, and was identified as *Pseudomonas* sp. by cultural, morphological and biochemical characteristics. Protocatechuic acid was isolated from the culture medium and was also shown to be oxidized by cells. Protocatechuate was further degraded through β-ketoadipate. Though, *p*-hydroxybenzoic acid was oxidized by cells grown on 4-hydroxyisophthalic acid, the relative rates of oxidation of these two compounds by whole cells as well as cell-free extracts suggests that *p*-hydroxybenzoate oxidizing enzymes may be gratuitously induced by 4-hydroxyisophthalic acid.

INTRODUCTION

PHTHALIC acids and their derivatives are very widely used in the plastic industry and are also known to be environmental contaminants[1]. Several studies on the microbial degradation of phthalic acids and phthalate esters showed the existence of diverse catabolic routes in different bacteria (2 and references cited therein). We have studied the degradation of isophthalate by a *Micrococcus* sp and showed the possible involvement of a hydrodiol intermediate in the formation of protocatechuate which is subsequently degraded by an ortho cleavage pathway[2]. Elmorsi and Hopper have suggested that 4-hydroxyisophthalic acid (4-HIP) may be an intermediate in the metabolism of isophthalate by a Pseudomonas[3]. However, the *Micrococcus* sp isolated by isophthalate enrichment failed to utilize 4-HIP as a carbon source. There are very few reports on the degradation of hydroxyisophthalic acids. 5-hydroxyisophthalate is degraded through 4,5-dihydroxyisophthalate and protocatechuate in a soil bacterium[4], whereas 4-HIP an intermediate in 2,4 xylenol metabolism by *Pseudomonas putida* is directly converted to protocatechuate by an oxidative decarboxylation reaction[5]. The enzyme catalyzing this reaction has been purified and studied[6]. In this communication, our results on the degradation of 4-HIP by a *Pseudomonas* sp are presented.

MATERIALS AND METHODS

Growth of the organism:

4-HIP utilizing organism was isolated from tap water and was purified by repeated plating on 4-HIP mineral salts agar plates. The organism was identified by its cultural, morphological and biochemical characteristics. Bergey's manual of determinative bacteriology (8th edition) was consulted for identification[7]. The organism was grown on a mineral medium[8] containing 0.2% 4-HIP as the sole carbon source in 500 ml Erlenmeyer flasks, on a rotary shaker at room temperature (around 25°C). Isolation and identification of phenolic compounds and oxygen uptake studies were carried out as described earlier[2], except that the concentration of aromatic substrate used in respirometer experiments is 5 μmol instead of 2 μmol.

Enzyme assays:

Cell-free extract was prepared in the following way. Cells (4 g wet weight) were suspended in 10 ml of phosphate buffer, pH 7.5 (0.05 M), sonicated for 5 min and centrifuged at 12,000 g for 20 min. The supernatant was further cleared by centrifuging at 1,05,000 g for 1 hr and was used for all the enzyme assays.

4-HIP hydroxylase activity was assayed on a Gilson Oxygraph fitted with a Clark's oxygen electrode. The total assay mixture (1.2 ml) contained phosphate buffer, pH 7.6 (120 μmoles) 4-HIP (0.5 μmoles) and NADPH (0.5 μmoles). *p*-Hydroxybenzoate (PHBA) hydroxylase assay was same as that of 4-HIP hydroxylase except that the substrate used is PHBA. Assay of protocatechuate dioxygenase and the identification of mode of ring cleavage are same as described[2]. Protein was estimated by the method of Lowry *et al*[9] and all the specific activities are expressed as μmoles of substrate disappeared/min/mg protein.

RESULTS AND DISCUSSION:

The organism isolated from tap water grew well on 4-HIP and its cultural, morphological and biochemical characters are given in table 1. It was identified as a species of *Pseudomonas*. The organism utilized 4-HIP rapidly reaching stationary phase within 16 hr. Analysis of the culture filtrate at various time intervals before the organism reached stationary phase, showed the accumulation of an ortho dihydroxy compound in the medium, which was identified as protocatechuic acid by its R_f values in different solvents, colour reactions and ultra-violet and infra red spectral characteristics (table 2). Oxygen uptake by whole cells showed increased oxygen consumption with 4-HIP, PHBA and protocatechuic acid. Salicylate did not result in any increase in oxygen uptake (figure 1). However, the rate of oxygen uptake with PHBA is much slower than that shown by 4-HIP. The formation of PHBA from 4-HIP involves a simple decarboxylation step and does not require any oxygen consuming reaction. If PHBA were to be an intermediate in 4-HIP degradation it is expected that PHBA should show atleast same rate of oxygen uptake as that observed with 4-HIP However, as the experiment was performed with the whole cells, some permeability barrier for PHBA could possibly

Table 1 *Cultural, morphological and biochemical characteristics of the organism*

Characteristic	Observation
1. Colony morphology	Blue, transparent, thin circular colonies on 4 HIP-agar plates.
Vegetative cells	
Shape	short rod
Motility	motile
Endospore	none
Gram reaction	negative
Growth in anaerobic agar	–
2. Biochemical tests	
Catalase	+
Oxidase	+
Amino acid deamination	–
Nitrate reduction	+
Indole production	–
Indole utilization	–
H_2S production	–
Starch hydrolysis	–
Gelatin liquefaction	–
Citrate utilization	+
Methyl red	–
Voges proskauer	–

\+ positive findings
– negative findings

Table 2 *Chromatographic and spectral properties of the compound isolated from the medium.*

Property	Isolated compound	Authentic protocatechuic acid
A. R_f values in		
Isopropanol/ ammonia/water (20:1:2)	0.03	0.03
Benzene/acetic acid/ water (6:7:3)	0.04	0.03
Formic acid/ water (2:98)	0.55	0.54
B. Colour reaction with		
(a) Diazotized *p*-nitroaniline	Pale grey	Pale grey
(b) Arnow's test	Cherry red	Cherry red
C. Ultra violet absorption	258	258
λ_{max} in ethanol	290	290

* Infra-red spectra of isolated compound and authentic protocatechuate are found to be superimposable (data not shown).

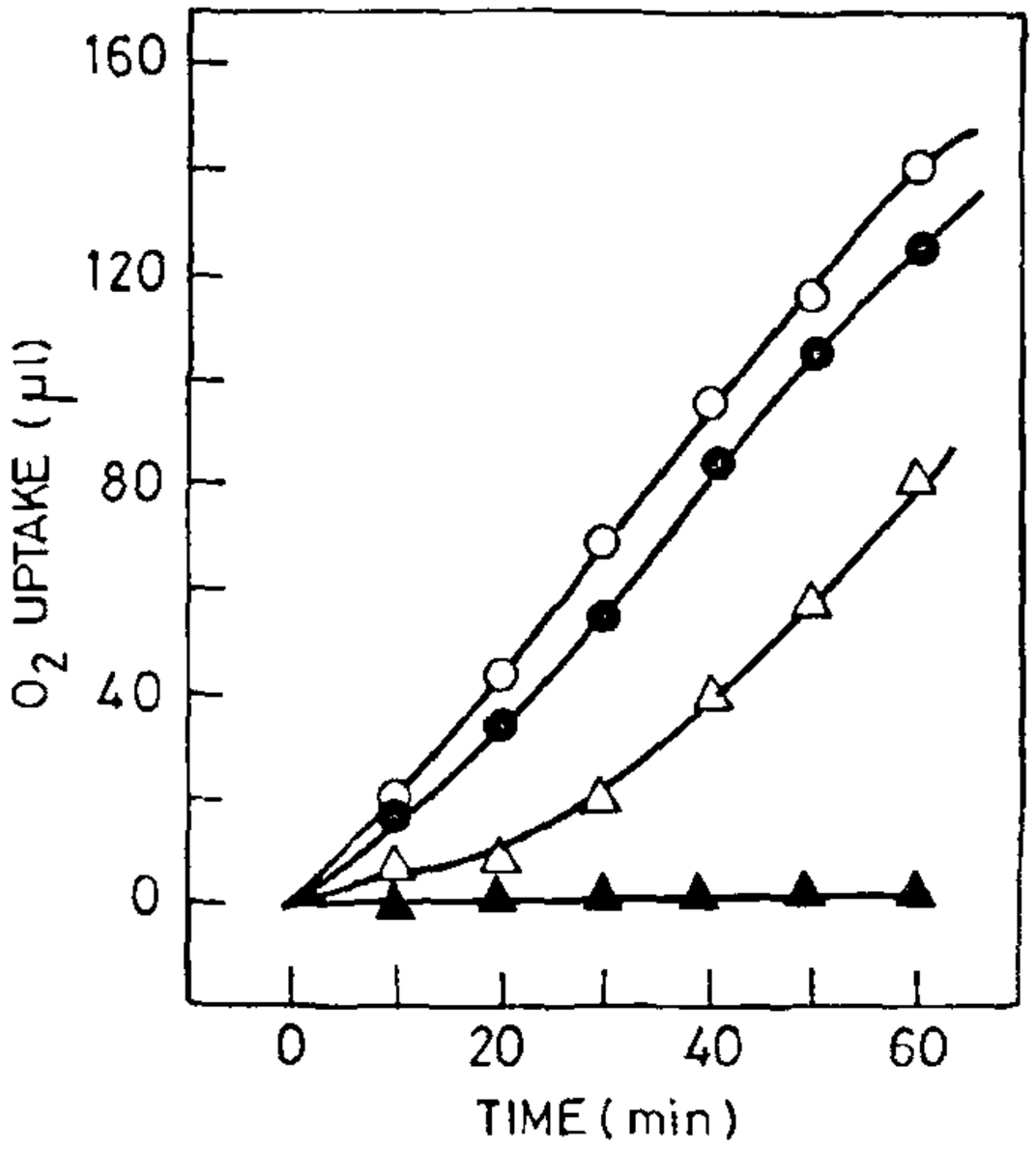

Figure 1. Oxygen uptake by cells grown on 4-hydroxyisophthalate with 4-hydroxyisophthalate (○–○–○) p-hydroxybenzoate (—Δ—Δ—), protocatechuate (—●—●—) and salicylate (—▲—▲—) oxygen uptake in the absence of any substrate was subtracted from all the values.

lower the rate of oxygen consumption. Though the cell-free extracts showed the presence of both 4-HIP hydroxylase (specific activity 0.24) and PHBA hydroxylase (specific activity 0.025) the former is ten times more active. The low level of PHBA hydroxylase in comparison with 4-HIP hydroxylase rules out the possibility of PHBA as an intermediate in 4-HIP degradation. The possibility of the same enzyme catalyzing the hydroxylation of both 4-HIP and PHBA at different rates was checked by undertaking the purification of 4-HIP hydroxylase activity (data not shown). The purified enzyme did not show any hydroxylating activity towards PHBA and the PHBA hydroxylase activity was completely lost even after the first DEAE cellulose chromatography. As observed by Elmorsi and Hopper[6] these results also indicate that a separate PHBA hydroxylase was induced when the cells are grown on 4-HIP. Protocatechuate is metabolized by ortho cleavage as evidenced by positive Rothera's reaction. No yellow coloured compounds appeared in the medium. The pathway for the catabolism of 4-HIP is shown in figure 2. The failure of the organism to grow on isophthalate did not allow us to look for the possibility of 4-HIP being an intermediate in isophthalate degradation as suggested by Elmorsi and Hopper[3]

Some organisms are known to utilize two or more pathways for the degradation of the same compound[10,11]. It is possible that part of 4-HIP may be decarboxylated to PHBA which may be subsequently degraded through protocatechuate by a separate hydroxylase. However, we failed to detect any 4-HIP decarboxylase activity and the anaerobic incubation of cells with 4-HIP did not show any accumulation of PHBA even after an 8 hr incubation period. These results clearly suggest that a separate PHBA hydroxylase was induced gratuitously when the cells are grown on 4-HIP.

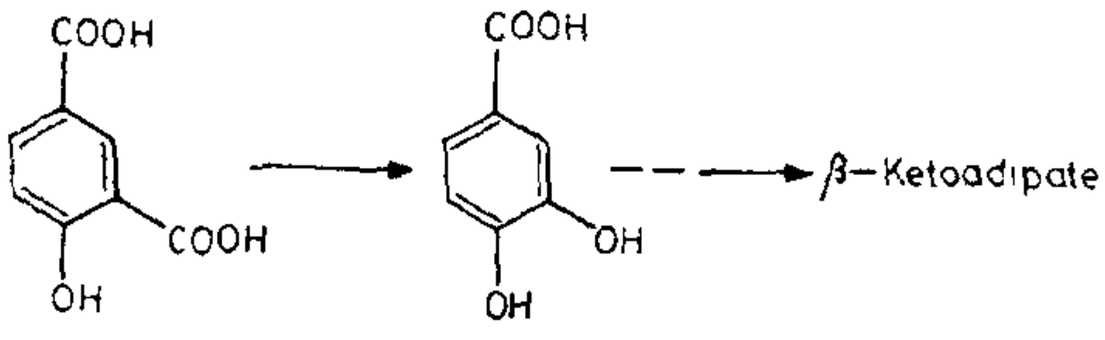

Figure 2. Proposed pathway for the 4-hydroxyisophthalate degradation.

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