MICROBIAL DEGRADATION OF PIPERONYLIC ACID BY *PSEUDOMONAS FLUORESCENS*

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1. Introduction

Although methylenedioxyphenyl compounds, such as safrole, isosafrole and piperonyl butoxide, are used commercially as insecticide synergists, little is known of the reactions undergone by the methylenedioxy ring during microbial metabolism. Mono-oxygenase activity towards safrole and dihydrosafrole was found by Harrison and Ribbons [1] in several pseudomonad strains grown on these substrates, and demethylenation by NADPH-dependent mono-oxygenases has been reported in rat liver microsomal fraction [2]. In these enzyme systems, however, oxygenation of the methylene bridge was not the major route by which the methylenedioxyphenyl compound, piperonylic (3, 4-methylenedioxybenzoic) acid was metabolised.

It was previously reported [3–5] that cell-free extracts of *Pseudomonas fluorescens,* strain PM3, which grows rapidly with p-methoxybenzoate as sole carbon and energy source would, in addition to demethylating the growth substrate, oxidatively attack piperonylate with the consumption of 2 moles of oxygen per mole substrate. Oxidation resulted in the accumulation of Rothera positive material, the 2, 4-dinitrophenylhydrazone of which was chromatographically identical with that of laevulinic acid, suggesting that degradation proceeded via protocatechuate which then underwent intradiol ring fission and further conversion to 3-oxoadipate. Observations are now presented on the stoichiometry of the initial oxidation and on the degradative pathway of piperonylate via protocatechuate.

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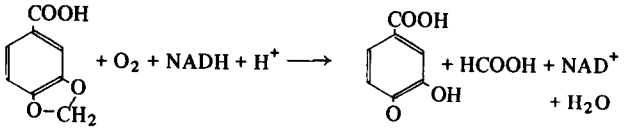
2. Materials and methods

Ps. fluorescens, strain PM3, was grown as described previously [4]. Cells were broken by sonic irradiation for 5 min and suspensions clarified [4]. The supernatant fraction, S2, produced by centrifugation at 78,000 *g* for 45 min was further centrifuged at 164,000 *g* for 3 hr to yield the soluble fraction containing the oxidative activity, fraction S3. Piperonylate mono-oxygenase and 4-*O*-demethylase activities were assayed polarographically and manometrically as indicated. All other conditions were reported previously [4].

3. Results and discussion

Oxidative attack to yield protocatechuate and formic acid involved the consumption of 1 mole of O_2 per mole of piperonylate and required NADH, or NADPH, as co-factor (fig. 1). The rate of oxygen consumption observed with NADPH as co-factor was about 80% of that found when NADH was used in the assay. The stoichiometry was that associated with a mono-oxygenase reaction:



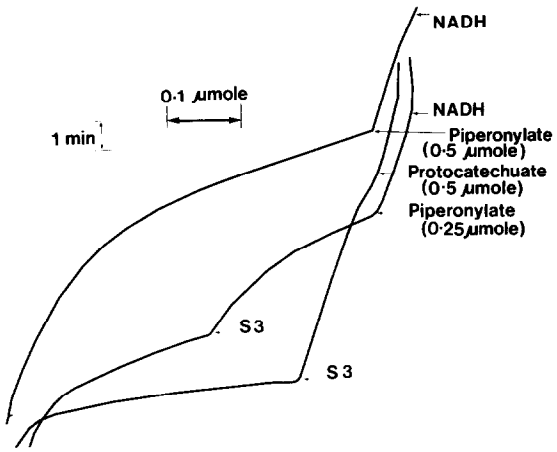


Fig. 1. Polarographic estimation of piperonylate and protocatechuate oxidation by extracts of *P. fluorescens*. The assay mixture contained: 95 μmoles Tris-HCl, pH 7.8; S3U fraction (equiv. to 8.5 mg protein); 0.5 μmole NADH and substrate as indicated in a total volume of 3 ml. Oxygen consumption was measured polarographically at 30°. S3 fraction (equiv. to 2.5 mg protein) was added where indicated.

Activity was proportional to the protein concentration above a threshold level of 1 mg/ml and was stable for several months when extracts were stored at −20°.

Protocatechuate was established as an intermediate in the catabolic pathway for piperonylate following the observation that protocatechuate 3,4-oxygenase sedimented more rapidly than piperonylate monooxygenase under specified conditions [4]. Centrifugation of the S2 fraction, adjusted to a protein concentration of 35 mg/ml, at 164,000 *g* for 6 hr resulted in an upper layer, S3U fraction, which exhibited greatly reduced dioxygenase activity (fig. 1). Oxygen consumption by incubation mixtures of S3U extract, NADH and piperonylate was allowed to proceed to completion after which the acidified and deproteinized supernatants were either extracted to yield a product which was confirmed as protocatechuate by m.p., and IR and UV spectrophotometry. This diphenol was further metabolized by protocatechuate 3,4-oxygenase, present in crude extracts with the eventual production of 3-oxoadipate. The keto acid was isolated after the action of S3 fraction on piperonylate by the method of Darrah and Cain [6], the IR spectrum of the isolated product being identical with that of authentic material. 3-oxoadipate production from piperonylate, estimated by the catalytic decarboxylation procedure using 4-aminoantipyrine [7], is shown in table 1.

Table 1
3-Oxoadipate production from piperonylic acid in the presence of S3 fraction

Substrate supplied	Yield of 3-oxoadipate (μmole)	Yield (% of expected value)*
Piperonylic acid (2.0 μmoles)	1.57	95.0%
Piperonylic acid (5.0 μmoles)	3.83	93.0%
Protocatechuate (2.0 μmoles)	1.36	82.5%

* Corrected for 82.5% yield of CO_2 obtained with authentic 3-oxoadipate (2.0 μmoles).
The assay mixture contained: 0.2 ml 20% (w/v) KOH in the centre well; the main wall contained 100 μmoles Tris-HCl, pH 7.8; 0.5 μmole NADH/mole substrate; S3 fraction (equiv. to 8.5 mg protein) and water to 3.0 ml total volume. Oxygen uptake was measured manometrically at 30°. After the cessation of oxygen uptake the 3-oxoadipate was assayed by adding 0.2 ml of acetic acid and 1.0 ml of 0.1 M-acetate buffer, pH 4.0, to the main well of each flask. KOH was swabbed from the centre wells and after re-equilibration the catalytic decarboxylation was initiated with 0.4 ml of 0.1 M 4-aminoantipyridine, tipped from the second sidearm.

The methylenedioxy carbon was eliminated as formate which was demonstrated in reaction mixtures containing aged S3 fraction, piperonylate and NADH. After oxidation was complete, as indicated manometrically, the incubation mixtures were deproteinized and steam distilled. Samples of distillates (12 vol, approx. 50 ml) were neutralized, concentrated and reduced with magnesium and HCl and treated with chromotropic acid. The presence of formate was established from the characteristic absorption spectrum [8], (peaks at 376, 490 and 580 nm) identical with that obtained when authentic formate was taken through the same procedure.

Determinations of formate in steam distillates of incubation mixtures were carried out manometrically by the action of a freshly prepared formate dehydrogenase preparation from *Escherichia coli*. However, yields of formate were not stoichiometric and amounted to 60–65% of theoretical over several assay experiments.

Piperonylate mono-oxygenase and 4-*O*-demethylase activities in extracts of *p*-methoxybenzoate-grown cells were probably catalyzed by the same

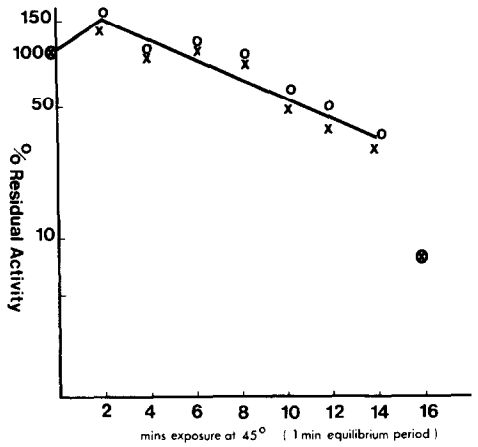


Fig. 2. Thermal denaturation of 4-methoxybenzoate-*O*-demethylase and piperonylate mono-oxygenase activities. The enzyme preparation was allowed 1 min to attain 45° before exposure time at this temperature was measured. After varying periods up to 20 min at 45°, an aliquot of the enzyme preparation was removed and rapidly cooled in melting ice before its activities were assayed. Oxygen uptake was measured polarographically at 30° in an assay mixture containing: 125 μmoles Tris-HCl, pH 7.8; 0.5 μmole NADH; 0.5 μmole substrate and 0.4 ml S3 fraction (equiv. to 10 mg protein). (X–X) Demethylase. (o–o) Mono-oxygenase.

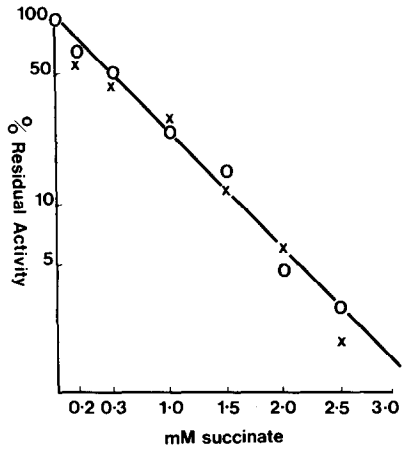


Fig. 3. Repression by succinate of *p*-methoxybenzoate-*O*-demethylase and piperonylate mono-oxygenase activities. Extracts were prepared as in [2] from cells grown to late log phase on medium containing 10 mM *p*-methoxybenzoate and varying amounts of succinate as shown. The assay mixture contained: 120 μmoles Tris-HCl, pH 7.8; 0.4 ml S3 fraction (equiv. to 12–14 mg protein in different preparations); 1.0 μmole NADH (0.1 ml) and 1.0 μmole substrate in a total volume of 3 ml. Oxygen was measured polarographically at 30°. (X–X) Demethylase. (o–o) Mono-oxygenase.

Table 2
Inhibition of 4-methoxybenzoate-*O*-demethylase and piperonylate mono-oxygenase activities.

Inhibitor	Concentration (mM)	Activity remaining(%) Demethylase	Mono-oxygenase
KCN	1	9	13
KCN	10	0	0
EDTA	0.1	78	64
EDTA	1	21	25
Sodium azide	10	99	98
Iodoacetamide	10	92	89
p-Chloromercuribenzoate	0.01	61	75
p-Chloromercuribenzoate	0.1	24	25
1,10-Phenanthroline	1	0	0
$MnCl_2$	1	18	19
$MgCl_2$	1	154	149
$ZnSO_4$	1	0	0
$CuCl_2$	1	0	0
$NiSO_4$	1	8	10
$CoCl_2$	1	0	0
$Pb(NO_3)_2$	1	2	1
$HgCl_2$	1	0	0

The assay mixture (3 ml) contained: 120 μmoles Tris-HCl, pH 7.8; S3 fraction (7.5 mg protein); 1.0 μmole substrate and inhibitor to give final conc. shown. Oxygen was measured polarographically at 30°. The inhibitor and enzyme were allowed to react for 5 min inside the reaction chamber before reaction was started by addition of substrate.

enzyme. The kinetics of thermal denaturation at 45° were similar when the extract was tested against anisate and piperonylate (fig. 2). The presence of inhibitors and metal ions to the assay led to closely correlated inhibition, or in the case of Mg^{2+} enhancement, of each activity (table 2). Both activities were strongly repressed when succinate was present in the growth medium and the extent of repression was identical for both activities (fig. 3). Furthermore, addition of either anisate or piperonylate to the assay mixture did not increase the rate of reaction with the other substrate present at saturating concentrations.

The S3 fraction will oxidise the 1-methyl group of *p*-toluic acid to the hydroxymethyl derivative *p*-carboxybenzyl alcohol, in which respect it is similar to other bacterial demethylases [9–10]. The data suggests, therefore, that piperonylate is, by analogy, oxidized by a mono-oxygenase to the hydroxymethyl-

enedioxy derivative which then undergoes spontaneous hydrolysis to protocatechuate and formate.

The catabolism of piperonylate by *Ps. fluorescens*, strain PM3, appears to be an example of co-oxidation. The methylenedioxy compound will not serve as the sole carbon and energy source and is only degraded in the presence of anisate.

Acknowledgements

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