Metabolism of styrene by *Rhodococcus rhodochrous*

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Abbreviations

The abbreviations recommended by the Biochemical Journal in its Instructions to Authors [Biochem. J. (1989) 257, 1-21] have been used.

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Summary

1. Eight strains of bacteria able to grow on styrene as a sole source of carbon and energy had been isolated prior to this project. The aim of the project was to discover the pathway for the metabolism of styrene in one of these strains, preferably a novel pathway.

2. Initial characterisation of these eight strains using growth experiments and simultaneous induction led to the selection of three strains for further work, and they were tentatively identified as coryneform bacteria. Simultaneous induction tests on one strain, a possible *Rhodococcus* sp., suggested that styrene was probably metabolised through phenylacetic acid, a well-established pathway for styrene degradation. The results from another of the three strains were not clear cut. The third strain was clearly using a novel pathway, and so it was selected for further study.

3. The organism which was selected for most of the work described in this thesis, strain 26, was identified as *Rhodococcus rhodochrous*. A method was developed to grow this strain in a fermenter containing a minimal salts medium, with styrene vapour supplied in the air supply. For larger quantities of cells, a nutrient broth medium was used, with styrene supplied as vapour in order to induce the enzymes involved in styrene degradation.

4. *R. rhodochrous* 26 is able to grow on styrene, toluene, ethylbenzene and 1-phenylethanol. Simultaneous induction experiments showed that growth on each of these substrates resulted in the ability to oxidise all of the others.

5. Oxidative responses to possible intermediates in known pathways of toluene metabolism showed high activities with toluene *cis*-glycol and 3-methylcatechol, with lower activities towards other possible substrates. This led to the proposition that a similar pathway was being used for styrene metabolism.

6. Styrene *cis*-glycol was produced by *Pseudomonas putida* from styrene, and identified by ^{1}H and ^{13}C n.m.r.. This substrate was oxidised by extracts of *R. rhodochrous* 26 which had been grown on styrene, in an NAD-dependent reaction. There was also an NAD-dependent oxidation of toluene *cis*-glycol. Ion exchange chromatography of extracts, coupled with a heat denaturation test, strongly indicated that both toluene *cis*-glycol and styrene *cis*-glycol were oxidised by the same enzyme.

7. Under anaerobic conditions, in the presence of NAD, extracts transformed styrene *cis*-glycol into a compound identified by g.c.-m.s. as vinylcatechol. Toluene *cis*-glycol was transformed to methylcatechol by the same system.

8. Intact cells incubated with styrene and 3-fluorocatechol (as an inhibitor of catechol oxygenase activity), accumulated a compound identified by g.c.-m.s. and ^{1}H n.m.r. as 3-vinylcatechol. 2-Fluoromuconic acid, identified by ^{1}H and ^{13}C n.m.r., also accumulated under these conditions.

9. When intact cells were incubated with styrene and 3-fluorocatechol in an atmosphere of 50 % $^{16}O_2$ and 50 % $^{18}O_2$, g.c.-m.s. analysis demonstrated the presence of two molecular ions for vinylcatechol, M and M+4, showing that initial ring attack is due to a dioxygenase.

10. When grown on styrene, *R. rhodochrous* 26 accumulated a compound identified by ^{1}H and ^{13}C n.m.r. as 2-vinylmuconic acid. Up to 40 % of the styrene substrate may be converted to this product. There was no sign of further metabolism of this product in either growth medium or cell extracts. A similar accumulation of muconic acid was noticed when *R. rhodochrous* 26 was grown on benzyl alcohol. Cell extracts did not seem to metabolise *cis, cis*-muconic acid, the expected product of benzyl alcohol breakdown through an *ortho* cleavage pathway.

11. It was not possible to detect any styrene dioxygenase activity in extracts using an oxygen electrode or spectrophotometric methods.

12. Extracts of *R. rhodochrous* 26 which had been grown on styrene contained catechol 1,2-oxygenase and catechol 2,3-oxygenase activities. The two enzymes were clearly separated by ion exchange chromatography, each appearing as a single peak. Both enzymes could oxidise catechol, 3-methylcatechol, 4-methylcatechol and vinylcatechol.

13. A yellow product was formed when ion exchange fractions containing catechol 2,3-oxygenase were incubated with 3-vinylcatechol. The absorbance characteristics of this product were consistent with it being a substituted 2-hydroxymuconic acid semialdehyde.

14. Extracts also possessed a 2-hydroxymuconic acid semialdehyde hydrolase activity, and this could be separated from the catechol oxygenases by ion exchange chromatography. This hydrolase activity was rapidly denatured by heating at 55 °C. Consecutive action of ion exchange fractions containing catechol 2,3-oxygenase activity and fractions containing 2-hydroxymuconic acid semialdehyde hydrolase activity on catechol or 3-methylcatechol led to the production of a compound tentatively identified by its absorbance characteristics as 2-hydroxypent-2,4-dienoate.

15. Consecutive action of ion exchange fractions containing catechol 2,3-oxygenase activity and 2-hydroxymuconic acid semialdehyde hydrolase activity on 3-vinylcatechol led to the production of acrylic acid, which was identified by h.p.l.c.. 2-Hydroxypent-2-4-dienoate was probably also formed, though the absorbance spectrum was confused by other components.

16. A small amount of acrylic acid was detected by h.p.l.c. in the medium of *R. rhodochrous* 26 while it was growing on styrene, but this never rose above a low level, and was not detectable at the end of growth. Growth did not occur on acrylic acid under the conditions tested, though *R. rhodochrous* 26 could grow on vinylacetic acid.

17. The results strongly suggest that *R. rhodochrous* 26 metabolises styrene to styrene *cis*-glycol using a dioxygenase, and then a *cis*-glycol dehydrogenase converts styrene *cis*-glycol to 3-vinylcatechol. This 3-vinylcatechol is cleaved by both catechol 1,2-oxygenase to give 2-vinylmuconic acid and by catechol 2,3-oxygenase to give 2-hydroxy-6-oxo-octa-2,4,7-trienoic acid. The 2-vinylmuconic acid does not appear to be metabolised. The 2-hydroxy-6-oxo-octa-2,4,7-trienoic acid is cleaved by 2-hydroxymuconic semialdehyde hydrolase to give acrylic acid and 2-hydroxypent-2,4-dienoate.

18. When intact cells, which had been grown on styrene, were incubated with toluene and 3-fluorocatechol, three products were identified by g.c.-ms: 3-methylcatechol, fluoromuconic acid and methylmuconic acid. When toluene was replaced by ethylbenzene, 3-ethylcatechol, ethylmuconic acid and fluoromuconic acid were identified.

19. Incubation of intact cells, which had been grown on styrene, with 3-fluorocatechol and acetophenone or 1-phenylethanol, led to the production of a compound identified as acetylcatechol. *R. rhodochrous* 26 was not able to grow on acetophenone. When grown on 1-phenylethanol, an odour similar to acetophenone was noted, suggesting that an alcohol dehydrogenase activity was present.

20. When intact cells, which had been grown on styrene, were incubated with 3-fluorocatechol and cinnamic acid, a compound identified by g.c.-m.s. as benzoic acid accumulated. When grown on cinnamic acid, *R. rhodochrous* 26 cells were able to oxidise benzoic acid and catechol, though not styrene, toluene or toluene *cis*-glycol.

21. When grown on benzyl alcohol *R. rhodochrous* 26 cells did not oxidise toluene or styrene, but could oxidise benzaldehyde and benzoic acid. These cells were also able to oxidise toluene *cis*-glycol.

22. *R. rhodochrous* 26 seems to metabolise toluene, ethylbenzene and probably benzene by the same general pathway as it uses to metabolise styrene. This strain

probably oxidises benzyl alcohol through a pathway similar to that encoded by the *Pseudomonas* mt-2 TOL plasmid. Cinnamic acid may be metabolised through benzoic acid.

Note: *Rhodococcus rhodochrous* 26 has been deposited at the NCIMB, with the name *Rhodococcus rhodochrous* NCIMB 13259.

1. Introduction

This introduction is divided into three sections, the first is a review of the genus *Rhodococcus*, the second an outline of aerobic aromatic breakdown by bacteria, and the third is a review of the bacterial metabolism of styrene.

1.1 The genus *Rhodococcus* and its degradative capacity

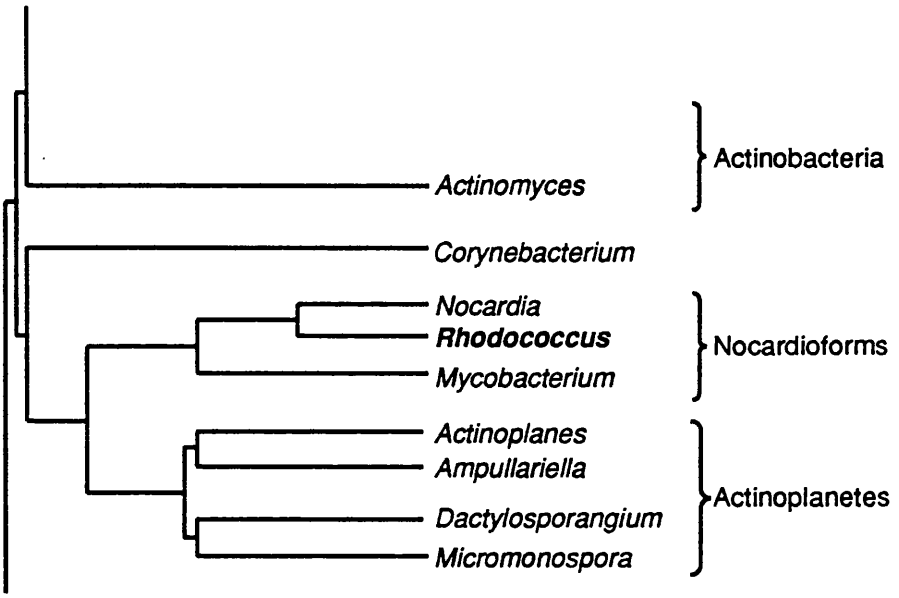
The genus *Rhodococcus* encompasses species with a wide range of metabolic capabilities, some of them novel. Although most research on the metabolic capabilities of this genus started only recently, the diversity of those capabilities discovered so far suggests that this genus could soon be seen to rival the pseudomonads in complexity. Another feature of the rhodococci is the rapidity with which basic research is being translated into practical applications, partly due to the robustness of many rhodococci in the natural environment. The biology and genetics of this genus have recently been reviewed by Finnerty (1992), and this genus was also included in a review of actinomycetes not belonging to the genus *Streptomyces* (Lechevalier & Lechevalier, 1985).

1.1.1 Classification

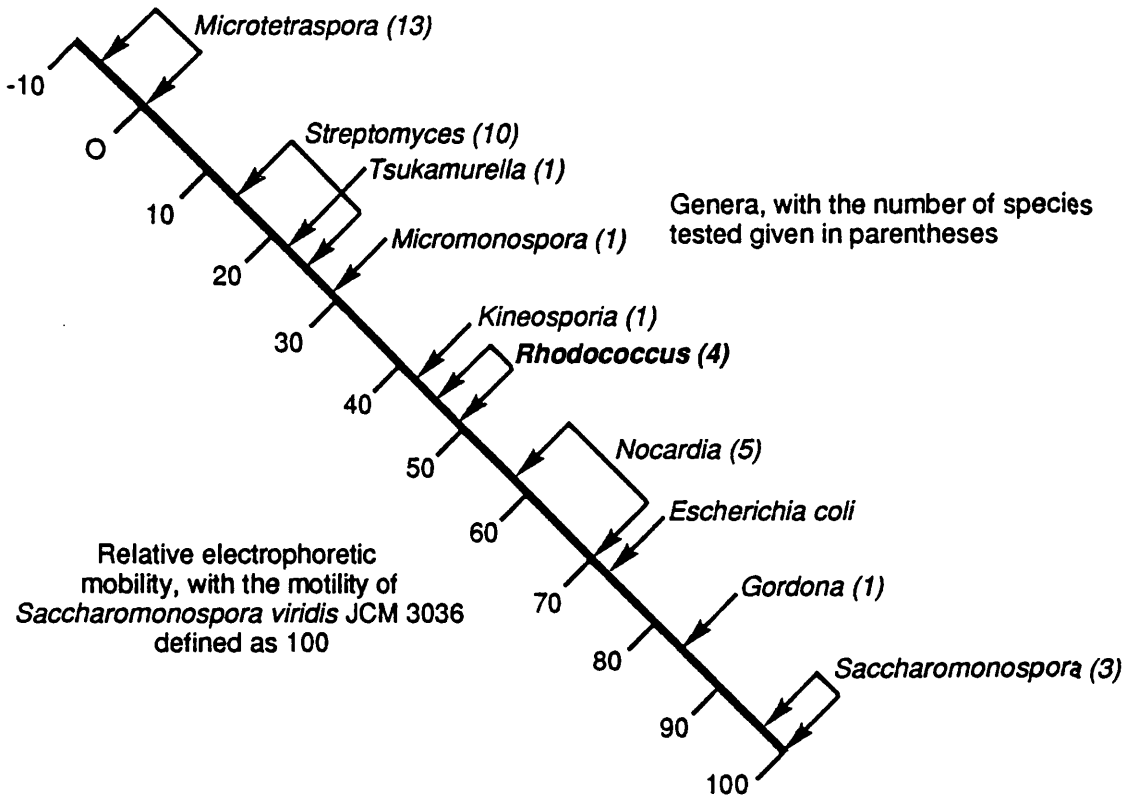
For many years, the classification of rhodococci has been confused; but now, with the use of new biochemical and genetic analyses, membership of the genus is becoming stable (Goodfellow, 1989b; Finnerty, 1992). Data from the partial sequencing of 16S RNA has enabled the suprageneric relationships of actinomycetes to be established (Figure 1.1a), and this places *Rhodococcus* beside *Nocardia* and *Mycobacterium* among the nocardioform actinomycetes (Goodfellow, 1989a). Other closely related genera include *Corynebacterium*, *Gordona* and *Tsukamurella*, and several species have been moved between these genera recently. Ochi (1992) has separated *Gordona* and *Tsukamurella* from *Rhodococcus* by measuring the electrophoretic heterogeneity of ribosomal protein AT-L30 (Figure 1.1b).

Figure 1.1: The classification of rhodococci

a) Evolutionary relationship between rhodococci and closely related bacteria, based on partial sequencing of 16S ribosomal ribonucleic acids (Goodfellow, 1989a)



b) The separation of rhodococci from other genera using the electrophoretic heterogeneity of ribosomal protein AT-L30 (Ochi, 1992)



Finnerty (1992) stated that the genus now includes 13 species: *R. aichiensis, R. chlorophenolicus, R. coprophilus, R. equi, R. erythropolis, R. fascians, R. globerulus, R. luteus, R. marionascens, R. maris, R. rhodnii, R. rhodochrous* and *R. ruber*. The genus *Gordona* contains the species previously classified as *R. bronchialis, R. rubropertinctus, R. sputi and R. terrae* (Finnerty, 1992). The genus *Tsukamurella* now contains the organisms previously classified as *R. aurantiacus* (Ochi, 1992). Many rhodococci remain to be assigned to a species, and several other species are described in the literature, for example *R. minimus* (Ermakova *et al.*, 1991).

1.1.2 Identification and basic physiology

Rhodococci are aerobic, Gram-positive and non-motile. They have a variety of growth patterns, starting with the coccus or short rod stage, and then may form filaments with side projections, show elementary branching or produce extensively branched hyphae (Goodfellow, 1989b). The definition of the genus *Rhodococcus* is based on cell wall composition, and rhodococcal walls must have the following (Finnerty, 1992):

1) A peptidoglycan containing N-acetylglucosamine, N-glycolylmuramic acid, D- and L-alanine, D-glutamic acid and meso-diaminopimelic acid.

2) Arabinose and galactose.

3) Phospholipids containing cardiolipin, phosphatidylethanolamine, phosphatidylinositol and phosphatidylinositol mannosides.

4) Tuberculostearic acid.

5) Mycolic acids with 34-52 carbon units.

6) Dihydrogenated menaquinones with eight isoprene units.

Rapid methods of rhodococcal identification are now being developed, including examination of rRNA restriction endonuclease patterns, using a digoxigenin-labelled *Escherichia coli* rDNA probe (Lasker *et al.*, 1992).

Most rhodococci form red, orange or pink colonies, and much of this pigmentation is believed to be the result of the presence of carotenoids; Takaichi *et al.* (1990) have identified four carotenoid pigments from *R. rhodochrous* RNMS1 (Figure 1.2a).

R. equi strains have acidic capsular polysaccharides, with different polysaccharides expressed in different serotypes. These are potential virulence factors (see below), and the structure of one is given in Figure 1.2b (Severn & Richards, 1992). Some rhodococci have been reported to produce antibiotics (Lechevalier & Lechevalier, 1985).

Membrane glycolipids have been found to change in composition and quantity during growth of both *R. rhodochrous* (Ioneda & Almeida, 1991) and an unidentified *Rhodococcus* sp. (Gordeev *et al.*, 1992), with the levels of trehalose dimycolate varying considerably.

Rhodococci can produce both emulsifiers (see below, 1.1.6a) and flocculents. *R. erythropolis* S-1 produces a bioflocculent, NOC-1 when grown on a wide variety of substrates (Kurane *et al.*, 1986b). NOC-1 is able to flocculate a wide range of materials, including kaolin clay, yeast and livestock wastewater (Kurane *et al.*, 1986a). The flocculating ability of NOC-1 is lost when it is heated or digested by proteases, suggesting that protein is involved in the activity of NOC-1 (Takeda *et al.*, 1991). The flocculent also lost activity when filtered through a 0.22 μm membrane, suggesting that it forms micelles (Takeda *et al.*, 1991).

1.1.3 Ecology and basic metabolism

Rhodococci have been isolated from a wide range of environments, varying from oil-contaminated sea water to human sputum (see below, 1.1.6a and 1.1.5b). They have even been isolated from a wall painting of the Nativity of the Virgin in the Ferapontov monastery, Russia (Petushkova et al., 1989). *R. rhodochrous* has been frequently isolated from scum at activated sludge plants (Sunairi *et al.*, 1993), and rhodococci are considered to be a significant part of the soil microbial community (Dobrovol'skaya & Tret'yakova, 1991).

There are two strategies for the growth and survival of bacteria in the environment: the R-selected strategy, in which organisms are opportunistic and grow fast, and the K-selected strategy, where organisms have a low growth rate but high substrate affinity and persistence (Salkinoja-Salonen *et al.*, 1991). Organisms can also be divided into autochthonous organisms, which are always present in an environment, and allochthonous (or zymogenous) organisms, which become abundant only after an increase in nutrients (Schlegel, 1986), and these characteristics can be equated with K-type and R-type strategies respectively (Williams, 1985). Rhodococci seem generally to exhibit a K strategy, with slow growth, but great persistence in the environment, i.e. they are autochthonous.

Koronelli *et al.* (1988) have investigated the survival of two hydrocarbon-degrading rhodococci, *R. erythropolis* Ar-25 and *R. maris* MST-32, under conditions of complete starvation after growth on a variety of compounds. For *R. maris*, the conditions of initial growth were important, with cultures grown initially on paraffin virtually dead after 4 months starvation at either 19 °C or 5 °C. However, if the growth substrate was glucose the number of cells increased over the four months of starvation. *R. erythropolis* displayed rather different behaviour, with small reductions in cell numbers during the four months if the initial substrate had been glucose or paraffin. However, if the cells were grown on beef extract bouillon medium, starvation led to a 17-fold increase in cells at 19 °C, and a 2-fold increase at 5 °C in four months. Microscopic examination showed that this increase in cell numbers was due to the formation of smaller cells, with diameters about half that of the originals, which were still able to reproduce in a nutrient medium (Koronelli *et al.*, 1988).

Pseudomonads and other Gram-negative bacteria generally exhibit catabolite repression, with the presence of an easily degradable substrate leading to an inhibition of the breakdown of other substrates. Rhodococci do not, generally, seem to exhibit this behaviour, and in several cases the presence of glucose actually accelerates the metabolism of other compounds, such as aromatics (see section 1.1.7c for an example).

Because of their persistence in the environment and apparent lack of catabolite repression, rhodococci have been found to be effective in bioremediation tests, for example in the removal of pentachlorophenol from natural soil (Briglia *et al.*, 1990). Two pentachlorophenol degrading bacteria, *R. chlorophenolicus* PCP-1 and *Flavobacterium* sp strain ATCC 39723 were immobilised on polyurethane foam and introduced into peaty soil with pentachlorophenol. In liquid culture the *Flavobacterium* sp. was able to tolerate 100 ppm of pentachlorophenol whereas the *Rhodococcus* tolerated only 2 ppm. The pentachlorophenol degrading ability of the *Flavobacterium* species declined within 60 days, and there was no enhancement of pentachlorophenol removal from the soil. However, the pentachlorophenol-degrading ability of *R. chlorophenolicus* was measurable for 200 days, even when an additional carbon source, distillery waste, was added. This ability was also retained for 200 days in soil uncontaminated with pentachlorophenol. The pentachlorophenol in the soil was removed and mineralised. Electron microscopic examination of the polyurethane foam after 290 days of soil burial still showed high numbers of bacteria morphologically similar to *R. chlorophenolicus*. These results indicate that *R. chlorophenolicus* behaves as an autochthonous soil bacterium, growing very slowly (the number of pentachlorophenol degrading cells remained about constant for almost a year), whereas *Flavobacterium* behaved as a allochthonous bacterium.

There have only been limited studies of the basic metabolism of rhodococci. *R. erythropolis* uses constitutive carrier-mediated transport in the uptake of glucose, and an inducible system for uptake of other compounds such as succinate (Lechevalier & Lechevalier, 1985). A variety of glucose assimilation pathways exist in rhodococci, including the Embden-Meyerhof-Parnas and the Entner-Doudoroff pathways (Lechevalier & Lechevalier, 1985).

The growth of *R. minimus* B-293 on glucose has been extensively investigated, and this strain shows very unusual kinetics (Baryshnikova *et al.*, 1985; Chemeris *et al.*, 1989; Ermakova *et al.*, 1991). Early research indicated that strain B-293 showed multiphasic growth kinetics on glucose, with at least three phases, which differed in the

efficiency of substrate utilisation (Chemeris *et al.*, 1989). This research suggested that there were several systems for glucose utilisation, some more efficient than others, which were induced at differing concentrations of the substrate. Two variants of *R. minimus* B-293 have been isolated, with one growing much more rapidly on glucose than the other (Ermakova *et al.*, 1991). The respiratory chain of the fast-growing variant, B-293M was active during exponential growth, and was sensitive to cyanide. The slow-growing variant had no respiratory sensitivity to cyanide; nor did B-293M in stationary phase. Cytochrome *d* seemed to be present at all times when cyanide-insensitive respiration was occurring, and this cytochrome is usually associated with a limitation of the oxidative activity of the respiratory chain (Ermakova *et al.*, 1991). These results suggest that the slow growth rate of strain B-293 is at least partially due to the inhibition of the respiratory chain, possibly as a mechanism of maintaining metabolism without multiplication.

1.1.4 Genetics

The genetics of rhodococci have recently been reviewed (Finnerty, 1992), though there is much new research in this field. Genetic systems for the transformation of rhodococci have only recently been developed, with the first report of plasmid transformation in 1988 (Singer & Finnerty, 1988). Most of the early systems were based on rhodococcal plasmids involving arsenic resistance (e.g. Dabbs *et al.*, 1990) or on genes derived from other genera (e.g. Singer & Finnerty, 1988). More recently rhodococcal plasmids encoding antibiotic resistance have been constructed (e.g. chloramphenicol resistance; Quan & Dabbs, 1993) or have been isolated (e.g. kanamycin and ampicillin resistance; Hashimoto *et al.*, 1992). The latter system was used successfully to transform *R. rhodochrous* ATCC 12674 with the nitrile hydratase and amidase genes from *Rhodococcus* sp. N-774, using electroporation. Other DNA cloned from rhodococci includes a fragment that codes for the decolorisation of sulphonated azo dyes (Heiss *et al.*, 1992) and a gene with extensive homology to the mammalian propionyl-CoA carboxylase beta chain. The latter gene was cloned from *Rhodococcus* sp. NI86/21, which was isolated from the rhizosphere of maize, and which

can grow on thiocarbamate herbicides as sole carbon and nitrogen source (Nagg *et al.*, 1992).

Bacteriophage NJL is one of the few phage isolated from *R. rhodochrous*. It is a temperate phage, and has a hexagonal head and a long, flexible tail. The genome consists of a 49 kb linear double-stranded DNA with cohesive ends. It contains unique target sites for *Hin*dIII and *Ssp*I, and two target sites each for *Nhe*I and *Sca*I. A physical map of the genome has been constructed using *cos* mapping (Sunairi *et al.*, 1993).

The gene encoding the chloramphenicol resistance protein of *R. fascians* NCPPB 1675, located on the conjugative plasmid pRF2, has been sequenced (Desomer *et al.*, 1992). Biochemical analysis has shown that resistance does not involve an antibiotic-modifying enzyme, but rather a hydrophobic, possibly membrane-associated protein. From sequence analysis, the protein appears to contain 12 membrane-spanning α-helices, homologous with the transmembrane tetracycline efflux proteins, suggesting that this protein provides chloramphenicol resistance by pumping out the antibiotic. A promoter region for this gene was sequenced, and did not contain any canonical *E. coli* or *B. subtilis* promoter sequence.

Genes from rhodococci have also been used to improve cloning techniques for *E. coli*. For example, the *E. coli* insertion-inactivation cloning vector pSLH8 contains a pigment gene, probably indole dioxygenase, from *Rhodococcus* sp. ATCC21145 (Hart & Woods, 1992) and *E. coli* colonies containing this gene produce insoluble indigo and indirubin, turning dark blue (Hart *et al.*, 1992). There are several cloning sites within the pigment gene, and if genes are inserted at any of these sites then colony pigmentation is lost.

1.1.5 Pathogenicity

a) Animal

Rhodococcus equi is an important pathogen of horses, causing severe bronchopneumonia in foals (Yager, 1987), with a worldwide morbidity of 5-17 % (Hillidge, 1987). The bacterium survives well in soils, especially those contaminated

with herbivore manure. It grows on acids produced by fermentation of herbivorous material in the guts of animals; for example it can use acetic or propionic acid as sole carbon and energy sources (Hughes & Sulaiman, 1987). This leads to a gradual build up of *R. equi* levels in the soil of horse farms; however, illness of animals is unevenly distributed among farms, with a few farms having endemic sickness and many others having no disease at all (Prescott, 1987). It seems that most horses are infected intestinally with *R equi* whilst they are young, but this form of infection does not harm the animal, though it does lead to the development of specific immunity. Harmful infection arises if the bacterium is inhaled into the lungs, producing severe, suppurative bronchopneumonia and suppurative lymphadenitis of regional nodes (Yager, 1987). Once foals develop these symptoms the mortality rates can be up to 80 %, though combinations of lipid-soluble antibiotics such as erythromycin and rifampin seem effective, with a success rate of up to 88 % (Hillidge, 1987). Fatal effects of *R. equi* are almost entirely specific to foals, occurring mainly between the ages of one month and four months, correlating with the loss of maternally-derived antibodies and the development of the foal's own immune system (Hillidge, 1987). Horses over six months old rarely develop disease unless they are immunocompromised. When experimentally infected, other animals such as piglets and mice develop some lung damage, but this is only temporary (Bowles *et al.*, 1987), though *R. equi* persists in the liver of mice for at least four weeks. *R. equi* is able to survive and multiply within the foal alveolar macrophages, which are the first line of defence against inhaled bacteria and the effector cells for pulmonary cell-mediated immunity. The bacteria appear to inhibit phagosome-lysosome fusion within these cells, and eventually the macrophages are irreversibly damaged and release bacteria into the medium (Zink *et al.*, 1987). In contrast, purified polymorphonuclear neutrophil leukocytes from foals (whether healthy or infected) are bactericidal against *R. equi*, showing a high uptake and destruction of bacteria (Yager *et al.*, 1987). However, the degranulation which occurs after phagocytosis of *R. equi* may add to tissue damage, because of the release of lysosomal enzymes and reactive oxygen species.

b) Human

Until recently there was little evidence of rhodococcal infections in humans. However, in the last few years several cases have been reported. The main bulk of these reports are *R. equi* infections connected with immune deficiencies, especially those caused by the Human Immunodeficiency Virus (HIV). The immune system of patients with HIV is progressively destroyed, allowing opportunistic pathogens which are usually unable to infect humans the opportunity to attack. These infections have been reviewed recently (Drancourt *et al.*, 1992). Between the first reported human *R. equi* infection in 1964 and the time of the Drancourt review, 50 cases had been reported, 40 of which were in immunocompromised patients, 18 of these having an HIV infection. The *R. equi* infections cause pneumonia in the human host, similar to the normal symptoms in foals (see section 1.1.5a), where infection also occurs during a period when their immune system is not fully active. The pneumonia can frequently be fatal to the human host, with 11 of the 18 HIV patients dying from the *R. equi* infection (Drancourt *et al.*, 1992).

There is evidence that other species of rhodococci may also be pulmonary pathogens in humans. Research in Nigeria has shown that rhodococci can frequently be isolated from the sputum of patients with chest disorders (Osoagbaka, 1989). Five species were isolated: four strains of *Rhodococcus aurantiacus*, and one each of *R. erythropolis*, *R. pellegrino*, *R. rubropertinctus* and *R. rhodnii*. These isolates were found to be pathogenic in immuno-suppressed white mice, and in addition, human case histories of infection strongly linked illness to rhodococcal infection.

Rhodococcal infections have also been found in other parts of the body, for example in a chronic corneal ulcer (Gopaul *et al.*, 1988). In this case *R. rhodochrous* was isolated from the infection, which was in an elderly woman. A new species of *Rhodococcus*, provisionally named *R. luganensis*, was isolated from contaminated packed red blood cells, after symptoms of fever and hypotension were produced in several patients following administration of the contaminated blood cells (de Clari *et al.*, 1992).

The insect vector of Chagas' disease (a disease of humans), *Rhodnius prolixus*, has a symbiotic relationship with *Rhodococcus rhodnii*, which lives in the gut of the insect (Goodfellow, 1989b). Genetic manipulation of *R. rhodnii* is being investigated as a possible method of altering the ability of *Rhodnius prolixus* to spread disease (Beard *et al.*, 1992).

It is clear that rhodococci are, in some circumstances, human pathogens. However, the extent of these infections in non-immunocompromised people is not yet clear.

c) Plant

One species of *Rhodococcus*, *R. fascians*, is a phytopathogen of both dicotyledonous and monocotyledonous plants. Infection of dicotyledonous plants, such as the sweet pea, causes fasciation, which is the loss of apical dominance and the development of leafy galls. In monocotyledonous plants, such as *Liliaceae*, cauliflower-like structures develop on the bulbs (Crespi *et al.*, 1992). Different strains of *R. fascians* show different ranges of host plant infectivity, though many strains can infect a variety of plant species(Hu *et al.*, 1992).

The molecular basis of fasciation is beginning to be elucidated. Crespi *et al.* (1992) have shown that virulence lies on a conjugative, 200 kb, linear extrachromosomal element. Insertion mutagenesis showed that three loci were necessary for normal fasciation, with other loci affecting the level of virulence. One of these loci was found to code for a protein with low homology to the isopentenyltransferase gene product produced by several Gram-negative phytopathogens, for example *Pseudomonas solanacearum*. This gene product is only expressed when *R. fascians* is exposed to extracts from fasciated tissue, and is likely to alter cytokinin levels within the plant, so affecting its growth. Another intriguing discovery in this research is the indication, from pulsed field electrophoresis, that the chromosome of this bacterium may be a linear 4 megabase molecule. If this is correct, it would make *R. fascians* the first free-living bacterium with a linear chromosome.

1.1.6 Growth on aliphatic hydrocarbons

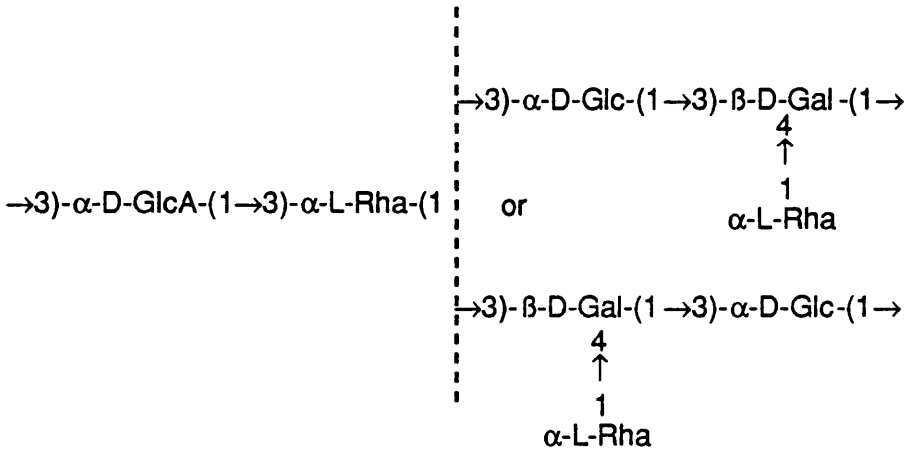
a) Long-chain hydrocarbons

Rhodococci are frequently isolated from oil-contaminated environments, from locations as diverse as Arctic waters and ice (Koronelli *et al.*, 1989), rivers in the Urals (Berdichevskaya *et al.*, 1991) and the soil and sea in Kuwait (Sorkhoh *et al.*, 1990). *R. rhodochrous* KUCC 8801, isolated in Kuwait, was found to be a very effective degrader of *n*-alkanes. This isolate was able to grow on straight chain alkanes containing from 12 to 20 carbon atoms and on the alkene tridecene, but it could not utilise short chain hydrocarbons, benzene or the branched chain hydrocarbon iso-octane (Sorkhoh *et al.*, 1990). *R. salmonicolor* metabolises *n*-alkanes by oxidation of the terminal methyl group to a carboxyl, followed by β-oxidation of the chain (Lechevalier & Lechevalier, 1985). This organism also attacks the side chains of 1-phenylalkanes by β-oxidation, producing phenylacetic acid, which is then hydroxylated to give homogentisic acid and cleaved (Lechevalier & Lechevalier, 1985). *Rhodococcus* sp. BPM 1613 can grow on a variety of isoprenoid hydrocarbons, such as pristane (2,6,10,14-tetramethylpentadecane), 1-pristene (2,6,10,14-tetramethyl-1-pentadecene) and farnesane (2,6,10-trimethyldodecane) as well as hexadecanene (Nakajima *et al.*, 1985a, b). Pristane is metabolised in this organism by two routes, to pristanic acid followed by β-oxidation, or from pristanic acid to pristanedioc acid (an ω-oxidation), followed by β-oxidation.

Sorkhoh *et al.* (1990) found that two strains of hydrocarbon-degrading *R. rhodochrous*, KUCC 8801 and KUCC 8802, had different effects on the dodecane on which they were growing, the former strain caused the oil to disintegrate into small granules, whereas the latter produced an emulsion. It is likely that these transformations were the result of extracellular surfactants, which are produced by many rhodococci. An emulsion-stabilising exo-polysaccharide produced by *Rhodococcus* strain 33 while growing on hexadecane is illustrated in Figure 1.3a (Neu *et al.*, 1992). Another biosurfactant, a trehalose tetraester (Figure 1.3b), is produced by a strain of *R. erythropolis* (Georgiou *et al.*, 1992). Extracellular glycolipids are also found, for

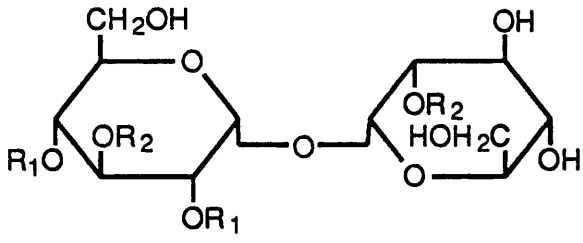
Figure 1.3: Some biosurfactants isolated from *Rhodococcus* spp.

a) A polysaccharide from *Rhodococcus* str. No 33 (Neu *et al.*, 1992)



The polysaccharide is *O*-acetylated, with one acetyl per repeated unit, though the acetylation may not be evenly distributed

b) A trehalose-2,3,4,2'-tetraester from *R. erythropolis* (Georgiou *et al.*, 1992)



R_1 = $OC(CH_2)_6CH_3$ and $OC(CH_2)_2COOH$

R_2 = $OC(CH_2)_8CH_3$

example in *Rhodococcus* sp. H13-A grown on alkanes and fatty alcohols, but not when the strain is grown on fatty acids or sugars (Singer & Finnerty, 1990). All these biosurfactants encourage the formation of emulsions, which should enable better access for the bacteria to the hydrophobic hydrocarbons. There is commercial interest in bacterial surfactants; their applications include enhancing oil recovery by mobilising remnants left in underground reservoirs, bioremediation of oil spills and use as dispersants in industry (Georgiou *et al.*, 1992).

b) Short-chain hydrocarbons

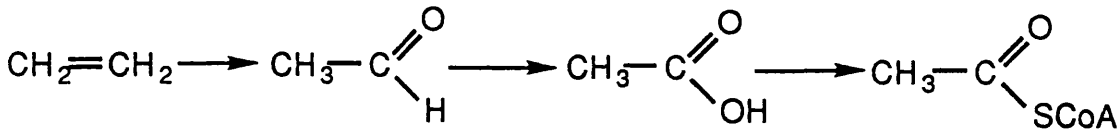
Rhodococci can be isolated that are able to grow on short-chain hydrocarbons such as acetylene and propane. Germon & Knowles (1988) isolated an acetylene and acetaldehyde utilising bacterium, *R. rhodochrous* E5, and found that it degraded the acetylene *via* acetaldehyde to acetyl CoA (Figure 1.4a). Rhodococci with acetylene-degrading capability have been shown to survive for at least eight years in soil with a past history of exposure to the gas (Terry & Leavitt, 1992). Previous studies into denitrification in crop soils had involved flooding the soils with acetylene as an inhibitor of N_2O reductase. These soils degraded acetylene in as few as seven days, even if they had not been exposed to the gas for four to eight years. Soils which had not been previously exposed needed 21 - 42 days to degrade the acetylene. Five acetylene-degraders were isolated, all rhodococci (Terry & Leavitt, 1992).

MacMichael & Brown (1987) isolated *R. rhodochrous* ATCC 21198, which was able to grow on propane. They found that growth on propane and propan-1-ol required the presence of CO_2, with the rate of propane utilisation directly related to the initial CO_2 concentration. The use of $^{14}CO_2$ confirmed the fixation of CO_2, and it was proposed that propane was metabolised through propan-1-ol, later followed by the methylmalonyl CoA pathway of CO_2 fixation (Figure 1.4b).

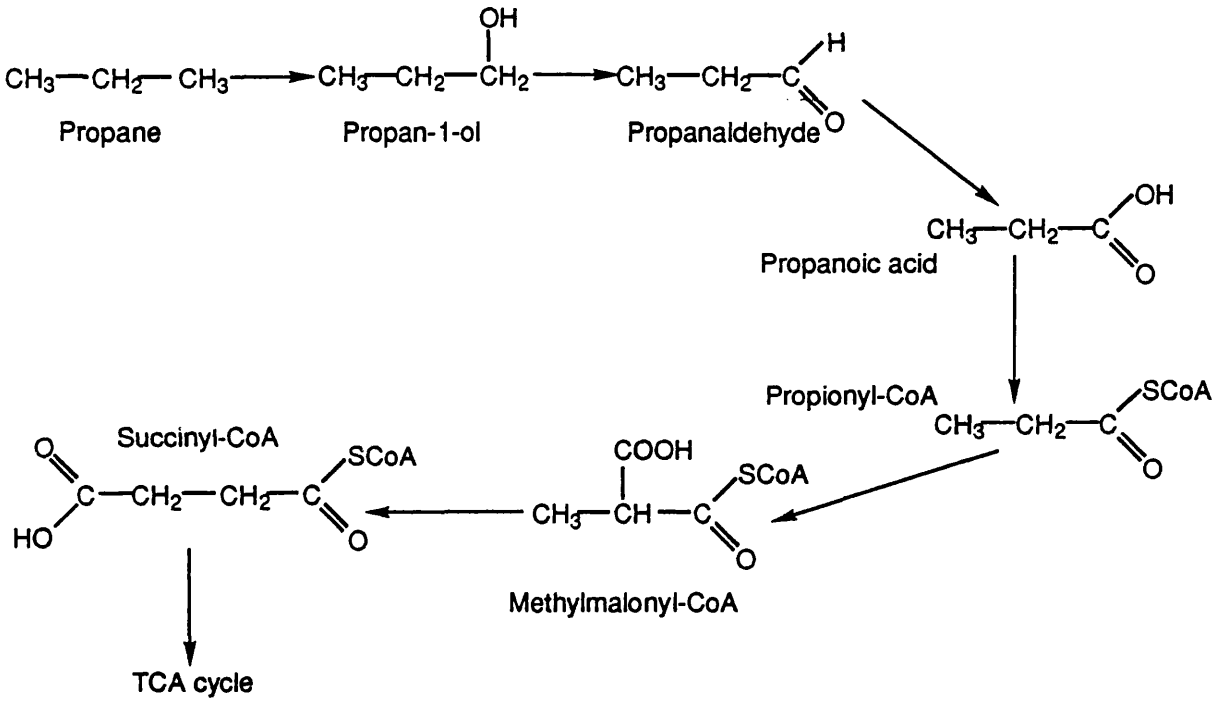
Another propane-utiliser, *R. rhodochrous* PNKb1, was isolated by Woods & Murrell (1989). This bacterium could not grow on any other short chain alkanes (C_1-C_8), or on alkenes. A propane oxygenase activity was found, which could co-oxidise

Figure 1.4: The metabolism of short-chain hydrocarbons by *Rhodococcus* spp.

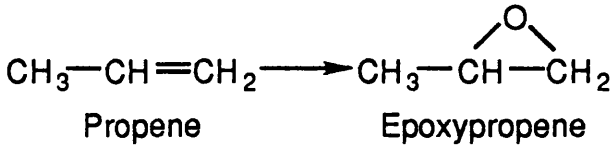
a) The breakdown of acetylene by *R. rhodochrous* E5 (Germon & Knowles, 1988)



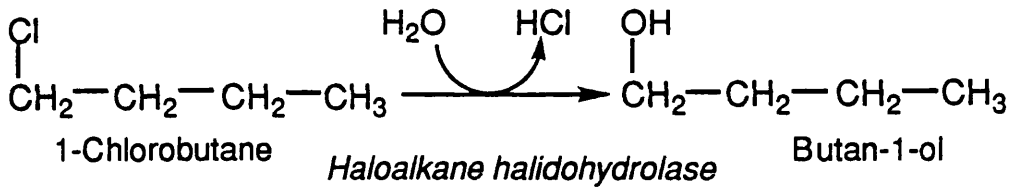
b) Metabolism of propane by *R. rhodochrous* ATCC 21198 (MacMichael & Brown, 1987)



c) The biotransformation of propene by *R. rhodochrous* PNKb1 (Woods & Murrell, 1989)



d) The dechlorination of 1-chlorobutane by *R. erythropolis* Y2 (Sallis *et al.*, 1990)



propene to epoxypropene (Figure 1.4c). Both terminal and sub-terminal alcohol dehydrogenase activities were induced by growth on propane. A secondary alcohol dehydrogenase was purified and characterised and found to use an NAD cofactor; it contained subunits with an Mr of 42 k, with the whole enzyme having an Mr of approximately 86 k (Ashraf & Murrell, 1990). This enzyme displayed a broad substrate specificity, oxidising 1-phenylethanol, cyclohexanol and primary alcohols. However, genetic, biochemical and immunological evidence has shown that there is an additional alcohol dehydrogenase also necessary for the metabolism of propane, and this dehydrogenase is induced by growth on propan-1-ol, but not by growth on propan-2-ol (Ashraf & Murrell, 1992). The precise roles of these alcohol dehydrogenases in the metabolism of propane has yet to be elucidated.

The ability of propane-grown *R. rhodochrous* to epoxidate alkenes has been used as a method of preparing epoxides. Woods & Murrell (1990) found that propane-grown *R. rhodochrous* could produce epoxides from ethene, propene and but-1-ene, though not from but-2-ene, pent-1-ene or phenylpropene. Kovalenko & Sokolovski (1992) found that propane-grown cells of *Rhodococcus* sp. 1r retained their propene-oxidising activity when immobilised by 'Double Immobilisation', which involved mixing cells with a gel forming system, followed by gelatinisation of this system within the pores of an inorganic support. *Rhodococcus* sp. DSM 43001 has been used in a model exhaust gas purification system to remove acetone and propionaldehyde vapour (Kirchner *et al.*, 1992)

The alcohol dehydrogenase activity of *R. equi* IFO 3730 has been used to produce 2-octanone from 2-octanol and hexanone from 2-methylcyclohexanol, this time using an 'Interface Bioreactor', in which the organism is grown on agar. The substrate is then dissolved in hexadecane and poured onto the agar surface (Oda & Ohta, 1992). This organism has also been used to oxidise allylic sulphides to sulphones and chiral sulphoxides (Ohta *et al.*, 1985)

Rhodococci have been isolated on some more complex hydrocarbon-based structures, for example Zambrizhiskii *et al.* (1991) isolated *Rhodococcus* sp. 22 which could grow on hydroxypropylated propylene glycol (Laprol 502). Several enzyme activities were detected, including laprol dehydrogenase, 1,2-propandiol dehydrogenase and lactate dehydrogenase.

c) Halogenated hydrocarbons

Sallis *et al.* (1990) isolated a 1-chlorobutane utilising *R. erythropolis*, strain Y2, which was also able to grow on a range of other α-substituted chloroalkanes, including 1-chloropropane, 1-chlorohexane and 1-chlorooctadecane. The ability to utilise these substrates was conferred by a halidohydrolase-type haloalkane dehalogenase (Figure 1.4d). This enzyme was purified and was found to be a monomer with a Mr of 34 k. The purified enzyme catalysed the cleavage of a wide range of mono- and di- substituted chloro- and bromo-alkanes, with the highest activity towards those which were terminally substituted.

Rhodococci have also been isolated that can degrade trichloroethene, one of the most commonly used degreasers in industry and a widespread pollutant of soil and groundwater (Ewers *et al.*, 1990). It has not yet been possible to find a strain which can grow on trichloroethene as a sole carbon and energy source, possibly due to energetic constraints (Dabrock *et al.*, 1992), since reducing equivalents are unlikely to be generated during trichloroethylene metabolism, due to the production of HCl. Other substrates are therefore used to isolate and grow these organisms. Ewers *et al.* (1990) used isoprene (2-methyl-1,3-butadiene) as an enrichment and growth substrate and isolated *R. erythropolis* JE77, which was able to co-oxidise *cis-* and *trans-* dichloroethene, 1,1-dichloroethene and vinylchloride in addition to trichloroethene, probably using epoxidation as the initial attack on the substrate. A similar strain, *R. erythropolis* BD1, was isolated on isopropylbenzene by Dabrock *et al.*(1992). This latter organism could also grow on toluene, phenol, protocatechuic acid, phenylpyruvate and isobutyrate.

1.1.7 Growth on aromatic compounds

Rhodococci have been isolated that can grow on many different aromatic compounds, and they appear to have most of the classical pathways for aromatic ring cleavage described in Section 1.2.2; *meta*- and *ortho*- cleavage of catechol and the *ortho*- cleavage of protocatechuic acid, gentisic acid and homogentisic acid (Rast *et al.*, 1980; Dugan & Golovlev, 1982, 1985). They also have a well developed ability to metabolise halogenated aromatic compounds.

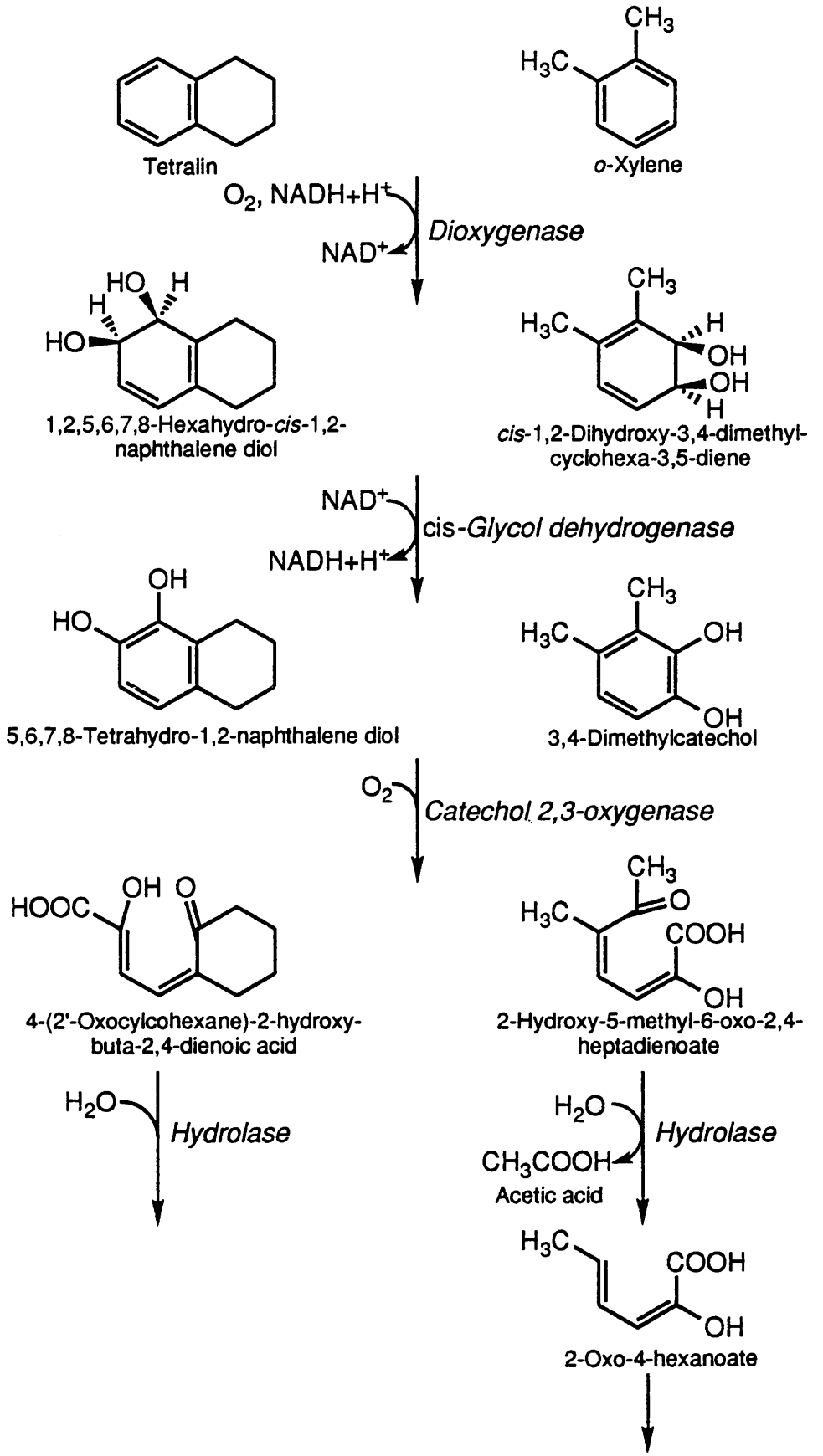
a) The diversity of substrates metabolised through a *cis*-glycol pathway by *Rhodococcus* sp. strain C125

Schraa *et al.* (1987) isolated *Rhodococcus* sp. strain C125 (originally *Corynebacterium* strain C125, renamed in van der Meer *et al.*, 1992) on *o*-xylene (1,2-dimethylbenzene). This strain can also grow well on toluene and ethylbenzene, and less well on benzene, tetralin, naphthalene, *o*-diethylbenzene, biphenyl and indane (Sikkema & de Bont, 1993). An NAD(P)H-dependent dioxygenase activity is present in extracts of cells grown on *o*-xylene or tetralin. This dioxygenase has a wide substrate range including (in order of decreasing activity): toluene, ethylbenzene, *o*-xylene, *o*-diethylbenzene, tetralin, naphthalene and benzene. Sikkema & de Bont (1993) have used a novel method for proving that the product of this dioxygenase is a *cis*-glycol, using toluene *cis*-glycol as a competitive inhibitor of the next enzyme, *cis*-glycol dehydrogenase. Whole cells incubated in the presence of toluene *cis*-glycol and tetralin accumulated an intermediate that produced 5,6,7,8-tetrahydro-1-naphthol and 5,6,7,8-tetrahydro-2-naphthol when acidified, consistent with the recyclisation of the *cis*-glycol of tetralin (Figure 1.5).

Extracts also contain non-specific *cis*-glycol dehydrogenase (NAD-dependent), catechol 2,3-oxygenase and hydrolase activities (Sikkema & de Bont, 1993). The *cis*-glycol dehydrogenase can oxidise benzene *cis*-glycol, toluene *cis*-glycol and the *cis*-glycol of tetralin. The catechol 2,3-oxygenase and hydrolase system is active with a range of substrates including catechol, 3-methylcatechol, 1,2-dimethylcatechol and

Figure 1.5: Metabolism of *o*-xylene and tetralin by *Rhodococcus* sp. strain C125

(Schraa *et al.*, 1987; Sikkema & de Bont, 1993)



5,6,7,8-tetrahydro-1,2-naphthalene diol. The products of the hydrolysis of 5,6,7,8-tetrahydro-1,2-naphthalene diol have not been determined.

Sikkema *et al.* (1992) observed an emulsification of the growth medium when this strain was grown on tetralin, and found that tetralin disturbed the ΔpH across the cell membrane. Van der Meer *et al.* (1992) monitored the breakdown of toluene and ethylbenzene by this strain in a soil column, and found that 90 % of the influent concentrations (about 500 µg/l) were degraded at 20 °C, though at 10 °C only 20-30 % were removed.

b) The metabolism of *p*-toluate by the modified 3-oxoadipate pathway

Bruce & Cain (1988) found that several rhodococci, *R. rhodochrous* N5 and N75, *R. corallinus* N657 and *Rhodococcus* strains BCN1, BCN2 and 4PH1, were able to grow on *p*-toluate, metabolising it through the *ortho*- cleavage pathway (Section 1.2.3d), but with a modification to cope with 4-methylcatechol (explained in the inset to Figure 1.6). Further research led to the pathway illustrated in Figure 1.6, and the purification of a new enzyme, 4-methylmuconolactonemethyl-isomerase, from *R. rhodochrous* N75 (Bruce *et al.*, 1989). This isomerase is very specific, and catalyses the conversion of (+)-(4S)-4-methylmuconolactone to (–)-(4S)-3-methylmuconolactone, with the putative intermediate of 1-methyl-2-enelactone (Figure 1.6). The enzyme has a native Mr of 75.5 k and is made up of monomers of 17 k.

c) Methylaniline and chloromethylaniline

R. rhodochrous strains CTM and Sb 4 have been isolated on 2-methylaniline (*o*-toluidine) as sole carbon and energy source. For fast breakdown in strain CTM an additional carbon source, such as glucose was necessary (Appel *et al.*, 1984; Fuchs *et al.*, 1991). Both strains degrade 2-methylalanine through 3-methylcatechol, followed by *meta*-cleavage in strain Sb4, and by both *ortho*- and *meta*- cleavage in strain CTM (Figure 1.7). In addition, strain CTM was able to co-metabolise 3-chloro-2-methylaniline and 4-chloro-2-methylaniline through the same pathway, with the chlorine atom being removed after *ortho*-ring cleavage (Figure 1.7; Fuchs *et al.*, 1991).

Figure 1.6: Metabolism of *p*-toluate via the modified 3-oxoadipate pathway in *R. rhodochrous* N75

(Bruce *et al.*, 1989)

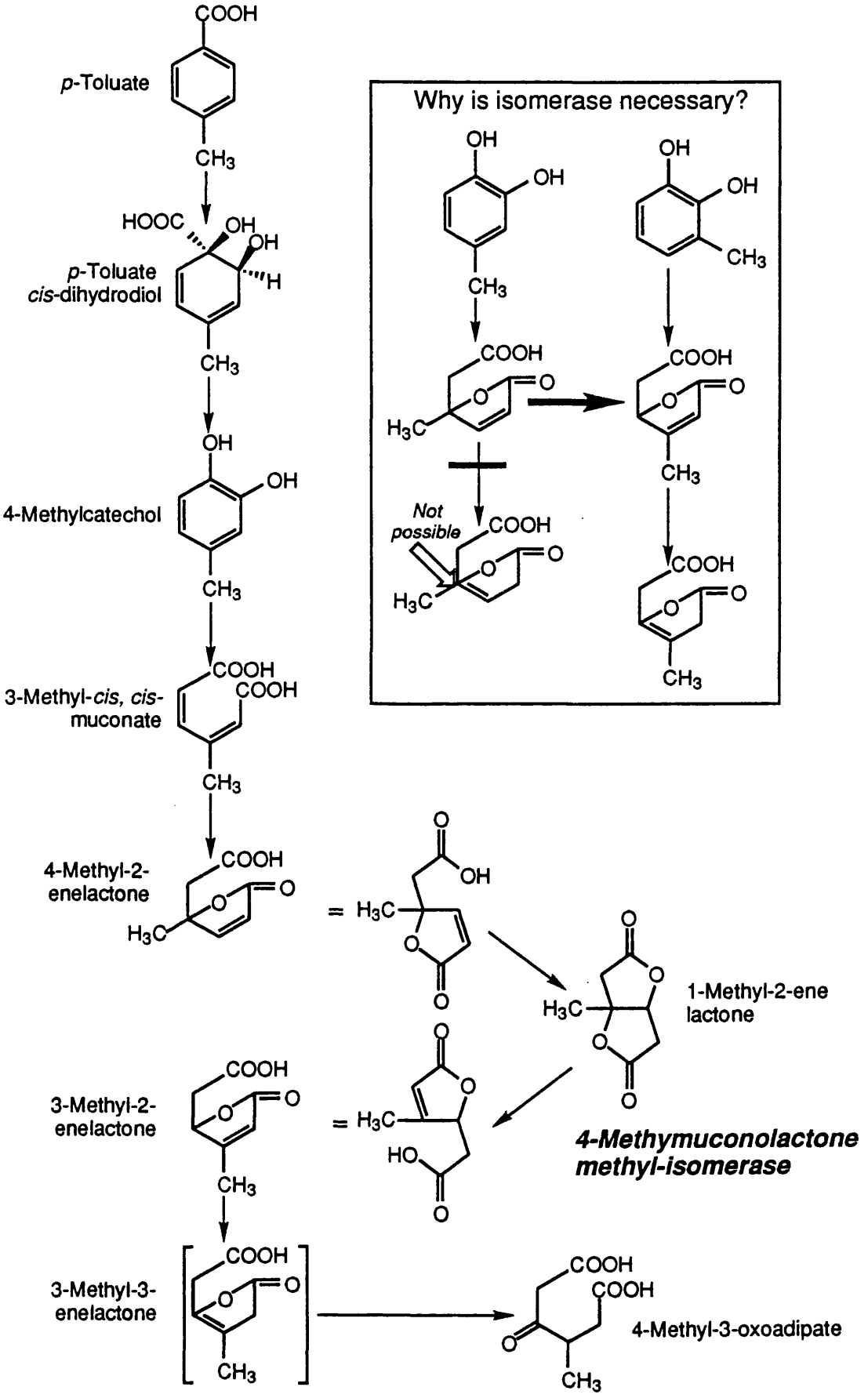
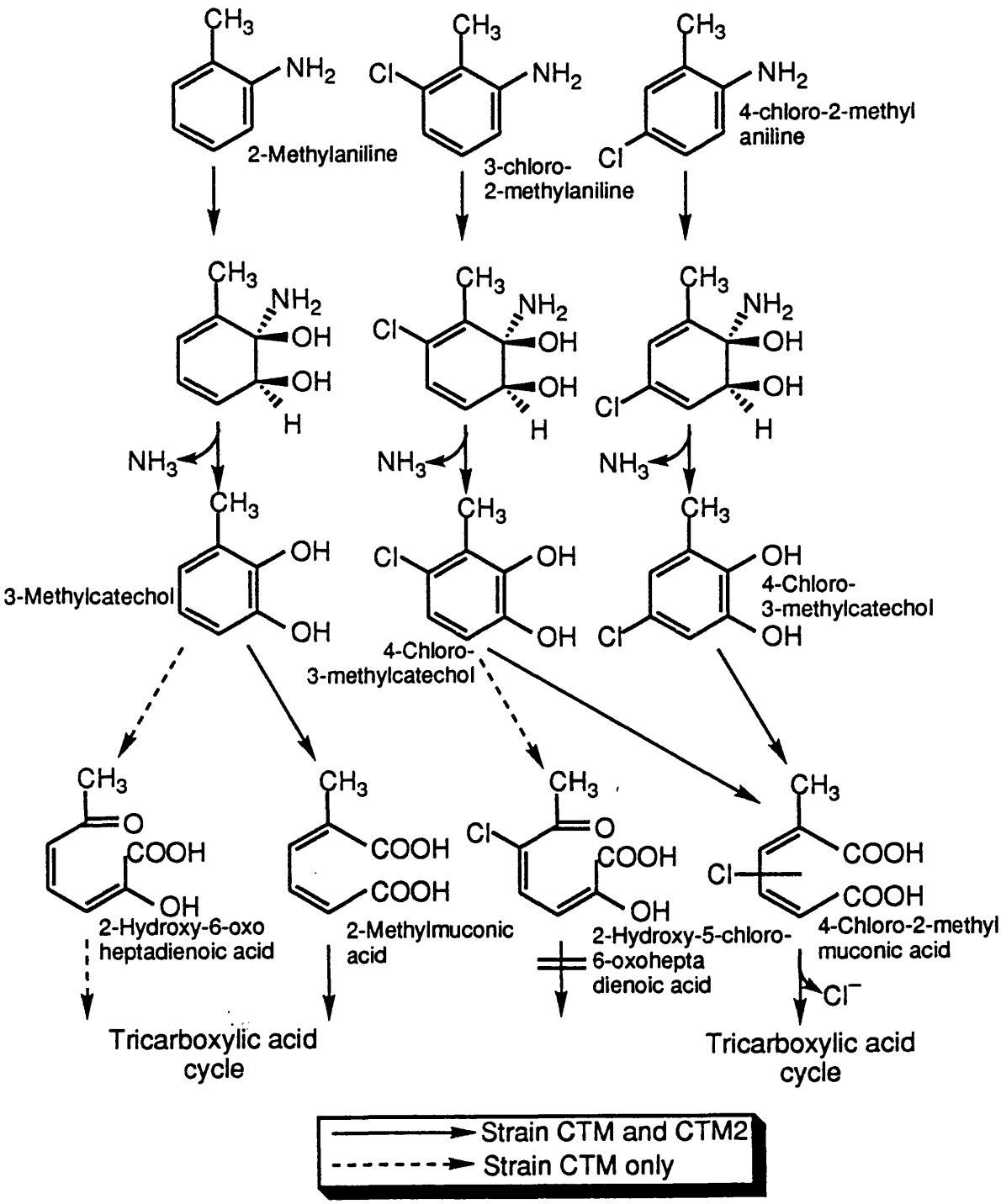


Figure 1.7: The breakdown of methylaniline and mono-chloromethyl anilines by *R. rhodochrous* CTM

(Fuchs *et al.*, 1991)



However, *meta*- cleavage of these chlorinated compounds led to an accumulation of 2-hydroxy-5-chloro-6-oxoheptadienoic acid as a dead-end metabolite. Prolonged cultivation with 2-methylaniline and 3-chloro-2-methylaniline produced a mutant, CTM2, which did not express catechol 2,3-oxygenase activity, so avoiding this problem.

Strain CTM was found to have two catechol 2, 3-oxygenases, C23OI and C23OII, which have been cloned into *E. coli* (Schreiner *et al.*, 1991). C23OI contained four identical 39 k subunits, whereas C23OII contained four 40 k subunits. The substrate ranges of the two enzymes were slightly different, with C23OII being able to accept all those substrates that C23OI could cleave, but it also cleaved 2,3-dihydroxybiphenyl and the catechols formed from phenylcarboxylic acids. There were also differences in relative activity, with C23OI preferentially cleaving methylcatechols; whereas C23OII preferred catechol.

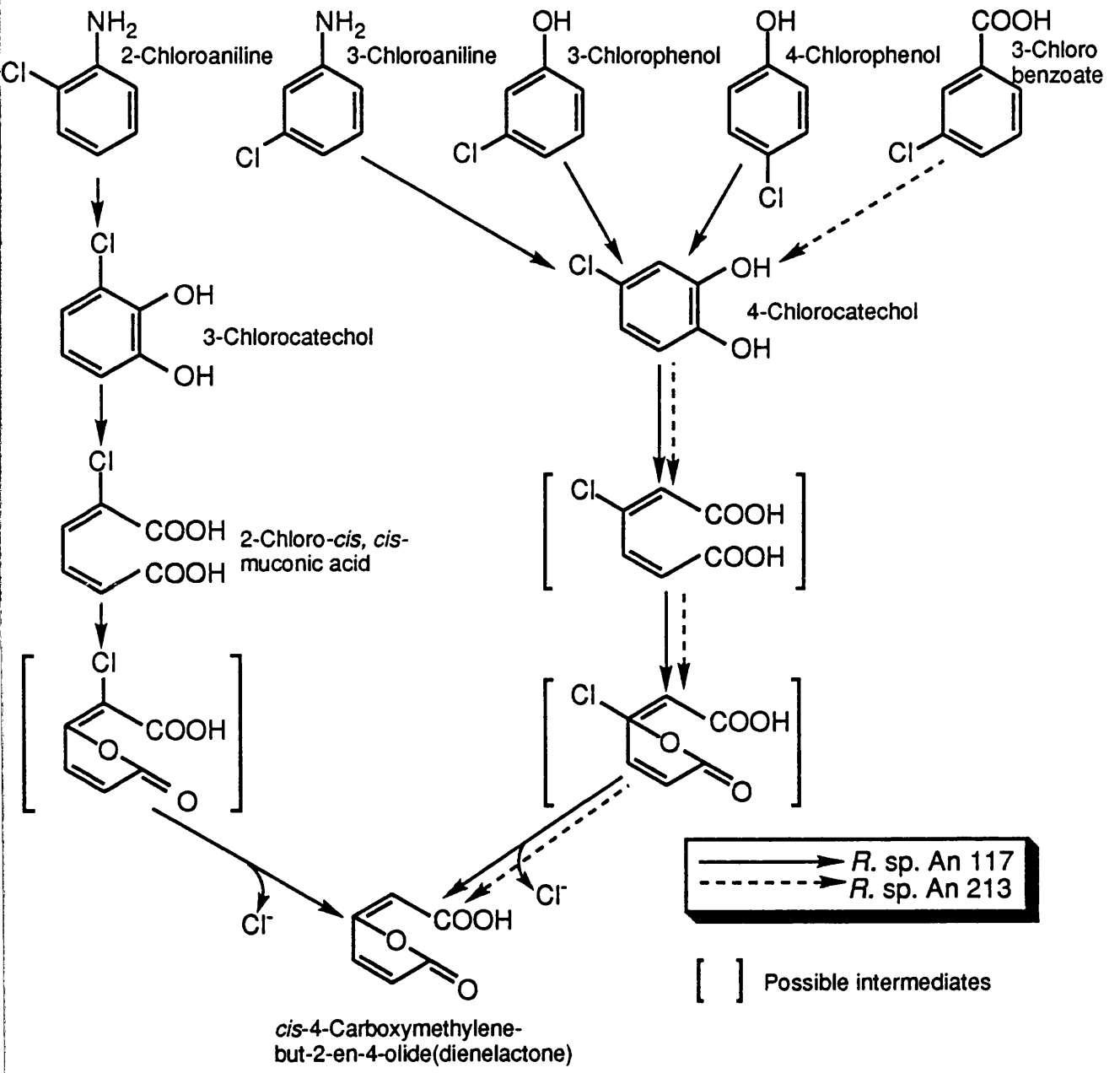
d) Phenols, monochlorophenols and related compounds

Many rhodococci are able to utilise phenol as a sole carbon and energy source. *Rhodococcus* sp. P1 was found to hydroxylate the ring adjacent to the phenolic hydroxyl group with an inducible phenol hydroxylase, producing catechol. The catechol was cleaved by a catechol 1,2-oxygenase to give *cis, cis*-muconic acid, which fed into the β-ketoadipate pathway (Straube, 1987). The kinetics of phenol degradation by this strain in both batch and continuous culture have been described; in batch conditions concentrations of up to 2.8 g phenol/l were degraded (Hensel & Straube, 1990; Straube *et al.*, 1990). This strain has been used as a biosensor for phenols and chlorophenols, using an oxygen electrode to measure oxygen uptake on exposure to various substrates (Riedel *et al.*, 1993).

Rhodococcus sp. An 117 and An213 are able to grow on phenol, benzoate, aniline and can co-metabolise their monochlorinated derivatives. These pathways have been extensively investigated, producing the results summarised in Figure 1.8 (Janke *et al.*, 1984, 1988a, b, 1989; Ihn *et al.*, 1989). The pathways used by these strains are similar

Figure 1.8: The breakdown of mono-chlorinated phenols, chlorobenzoate and chloroanlline by *Rhodococcus* sp. An 117 and An 213

(Janke *et al.*, 1989)



to those described above (section 1.1.7c) for the metabolism of chloromethylanilines by *R. rhodochrous* strain CTM, with the chlorine being removed after ring cleavage. A mutant strain, Am 144, has been isolated, which has no catechol 1,2-oxygenase activity, so it accumulates catechols and chlorocatechols from aniline, phenol and chlorinated derivatives (Janke & Ihn, 1989).

R. erythropolis strain 1CP can grow on 4-chlorophenol and 2,4-dichlorophenol as sole carbon and energy sources, though co-substrates such as glucose accelerated the breakdown of the chlorophenols (Gorlatov *et al.*, 1989; Gorlatov & Golovleva, 1992). Two catechol 1,2-oxygenases have been purified from this strain: C12OI with a native Mr of 67 k and C12OII, which had a much higher affinity for chlorocatechols, with a Mr of 64 k (Mal'tseva *et al.*, 1991).

The side chain fluorinated aromatic, 3-trifluoromethylbenzoate, completely inhibits the catechol 1,2-oxygenase of *R. rubropertinctus* N657 (Engesser *et al.*, 1988).

e) Phloroglucinol

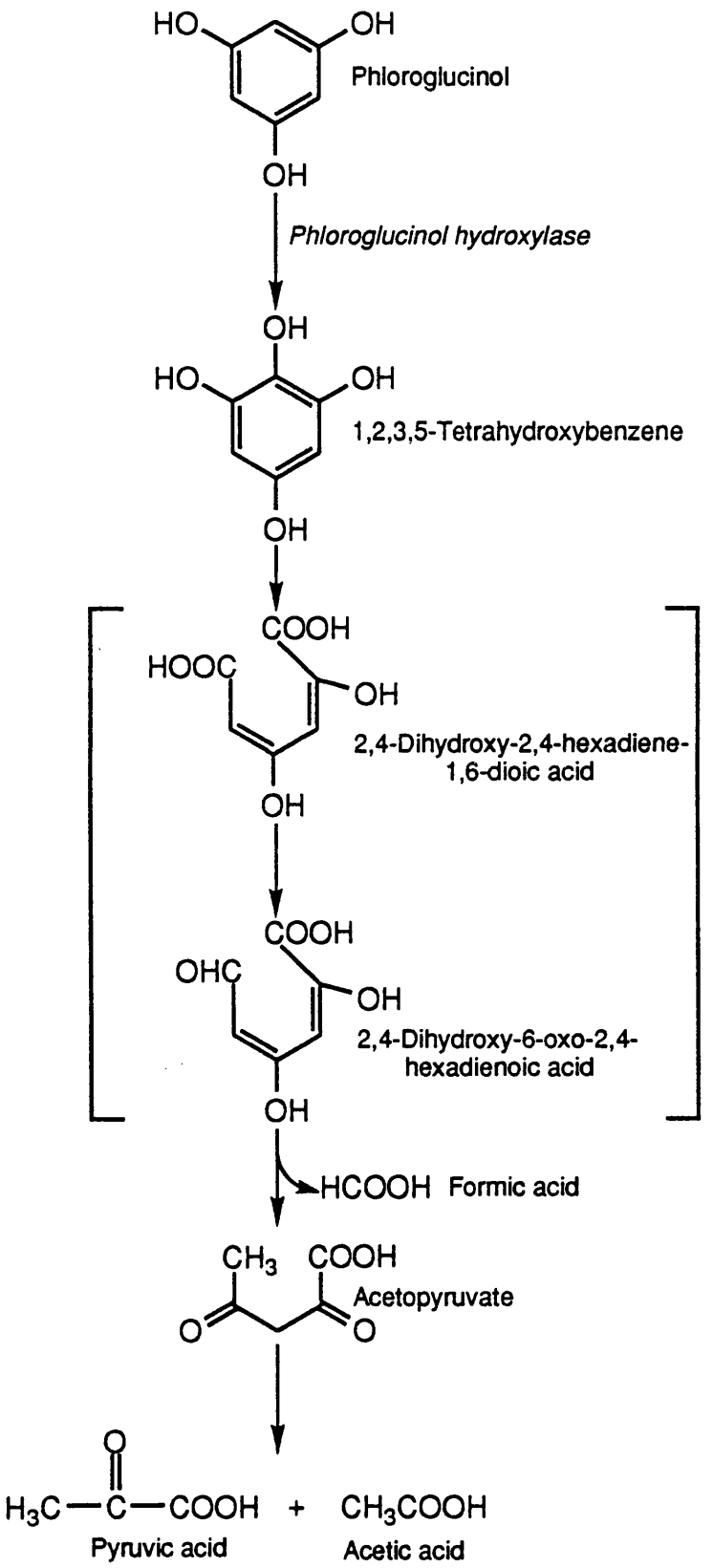
Armstrong & Patel (1993) have isolated *Rhodococcus* sp. BPG-8 on phloroglucinol (1,3,5-trihydroxybenzene). This substrate is hydroxylated by an NAD(P)H-dependent hydroxylase, to give 1,2,3,5-tetrahydroxybenzene, followed by dioxygenative cleavage and hydrolysis of the ring cleavage product to give formic acid , acetic acid and pyruvic acid (Figure 1.9). This strain also metabolises resorcinol (1,3-dihydroxybenzene) *via* 1,2,4 trihydroxybenzene (see below, 1.1.7f).

f) Pentachlorophenol

Chlorophenols were widely used as biocides in the past; some are recalcitrant, notably pentachlorophenol, and so have accumulated in the environment (Haggblom, 1992). Although monochlorophenols are often degraded by ring cleavage before chloride elimination (above, section 1.1.7d), this does not occur with substrates such as pentachlorophenol, where at least some of the chlorine atoms are usually removed before ring cleavage. Extensive research has been done on *R. chlorophenolicus* PCP-1 (DSM 43826), a strain which can grow on pentachlorophenol as a sole carbon and

Figure 1.9: Metabolism of phloroglucinol by *Rhodococcus* sp. BPG-8

(Armstrong & Patel, 1993)

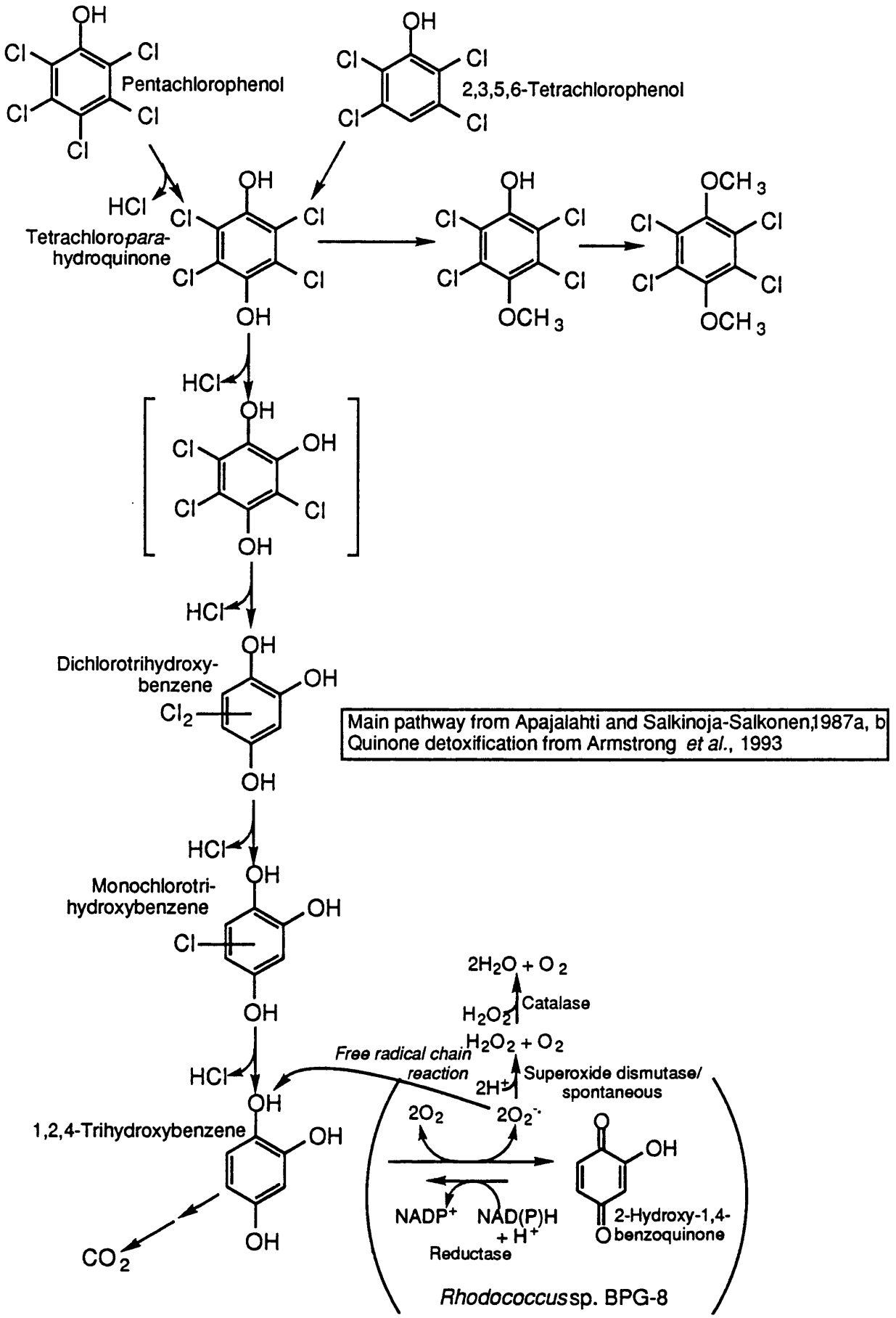


energy source (Apajalahti & Salkinoja-Salonen, 1987a). This organism uses the pathway illustrated in Figure 1.10 to remove all the chlorines on pentachlorophenol before the ring is cleaved (Apajalahti & Salkinoja-Salonen, 1987b). This dechlorination is undertaken by two enzyme activities, first dechlorinating *para*-hydroxylation by a membrane-bound, FAD and NADPH-dependent enzyme, then dechlorination by a soluble enzyme (Uotila *et al.*, 1991). The dechlorinating *para*-hydroxylation enzyme involves cytochrome P-450, and in normal aerobic conditions the oxygen in the hydroxyl comes from O_2, though in the presence of iodosobenzene and Na_2SO_3 it can come from H_2O (Uotila *et al.*, 1992). These reagents also enable anaerobic dechlorination in cell-free extracts, possibly by activating the P-450 peroxide shunt. This organism also contains a constitutive pentachlorophenol and tetrachloro-*para*-hydroquinone O-methylation activity, which may be a detoxification mechanism (Figure 1.10).

Other rhodococcal strains have been isolated which seem to have a similar pentachlorophenol-degrading ability; these include *Rhodococcus* strain CP-2/DSM 4598 (Haggblom *et al.*, 1989) and strain CG-1 (Haggblom *et al.*, 1988). The latter strain has been found to methylate pentachlorophenol, like strain PCP-1, as has another *Rhodococcus* sp. (Allard *et al.*, 1987).

The mechanism of metabolism of 1,2,4-trihydroxybenzene by pentachlorophenol-degrading strains has not yet been elucidated, though it must be metabolised, otherwise the strains could not grow on pentachlorophenol. As mentioned above (1.1.7e), Armstrong & Patel (1993) have isolated *Rhodococcus* species BPG-8 on a similar compound, 1,3,5-trihydroxybenzene, and cell-free extracts of this strain produced 1,2,4-trihydroxybenzene during the transformation of resorcinol (1,3-dihydroxybenzene). The oxidation of 1,2,4-trihydroxybenzene to 2-hydroxy-1,4-benzoquinone would produce superoxide radicals, which could damage cell structures *via* free-radical chain reactions. Armstrong *et al.* (1993) found that superoxide dismutase and catalase (both present in the extract) retarded this reaction, and they also found that extracts of this strain contain an NAD(P)H-dependent reductase activity that can convert the benzoquinone back to

Figure 1.10: The breakdown of pentachlorophenol by *R. chlorophenolicus* PCP-1



1,2,4-trihydroxybenzene (Figure 1.10). These reactions may stabilise the trihydroxybenzene and thus allow *meta*- cleavage, though no evidence exists of these processes in pentachlorophenol-degrading strains.

R. chlorophenolicus PCP-1 and *Rhodococcus* sp. CP-2 have been used to treat simulated pentachlorophenol-contaminated ground water (Valo *et al.*, 1990). The rhodococci were immobilised on a polyurethane carrier in a column, and fed with pentachlorophenol, 2,4,6-trichlorophenol and 2,3,4,6-tetrachlorophenol at 3 - 130 mg/l. The chlorophenol concentration in the effluent was reduced to 10^{-3} to 10^{-4} that of the influent concentration. Removal of chlorophenol was effective even at 4 °C, due to absorption, as long as the temperature was intermittently raised to 25 °C to allow degradation. The immobilised bacteria continued to degrade chlorophenols for 4 months, with little contamination by other bacteria.

R. chlorophenolicus PCP-1 has also been used to degrade pentachlorophenol in non-sterile natural soil, whether lightly (30 mg pentachlorophenol/kg) or heavily (600 mg pentachlorophenol/kg) contaminated (Middeldorp *et al.*, 1990). In sandy soil only 500 cells/g were necessary to start mineralisation, and inoculum-induced mineralisation continued for more than four months. The rate of mineralisation was enhanced by an additional carbon source, such as distillery waste. The soils used, a natural sandy loam and a peaty soil, showed no pentachlorophenol degradation activity before inoculation, and none developed in control soils during the four months of the experiment. As mentioned earlier (Section 1.1.3), rhodococci have been found to be more effective at degrading chlorophenols in natural situations than other chlorophenol-degrading bacteria.

g) Nitroaromatics

Little is known about the biodegradation and biotransformation of dinitro- and polynitro- aromatics, because of their toxicity, though they are often reduced and cross linked (Fewson, 1981). *Rhodococcus* sp. QT1 is able to grow on 1,3-dinitrobenzene, though very slowly, and can also use it as a sole nitrogen source while growing on

succinate (Dickel & Knackmuss, 1991). This strain is also able to grow on phenol, resorcinol and benzoate, though not on salicylate or toluene. 4-Nitrocatechol was found to be an intermediate in the catabolism of 1,3-dinitrobenzene, with the first nitro group removed as nitrite during re-aromatisation of the dihydrodiol (Figure 1.11a). The other nitro group is removed after ring cleavage, though it is not clear when.

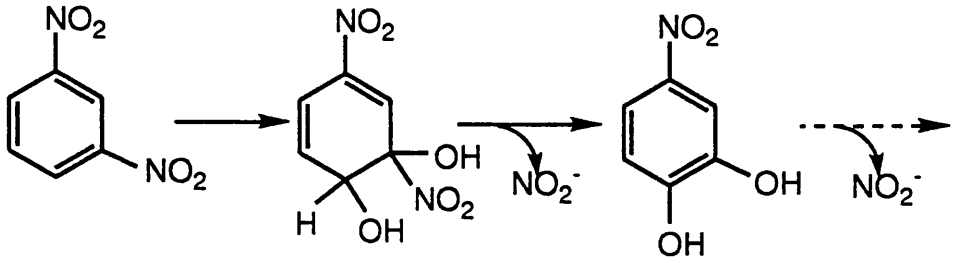
R. erythropolis strains HL 24-1 and HL 24-2 were isolated on the basis of their ability to use 2,4-dinitrophenol as sole nitrogen source, with succinate as a carbon source, though both strains are also able to use 2,4-dinitrophenol as a sole carbon source (Lenke *et al.*, 1992). Nitrite and 4,6-dinitrohexanoate (a minor dead-end metabolite) were released during growth, with the sum of these compounds indicating stoichiometric conversion of 2,4-dinitrophenol, but the metabolic pathway was not elucidated (Figure 1.11b). The transformation of picric acid by strain HL24-2 was found to produce 2,4,6-trinitrocyclohexanone as a dead end metabolite (Figure 1.11c), though this metabolite further degraded to 1,3,5-trinitropentane during extraction (Lenke & Knackmuss, 1992). These results indicate that the initial ring attack is probably nucleophilic, rather than electrophilic (as in an oxygenase), possibly because of the presence of the electrophilic nitro substituents (Figure 1.11c).

A *Rhodococcus* sp. was isolated from a soil sample in which 2,4-dinitrophenol was degraded (Schmidt & Gier, 1990). In this soil sample the 2,4-dinitrophenol was degraded with multiphasic mineralisation kinetics. It was found that the sample contained another 2,4-dinitrophenol degrader, a *Janthinobacterium* sp. This bacterium grew slowly on very low concentrations of 2,4-dinitrophenol, whereas the *Rhodococcus* sp. grew faster, but only on higher concentrations of the substrate, leading to the complex mineralisation kinetics. Further research on these organisms has shown that glucose at 100 mg/l promotes the metabolism of 2,4-dinitrophenol, though a concentration of 1000 mg/l inhibits breakdown (Hess *et al.*, 1993).

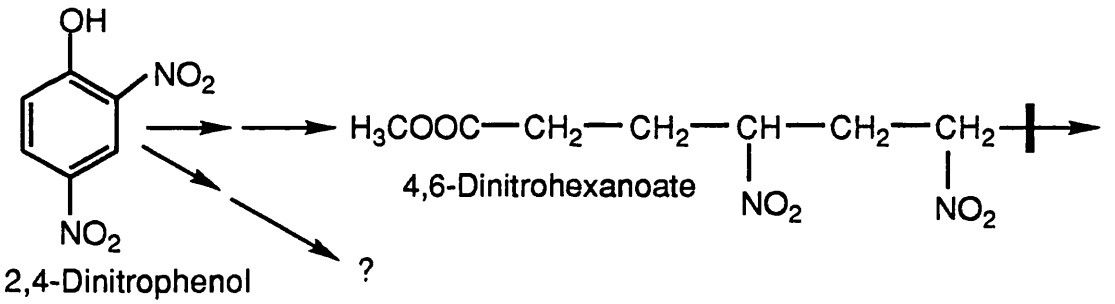
R. rhodochrous IFO 3338 has been found to hydrogenate the nitroolefin 1-phenyl-2-nitropropene to 1-phenyl-2-nitropropane (Figure 1.12). The product was in the optically

Figure 1.11: The breakdown of nitroaromatics by *Rhodococcus* sp.

a) 1,3-Dinitrobenzene catabolism by *Rhodococcus* sp QT-1 (Dickel & Knackmuss, 1991)



b) 2,4-Dinitrophenol cataboiism by *R. erythropolis* HL24-1 and HL24-2 (Lenke *et al.*, 1992)



c) Proposed route for the removal of nitrite and the production of 1,3,5-trinitropentane from picric acid by *R. erythropolis* HL24-2 (Lenke & Knackmuss, 1992)

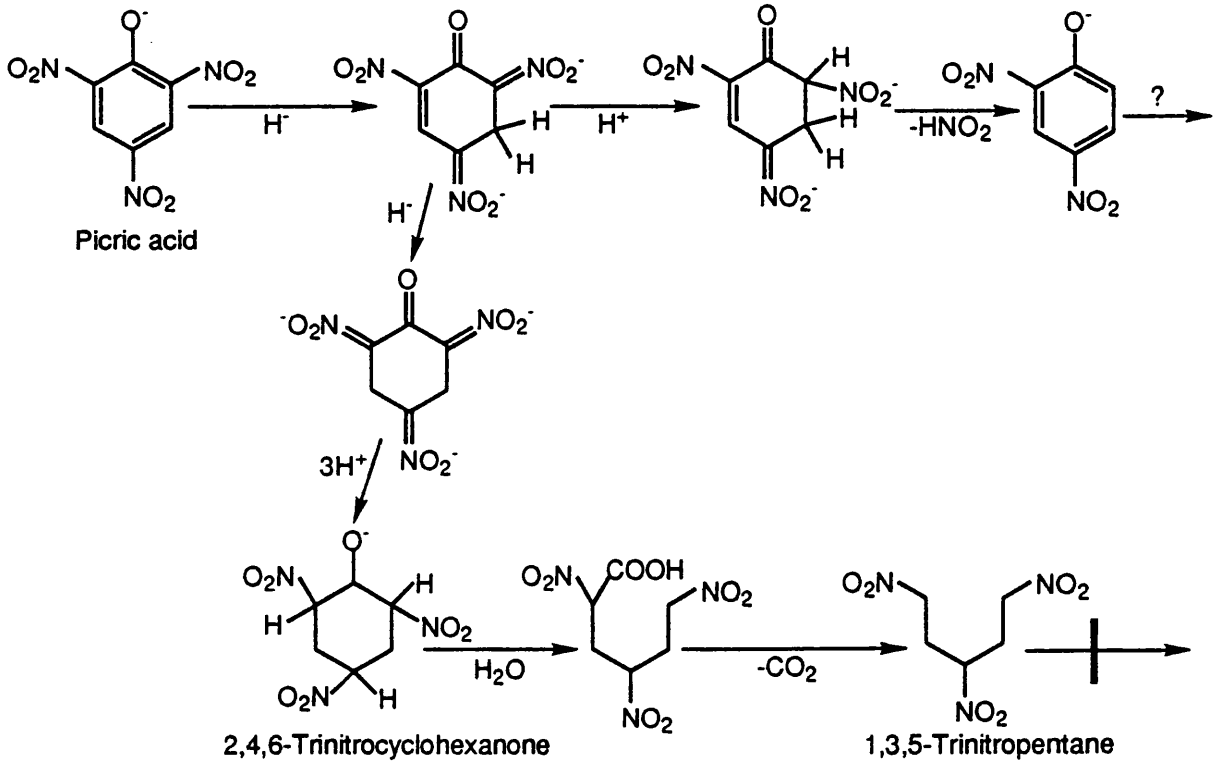
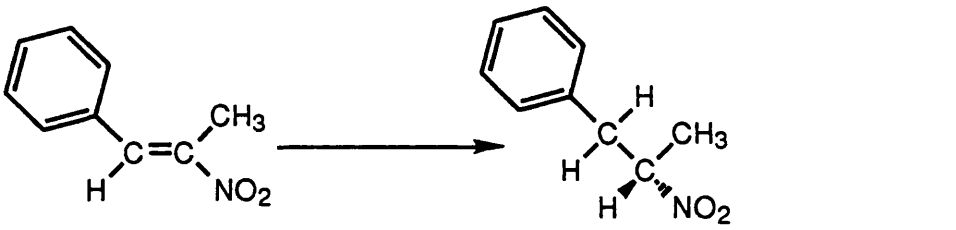


Figure 1.12: Hydrogenation of nitrooiefins by *R. rhodochrous* IFO 3338

(Sakai *et al.*, 1985)



1-Phenyl-2-nitropropene (S)-1-Phenyl-2-nitropropane

active, (S) configuration, though it isomerised in aqueous solution unless derivatised (Sakai *et al.*, 1985).

h) Lignin-related and alkoxy aromatics

R. erythropolis DSM 1069 is able to grow on a wide range of lignin-related monomers, and possible routes of metabolism of these compounds have been proposed (Figure 1.13), partly as a result of studies with mutants (Eggeling & Sahm, 1980). The coniferyl alcohol dehydrogenase from this organism has been purified, and is NAD-dependent (Jaeger, 1988). A formaldehyde dehydrogenase activity was also expressed in this strain when growing on compounds containing methyl groups, such as methoxybenzoic acids (Eggeling & Sahm, 1985). This formaldehyde dehydrogenase has been purified, and the native Mr was 130 k, made up of three identical subunits of 44 k. The purified enzyme required NAD and an additional reduced cofactor for activity with formaldehyde, but could oxidise ethanol to acetaldehyde with only NAD present. The enzyme was also active with primary alcohols up to octanol.

A *R. rhodochrous* strain was isolated on cinnamic acid (Andreoni *et al.*, 1991) , and when grown on this substrate benzoic acid transiently accumulated in the medium. In addition, cells grown on cinnamic acid possessed an inducible catechol 1,2-oxygenase activity, so the pathway shown in Figure 1.14a was proposed for cinnamic acid metabolism in this strain. This strain also converted the lignin-related dimer (R)-anisoin into (1R, 2R)-1,2-bis(4-methoxyphenyl)ethane-1,2-diol, by reduction of a carbonyl to an alcohol (Figure 1.14b). The strain did not transform (S)-anisoin, and so this reaction could be used to produce chiral intermediates.

Karlson *et al.* (1993) have isolated *R. rhodochrous* strain 116, which is able to grow on a variety of alkoxyphenols and alkoxybenzoates as sole carbon and energy source. These substrates induced cytochrome P-450s, with a different P-450 induced on growth on 4-methoxybenzoate or 4-ethoxybenzoate ($P\text{-}450_{RR2}$) from that which was induced by growth on 2-methoxyphenol or 2-ethoxyphenol ($P\text{-}450_{RR1}$). Each of these cytochromes bound only the substrates that induced it, suggesting that they catalyse the

Figure 1.13: Proposed pathways for the breakdown of lignin-related aromatic compounds by *R. erythropolis* DSM 1069

(Eggeling & Sahm, 1980)

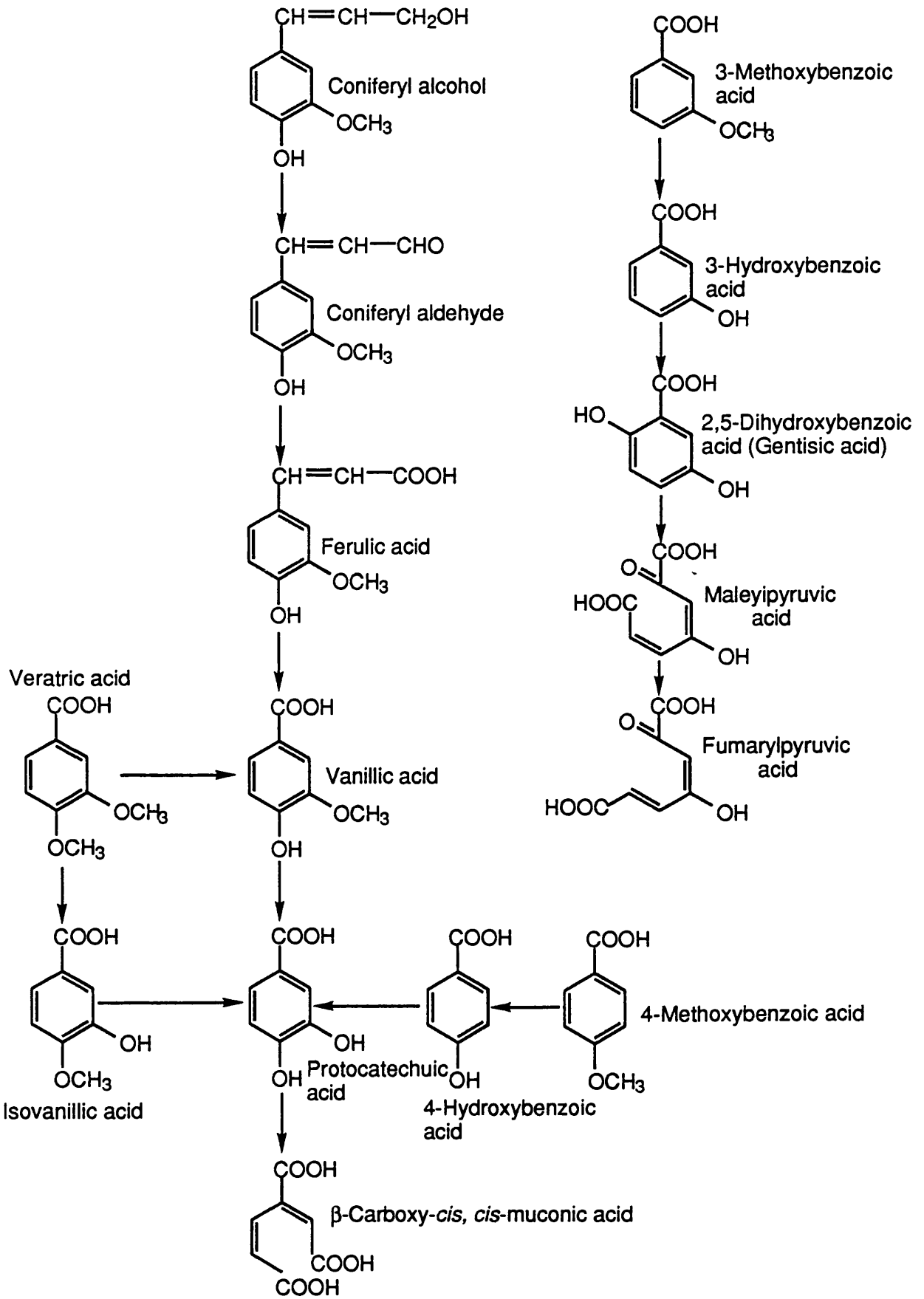
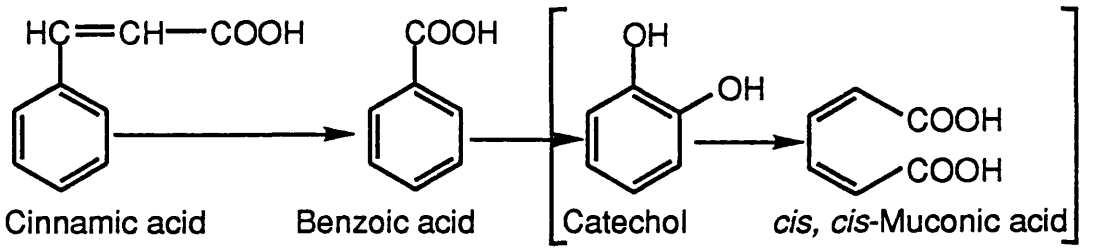


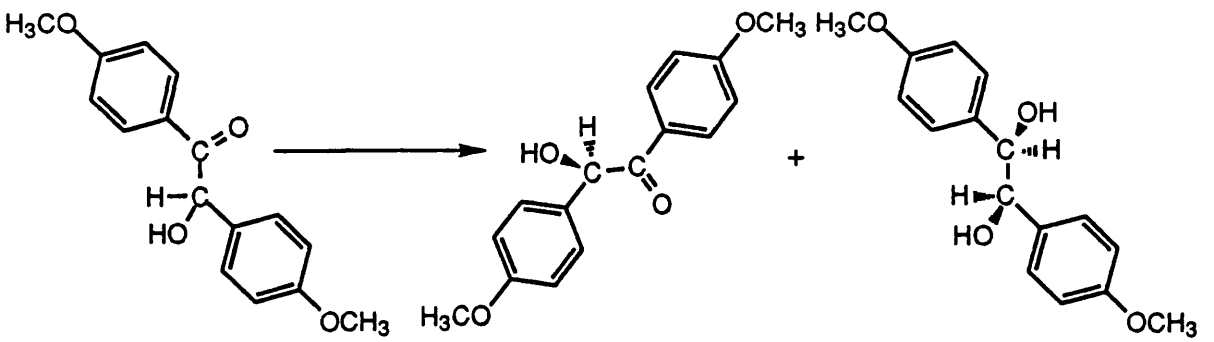
Figure 1.14: The breakdown of cinnamic acid and biotransformation of (R, S)-anisoin by *R. rhodochrous*

(Andreoni *et al.*, 1991)

a) The breakdown of cinnamic acid



b) The biotransformation of (R, S)-anisoin to (1*R*, 2*R*)-1,2-bis(4-methoxyphenyl)ethane-1,2-diol.



O-dealkylation of the substrate (Figure 1.15a). The enzymes of further metabolism (through catechol and protocatechuic acid) were induced by the aromatic ethers. Cytochrome P-450_{RR1} has been purified, and has a subunit Mr of 44.5 k, and displays similar electronic absorption spectra to cytochrome P-450_{cam} (Eltis *et al.*, 1993). The enzyme binds a variety of 2-substituted phenols, but will not bind 3- and 4-substituted ether phenols.

l) Terephthalates

Esters of phthalic acids are widely used in the production of plastics and rubber, and are found in both water reservoirs and soil. Slizen *et al.* (1989) have isolated *R. erythropolis* strain DSS-31, which can grow on terephthalate, monomethyl-terephthalate and dimethylterephthalate. While growing on dimethylterephthalate, terephthalate, monomethylterephthalate and protocatechuic acid were detected in the medium, and cell free extracts possessed a protocatechuate 3,4-dioxygenase activity and produced 3-ketoadipic acid from protocatechuic acid. The pathway shown in Figure 1.15b was proposed for the breakdown of dimethylterephthalate by this strain.

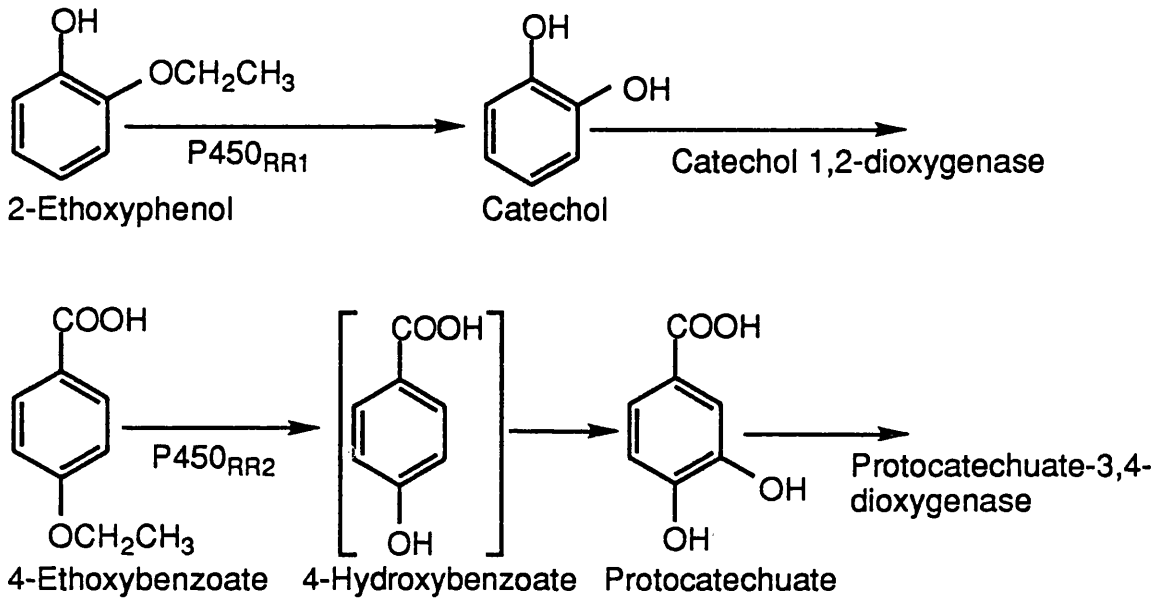
R. rubropertinctus M3 can also grow on terephthalate, and the metabolism of terephthalate in this strain appears to be complex, with benzoate, 4-hydroxybenzoate, catechol and protocatechuate all identified from cultures (Golovleva *et al.*, 1992). This strain forms aggregates up to 50 μm in size, enabling easy separation of cells from effluent in a bioreactor-based water treatment system (Golovleva *et al.*, 1992).

j) Polycyclic aromatic compounds

Rhodococci have been isolated that can use naphthalene, pyrene, phenanthrene, anthracene, fluoranthrene and chrysene as sole sources of carbon and energy. *Rhodococcus* sp. strain B4 can grow on naphthalene, and Grund *et al.* (1992) have proposed the pathway in Figure 1.16 for the metabolism of this substrate. Like many naphthalene-degraders, this strain has salicylate as an intermediate in the catabolic pathway, but more unusually, this is then metabolised through gentisate. The best defined naphthalene pathway, that encoded by the *Pseudomonas putida* NAH7 plasmid,

Figure 1.15: The breakdown of alkoxyaromatics and dimethylterephthalate

a) Cytochrome P-450 dependant degradation of 2-alkoxyphenols and 4-alkoxybenzoates by *R. rhodochrous* str. 116 (Karlson *et al.*, 1993)



b) The breakdown of dimethylterephthalate by *R. erythropolis* DSS-31 (Slizen *et al.*, 1989)

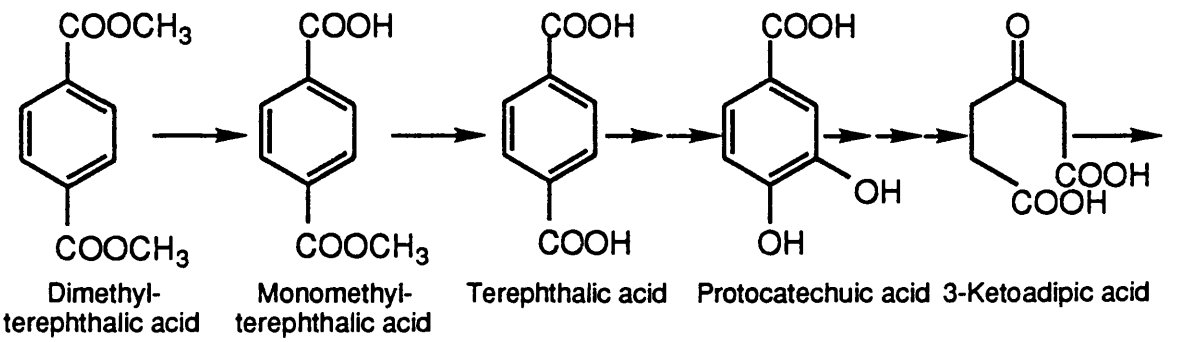
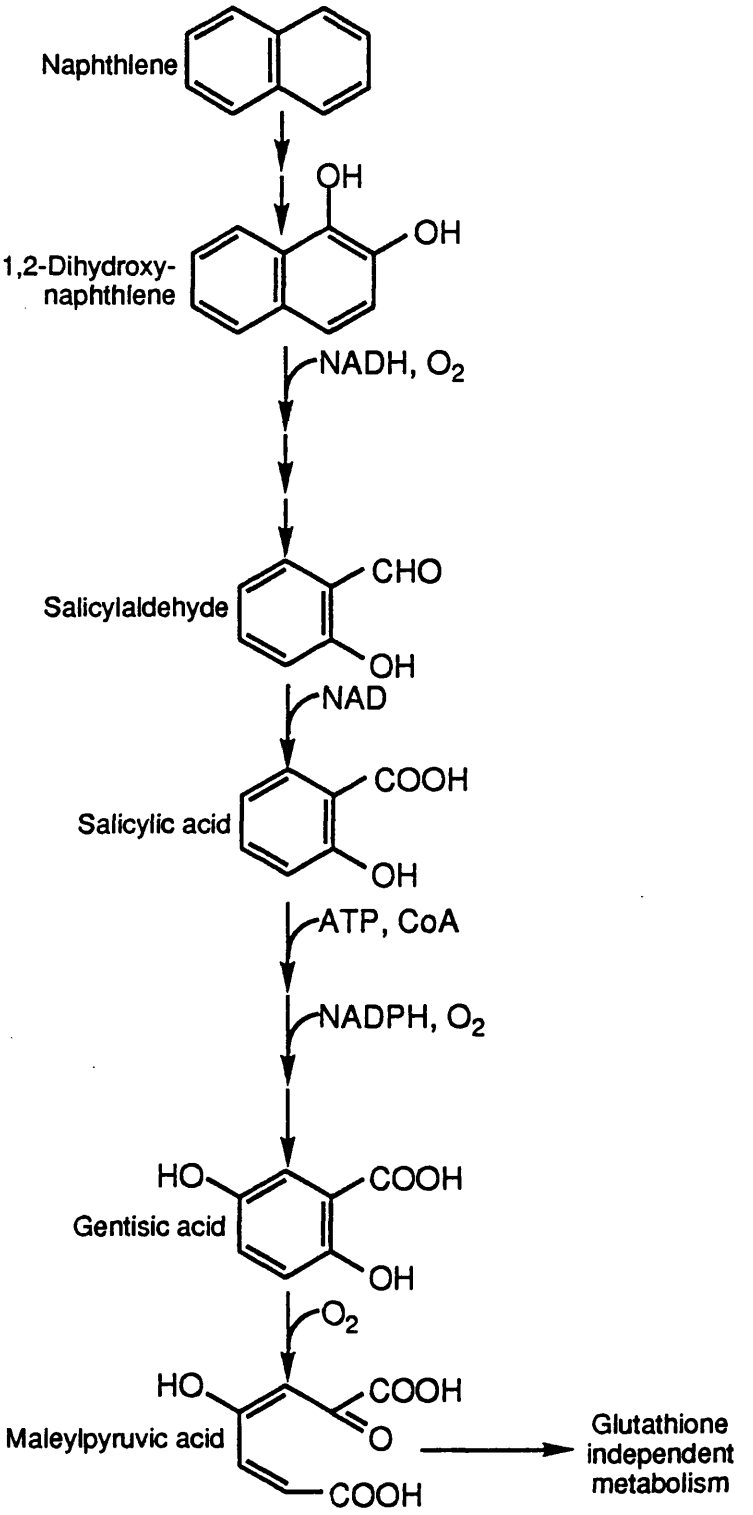


Figure 1.16: Metabolism of naphthalene by *Rhodococcus* sp. strain B4

(Grund *et al.*, 1992)



produces salicylate, which is then metabolised *via* catechol, though other organisms have been shown to use gentisate. The cofactor requirements of some of the enzymes in *Rhodococcus* sp. strain B4 are also unusual, with 1,2-dihydroxynaphthalene oxygenase needing NADH, and the salicylate 5-hydroxylase requiring NADPH, ATP and coenzyme A, suggesting that salicylic acid is initially activated to salicyl CoA.

A 1-naphthol-degrader, *Rhodococcus* sp. NCIB 12038, has been found to have a specific 1-naphthol oxygenase, though this organism showed only slow growth with naphthalene (Larkin, 1988).

Walter *et al.* (1991) isolated *Rhodococcus* sp. UW1, which is able to grow on pyrene, phenanthrene, anthracene, fluoranthrene and chrysene, and it can co-metabolise naphthalene, dibenzofuran, fluorene and dibenzothiophene. The initial steps in pyrene breakdown were suggested by the identification of a compound transiently present in the growth medium, a result of recyclisation of the ring cleavage product (Figure 1.17). However, it was not clear whether initial ring attack occurred at the 1,2- or 4,5-position, though the wide substrate range may imply that both systems of hydroxylation are used.

A *R. rhodochrous* strain has been isolated that can grow on polychlorinated biphenyls, a group of recalcitrant, lipophilic compounds used in the past as hydraulic fluids, dielectric fluids, plasticizers and heat exchangers (Boyle *et al.*, 1992). This strain was able to degrade 25 % of the polychlorinated biphenyl congeners in the blend Aroclor 1242. A range of intermediates were identified by mass spectra in the culture fluid, suggesting that metabolism of the polychlorinated biphenyls proceeds through ring hydroxylation and dioxygenase ring cleavage.

k) Heteroaromatics

Rhodococcus sp. B1 has been isolated on quinoline, which it metabolises *via* the pathway illustrated Figure 1.18 (Schwarz *et al.*, 1989). The first enzyme of this pathway, quinoline oxidoreductase, has been purified and contains 3 different subunits, with relative molecular masses of 82 k, 32 k and 18 k, and a native Mr of 300 k (Peshke

Figure 1.17: The breakdown of pyrene by *Rhodococcus* sp. UW1

(Walter *et al.*, 1991)

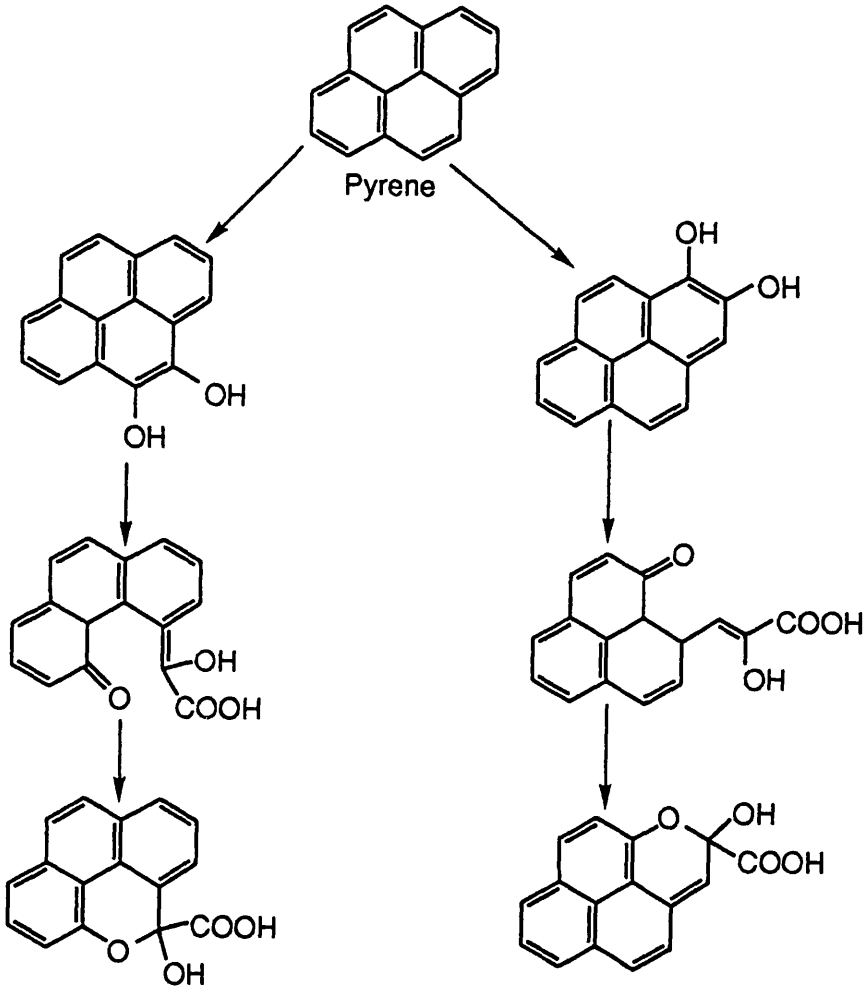
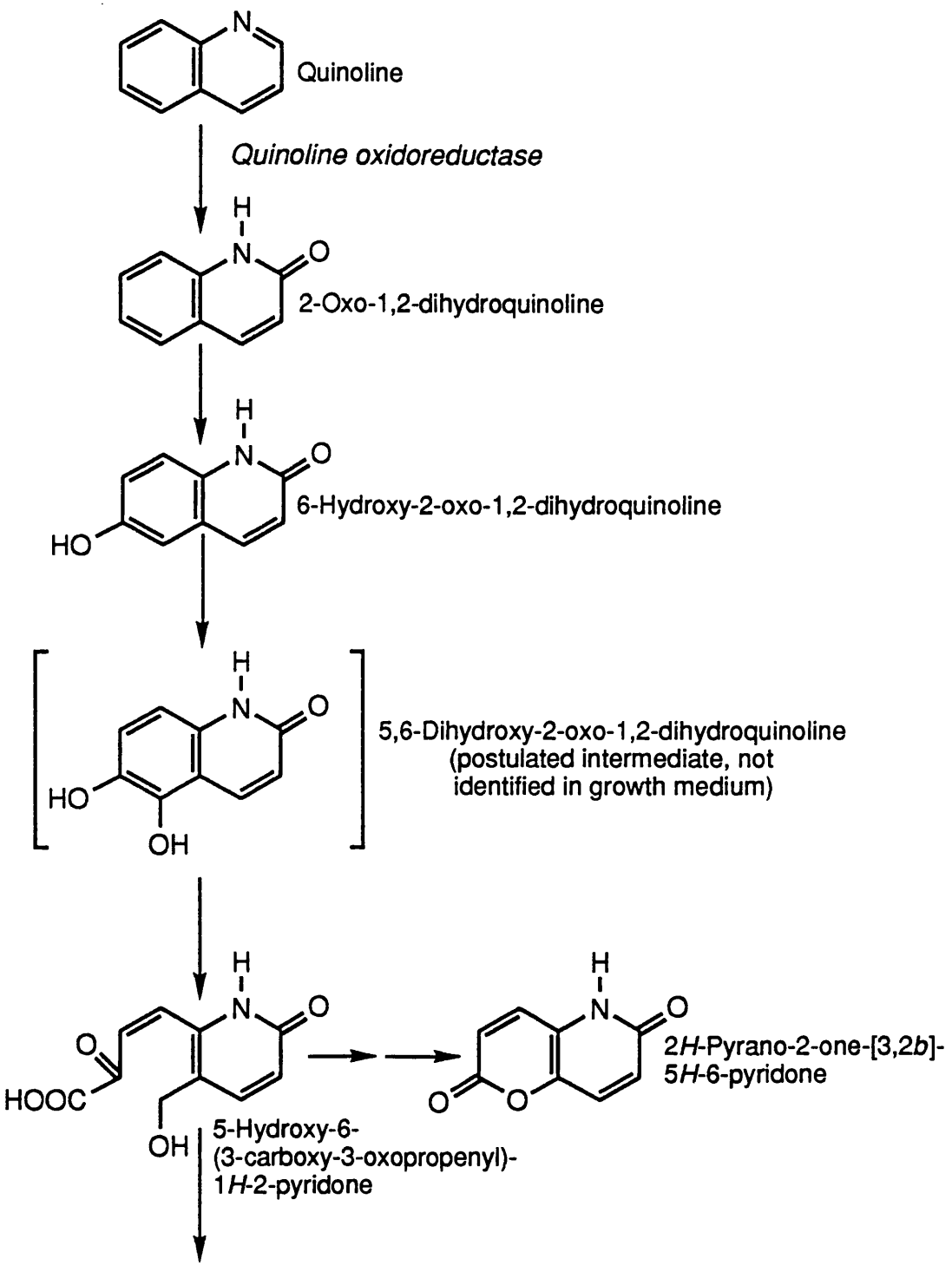


Figure 1.18: The breakdown of quinoline by *Rhodococcus* sp. B1

(Schwarz *et al.*, 1989)



& Lingens, 1991). Each molecule of the native enzyme contains 1.3 atoms of molybdenum, 8 atoms of iron, 8 atoms of acid-labile sulphur, 2 molecules of FAD and 2 molecules of molybdopterin cysteine dinucleotide. A similar enzyme has been characterised from *P. putida* 86, and the *N*-terminal amino acid sequences of the two enzymes are identical (Peshke & Lingens, 1991).

l) Hydroaromatics

R. rhodochrous N75 (the strain with the modified 3-oxoadipate pathway, 1.1.7b) can grow on both quinate and shikimate (Figure 1.19). Growth on both of these substrates, but not benzoate, induces a soluble 31.5 - 44 k NAD-dependent quinate/shikimate dehydrogenase (Bruce & Cain, 1990). Catabolism of hydroaromatics by most bacteria is initiated by a membrane associated and PQQ-mediated enzyme, so the NAD-dependence of this enzyme is unusual. Low levels of the biosynthetic shikimate: NADP 3-oxidoreductase were also found. Quinate-grown cells also showed high levels of dehydroquinate dehydratase, dehydroshikimate dehydratase, protocatechuate 3,4 oxygenase and the other enzymes of the metabolism of protocatechuate.

m) Steroids

Watanabe *et al.* (1986) found that many strains of *Rhodococcus* were able to grow on cholesterol as a sole carbon and energy source. They isolated 16 bacterial strains on cholesterol from foods of animal origin, e.g. butter, and found that they were all rhodococci, mainly *R. equi* and *R. rhodochrous*. They also found that many rhodococcal strains in culture collections could degrade cholesterol, including *R. erythropolis*, *R. fascians* and *R. rhodnii*. The bacteria were found to produce both an extracellular and an intracellular cholesterol oxidase activity.

Cholesterol oxidase catalyses the conversion of cholesterol to 4-cholesten-3-one (Figure 1.20), the first step in the breakdown of cholesterol, and has been purified from *R. equi* ATCC 33706 (Johnson & Somkuti, 1991), *R. equi* No. 23 (Watanabe *et al.*, 1989) and *R. erythropolis* IMET 7185 (Atrat *et al.*, 1992). The *R. equi* No. 23 enzyme

Figure 1.19: The metabolism of shikimic acid by *R. rhodochrous* N75

(Bruce & Cain, 1990)

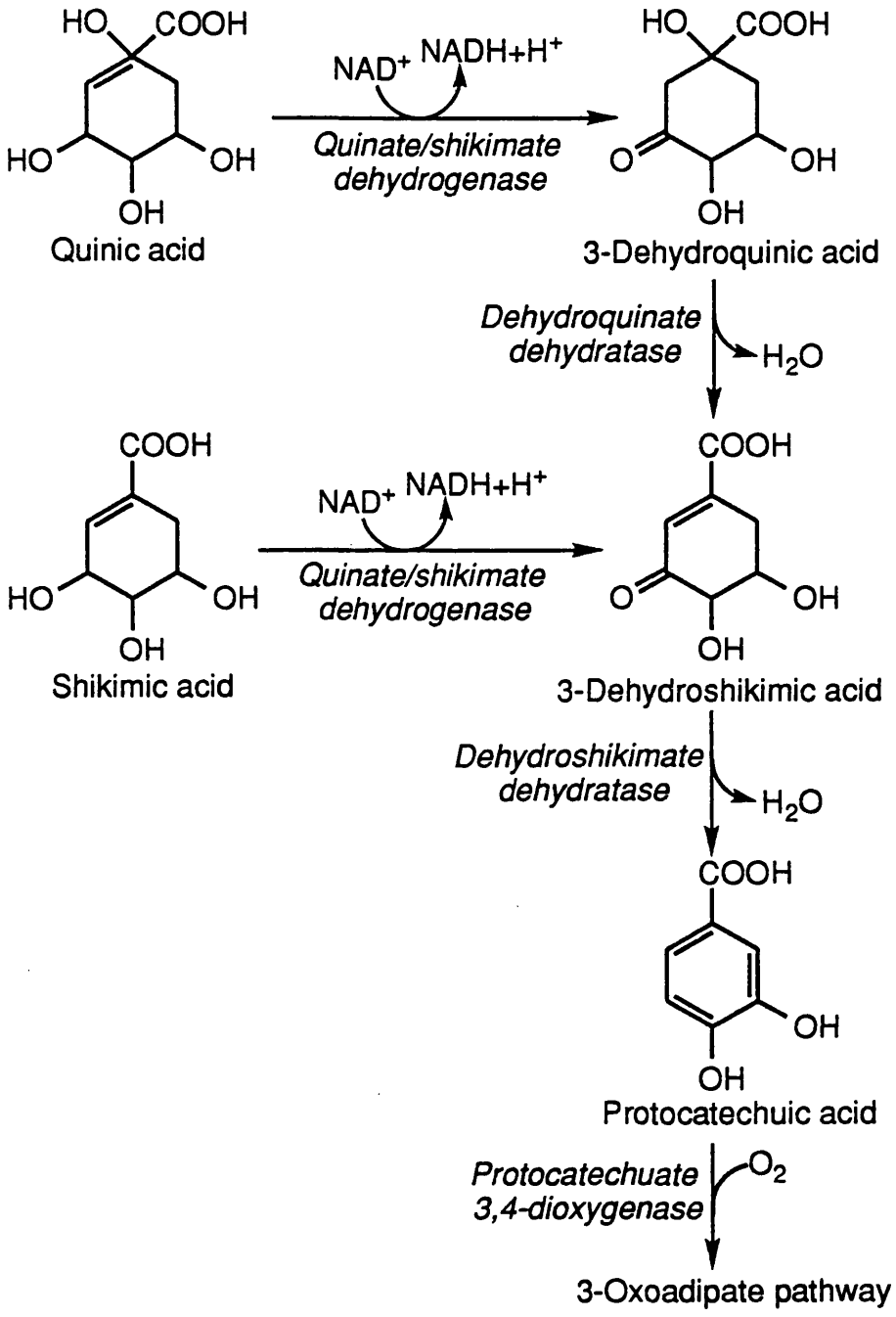
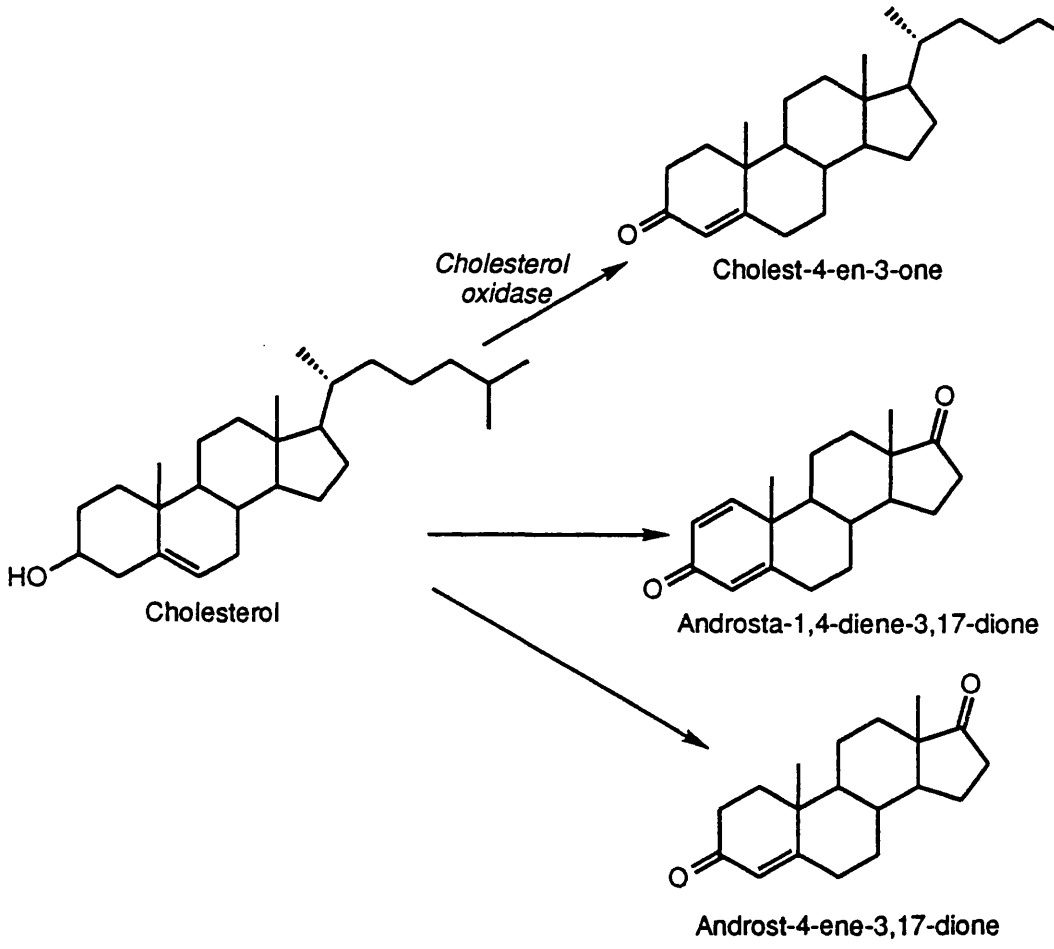


Figure 1.20: Some biotransformations of cholesterol catalysed by *Rhodococcus* spp.

(Ahmad *et al.*, 1991; Atrat *et al.*, 1992)



was purified from culture medium, and had a Mr of 56 k. Both the extracellular and membrane-bound enzymes from *R. equi* ATCC 33706 were purified, and had slightly different pH optima, though they may be two forms of the same enzyme. The enzyme from *R. erythropolis* IMET 7185 had a Mr of 62 k, and immunoelectron microscopy showed that it was present in two locations: an extractable component located at up to 80 nm above the cell surface in a surface layer which also contained a lot of carbohydrate, and a non-extractable component, some of which was closer to the cell wall, the rest being within the cytoplasmic membranes and peripheral cytoplasm (Atrat *et al.*, 1992).

The oxidation of cholesterol by cells of *R. erythropolis* ATCC 25544 has been investigated in a liposomal medium, with the aim of optimising bioconversion to cholestenone (Goetschel *et al.*, 1992). In addition, cells of *R. erythropolis* IMET 7185 have been used in the construction of a membrane electrode for the detection of cholesterol (Wollenberger *et al.*, 1980).

Another steroid metabolising enzyme that has been characterised in *R. erythropolis*, in this case strain IMET 7030, is an inducible, 56 k, steroid-1-dehydrogenase which has FAD as cofactor (Kaufmann *et al.*, 1992). Immunogold labelling has shown that this enzyme is mainly present in the peripheral region of the cytoplasm, the inner side of the cytoplasmic membrane and in channels connecting the cytoplasm with the cell surface (Wagner *et al.*, 1992a). This protein has been over expressed in *E. coli*, but was not found to be active, with most of it being present in inclusion bodies (Wagner *et al.*, 1992b).

R. equi DSM 89-133 has been found to modify cholesterol and other sterols to androst-4-ene-3,17-dione and androsta-1,4-diene-3,17-dione (Figure 1.20; Ahmad *et al.*, 1991). Miclo & Germain (1992) have postulated a pathway for the breakdown of androst-4-ene-3,17-dione to 3aα-H-4α(3'α-hydroxy-3'-propionic acid)-5α-hydroxy-7aβ-methylhexahydro-1-indanone by *R. equi* ATCC 14887. Further research on this pathway should explain the mechanism of cholesterol breakdown in *R. equi*.

Other steroid transformations by *Rhodococcus* sp. have been recorded, for example the transformation of 3β-hydroxy-5α-H-pregnanes into their Δ^4-3-keto-α-α-hydroxy derivatives (Voishvillo *et al.*, 1992).

1.1.8 Growth on other miscellaneous compounds

a) Dioxane and tetrahydrofuran

Bernhardt & Diekmann (1991) isolated several *Rhodococcus* strains which could grow on dioxane and tetrahydrofuran. From growth studies a pathway was proposed (Figure 1.21a) for the breakdown of tetrahydrofuran and butan-1,4-diol.

b) 1,8-Cineole

Rhodococcus sp. strain C1 was isolated by Williams *et al.* (1989) on 1,8-cineole as sole carbon and energy source. The pathway in Figure 1.21b was proposed for the metabolism of this substrate, based on the transient accumulation of 6-*endo*-hydroxycineole and 6-oxocineole in growth media, simultaneous induction experiments and enzymological investigations in extracts. The 6-*endo*-hydroxycineole dehydrogenase and 6-oxocineole oxygenase activities were measured in extracts. No transformation of 1,8-cineole could be detected in extracts, probably because of the instability of the initial oxygenase.

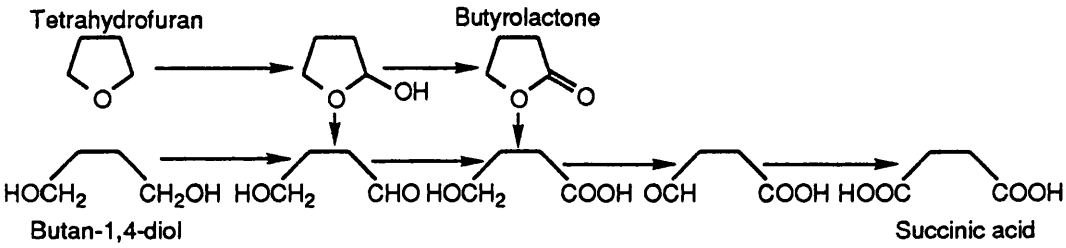
c) Alicyclic hydrocarbons

A *Rhodococcus* sp. was identified as a member of a three-organism consortium which was able to grow on a wide range of alicyclic hydrocarbons including methylcyclohexane, though its role was not determined (Lloyd-Jones & Trudgill, 1989)

Shipston *et al.* (1992) found that whole cells of *R. coprophilus* and *R. fascians*, strains isolated on cyclohexanol, were able to transform bicyclo(3.2.0)hept-2-en-6-one with a cyclohexanone monooxygenase activity. The (+) enantiomer of this substrate was converted to (–)-1(S),5(R)-2-oxabicyclo(3.3.0)oct-6-en-3-one with high enantiomeric excess, and the (–) enantiomer was converted to (–)-1(R),5(S)-3-oxabicyclo(3.3.0)oct-6-en-2-one, also with high enantiomeric excess (Figure 1.22a).

Figure 1.21: Metabolism of tetrahydrofuran and 1,8-cineole by *Rhodococcus* spp.

a) Hypothetical catabolic pathway for C_4 substrates in *Rhodococcus* sp. strain 219 (Bernhardt & Diekmann, 1991)



b) Metabolism of 1,8-cineole by *Rhodococcus* C1 (Williams *et al.*, 1989)

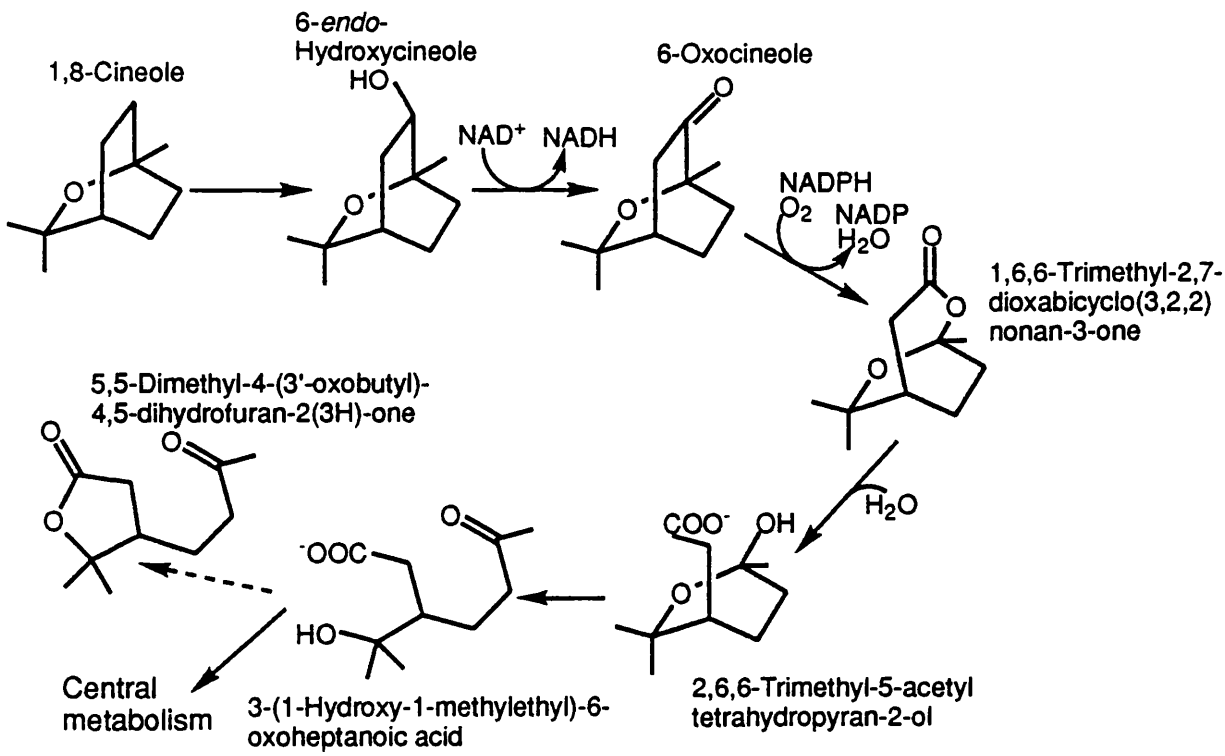
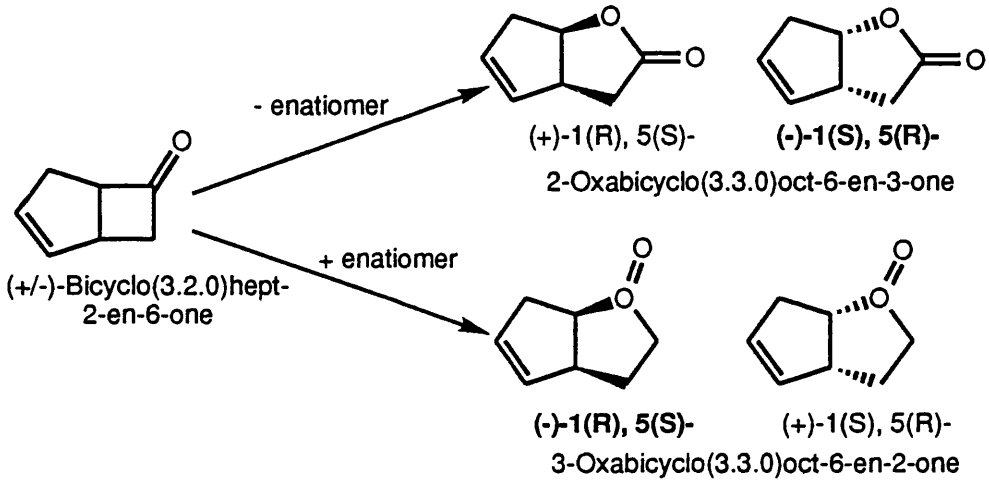
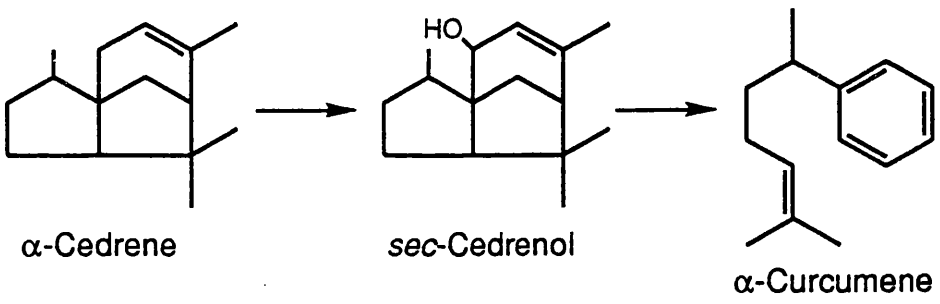


Figure 1.22: Metabolism of bicyclo(3.2.0)hept-2-en-6-one, α-cedrene and atrazine by *Rhodococcus* spp.

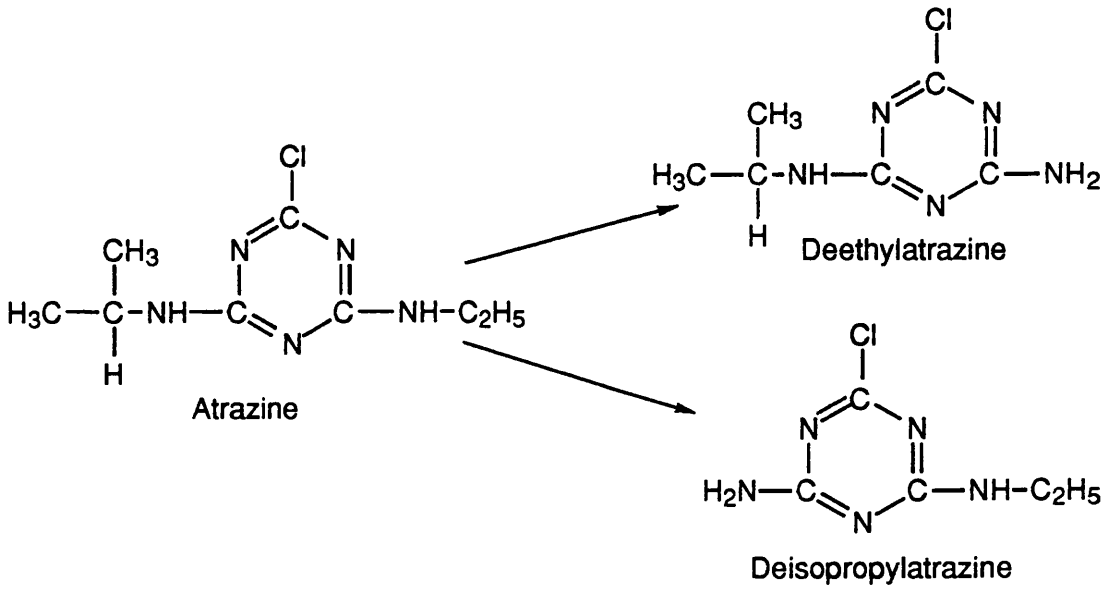
a) The oxidation of bicyclo(3.2.0)hept-2-en-6-one by *R. coprophilus* and *R. fascians* (Shipston *et al.*, 1992)



b) Oxidation of α-cedrene to *sec*-cedrenol by *Rhodococcus* strain KSM-7358 (Takigawa *et al.*, 1993)



c) Metabolism of atrazine by *Rhodococcus* strain TE1 (Behki *et al.*, 1993)



d) Cyclic sesquiterpenes

Takigawa *et al.* (1993) have isolated *Rhodococcus* sp. strain KSM-7358, which is the first identified organism to grow on cyclic sesquiterpenes such as α-cedrene and caryophyllene. When grown on α-cedrene, a component of cedar wood oil, *Rhodococcus* sp. strain KSM-7358 produces *sec*-cedrenol and α-curcumene with high yields (Figure 1.22b), though the metabolic pathway for growth on this substrate has not yet been elucidated. Takigawa *et al.* (1993) also report that *sec*-cedrenol has biological vasodilation and antihistamine activities.

e) Atrazine and *s*-ethyldipropyithiocarbamate

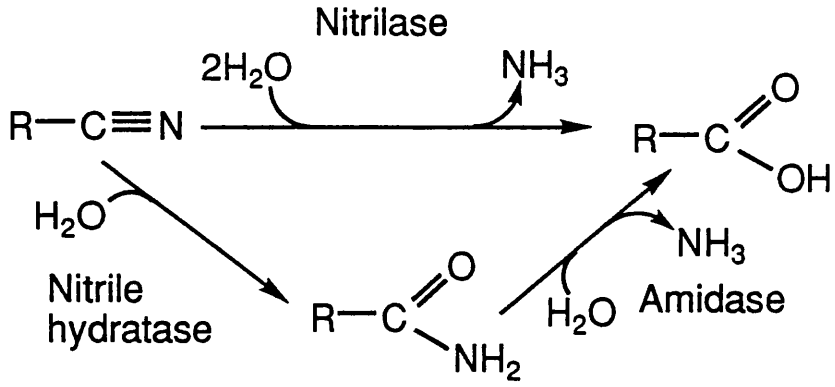
The herbicide *s*-ethyldipropylthiocarbamate (EPTC) can be utilised by several rhodococcal strains. *Rhodococcus* JE1 metabolises it through propionaldehyde and *N*-depropyl EPTC, which is further degraded to mercaptan (Chapalamadugu & Chaudry, 1992).In contrast, *Rhodococcus* TE1 cleaves EPTC at the thioester linkage, forming dipropylamine which is then further metabolised (Behki & Khan, 1990). This strain is also able to degrade another herbicide, atrazine, as are several other rhodococcal strains which are able to metabolise EPTC (Behki *et al.*, 1993). *Rhodococcus* TE1 metabolises atrazine to deethyl- and deisopropylatrazine (Figure 1.22c), though these products are not degraded further. This *N*-dealkylation activity is oxygen dependent and was found to require a 77-kb plasmid, which is also necessary for the metabolism of EPTC (Behki *et al.*, 1993).

1.1.9 Biotransformation of nitriles

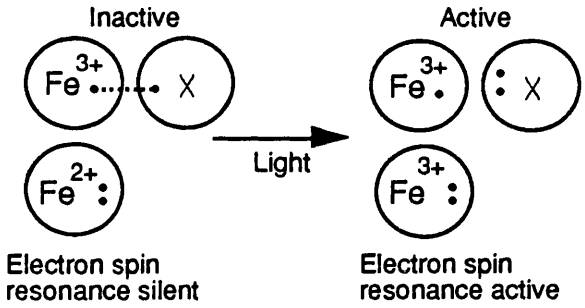
There has been much research on the nitrilase system of rhodococci because of the significant commercial applications of these enzymes (Kobayashi *et al.*, 1992a). The conversion of a nitrile group to a carboxylic acid can occur *via* two routes, either by direct deamination to the carboxylic acid or by a two stage conversion, *via* an amide (Figure 1.23). Both of these routes have been found in rhodococcal strains, and several nitrilases and nitrile hydratases have been purified and characterised (Table 1.1).

Figure 1.23: The metabolism of nitriles by *Rhodococcus* spp.

a) The nitrilase system

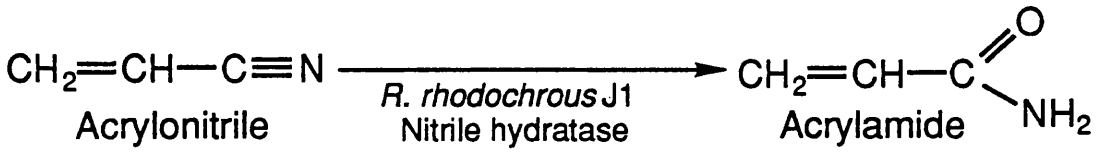


b) Hypothetical model for the photoactivation reaction of the nitrile hydratase from *Rhodococcus* sp. N-771 (Honda *et al.*, 1992)



• = Electron
X = Pyrroloquinoline quinone?

c) The production of acrylamide from acrylonitrile (Kobayashi *et al.*, 1992a)



d) The transformation of fumaronitrile to 3-cyanoacrylic acid (Bengis-Garber & Gutman, 1989)

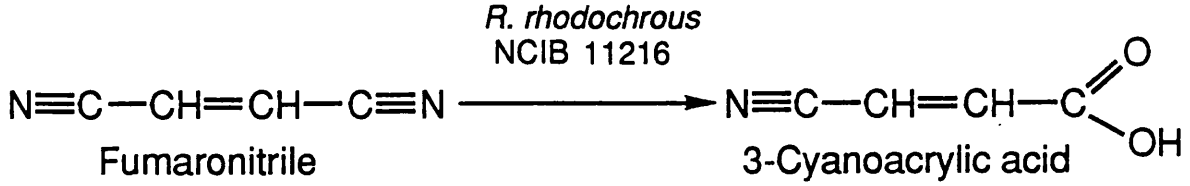


Table 1.1: Characteristics of nitrilases and nitrile hydratases isolated from *Rhodococcus* spp.

a) Nitrilases

Strain	Structure
R. rhodochrous J1 (Kobayashi *et al.*, 1989)	Native Mr = 78 k, two identical subunits
R. rhodochrous K22 (Kobayashi *et al.*, 1990, 1992b)	Subunit Mr = 41 k, native enzyme has 15 to 16 of these, so Mr = 600 - 650 k
R. rhodochrous PA34 (Bhalla *et al.*, 1992)	45 k, monomer
Rhodococcus ATCC 39484 (Stevenson *et al.*, 1992)	Subunit 40 k, in presence of substrate, forms 560 k complex

b) Nitrile hydratases

Strain	Structure
R. rhodochrous J1 High Mr (Nagasawa *et al.*, 1991)	Native Mr: 500-530 k α subunit: 26 k ß subunit: 29 k 11-12 mol cobalt/mol enzyme
R. rhodochrous J1 Low Mr (Kobayashi *et al.*, 1991)	Native Mr: 130 k α subunit: 26 k ß subunit: 29 k Contains cobalt
Rhodococcus sp. N-771 (Nagamune *et al.*, 1990; Honda *et al.*, 1992)	Native Mr: 60 k α subunit: 27.5 k ß subunit: 28 k Two atoms of iron/enzyme Pyrroloquinoline quinone present Photoresponsive
Rhodococcus sp. N-774 (Endo & Watanabe, 1989; Ikehata *et al.*, 1989)	Native Mr: 70 k α subunit: 28.5 k ß subunit: 29 k Contains iron and pyrroloquinoline quinone Photoresponsive
Rhodococcus sp. (Hjort *et al.*, 1990)	Native Mr: 52 k Two subunits, 26 and 23 k

Four nitrilases have so far been characterised, and though the native enzymes are of varying Mr, they are all made up of subunits of similar size, 39-45 k (Table 1.1a). In *R. rhodochrous* J1 cells induced by isovaleronitrile, up to 30 % of the soluble protein was found to be nitrilase (Kobayashi *et al.*, 1989). None of these enzymes has any associated metal ions. Thiol reagents caused inhibition of the enzymes from *R. rhodochrous* J1 and PA34 (Kobayashi *et al.*, 1989; Bhalla *et al.*, 1992) and reductants such as dithiothreitol were found to stimulate the enzyme from *R. rhodochrous* J1 (Kobayashi *et al.*, 1989). The enzyme from *R. rhodochrous* K22 was found to have a single cysteine, Cys-170, and when this was replaced by Ala or Ser by site-directed mutagenesis the enzyme lost all nitrilase activity (Kobayashi *et al.*, 1992b). The nitrilase from *Rhodococcus* ATCC 39484 has two cysteines, one of which is catalytically essential (Stevenson *et al.*, 1992). These results strongly suggest that all four nitrilases have a cysteine residue at or near their active sites.

The five nitrile hydratases characterised so far show a wide range of properties, with different metal ions associated with the enzymes, though they all have two dissimilar subunits (Table 1.1b). The two cobalt-containing hydratases from *R. rhodochrous* J1 are produced only when cobalt is present in the medium; no other transition metal ions could replace the cobalt (Kobayashi *et al.*, 1991; Nagasawa *et al.*, 1991). The nitrile hydratases from *Rhodococcus* sp. N-771 and N-774 are both iron associated and photoresponsive; they lose activity when stored in the dark as intact cells or crude extracts, and this activity can be recovered by irradiation with u.v. light. Endo & Watanabe (1989) found that the purified nitrile hydratase from *Rhodococcus* sp. N-774 was not inactivated in the dark when it was purified with n-butyrate as a stabilising agent. Nagamune *et al.* (1990) purified the enzyme from strain N-771 in its inactivated form, and were able to note a change in absorption characteristics on irradiation. Honda *et al.* (1992) found that photoreactivation of the nitrile hydratase of this strain takes less than 1 μs, and a hypothetical model for the photoactivation reaction was proposed, based on the results of Mössbauer and electron spin resonance spectra (Figure 1.23b).

It has recently been found that the amino acid sequences of the nitrile hydratases from strains N-771 and N-774 are identical (Honda *et al.*, 1992). There is a high level (approximately 40%) of amino acid sequence identity between the two *R. rhodochrous* J1 nitrile hydratases and that from *Rhodococcus* sp. N-774 (Kobayashi *et al.*, 1991).

There are many examples of nitrilase or nitrile hydratase biotransformations performed by either whole cells or purified enzymes, and one of the main advantages of using these enzymes rather than chemical methods is the specificity of the reactions. Some enzymes are already in commercial use, for example the high Mr nitrile hydratase from *R. rhodochrous* J1 is being used to produce more than 30, 000 tonnes/annum of acrylamide from acrylonitrile (Figure 1.23c; Kobayashi *et al.*, 1992a). Bengis-Garber & Gurman (1989) produced the novel compound 3-cyanoacrylic acid from fumaronitrile using whole cells of *R. rhodochrous* NCIB 11216 (Figure 1.23d); this chemical is not available through any other route, and may have important applications as a co-polymer due to its having two different functional groups (Bengis-Garber & Gutman, 1989). Whole cells of *R. rhodochrous* PA-34 have been used to hydrolyse α-aminonitriles asymmetrically in order to produce optically active amino acids, generally the L-forms (Bhalla *et al.*, 1992). Novo Industrie (Denmark), supply an immobilised nitrile hydratase catalyst obtained from a *Rhodococcus* sp., and this has been used by Klempier *et al.* (1992) for the hydrolysis of heterocyclic nitriles.

1.1.10 Other biotransformations

a) Phenylalanine dehydrogenase

A phenylalanine dehydrogenase has been purified from *R. maris* K-18, with an Mr of 70 k and two identical subunits (Misono *et al.*, 1989). NAD^+ was the natural cofactor, and the enzyme catalysed the oxidative deamination of L-phenylalanine and several other L-amino acids, and the reductive amination of phenylpyruvate and *p*-hydroxyphenylpyruvate. Kinetic and inhibition studies found that NADH bound first to the enzyme, then phenylpyruvate, then ammonia, and the products were released in the order L-phenylalanine and then NAD^+.

A similar L-phenylalanine dehydrogenase has been partially purified from *Rhodococcus* sp. M4, and this enzyme has been used in an experimental enzyme-membrane-reactor for the continuous production of L-phenylalanine (Hummel *et al.*, 1987).

b) Biodesulphurisation

One of the environmental problems caused by the burning of coal is the production of oxides of sulphur, which can pollute the surrounding area, and combine with water to form acid rain. One method of avoiding this pollution is to remove sulphur from the coal before burning, and microorganisms present possibilities for this process. *R. rhodochrous* IGTS8 is able to use dibenzothiophene (considered to be representative of a portion of organic sulphur in coal) as sole sulphur source, but not as a carbon source (Kilbane & Jackowski, 1992). The mechanism of desulphurisation of dibenzothiophene has been determined, and is shown in Figure 1.24 (Gallagher *et al.*, 1993). Growing cells metabolised dibenzothiophene to 2,2'-dihydroxybiphenyl, whereas stationary phase cells transformed it to 2-hydroxybiphenyl, and these two substrates were not metabolised further. Experiments with water-soluble material derived from coal (made by grinding it to powder then dissolving it in strong alkali) has shown that sulphur is effectively removed by the bacteria and incorporated in the biomass, suggesting that this method could be used for desulphurisation of the water-soluble portion of coal (Kilbane & Jackowski, 1992).

c) Glycosphingolipid-specific endoglycosidases

Two glycosphingolipid- specific endoglycosidases (endoglycoceramidases) have been purified from a mutant of *Rhodococcus* sp G74-2, strain M750 (Ito & Yamagata, 1989). The relative molecular masses of the two enzymes were 55.9 k and 58.9 k, and they both displayed similar properties, hydrolysing the glucosylceramide linkage of ganglio-type, lacto-type and globo-type glycosphingolipids to give intact polysaccharides and ceramides (Figure 1.25a). A third, minor species of endoglycoceramidase was also found, and this could hydrolyse the galactosylceramide linkage of galactose-type glycosphingolipids. Experiments with $H_2{}^{18}O$ showed that the

Figure 1.24: The desulphurisation of dibenzothiophene by *R. rhodochrous* IGTS8

(Gallagher *et al.*, 1993)

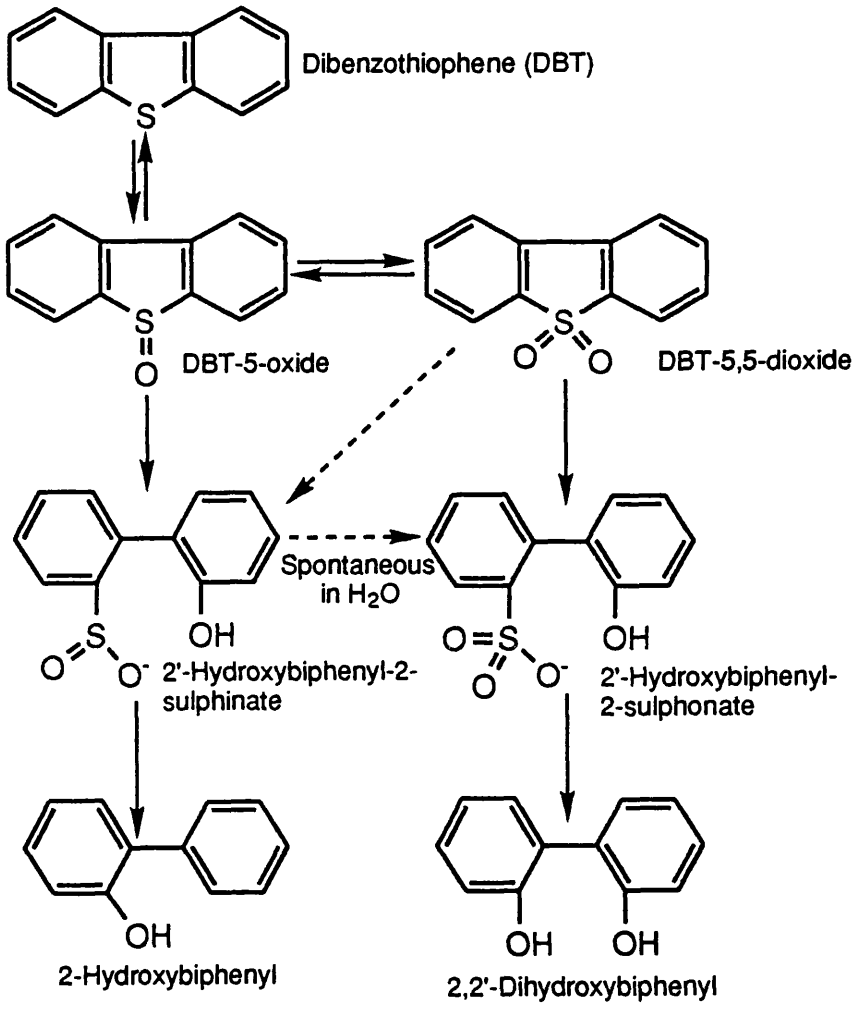
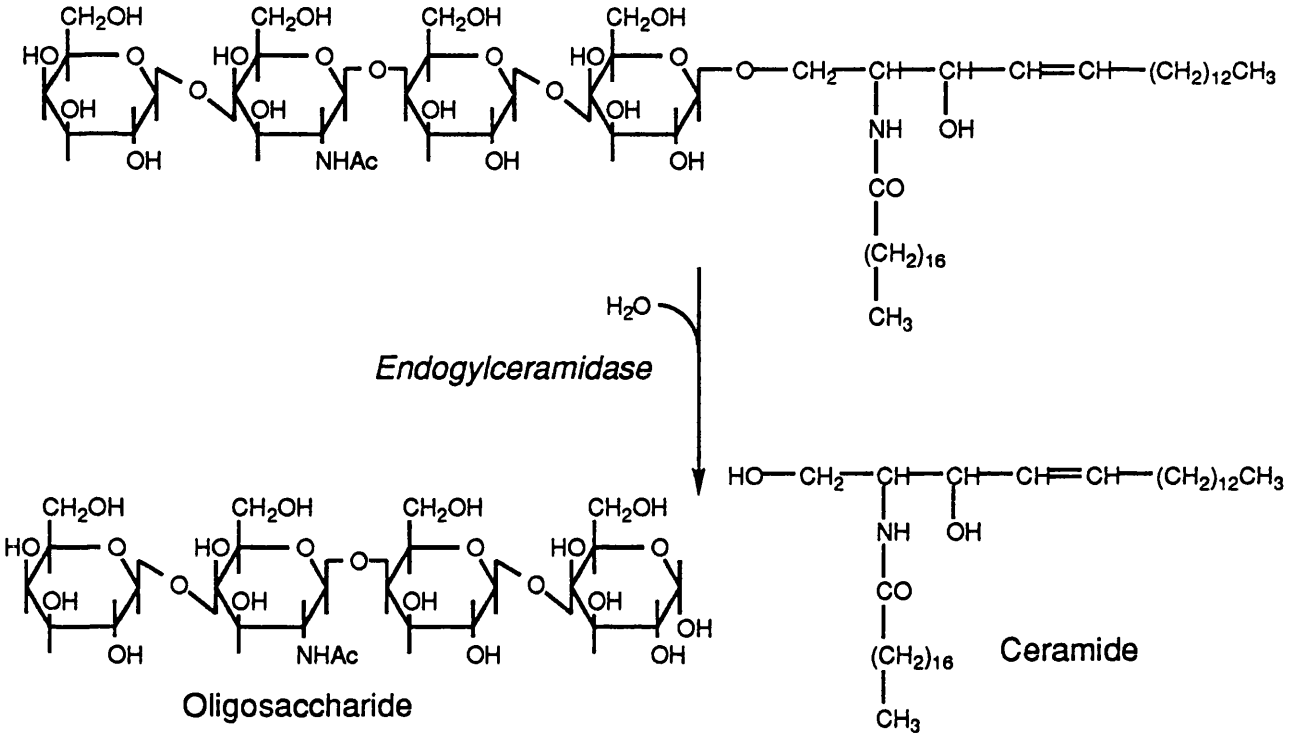
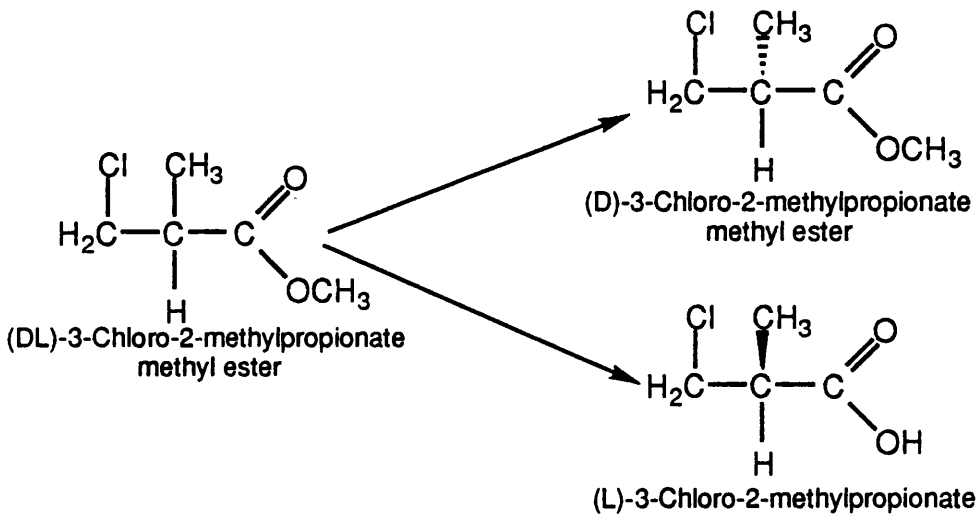


Figure 1.25: Further biotransformations catalysed by *Rhodococcus* spp.

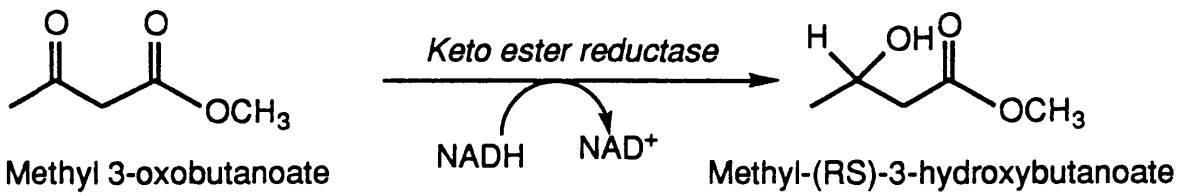
a) Endoglycoceramidase activity of *Rhodococcus* sp. M-750 (Ito & Yamagata, 1989)



b) Enantioselective resolution of methyl-3-chloro-2-methylpropionate by *Rhodococcus* sp. ME6 (Smith *et al.*, 1992)



c) Reduction of methyl 3-oxobutanoate (Peters *et al.*, 1992)



first two enzymes hydrolyse the glycosidic linkage between the oligosaccharide and ceramide.

d) Enantioselective resolution of methylesters

Smith *et al.* (1992) have isolated *Rhodococcus* sp. ME6, which is able to grow on methyl-3-chloro-2-methylpropionate. The first step of the breakdown of this substrate is the esterase-mediated transformation of methyl-3-chloro-2-methylpropionate to 3-chloro-2-methyl propionate, and it was found that the L(+) isomer of the ester was transformed at twice the rate of the D(–) isomer (Figure 1.25b). This difference in rate allows kinetic resolution to maximise the proportion of the D(–) isomer present, which is a possible precursor for the antihypertensive agent Captopril.

e) Reduction of keto esters

Peters *et al.* (1992) have investigated the keto ester reductase activity of *R. erythropolis* (Figure 1.25c). This activity has a wide substrate specificity, and is induced best by growth on *n*-alkanes. Enantiomeric purity of the product varied, probably due to the presence of several enzyme activities.

f) Accumulation of caesium

Tomioka *et al.* (1992) measured the accumulation of caesium by soil bacteria plated on agar plates using ^{137}Cs autoradiography. All 13 colonies which accumulated caesium were identified as rhodococci, and uptake of caesium in two of these strains, *R. erythropolis* CS98 and *Rhodococcus* sp. CS402, was investigated. *R. erythropolis* CS98 could remove 90 % of the caesium in a 10 μM solution in 24 hours, and could accumulate up to 52 μmol caesium/g dry weight of cells. Accumulation occurred during exponential growth, and the caesium was released as the cells passed into stationary phase.

1.1.11 Production of poly(3-hydroxyalkanoate)s

Several species of bacteria produce a poly(3-hydroxyalkanoate)s as storage compounds. *Alcaligenes eutrophus* is already being used by ICI Plc. to produce a copolyester of 3-hydroxybutyrate and 3-hydroxyvalerate, 'Biopol' (Byrom, 1987).

Biopol is a plastic material which is fully biodegradable, both in soil and within the human body, and consequently has many possible applications. *Rhodococcus ruber* NCIMB 40126 has been found to produce similar copolyesters (Haywood *et al.*, 1991). Studies on the copolyesters produced by this organism have shown that a high proportion of the polymer is made up of 3-hydroxyvalerate, even when single carbon sources such as glucose are used (Anderson *et al.*, 1992). This is in contrast to *A. eutrophus*, where two carbon sources are required to produce 3-hydroxyvalerate units, and lower amounts of 3-hydroxyvalerate are present in copolymers. It is possible that copolymers produced by *R. ruber* could be blended with those from *A. eutrophus* to provide materials with novel characteristics.

The gene encoding the poly(3-hydroxyalkanoate) synthase in *R. ruber* has been cloned and sequenced, and was found to have 38.4 % amino acid identity and 55.4 % similarity to the *A. eutrophus* synthase (Pieper & Steinbüechel, 1992).

1.1.12 Patents

According to the World Patents Index (WPI), there were 10 patent families submitted concerning rhodococci in each of 1991 and 1992. Many of these patents concern biotransformations, particularly those using the nitrilase system. There are also some patents for waste treatment, for example the bio-leaching of metal oxides from fly ash using *Rhodococcus* strain GIN-1 (WPI Acc. No: 92-285018/35, Israel Electric Corp. Ltd.), and the decomposition of isoprene rubber (WPI Acc. No: 90-128238/17, Agency of Indian Science and Technology, Fuji Latex KK). As research in this field expands it is likely that the patenting rate will increase.

1.2 Metabolism of aromatic compounds by microorganisms

1.2.1 Aromatics in nature

Aromatic chemicals are synthesised naturally in large amounts, fulfilling many purposes within and outside the cells of organisms. The commonest, and largest, natural

aromatic structure is lignin, the main structural element of trees and plants, made up of aromatic residues cross-linked together in a complex matrix . Aromatics are also involved in the most basic cell functions, for example the amino acid phenylalanine. Microorganisms have therefore been exposed to aromatic chemicals within the natural environment, well before man started manufacturing and releasing new (xenobiotic) aromatic chemicals.

1.2.2 Principle routes of breakdown

The breakdown of aromatics by microorganisms occurs under both aerobic and anaerobic conditions, though for this study only aerobic breakdown is relevant. The basic principle of aerobic bacterial aromatic breakdown is the conversion of diverse aromatic compounds into a more restricted range of, usually, dihydroxylated aromatic compounds. The aromatic ring is then cleaved with O_2, adjacent to one or both of the hydroxyl groups, using a dioxygenase enzyme. A variety of common ring cleavage substrates is shown in Figure 1.26. An aromatic compound with one or more side chain substituents can either have the substituent modified before ring cleavage, or it may be retained after ring cleavage. Anaerobic breakdown of aromatics generally proceeds through the sequential reduction (dearomatisation) and hydroxylation of the rings (Fewson, 1981); the anaerobic metabolism of styrene is given in Figure 1.37.

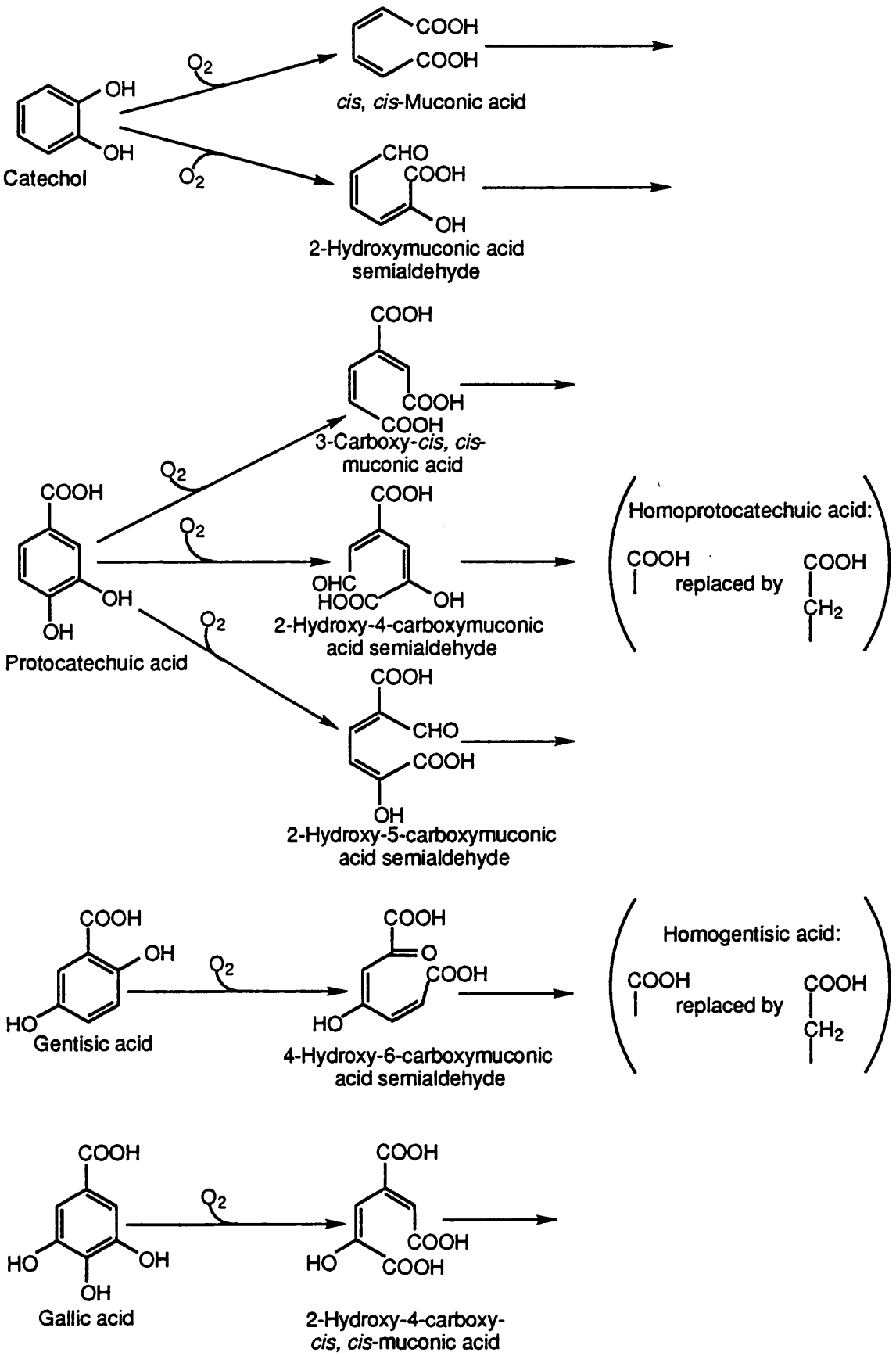
A good example of the range of the options for aerobic degradation is the breakdown of toluene, which has been investigated in a wide variety of bacteria. Toluene breakdown is also a good model for the degradation of styrene, since both are monosubstituted aromatic chemicals.

1.2.3 Toluene breakdown

The metabolism of toluene (methylbenzene) by aerobic bacteria has been shown to proceed by at least five different routes, involving different combinations of side chain modification and ring hydroxylation.

Figure 1.26: Common ring cleavage reactions

(Fewson, 1981)



a) TOL plasmid route, *via* side chain oxidation

The most extensively researched route of toluene breakdown is that coded for on the 117 kbp TOL plasmid pWW0, from *Pseudomonas putida* mt-2 (Figure 1.27). This pathway is also encoded by many other plasmids, including pWW5, pWW14, pWW74 and pWW88 (Chatfield & Williams, 1986) and in many other organisms, including *Alcaligenes eutrophus* (Burlage *et al.*, 1989). The methyl side chain of the toluene is progressively oxidised to benzoic acid, then catechol is formed by the actions of toluate oxygenase and benzoate 1,2-*cis*-diol dehydrogenase. This catechol can then be *meta*-cleaved by enzymes encoded by the plasmid, or *ortho*- cleaved by chromosomally encoded enzymes (see below).

b) Toluene dioxygenase route

An alternative route of toluene breakdown involving no modification of the side chain methyl group is the toluene dioxygenase or TOD pathway. Toluene breakdown is initiated by a dioxygenase-mediated addition of O_2 to the ring, giving toluene *cis*-glycol (Figure 1.28). This *cis*-glycol is then rearomatised to give 3-methylcatechol. The catechol is usually cleaved and metabolised by the *meta*- pathway, though some organisms can use the *ortho*- pathway (below). This breakdown route has been extensively studied in *Pseudomonas putida* F1 (Rogers & Gibson, 1977; Yeh *et al.*, 1977; Subramanian *et al.*, 1985) but is also found in a variety of other organisms, including a thermotolerant *Bacillus* species (Simpson *et al.*, 1987).

c) Monooxygenase routes

There are three possible positions for a monooxygenase attack on the aromatic ring of toluene, and organisms have been isolated which use all these routes. In one of these organisms, side chain attack also occurs, whereas in the other two the methyl group is retained (Figure 1.29).

The toluene-2-monooxygenase in *P. cepacia* G4 attacks the aromatic ring to produce *o*-cresol, and the same enzyme has also been found to catabolise trichloroethylene (Shields *et al.*, 1989, 1991). The ring is then hydroxylated by another monooxygenation, to give 3-methylcatechol, which is then metabolised by *meta*-

Figure 1.27: The metabolism of toluene encoded by the TOL plasmid pWW0 from *P. putida* mt-2

(Burlage *et al.*, 1989)

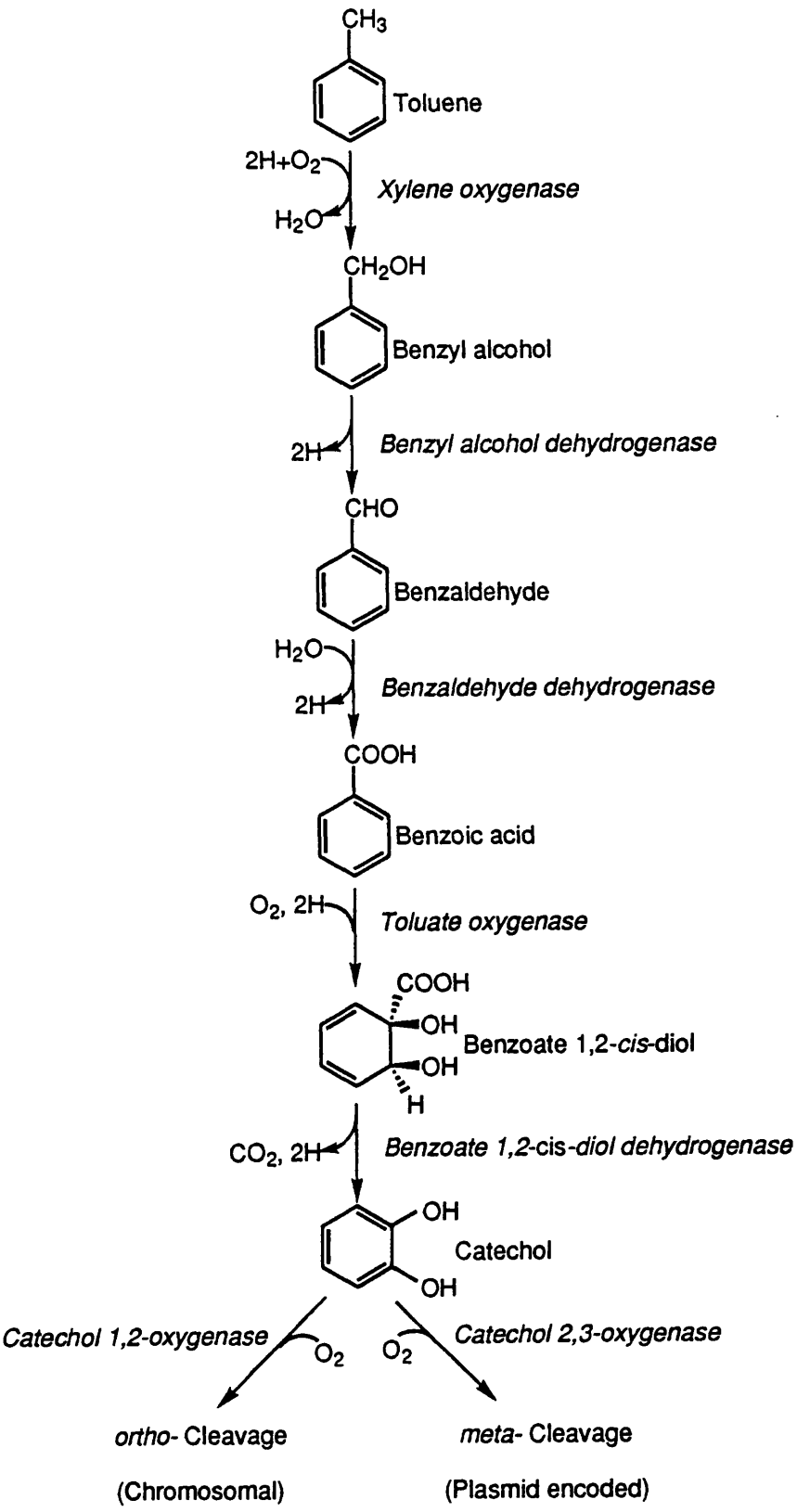


Figure 1.28: The breakdown of toluene *via* toluene *cis*-glycol

(Subramanian *et al.*, 1985)

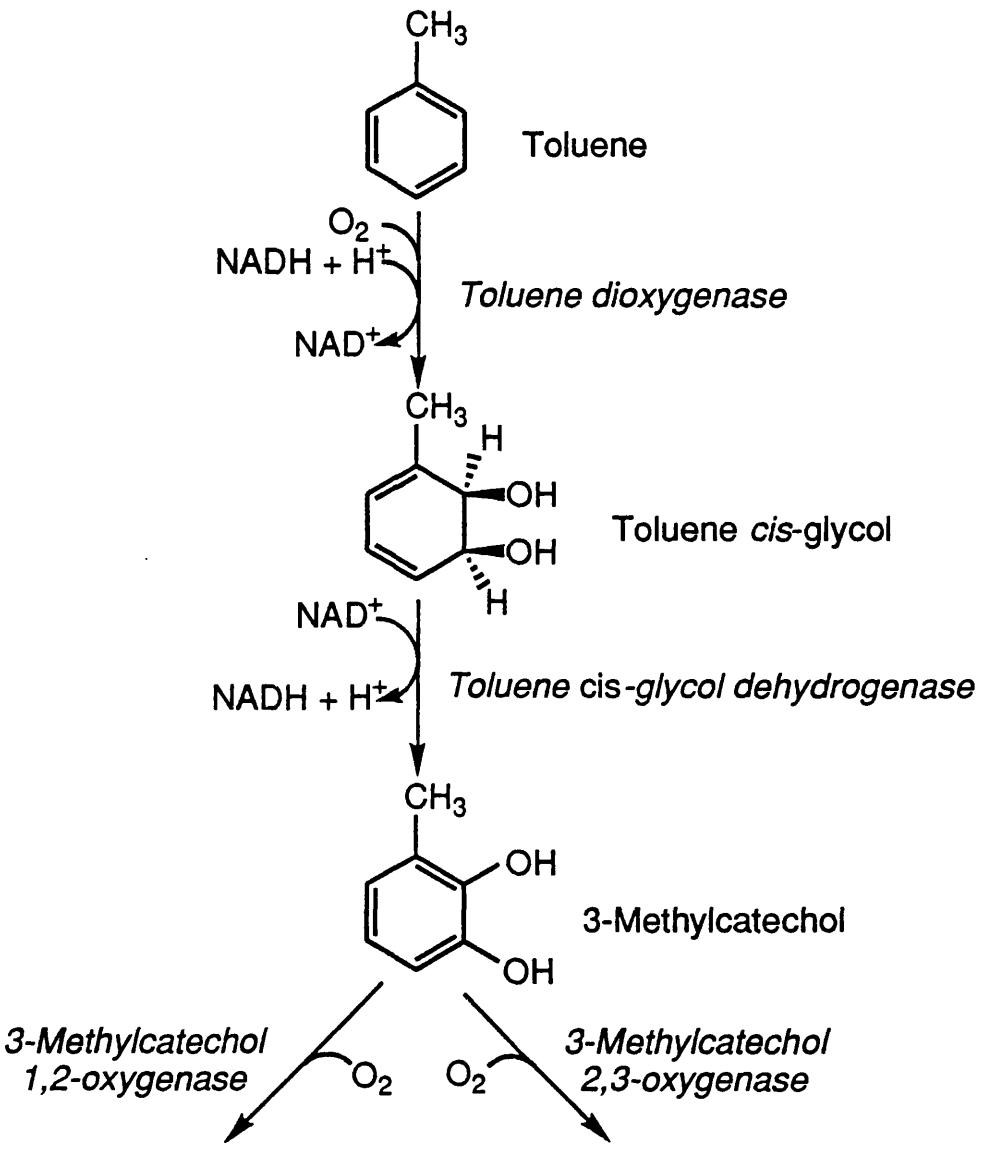
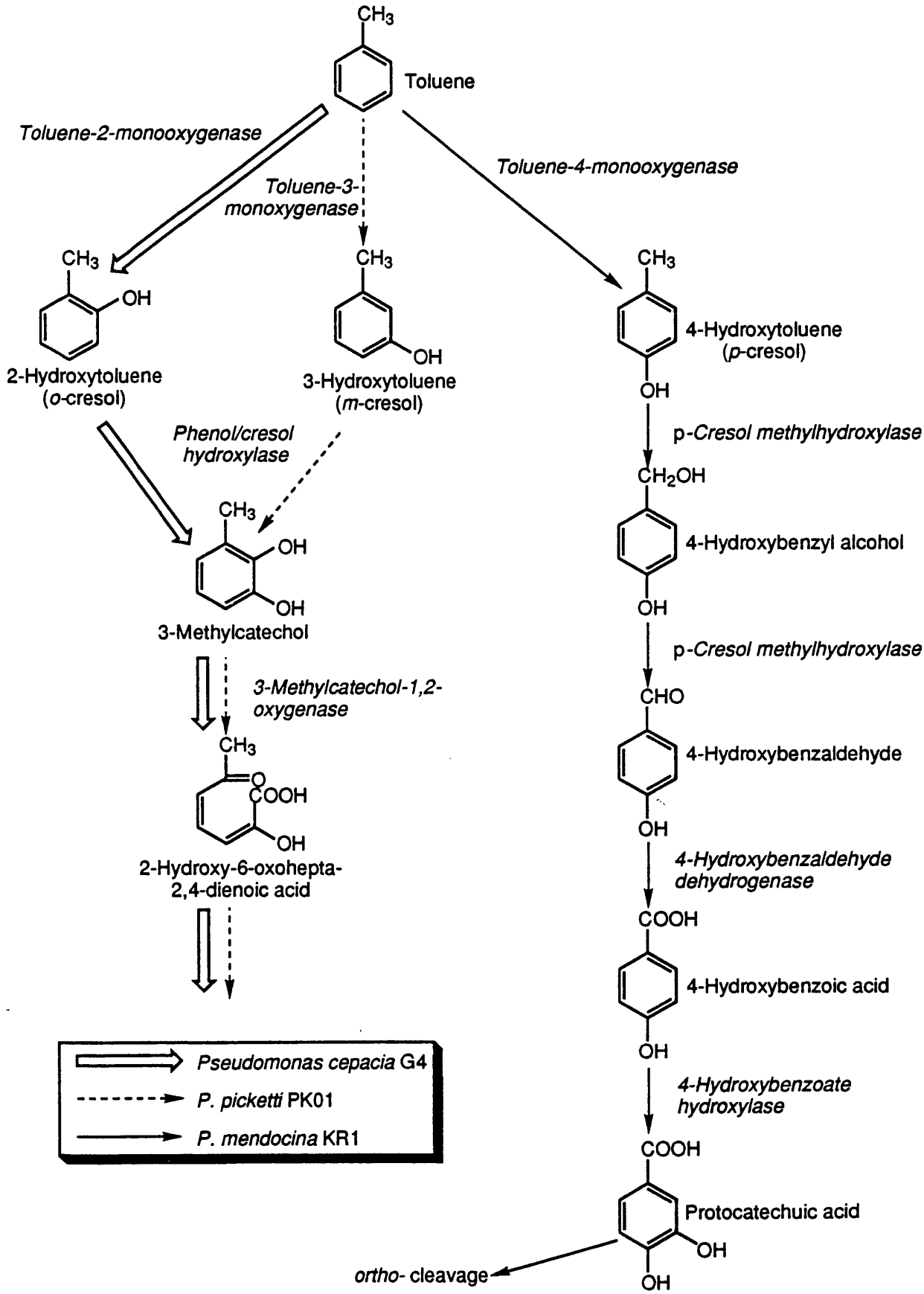


Figure 1.29: The breakdown of toluene *via* initial ring monohydroxylation

(Shields *et al.*, 1989; Kukor & Olsen, 1991; Whited & Gibson, 1991)



cleavage. A similar pathway is used by *P. picketti* PK01, though in this organism the initial attack is on the 3-position of the aromatic ring (Kukor & Olsen, 1991).

In contrast, *P. mendocina* KR1 oxidises the methyl group of the toluene before ring cleavage, but after the first ring monooxygenation, which is at the 4-position (Whited & Gibson, 1991; Yen *et al.*, 1991; Yen & Karl, 1992). The ring-cleavage substrate in this organism is protocatechuic acid, which is oxidised by *ortho*- ring cleavage (below).

d) Metabolism of catechol, 3-methylcatechol and protocatechuic acid by *ortho*- routes

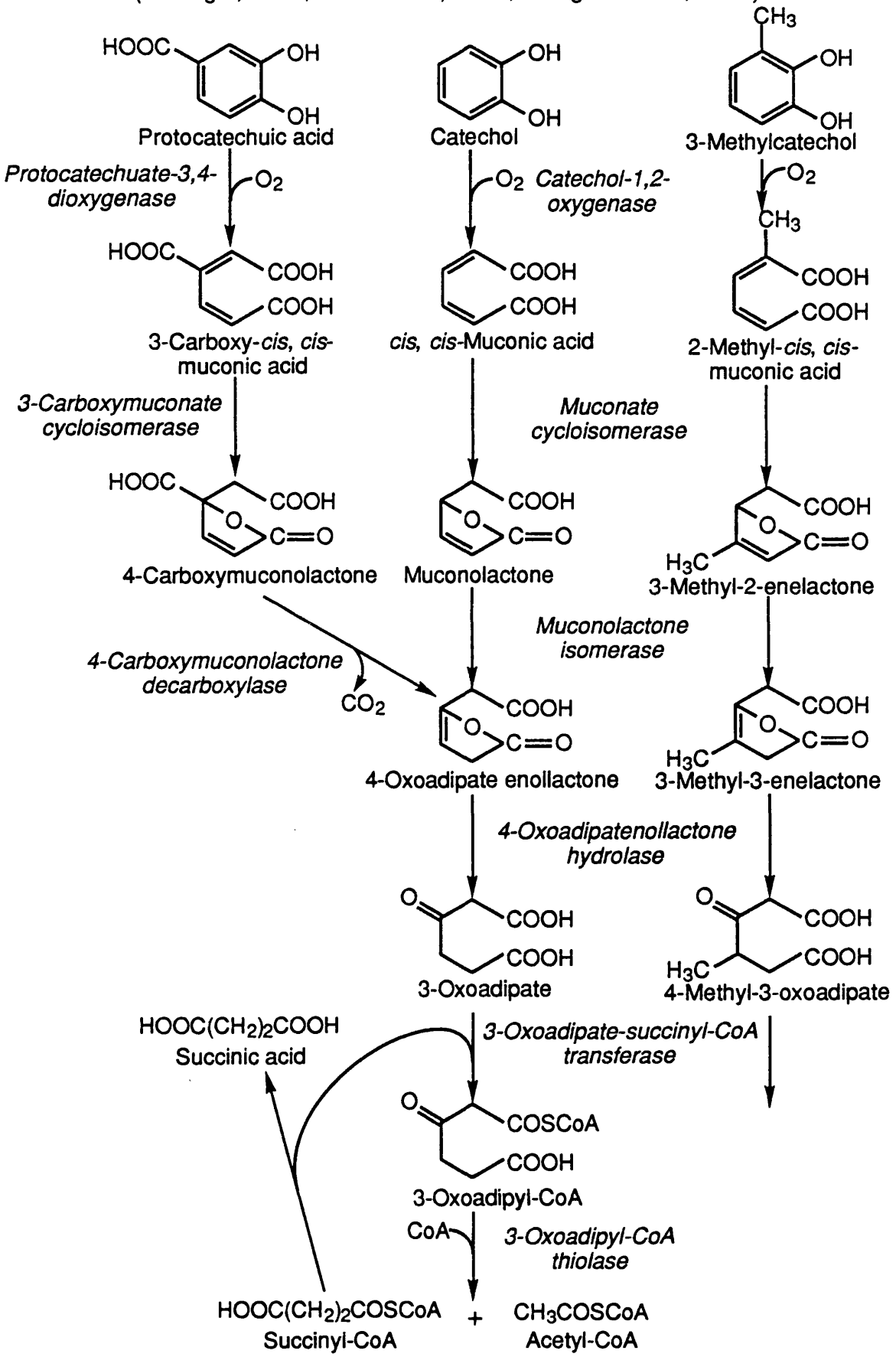
The metabolism of catechol and protocatechuate through *ortho*- (intra-diol) cleavage is shown in Figure 1.30. Many of the investigations into these pathways have been done in pseudomonads, but other genera have been found to use the same route (Schlegel, 1986).

Although 3-methylcatechol is usually metabolised by catechol 2,3-oxygenase and the *meta*- cleavage pathway, it can also be degraded through *ortho*- cleavage (Figure 1.30). The pathway illustrated is that proposed by Bruce *et al.* (1989), for the breakdown of 4-methylcatechol through the modified *ortho*- pathway in *Rhodococcus rhodochrous* N75, a pathway which isomerises the 4-methylcatechol metabolite 4-methyl-2-enelactone to the equivalent 3-methylcatechol metabolite 3-methyl-2-enelactone (see Figure 1.6).

Other organisms have been found to degrade 3-methylcatechol through *ortho*-cleavage. Pettigrew *et al.* (1991) isolated a mutant of *Pseudomonas* sp. strain JS6, strain JS62, which was deficient in catechol 2,3-oxygenase activity, but was still able to grow on toluene *via* 3-methylcatechol and *ortho*- cleavage. The transient metabolite 2-methyllactone was identified in the medium of this mutant when grown on toluene. A similar transient intermediate, 2-methyldienelactone, was isolated from the original organism, strain JS6, when it was grown on *p*-chlorotoluene (Haigler & Spain, 1989). In this case the lactone was transformed into 5-methyl-3-oxoadipate. Another mutant deficient in catechol 2,3-oxygenase activity, *Rhodococcus rhodochrous* CTM2 (see

Figure 1.30: The breakdown of catechol, 3-methylcatechol and protocatechuic acid by *ortho*-ring cleavage

(Schlegel, 1986; Bruce *et al.*, 1989; Pettigrew *et al.*, 1991)



section 1.1.7c) can also degrade 3-methylcatechol through an *ortho-* cleavage pathway (Fuchs *et al.*, 1991).

e) Metabolism of catechol and 3-methylcatechol by *meta-* routes

The *meta-* (extra-diol) cleavage pathway (Figure 1.31) is the other main pathway of catechol breakdown. It is present in many bacteria, for example it is encoded by the *P. putida* mt-2 TOL plasmid pWW0 (Burlage *et al.*, 1989), and by the chromosome of *P. picketti* PK01 (Kukor & Olsen, 1991). The 2-hydroxymuconic semialdehyde hydrolase route is the usual method of metabolising 3-substituted catechols, whereas 4-substituted catechols are usually transformed through the oxalocrotonate route. Protocatechuic acid can also be metabolised by a *meta-* route, following cleavage by either protocatechuate 2,3-oxygenase or protocatechuate 4,5-oxygenase (cleavage products in Figure 1.26).

1.3 Metabolism of styrene

Styrene (phenylethene, phenylethylene, vinylbenzene) is a major industrial chemical, with many uses, such as the manufacture of polystyrene (Figure 1.32a), plastics and styrene-butadiene rubbers. It is one of the most important aromatic chemicals produced by industry, with 3.64×10^9 kg manufactured in 1990 in the United States alone (Fu & Alexander, 1992). The release of man-made styrene into the environment can occur by a variety of routes, include factory wastewater, evaporation and the pyrolysis of polystyrene; at 500 °C about 6 mg of styrene are released for every 100 mg of polystyrene decomposed (Snyder, 1984).

Styrene can also be produced naturally , and styrenes are found in wine, beer, soya sauce and blueberries (Arfmann & Abraham, 1989). Naturally, styrene is usually produced by the decarboxylation of cinnamic acid (Figure 1.32b), a common plant acid (Herzog & Ripke, 1908). Cinnamic acid is also used as an anti-bacterial agent in food products, which has led to cases of food becoming contaminated with styrene, due to the activity of yeasts such as *Pichia carsonii* (Shimada *et al.*, 1992). After growth on

Figure 1.31: The breakdown of catechol and 3-methylcatechol by *meta*- ring cleavage

(Burlage *et al.*, 1989; Harayama *et al.*, 1989; Kukor & Olsen, 1991)

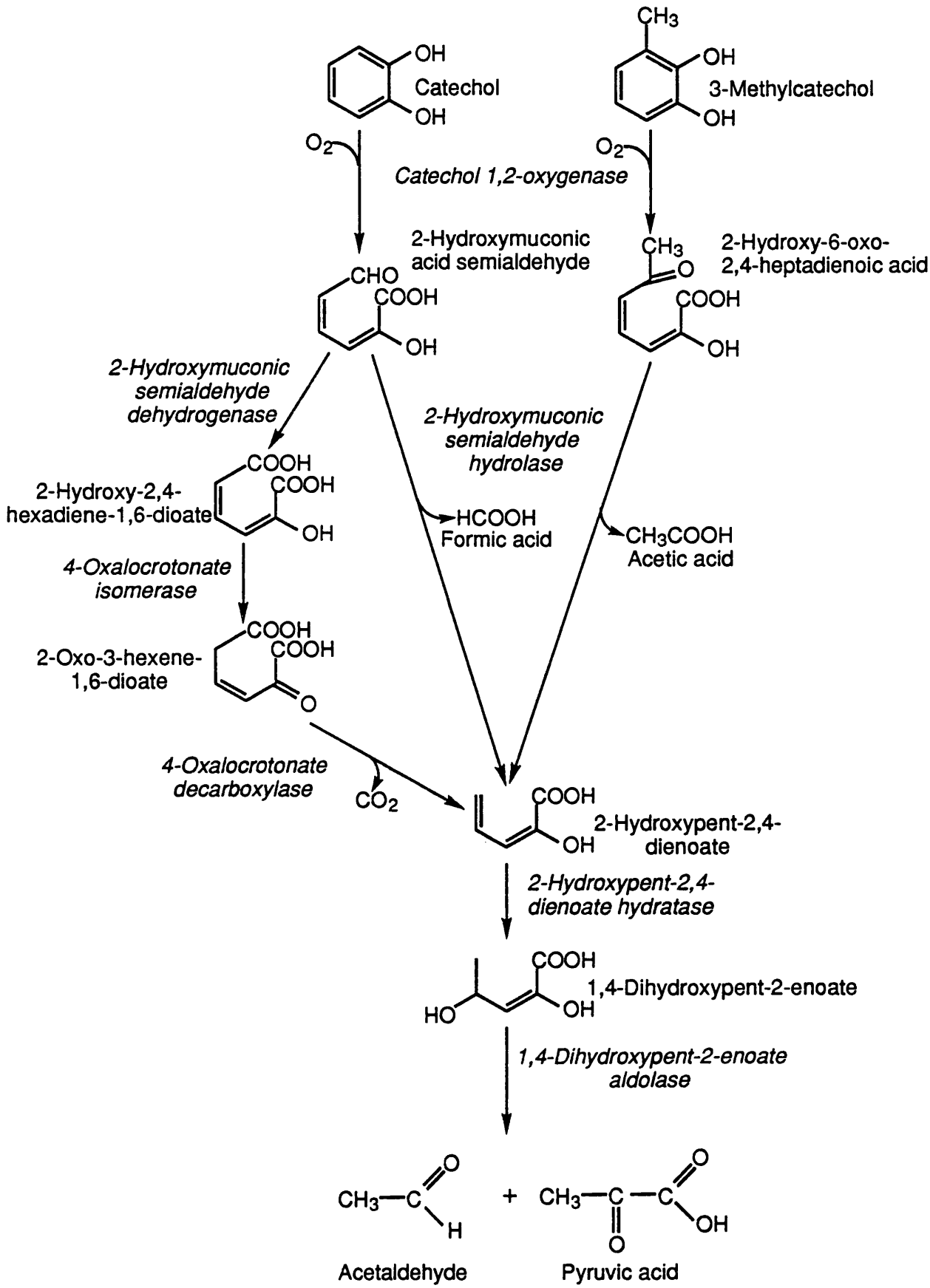
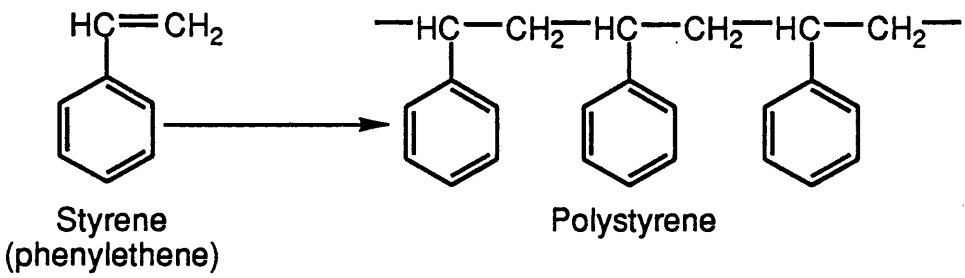
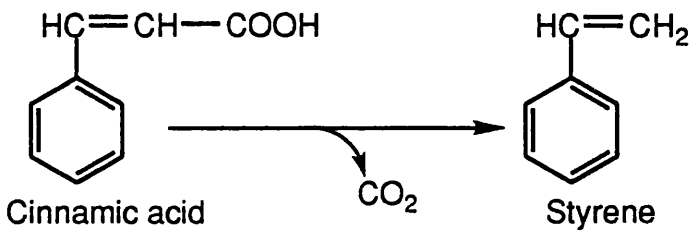


Figure 1.32: Uses and production of styrene

a) The production of polystyrene from styrene



b) The decarboxylation of cinnamic acid to produce styrene (Shimada *et al.*, 1992)



glucose, once the glucose is exhausted, *Penicillium caseicolum* synthesises styrene; the route of synthesis is not known (Spinnler *et al.*, 1992).

Styrene (Mr = 104.14) is a colourless, volatile, strong smelling, aromatic compound which is slightly soluble in water. It is toxic in fairly low quantities, mainly due to membrane effects (Bond, 1989). In humans, high levels of styrene vapour irritate the eyes, nose and respiratory tract, and also have effects on the nervous system, liver, lungs and kidneys (Fielder & Lowing, 1981). The UK occupational exposure limit (OEL) is 50 ppm over an 8 hour working day. The smell is obvious at less than 0.5 ppm.

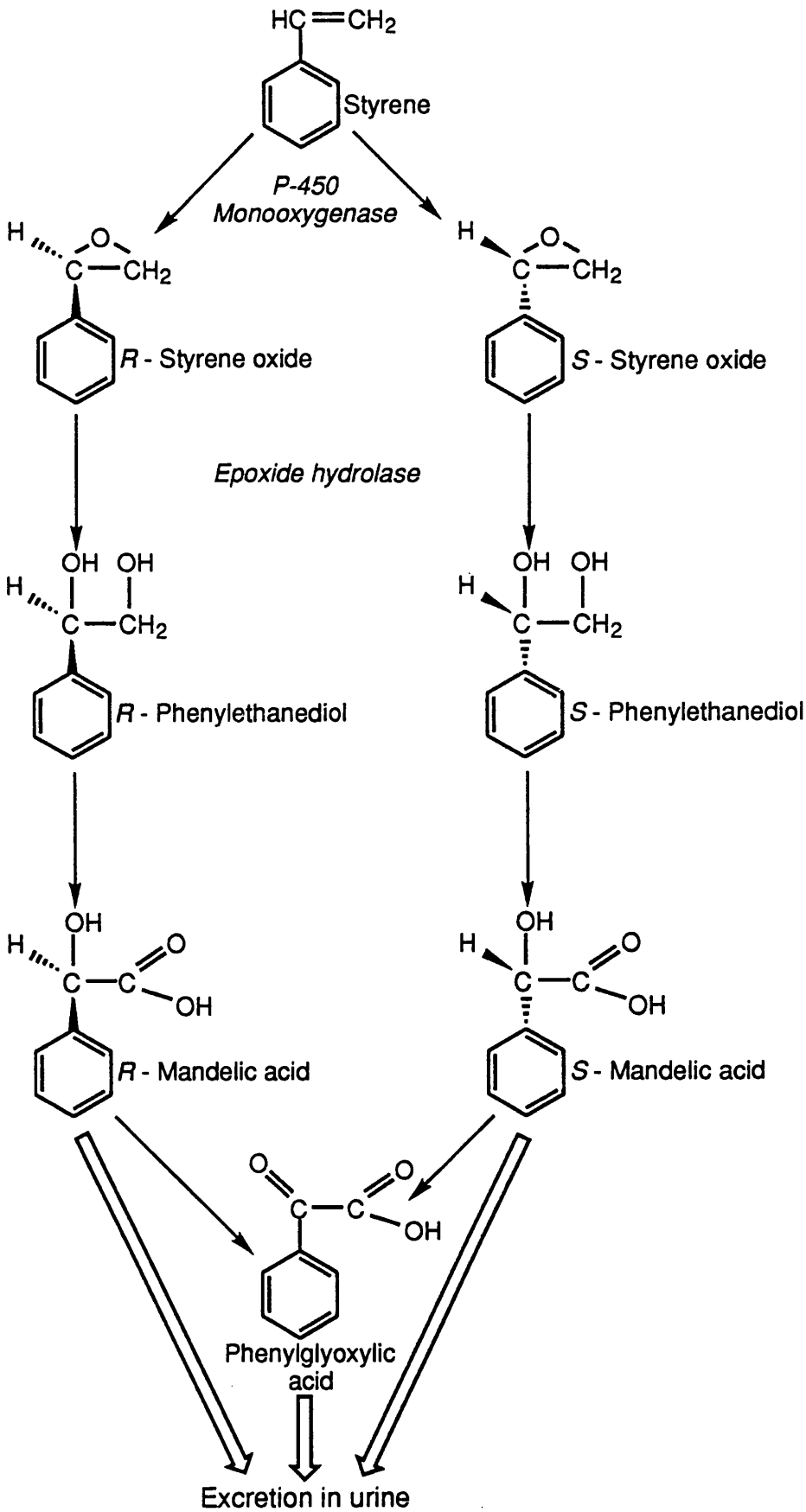
1.3.1 Mammalian metabolism of styrene

In the mammalian liver styrene is oxidised by cytochrome P-450 mixed function oxidases to styrene oxide, which is hydrated to phenylethanediol, then oxidised to mandelic acid and phenylglyoxylic acid, which are excreted in the urine(Figure 1.33, Korn *et al.*, 1984). A variety of other detoxification procedures also occur, for example conjugation of glutathione to styrene oxide (Elovaara *et al.*, 1991). Styrene oxide is a known carcinogen, and the *R*- enantiomer has been shown to be more mutagenic to *Salmonella typhimurium* strain TA100 than the *S*- enantiomer (Foureman *et al.*, 1989), so the stereospecificity of styrene breakdown has toxicological implications. Foureman *et al.* (1989) investigated the stereospecificity of rat hepatic microsomes and isolated rat liver cytochrome P-450s in their attack on styrene. They found that microsomes from rats exposed to styrene produced more *S*- styrene oxide, but if rats had been pre-treated with β-naphthoflavone, more of the *R*- enantiomer was formed. Some of the cytochrome P-450 isozymes purified from the rat liver showed no enantiomeric specificity, whereas others preferentially produced either *R*- or *S*- styrene oxide. Similar variations in cytochrome P-450 isozymes have been found in rabbit pulmonary cells (Harris *et al.*, 1986).

Cytochrome P-450$_{cam}$, a cytosolic enzyme from *Pseudomonas putida* that catalyses the 5-hydroxylation of camphor, can also produce styrene oxide from styrene (Fruetel *et*

Figure 1.33: The metabolism of styrene in the human liver

(Watabe *et al.*, 1981; Korn *et al.*, 1984)



al., 1992). The styrene oxide produced is predominantly in the *S*- form, by a ratio of approximately five to one.

The epoxide hydrolase activity of rat liver microsomes has also been shown to be stereoselective, with *S*-styrene oxide hydrated at four times the rate of *R*-styrene oxide (Watabe *et al.*, 1981).

Exposure of humans to styrene is monitored by measurement of the concentrations of mandelic acid and phenylglyoxylic acid in the urine (Sollenberg *et al.*, 1988). The mandelic acid excreted in human urine is usually virtually racemic, with just a small excess of the *R*- form (Drummond *et al.*, 1989). Exposure to other chemicals can have substantial effects on both the enantiomeric specificity and the rate of styrene breakdown. Elovaara *et al.* (1991) showed that the *R/S* ratio of mandelic acid isomers in the urine of rats exposed to styrene varied between two in rats exposed to only styrene and four in rats pretreated with phenobarbital. Wilson *et al.* (1983) found that intake of alcohol could delay peak mandelic acid excretion in workers exposed to styrene by up to three hours.

1.3.2 The fate of styrene in the environment

Large quantities of styrene are released into the environment during its production and use (Fu & Alexander, 1992). Because of the volatility of styrene, much of this is either released as vapour or vaporises after release. The fate of styrene in the environment was studied by Fu & Alexander (1992), who found that the extent of volatilisation varied in different environments, with 50 % being lost within three hours in shallow layers of lake water, compared with only 26 % lost from a 1.5 cm soil depth in 31 days. They also detected rapid mineralisation of the styrene in sewage, neutral mineral soil (pH 7.23) and organic soil, with lower rates in groundwater and lake water, and the lowest rates were recorded in aquifer sand, waterlogged soil and acidic mineral soil (pH 4.87). There was no mineralisation in sterile soil, and though a high percentage of the styrene was sorbed to the soil, this had no effect on degradation of the styrene. The percentage mineralised per hour decreased with decreasing styrene concentration in

the lake water and aquifer sand, suggesting that a threshold concentration for mineralisation may exist in these environments. In the other soils, the rate of mineralisation was directly proportional to concentration at concentrations of styrene below 1.0 mg/kg, though it was non-linear at concentrations above this, suggesting that there may be no threshhold concentration in these conditions. The lack of any mineralisation in sterile soil showed that styrene breakdown was undertaken by microorganisms.

1.3.3 Microbial metabolism of styrene

Initial attempts to isolate organisms that could grow on styrene were unsuccessful, probably because the concentrations of styrene used for the isolation were too high, for example 0.1 M styrene used by Sielicki *et al.* (1978b). More recent research has shown that it is possible to isolate large numbers of styrene utilising organisms from soil if concentrations are kept low; for example Hartmans *et al.* (1990) isolated 14 strains of aerobic bacteria and two fungal strains from normal soils using a styrene concentration of about 0.5 mM. High concentrations of styrene are not, however, invariably toxic to microorganisms, since Inoue & Horikoshi (1989) have isolated *Pseudomonas putida* IH-200 which can grow on glucose in the presence of 50 % styrene. Most research into styrene metabolism has focused on aerobic bacteria, with no research reported on fungi and little on anaerobic metabolism.

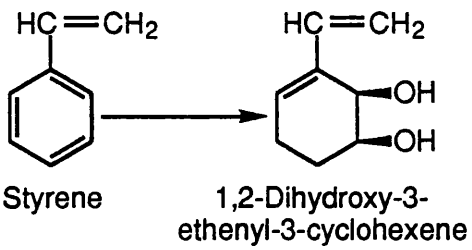
a) Aerobic breakdown

Aerobic breakdown of styrene must initially proceed *via* an attack on either the aromatic ring or on the side chain. No pathway involving initial ring attack has yet been proven, though (–)-1,2-dihydroxy-3-ethenyl-3-cyclohexene (Figure 1.34a) has been isolated from the medium of *P. putida* MST growing on styrene (Bestetti *et al.*, 1989). A ring attack route has also been suggested in other studies, for example in *Xanthobacter* strain 124X (below).

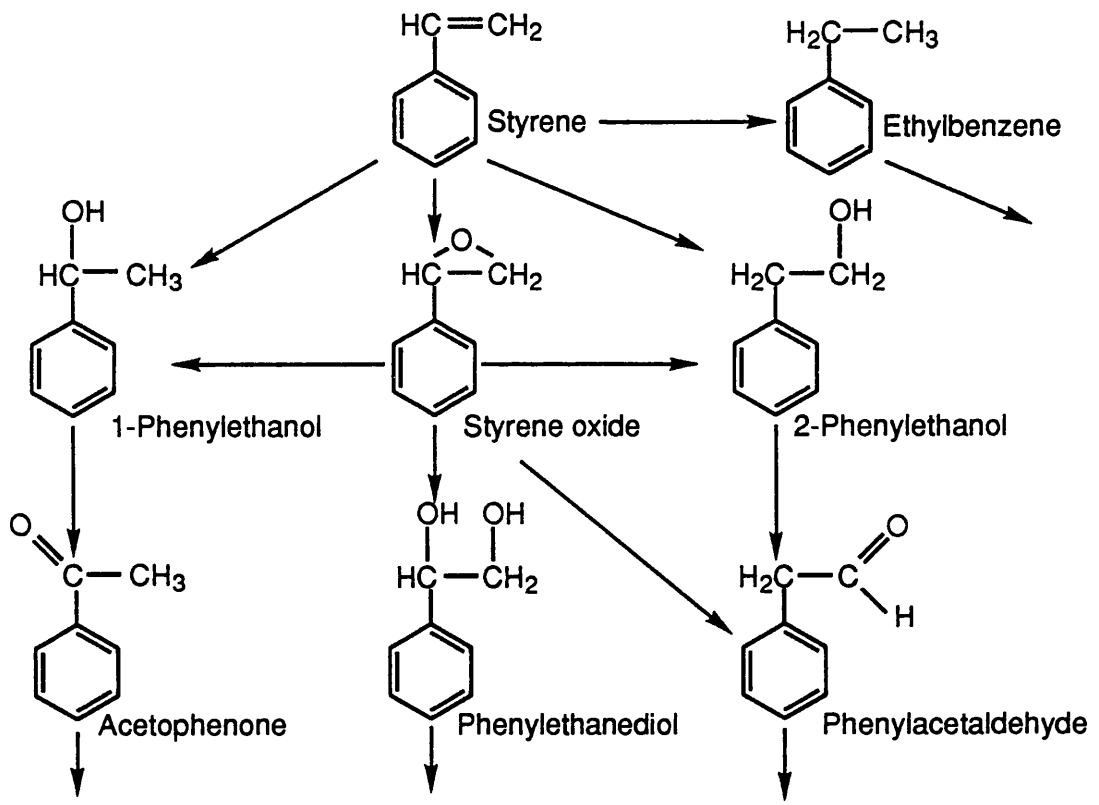
Possible reactions of the vinyl side chain are illustrated in Figure 1.34b. The initial attack could be a monooxygenation to give styrene oxide, as in the human liver, which

Figure 1.34: Routes used by bacteria to metabolise styrene

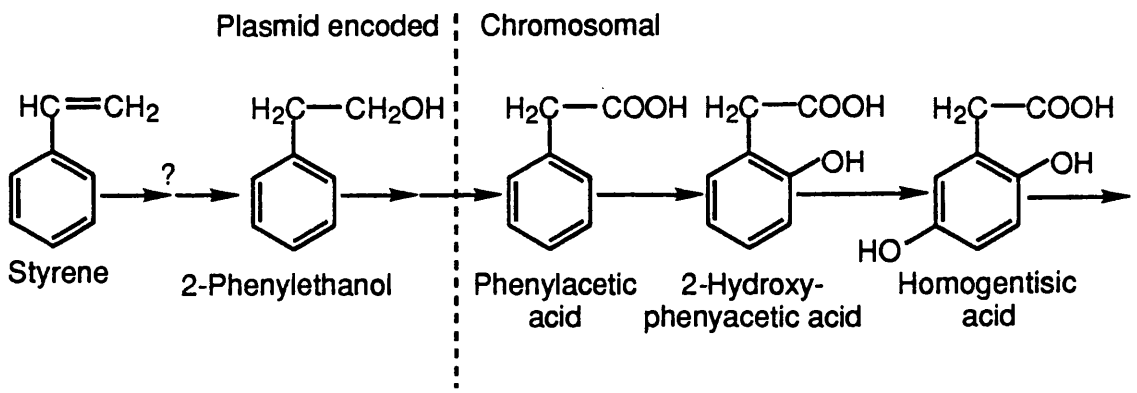
a) The transformation of styrene by *P. putida* str. MST (Bestetti *et al.*, 1989)



b) Possible transformations of styrene through side chain attack



c) The metabolism of styrene by *P. fluorescens* ST (Bestetti *et al.*, 1984)



could then be reduced to either 1- or 2-phenylethanol, hydrolysed to phenylethanediol or isomerised to phenylacetaldehyde. Alternatively, water could be added across the alkene bond of styrene to give, again, either 1- or 2-phenylethanol. Finally, the side chain could be reduced, to give ethylbenzene.

b) Metabolism through phenylethanediol

The route *via* phenylethanediol and mandelic acid, as used by the human liver, is a feasible bacterial route, but it was only recently that a *Pseudomonas putida* R1 culture was found to produce phenylethanediol and mandelic acid from styrene (Rustemov *et al.*, 1992). Though the presence of styrene oxide as an intermediate in this organism was not proven, it seems to be the only reasonable intermediate between styrene and phenylethanediol. The authors suggest that the mandelic acid produced from styrene may be metabolised through benzoic acid and catechol, though the only evidence for this is the presence of catechol 2,3-oxygenase activity when the organism was grown on styrene, α-methylstyrene or toluene.

c) Breakdown *via* phenylacetic acid

The majority of organisms investigated have been found to use a pathway that coverts styrene to 2-phenylethanol or phenylacetic acid. Sielicki *et al.* (1978b) isolated a mixed culture that could degrade styrene, and found that phenylacetic acid and 2-phenylethanol were present in the growth medium. Shirai & Hisatsuka (1979a) isolated *Pseudomonas* 305-STR-1-4 on styrene, and found that 2-phenylethanol was produced from both styrene and styrene oxide, and that small quantities of styrene oxide were detectable in the growth medium when the metabolic inhibitor pyrazole was present. The same group also isolated another pseudomonad which produced 2-phenylethanol from styrene (Shirai & Hisatsuka, 1979b). The further metabolism of 2-phenylethanol and phenylacetic acid was not investigated.

Baggi *et al.* (1983) isolated *P. fluorescens* strain ST on styrene, and found that phenylacetic acid and 2-hydroxyphenylacetic acid could be detected in the medium when this strain degraded styrene. It could grow on these compounds, and in addition 2-

phenylethanol and phenylacetaldehyde. The ability to grow on styrene and 2-phenylethanol was found to be dependent on the presence of a plasmid, whereas growth with phenylacetic acid did not depend on the presence of this plasmid (Bestetti *et al.*, 1984). Growth on styrene, phenylacetic acid or 2-hydroxyphenylacetic acid led to the induction of homogentisate 1,2-dioxygenase. The pathway in Figure 1.34c was proposed for styrene breakdown in this strain. The plasmid, pEG, was identified as a self-transmissible, 37 kbp plasmid, which encodes the enzymes transforming styrene to 2-phenylethanol and 2-phenylethanol dehydrogenase, possibly in a transposon-like region, which is also able to integrate into the chromosome (Bestetti *et al.*, 1989). Isolation of revertants of *sty* $^-$ mutants which do not express enzyme activities for attack on the side chain has indicated that oxidation of the aromatic nucleus may occur in some revertants (Bestetti *et al.*, 1991).

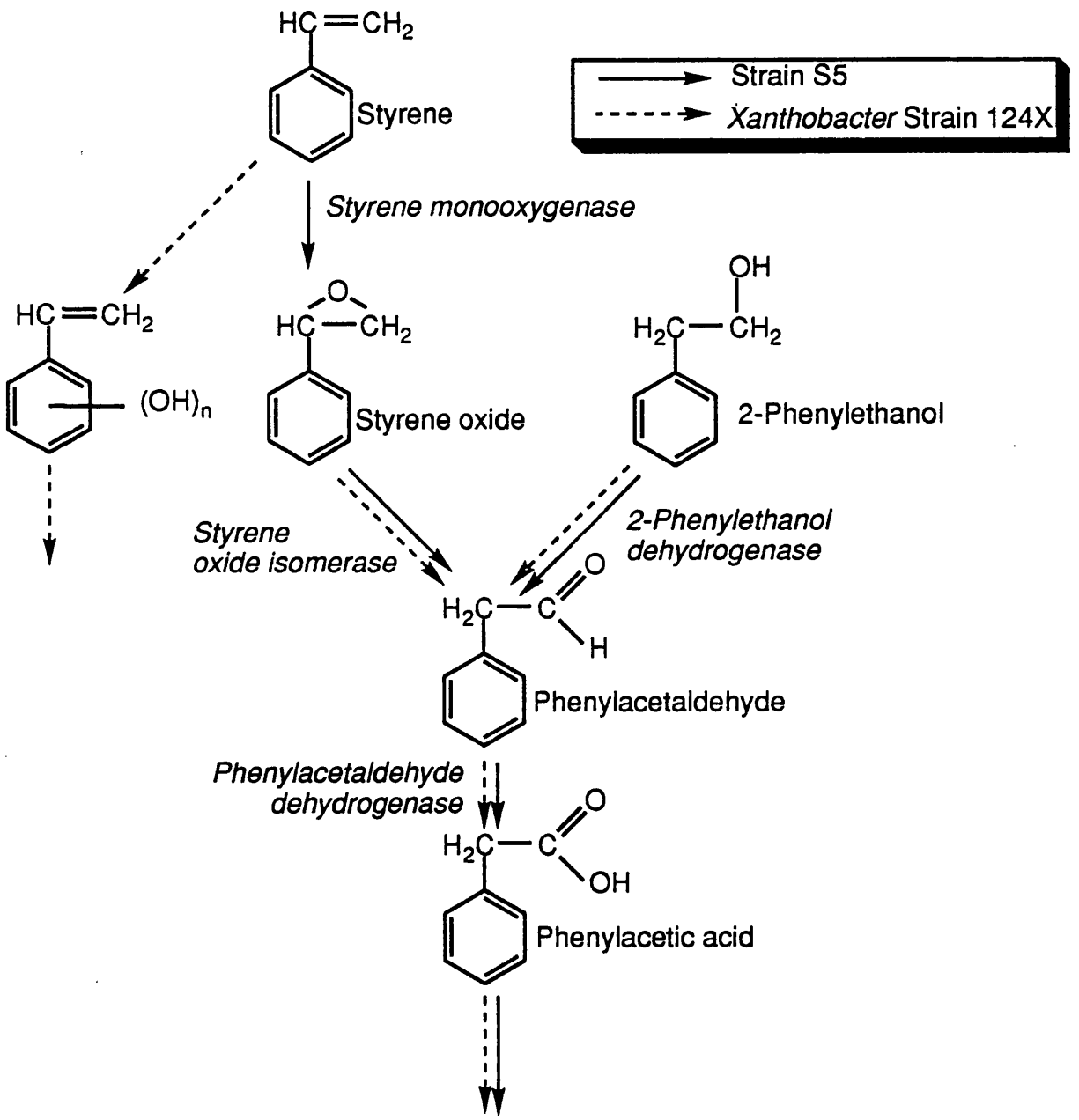
The production of styrene oxide, 2-phenylethanol and 2-hydroxyphenylacetic acid from styrene has also been shown to occur in two thermophilic *Bacillus* strains (Srivastava, 1990). A pseudomonad, strain Y2 has been isolated which can grow on styrene and ethylbenzene, with both of these substrates apparently metabolised through 2-phenylethanol, phenylacetic acid and 2-hydroxyphenylacetic acid (Utkin *et al.*, 1991).

The most detailed enzymological investigation of styrene breakdown through phenylacetic acid has been undertaken by Hartmans *et al.* (1989, 1990). This group found that *Xanthobacter* strain 124x was able to degrade styrene oxide and 2-phenylethanol *via* phenylacetaldehyde and phenylacetic acid, though there was no evidence of styrene being degraded by this route (Figure 1.35). The initial step in styrene breakdown was oxygen dependent, so was probably a ring attack. In addition, when Strain 124x was grown on styrene and 1-phenylethanol, but not on styrene oxide, a transient yellow colour accumulated in the medium, suggesting that a ring attack similar to that used by *Nocardia* T5 could be occurring (see below).

Styrene oxide was isomerised to phenylacetaldehyde by a novel enzyme, styrene oxide isomerase, which required no cofactors. The partially purified enzyme activity

Figure 1.35: The metabolism of styrene, styrene oxide and 2-phenylethanol by Strain S5 and *Xanthobacter* strain 124X

(Hartmans *et al.*, 1989; Hartmans *et al.*, 1990)



was found to be specifically active with styrene oxide; 1,2-epoxypropane, *trans*-2,3-epoxybutane, epichlorohydrin and 1-phenyl-1,2-epoxypropane were not used as substrates. A PMS-dependent 2-phenylethanol dehydrogenase activity was measured, as were PMS and NAD-dependent phenylacetaldehyde dehydrogenase activities (Hartmans *et al.*, 1989).

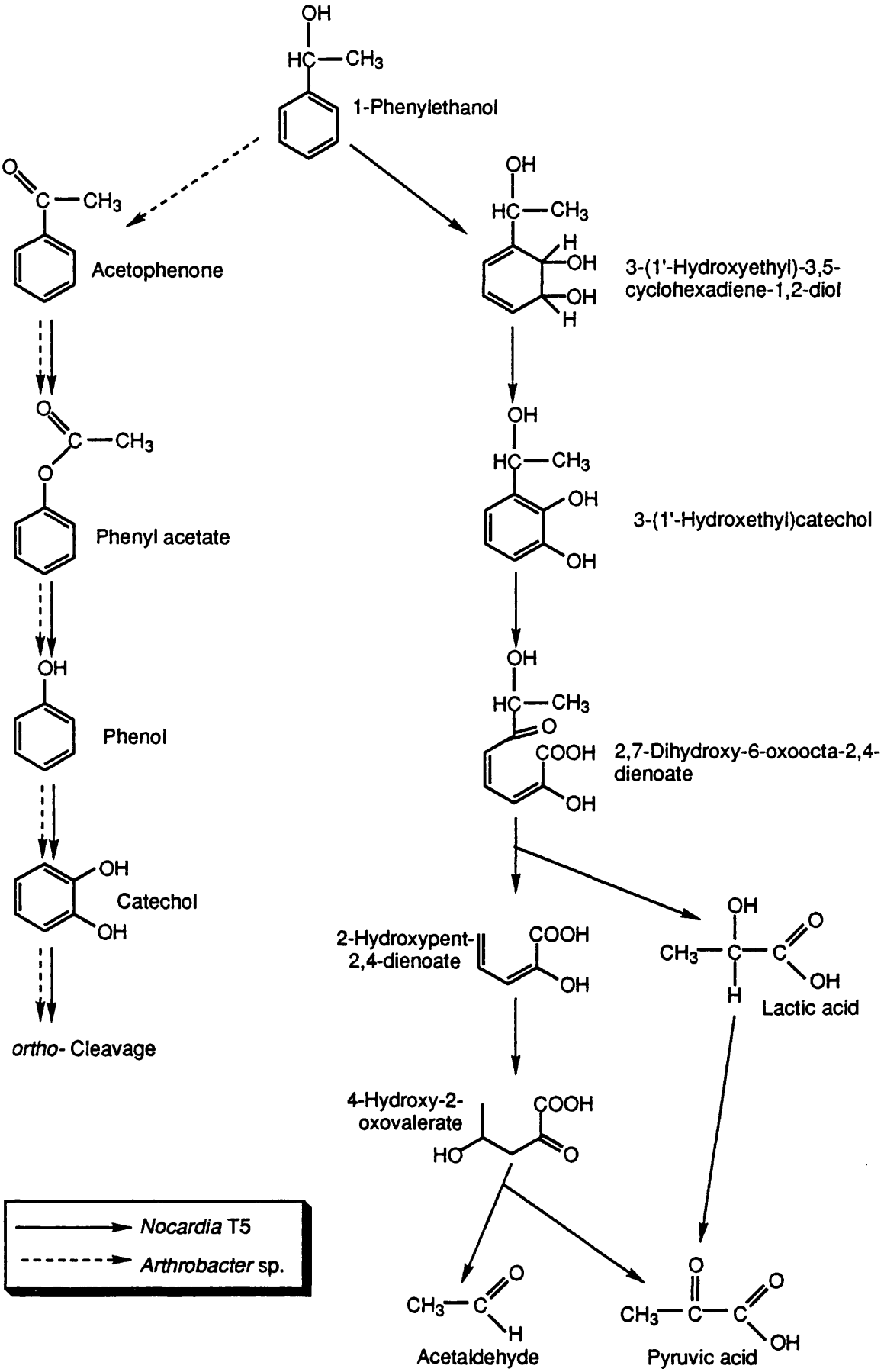
Hartmans *et al.* (1990) later isolated 14 strains of bacteria on styrene, four of which were Gram-negative and the rest Gram-positive. Extracts of 11 of these strains had a FAD-requiring styrene monooxygenase activity, which transformed styrene to styrene oxide. One of the isolates, strain S5, a Gram-positive, pink organism, was characterised further, and was found to also posses styrene oxide isomerase, NAD, NADP and PMS-dependent phenylacetaldehyde dehydrogenase and NAD-dependent 2-phenylethanol dehydrogenase activities. The combination of these activities, and the chemical identification of phenylacetaldehyde, 2-phenylethanol and phenylacetic acid as products of styrene breakdown, led to the proposal that styrene was metabolised by the pathway illustrated in Figure 1.35. The styrene monooxygenase activity in strain S5 was dependent on NADH, O_2 and FAD, though in one strain, S14, the equivalent enzyme was NADPH-dependent. None of the isolates could oxidise phenylethanediol after growth on styrene, and only one, the Gram-positive strain S3, could oxidise acetophenone and ethylbenzene and this was also the only strain which could not oxidise phenylacetic acid. No styrene degradation was detected in cell-free extracts of this strain. It is possible this strain degrades styrene *via* an initial ring attack.

d) Breakdown *via* 1-phenylethanol

No bacteria have been isolated which can degrade styrene through side chain attack to 1-phenylethanol, though bacteria are known to be able to metabolise this substrate. Cripps *et al.* (1978) established two pathways of 1-phenylethanol oxidation, one through an initial attack on the side chain, and the other through an initial attack on the ring (Figure 1.36).

Figure 1.36: The breakdown of 1-phenylethanol by *Nocardia* T5 and an *Arthrobacter* sp.

(Cripps *et al.*, 1978)



e) Anaerobic breakdown

The anaerobic metabolism of styrene has been investigated by Grbic-Galic *et al.* (1990). Methanogenic microbial consortia were isolated from anaerobic sewage sludge with ferulic acid or styrene as sole organic carbon or energy sources. Two further consortia were developed from the ferulic acid consortium, using benzene and toluene to support growth. Styrene was extensively degraded by the ferulic acid, the toluene and the styrene consortia, with 8 mM styrene disappearing within eight months, forming CO_2 as the final product. No methane production was observed during styrene degradation, possibly due to the toxicity of styrene or products of its metabolism to methanogens. The benzene consortium was unable to degrade styrene. Analysis of intermediates accumulating during growth was used to suggest tentative routes for anaerobic transformation of styrene (Figure 1.37). The intermediates found in the highest transient concentrations were benzoic acid and phenol, but many other aromatics were also isolated, including all those shown in Figure 1.37.

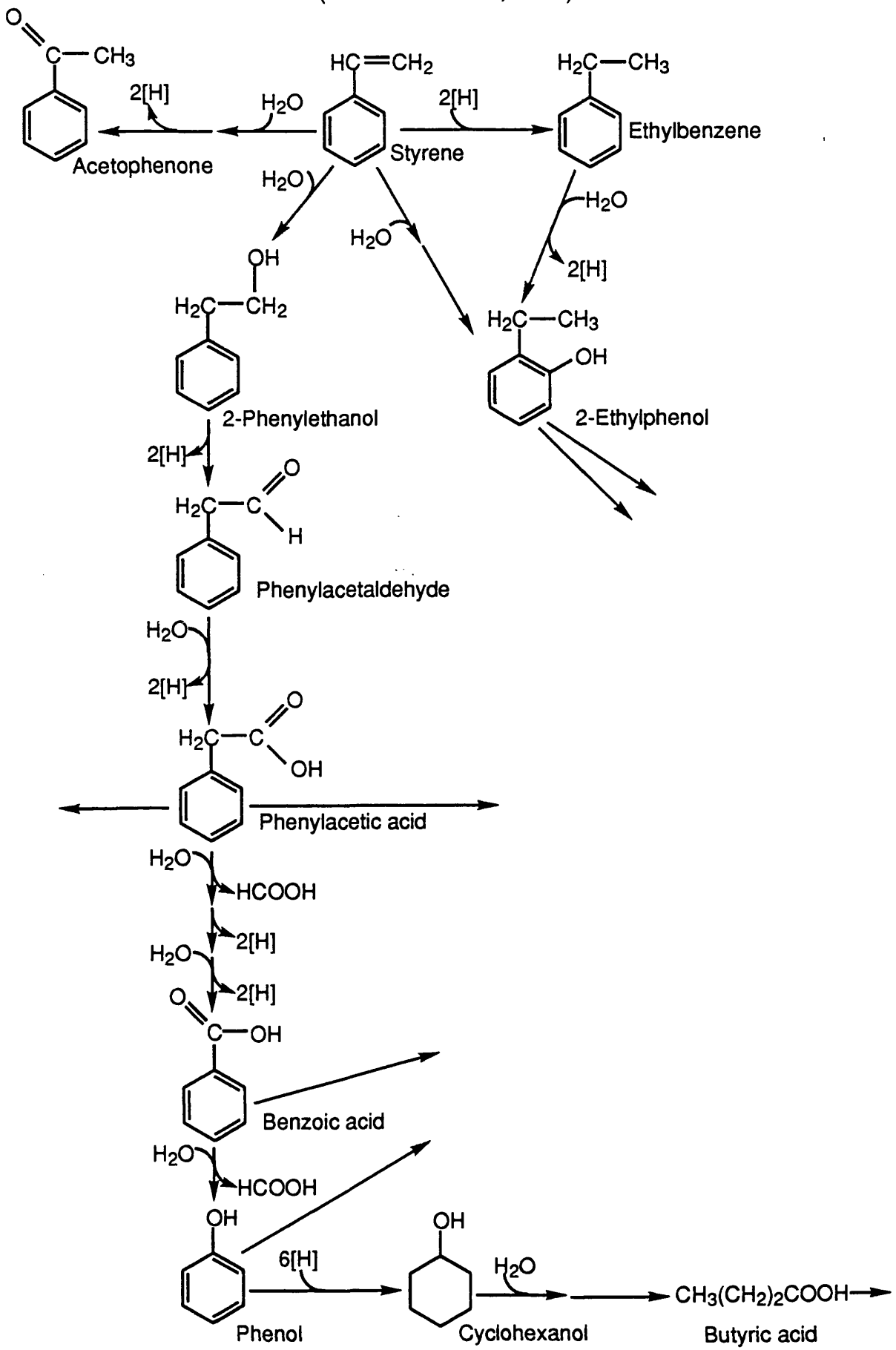
Fifteen bacterial strains were isolated from the styrene degrading consortia, six from the styrene enrichment, six from the toluene consortium and three from the ferulate consortium. All except two strains were Gram-negative rods, tentatively assigned to the genus *Enterobacter*, with the others sporogenous rods resembling *Clostridium*. Of six strains tested for the ability to grow on styrene, two *Enterobacter*-like strains, isolated from the toluene consortium, could grow on 0.1 mM styrene, with a generation time of 24 hours. In addition, *Enterobacter cloacae* DG-6 transformed styrene into a wide variety of aromatic compounds, though no aliphatics were detected (Grbic-Galic *et al.*, 1990).

1.3.4 Bacterial metabolism of styrene oligomers

The commonest derivative of styrene in the natural environment is polystyrene, an insoluble, stable structure. Sielicki *et al.* (1978a) found that only 1.5-3.0 % of ^{14}C-labelled polystyrene was degraded to $^{14}CO_2$ in soil over a four month period. By enriching with 1,3-diphenylbutane, a saturated styrene dimer, they were able to isolate a mixed culture containing *Bacillus*, *Pseudomonas*, *Micrococcus* and *Nocardia* species.

Figure 1.37: Possible routes of anaerobic metabolism of styrene

(Grbic-Galic *et al.*, 1990)



By isolating and identifying intermediates accumulating in the medium the pathway given in Figure 1.38 was proposed for the breakdown of this substrate. Tsuchii *et al.* (1977) found that soil samples could rapidly degrade styrene dimers and showed some ability to degrade trimers, but higher oligomers were unaffected after a month's incubation. *Alcaligenes* sp. 559 was isolated from one of these soil samples, and this strain could utilise unsaturated styrene dimer (1,3-diphenylbut-1-ene) as a sole carbon source, and saturated styrene dimer was co-oxidised. This strain could not grow on styrene. The breakdown of styrene dimers which are ring-substituted with a methyl group has been investigated with this strain and another strain isolated from the same soil samples, *Pseudomonas* sp. strain 419 (Higashimura *et al.*, 1983). This study showed that styrene-methylstyrene co-dimers could be degraded by both organisms, but methylstyrene homo-dimers could not be degraded.

1.3.5 Metabolism of methylstyrenes

a) α-Methylstyrene

Waste from the production of synthetic rubber can contain α-methylstyrene, which is toxic to both humans and the biological systems of rivers. Research into its metabolism by bacteria is still in a preliminary stage, with most work concentrating on the isolation of intermediates, though it seems that isolation of bacteria that can grow on α-methylstyrene is straightforward. Ilyateldinov *et al.* (1984) isolated two α-methylstyrene-utilising bacteria from the sewage of a synthetic rubber plant, and they were identified as *Bacillus cereus* 3 and *Pseudomonas aeruginosa* 21. Omori *et al.* (1974) isolated a bacterium which could grow on α-methylstyrene, and it was later identified as *Pseudomonas convexa* S107B1(Omori *et al.*, 1975). When this bacterium was grown on α-methylstyrene, (–)-*cis*-1, 2-dihydroxy-3-isopropenyl-3-cyclohexene was found in the medium. Bestetti *et al.* (1989) found that *P. putida* MST, able to grow on α-methylstyrene as sole carbon and energy source, also produced this chemical from α-methylstyrene. This strain also produced 2-phenyl-2-propen-1-ol (Figure 1.39a).

The most extensively researched α-methylstyrene degrader is *Pseudomonas aeruginosa* DS13, which has been found by Rustemov *et al.* (reported in Golovleva *et*

Figure 1.38: The metabolism of a styrene dimer by a mixed culture

(Sielicki *et al.*, 1978a)

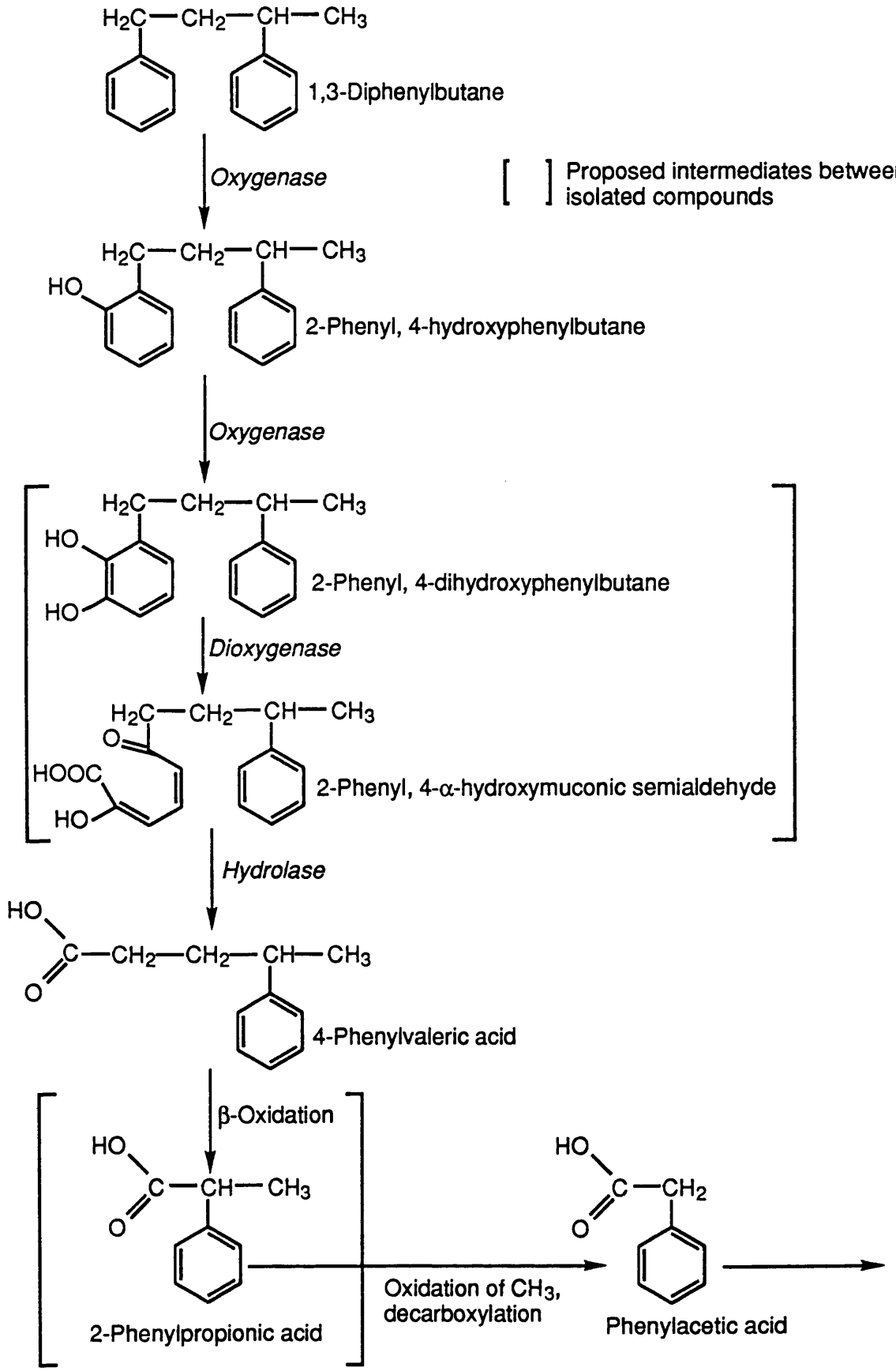
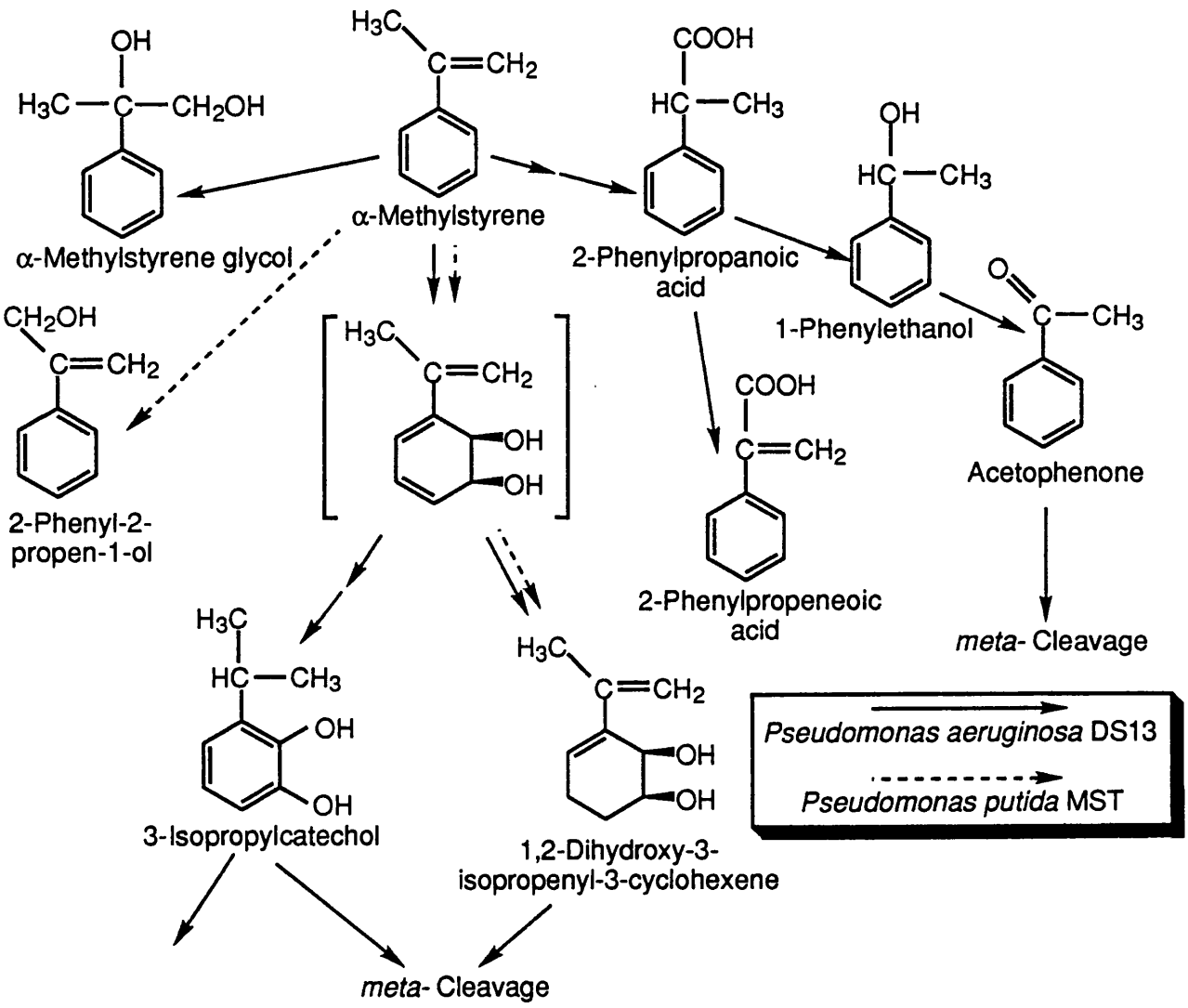
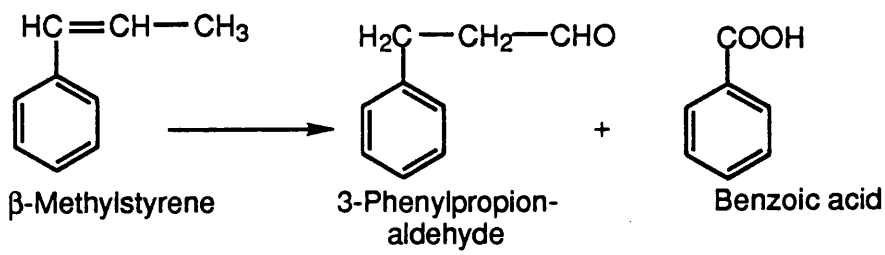


Figure 1.39: Transformations of methylstyrenes

a) Transformation and metabolism of α-methylstyrene by *Pseudomonas aeruginosa* DS13 and *P. putida* MST (Bestetti *et al.*, 1989; Golovleva *et al.*, 1992)



b) Transformation of β-methylstyrene by *Pseudomonas convexa* S107B1 (Omori *et al.*, 1974)



al., 1992) to produce a wide variety of compounds from α-methylstyrene (Figure 1.39a). The isolation of 1, 2-dihydroxy-3-isopropenyl-3-cyclohexene was again reported. In strain DS13 this compound, along with 3-isopropylcatechol and acetophenone, can be metabolised by a catechol 2,3-oxygenase with a relaxed substrate specificity. Strain DS13 and the similar strain *P. aeruginosa* BS176, both have at least some of the enzymes involved in the breakdown of α-methylstyrene encoded by a plasmid (Golovleva *et al.*, 1988a, b). The ability to metabolise α-methylstyrene is retained in strain DS13 when it is studied under conditions of industrial sewage purification (Golovleva *et al.*, 1988a).

b) β-Methylstyrene

Little work has been done on the bacterial breakdown of β-methylstyrene, though Omori *et al.* (1974) found that *P. convexa* S107B1 (above) produced 3-phenylpropionaldehyde and benzoic acid from β-methylstyrene (Figure 1.39b).

1.3.6 Applications of the bacterial transformation of styrene

The application of styrene biodegradation and biotransformation to practical projects is still in its infancy. Ottengraf *et al.* (1986) used a *Nocardia* species, able to grow on styrene and isolated from sewage sludge, to remove styrene vapour in a biofilter. The biofilter consisted of a compost-based structure, and the *Nocardia* sp. was added to the existing bacterial flora of the compost. The filter could eliminate up to 80 g of styrene/m^3/h.

The epoxidation of styrene to styrene oxide has been studied in several organisms with non-specific epoxidases. Furuhashi *et al.* (1986) produced styrene oxide using *Nocardia corallina* B276, and Higgins *et al.* (1979) used *Methylosinus trichosporium* OB 3b. Burback & Perry (1993) found that *Mycobacterium vaccae* ATCC 29678 could degrade styrene in a mixture of styrene and toluene in addition to degrading it as a sole carbon source. The breakdown of styrene was stimulated by the addition of toluene.

1.4 Aims and scope of this thesis

At the start of this project, eight strains of bacteria had been isolated that were all able to grow on styrene as a sole carbon and energy source. The aims of the project were:

1) to carry out a preliminary characterisation of these strains, and then to choose the one that appeared to have the most novel and interesting pathway for the breakdown of styrene;

2) to elucidate the pathway of styrene breakdown in this strain, and to identify the enzymes involved;

3) to carry out a preliminary survey of possible metabolic routes for the degradation of other aromatics by this strain.

2. Materials and Methods

2.1 Materials

Chemicals used were generally analytical grade, and were obtained from the suppliers listed below.

1-Phenylethanol, 2-phenylethanol, acetaldehyde, acetophenone, ammonium sulphate, benzaldehyde, benzene, benzoic acid, benzyl alcohol, catechol, cinnamic acid, Coomassie Brilliant Blue G250, DL-mandelic acid, ethylbenzene, iron (III) chloride, iron (II) sulphate, *N*-methylphenazonium methosulphate (PMS), *n*-octane, orthophosphoric acid, *o*-cresol, *m*-cresol, *p*-cresol, *p*-xylene, phenylacetic acid, polypolypropylene glycol 2025, propionic acid, styrene, toluene, triethylamine and hexane were from BDH chemicals, Poole, Dorset, UK.

2-Hydroxyphenylacetate, 3-fluorocatechol, 3-hydroxyphenylacetate, 3-methylcatechol, 4-methylcatechol, α-methylstyrene, acrylic acid, β-methylstyrene, methanol, methylsuccinic acid, phenylethanediol, phenylacetaldehyde, sodium dithionite, styrene oxide, *bis*-trimethylsilylacetamide and vinylacetic acid were from Aldrich Chemical Co., Gillingham, Dorset, UK.

4-Hydroxybenzoate, L-lactate, D-lactate, 2-(*N*-morpholino) ethanesulphonic acid (Mes), oct-1-ene, phenyl acetate, protocatechuic acid and sodium pyruvate were from Sigma Chemical Co., Poole, Dorset, UK.

Acetic acid, acetone, chloroform, diethyl ether, glucose, glycerol, diaminoethanetetra-acetic acid disodium salt (EDTA), hydrochloric acid, magnesium sulphate, nitriloacetic acid, phenol, sodium chloride, potassium dihydrogen ortho-phosphate, sodium hydroxide, sodium sulphate (anhydrous), succinic acid and trichloroacetic acid (TCA) were from Fisons Scientific, Loughborough, UK.

Dithiothreitol (DTT), 2-amino-2-(hydroxymethyl)-1,3-propandiol (Tris buffer), β-nicotinamide adenine dinucleotide (NAD), reduced β-nicotinamide adenine

dinucleotide (NADH) and β-nicotinamide adenine dinucleotide phosphate, disodium salt (NADP) were from Boehringer Mannheim GmbH, Germany.

DL-Lactic acid, 2-mercaptoethanol and sodium acetate were from Koch Light, Haverhill, Suffolk, UK.

Homogentisic acid, 4-hydroxymandelic acid, D-mandelic acid, L-mandelic acid and phenylglyoxylic acid were from Fluka Ab, Switzerland.

Ammonia and sulphuric acid were from May and Baker, Dagenham, Essex, UK.

m-Xylene and *o*-xylene were from Hopkin and Williams, Chadwell Heath, Essex, UK.

Ethanol was from A. R. Hayman Ltd, Whitham, UK.

Ethyl acetate was from Rhone-Poulenc Ltd, Manchester, UK.

Nitrogen gas (oxygen free) was from BOC Ltd, Guildford, UK.

Toluene *cis*-glycol was from ICI Biological Products (now Zeneca), Billingham, UK.

The cylinder of approximately 50 % $^{18}O_2$ / 50 % $^{16}O_2$ was obtained from ICON Services Ltd., 19 Ox Bow Lake, Summit, NJ 07910, USA.

Deuterochloroform was obtained from CEA, Gif-sur-Yvette, France.

Bovine serum albumin (Fraction V from bovine plasma) was obtained from Wilfrid Smith Ltd., London, UK.

Nutrient broth, nutrient agar and bacteriological agar No. 1 were from Oxoid, Basingstoke, Hampshire, UK.

Catalase (crystalline, from beef liver) was obtained from Sigma Chemical Co., Poole, Dorset, UK.

DEAE-Sephacel super fine was from Pharmacia, Uppsala, Sweden.

2.2 Safety

All experimental procedures were assessed as required by the Control of Substances Hazardous to Health (COSHH) regulations, and full risk assessments were compiled. In addition, the regulations described in the document 'Safety code and radiation rules' published by the Department of Biochemistry, University of Glasgow, were adhered to.

2.3 General methods

2.3.1 pH measurements

Routine measurements of pH were made with a Kent Electronic Instruments Ltd. (Chertsey, Surrey, UK), type 7010 meter. Measurements in small volumes (e.g. cuvettes) were made with a Radiometer (Copenhagen, Denmark) type M26 pH monitor fitted with a GK 2302 micro pH electrode.

2.3.2 Conductivity measurements

Conductivity measurements were made with a radiometer (Copenhagen, Denmark) type CDM2e meter.

2.3.3 Glassware

Glassware was washed in a solution of Haemosol (Meinecke & Co. Inc., Baltimore, USA), then rinsed thoroughly with glass-distilled water.

2.3.4 Sterilisation

a) Moist heat

Media were sterilised by autoclaving at 110 °C for 30 min, and the success of the sterilisation was verified using a Browne's tube (Albert Browne Ltd., Leicester, UK).

b) Dry heat

Glass pipettes for inoculations were sealed in Kraft paper and heated in an oven at 160 °C for 1.75 h.

c) Filtration

Volatile or heat labile compounds, such as glucose, were filtered through Millex-GS (if a small volume) or Millex-HV sterile filters (Millipore S.A., 67 Molsheim, France).

d) Ethylene oxide

Plastic pipettes for inoculations were sterilised in an Anprolene steriliser by exposure to ethylene oxide (H.W. Anderson products Inc., New York, USA).

2.3.5 Protein estimation

Protein concentrations were estimated by a modification of the Bradford (1976) method. The Bradford's reagent was made according to the following method: 100 mg of Coomassie Brilliant Blue G250 was stirred for 1 h in 50 ml of ethanol, then 100 ml of 88 % (w/v) orthophosphoric acid was added. The solution was made up to 1 l with distilled water, then it was filtered three times before use. The reagent was stored at room temperature in a dark glass bottle.

Samples were prepared by a digestion method, to dissolve any cell membrane material. The sample was diluted with the appropriate buffer, then 0.1 ml of it was placed in a glass test tube with 0.2 ml of 1 M NaOH, and then was left overnight at 37 °C. Standards of 0, 0.1, 0.2, 0.3, 0.5, 0.7 and 1.0 mg of BSA/ml buffer were also made up, and 0.1 ml of each was digested in the same way as the sample.

To measure the protein concentration of the samples, 5 ml of Bradford's reagent was added to each sample and gently mixed. The A_{595} of this solution was measured five min later, and protein concentrations were calculated by reference to a standard curve produced from the standards, which were repeated on each occasion the assay was done.

2.3.6 Buffers and solutions

All buffers and solutions were prepared using glass-distilled water. Buffers were prepared at room temperature, with the pH of the solution adjusted using the appropriate acid and base, usually NaOH and HCl. The commonest buffer used was a 50 mM phosphate buffer at pH 7.0, made with 13.6 g KH_2PO_4/l.

2.3.7 Centrifugation

Centrifugation was routinely performed at 4 °C. The machines and conditions usually used are described below:

1) For the harvest of whole cells from growth media, either to obtain whole cells or the culture supernate: for small volumes a Beckmann (Palo Alto, California, USA) Model J2-21 with JA20 rotor was used, at 12000 g for 10 min, for larger volumes an MSE (Crawley, UK) High speed 18 with 6*250 fixed angle rotor was used, at 8000 g for 20 min. For very large volumes (e.g. harvesting a 10 litre fermenter), an MSE Mistral was used, with a 6 l rotor, at 6000 g for 30 min.

2) For the removal of precipitated protein from cell extracts the sample, if small, was spun in an Eppendorf tube for 2 min in an Eppendorf Centrifuge 3200 (Hamburg, Netherlands), at 15, 000 g. Larger samples were spun as described in 1) above.

The production of cell-free extracts using an ultracentrifuge is described later (Section 2.4.6).

2.3.8 Use of the spectrophotometer

A Phillips (Cambridge, UK) PU 8730 u.v./visible scanning spectrophotometer was used throughout this investigation. Simple assays were routinely done in disposable plastic 3 ml cuvettes, except when monitoring absorbances of less than 340 nm, when quartz 3 ml cuvettes were used instead.

Absorbance scans and repeated absorbance scans were performed automatically by the machine, the baseline having earlier been set on appropriate buffer.

2.3.9 Solvent extractions

The extraction of substances from aqueous solution was routinely undertaken using a simple solvent extraction, using ethyl acetate to extract from an acidified solution. Diethyl ether was used when small volumes were being extracted. Extraction was done in a separating funnel of appropriate volume, by shaking the aqueous portion and the solvent together for approximately five min. The solvent was then usually dried by adding anhydrous sodium sulphate. The extraction was repeated 3 to 5 times.

2.3.10 Solvent removal

Small volumes of a few ml of solvent (ethyl acetate or diethyl ether) were removed by evaporation under stream of oxygen-free N_2, either at room temperature or warmed slightly.

Larger volumes of solvent were removed using a Buchi Rotavapor R rotary evaporator (Buchi, Flawil, Switzerland). The evaporation was routinely done at 25 °C.

2.3.11 Thin layer chromatography

Thin layer chromatography was performed on Merck silica gel 60 F-254 plates (BDH, Poole, Dorset, UK), which included a UV indicator. The solvent system used was 180 ml chloroform: 20 ml glacial acetic acid, which was placed in a glass, lidded, tank. Samples were loaded on the plate using a syringe, usually in methanol, allowed to dry for a few minutes, then placed in the tank. Plates were usually run for approximately 90 min, until the solvent front was approaching the top of the plate. The plates were then removed from the tank, and allowed to dry for a few minutes.

Components on plates were visualised using u.v. light, routinely from a Transilluminator gel-viewer (λ_{max} = 302 nm), though selected plates were also checked under a Model UVGL-58 u.v. lamp with a λ_{max} = 254 nm (both from Ultraviolet products Inc., California, USA). If components on the plate were not needed for further analysis, then the plate was placed in a glass tank containing iodine crystals, to allow staining to develop. The results of these tests were recorded on a tracing of the plate.

When components on the plate were needed for further analysis, the area of the plate suspected of containing the component (from the u.v. results) was scraped off on to fresh filter paper, and then placed in a sintered glass funnel. The component was then eluted using ethyl acetate, using suction if necessary to pull the ethyl acetate through the funnel.

2.4 Isolation, maintenance and growth of bacteria

2.4.1 Isolation of bacteria by enrichment on styrene

Eight strains of bacteria were isolated by enrichment culture on styrene (C.A. Fewson, personal communication). The source of the enrichment for each strain is given below:

Strain 26: Unidentified waste from a Ciba-Geigy production plant in Germany

Strains 67, 68, 79, 80, 125, 126, 137: Puddle in unlabelled barrels in a waste disposal area in Switzerland.

2.4.2 Maintenance of bacterial cultures

Cultures were maintained either streaked on nutrient agar slopes or in nutrient broth in universal bottles (see below). After a few days growth at 30 °C the cultures were stored at 4 °C.

a) Cloning

The cultures were cloned every few months, to ensure that they were still able to grow on styrene. The strain was streaked on a Z1 agar plate (see below), and placed in a glass desiccator in which there was a small, open, container of styrene. This desicator was placed at 30 °C for a few weeks to enable the bacteria to grow on the styrene vapour, then colonies were picked off and streaked on nutrient agar plates, incubated at 30 °C for several days, then transferred to nutrient agar slopes or nutrient broth.

2.4.3 Identification of bacterial cultures

a) Gram test

Gram stains were done by a modification of the method described by Gram (Lillie, 1928).

b) Oxidase test

The presence of cytochrome oxidase in the bacterium was tested for by placing some of a slope culture on a test stick (F. Hoffman - La Roche & Co. Ltd. Diagnostica, Basle, Switzerland). If the strip went purple, then the test was positive. A positive control of a *Pseudomonas putida* strain and a negative control of an *Escherichia coli* strain were also tested.

c) NCIMB identification

Further identification was performed by NCIMB Ltd. (Aberdeen, UK).

2.4.4 Growth of bacteria

Bacteria were usually grown at 30 °C, on a rotary shaker moving at 180 rpm.

a) Growth in nutrient broth

Cultures of 50 ml and 500 ml volume were grown in 250 ml and 2 l conical flasks respectively. Cultures were also maintained in 25 ml universal bottles containing 10 ml of nutrient broth. The nutrient broth medium was made up as described in the manufacturers instructions, using 13 g/l of distilled water. The inoculum used varied with the experiment, but was often 0.1 ml of a nutrient broth culture.

b) Nutrient agar

Nutrient agar was made up as specified by the manufacturers, at a concentration of 28 g/l. After autoclaving it was poured aseptically into sterile plastic petri dishes. To produce slopes the agar was poured into 25 ml universal bottles, 10 ml/bottle, before autoclaving. After autoclaving the universals were laid at an angle as the medium set.

c) Z1 minimal salts medium

The standard minimal salts medium used for growth on many substrates was the Z1 medium (Livingston & Fewson, 1972). This consisted of 2 g KH_2PO_4 and 1 g $(NH_4)_2SO_4$ in 1 l of distilled water, adjusted to pH 7.0. After autoclaving 20 ml/litre of a 2 % (w/v) $MgSO_4$ solution (which had also been sterilised by autoclaving) was added aseptically, unless an iron supplement was added, when 20 ml/litre of a solution containing 2 % (w/v) $MgSO_4$ and 0.22 g/l $FeSO_4$ was added (this had been adjusted to pH 2.0 and autoclaved).

d) Z1 agar

This was made using the Z1 medium as described above, with the addition of 15 g of bacteriological agar no. 1 per 1 before autoclaving, but after the pH had been adjusted. The $MgSO_4$ was added as described above, then the agar was poured aseptically into sterile plastic petri dishes.

e) Growth on glucose/salts medium

Cultures were grown on glucose in similar flasks to those used for nutrient broth, in a Z1 medium as described above, with the glucose added in aqueous solution through a membrane filter after autoclaving, to give a final concentration of 5 mM. The inoculum was usually 1 ml of a nutrient broth culture.

f) Growth on volatile chemicals in sealed flasks

Cultures of 100 ml or 200 ml in volume were grown in 500 ml and 1 l gas-tight screw-top flasks (Scotlab, Bellshill, Scotland) on Z1 medium. The substrate was usually added at a final concentration of 1 mM after the flasks were autoclaved, using sterilised eppendorf tips. In some experiments further substrate additions were made after growth had started. The inoculum was usually 5 ml of a nutrient broth culture. For growth on substrate concentrations of more than 1 mM an iron supplement was routinely added as described in Section 2.4.4c.

g) Growth in flasks on miscellaneous non-volatile substrates

Cultures of 50 ml in volume were grown in 250 ml conical flasks on Z1 medium. The substrate was added at a final concentration of 5 mM before autoclaving, except for heat sensitive substrates. These substrates (glucose, phenylethanediol, 2-hydroxyphenylacetic acid, 3-hydroxyphenylacetic acid and phenol) were dissolved in 5 ml of Z1 buffer and membrane filtered into the flasks after they had been autoclaved. The inoculum was 2.5 ml of a nutrient broth culture.

h) Growth on benzyl alcohol in large flasks

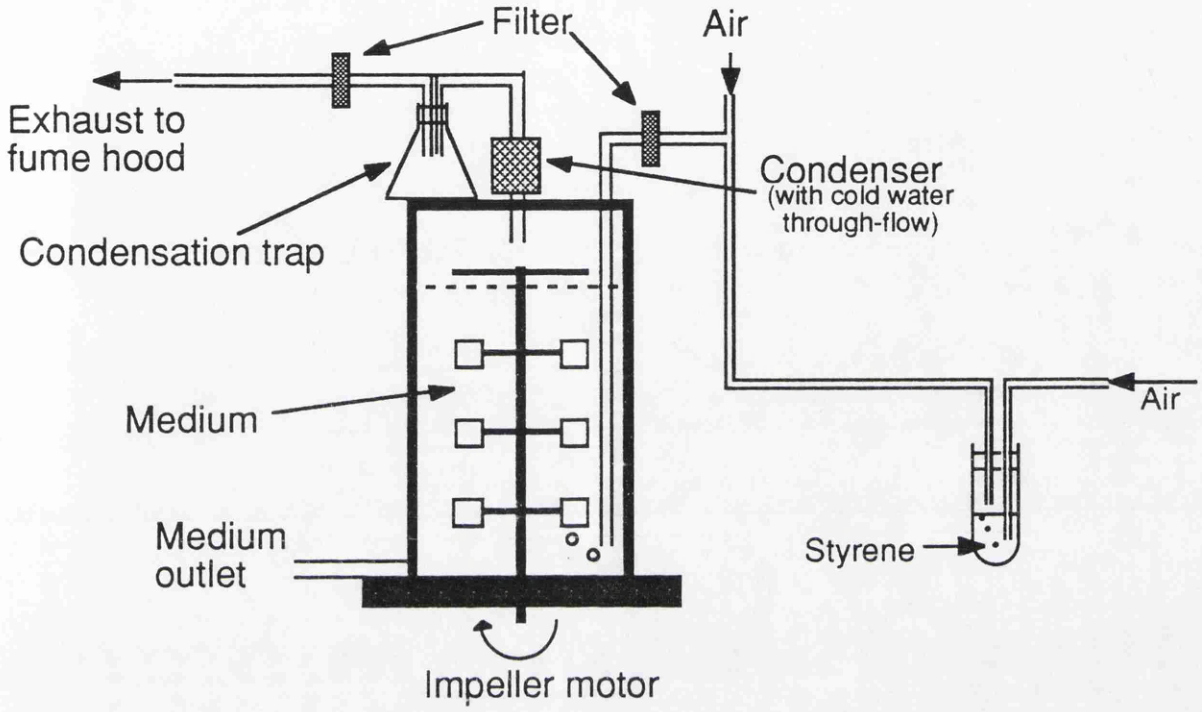
For large quantities of cells grown on benzyl alcohol, 3 l of Z1 medium was autoclaved in a 10 l round-bottomed flask, with the flask's contents including a magnetic bar. After autoclaving, 60 ml of sterile 20 g/l $MgSO_4$, 5 ml of nutrient broth culture and 1.6 ml of benzyl alcohol were added. The flask was then placed in the warm room at 30 °C on a magnetic stirrer at 80 rpm. Air was pumped into the flask *via* a pipette plugged with cotton wool, at a rate of 4 l/min.

After 3 days growth additional substrate was provided by the sterile addition of 1 l of Z1, which had $MgSO_4$ and 0.5 ml of benzyl alcohol added to it. Five h after this addition the contents of the flask were harvested.

i) Growth of *R. rhodochrous* 26 on styrene in a 10 l fermenter

R. rhodochrous 26 was grown in a Z1-based medium with a 50 mM Mes buffer to control the pH, in a 10 l Biostat V fermenter (B. Braun, Melsungen AG, Germany), set up as illustrated in Figure 2.1. All tubing exposed to styrene vapour was solvent-resistant Viton (BDH, Poole, Dorset, UK). The bulk of the medium was autoclaved in the fermenter : 19 g of KH_2PO_4 and 9.5 g $(NH_4)_2SO_4$ in 9.5 l of distilled water, with the pH adjusted to 7.0. Heat-sensitive additions of 106.6 g Mes, 2g $MgSO_4$ and 20 ml of a chelated metals solution (see below) were dissolved in 500 ml of distilled water and adjusted to pH 7.0, and this solution was membrane filtered and added to the fermenter after autoclaving.

Figure 2.1: A simplified diagram of the fermenter system used to grow *R. rhodochrous* 26 on styrene



The inoculum was prepared by growing cells on 1 mM styrene in a gas-tight flask, as described in Section 2.4.4f, with 100 ml of medium and a nutrient broth inoculum of 10 ml. After two days growth the contents of the flask were added to the fermenter. The fermenter was kept at 30 °C using a circulating hot-water element within the fermenter. The medium was stirred, and air was bubbled through at 4 l/min. In addition to the main air supply, 200 ml/min was bubbled through styrene before entering the fermenter, thus providing the styrene substrate in the vapour phase.

After approximately 5 days growth the flow of air bubbling through styrene was increased to 400 ml/min, and the fermenter was harvested a day later, as described in Section 2.4.5.

j) Chelated metals solution

The chelated metals solution was made as follows: 50 g of nitriloacetic acid was dissolved in 500 ml of distilled water, and 125 ml of 5 M NaOH was added. The solution was adjusted to pH 7.0 with 5 M HCl, then the following compounds were added: 1.10 g $FeSO_4.7H_2$; 50 mg $MoO_4.2H_2O$; 50 mg $MnSO_4.4H_2O$; 50 mg $ZnSO_4.7H_2O$; 25 mg $CuSO_4.5H_2O$ and 25 mg $CoCl_26H_2O$ (Beggs & Fewson, 1977). The solution was then made up to 1 l and autoclaved.

k) Growth of *R. rhodochrous* 26 on styrene and nutrient broth in a 10 l fermenter

In order to obtain higher yields of cells grown in the presence of styrene, a nutrient broth based medium was used in order to provide an additional growth substrate. The medium consisted of: 130 g nutrient broth, 20 g KH_2PO_4, 10 g $(NH_4)_2SO_4$, 4 g $MgSO_4$, 104.4 g Mes, 20 ml chelated metals solution (as above) and 0.5 ml of anti-foam (polypolypropylene glycol 2025), all in 10 l of distilled water, with the pH adjusted to 7.0 with 5 M NaOH. This medium was autoclaved in the fermenter, as described above.

The fermenter was set up as shown in Figure 2.1, air was supplied at 4 l/min and the medium was agitated by the impeller. The inoculum was 500 ml of a 3 d old nutrient broth culture. After 48 h of growth on the nutrient broth-based medium, 400 ml air/min

was bubbled into the fermenter through styrene. After a further 24 h the fermenter was harvested as described below.

2.4.5 Harvesting of bacteria and collection of growth media

Cultures to be harvested were chilled on ice before being centrifuged as described in Section 2.3.7 above. The supernate was decanted off, and disposed of unless it was needed for experiments, when it was stored on ice. The pellet was then washed by resuspending it in ice-cold 50 mM phosphate buffer, pH 7.0, to a volume equal to the original culture. The centrifugation was then repeated, the supernate was decanted, and this washing was normally repeated another twice. Pellets of bacteria were then stored at -20 °C.

2.4.6 Production of cell-free extracts

The buffer used for extract preparation was usually 50 mM phosphate pH 7.2, 10% glycerol and 10% ethanol (PEG) This buffer has previously been used in similar investigations (Yeh *et al.*, 1977). The buffer was usually de-gassed, by repeated evacuation (usually 3 times) and flushing with oxygen-free nitrogen. Frozen cells were thawed and slowly resuspended in buffer (0 °C). They were then pressed between three and five times in an Aminco French pressure cell press (SLM Instruments Inc., American Instrument Co, Urbana, Illinois 61801, USA) at 117 MPa. The body of the press was cooled on ice before use. The pressed extract was then centrifuged at 12,000 g (in a Beckman model J 2-21, see Section 2.3.7) for 10 min, and the supernatant retained. This was further centrifuged at 145,000 g for 1 h in a Beckman L5-65 ultracentrifuge with a Type 65 rotor, and the supernate was decanted. This supernate was used as the cell-free extract, and it was stored at -20 °C. If the pellet was required it was resuspended in a volume of PEG equal to the volume of the original sample and stored in the same way.

In some experiments a Mes-based buffer (MEG) was used instead of PEG: 50 mM Mes, 10 % ethanol, 10 % glycerol and 1 mM DTT, all in distilled water and adjusted to pH 6.0.

2.4.7 Heat treatment of cell-free extract

The sample of extract was thawed and placed in an eppendorf tube. This was then placed in a holder and floated for 5 min in a water bath at 55 °C. The tube was then put on ice for a few minutes, then any precipitated protein was centrifuged off (Section 2.3.7). The supernate was usually transferred to another eppendorf tube, and stored on ice until needed.

2.5 Production, extraction and analysis of intermediates

2.5.1 Analysis

a) Gas chromatography - mass spectrometry (g.c.-m.s.)

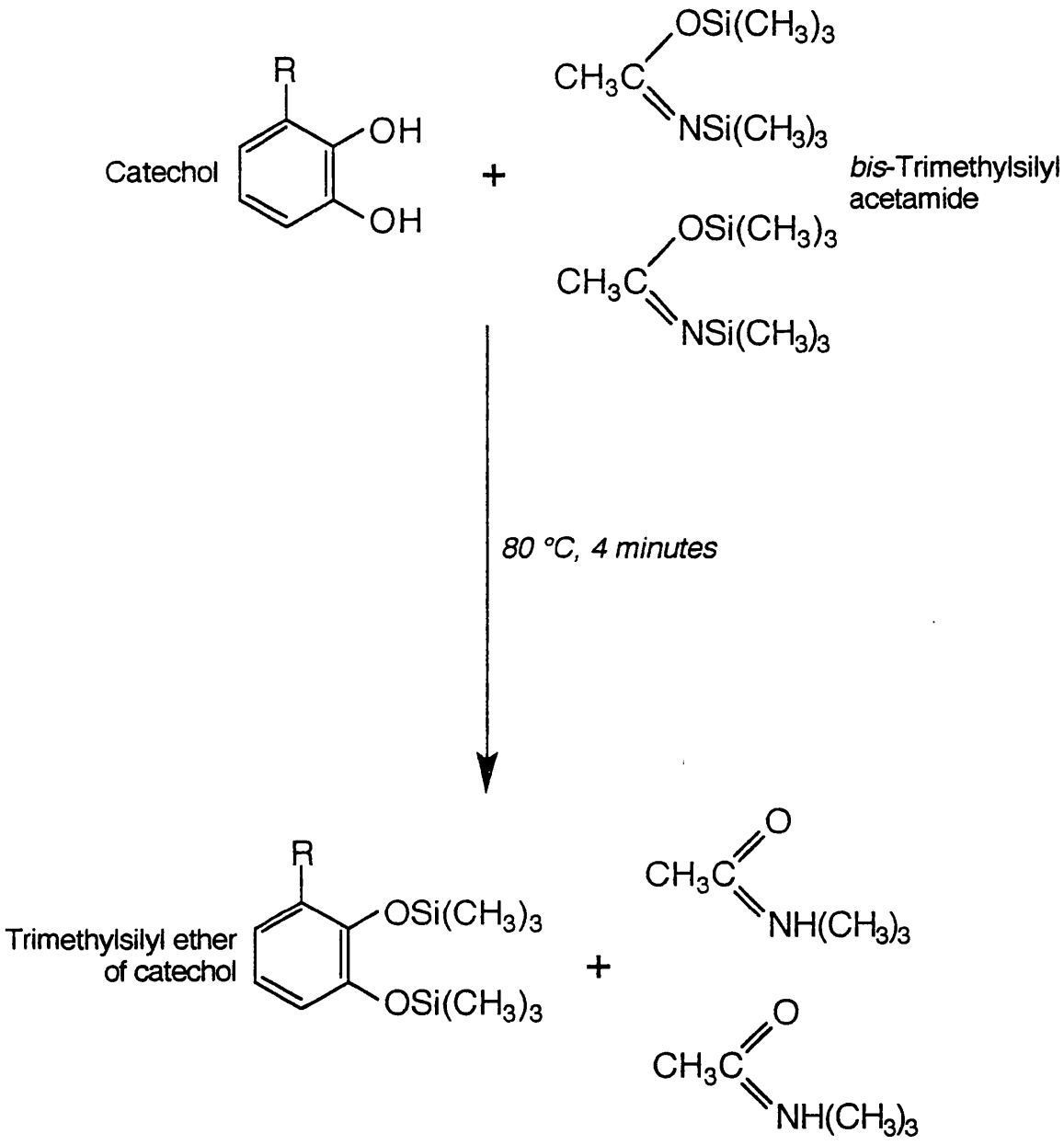
All g.c.-m.s. analysis was done by Mr. Ken Clarke, British Carbonisation Research Association, Mill Lane, Wingerworth, Chesterfield, Derbyshire, S42 6NG.

The samples were sent to Chesterfield as residues in glass bottles. The samples were treated with a small volume, usually 0.3 ml, of *bis*-trimethylsilylacetamide and heated at 80 °C for 4 min. This produced trimethylsilyl ether derivatives of phenolic compounds (Figure 2.2). The samples were analysed in a Hewlett-Packard 5890 series 2 chromatograph coupled to a Hewlett-Packard 5791 quadrapole mass spectrometer (Hewlett-Packard, Bracknell, UK). The g.c. column was a 25 m * 0.22 mm internal diameter fused silica capillary column. The stationary phase was phase bonded methyl silicone BPI (Scientific and Glass Engineering, Milton Keynes, UK). The carrier gas was He, inlet pressure 10 p.s.i.. The g.c. was normally operated with the split open. The m.s. was usually operated in full scan mode, 50 - 550 a.m.u.. Fragmentation patterns were compared with the MBS 54K library, from Hewlett-Packard.

b) Nuclear magnetic resonance analysis

N.m.r. analysis was carried out in the Department of Chemistry, University of Glasgow on a Bruker WP-200 SY 200 Hz n.m.r. spectrometer, with tetra-methylsilane as an internal standard. Samples were dissolved in $CDCl_3$. Assignments were calculated by Dr. R.A. Hill.

Figure 2.2: The derivatisation of catechols with *bis*-trimethylsilyl acetamide



c) Use of h.p.l.c. to detect acrylic acid

Analysis of the accumulation of acrylic acid in solutions was performed by h.p.l.c. analysis, using a Gilson h.p.l.c. system (Anachem Ltd., Luton, UK), with a Aminex HPX-87H 300*7.8 mm organic acids column (Bio-rad, Richmond, California, USA) protected by a Micro-guard Cation H guard column (also Bio-rad). The buffer was 25 mM H_2SO_4, at a flow rate of 1 ml/min. Components were detected using a u.v. detector at 210 nm, with the sensitivity set at 0.05, and recorded on a chart recorder running at 5 mm/min.

Samples of 50 µl of the solution to be tested were injected onto the column *via* a 100 µl loop. Standards of acrylic acid were made up as follows: a 1 mM solution was made up in a gas tight flask, 22 µl in 300 ml distilled water, then this was diluted to make up a 0.1 mM solution (1 ml to 9 ml distilled water) or a 0.05 mM solution (1 ml to 19 ml distilled water).

d) Total organic carbon (t.o.c.) analysis

Total organic carbon analysis was done using a Shimadzu TOC-500 analyser (Dyson Instruments, Hetton, Tyne and Wear, UK) with an autosampler, and a sample size of 5 µl. The low inorganic carbon and organic carbon standards were both distilled water. The high inorganic carbon standard was 400 ppm sodium carbonate, the high organic carbon standard was 400 ppm sodium phthalate. Each sample was analysed 5 times, and a mean was calculated.

To monitor the t.o.c. of cells grown on styrene, 6*500 ml flasks containing 100 ml of Z1 and Fe medium were inoculated with 5 ml of nutrient broth culture. A 2 ml sample was removed aseptically from each flask for analysis (see below), then 10 µl of styrene was added to four of the flasks. The flasks were left to grow on a shaker at 30 °C for 4 days, when the two flasks with no styrene in and two of the other flasks were harvested, as described below. The final two flasks were harvested one day later.

Each sample was divided into two parts, one of which was t.o.c. analysed, and the other part of which was centrifuged as usual to remove cell material. The supernate was

then t.o.c. analysed, and the pellet was resuspended in buffer to the original sample volume, and t.o.c. analysed. Controls of 5 ml nutrient broth diluted by 100 ml Z1 and 10 µl styrene dissolved in 600 ml of distilled water were also analysed.

2.5.2 Production of styrene *cis*-glycol by *Pseudomonas putida* UV4

The production of styrene *cis*-glycol was done in the laboratories of ICI Biological Products (now Zeneca, Billingham, UK), with assistance from Dr R.A. Holt. *P. putida* UV4 was provided by ICI.

a) Media

The basic minimal medium used was Seed 2 (S2) : 20 ml of phosphate buffer stock (95 g KH_2PO_4 and 78 g NaH_2PO_4 per litre of distilled water, adjusted to pH 7.2), 5 ml of $(NH_2)_2SO_4$ stock (36 g/100 ml), 0.5 ml $MgSO_4.7H_2O$ stock (40 g/100 ml), 0.1 ml $FeCl_3$ stock (0.972 g/100 ml) and 1 ml of trace elements (Fisons, UK), made up to 1 l with distilled water and sterilised by autoclaving. For S2/pyruvate medium, 1.2 ml of membrane-filtered 25 % sodium pyruvate solution was added per 50 ml of S2 medium, after autoclaving. Seed 2 glucose case amino acids agar plates were made by adding 20 ml of sterile 27 % w/v glucose solution and 20 ml of sterile 20 % w/v (casamino) acids solution to 400 ml of molten Seed 2 agar (Seed 2 medium with 15 g/l of bacteriological agar no. 1).

b) Selection of active *P. putida* UV4

Unfortunately this mutant is not stable, so it must be constantly re-selected for maximum activity (usually weekly). It must not be stored at 4 °C as it loses activity under these conditions. The first re-selection stage was the constitutive assay, to ensure that the aromatic breakdown pathway was constitutively expressed:

A culture of UV4 was serially diluted to 10^{-8} with S2 medium. The 10^{-4}, 10^{-6} and 10^{-8} dilutions were plated out on S2/glucose/case amino acids agar, and incubated at 28 °C for 48 h. The colonies were then sprayed with sterile 0.5 M catechol solution. Those colonies turning green within 5 min were considered to have constitutive

catechol oxygenase expression, and were assumed to have a constitutive aromatic breakdown pathway. Several active colonies were picked off and used to inoculate baffled 250 ml flasks containing 50 ml of S2/pyruvate medium. These were incubated on an orbital shaker overnight at 28 °C, when the activity level of the culture was measured, by measuring the rate of transformation of toluene to toluene *cis*-glycol:

The cells from 4 ml of culture were centrifuged off and resuspended in 50 mM phosphate buffer, pH 7.2. The centre well of a baffled 250 ml quickfit conical flask was filled with 0.5 ml toluene and sealed with a foam bung, to allow some equilibration of the toluene with the atmosphere. The cell density was measured using OD_{600} and the A_{265} was measured, to give a baseline to measure the accumulation of toluene *cis*-glycol at this wavelength. An ethanol co-substrate, 40 µl, was added, then the suspension was placed in the baffled flask, and put on the shaker at 28 °C. After 2 h, 1 ml of solution was removed and the OD_{600} and the A_{265} were measured. The 'activity' was then calculated: sample(A_{265}/OD_{600}) -start (A_{265}/OD_{600}). The flask with the highest activity was then used to inoculate the growth flasks for the transformation, or was streaked on S2/pyruvate agar for future use.

c) Transformation and extraction

Ten baffled 1 l flasks containing 200 ml S2/pyruvate medium were inoculated with a loop full of an active *P. putida* UV4 culture, before being placed on a shaker at 120 rpm and 28 °C. After 24 h growth the contents of the flasks were centrifuged to obtain the cells. These cells were resuspended in 1 l of 50 mM phosphate buffer, pH 7.6, and 4 ml of ethanol was added. The cell suspension was placed in baffled flasks with centre wells; 9*500 ml flasks with 100 ml of suspension and 2*250 ml flasks with 50 ml of suspension. Styrene (Aldrich, 99%+, 'gold label') was placed in the centre wells, 0.5 ml in each. The flasks were then incubated at 28 °C on the orbital shaker. After 24 h the cell suspension was removed from the flasks and the cells were centrifuged off. The supernate was retained and its pH was adjusted to 8.0 with 5 M NaOH. The volume of the supernate was reduced to approximately 50 ml by rotary evaporation at 40 °C. The supernate was then saturated with $(NH_4)_2SO_4$ and filtered. The pH was again adjusted

to 8.0, and the solution was stored overnight at 4 °C. The next day the solution was allowed to warm to room temperature, then it was filtered again, before being extracted (Section 2.3.9) with 4*50 ml portions of ethyl acetate. The ethyl acetate was evaporated down to approximately 1 ml in the rotary evaporator at 30 °C, then was transferred to a test tube and placed in ice. Approximately 4 ml of iced hexane was added, and the solution was immediately filtered through Whatman No.1 filter paper, trapping a dirty yellow precipitate, which was discarded. The solution was kept on ice as pure white crystals appeared, which were filtered off, then dissolved in ethyl acetate with 0.1 % triethylamine stabiliser. Some crystals were dissolved in deuterochloroform for n.m.r. analysis.

d) Storage and use

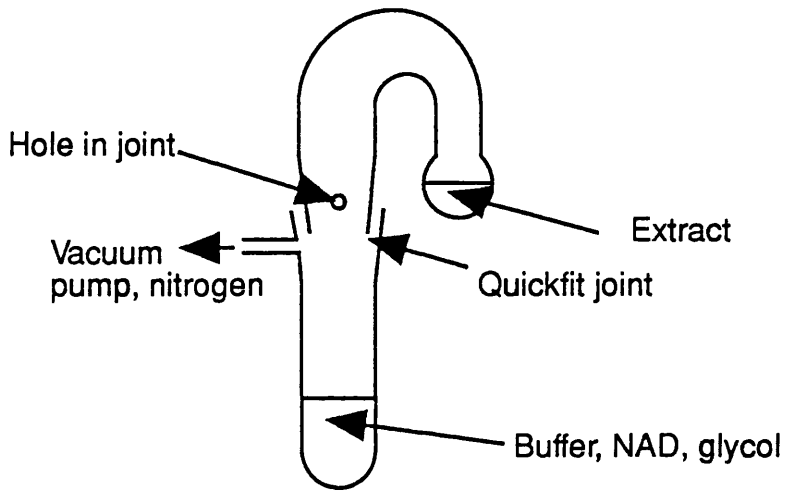
Styrene *cis*-glycol was stored in ethyl acetate with 0.1 % triethylamine at 4 °C. It was generally used as a solution in ethyl acetate. If larger quantities were used, then it was extracted from the ethyl acetate into 50 mM phosphate buffer, pH 7.6, by shaking in a test tube.

2.5.3 Anaerobic transformations using Thunberg tubes

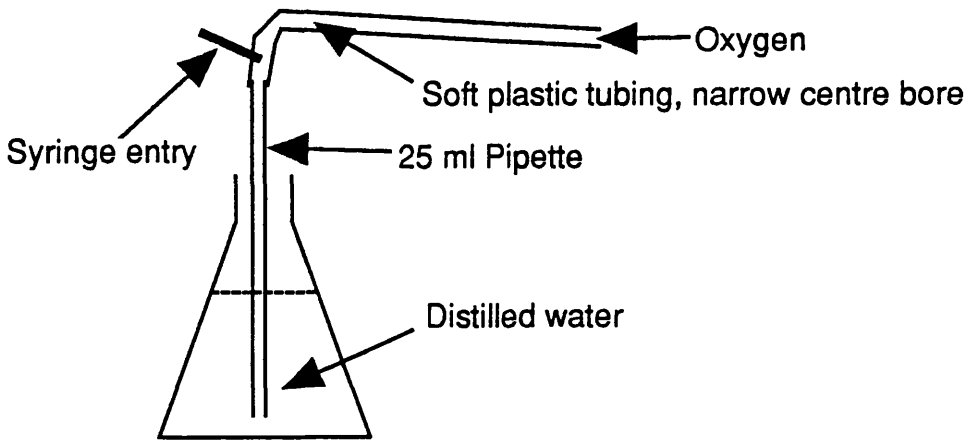
Buffer (50 mM phosphate pH 7.6), NAD and *cis*-glycol were placed in the test tube portion of a Thunberg tube (Figure 2.3a), chilled on ice. Extract from cells grown on nutrient broth and styrene was placed in the bulb, and the two parts of the tube were connected, with a small amount of Vaseline on the quickfit joint. The hole in the joint was aligned with the outlet tube and this tube was connected to a two-way joint connected to both an oxygen-free nitrogen cylinder and a vacuum pump. The vacuum pump was used to evacuate and de-gas the contents of the Thunberg tube, then the tube was refilled with nitrogen gas. This procedure was repeated twice, leaving the tube full of nitrogen. The bulb was then twisted so that the hole in the joint was no longer aligned with the outlet tube, and the contents of the bulb were mixed with those of the test tube by inversion. The tubes was placed in a water bath at 30 °C for 30 minutes. The reaction was stopped with 300 µl of 1 M HCl, immediately after the top was removed. The reaction mixture was then centrifuged to remove precipitated protein. If

Figure 2.3: Thunberg tube and equipment used to provide oxygen for the production of 3-vinylcatechol in a controlled atmosphere

a) Thunberg tube, set up for the NAD-linked transformation of a *cis*-glycol



b) Apparatus used to provide the oxygen for the production of 3-vinylcatechol in a controlled atmosphere



required, the supernate was then extracted with 3* the volume of ethyl acetate or diethyl ether.

2.5.4 Production of intermediates by whole cells in the presence of fluorocatechol

Intact frozen cells grown on nutrient broth and styrene were suspended in phosphate buffer at 1 g per 100 ml in a gas-tight shake flask. 3-Fluorocatechol was added at a final concentration of 2 mM, along with 20 µl of styrene, then the flask was incubated at 30°C on the orbital shaker for 2 h. The reaction was stopped by acidification, the cells were removed by centrifugation, and the medium was extracted with ethyl acetate. For production of pure compound, most of the ethyl acetate extract was evaporated off and the remainder separated by t.l.c..

a) Bulk production of 3-vinylcatechol for n.m.r.

Phosphate buffer, 185 ml of 50 mM, was placed in each of three 1 litre screw-top flasks. Six grams of frozen cells grown in a nutrient broth/styrene fermenter were thawed and resuspended in 30 ml of buffer, and 10 ml of the suspension was placed in each flask. 3-Fluorocatechol, 15.4 mg, was dissolved in 15 ml of buffer, and 5 ml of this solution was added to each flask (giving a final concentration of 2 mM). Then 40 µl of styrene was added to each flask, and the flasks were put on the shaker at 30 °C for 2 h. The flasks were placed on ice, and 8 ml of 5 M HCl was added to each, and they were centrifuged to remove cell material (Section 2.3.7), and the supernate was stored in the cold room at 4 °C overnight. The supernate was then extracted in two halves, 2*300 ml, each extracted with 5*50 ml ethyl acetate. The ethyl acetate was then dried over anhydrous sodium sulphate, and evaporated down to approximately 1 ml using the rotary evaporator at 25 °C. The sample was then streaked on eight t.l.c. plates, and run in the normal solvent system, as in Section 2.3.11. After visualisation under u.v., the appropriate band was scraped off and eluted with ethyl acetate. The ethyl acetate was evaporated off under nitrogen at room temperature.

2.5.5 Use of 50% $^{18}O_2$ / 50% $^{16}O_2$ atmosphere to produce 3-vinylcatechol

Two 50 ml side-arm flasks had 19 ml buffer (50 mM phosphate, pH 7.0) placed in them, with 0.4 g of thawed cells which had been grown in the fermenter on nutrient broth and styrene. 3-Fluorocatechol was added to each flask, 0.5 mg in 1 ml buffer per flask (final concentration = 2 mM). Suba-seals (BDH, Poole, Dorset, UK) were used to seal both flasks, and they were evacuated using a vacuum pump connected to the side-arm, then flushed with oxygen-free nitrogen. This was repeated three times, and the flasks were evacuated again. The control flask was injected through the suba-seal with 20 ml of $^{16}O_2$ (see below), then the remaining vacuum was filled by oxygen-free nitrogen gas. The tube attached to the side-arm was then closed with a clip. The other flask was injected with 20 ml of an approximately 50:50 mixture of $^{18}O_2$ and $^{16}O_2$ (see below), then oxygen-free nitrogen was allowed in to release the vacuum. Both flasks then had 10 µl of styrene added to them through the suba-seals, and they were placed on the orbital shaker at 30 °C.

After 2 hours the flasks were acidified with 1 ml of 5M HCl, and chilled for 5 minutes. The contents were centrifuged to remove the precipitated protein, and the supernates were each extracted with 4*20 ml ethyl acetate. The extract was then dried over anhydrous sodium sulphate, and the ethyl acetate was removed, first using the rotary evaporator at 25 °C, followed by evaporation under a stream of N_2.

a) Addition of oxygen to the reaction mixture

Additions of oxygen were made using a 25 ml syringe fitted with a disposable needle. The syringe was filled with oxygen using the apparatus depicted in Figure 2.3b. Initially the pipette was filled with water, using a pipette-filler to suck up the water, after which the tubing was closed with a clip. Then an oxygen cylinder, containing either 100 % $^{16}O_2$ or a mixture of approximately 50% $^{16}O_2$ and 50% $^{18}O_2$, was connected to the tube and used to fill the pipette with oxygen. The needle of the syringe was carefully passed through the wall of the tube to the central channel, retaining a seal

around the needle, and 20 ml of oxygen was removed. This oxygen could then be added to the flasks.

2.5.6 Extraction of putative muconic acid from culture supernate

Intact cells, 20 g, which had been grown on nutrient broth and styrene were thawed and suspended in 1.8 l of 50 mM phosphate buffer, pH 7.0. This suspension was divided into 12*500 ml conical flasks (100 ml in each) and 3*1 l flasks (200 ml in each), and 30 µl styrene was added to each of the 500 ml flasks and 60 µl styrene was added to each of the 1 l flasks. A foam bung was used to seal each flask, and they were placed on the orbital shaker at 30 °C for 4 hours. The media were accumulated, then acidified with 20 ml of 5 M HCl, and cells were centrifuged off using the MSE Mistral 6l rotor (Section 2.3.7).

The supernate was extracted in two 900 ml batches, each with 3*200 ml of ethyl acetate. The ethyl acetate was then dried over anhydrous sodium sulphate, filtered and evaporated down to approximately 0.5 ml in the rotary evaporator at 25 °C. Methanol (1 ml) was then added and the solution was streaked on five t.l.c. plates and run in the normal solvent system (Section 2.3.11). The relevant band was marked under u.v. illumination before being scraped of and eluted with approximately 50 ml of ethyl acetate (Section 2.3.11). The ethyl acetate was dried, filtered and evaporated down (at 25 °C) to as small a volume as possible (approximately 0.5 ml) before having 1 ml of methanol added to facilitate transfer to a pre-weighed sample tube. The remaining solvent was evaporated off under nitrogen.

2.5.7 Monitoring of accumulation of putative muconic acids in medium of cells grown on styrene or benzyl alcohol.

Flasks of 500 ml volume containing 100 ml of Z1 medium with iron supplement were inoculated with 5 ml of a nutrient broth culture and 1 mM of the test substrate. The flasks were fitted with mini-inert valves (Pierce & Warriner Ltd., Chester, UK) to enable easy sampling using sterile disposable needles, with minimal loss of volatile components. The flasks were incubated at 30 °C on an orbital shaker during growth, as

usual. Samples of 3 ml were taken at the start and at various later times. This samples were placed in two eppendorf tubes and spun in the eppendorf centrifuge (Section 2.3.7) to remove cell material. The supernate then had its absorbance scanned between 200 and 300 nm.

2.5.8 Production of 2,3-cleavage product from 3-vinylcatechol

NAD-linked transformation of styrene *cis*-glycol to 3-vinylcatechol was carried out as described in Section 2.5.3 above, using four Thunberg tubes. Each tube contained 20 mg of NAD and approximately 2.5 mg of styrene *cis*-glycol (extracted from ethyl acetate into buffer, as in Section 2.3.9) and 1.5 ml of induced extract in the bulb. After evacuation and mixing, the tubes were incubated for 45 min, before the reaction was stopped with acid, spun, and the supernate retained.

Ion exchange column fractions of extracts containing high levels of catechol 2,3-oxygenase activity were pooled and heat treated at 55 °C for 2 minutes to remove any hydrolase activity (see Results, 5.3.4). The 3-vinylcatechol solution was made up to 100 ml with 50 mM phosphate buffer, pH 7.0, and adjusted to pH 7.0 with 5M NaOH, and then incubated at 30 °C in a water bath, aerated continuously through a sintered glass sparger. The ion exchange fractions were then gradually added, and the solution went a deep yellow. Sampling of small amounts by absorbance measurements was used to monitor the accumulation of the semialdehyde. When accumulation had ceased, the reaction was stopped with 10% TCA (0 °C), upon which the colour changed from deep yellow to deep red. Precipitated protein was centrifuged off, and the supernate was extracted with 5*50 ml ethyl acetate, and dried with anhydrous sodium sulphate. The ethyl acetate extract was then evaporated down to 25 ml in the rotary evaporator, and dried again. This extract was then extracted with 50% concentrated NH_3, 4*3 ml. The first two extractions were deep red, the next yellow and the last colourless, and when all were mixed they were red.

2.6 Measurements of rates of oxygen uptake by intact cells and extracts

2.6.1 Simultaneous induction experiments

Simultaneous induction experiments were carried out using a Clark-type oxygen electrode (Clark, 1956) obtained from Rank Brothers (Cambridge, UK). The electrode was kept at a constant temperature of 30 °C by means of a warm water jacket.

The reaction buffer was usually 50 mM phosphate, pH 7.0, and it was kept aerated by stirring vigorously in a 30 °C water bath during the experiment. The well in the oxygen electrode was filled with 2.8 ml of this buffer, and left for some minutes until the oxygen concentration, as displayed on a chart recorder, had stabilised. The chart recorder scale was adjusted to 100 %. The addition of 100 µl of cell suspension (see below) was followed by the stopper being screwed on until the solution level was part way up the narrow channel, avoiding air bubbles.

The rate of oxygen consumption without substrate - the endogenous consumption - was then measured over at least 5 minutes. The substrate, routinely 100 µl of a 10 mM solution (final concentration 0.33 mM) was then added through the narrow channel, and the height of the stopper was adjusted to leave the reaction mixture at the same level within it as before. The rate of oxygen consumption was then plotted for at least five minutes. The difference between this rate and the endogenous rate was taken to be the increase in oxygen consumption due to the substrate. The rates, in nmol O_2/min/mg protein were calculated from the gradients plotted on the chart recorder, the concentration of oxygen in the vessel (see below), and the known protein concentration of the sample.

After each experiment the reaction mixture was sucked out and the vessel was rinsed at least three times with distilled water.

a) Preparation of cell suspension

Cells were harvested and washed as described earlier (Section 2.4.5), then they were resuspended in 50 mM phosphate buffer, pH 7.0, to give a protein concentration usually

between 0.5 and 5.0 mg /ml; usually x g of cells were resuspended in 3x ml of buffer. Cell suspensions were stored on ice during the experiment.

b) Calibration

The oxygen electrode was calibrated by use of the oxidation of known concentrations of NADH by *N*-methylphenazonium methosulphate (PMS), in the presence of catalase to remove any H_2O_2 produced. This leads to the following stoichiometry (Robinson & Cooper, 1970):

$$NADH + H^+ + 0.5\ O_2 \rightarrow NAD^+ + H_2O$$

The oxygen electrode was set up as described above, with 2.8 ml of phosphate buffer. Catalase was then added (400 µg in 100 µl of buffer) and the top was screwed on the electrode, then the mixture was left for a few minutes until the chart recorder was stable. PMS was then added (20 µg in 100 µl of buffer) and the mixture was again left to stabilise. Additions of NADH were then made (concentrations calculated from A_{340} and using $\varepsilon = 6.3\ mM^{-1}cm^{-1}$ (Boehringer, 1987)), either 0.083 µmoles in 10 µl buffer or 0.176 µmoles in 20 µl buffer. This led to a rapid consumption of oxygen, followed by a stabilisation, and was repeated several times during a single experiment. By measuring the changes in the chart recorder reading on addition of the known quantities of NADH it was possible to calculate the oxygen concentration in a typical oxygen electrode assay as 277 nmol/ml.

2.6.2 Attempted re-activation of oxygenase activity

This method is a modification of that used by Bernhardt & Meisch (1980). Cell-free extracts in MEG buffer (Section 2.4.6) of cells grown on styrene and nutrient broth in a fermenter and cells grown on nutrient broth were treated as described below:

1) A re-activation mixture was made up, consisting of 15 mg of EDTA, 0.1 g DTT, 0.3 ml 2-mercaptoethanol and 26 mg of sodium dithionite in 20 ml of MEG buffer.

2) An equal volume of extract and re-activation mixture were mixed, and a top was placed on the vessel, after the air space had been filled with nitrogen gas. This was then left on ice for 1 hour.

3) $FeSO_4$ was added to the reaction mixture, to a final concentration of 2 mM. The mixture was left on ice for a further hour.

2.7 Enzyme assay procedures

All spectrophotometric assays were done at 30 °C.

2.7.1 *cis*-Glycol dehydrogenase assay

The reaction mixture consisted of 2.8 ml of 50 mM phosphate buffer (pH 7.0), 50 µl of extract and 50 µl of 7 mM toluene *cis*-glycol (final concentration of 0.12 mM, dissolved in buffer, having been re-crystallised from ethyl acetate, as below) or 5 µl of approx. 9 mg/ml styrene *cis*-glycol in ethyl acetate (final concentration approx. 0.1 mM). The reaction was started with 100 µl of 50 mM NAD (final concentration 1.67 mM). The rate of production of NADH was measured at 340 nm, and rates were calculated using an $\varepsilon = 6.3\ mM^{-1}cm^{-1}$ (Boehringer, 1987).

a) Re-crystallisation of toluene *cis*-glycol

Toluene *cis*-glycol was supplied by ICI as a 20 % solution in ethyl acetate, with 0.1 % triethylamine as stabiliser. The toluene *cis*-glycol was re-crystallised from this solution using cold hexane. An ice-salt slurry was made up, with a temperature of approximately -10 °C, and this was used to cool hexane and 1 ml of toluene *cis*-glycol in ethyl acetate. Once cooled, 4 ml of hexane was added to the *cis*-glycol, and as a brown precipitate formed the solution was immediately filtered using Whatman No. 1 Qualitative filter paper, which was disposed of. A white crystalline suspension then formed in the hexane. These crystals were filtered off and rinsed with hexane, and were stored at -20 °C. This procedure gave 51 mg of crystalline toluene *cis*-glycol.

2.7.2 Catechol 1,2-oxygenase assay

The reaction mixture consisted of 2.85 ml of 50 mM phosphate buffer (pH 7.0), 50 µl of extract and the reaction was started by addition of 100 µl of a 3 mM solution of the substrate (catechol, 3-methylcatechol or 4-methylcatechol) in buffer, giving a final concentration of 0.1 mM (Gibson, 1971). EDTA was added, when specified, at a final concentration of 1.3 mM. The reaction was monitored by measuring the accumulation of the 1,2-cleavage products at 260 nm. Absorption coefficients of cleavage products used in calculating rates were: catechol, ε=16.8 $mM^{-1}cm^{-1}$; 3-methylcatechol, ε=18.0 $mM^{-1}cm^{-1}$; 4-methylcatechol, ε=13.9 $mM^{-1}cm^{-1}$ (Dorn & Knackmuss, 1978b).

2.7.3 Catechol 2,3-oxygenase assay

The reaction mixture consisted of 2.85 ml of 50 mM phosphate buffer (pH 7.0) and 50 µl of extract which had been heat treated at 55 °C for 5 minutes (Section 2.4.7). The reaction was started by addition of 100 µl of a 10 mM solution of the substrate (catechol, 3-methylcatechol or 4-methylcatechol) in buffer, giving a final concentration of 0.3 mM (Gibson, 1971). The reaction was monitored by measuring the accumulation of the 2,3-cleavage products of the catechols. Absorption coefficients used for calculating rates were: catechol, ε=48.4 $mM^{-1}cm^{-1}$ at 375 nm; 3-methylcatechol, ε=19.8 $mM^{-1}cm^{-1}$ at 390 nm; 4-methylcatechol, ε=33.2 $mM^{-1}cm^{-1}$ at 380 nm (Wallis & Chapman, 1990).

2.7.4 2-Hydroxymuconic acid semialdehyde hydrolase

The reaction mixture consisted of 2.85 ml of 50 mM phosphate buffer (pH 7.0) and 2-hydroxymuconic acid semialdehyde, which was prepared from heat treated extract and catechol, as described below, and was added at the concentrations specified. The assay was pre-incubated for 5 minutes to allow the A_{375} to stabilise before the reaction was started by addition of 50 µl of extract. The reaction was monitored by following the removal of 2-hydroxymuconic acid semialdehyde at 375 nm, using ε_{375}=48.4 $mM^{-1}cm^{-1}$ (Wallis & Chapman, 1990).

a) Production of 2-hydroxymuconic acid semialdehyde

This substrate was produced when required by the action of 1 ml of heat treated extract (from cells grown on styrene and nutrient broth) on catechol. The extract was heat treated as described in Section 2.4.7. To 2 ml of 50 mM phosphate buffer, pH 7.0, was added 0.5 ml of a solution containing 5 mg catechol/ml (i.e. 0.25 mg) and 600 µl of heat treated extract. This mixture was allowed to react at 30 °C and stirred regularly. The accumulation of yellow 2-hydroxymuconic acid semialdehyde was monitored by taking 50 µl samples, diluting them with 2.95 ml of buffer, then measuring A_{375}. Once the absorbance had stabilised the reaction was chilled on ice and stopped with 3 drops of 5 M HCl. The precipitated protein was then removed using an eppendorf centrifuge (Section 2.3.7). The supernate was then stored on ice until used.

2.8 Ion exchange chromatography of extracts

All procedures were carried out at 4 °C.

Cells which had been grown in the fermenter on nutrient broth/styrene (7.7 g) were suspended in 23 ml of a buffer containing 20 mM Tris pH 7.5, 10 % ethanol, 10 % glycerol and 1 mM DTT (TEGD), before being broken by 3 passes in a French press and centrifuged as normal to obtain 17 ml of high-spin supernate (Section 2.4.6)

Extract (15 ml) was applied at 20 ml/hr to a column packed with DEAE sephacel super fine (7 cm * 2.6 cm, volume 20 ml) which had been pre-equilibrated with TEGD buffer. The column was washed with approximately two column volumes of TEGD buffer at 20 ml/hr, and then with approximately two column volumes of 0.15 M NaCl/TEGD buffer. A linear 0.15 - 1 M NaCl/TEGD gradient was used to elute enzyme activities. The gradient volume was 300 ml, and the flow rate was 20 ml/hr. When the gradient had finished, 2M NaCl/TEGD was used to remove any traces of bound material. Fractions of 5 ml were collected until fraction 30, when the fraction size was reduced to 2 ml.

2.8.1 Enzyme assays

Enzyme activities in fractions were assayed using essentially the same procedures as described above (Sections 2.7 - 2.7.4), but with slight differences in the reaction mixtures:

a) Toluene *cis*-glycol dehydrogenase

The reaction mixture consisted of 2.75 ml of buffer (50 mM phosphate, pH 7.0), 100 µl of the fraction under test and 100 µl of 50 mM NAD, and was started with 50 µl of 7 mM toluene *cis*-glycol.

b) Styrene *cis*-glycol dehydrogenase

The reaction mixture contained 2.75 ml of buffer, 100 µl of the fraction under test and 100 µl of 50 mM NAD, and was started with 5 µl of 9 mg/ml styrene *cis*-glycol in ethyl acetate.

c) Catechol 2,3-oxygenase

The reaction mixture contained 2.85 ml buffer, 50 µl of the fraction under test, and the reaction was started with 100 µl of a 10 mM solution of catechol.

d) Catechol 1,2-oxygenase

The reaction mixture contained 2.8 ml buffer, 100 µl of the fraction under test, and the reaction was started with 100 µl of a 10 mM solution of catechol.

e) 2-Hydroxymuconic acid semialdehyde hydrolase

The reaction mixture contained 2.7 ml buffer, 100 µl of the fraction under test, and the reaction was started with 200 µl of a solution of 2-hydroxymuconic acid semialdehyde , made as described above (Section 2.7.4a).

2.8.2 Heat inactivation of *cis*-glycol dehydrogenase activity

Column fractions with high *cis*-glycol dehydrogenase activity were mixed and then heated in a water bath at 34 °C, with samples taken for assay at various times. The styrene and toluene *cis*-glycol dehydrogenase activities were assayed as described above, except the assay was started with 50 µl of the heat treated extract.

3. Characterisation of the organisms and evaluation of possible pathways for the metabolism of styrene

3.1 Initial characterisation

Preliminary tests were done on all of the eight strains that had been isolated by enrichment on styrene (Methods 2.4.1) in order to eliminate those strains which had similar characteristics or which had problems with growth (e.g. very slow growth, clumping etc.). All strains were found to be Gram positive (Methods 2.4.3a) and oxidase negative (Methods 2.4.3b). Growth was tested on a variety of possible breakdown products of styrene, and a few simultaneous induction tests were done. From these results it was decided that strains 79 and 80 were similar to each other, as were strains 68, 125, 126 and 137. Initially, four strains were selected for further work: strains 26, 67, 80 and 137. However, strain 67 was subsequently eliminated after problems with growth.

3.2 Further characterisation of three strains

3.2.1 Identification

Preliminary identification of the three selected strains was made by NCIMB Ltd, Aberdeen (Table 3.1). Photographs of the three strains growing on nutrient agar are in Figure 3.1, showing the morphological variation of these strains during growth. All three strains were found to be Gram positive, and appear to be coryneform bacteria.

3.2.2 Growth tests

Each of the three strains was grown on a selection of substrates. Most substrates chosen are possible intermediates on the pathway of styrene breakdown (Introduction, Figure 1.34). Toluene and ethylbenzene were also used, to test the versatility of aromatic breakdown. Growth experiments provide an indication of substrates that an

Table 3.1: Preliminary identification of strains 26, 80 and 137

Test	Strain 26	Strain 80	Strain 137
Cell morphology (see also Fig 3.1) [1]	1 day: rods, chains 14 days: oval	1 day: rods, chains 14 days: rods	1 day: rods, chains 14 days: oval
Gram stain [2]	+	+	+
Spores	-	-	-
Motility	-	-	-
Colony morphology [3]	Salmon pink, opaque, round, regular, entire, shiny, convex <0.5 mm diameter	Salmon pink, opaque, round, regular, entire, matt, convex <0.5 mm diameter	Cream, opaque, round, regular, entire, shiny, convex <0.5 mm diameter
Growth temperature	25°C + 37°C + 45°C (+)	25°C + 37°C + 45°C (+)	25°C + 37°C + 45°C -
Catalase	+	+	+
Oxidase	-	-	-
Fermentative in glucose OF	-	-	-
Preliminary identification	**coryneform species; possibly *Rhodococcus***	**coryneform species; possibly *Rhodococcus***	**coryneform species**

Identification performed by NCIMB Ltd, Aberdeen (Methods, 2.4.3c).

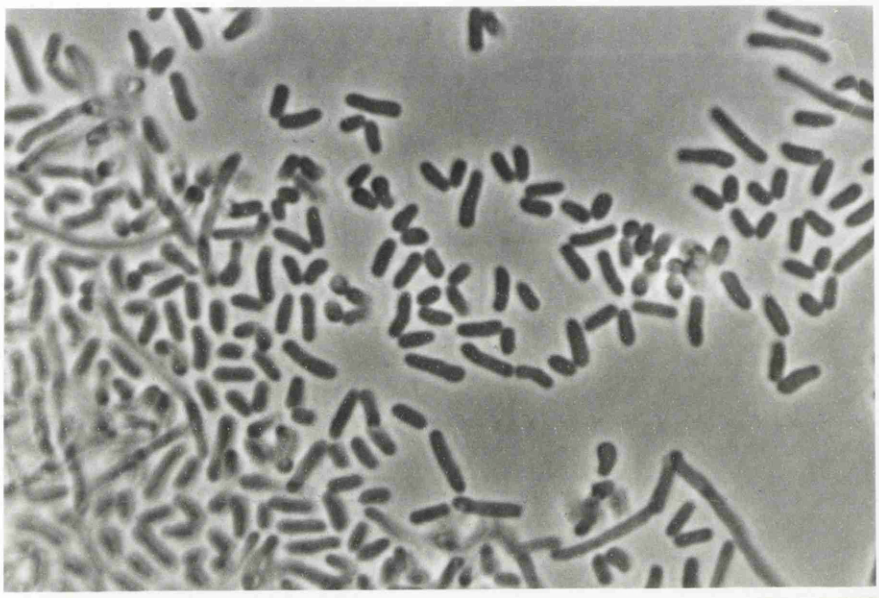
[1] Growth on nutrient agar
[2] Gram test initially performed as Methods 2.4.3a, then repeated by NCIMB
[3] After 48 hours growth on nutrient agar

Figure 3.1: Photographs of the three isolates after growth on nutrient agar for 1 day and 14 days

Strain 26

1 day:

10 μm



14 days:

10 μm

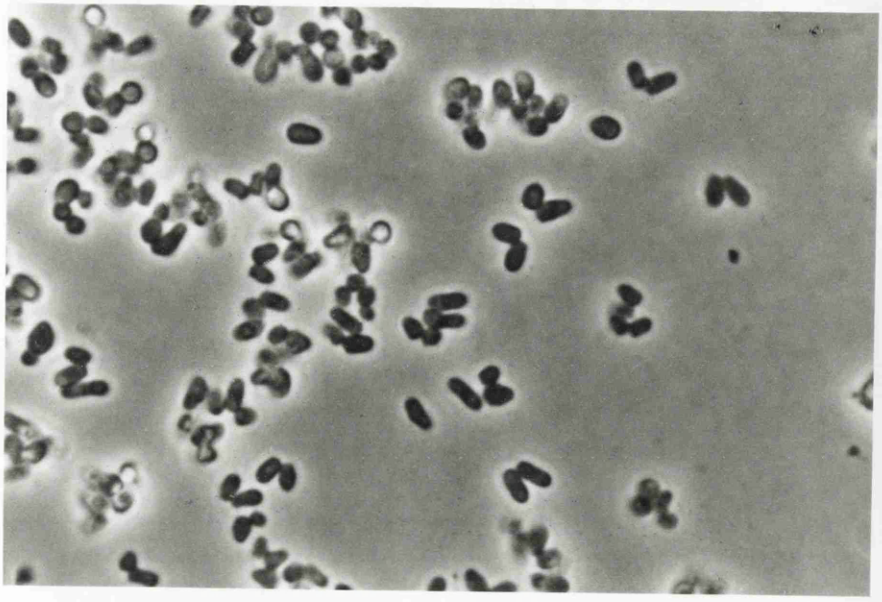
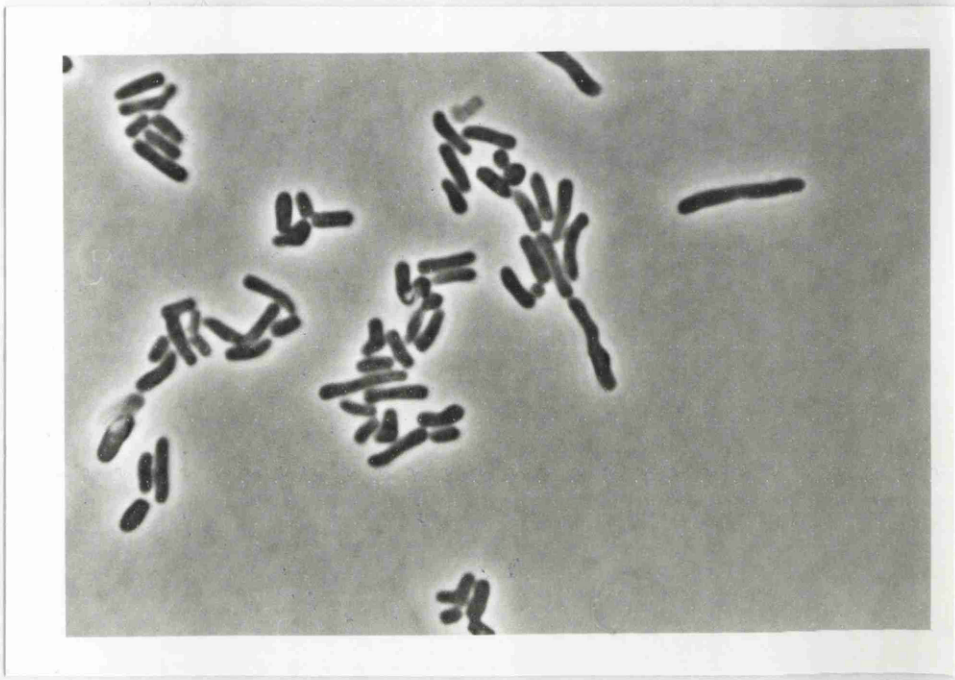


Figure 3.1 continued

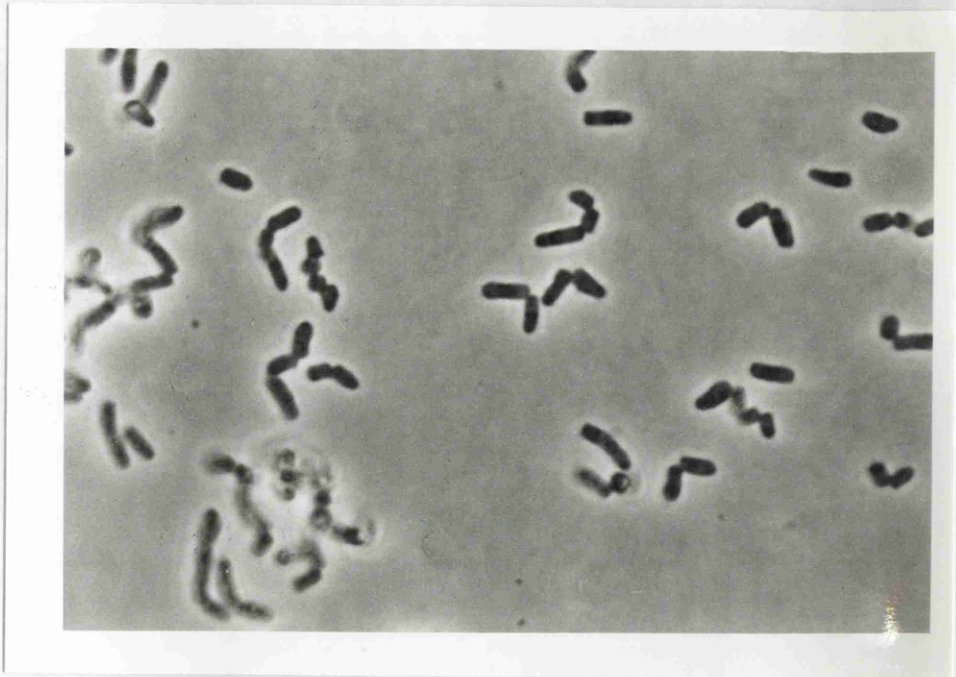
Strain 80

1 day:



10 μm

14 days:



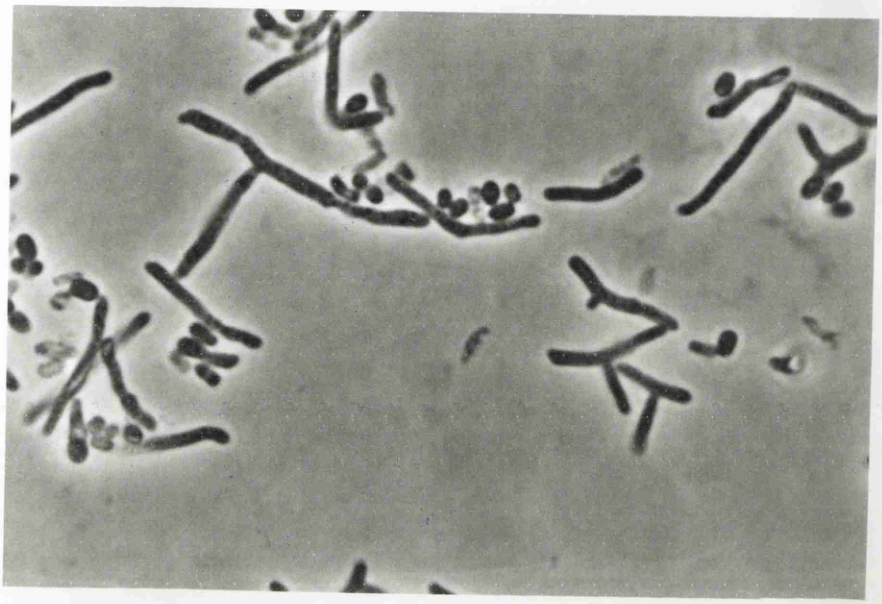
10 μm

Figure 3.1 continued

Strain 137

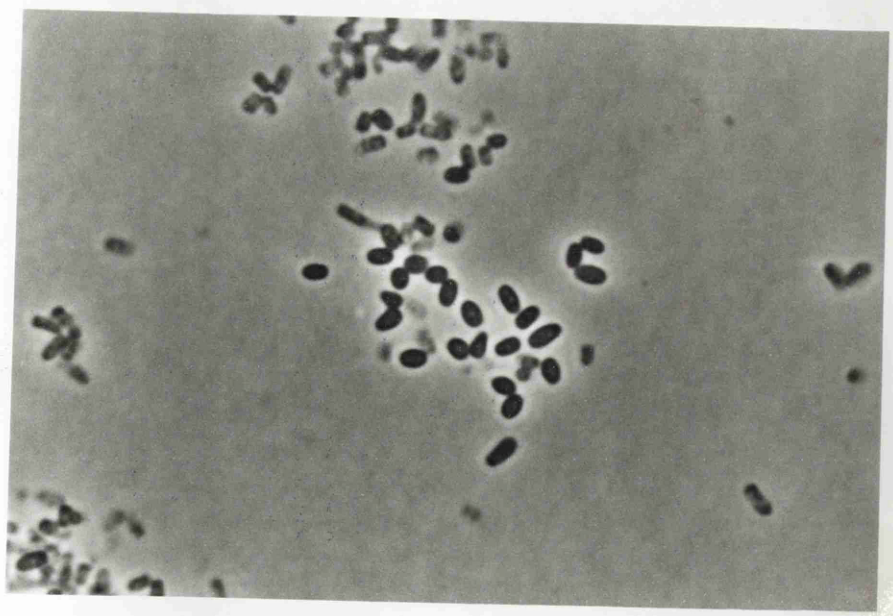
1 day:

10 μm



14 days:

10 μm



organism is capable of metabolising, though substrates that are toxic or do not pass through the cell membrane will score negative, even if metabolism within the cell is possible.

Strains 80 and 137 could grow on the widest range of substrates (Table 3.2). All strains could grow on ethylbenzene, and strains 26 and 137 grew on toluene. Yellowing was noted in the medium when strain 137 was grown on acetophenone or phenylethanediol, and when strain 26 was grown on acetophenone or 1-phenylethanol. A smell similar to acetophenone was noted when strain 26 was grown on 1-phenylethanol.

No strain was able to grow on mandelic acid, a possible breakdown product of styrene, but both strains 80 and 137 were able to grow on the compounds produced by the breakdown of styrene via either the phenylacetaldehyde/phenylacetic acid route or the 1-phenylethanol/acetophenone route (Introduction, Figures 1.34, 1.35, 1.36). Strain 26, however, was unable to grow on styrene oxide, phenylacetaldehyde, phenylacetic acid or acetophenone.

The growth of all three strains on styrene in a fermenter was tested (for full details of growth in a fermenter see 3.3.2b). Strains 26 and 137 grew normally, but strain 80 did not grow within the fermenter medium, but instead grew attached to the rotor and the glass walls of the fermenter, above the level of the medium.

3.2.3 Simultaneous induction experiments

Simultaneous induction is another method of elucidating pathways, measuring induction of the ability to oxidise substrates rather than growth. Cells grown on one substrate, in this case styrene, are washed and then their oxygen consumption is monitored with styrene and other substrates in a Clarke-type oxygen electrode (Methods 2.6.1). If a substrate results in an increase in oxygen consumption above the endogenous rate, it can be hypothesised that enzymes involved in its metabolism have been induced. It may then be suggested that those substrates that are oxidised are on the pathway of breakdown of the growth substrate, whereas substrates that are not oxidised

Table 3.2: Growth of strains 26, 80 and 137 on various substrates

Growth substrates were all tested at 1 mM except where shown otherwise. Cultures were grown in flasks as in Methods 2.4.4f for 5 days, then the OD_{500} was measured. All substrates were tested at least twice.

Key: - Not significantly different from control (no substrate)
+ OD_{500} less than 0.5
++ OD_{500} more than 0.5

Substrate	Strain 26	Strain 80	Strain 137
Styrene	++	++	++ [4]
Styrene oxide	- [2]	++	+
1-Phenylethanol	++ [6]	++	+
2-Phenylethanol	- [5]	++	++
Acetophenone	- [4,2]	++	+ [4]
Phenylacetaldehyde	- [2]	++	- [5]
Phenylethanediol [3]	++	++	+ [4]
Phenylacetic acid [3]	- [2]	++	++
Mandelic acid [3]	-	-	-
Toluene	++	-	+
Ethylbenzene	+ [1]	++	++

[1] In some experiments no visible growth occurred, probably because bacteria were killed by the substrate
[2] Substrate concentration 0.5 mM
[3] Substrate concentration 5 mM, 50 ml flask as Methods 2.4.4g
[4] Yellowing noted in medium
[5] Growth occurred on vapour (Methods 2.4.2a, with substrate replacing styrene)
[6] Yellowing noted in medium, odour similar to acetophenone

are not on the breakdown pathway. Problems arise if a substrate cannot be transported into the cell, giving a false negative result, even though it is part of a breakdown route within the cell. False positives can arise due to substrate and inducer ambiguity of enzymes.

Strain 26 gave high rates of oxygen uptake with the phenylethanols, toluene, ethylbenzene and toluene *cis*-glycol, but much lower rates of oxidation of acetophenone, styrene oxide and phenylacetaldehyde (Table 3.3). Strain 80, however, showed very high rates of oxygen uptake with styrene oxide, phenylacetaldehyde and phenylacetic acid. An 'aldehydic smell' was noticed when styrene oxide was added to strain 80. Strain 137 showed the properties of both strains 26 and 80, giving high rates of oxygen uptake with a wide range of substrates.

3.2.4 Discussion of growth and simultaneous induction of the three strains

The aim of these two sets of experiments was to provide pointers towards establishing the pathway of styrene breakdown in the three strains. The growth experiments suggested that both strains 80 and 137 were capable of growth on the intermediates of either the acetophenone pathway or the phenylacetaldehyde pathway. In contrast, strain 26 was unable to grow on any of the substrates from lower down the three side chain oxidation pathways (Introduction, Figures 1.34, 1.35, 1.36).

The occurrence of yellowing in the medium of strain 137 when grown on styrene, acetophenone and phenylethanediol and of strain 26 when grown on acetophenone and 1-phenylethanol suggest the formation of a *meta*- cleavage product. When strain 26 was grown on 1-phenylethanol, an acetophenone-like smell was noted. Acetophenone does not support growth in this strain, so it is possible that an alcohol dehydrogenase activity is transforming the 1-phenylethanol to acetophenone, which then accumulates.

The simultaneous induction responses are rather more revealing. If the results are superimposed on the various possible pathways for degradation of styrene, clear patterns are visible. Strain 80 has the most straightforward results (Fig 3.2) which show

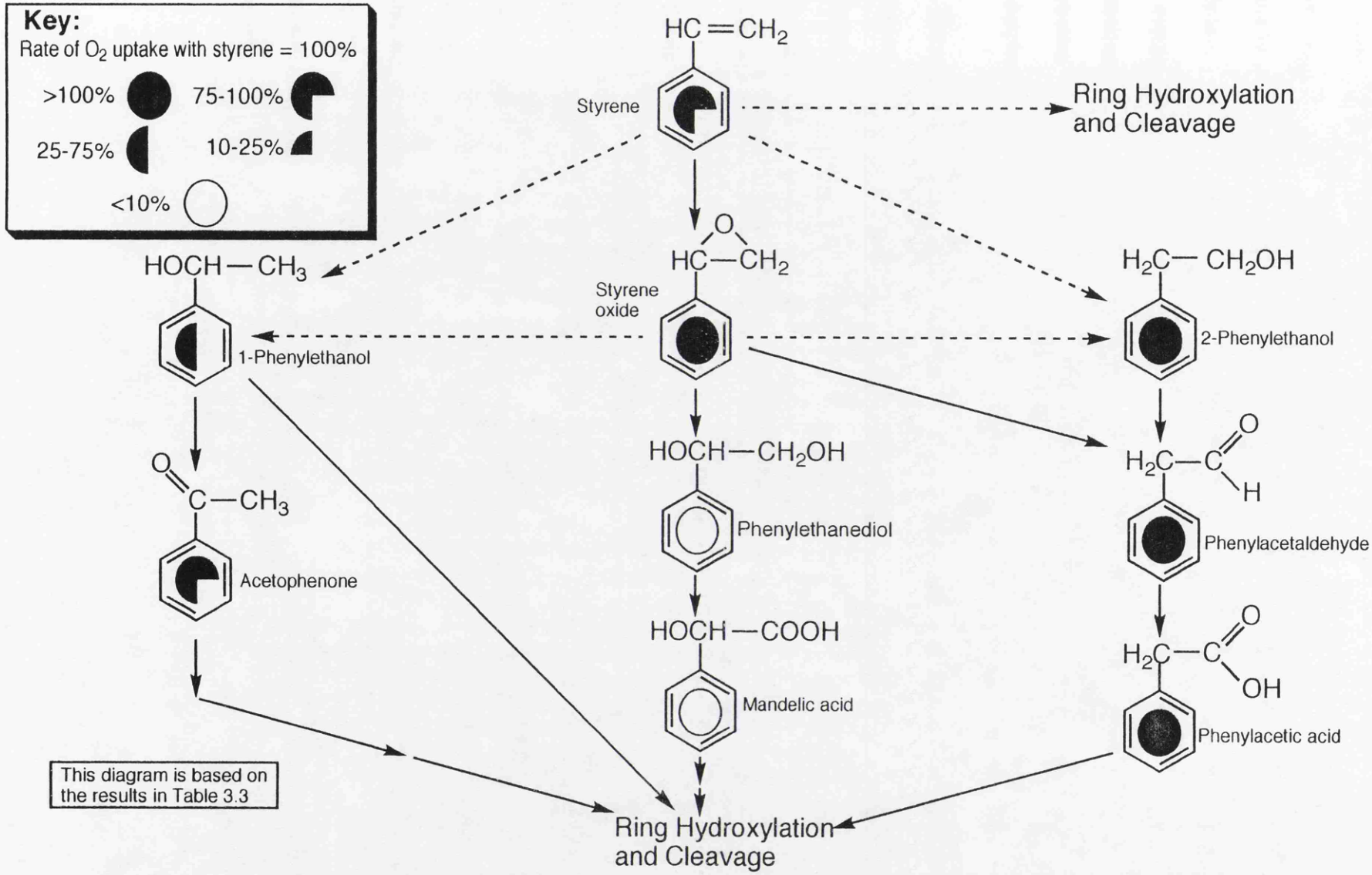
Table 3.3: Simultaneous induction tests on strains 26, 80 and 137 grown on styrene

Strains 80 and 137 were grown on styrene in flasks as described in Methods 2.4.4f, strain 26 was grown in a fermenter as described in Methods 2.4.4i Substrates were prepared as in Methods 2.6.1 and 100 µl of 10 mM (or saturated, if solubility less than 10 mM) solutions were tested (final concentration = 0.33 mM), except for toluene *cis*-glycol, for which 50 µl of 7 mM solution (final concentration = 0.12 mM) was used. All results are means of at least two determinations. The results for strain 137 are shown for two batches, because of a large difference in oxygen consumption with styrene between the batches. Protein was measured by the modified Bradford method, Methods 2.3.5.

Substrate	Rate of oxygen uptake (nmol/min/mg protein)			
	Strain 26	Strain 80	Strain 137	
Styrene	140	120	690	160
Styrene oxide	40	410 [1]	180	
1-Phenylethanol	90	60	330	
2-Phenylethanol	120	130	360	
Acetophenone	20	100		30
Phenylacetaldehyde	40	460		110
Phenylethanediol	50	<10		20
Phenylacetic acid	0	340		100
Mandelic acid	<10	<10		<10
Toluene	140	20	440	
Toluene *cis*-glycol	270	<10		640
Ethylbenzene	180	20	600	180
Acrylic acid	20	40		80

[1] 'Aldehydic' smell noticed after substrate addition

Figure 3.2: Ability of strain 80 to oxidise various substrates after growth on styrene



that cells grown on styrene have a very high rate of oxygen consumption when given substrates from the styrene oxide/ phenylacetaldehyde pathway, compared with much lower responses for substrates from the 1-phenylethanol/acetophenone pathway, and virtually no response with intermediates of the phenylethanediol/mandelic acid route. In addition, an 'aldehydic' smell was noted when styrene oxide was added to strain 80 in the oxygen electrode. This could be phenylacetaldehyde, possibly produced by isomerisation from styrene oxide as found in a Xanthobacter strain (Figure 1.35; Hartmans *et al.*, 1989) These results strongly suggest the styrene oxide/phenylacetaldehyde route as the pathway of styrene breakdown in strain 80.

The results for strain 26 are far less clear (Fig 3.3). The cells showed some oxidation of the first or second substrates in the pathways, but there was little or no response to those at a stage lower eg. acetophenone and mandelate (on which it cannot grow; Table 3.2), or phenylacetaldehyde. These results suggest that strain 26 does not use any of the routes of styrene breakdown illustrated in Fig 3.3.

Strain 137 (Fig 3.4) is rather similar to strain 26, with little response to the substrates lower down the pathways. However, it is possible that 137 could be using the phenylacetaldehyde route, since there is a response to every substrate on this route.

At this stage it was decided that one organism should be chosen for further work from the three under investigation. Strain 80 appears to degrade styrene *via* the established phenylacetaldehyde route, so it was eliminated. Strain 137 could also be using this route, though this is less clear; but strain 26 almost certainly uses a different route and so it was chosen for further research, because of the apparent novelty of its styrene breakdown pathway.

3.3 Characterisation of Strain 26

3.3.1 Identification

Preliminary identification of Strain 26 suggested that it could be a *Rhodococcus* species (Table 3.1). Further identification to species level was quite straightforward

Figure 3.3: Ability of strain 26 to oxidise various substrates after growth on styrene

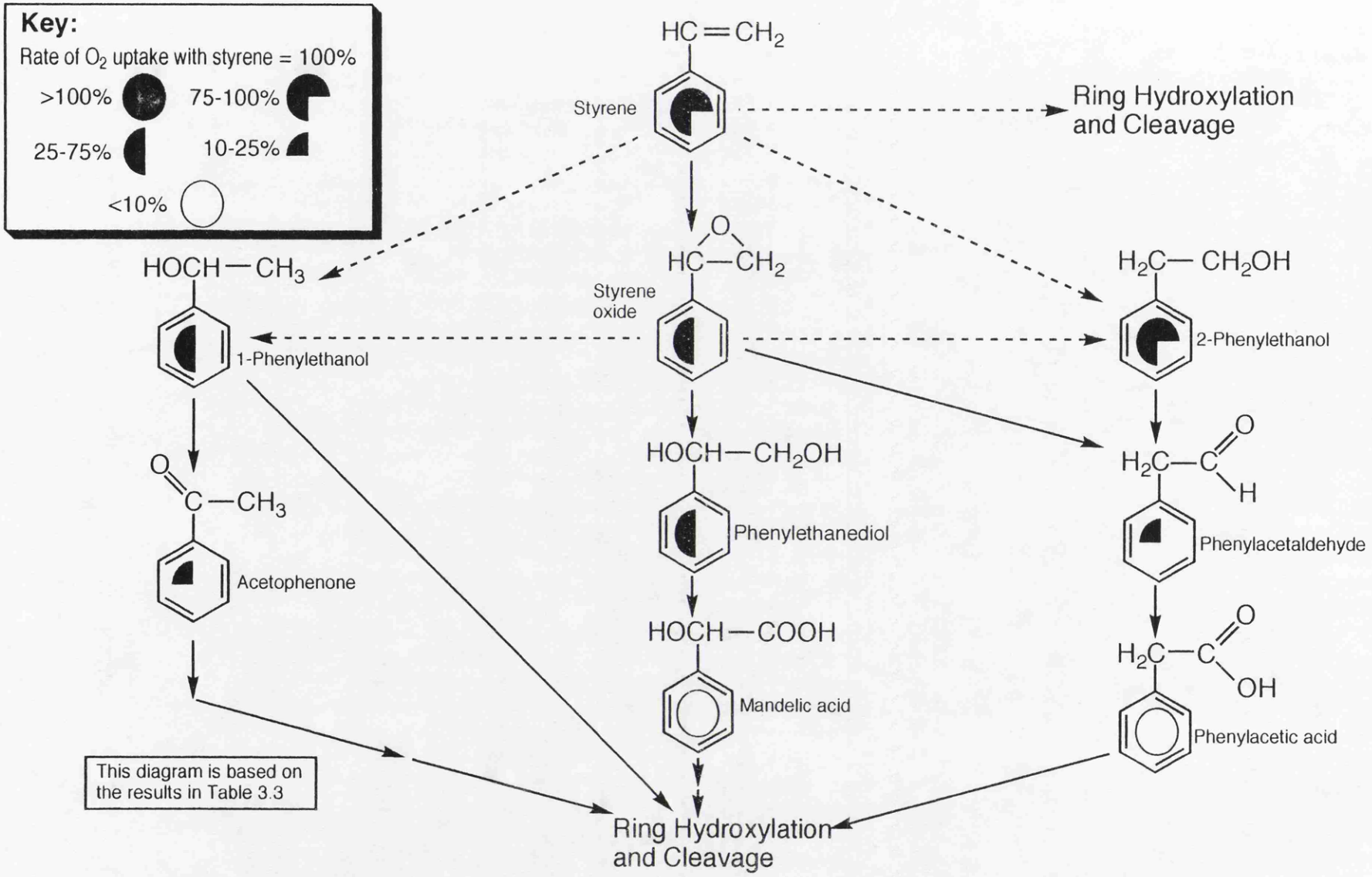
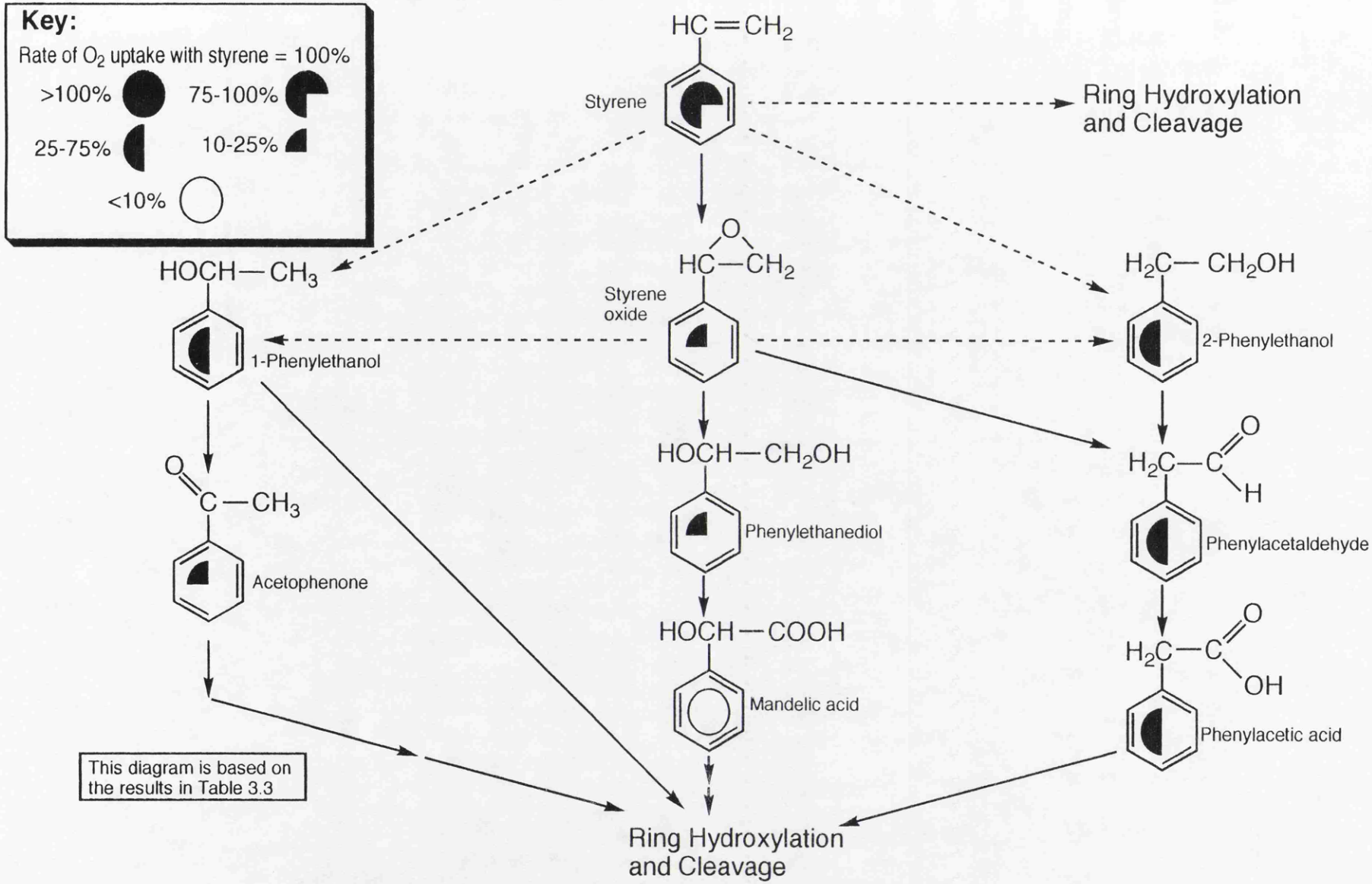


Figure 3.4: Ability of strain 137 to oxidise various substrates after growth on styrene



using analysis of the cell wall and fatty acid profiles (see Introduction, 1.1.2). These tests were performed by the NCIMB in Aberdeen (results in Table 3.4). Apart from two growth results, all the tests indicated that strain 26 is a strain of *Rhodococcus rhodochrous*.

3.3.2 Growth methods

In order to produce sufficient biomass for experiments, methods had to be developed to grow *Rhodococcus rhodochrous* 26 on styrene. Many problems were encountered in developing appropriate systems, largely because of the toxicity of the substrate. It was found that apparent concentrations of styrene of greater than 1 mM generally led to cell death or pelleting of the bacterium. The solubility of styrene in water at 30°C is only about 1.5 mM (Hartmans *et al.*, 1990), so a two phase system exists above this concentration, which is likely to damage cell membranes. Iron was also found to improve growth, so was routinely added to media.

a) Flask

Cells were grown in gas-tight conical flasks as in Methods 2.4.4f. Styrene was added gradually, starting with 1 mM, with more added when growth was observed. Growth was very variable between and within batches of inoculum. Small inocula (1 ml nutrient broth culture per 100 ml medium) often led to cell death. Higher levels of inoculation, for example 5 ml nutrient broth culture per 100 ml medium, were used to try to reduce this variability. Some growth curves are shown in Figure 3.5a, demonstrating the variability of cultures, even using a 5 ml inoculum, in otherwise apparently identical experiments.

This method was also used to grow *R. rhodochrous* 26 on other toxic substrates, such as toluene, ethylbenzene and benzene.

b) Fermenter

A 10 l fermenter was used to produce larger quantities of induced cells. A minimal salts medium was used, with a 50 mM MES buffer at pH 7.0, to prevent the fall in pH that occurred with only phosphate buffering in the styrene salts medium. Styrene was

Table 3.4: Identification of strain 26

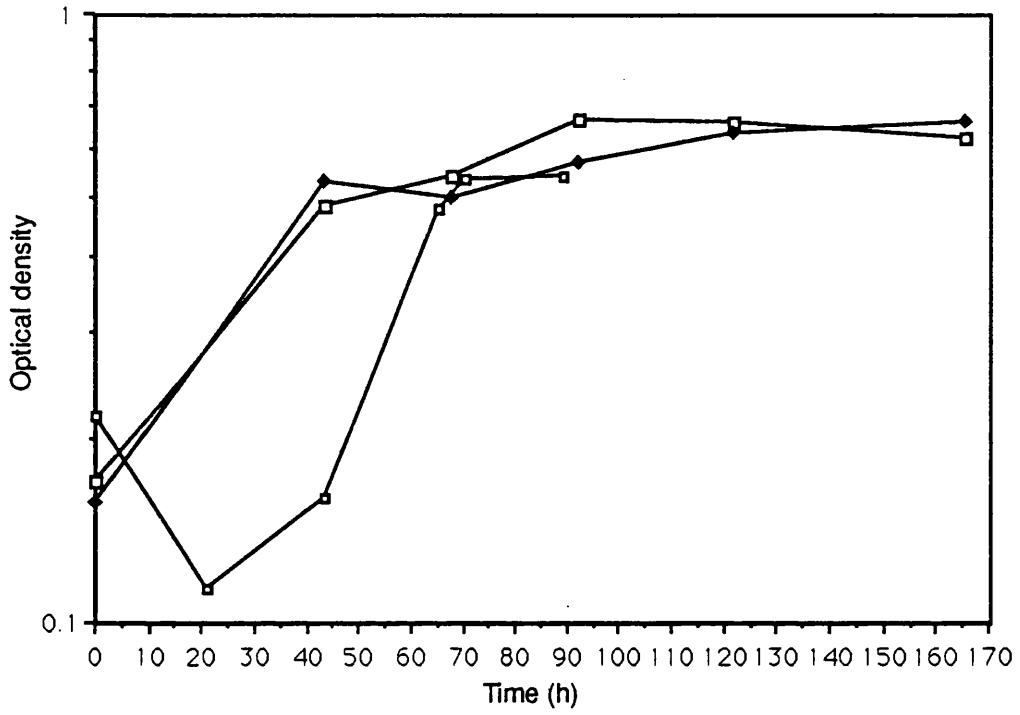
Test	Result
Analysis	Mycolic acids present Cell wall diamino acid *meso*-DAP
GC Fatty acid profile	Tetradecanoic acid, $C_{14:0}$ Pentadecanoic acid, $C_{15:0}$ Hexadecenoic acid, $C_{16:1}$ Hexadecanoic acid, $C_{16:0}$ Heptadecenoic acid, $C_{17:1}$ Heptadecanoic acid, $C_{17:0}$ Octadecenoic acid, $C_{18:1}$ Octadecanoic acid, $C_{18:0}$ Tuberculostearic acid, $10MeC_{18:0}$
All results below were recorded after 10 days at 30 °C	
Decomposition of:	Adenine - Tyrosine + Urea -
Growth on sole carbon sources	Inositol [1] - Maltose - Mannitol + Rhamnose - Sorbitol + *m*-Hydroxybenzoic acid [2] + Sodium adipate (+) Sodium benzoate + Sodium citrate + Sodium lactate + Testosterone - L-Tyrosine + Glycerol [1] + Trehalose + *p*-Hydroxybenzoic acid [2] +
Growth in 5% NaCl	+
Growth at 10°C	+
Growth in azide [3]	-
ONPG	-
Conclusion	**Most closely resembles biochemical profile of *Rhodococcus rhodochrous*.** Atypical results: cannot grow on maltose or testosterone

Identification performed by NCIMB Ltd, Aberdeen.

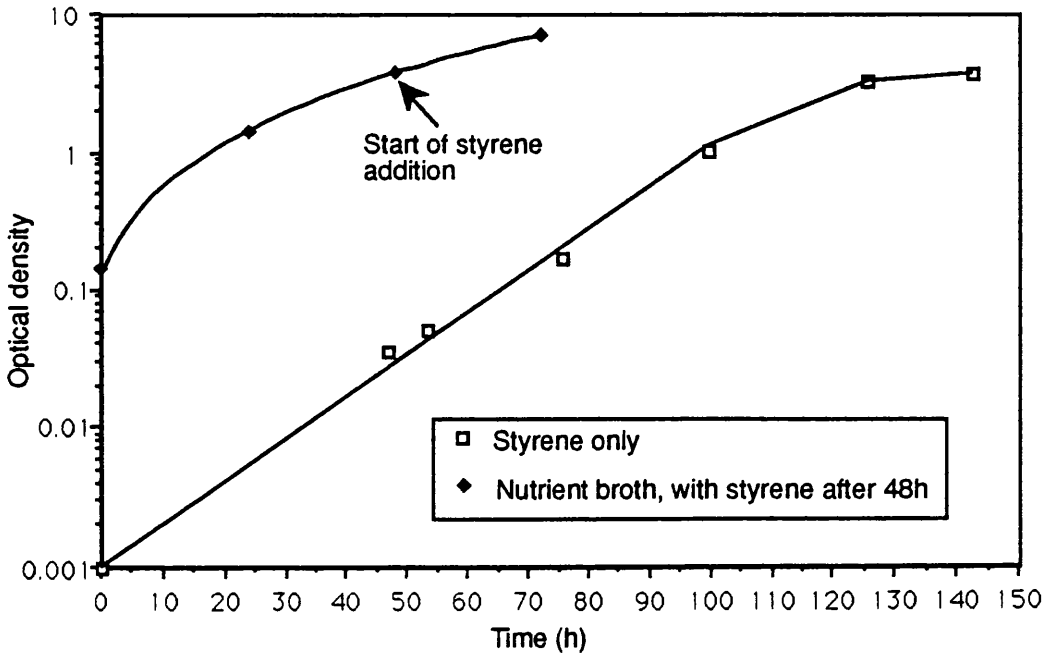
[1] 1% w/v
[2] 0.1% w/v
[3] 0.02% w/v

Figure 3.5: Growth of *R. rhodochrous* 26 on styrene

a) Three examples of growth on styrene in flasks (as Methods 2.4.4f, with a 5 ml inoculum)



b) Growth in the fermenter on styrene or nutrient broth and styrene (as in Methods 2.4.4i, k)



provided by bubbling about 10% of the air supply to the fermenter through liquid styrene (Methods 2.4.4i). The doubling time was approximately ten hours (Figure 3.5b), and after growth for 6 days around 15g wet weight of cells could be harvested from the 10 litres of culture medium. However, this culture method was not reliable, since on several occasions the bacteria failed to grow.

For even larger quantities of induced cells, cells were grown for 48 h on nutrient broth based medium in the fermenter before a flow of styrene-saturated air was started, then the cells were harvested 24 h later (Methods 2.4.4k; Figure 3.5b). This produced a yield of around 90 g wet weight per 10 l (Figure 3.5b), and using this procedure there were no problems with the culture not growing.

Activities of cells grown on styrene, using either fermenter method, towards styrene (measured using oxygen consumption in an oxygen electrode) were found to be similar, though with substantial variation between batches (Table 3.7). When grown in the fermenter on styrene, *R. rhodochrous* 26 always produces a yellowing of the medium (λ_{max} =401 nm), and another absorbance peak, λ_{max} = 283 nm.

3.3.3 Detailed growth tests

Preliminary experiments to discover the range of growth substrates had been performed earlier (Table 3.2). Results for a wider range of substrates are shown in Table 3.5. Although growth was quantified in these experiments, the values should be regarded with some caution because of the difficulties in measuring the OD_{500} due to occasional clumping, and also substantial inter-batch variation. *R. rhodochrous* 26 can grow on a wide range of aromatic compounds, for example toluene, benzene, ethylbenzene, α-methylstyrene, 1-phenylethanol, benzyl alcohol, benzaldehyde, benzoic acid, cinnamic acid and phenol. When grown on 1-phenylethanol, an odour similar to acetophenone was noted. *R. rhodochrous* 26 is unable to grow on the short chain alkane octane or the short chain alkene oct-1-ene (containing the -CH=CH_2 group, as in styrene), but it is able to grow on acetaldehyde or vinylacetic acid. *R. rhodochrous* 26 cannot grow on any of the xylenes, mandelic acid, β-methyl styrene or acrylic acid.

Table 3.5: Ability of *R. rhodochrous* 26 to grow on various potential substrates

After 4 days growth in either 100 ml gas tight flasks (1 mM and 0.5 mM substrates), or in 50 ml flasks (5 mM substrates), as Methods 2.4.4f and g, the OD_{500} was measured. The inoculum was 2.5 ml of nutrient broth culture for the 50 ml flasks and 5 ml of nutrient broth culture for the 100 ml flasks. When no substrate was added, the OD_{500} after 4 days was about 0.21 in 100 ml flasks and about 0.30 in 50 ml flasks. All substrates were tested at least twice.

Positive resuits:

Growth substrate	OD_{500} after 4 days		
	5 mM	1 mM	0.5 mM
1-Phenylethanol		0.58[1]	
2-Hydroxyphenylacetate	1.15		
2-Phenylethanol		0.33	0.17 [2]
3-Hydroxyphenylacetate	1.38		
4-Hydroxybenzoate	1.10		
α-Methylstyrene		0.35	0.38
Acetaldehyde	0.68		
Acetate	0.89		
Benzaldehyde		0.75	
Benzene		0.50	
Benzoic acid	1.34		
Benzyl alcohol		0.58	
Cinnamic acid		0.56	
Ethylbenzene			0.47 [3]
Glucose	1.33		
Phenol	1.02		
Phenyl acetate		0.55	
Phenylethanediol	0.59		
Styrene		0.59	
Succinate	0.56		
Toluene		0.50	
Vinylacetic acid	0.97		

[1] Odour similar to acetophenone, yellowing in medium
[2] Growth recorded on vapour, as Methods 2.4.2a
[3] In some experiments no visible growth occurred, probably because the bacteria were killed by the substrate

No growth was recorded on the following substrates:

At 5 mM, as Methods 2.4.4g: Phenylacetic acid *, DL-Mandelic acid, D-Mandelic acid *, L-Mandelic acid *, Phenylglyoxylic acid *, Methylsuccinic acid *, 4-Hydroxymandelic acid *

At 1 mM, as Methods 2.4.4f: β-Methylstyrene *, Phenylacetaldehyde *, Acetophenone * [1], *m*-Xylene *, *o*-Xylene *, *p*-Xylene *, Oct-1-ene *, Octane *, Acrylic acid (at 5 mM) *

* Also tested at 0.5 mM
[1] Yellowing in medium

3.3.4 Metabolism of toluene

The simultaneous induction results for *R. rhodochrous* 26 that had been grown on styrene (Section 3.2.3) did not give any positive indication of a possible breakdown pathway. However, *R. rhodochrous* 26 that had been grown on styrene could also oxidise toluene (Table 3.2). As mentioned earlier, toluene breakdown pathways have been extensively studied in a variety of organisms, and also have the advantage that most of the intermediates are easily available (Introduction 1.2.3). It was considered possible that both toluene and styrene might be degraded by similar pathways, so if the route of toluene breakdown could be established, the equivalent pathway for the breakdown of styrene could be hypothesised and then tested.

Two batches of *R. rhodochrous* 26 cells were tested for the ability to oxidise intermediates of various pathways of toluene degradation; one of cells grown in the fermenter on styrene, and the other of cells grown in a flask on toluene (Table 3.6). It is clear that there is very little difference in induction patterns between cells grown on styrene and those grown on toluene, after allowing for possible variation due to differences in growth stage and concentration of the growth substrates.

The pattern of oxygen consumption superimposed on the known pathways of toluene degradation (Fig 3.6) indicates that toluene is probably degraded through toluene *cis*-glycol and 3-methylcatechol, both by styrene-grown and toluene-grown cells. All the other routes seem to be ruled out because of low or very low oxygen consumption with at least some of the potential intermediates. It therefore appeared possible that styrene might be degraded through a similar route; this idea is explained further in subsequent sections.

3.3.5 Further induction experiments

a) Cells grown on glucose or nutrient broth

Few of the substrates tested were oxidised by cells grown on glucose, the exceptions being glucose, acetate, acetaldehyde, acrylate and propionate (Table 3.7).

Table 3.6: Comparison of simultaneous induction patterns of *R. rhodochrous* 26 grown on styrene and toluene

R. rhodochrous 26 was grown in flasks on toluene as in Methods 2.4.4f, with an extra 2 mM toluene added the evening before harvesting. Cells were also grown on styrene in a fermenter as in Methods 2.4.4i. Substrates were prepared as in Methods 2.6.1, and 100 µl of 10 mM (or saturated, if solubility less than 10 mM) solutions were tested (final concentration = 0.33 mM), except for toluene *cis*-glycol, for which 50 µl of 7 mM solution (final concentration = 0.12 mM) was used. All results are means of at least two determinations.

Test substrate	Grown on styrene		Grown on toluene	
	Oxygen uptake nmol/min/mg protein	%	Oxygen uptake nmol/min/mg protein	%
Toluene	140	100	220	100
Benzyl alcohol	60	43	120	55
Benzaldehyde	10	7	50	23
Benzoic acid	0	0	30	14
o-Cresol	<10	<7	10	5
m-Cresol	<10	<7	20	9
p-Cresol	<10	<7	<10	<5
Protocatechuic acid	10	7	<10	<5
Catechol	360	260	470	210
3-Methylcatechol	1500	1100	1560	710
4-Methylcatechol	140	100	90	41
Toluene *cis*-glycol	270	190	440	200
Styrene	140	100	240	110

Figure 3.6: Ability of *R. rhodochrous* 26 grown on toluene to oxidise possible toluene metabolites

(Data from Table 3.6)

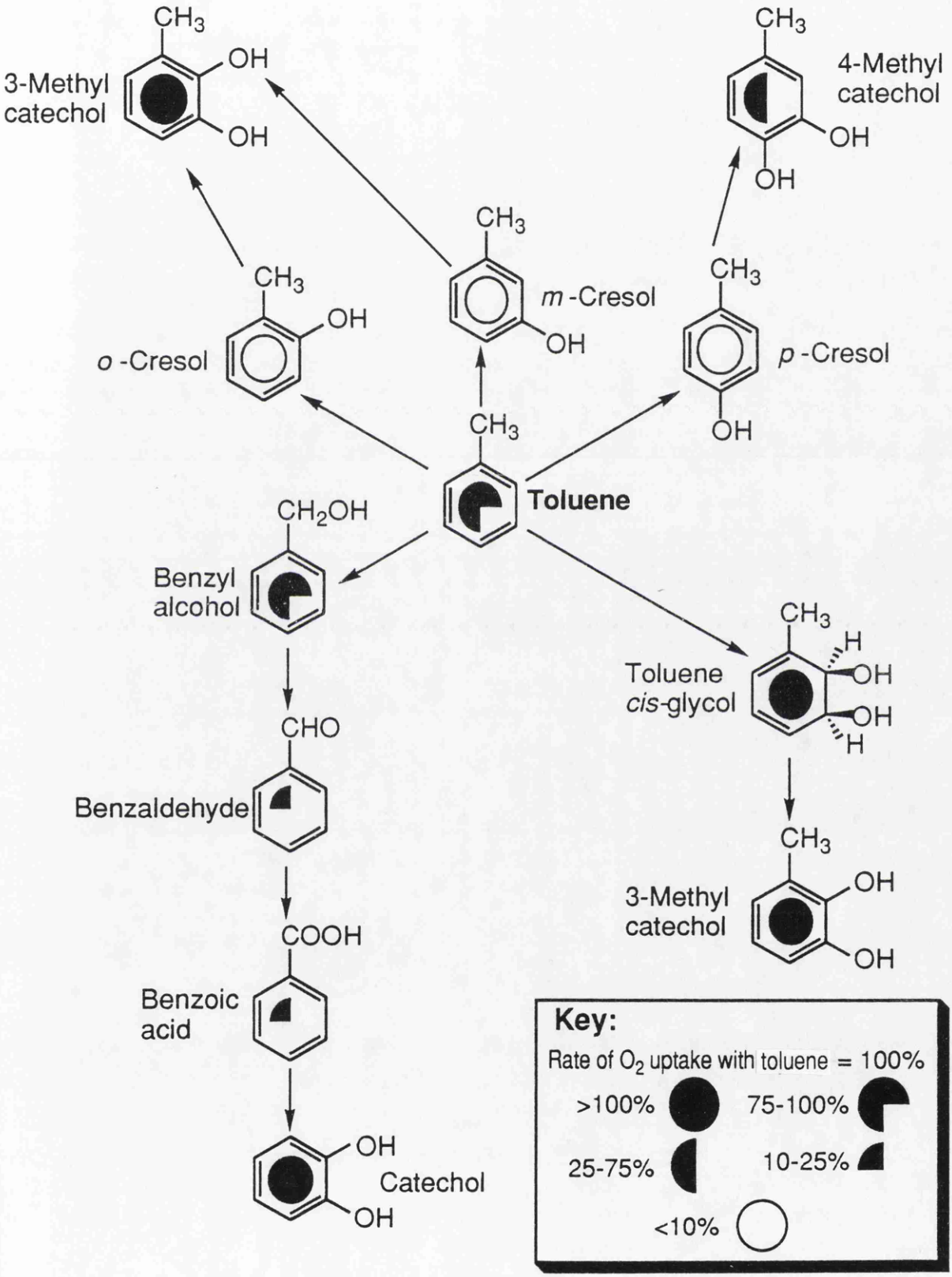


Table 3.7: Induction results of *R. rhodochrous* 26 grown on glucose, styrene and nutrient broth

Glucose and nutrient broth cells were grown in flasks as in Methods 2.4.4a and e. Cells grown on styrene and nutrient broth/ styrene were grown in a fermenter, as in Methods 2.4.4i. Substrates were prepared as in Methods 2.6.1, and 100 µl of 10 mM (or saturated, if solubility less than 10 mM) solutions were tested (final concentration = 0.33 mM), except for toluene *cis*-glycol, for which 50 µl of 7 mM solution (final concentration = 0.12 mM) was used. All results are means of at least two determinations. Some results are repeated from Tables 3.3 and 3.6

Substrate	Oxygen consumption (nmol O_2/min/mg protein) of *R. rhodochrous* 26 cells grown on:			
	Glucose	Styrene	Nutrient Broth/ Styrene	Nutrient Broth
1-Phenylethanol	<10	90	60	10
2-Phenylethanol	<10	120		
3-Fluorocatechol		1		
3-Methylcatechol	10	1500	540	<10
4-Methylcatechol	10	140		30
α-Methylstyrene	0	150		
Acetaldehyde	30	60		
Acetate	60		100 [1]	
Acetophenone	<10	20		
Acrylic acid	20	20	20 [1]	
β-Methylstyrene	<10	140		
Benzaldehyde	10	10		
Benzene	<10	40	20	
Benzoic acid	0	0		
Benzyl alcohol	0	60		
Catechol	0	360		80
Cinnamic acid	10	20	40 [1]	
D-Lactate	<10	10		
Ethylbenzene	<10	180	90	<10
Glucose	40		30 [1]	
Homogentisic acid	<10	<10		
L-Lactate	<10	10		
m-Cresol	<10	<10		
DL-Mandelic acid	<10	<10		
Nutrient broth				90
o-Cresol	<10	<10		
p-Cresol	<10	<10		
Phenol	<10	<10		
Phenylacetaldehyde	<10	40		
Phenylacetic acid	<10	0		<10
Phenylethanediol	<10	50		
Phenylglyoxylic acid	<10	10		
Propionic acid	30	80		
Protocatechuic acid	<10	10		
Styrene	0	140	60	<10
Styrene oxide	<10	40		
Toluene	<10	140	40	<10
Toluene cis-glycol	20	270	300	<10
Vinylacetic acid	20	110		

[1] Cells which had been frozen and thawed used, oxygen consumption with styrene = 60 nmol O_2/min/mg protein

R. rhodochrous 26 grown on nutrient broth (Table 3.7) showed little response to styrene, toluene, toluene *cis*-glycol, ethylbenzene, 3-methylcatechol and 1-phenylethanol. However there was some response to catechol and 4-methylcatechol.

b) Cells grown on styrene or nutrient broth/styrene

The induction pattern of *R. rhodochrous* 26 on styrene has already been largely covered (Tables 3.3 and 3.6); however, the full results are presented in Table 3.7. Oxygen consumption with catechols was in the order 3-methylcatechol > catechol > 4-methylcatechol. Cells of *R. rhodochrous* 26 grown in the fermenter on nutrient broth and styrene showed a similar pattern of responses to those of cells grown on styrene alone. It is possible that the rate of oxidation of cinnamic acid was higher when nutrient broth and styrene were used to support growth.

c) Cells grown on 1-phenylethanol, benzene or ethylbenzene

Cells that had been grown on 1-phenylethanol, benzene and ethylbenzene were tested with a small number of substrates (Table 3.8). In addition to a high oxygen consumption with the growth substrate, there was also induction towards styrene, toluene, toluene *cis*-glycol, catechol, 3-methylcatechol and 4-methylcatechol. Oxygen consumption with added catechols was in the order 3-methylcatechol > catechol > 4-methylcatechol, for cells grown on either ethylbenzene or 1-phenylethanol.

d) Cells grown on benzyl alcohol

In addition to a high rate of oxygen consumption with benzyl alcohol, there were also high responses to benzaldehyde, benzoic acid, the catechols and toluene *cis*-glycol (Table 3.8). Styrene and toluene caused little or no oxygen consumption. Oxygen consumption with added catechols was in the order catechol > 3-methylcatechol > 4-methylcatechol. When grown on benzyl alcohol in large flasks a smell similar to benzaldehyde was noticed.

Table 3.8: Simultaneous induction tests on *R. rhodochrous* 26 grown on various substrates

R. rhodochrous 26 was grown on the substrates below in screw-top flasks, as in Methods 2.4.4f, except for those cells grown on benzyl alcohol, which were grown in 4l flasks as in Methods 2.4.4h. Substrates were prepared as in Methods 2.6.1, and 100 µl of 10 mM (or saturated, if solubility less than 10 mM) solutions were tested (final concentration = 0.33 mM), except for toluene *cis*-glycol, for which 50 µl of 7 mM solution (final concentration = 0.12 mM) was used. All results are means of at least two determinations.

Substrate	Oxygen consumption (nmol O_2/min/mg protein) of *R. rhodochrous* 26 cells grown on:				
	Ethylbenzene	1-Phenylethanol	Benzene	Benzyl alcohol	Cinnamic acid
Styrene	70	70	140	<10	0
Toluene	190	30	200	0	0
Benzene			60		
Ethylbenzene	130	90			
1-Phenylethanol	60	50	140		
2-Phenylethanol		30			
Toluene *cis*-glycol	270	110	220	100	<10
3-Methylcatechol	1220	300	1470	460	
4-Methylcatechol	80	70		260	
Catechol	530	100		620	
Benzyl alcohol				280	
Benzaldehyde				400	
Benzoic acid				430	280
Phenylacetic acid				<10	
Protocatechuic acid				10	
Cinnamic acid					300

e) Cells grown on cinnamic acid

A limited range of substrates were tested with cells grown on cinnamic acid (Table 3.8). There was little induction towards styrene, toluene and toluene *cis*-glycol, but a high level of oxygen consumption with both cinnamic acid and benzoic acid.

3.4 Discussion

3.4.1 Styrene breakdown by *R. rhodochrous* 26

As hoped, the combination of growth tests and simultaneous induction experiments resulted in the identification of a possible pathway for styrene breakdown in the selected strain. The established pathway of toluene breakdown *via* toluene *cis*-glycol is shown in Figure 3.7. By replacing the methyl group of toluene with the vinyl group of styrene a possible pathway for the breakdown of styrene is produced.

3.4.2 Styrene breakdown by strains 80 and 137

Re-examination of the simultaneous induction results of the three selected strains (see Section 3.2.3) in the light of the results for oxygen uptake with toluene *cis*-glycol clarifies the earlier discussions about possible pathways (3.2.4). In Figure 3.8a the results for relative rates of oxygen consumption by *R. rhodochrous* 26 and strain 80 exposed to various substrates are compared. For *R. rhodochrous* 26, toluene *cis*-glycol has the highest response, closely followed by toluene and ethylbenzene. However, strain 80 did not oxidise toluene *cis*-glycol, and it oxidised toluene and ethylbenzene at only a low rate. Strain 80 oxidised styrene oxide, phenylacetaldehyde and phenylacetic acid at very high rates. The result from oxygen consumption with toluene *cis*-glycol supports the hypothesis that strain 80 metabolises styrene *via* the phenylacetic acid pathway (Introduction, 1.3.3c).

A comparison of the results of *R. rhodochrous* 26 and strain 137 (Figure 3.8b) shows the induction patterns of the two organisms are very similar. Strain 137 grown on styrene, like strain 26, has high rates of oxygen consumption with toluene *cis*-glycol, toluene and ethylbenzene. Intermediates on the phenylacetic acid pathway produce a

Figure 3.7: Breakdown of styrene and toluene *via* *cis*-glycol

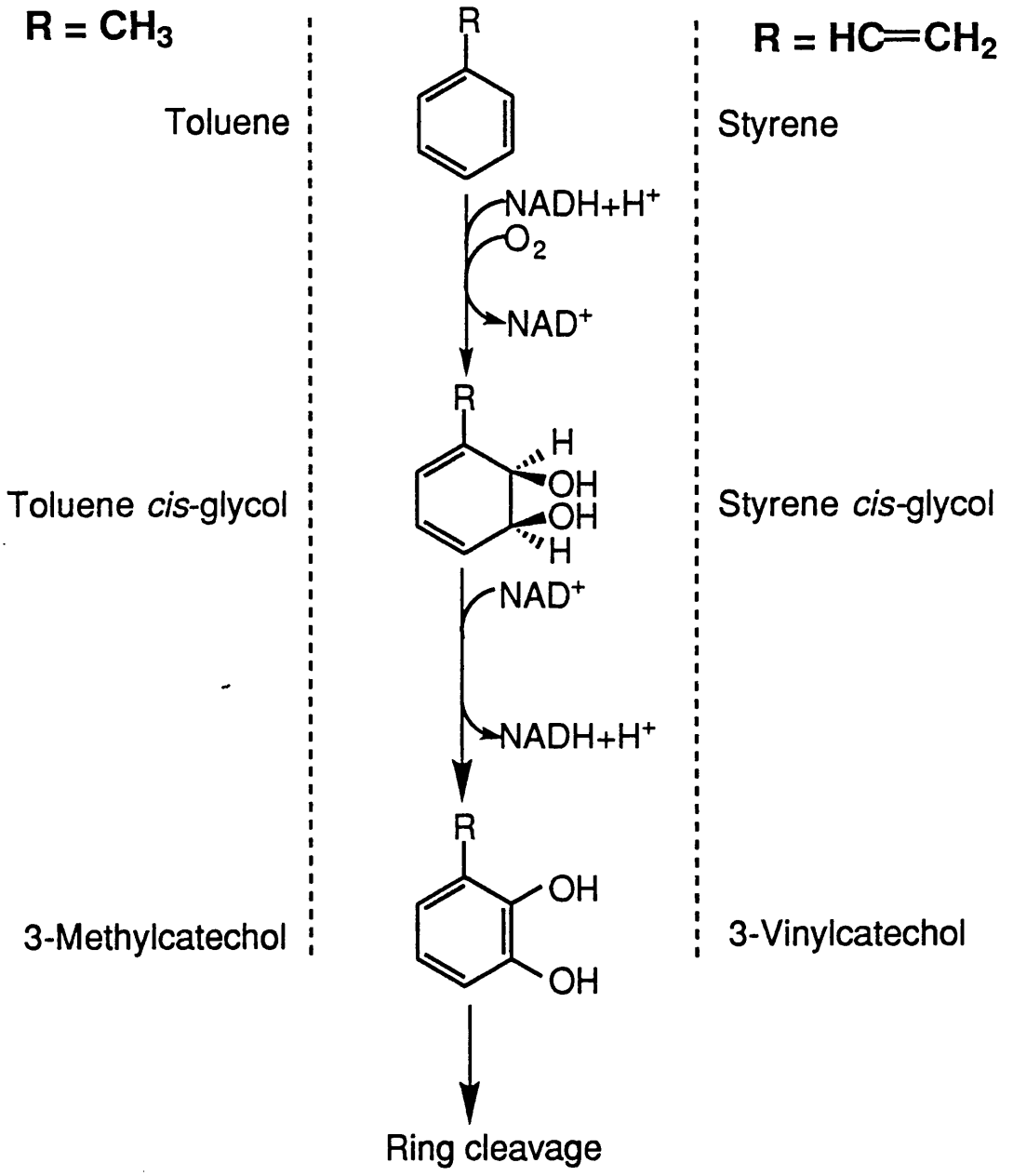
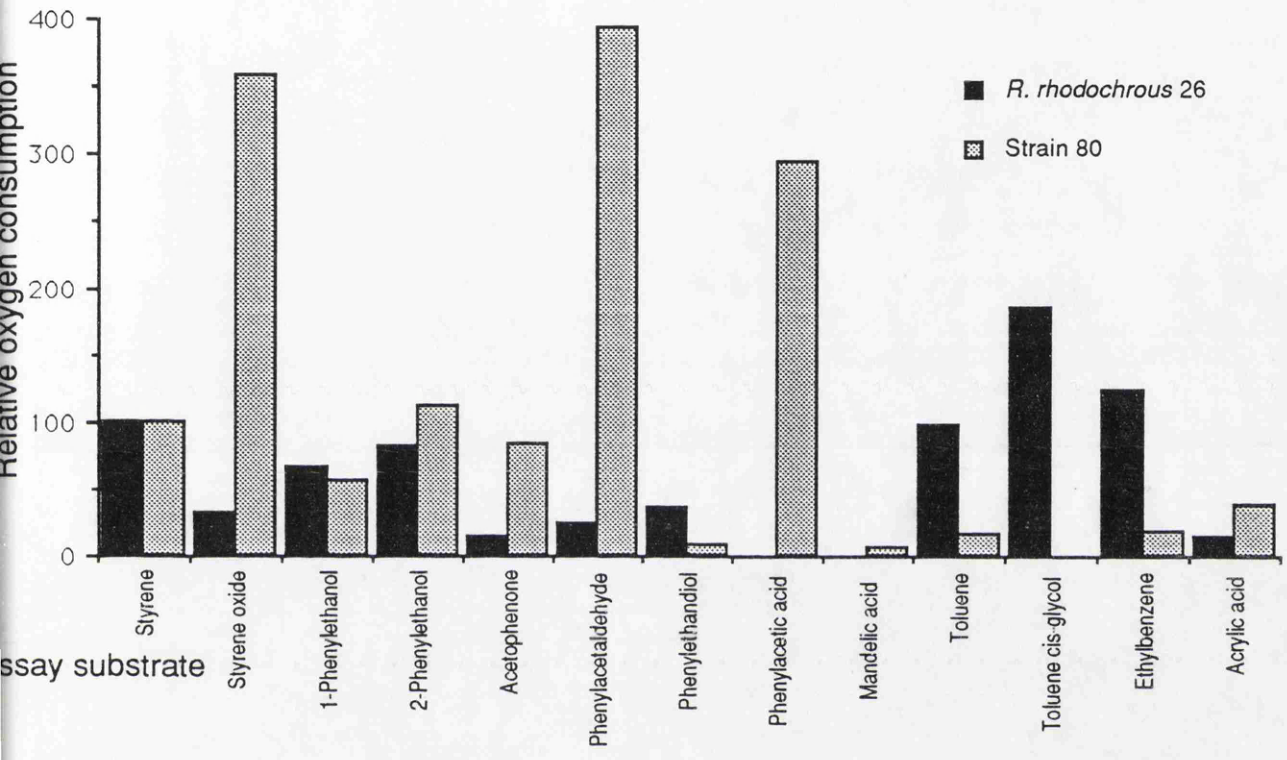
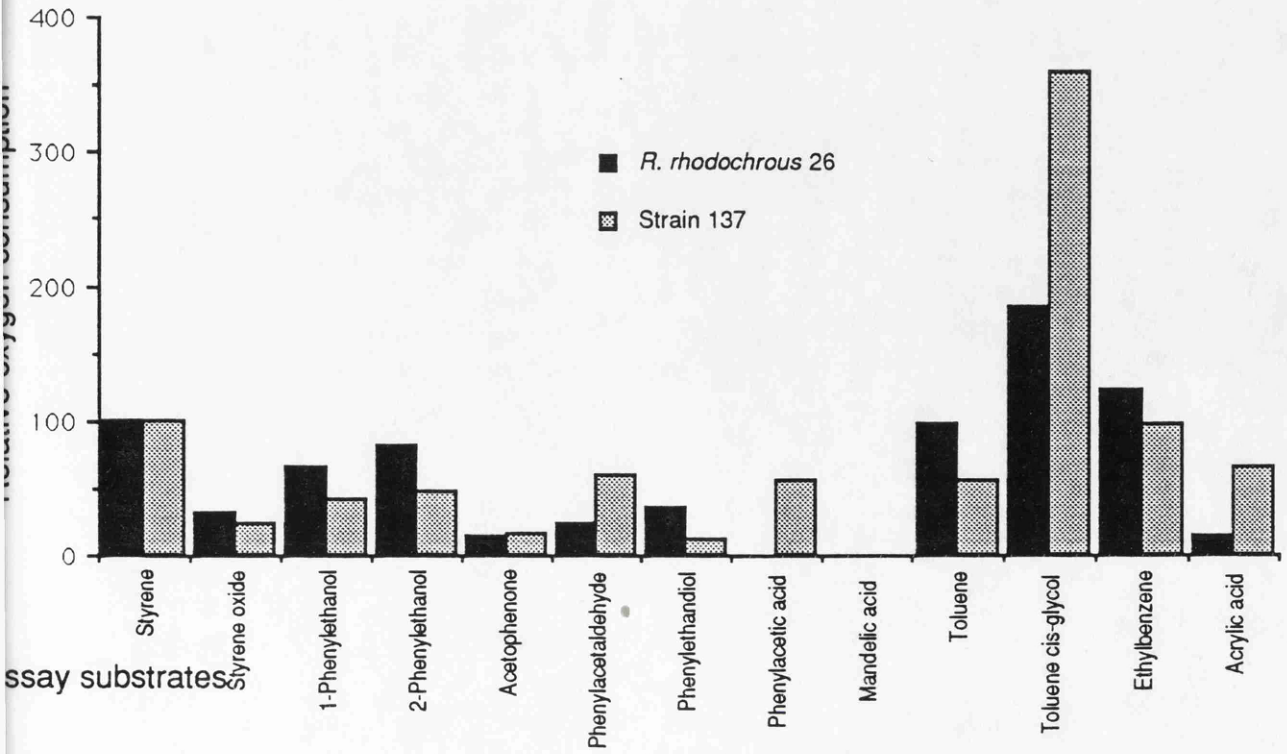


Figure 3.8: A comparison of relative rates of oxygen consumption with various substrates for cells of *R. rhodochrous* 26, strain 80 and strain 137 grown on styrene

a) *R. rhodochrous* 26 and strain 80 compared (rate with styrene = 100)



b) *R. rhodochrous* 26 and strain 137 compared (rate with styrene = 100)



much lower rate of oxygen consumption, indicating that strain 137 may also use a *cis*-glycol pathway to degrade styrene.

3.4.3 *R. rhodochrous* 26 grown on other substrates

Comparing the simultaneous induction results for cells grown on styrene and those grown on glucose, it is clear that the enzymes (and/or uptake mechanisms) involved in styrene breakdown are inducible, with the exception of those involved in short carbon chain metabolism. Cells grown on nutrient broth cannot oxidise aromatics, with the exception of some catechol breakdown activity. Cells grown on both nutrient broth and styrene show a similar oxygen consumption rates as those grown on styrene, suggesting that there is little or no catabolite repression from the nutrient broth.

When *R. rhodochrous* 26 was grown on styrene, toluene, benzene, ethylbenzene and 1-phenylethanol, the induction results were similar (Table 3.8). This suggests that an identical, non-specific aromatic breakdown pathway may be involved in degrading all these substrates.

R. rhodochrous 26 grown on benzyl alcohol shows a different pattern, unable to oxidise styrene and toluene. A common route of benzyl alcohol metabolism, as encoded by the TOL plasmid, proceeds *via* benzaldehyde and benzoic acid (Introduction, 1.2.3a). These two substrates were oxidised by cells grown on benzyl alcohol, indicating that *R. rhodochrous* 26 could be using this pathway to metabolise benzyl alcohol. This route is also supported by the odour of benzaldehyde present in growth flasks when high concentrations of benzyl alcohol are used. This pathway can include a dihydrodiol dehydrogenase step (in the conversion of benzoate to catechol, see Figure 1.27 in Introduction), which could explain the oxidation of toluene *cis*-glycol by cells grown on benzyl alcohol. The organism could either have one *cis*-glycol dehydrogenase, induced by growth on both styrene and benzyl alcohol, or a separate, but non-specific, benzoate 1,2-*cis*-diol dehydrogenase which can also oxidise toluene *cis*-glycol.

The relative rates of oxygen consumption of cells grown on benzyl alcohol with the three catechols (catechol > 3-methylcatechol > 4-methylcatechol) are different from

those for styrene grown cells (3-methylcatechol > catechol > 4-methylcatechol). Previous work has shown that different catechol 1,2-oxygenases and catechol 2,3-oxygenases have different relative rates of oxidation of catechol and methylcatechols (Chatfield & Williams, 1986; Mal'tseva *et al.*, 1991). It is possible that *R. rhodochrous* 26 may express a different combination of catechol 1,2- and 2,3-oxygenases when grown on benzyl alcohol rather than on styrene.

Cinnamic acid was included as a growth substrate because styrene can be formed from it by decarboxylation (Introduction, 1.3). However, the induction results (Table 3.8) show that growth on it does not induce the styrene pathway. There is, however, a high level of induction towards benzoic acid, a breakdown intermediate in an established pathway (Introduction, 1.1.7h). In this case there is little induction towards toluene *cis*-glycol.

4. Production and isolation of pathway intermediates

4.1 Introduction

At this stage a possible pathway for the breakdown of styrene by *R. rhodochrous* 26 had been postulated, *via* styrene *cis*-glycol and 3-vinylcatechol. Because of the unavailability of these intermediates commercially, some methods were developed for their production. The production of possible intermediates by whole cells and extracts of *R. rhodochrous* 26 was then examined.

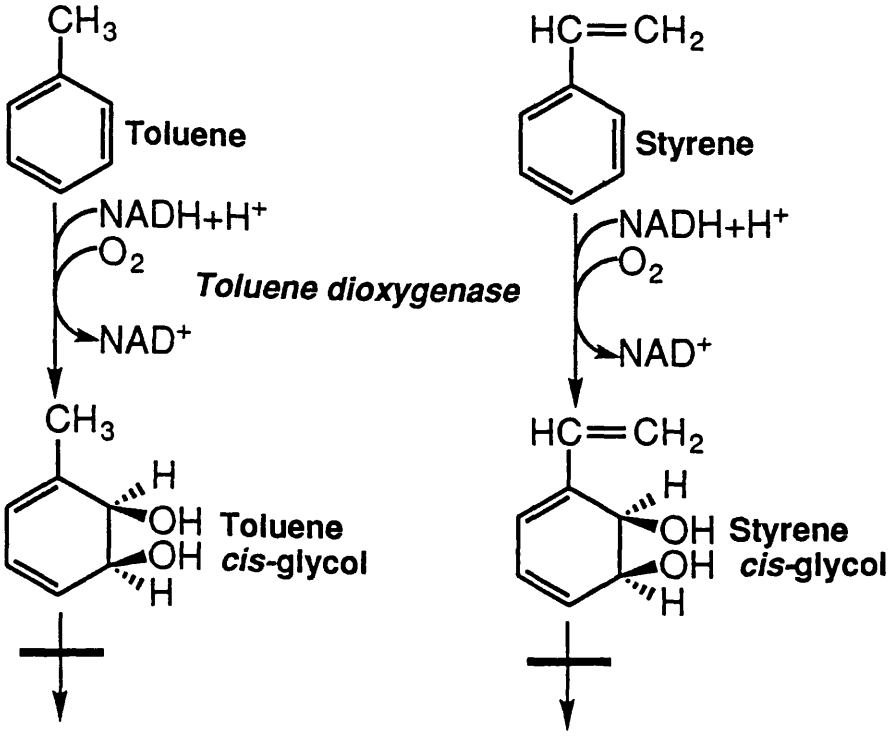
4.2 Production of styrene *cis*-glycol by *Pseudomonas putida* UV4

4.2.1 Introduction

The toluene *cis*-glycol used in the previous chapter was obtained from ICI, where it had been produced from toluene by *Pseudomonas putida* UV4 which is a mutant strain derived from an organism with a full toluene dioxygenase pathway (Introduction, 1.2.3b). The mutant does not have a dihydrodiol dehydrogenase activity, so intact cells accumulate toluene *cis*-glycol when fed toluene and ethanol, as a co-metabolite to convert the NAD^+ to NADH. The mutant constitutively expresses the remaining enzymes of the aromatic breakdown pathway. Other similar mutants have been produced, for example *Pseudomonas putida* 39D by Gibson (1970).

The toluene dioxygenase of *P. putida* UV4 has a very wide substrate range, and has already been used to produce many different *cis*-glycols, for example benzene *cis*-glycol (Harrop *et al.*, 1992). *P. putida* 39D has been used to make styrene *cis*-glycol (Hudlicky *et al.*, 1989), so it seemed likely that *P. putida* UV4 would also be able to perform this biotransformation (see Figure 4.1).

Figure 4.1: The production of *cis*-glycols by *Pseudomonas putida* UV4



4.2.2 Transformation of styrene to styrene *cis*-glycol

An active culture of *P. putida* UV4 was selected (Methods 2.5.2b), grown in 2 litres of pyruvate minimal medium for 24 hours (Methods 2.5.2a), and then harvested and resuspended in 1 litre of buffer (Methods 2.5.2c). The transformation and extraction were performed as described in Methods 2.5.2c, resulting in the production of about 30 mg of crystalline styrene *cis*-glycol, which was immediately dissolved in ethyl acetate with triethylamine as a preservative.

The structure of the styrene *cis*-glycol was confirmed by both ^{1}H n.m.r. and ^{13}C n.m.r. (Figure 4.2), with the results in close agreement with those obtained for the styrene *cis*-glycol produced by *P. putida* 39D (Hudlicky *et al.*, 1989). Although this n.m.r. data could not distinguish between styrene *cis*-glycol and styrene *trans*-glycol, it is very unlikely that the *trans*- isomer would be formed by *P. putida* UV4, since other workers have found that bacterial dioxygenase enzymes produce *cis*-glycols (Ziffer *et al.*, 1977). Enzyme assays using styrene *cis*-glycol are described in the next chapter, Section 5.2.1.

4.3 Production of vinylcatechol from styrene *cis*-glycol

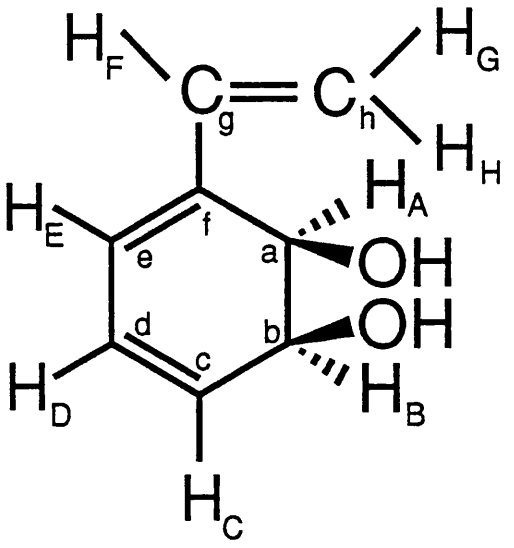
4.3.1 Introduction

As can be seen in the pathway depicted in Figure 4.3, it would be expected that the product of the postulated *cis*-glycol dehydrogenase reaction of *R. rhodochrous* 26 would be a catechol. It is then likely that the aromatic ring of catechol would be split with O_2, either in the 1,2- or 2,3- position. If the dehydrogenase reaction could be performed under anaerobic conditions, then the catechol should accumulate.

4.3.2 Test run with toluene *cis*-glycol

An extract of *R. rhodochrous* 26 which had been grown on nutrient broth and styrene (400 µl of an extract containing 4.7 mg protein/ml), toluene *cis*-glycol (0.2 µmoles) and NAD (4 µmoles) in 1 ml of 50 mM phosphate buffer pH 7.6, were mixed

Figure 4.2: Nuclear magnetic resonance analysis of styrene *cis*-glycol formed from styrene by *Pseudomonas putida* UV4



δ_H ($CDCl_3$)

6.40 (1H, dd, *J* 11 and 18 Hz, H_D)
5.95 (3H, m, $H_{F,G,H}$)
5.50 (1H, d, *J* 18 Hz, H_C)
5.12 (1H, d, *J* 11 Hz, H_E)
4.50 (1H, m, H_A)
4.47 (1H, m, H_B)
2.72 (1H, brs, OH)
1.81 (1H, brs, OH)

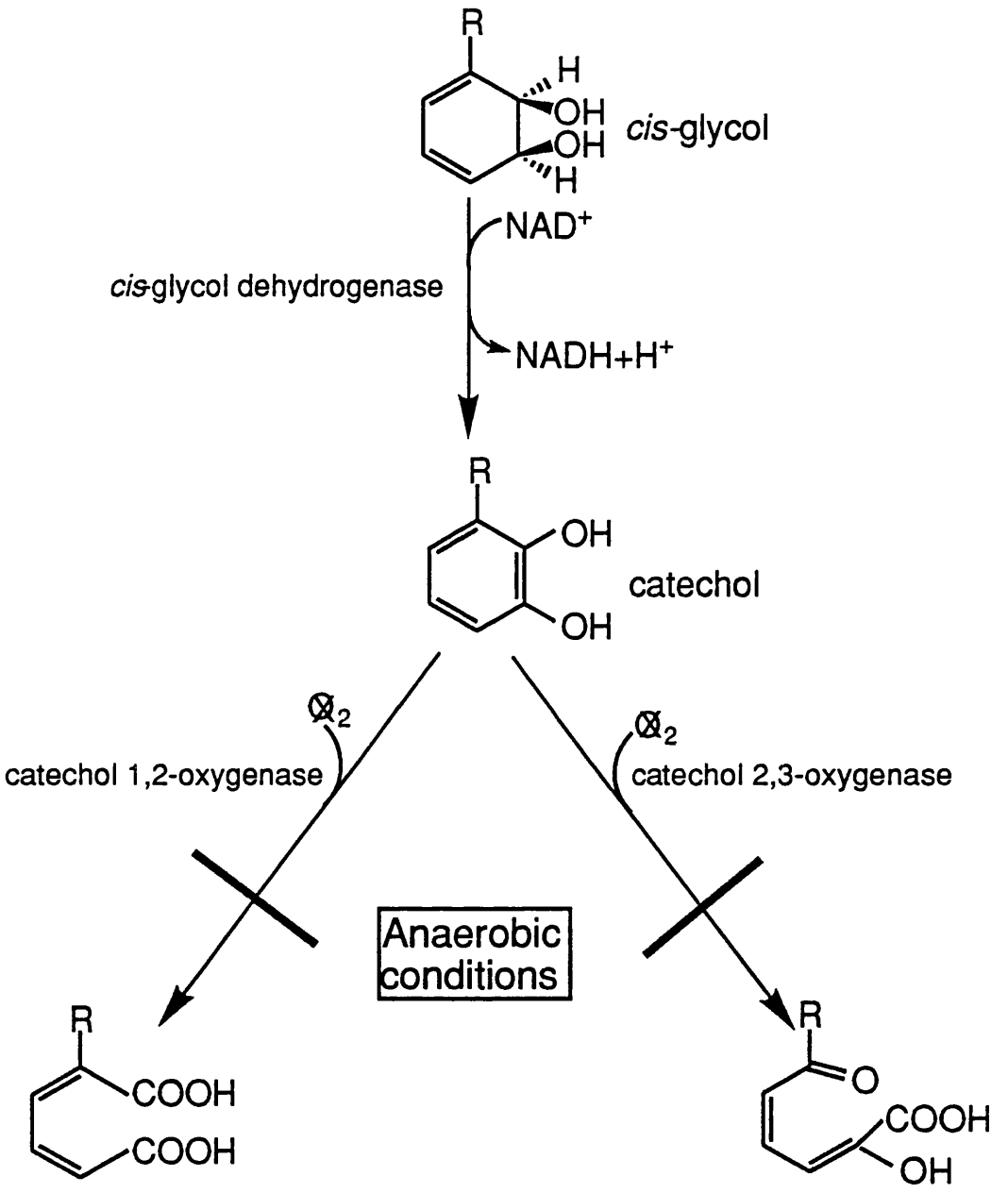
δ_C ($CDCl_3$)

65.4	C_b
70.6	C_a
114.0	C_h
123.5	C_g *
124.6	C_c *
132.4	C_d *
135.7	C_e *
136.8	C_f

* assignments may be interchanged

n.m.r. analysis performed as in Methods 2.5.1b

Figure 4.3: Catechol production from *cis*-glycol



under anaerobic conditions in a Thunberg tube (Methods 2.5.3), and allowed to react for 30 minutes. The reaction was stopped, and the product was extracted with ethyl acetate (Methods 2.3.9). When run on t.l.c. with solvent system 1 (Methods 2.3.11) a spot was visible under u.v. illumination and after exposure to iodine vapour. The spot had an R_F value of approximately 0.7, which was very close to that of authentic 3-methylcatechol.

Further characterisation of the putative 3-methylcatechol was performed by g.c.-m.s. (Methods, 2.5.1a). The sample produced above was combined with another produced with the same method, and the trimethylsilyl derivative was formed before analysis. The fragmentation pattern observed (Figure 4.4) was identical with that of the known spectrum of the trimethylsilyl ether of 3-methylcatechol (contained in a library on the g.c.-m.s. computer at the BCRA), with *m/z* 268 (M^+, 19 %), 253 (M-15, 4 %), 73 ($(Si(CH_3)_3)^+$, 100 %).

4.3.3 Vinylcatechol production from styrene *cis*-glycol

An extract of *R. rhodochrous* 26 which had been grown on nutrient broth and styrene (1.2 ml of an extract containing 4.7 mg protein/ml), styrene *cis*-glycol (approximately 3.6 µmoles, as 100 µl of a 5 mg/ml solution in ethyl acetate) and NAD (12 µmoles) in 3 ml of 50 mM phosphate buffer pH 7.6, were mixed under anaerobic conditions in a Thunberg tube (Methods 2.5.3), and allowed to react for 30 minutes. After acidification and centrifugation the sample was divided into four portions, two of which were frozen in aqueous form. The other two samples were extracted and evaporated as described in Methods 2.3.9 and 2.3.10. One sample then had methanol added, and was run on t.l.c. using solvent system 1 (Methods 2.3.11). There were few spots visible under u.v. on the plate, and only one spot showed similar characteristics to 3-methylcatechol:- a permanent stain when exposed to iodine vapour, an R_F (approximately 0.5) close to that of 3-methylcatechol and a browning on prolonged exposure to air. This spot was probably vinylcatechol.

The second sample of putative vinylcatechol was derivatised and analysed by g.c.-m.s. (Methods 2.5.1a), and the mass spectrum is illustrated in Figure 4.5a. A component

Figure 4.4: Mass spectrum of 3-methylcatechol produced from toluene *cis*-glycol by extracts of *R. rhodochrous* 26

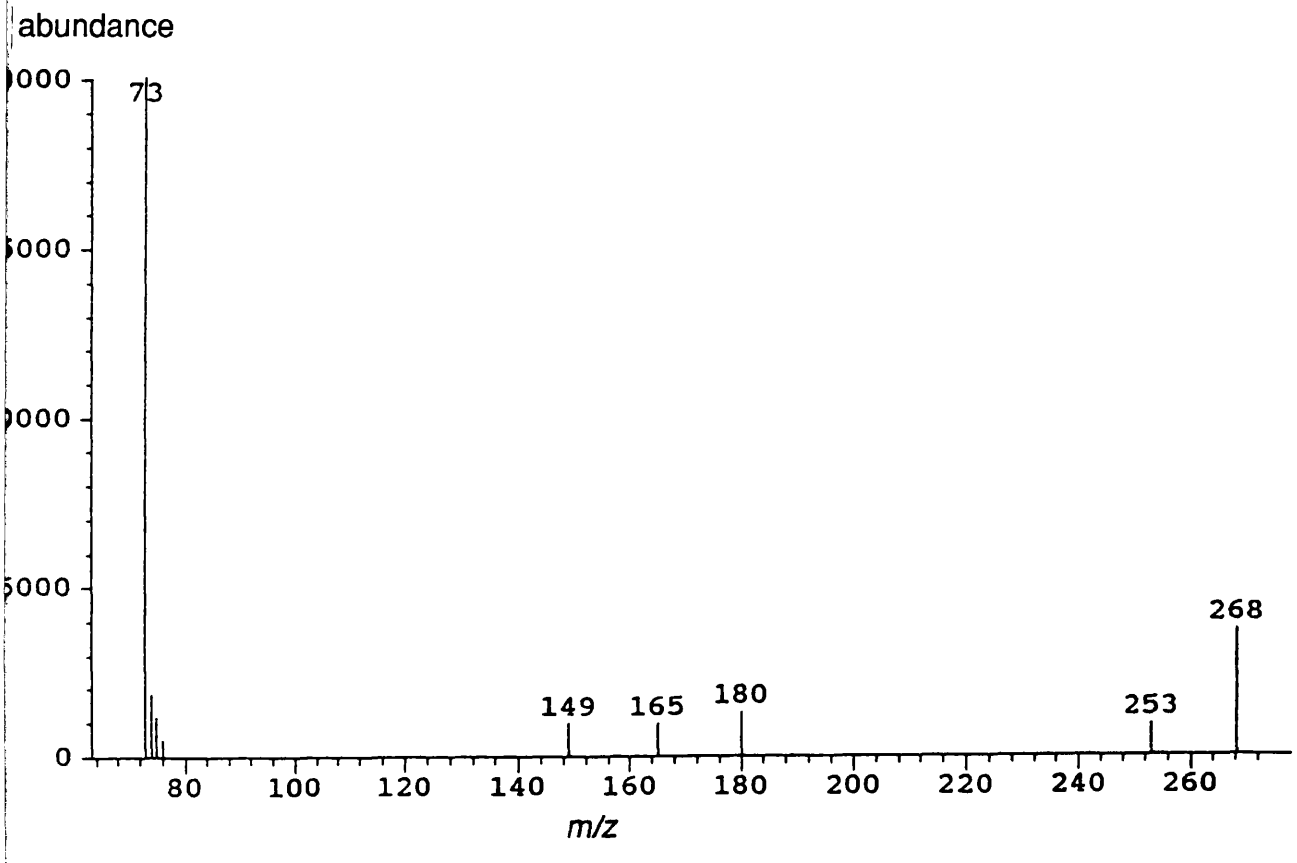
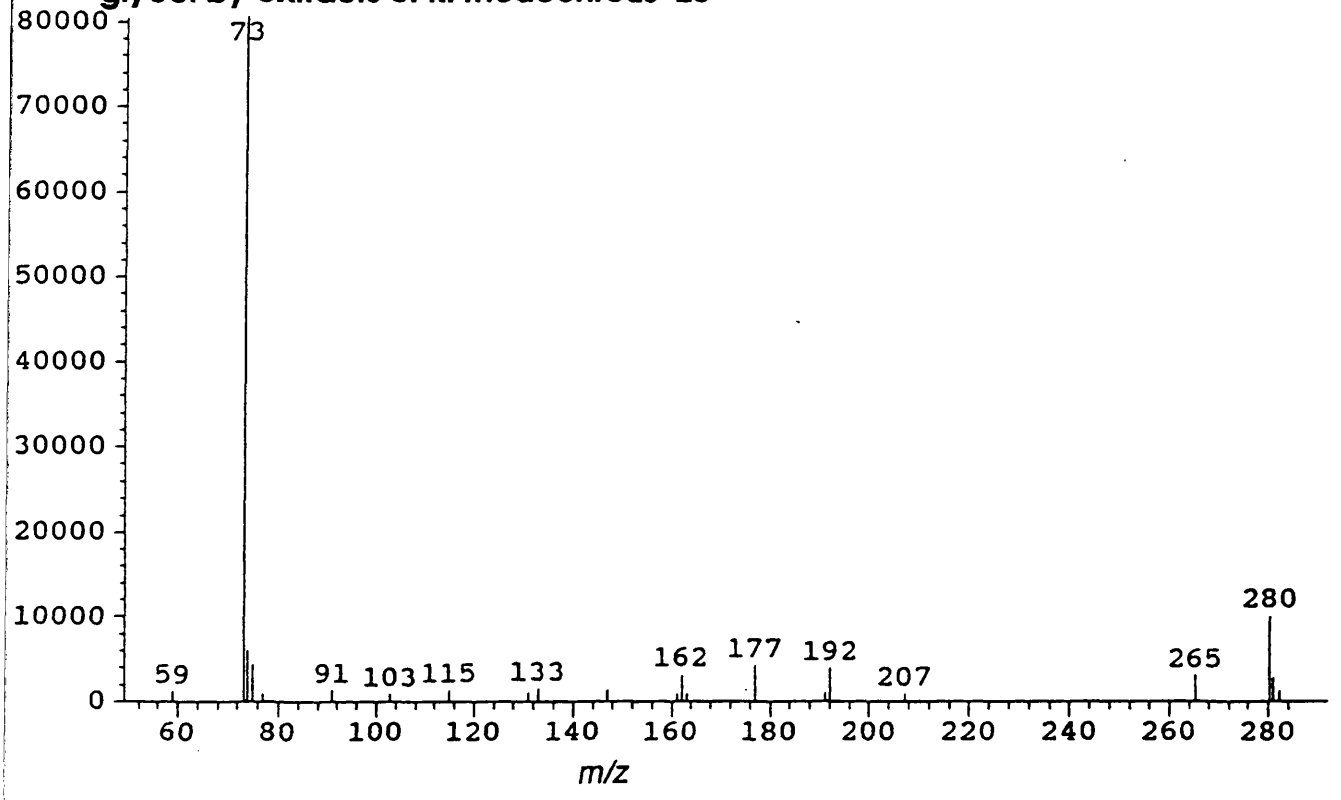
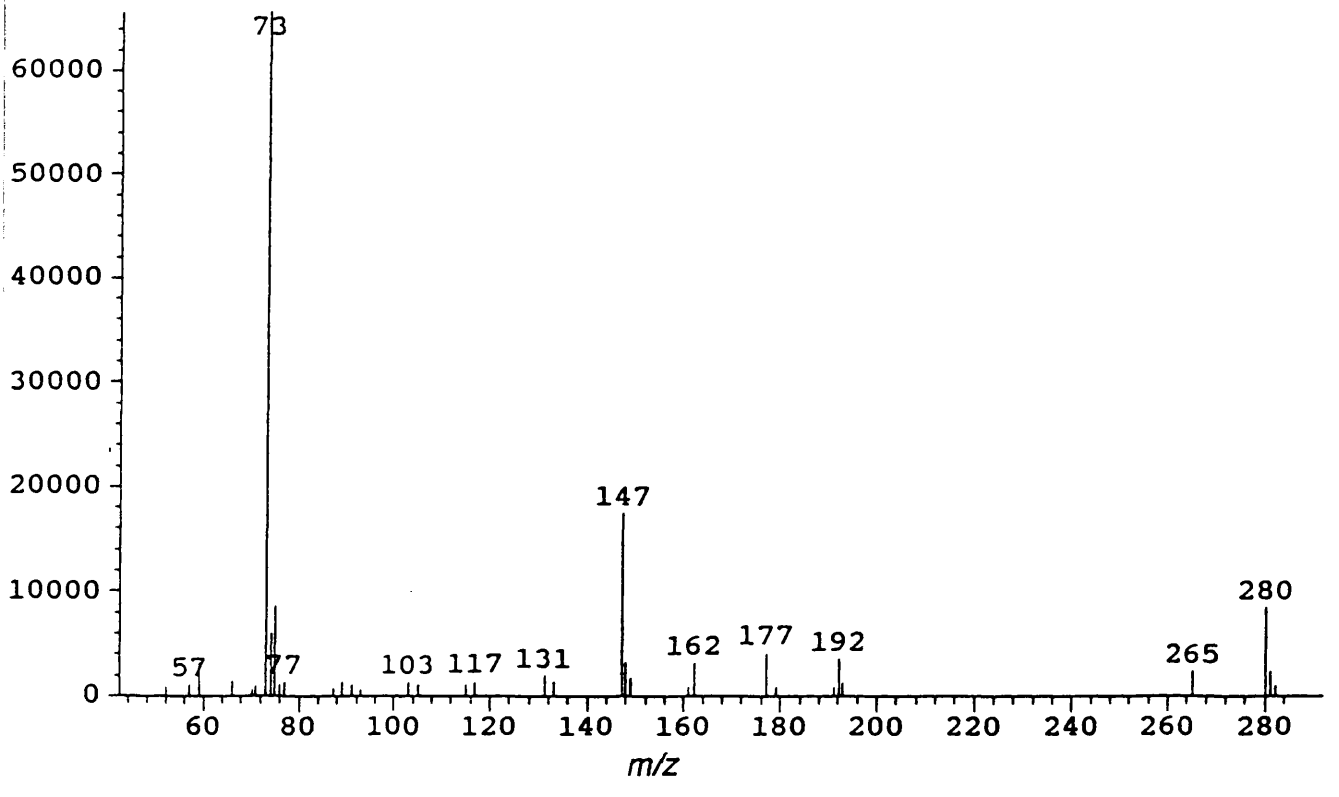


Figure 4.5: Vinylcatechol mass spectra

a) Mass spectrum of vinylcatechol from the transformation of styrene *cis*-glycol by extracts of *R. rhodochrous* 26



b) Mass spectrum of catechol accumulated in the medium when intact cells of *R. rhodochrous* 26 were incubated with styrene and 3-fluorocatechol



was present which produced a fragmentation pattern consistent with a trimethylsilyl ether derivative of vinylcatechol, with *m/z* 280 (M^+, 12 %), 265 (M-15, 3 %), 73 ($(Si(CH_3)_3)^+$, 100 %). However, g.c.-m.s. does not give reliable information as to the position of the vinyl group on the aromatic ring.

4.4 Accumulation of catechols by whole cells in the presence of 3-fluorocatechol

4.4.1 Introduction

Various workers have shown the accumulation of catechols from a range of substrates in the presence of appropriate inhibitors (Smith & Ratledge, 1989b; Smith & Ratledge, 1989a). Several reports in the literature have documented the inhibitory effects of 3-fluorocatechol on catechol oxygenases (Dorn & Knackmuss, 1978b; Bartels *et al.*, 1984). Preliminary tests on extracts of *R. rhodochrous* 26 which had been grown on nutrient broth/styrene showed substantial inhibition (>95%) of 3-methylcatechol 2,3-oxygenase activity with 340 μM 3-methylcatechol as a substrate and concentrations of 3-fluorocatechol as low as 17 μM, even without pre-incubation (for more details on catechol oxygenase assays see Sections 5.2.2 and 5.2.3). The effect on 3-methylcatechol 1,2-oxygenase activity was found to be less substantial; e.g. an assay containing 340 μM 3-methylcatechol, 17 μM 3-fluorocatechol and 0.1 mg protein/ml had a rate one-sixth that of an assay without the 3-fluorocatechol. It was therefore hypothesised that 3-fluorocatechol could be used as a catechol oxygenase inhibitor with whole cells, to produce an accumulation of catecholic intermediates.

4.4.2 Catechol accumulation by intact cells with styrene as substrate and 3-fluorocatechol as inhibitor

Initial tests of combining styrene, intact cells and 3-fluorocatechol in buffer for a period of time, then extraction and t.l.c. showed that a band was visible on the t.l.c. plate with similar characteristics to the band predicted to be vinylcatechol in Section 4.3.3. Since vinylcatechol was found by g.c.-m.s. in that sample, this strongly indicated that this band was vinylcatechol. Test runs were undertaken to discover conditions for

optimum production of this band on t.l.c.. The optimal procedure is described in Methods 2.5.4. Gas-tight flasks were used, containing phosphate buffer at pH 7.0, thawed cells (which had been grown on nutrient broth/styrene in the fermenter), 3-fluorocatechol and styrene. After 2 hours on a shaker, the culture was acidified, cells were spun off and the medium was extracted with ethyl acetate (Methods 2.3.9). The extract was then run on t.l.c. using system 1 (Methods 2.3.11). A tracing of a typical t.l.c. plate is shown in Figure 4.6, including controls.

As a first identification step, the entire extract was derivatised and subjected to g.c.-m.s. (Methods 2.5.1a, but run with no split on the g.c,). A component was identified that had an identical R_F value and fragmentation pattern to that identified as the trimethylsilyl ether derivative of vinylcatechol in Section 4.3.3 (Figure 4.5b), with *m/z* 280 (M^+, 13 %), 265 (M-15, 3 %), 73 ($(Si(CH_3)_3)^+$, 100 %). No vinylcatechol was detectable in a sample prepared in the same way, but omitting the 3-fluorocatechol during the accumulation.

In order to obtain a firm identification of the vinylcatechol, including the ring position of the vinyl group, it was necessary to use ^1H n.m.r.. However, this technique required a pure sample, and a lot more material (usually around 5 mg minimum). A much larger quantity of cells (6 g) was used to produce enough material and the t.l.c. system already described was used to separate the vinylcatechol. However, there were several failed preliminary experiments, apparently because vinylcatechol is unstable. This could be due to the formation of polymeric material. Success was eventually obtained using the method described in 2.5.4a. The sample was then analysed by ^1H n.m.r. (Methods, 2.5.1b) and the results (Figure 4.7) confirm the structure of the isolated substance to be 3-vinylcatechol.

4.4.3 Catechol accumulation with other substrates

It was decided to use the above procedure, along with g.c.-m.s. analysis, to examine catechol accumulation when *R. rhodochrous* 26 was incubated with other substrates in the presence of 3-fluorocatechol. The only change to the procedure described in

Figure 4.6: Production of vinylcatechol from styrene by intact cells of *R. rhodochrous* 26 in the presence of 3-fluorocatechol; representation of a t.i.c. plate, with various controls

Chromatography was performed as in Methods 2.3.11. Samples were applied to the plate (which contained a fluorescent dye) dissolved in methanol, the plate was then allowed to dry for a few minutes before being placed in solvent system 1 (acetic acid: chloroform, 1:9). After approximately 90 min, the plate was removed from the solvent and left to dry for a few minutes. The plate was then illuminated by UV light (λ= 302 nm), and the areas of the plate which did not fluoresce were marked. The plate was then placed in an iodine tank for approximately 1 hour, then the staining was observed. Some spots stained permanently, others only transiently, the stain vanishing after approximately 30 minutes.

This figure is an idealised representation of two plates, with the right hand track being from a different plate from the others. The labels beside spots are based on identities discussed later in the chapter. The samples were prepared as in Methods 2.5.4:

Blank	Extraction of buffer
Cells	Extraction of buffer plus cells
3-Fluorocatechol	Extraction of buffer, cells and 3-fluorocatechol
Styrene	Extraction of buffer, cells and styrene
Styrene + 3-fluorocatechol	Extraction of buffer, cells, styrene and 3-fluorocatechol

Figure 4.6: Production of vinylcatechol from styrene by intact cells of *R. rhodochrous* 26 in the presence of 3-fluorocatechol; representation of a t.l.c. plate, with various controls

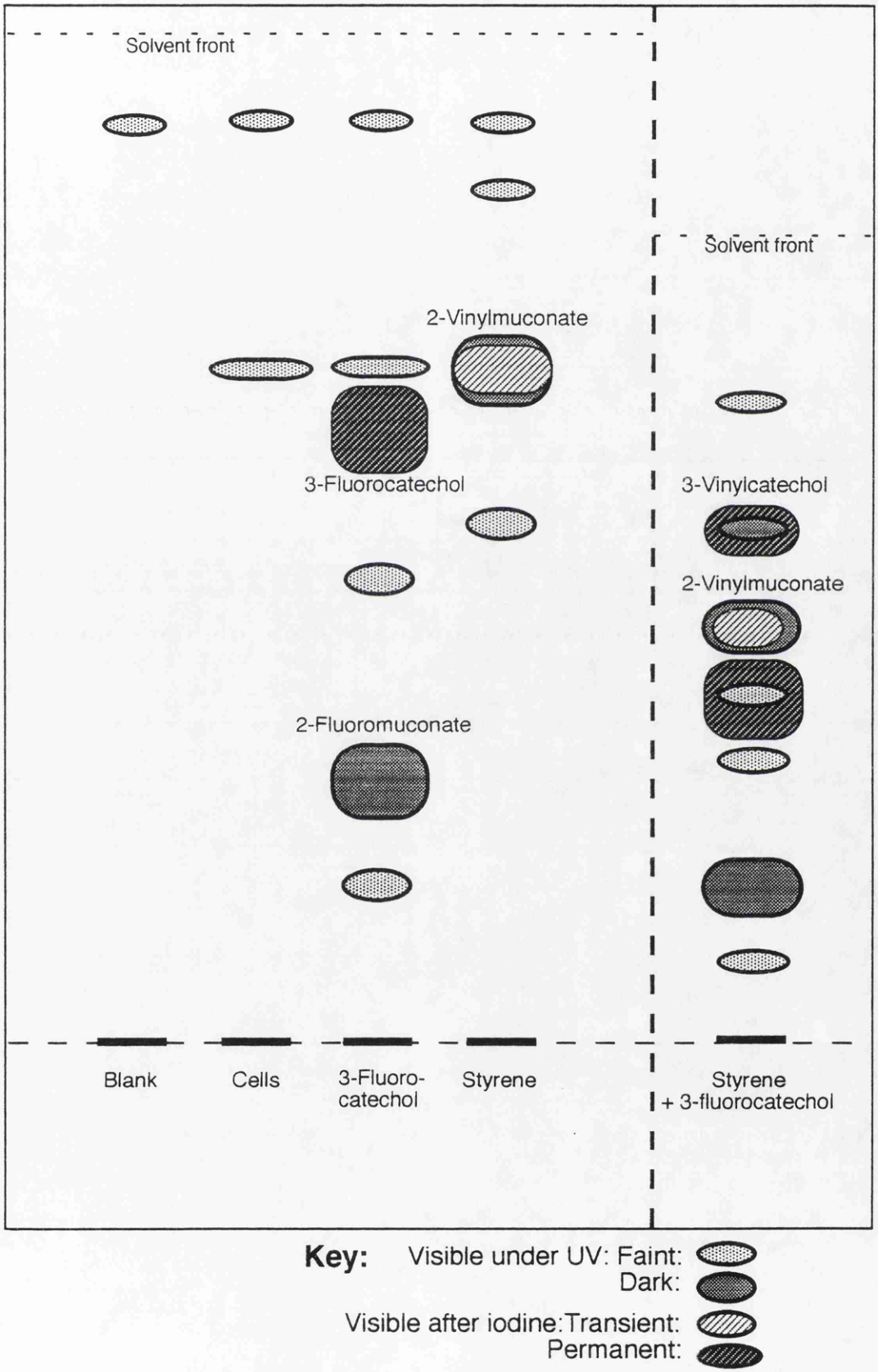
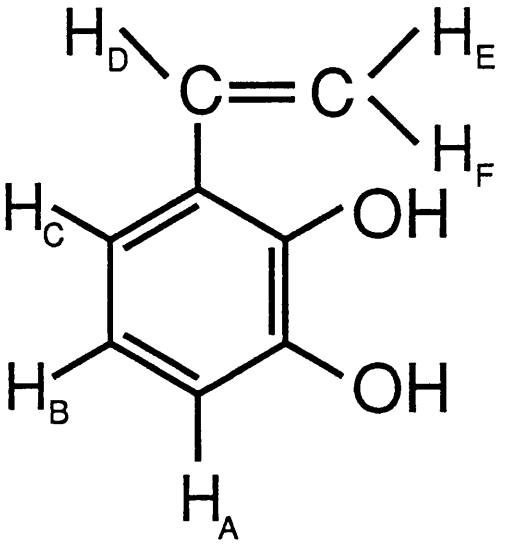


Figure 4.7: Nuclear magnetic resonance analysis of 3-vinylcatechol accumulated by intact cells of *R. rhodochrous* 26 incubated with styrene in the presence of 3-fluorocatechol



$\delta_{H((CD_3)_2CO)}$

7.03 (1H, dd, *J* 11.1 and 17.8 Hz, H_D)
6.96 (1H, dd, *J* 1.6 and 7.7 Hz, H_C)
6.75 (1H, dd, *J* 1.6 and 8.0 Hz, H_A)
6.63 (1H, t, *J* 8.0 Hz, H_B)
5.74 (1H, dd, *J* 1.7 and 17.8 Hz, H_F)
5.17 (1H, dd, *J* 1.7 and 11.1 Hz, H_E)

n.m.r. analysis performed as in Methods 2.5.1b

Methods 2.5.4 (with 1g cells per 200 ml medium per flask, one flask per substrate) was the use of an equivalent amount of the test substrate instead of styrene. A small amount of each ethyl acetate extract was spotted on a t.l.c. plate and the plate was run in solvent system 1 (Figure 4.8). The remainder of the extracts were derivatised and subjected to g.c.-m.s. (Methods 2.5.1a):

a) Toluene

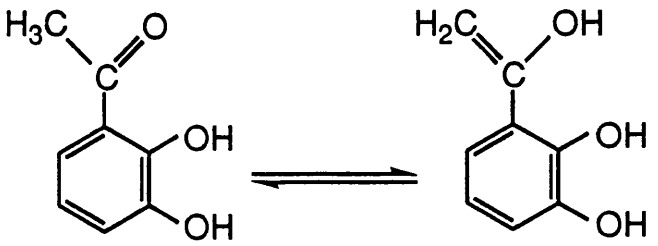
A component was detected which had a fragmentation pattern identical with that of the known spectrum of the trimethylsilyl ether of 3-methylcatechol, with an *m/z* 268 (M^+, 15 %), 253 (M-15, 2 %), 73 ($(Si(CH_3)_3)^+$, 100 %).

b) Ethylbenzene

A component was detected which had a fragmentation pattern identical with that of the known spectrum of the trimethylsilyl ether of 3-ethylcatechol, with an *m/z* 282 (M^+, 30 %), 267 (M-15, 9 %), 73 ($(Si(CH_3)_3)^+$, 100 %).

c) Acetophenone

No component was detected with a molecular ion corresponding to the trimethylsilyl derivative of 3-acetylcatechol. However, a component was detected which had a molecular ion of 368, which corresponds to the trimethylsilyl ether of the enol form of 3-acetylcatechol, with an *m/z* 368 (M^+, 15 %), 353 (M-15, 36 %), 73 ($(Si(CH_3)_3)^+$, 100 %). Acetophenone is known to undergo keto-enol tautomerisation (Loudon, 1984), so it is likely that acetyl catechol also undergoes this transformation:



Acetylcatechol (keto form) ⇌ Acetylcatechol (enol form)

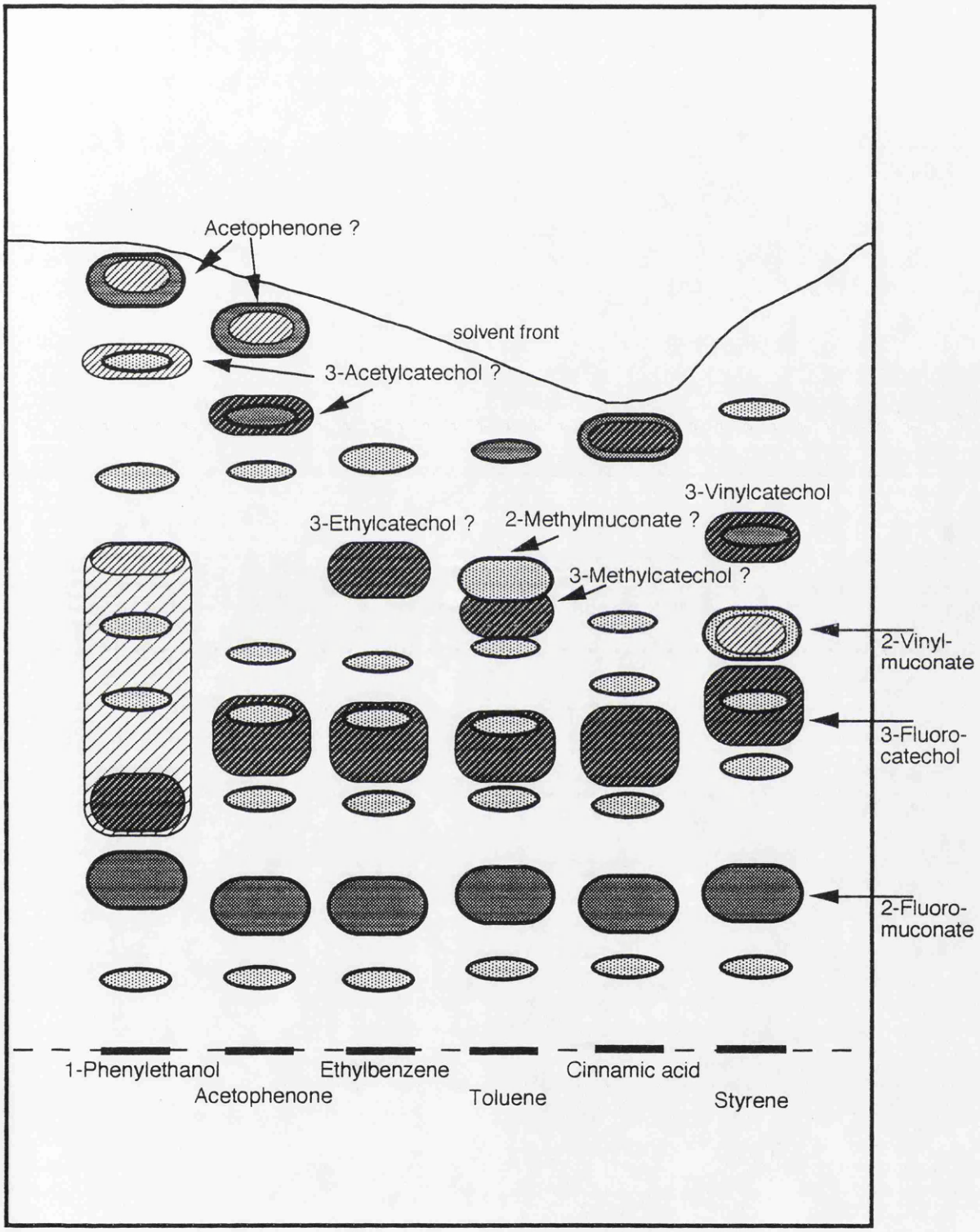
If the enol form derivatised faster than the keto form, or if there was more enol than keto under the conditions of derivatisation, then the enol form would predominate in the derivatised mixture.

Figure 4.8: Production of catechols from various substrates by intact cells of *R. rhodochrous* 26 in the presence of 3-fluorocatechol; representation of a t.l.c. plate

Chromatography was performed as in Methods 2.3.11. Samples were applied to the plate (which contained a fluorescent dye) dissolved in methanol, the plate was then allowed to dry for a few minutes before being placed in solvent system 1 (acetic acid: chloroform, 1:9). After approximately 90 min, the plate was removed from the solvent and left to dry for a few minutes. The plate was then illuminated by UV light (λ= 302 nm), and the areas of the plate which did not fluoresce were marked. The plate was then placed in an iodine tank for approximately 1 hour, then the staining was observed. Some spots stained permanently, others only transiently, the stain vanishing after approximately 30 minutes.

This figure is an idealised representation of one plate, with each track having a different test substrate, as specified. The samples were prepared as described in the text. The labels beside spots are the same as those in Figure 4.6, except those which are queried, which are based on the similarity in R_F to standards, in the case of acetophenone and 3-methylcatechol, or on predicted identity, based on the characteristics of the iodine stain.

Figure 4.8: Production of catechols from various substrates by intact cells of *R. rhodochrous* 26 in the presence of 3-fluorocatechol; representation of a t.l.c. plate



Key: Visible under UV: Faint:
Dark:
Visible after iodine: Faint or Transient:
Permanent:
some smearing:

d) 1-Phenylethanol

A component was detected which was identical to that present in the acetophenone sample, i.e. probably the trimethylsilyl derivative of the enol form of 3-acetylcatechol, with an *m/z* 368 (M^+, 6 %), 353 (M-15, 14 %), 73 ($(Si(CH_3)_3)^+$, 100 %). There was no detectable trimethylsilyl derivative of 1-(2,3-dihydroxyphenyl)ethanol which would be the catechol expected to be formed from 1-phenylethanol. A smell similar to that of acetophenone was noticed in the screw top flask when it was harvested, and the t.l.c. of the extract (Figure 4.8) showed a band that ran at the solvent front, as does acetophenone.

e) Cinnamic acid

There was no detectable catechol (other than 3-fluorocatechol) in the sample. However, a component was detected which produced a fragmentation pattern identical with the library sample of the trimethylsilyl derivative of benzoic acid, with an *m/z* 194 (M^+, 9 %), 179 (M-15, 100 %). There was no benzoic acid detectable by g.c-m.s. in the cinnamic acid used as substrate.

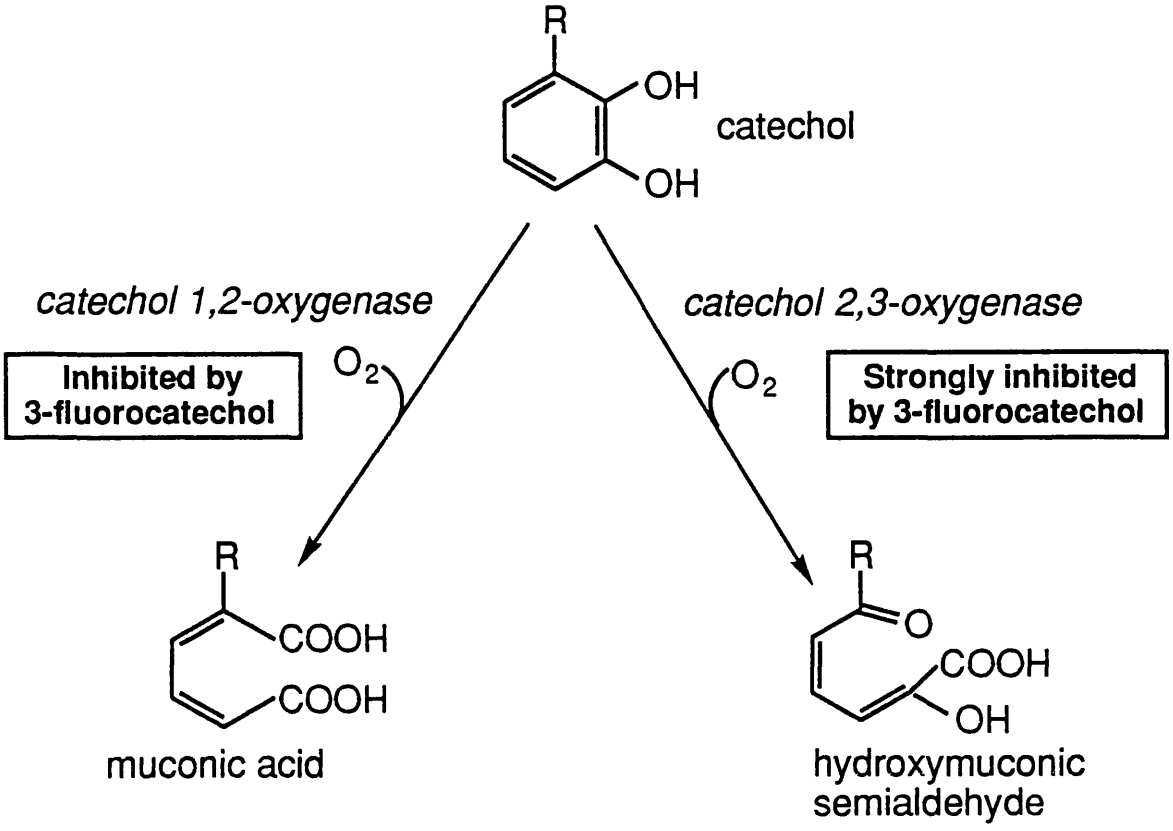
4.5 Identification of the muconic acid accumulating in the growth medium

It had been noticed that there was frequently an absorbance between 250 and 300 nm in growth medium after growth or *R. rhodochrous* 26 on styrene and other aromatics. There was also acidification in the growth medium unless Mes was used as a buffer (Section 3.3.2). These results suggested that some acidic product was accumulating in the medium.

4.5.1 Isolation and identification of 2-fluoromuconate

Several u.v.-absorbing bands are visible on the t.l.c. plate in Figure 4.6, whether the cells had been exposed to styrene or 3-fluorocatechol. The assays performed in Section 4.4.1 showed that 3-fluorocatechol strongly inhibits catechol 2,3-oxygenase, but inhibits catechol 1,2-oxygenase only slightly. Therefore, the most likely transformation of 3-fluorocatechol is probably 1,2-cleavage, producing 2-fluoromuconic acid (Figure 4.9).

Figure 4.9: The cleavage of catechol



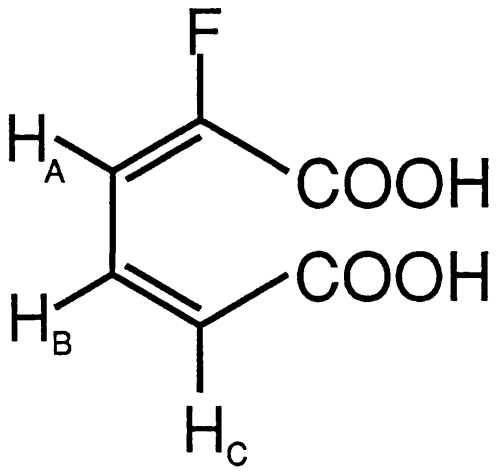
One of the u.v.-absorbing bands on the t.l.c. plate could therefore be 2-fluoromuconic acid, possibly the largest band. Muconic acids generally have an absorbance in the region of 250-270 nm (Dorn & Knackmuss, 1978b), so the substances that accumulated in the growth media and absorbed in this region could be muconic acids.

In order to confirm the identity of the fluoromuconic acid, the equivalent bands were scraped off a set of plates which had also been used to produce 3-vinylcatechol, as described in Section 4.4.2. The material was eluted from the silica as in Methods 2.3.11. After evaporation of the ethyl acetate, a reddy-brown powder was produced, with an absorbance peak of 262.9 nm at pH 6.8 in phosphate buffer. This sample was then analysed by ^{1}H n.m.r. (Figure 4.10), which confirmed the identity of the compound as 2-fluoromuconic acid. These ^{1}H n.m.r. results were in agreement with those published for 2-fluoromuconic acid by Schmidt *et al.* (1980) and by Schreiber *et al.* (1980).

4.5.2 Isolation and identification of 2-vinylmuconate

When *R. rhodochrous* 26 was grown on styrene in a fermenter (Section 3.3.2) the medium showed an absorption in the u.v. (λ_{max} = 283 nm). In addition, a thick band was visible on the t.l.c. plate in Figure 4.6, extracted from a reaction medium in which intact cells had been incubated with styrene, but with no fluorocatechol. It was decided that this band could be a muconate, accumulated when cells were incubated with styrene. A batch of frozen cells (20 g) was thawed, suspended in buffer, and styrene was added (Methods 2.5.6). After 4 hours on a shaker at 30 °C the ensuing suspension was acidified, spun, extracted and separated by t.l.c. (Methods 2.5.6). The major u.v. absorbing band was eluted using ethyl acetate, which was then evaporated off. This produced 12 mg of a yellow liquid, which was analysed by ^{1}H n.m.r. and ^{13}C n.m.r. , and the analysis (Figure 4.11) confirmed the structure as 2-vinylmuconic acid (2-ethenylhexa-2,4-dienedioic acid). An absorbance spectrum was taken of a sample from an identical experiment (Figure 4.12), which showed a major peak in the u.v., varying little with pH: pH 1.5, λ_{max} = 287.2 nm; pH 6.4, λ_{max} = 283.6 nm; pH 12.9, λ_{max} = 282.8 nm.

Figure 4.10: Nuclear magnetic resonance analysis of 2-fluoromuconic acid accumulated by intact cells of *R. rhodochrous* 26 incubated with styrene in the presence of 3-fluorocatechol

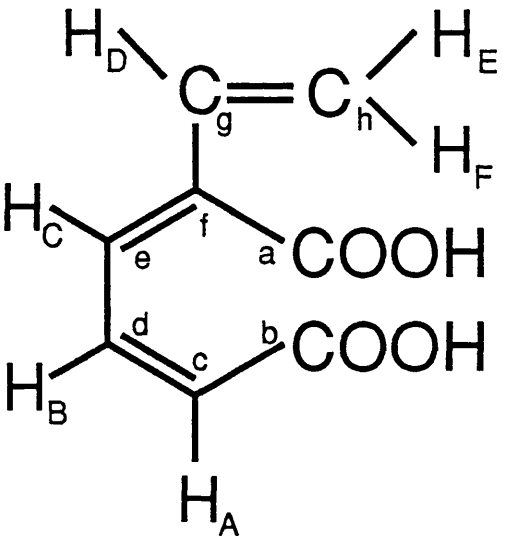


$\delta_H((CD_3)_2CO)$

7.86 (1H, dd, *J* 11.0 Hz, J_{HF} 19.2 Hz, H_A)
7.69 (1H, t, *J* 11.0 Hz, H_B)
5.74 (1H, d, *J* 11.0 Hz, H_C)

n.m.r. analysis performed as in Methods 2.5.1b

Figure 4.11: Nuclear magnetic resonance analysis of 2-vinylmuconic acid accumulated by intact cells of *R. rhodochrous* 26 incubated with styrene



$\delta_H((CD_3)_2CO)$

7.28 (6H, m)

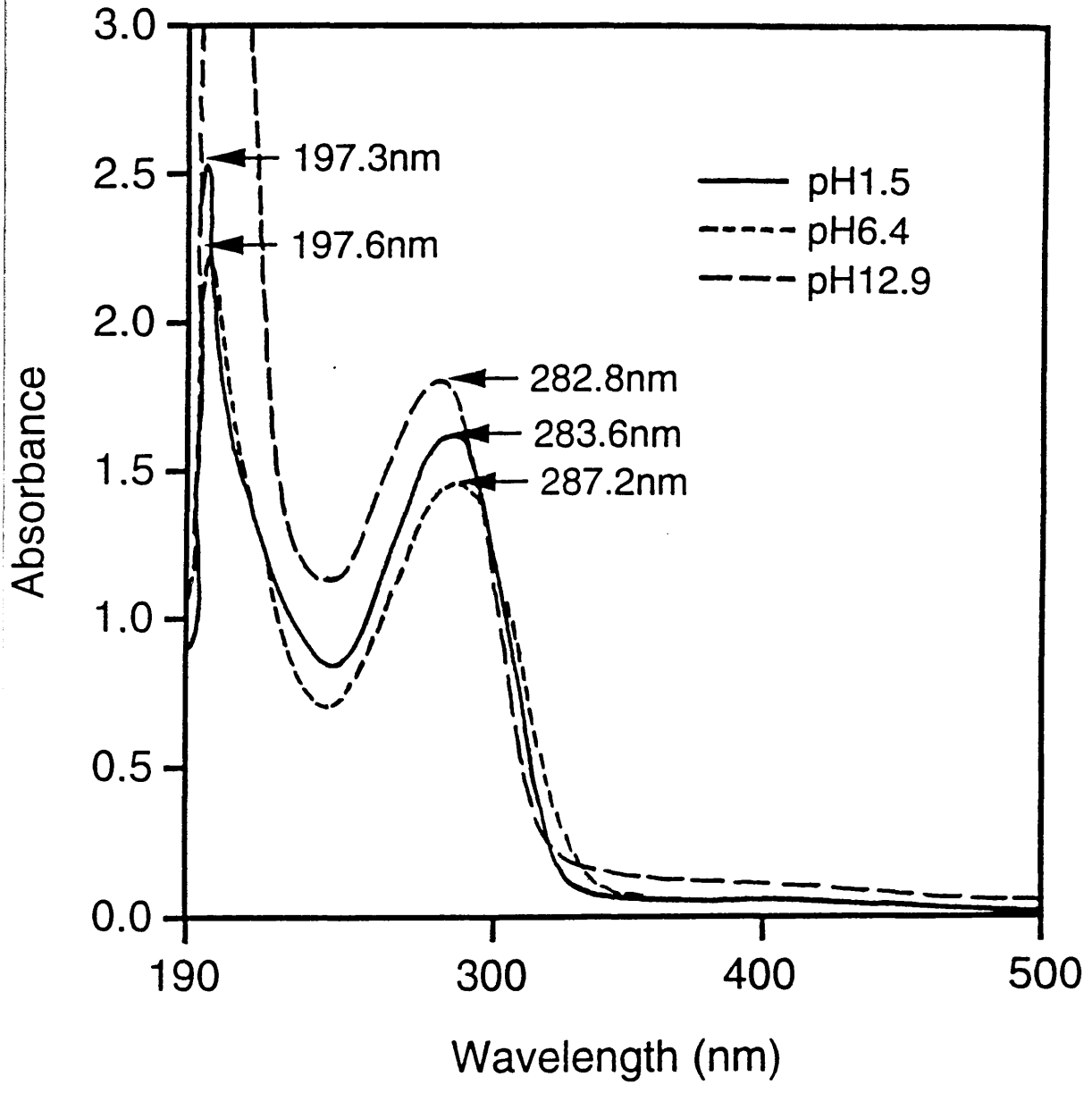
$\delta_C((CD_3)_2CO)$

7.6	C_h
3.9	C_g
26.4	C_c *
26.9	C_d *
28.0	C_e *
43.5	C_f
67.2	COOH
72.3	COOH

* assignments may be interchanged

n.m.r. analysis performed as in Methods 2.5.1b

Figure 4.12: Absorbance scan of vinylmuconic acid at various pHs



4.5.3 Identification of muconic acids formed from other substrates

Further analysis of the g.c.-m.s. results from the fluorocatechol accumulation experiments with toluene and ethylbenzene substrates (see above, 4.4.3a and b) led to the identification of fragmentation patterns similar to a standard of trimethylsilyl muconic acid, with molecular ions corresponding to the trimethylsilyl derivatives of fluoromuconic acid, methylmuconic acid and ethylmuconic acid:

a) Fluoromuconic acid

Both the toluene and the ethylbenzene samples contained a component which appeared to be the trimethylsilyl derivative of fluoromuconic acid, with an *m/z* 304 (M^+, 3 %), 289 (M-15, 58 %), 73 ($(Si(CH_3)_3)^+$, 100 %) in the toluene sample and with an *m/z* 304 (M^+, 5 %), 289 (M-15, 100 %) in the ethylbenzene sample

b) Methylmuconic acid

The toluene sample contained a component which appeared to be the trimethylsilyl derivative of methylmuconic acid, with an *m/z* 300 (M^+, 6 %), 285 (M-15, 39 %), 73 ($(Si(CH_3)_3)^+$, 100 %).

c) Ethylmuconic acid

The ethylbenzene sample contained a component which appeared to be the trimethylsilyl derivative of ethylmuconic acid, with an *m/z* 315 (M^+, 1 %), 300 (M-15, 39 %), 197 (?, 100 %).

d) Absorption characteristics

Further evidence for the production of muconic acids by *R. rhodochrous* 26 when grown on many substrates was the presence of accumulated u.v.-absorbing medium components. The u.v. characteristics of the putative muconic acids accumulated in the growth medium when the organism was grown on various substrates are listed below (at approximately pH 6.6):

Toluene, λ_{max} = 262 nm; **Ethylbenzene**, λ_{max} = 258 nm; **Benzene**, λ_{max} = 255 nm; **Cinnamic acid**, λ_{max} = 259 nm; **Benzyl alcohol**, λ_{max} = 259 nm

4.5.4 Monitoring the accumulation of muconic acids in cultures of *R. rhodochrous* 26 growing on styrene or benzyl alcohol.

The presence of 2-vinylmuconic acid in the growth medium of *R. rhodochrous* 26 grown on styrene, and of other muconic acids formed from other substrates, raised the question as to why these compounds accumulated. Were they being formed more quickly than they were utilised, or were they dead-end products that were not metabolised further? To test this, cells were grown on styrene while the approximate concentration of muconic acids was monitored by following the absorbance spectrum of the medium (Methods 2.5.7).

When styrene was used as the growth substrate it was found that the apparent concentration of muconic acid increased approximately in line with the growth of *R. rhodochrous* 26 (Figure 4.13). The rapid increase in the apparent concentration of muconate in flask 1 at the end of the experiment was probably due to cell lysis, resulting in the release of u.v.-absorbing compounds. The concentration of the putative muconate plateaued at the end of growth and the fact that it never fell suggests that it is not metabolised.

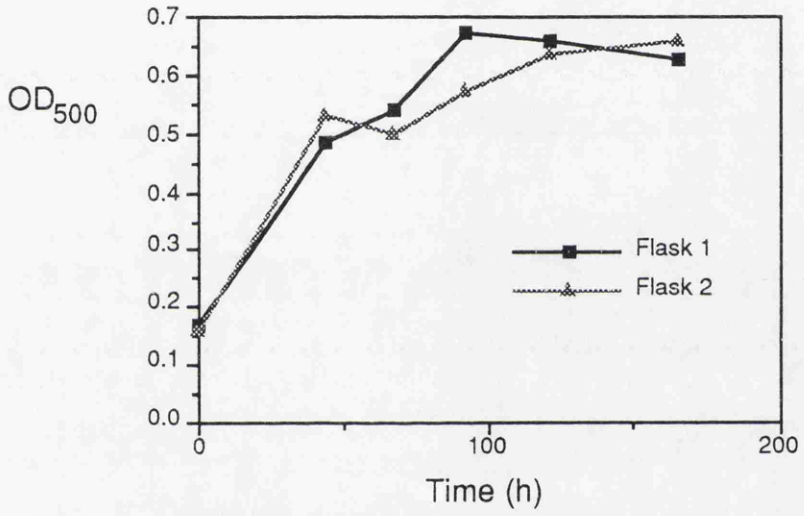
The further metabolism of substituted muconic acids is known to cause particular problems for organisms (Introduction, 1.2.3d)). The accumulation of muconic acids during growth on benzyl alcohol was therefore tested, since it was thought likely that benzyl alcohol would be metabolised to the unsubstituted *cis, cis*-muconic acid. However, because benzyl alcohol itself has an absorbance in the 260 nm region this method did not give clear results. The absorbance in this region remained fairly constant, but the peak did shift slightly during growth, moving from 257.3 to 258.7 nm, suggesting that muconic acid had also accumulated during growth on benzyl alcohol.

In order to get a clearer idea of the quantity of styrene which was being converted into cellular material and into muconic acid during growth, a total carbon analysis was attempted (Methods 2.5.1d). However, due to the low yields obtained with the concentration of styrene used (1 mM), it was not possible to obtain reliable results.

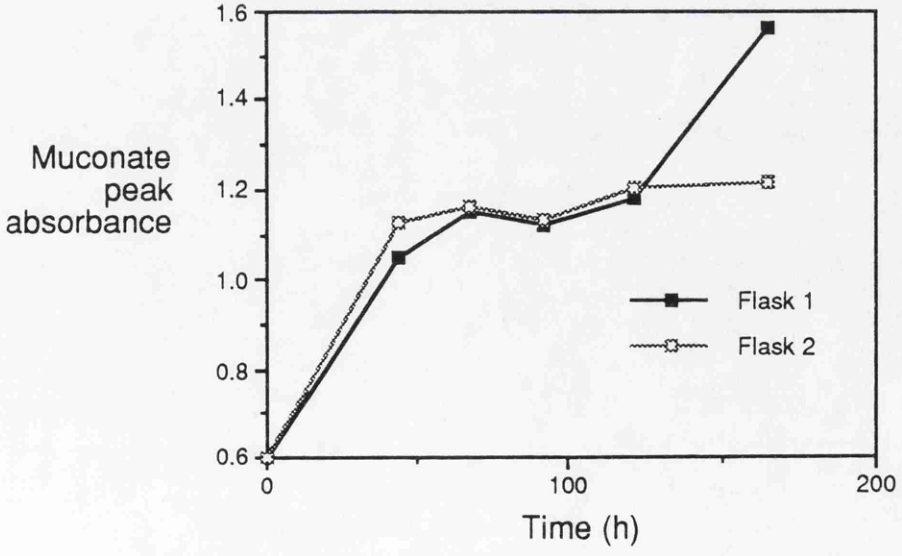
Figure 4.13: Monitoring of muconic acid accumulation during growth on styrene

Cells were grown on styrene in screw top flasks, as in Methods 2.4.4f Two replicate flasks were inoculated with 5 ml of nutrient broth culture and were sampled regularly. Sampling involved the aseptic removal of medium through a mini-inert valve (Methods 2.5.7), the optical density at 500 nm was taken, to measure growth, then the sample was spun to remove cells, and the absorbance of the supernate was measured between 200 and 300 nm. The absorbance and wavelength of the peak nearest to 280 nm (275.4 - 277.8 nm) was recorded, except for t=0, in which there was no peak, so the absorbance at 270 nm was taken as a starting point.

a) Growth of cells on styrene (these growth curves are also plotted in Figure 3.5)



b) The production of muconic acid during growth on styrene



However, it was clear that maybe as much as 40% of the styrene growth substrate was accumulated as muconic acid.

4.6 Discussion

4.6.1 The metabolism of styrene by *R. rhodochrous* 26

1) Extracts of *R. rhodochrous* 26 grown on nutrient broth/styrene were able to transform styrene *cis*-glycol, in an NAD-dependent reaction, into a compound identified by g.c.-m.s. to be vinylcatechol (Section 4.3.3, Figure 4.5).

2) Cells metabolising styrene in the presence of 3-fluorocatechol, as a catechol oxygenase inhibitor, accumulate a compound which g.c.-m.s. and ^{1}H n.m.r. have identified as 3-vinylcatechol (Section 4.4.2, Figure 4.7).

3) *R. rhodochrous* 26 accumulates a substance while growing on styrene, which has been shown by ^{1}H and ^{13}C n.m.r. to be 2-vinylmuconate (Section 4.5.2, Figure 4.11).

4) It is likely that 2-vinylmuconic acid is not metabolised, and that up to 40% of the styrene provided as a growth substrate may accumulate as muconic acid.

These results confirm the pathway postulated at the end of the last chapter (Figure 3.7), and in addition show that 3-vinylcatechol is cleaved by a catechol 1,2 oxygenase type enzyme, producing 2-vinylmuconic acid, which appears to be a dead end product. However, *R. rhodochrous* 26 is able to grow on styrene, so there must be an active pathway to break down the 3-vinylcatechol, and this is covered in the next chapter.

4.6.2 The metabolism of other substrates

a) The formation of catechols

The catechols produced from toluene, ethylbenzene and acetophenone (methylcatechol, ethylcatechol and acetylcatechol respectively) are those that would be expected from a pathway similar to that in Figure 3.6. Earlier growth experiments (Table 3.2) had shown that *R. rhodochrous* 26 is unable to grow on acetophenone

under the conditions tested, but it appears that some transformation of this substrate is possible.

The results using 1-phenylethanol as a substrate were unexpected, as no catechol derived from the dihydroxylation of 1-phenylethanol was detected, in spite of the fact that 1-phenylethanol is a growth substrate (Table 3.2). However, it is possible that this catechol was present, and it was either very unstable or present only in small quantities. However, acetylcatechol was detected, as when acetophenone was the substrate. A smell similar to acetophenone had been noticed when the organism was grown on 1-phenylethanol (Table 3.2). There was also a spot present on the t.l.c. plate of this sample (Figure 4.8) which ran at the solvent front, as does authentic acetophenone. The simplest explanation for this is that there is an alcohol dehydrogenase present which is transforming the ring substituent from an alcohol to a ketone. This alcohol dehydrogenase must be acting on the 1-phenylethanol to produce acetophenone, but it could also act on any or all of the intermediates between 1-phenylethanol and acetylcatechol (Figure 4.14).

No catechol was detected in the cinnamic acid sample, though for the reasons mentioned in the previous paragraph this is not proof that no catechol whatsoever was produced. However, the presence of a compound tentatively identified as benzoic acid, indicates that the cinnamic acid could be being transformed by the pathway shown in Figure 1.14a, and that this may be the pathway used when *R. rhodochrous* 26 grows on cinnamic acid, since cells grown on cinnamic acid can oxidise benzoic acid (Table 3.8).

b) The accumulation of muconic acids in growth media

The identification by g.c.-m.s. of methylmuconate and ethylmuconate in the medium of cells exposed to 3-fluorocatechol and toluene or ethylbenzene strongly indicates that these substrates are metabolised by the same route as styrene (Figure 4.15a). The absorbance maximum of the compound accumulating when *R. rhodochrous* 26 is grown on toluene, λ_{max} of 262 nm (4.5.3d), compares well with the value reported in the literature for 2-methylmuconic acid, a λ_{max} of 260 nm at pH 8.0 (Dorn & Knackmuss, 1978b). The absorption maximum of 2-fluoromuconic acid also compares

Figure 4.14: Possible routes for the production of 3-acetylcatechol from 1-phenylethanol

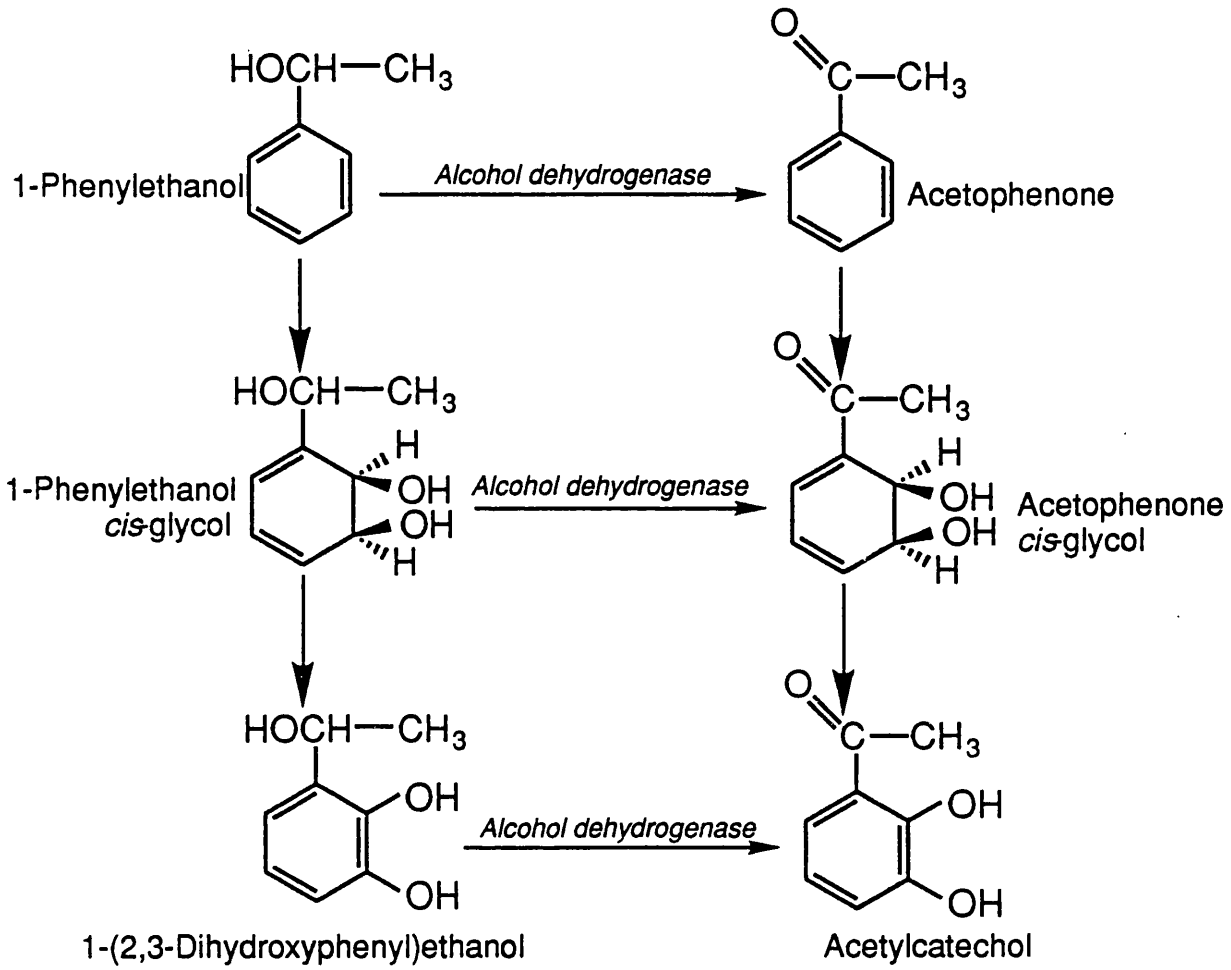
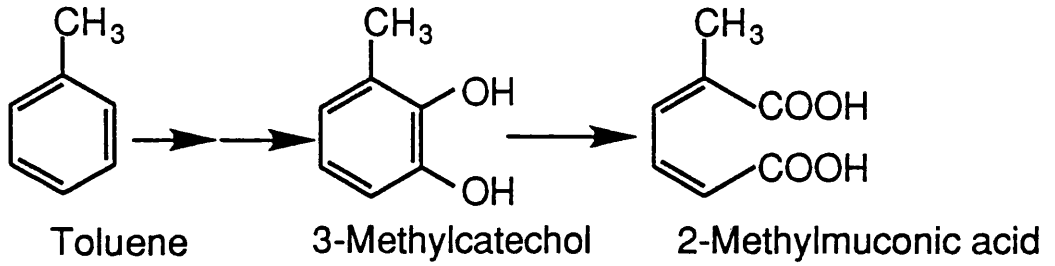
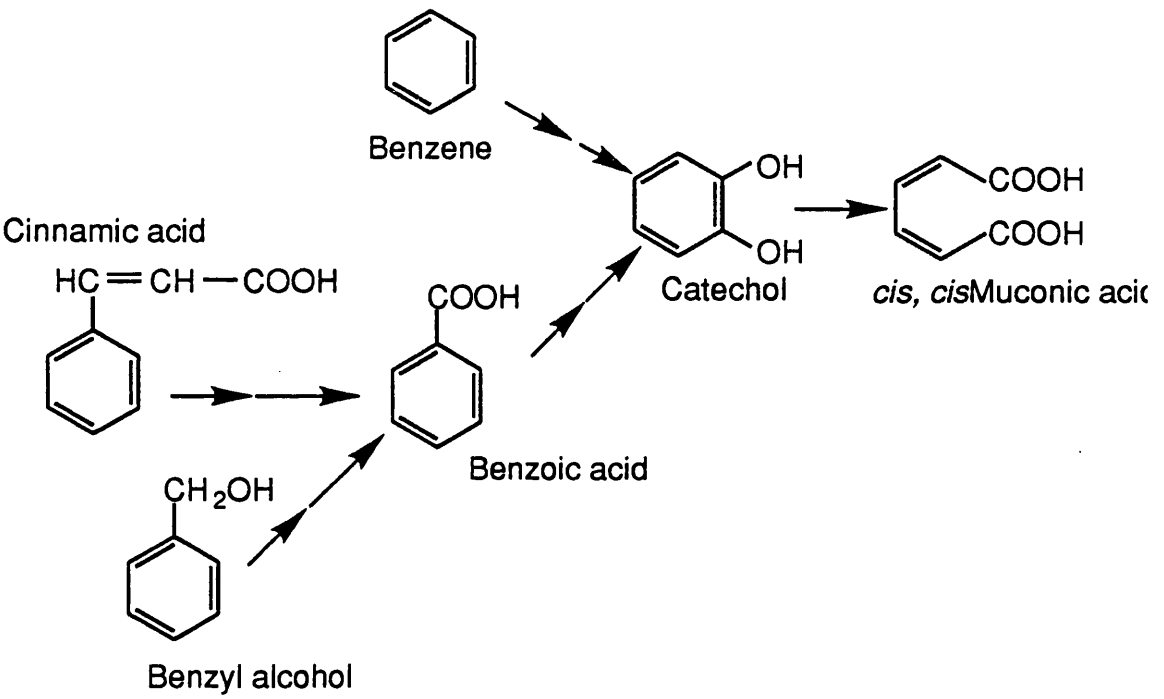


Figure 4.15: The production of catechols and muconates from various substrates

a) The production of 2-methylmuconic acid from toluene



b)The production of *cis, cis*-muconic acid from benzene, cinnamic acid and benzyl alcohol



well with information reported previously, with an absorbance peak of 262.9 nm at pH 6.8 (4.5.1), compared with a reported peak of 261 nm at pH 8.0 (Dorn & Knackmuss, 1978b).

R. rhodochrous 26 might be expected to transform several substrates to *cis, cis*-muconic acid (Figure 4.15b). The reported λ_{max} of this substance is 257 nm at pH 8.0 (Dorn & Knackmuss, 1978b), which compares well with the observed absorbance characteristics of media from cells grown on the relevant substrates: benzyl alcohol, λ_{max} = 259 nm; benzene, λ_{max} = 255 nm and cinnamic acid, λ_{max} = 259 nm (Section 4.5.3d).

It is likely that even the simplest muconate, *cis, cis*-muconate, is accumulated by cells growing on benzyl alcohol, indicating that either the organism is totally unable to metabolise muconic acids or the breakdown proceeds at an extremely low rate.

c) Conclusions

The identification of catechols accumulating in the media of cells grown on substrates other than styrene, coupled with the identification of muconic acids further supports the hypothesis that substrates such as toluene and ethylbenzene are being metabolised by the same pathway as styrene.

5. Identification of enzymes involved in the degradation of styrene

The previous chapter described the identification of certain pathway intermediates that are apparently involved in the degradation of styrene by *R. rhodochrous* 26 and this provided further evidence for the catabolic pathway that might be involved (Figure 3.7). That postulated pathway can then be extended to suggest further possible steps in metabolism of the ring cleavage products, based on established routes (Introduction, Figures 1.28, 1.30, 1.31), and this is illustrated in Figure 5.1. This chapter describes an investigation of the enzymes of this pathway.

5.1 Ring oxygenases in whole cells and extracts

5.1.1 The use of $^{18}O_2$ to distinguish between an initial monooxygenase or dioxygenase ring attack.

a) Introduction

Earlier results had indicated that styrene *cis*-glycol was probably involved in the metabolism of styrene, which would strongly indicate that the first enzymatic attack on the styrene was a dioxygenase, adding one molecule of oxygen (Figure 5.2a). Another possible metabolic route to 3-vinylcatechol could be *via* two consecutive monooxygenase reactions (Figure 5.2a). One method of distinguishing between these two pathways is the use of $^{18}O_2$.

A method had already been developed (Section 4.4.2) to isolate 3-vinylcatechol from the medium of intact cells exposed to styrene in the presence of 3-fluorocatechol. This 3-vinylcatechol had already been identified by its derivatised mass spectrum (Chapter 4.4.2). If the $^{16}O_2$ present in the reaction mixture producing this 3-vinylcatechol was replaced by a blend of $^{18}O_2$ and $^{16}O_2$ then the molecular masses of the 3-vinylcatechols produced would give information as to the nature of enzymatic attack on styrene, as explained in Figure 5.2b.

Figure 5.1: Generalised scheme for the breakdown of an aromatic compound *via* a *cis*-glycol

(for styrene, R = -CH=CH$_2$)

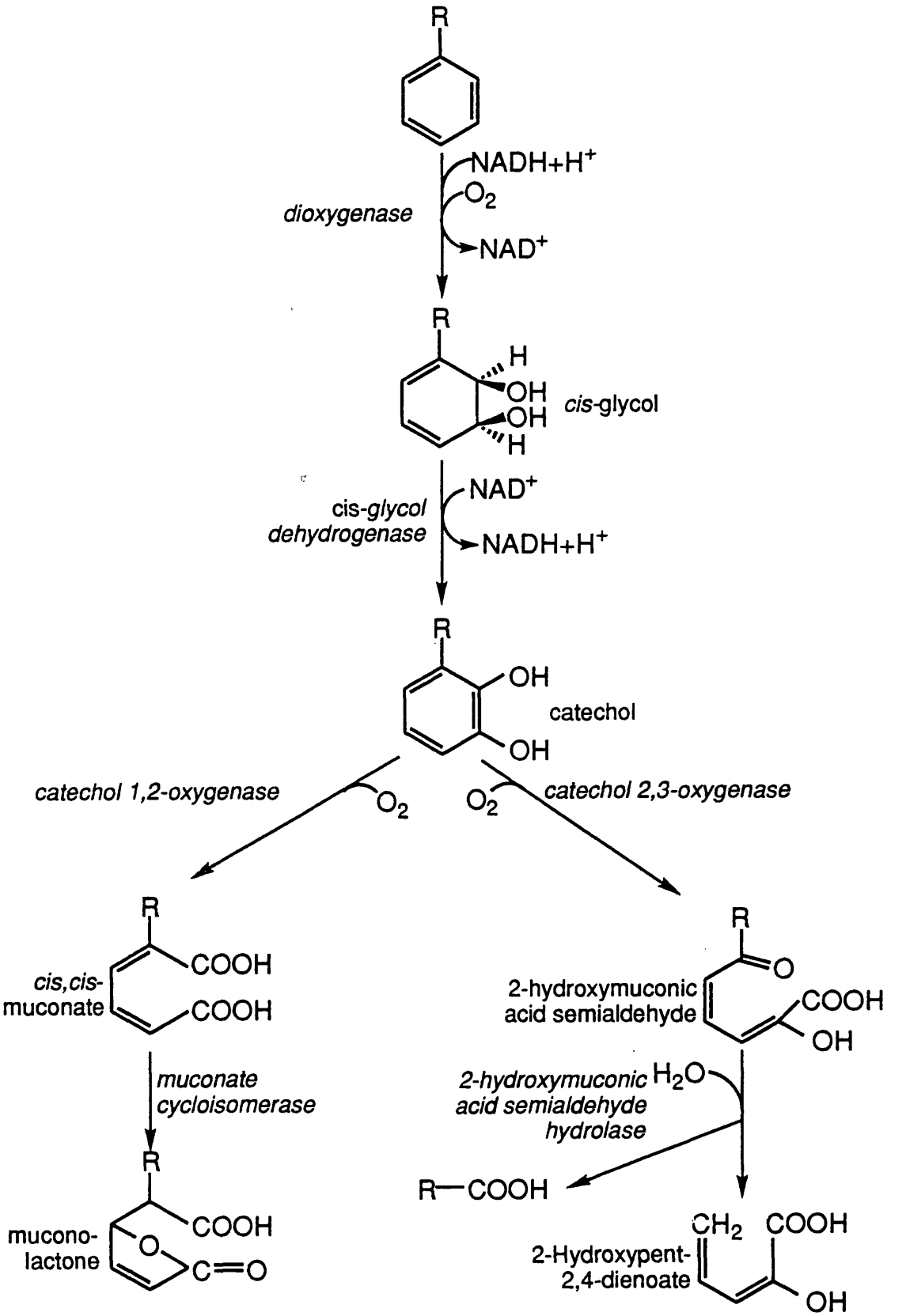
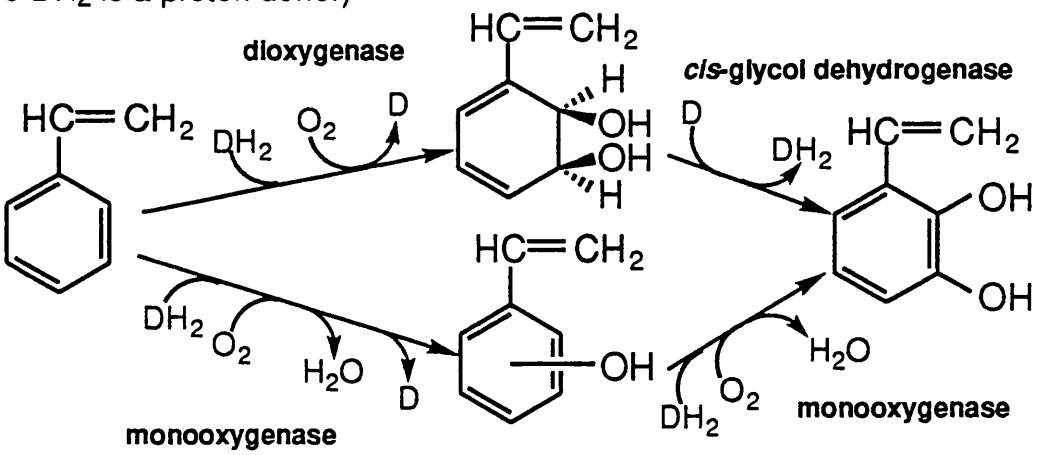


Figure 5.2: The use of $^{18}O_2$ to distinguish between initial monooxygenase or dioxygenase ring attack

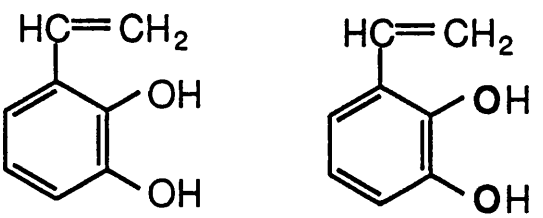
a) The production of 3-vinylcatechol from styrene *via* both monooxygenase and dioxygenase ring attack

(Where DH_2 is a proton donor)



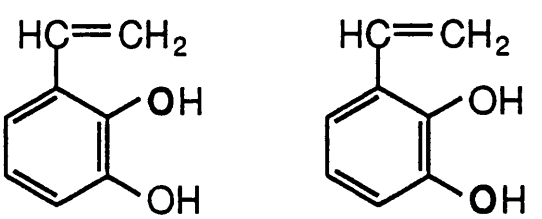
b) Comparison of the molecular weights of 3-vinylcatechols produced in an atmosphere containing both $^{16}O_2$ and $^{18}O_2$

If the ring of styrene is attacked by a dioxygenase, then both added oxygen atoms must come from the same oxygen molecule, so the two possible catechol structures are:
(^{18}O = **O**, ^{16}O = O)



M_r = 136 M_r = 140

If the ring is attacked by two consecutive mono-oxygenases, then the added oxygen atoms can come from different oxygen molecules, so in addition to the above catechols, two others are possible:



M_r = 138 M_r = 138

b) Method and results

The basic method used was the same as that used to accumulate 3-vinylcatechol for analysis in the previous chapter (Section 4.4.2), with modifications to allow the transformation to occur in a controlled oxygen atmosphere (Methods 2.5.5). One sample of 3-vinylcatechol was generated in an atmosphere of approximately 50 % $^{18}O_2$ and 50 % $^{16}O_2$, the other in 100 % $^{16}O_2$.The two samples were then derivatised to trimethylsilyl derivatives and analysed by g.c.-m.s. (Methods 2.5.1a).

The full fragmentation spectrum of the trimethylsilyl derivative of 3-vinylcatechol produced in an atmosphere of 50 % $^{18}O_2$ / 50 % $^{16}O_2$ is illustrated in Figure 5.3. The spectrum in the region of the molecular ion is shown in Figure 5.4a, compared to that of the 3-vinylcatechol produced in an atmosphere of 100 % $^{16}O_2$ in Figure 5.4b. These results clearly show that the molecular ion at 280 in the control sample is also present in the 50 % $^{18}O_2$ / 50 % $^{16}O_2$ sample, but this sample has an additional ion at 284, four mass units higher. The derivatised molecular ions of 280 and 284 are equivalent to underivatised molecular ions of 136 and 140 respectively. The smaller fragments at M+1 and M+2 are present in both samples, and are due to isotopes of silicon and carbon. The relative abundance of the peak at m/z = 282.3 is no higher in the sample from 50 % $^{18}O_2$ / 50 % $^{16}O_2$ than in the sample produced in an atmosphere of 100 % $^{16}O_2$. Apart from these isotope effects, there is no sign of an M+2 peak in the $^{18}O_2$ sample.

The production of M+4 fragments but no M+2 fragments when intact cells are exposed to styrene in the presence of 3-fluorocatechol and in an atmosphere of approximately 50% $^{18}O_2$ strongly supports the hypothesis that the initial ring oxygenation of styrene is carried out by a dioxygenase (as explained in Figure 5.2b).

5.1.2 Attempts to detect oxygenase activity in extracts using an oxygen electrode

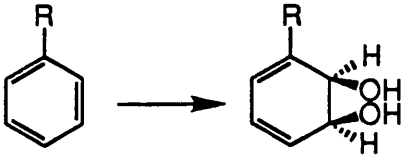


Figure 5.3: Fragmentation pattern of 3-vinylcatechol produced from styrene by intact cells in an atmosphere of approximately 50% $^{18}O_2$/50% $^{16}O_2$

The sample of 3-vinylcatechol was produced as explained in the text, and was derivatised as described in Methods 2.5.1a. The mass spectrum of the sample was measured using the method described in Methods 2.5.1a.

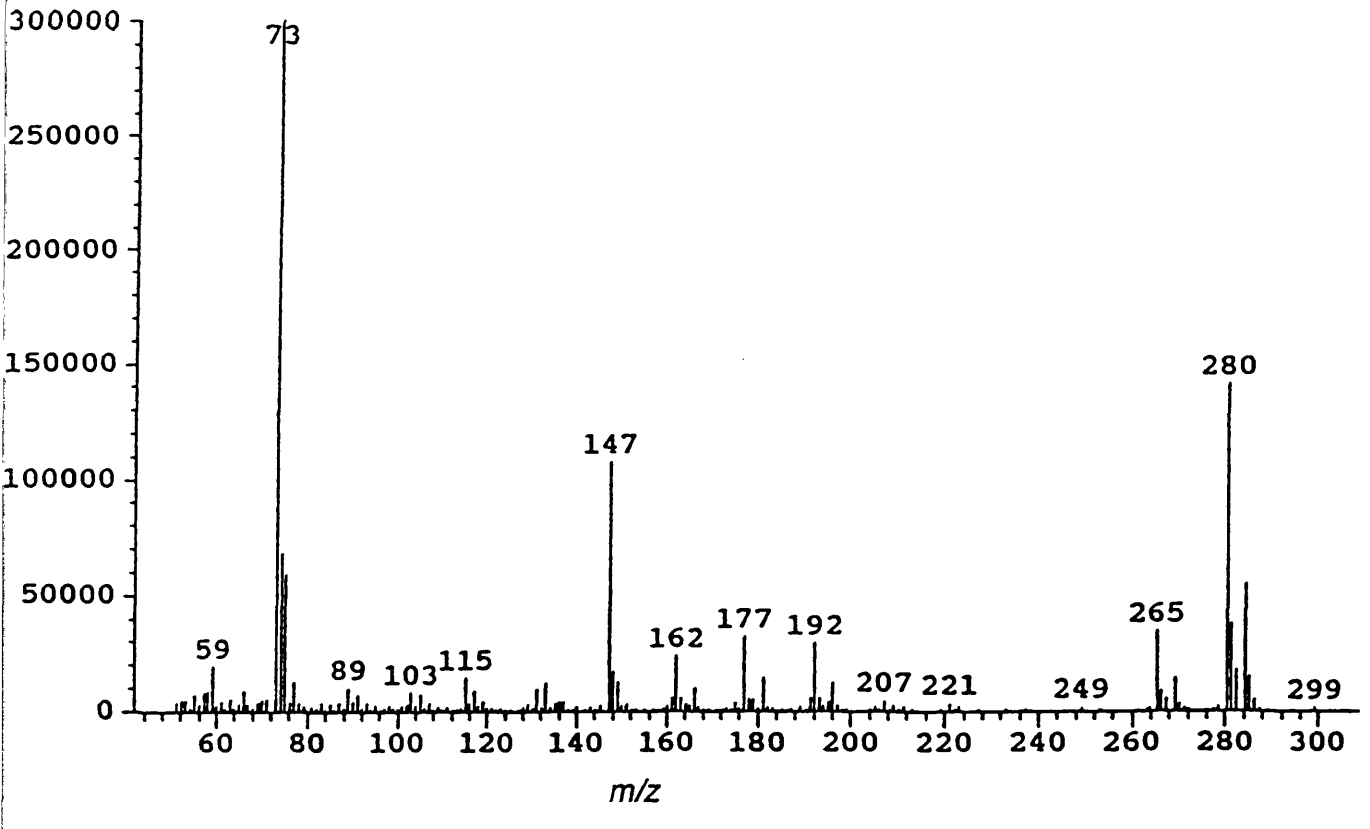
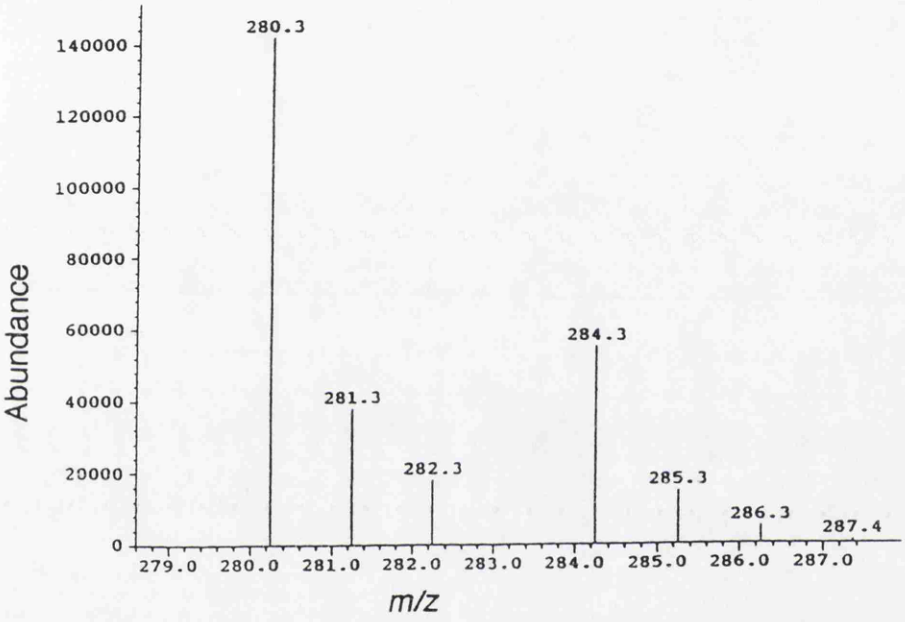


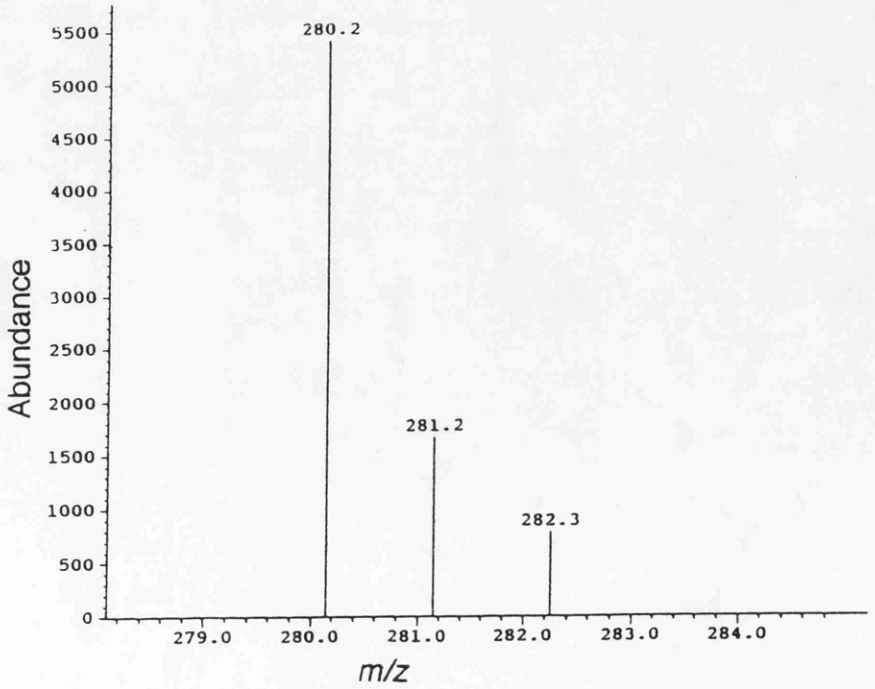
Figure 5.4: Comparison of the fragmentation patterns in the region of the molecular ion of 3-vinylcatechol produced from styrene by intact cells in an atmosphere of approximately 50% $^{18}O_2$/ 50% $^{16}O_2$ and of 100% $^{16}O_2$

Samples were produced as described in the text, and were derivatised and analysed by g.c.-m.s. as described in Methods 2.5.1a.

a) The mass spectrum in the region of the molecular ion for the trimethylsilyl derivative of 3-vinylcatechol produced from styrene in an atmosphere of 50% $^{18}O_2$/ 50% $^{16}O_2$



b) The mass spectrum in the region of the molecular ion for the trimethylsilyl derivative of 3-vinylcatechol produced from styrene in an atmosphere of 100% $^{16}O_2$



The previous experiment had shown that ring oxygenation occurs as a result of an attack by molecular oxygen. It might therefore be expected that a measurable increase in oxygen consumption in the oxygen electrode would be detected when styrene was added to a reaction mixture containing extract. Increased oxygen consumption after addition of styrene had been noticed using intact cells in an oxygen electrode (Table 3.3), but when an equivalent preliminary experiment was done using cell-free extract instead of cells in the oxygen electrode (Methods 2.6.1), there was no measurable increase in the rate of consumption of oxygen when styrene or toluene (100 µl of saturated solution in a 3 ml assay) were added. Dioxygenases are often unstable (Haigler & Gibson, 1990a), so attempts were made to develop a method that gave the maximum chance of retaining activity. However, although many different experimental methods and buffers were tested, no activity was ever detected. One example follows.

a) The production of cell-free extract

Since oxygenases are frequently inactivated by exposure to oxygen, all French pressing was performed using buffers that had been degassed and then saturated with nitrogen (Methods 2.4.6), and all procedures were carried out at 4°C. Phosphate buffer was replaced by Mes (50 mM, pH 6.0), since some reports have suggested that phosphate buffer may damage oxygenases (Powlowski & Shingler, 1990). To minimise possible inactivation of any enzymes, cells were harvested fresh from a fermenter, where they had been growing on nutrient broth/styrene (i.e. induced). The cells were rinsed with 50 mM Mes buffer (pH 6.0), then homogenised as gently as possible in degassed MEG buffer (also pH 6.0, Methods 2.4.6). The suspension was then French pressed as usual, and the high spin supernate was retained, which had a protein concentration of 4.3 mg/ml. A batch of cells which had been grown on nutrient broth (i.e. uninduced) were also pressed in the same way, as a control, the high spin supernate of which had a protein concentration of 0.7 mg/ml.

b) Assaying for cell-free oxygenase activity

Assays were performed in the oxygen electrode, with slight variations on the normal method. One aim was to get as high a concentration of protein in the assay as possible;

this was achieved by limiting the assay volume to 2 ml, and using 500 µl of extract per assay (i.e. approximately 1.0 mg protein/ml in the induced assays and 0.2 mg protein/ml in the uninduced). The following procedures were used:

i) Mes buffer (1.2 ml, pH 7.0) was placed in the oxygen electrode well, and was left for five minutes to allow saturation with oxygen. The top was screwed on, and the chart recorder set to 100 % oxygen saturation. Then 500 µl of either induced or non-induced extract and 100 µl 2 mM $FeSO_4$ were added, and left for 5 minutes to allow the rate of oxygen consumption to stabilise. Then 100 µl of 3.3 mM NADH was added and the reaction mixture was left again for 5 minutes. Finally, 100 µl of saturated styrene solution was added. There was no change in the rate of oxygen consumption when styrene was added with either the induced or the non-induced extract.

ii) A reaction was done in the same way as (i), but the concentration of the added NADH was 50 mM instead of 3.3 mM. There was still no change in the rate of oxygen consumption on addition of the styrene solution

iii) A reaction was done in the same way as (i), but 1 µl styrene was added instead of the styrene solution. However, there was no change in the rate of oxygen consumption

In order to test if the styrene dioxygenase activity was present in the membrane fraction, the pellets from the ultracentrifugation of the extract (Methods 2.4.6) were resuspended in MEG buffer, which gave 3.5 mg protein/ml of induced pellet suspension, and 0.7 mg protein/ml of uninduced pellet suspension. These samples were then tested for any change in oxygen consumption when styrene was added, using the following assay:

Mes buffer (1.6 ml, pH 7.0) was placed in the oxygen electrode well, and was left for five minutes to allow saturation with oxygen. The top was screwed on, and the chart recorder set to 100 % oxygen saturation. Then 500 µl of either induced or non-induced pellet suspension and 100 µl 2 mM $FeSO_4$ were added, and left for 5 minutes to allow the rate of oxygen consumption to stabilise. Then 100 µl of 3.3 mM NADH was added

and the reaction mixture was left again for 5 minutes. Finally 100 µl of saturated styrene solution was added. There was no change in the rate of oxygen consumption when styrene was added with either the induced or the non-induced extract.

These results suggest that the putative dioxygenase indicated by the $^{18}O_2$ results is too unstable to be active under any of these conditions.

c) An attempt to reactivate the oxygenase

Previous work with purified oxygenases has shown that it is sometimes possible to activate and deactivate the enzymes by reducing and oxidising them (Bernhardt & Meisch, 1980). A similar re-activation method was developed for use with the extracts produced above (Section 5.1.2a). This involved the addition of reducing agents, storage on ice for 1 h, then the addition of Fe^{2+}, followed by 1h storage on ice (Methods 2.6.2). This 'activated' extract was then assayed in the oxygen electrode in the following way:

(i) Mes buffer (1.2 ml, pH 7.0) was placed in the oxygen electrode well, and was left for five minutes to allow saturation with oxygen. The top was screwed on, and the chart recorder set to 100 % oxygen saturation. Then 500 µl of either induced or non-induced extract which had been 'activated' was added. The chart recorder trace immediately showed that the oxygen in the assay was being rapidly reduced by the extract, so the assay could not be continued.

(ii) This reaction was performed in the same way as (i), except 1.6 ml of MES buffer was used and only 100 µl of 'activated' extract was added. However, the oxygen was again rapidly consumed by the extract.

Clearly this method is not suitable for use with an oxygen electrode, because of the large amount of reducing power involved, which causes immediate reduction of free oxygen in the reaction mixture.

5.1.3 Other attempts at measuring oxygenase activity

In addition to the use of oxygen consumption changes to detect cell-free oxygenase activity, several other assay systems were tried:

(i) **Gas headspace analysis**: Samples of the gas above a reaction mixture were analysed by g.c, to detect any changes in the concentration of styrene. However, no significant changes were detected.

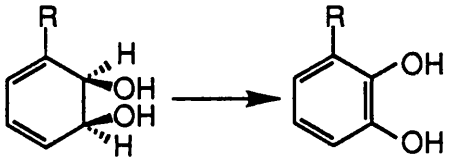
(ii) **Spectrophotometric measurement of NAD(P)H oxidation**: The dioxygenase reaction is likely to be either NADH or NADPH linked, so theoretically it should be possible to monitor the reaction by changes in A_{340}. Unfortunately, the level of endogenous NAD(P)H oxidase activity masked any changes due to the presence of styrene.

(iii) **Spectrophotometric measurement of the oxidation of indole**: Other workers have shown that toluene dioxygenase from *Pseudomonas putida* can oxidise indole to a yellow dye, which eventually air-oxidised to indigo(Jenkins & Dalton, 1985). This reaction was attempted with extracts of *R. rhodochrous* 26, but no transformation of indole was detected.

5.2 Identification of enzyme activities in extracts

All assays were performed with extracts in PEG buffer (Methods 2.4.6) of cells which had been grown on nutrient broth/styrene (induced extract) in a fermenter (Methods 2.4.4k) or on nutrient broth (uninduced extract) in a flask (Methods 2.4.4a). These assay procedures have not been completely optimised.

5.2.1 Toluene *cis*-glycol and styrene *cis*-glycol dehydrogenase



An NAD-linked toluene *cis*-glycol dehydrogenase activity was found in extracts of *R. rhodochrous* 26 (prepared as in Methods 2.4.6) which had been grown on nutrient broth and styrene, but not when the cells had been grown on nutrient broth only (Table 5.1). Rates of up to 130 nmol/min mg protein were found in some extracts (results not

Table 5.1: Measurements of styrene and toluene *cis*-glycol dehydrogenase activities

Assays were done as in Methods 2.7.1, in 50 mM phosphate buffer (pH 7.0). Toluene *cis*-glycol (dissolved in buffer, having been re-crystallised from ethyl acetate, as in Methods 2.7.1a) was added at a final concentration of 0.12 mM; styrene *cis*-glycol was added in ethyl acetate (5 µl of approx. 9 mg/ml), final concentration approx. 0.1 mM. The extract (in PEG buffer, Methods 2.4.6) from cells grown on nutrient broth contained 0.7 mg protein/ml (assay concentration 0.01 mg protein/ml), and the extract from cells grown on nutrient broth/styrene contained 4.0 mg protein/ml (assay concentration 0.07 mg protein/ml). The reaction was started with NAD, at a final concentration of 1.67 mM. Controls were done without *cis*-glycol, without NAD and with extract replaced with PEG buffer, with no activity detected in any control. All results are means of at least two determinations. Because of the presence of ethyl acetate in the styrene *cis*-glycol assay, the effect of the same quantity (5 µl) of ethyl acetate was tested on the toluene *cis*-glycol reaction.

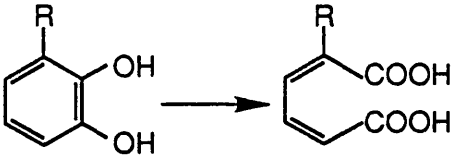
The rate of production of NADH was measured at 340 nm, and rates were calculated using $\varepsilon = 6.3\ mM^{-1}cm^{-1}$ (Boehringer, 1987)

Substrate	Rate of NAD reduction (nmol/min/mg protein)		
	Extract of cells grown on nutrient broth	Extract of cells grown on nutrient broth/styrene	Extract of cells grown on nutrient broth/styrene, with ethyl acetate present in assay
Toluene *cis*-glycol	<1.4	50	36
Styrene *cis*-glycol	0		44

shown). No activity was found when NADP was used as the cofactor. A similar NAD-linked activity was found when styrene *cis*-glycol was used instead of toluene *cis*-glycol as the substrate.

During incubation of induced extract and styrene *cis*-glycol a yellow colour accumulated in the reaction mixture (λ_{max} = 334 nm and 423 nm), but only if NAD was present. This colour later disappeared.

5.2.2 Catechol 1,2-oxygenase



Catechol 1,2 oxygenase assays were done as described in Methods 2.7.2, with catechol, 3-methylcatechol and 4-methylcatechol as substrates (Table 5.2). With induced extract, the rate of catechol oxygenation was higher than that of 3-methylcatechol. The rate of oxidation of 4-methylcatechol was substantially slower. The rate of catechol oxidation in uninduced extract was less than 10% of that in induced, though the relative rates with the different catechols were similar.

The standard catechol 1,2-oxygenase assay as described in the literature (Dorn & Knackmuss, 1978a) includes EDTA, which inactivates the next enzyme, muconate cycloisomerase (Introduction, Figure 1.30). The discovery of an accumulation of muconic acids in the medium (Section 4.5) suggested that this enzyme activity might not be present in *R. rhodochrous* 26, and indeed EDTA had little effect on the activity of catechol 1,2-oxygenase (Table 5.2), suggesting that either this organism has no cycloisomerase activity, a very low cycloisomerase activity unaffected by EDTA, or - although much less likely - that it has a muconate cycloisomerase that is unaffected by EDTA.

Another way of testing for cycloisomerase activity is to monitor a catechol 1,2-oxygenase reaction mixture over a longer period of time, in order to see if there is any decrease in A_{260}. When an assay mixture containing catechol and extract from cells

Table 5.2: Measurement of catechol 1,2-oxygenase activity with various substrates

Assays were done as described in Methods 2.7.2, in a final volume of 3 ml of 50 mM phosphate buffer (pH 7.0). The uninduced extract contained 0.7 mg protein/ml (assay concentration 0.01 mg protein/ml), and the induced extract contained 4.0 mg protein/ml (assay concentration 0.07 mg protein/ml). Extract from cells grown on benzyl alcohol contained 2.6 mg protein/ml (assay concentration 0.04 mg protein/ml). The reaction was started by addition of the catechol, at a final concentration of 0.1 mM. EDTA was added, when specified, at a final concentration of 1.3 mM. Controls were carried out without the catechol and with extract replaced with PEG buffer, both of which gave no measurable rate. All results are means of at least two determinations.

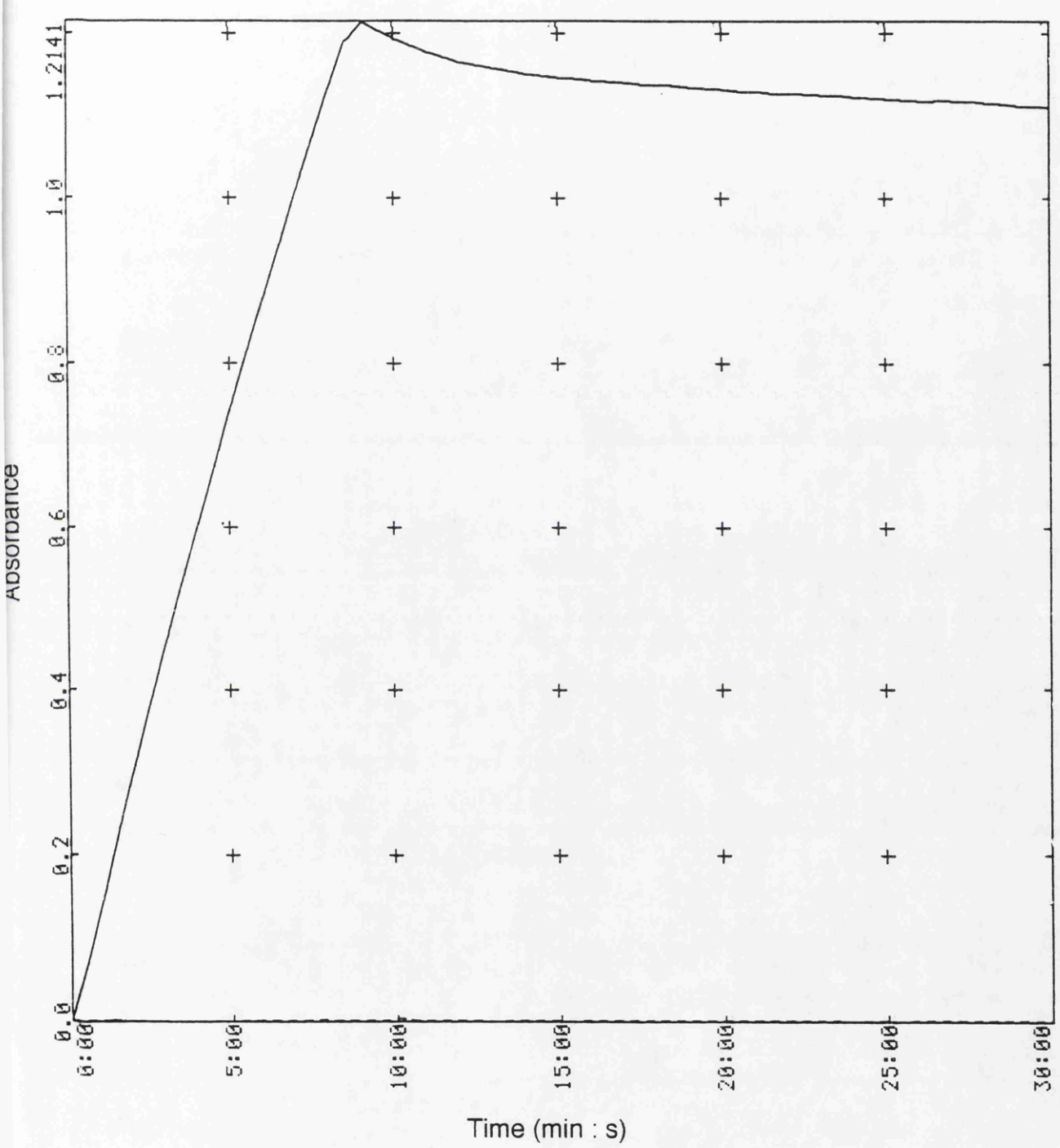
Absorption coefficients of the 1,2-cleavage products in Tris pH 8.0 , all at 260 nm: catechol, ε=16.8 $mM^{-1}cm^{-1}$; 3-methylcatechol, ε=18.0 $mM^{-1}cm^{-1}$; 4-methylcatechol, ε=13.9 $mM^{-1}cm^{-1}$ (Dorn & Knackmuss, 1978b). n.d. = not done.

	Rate of product formation (nmol/min/mg protein)		
Substrate	Extract of cells grown on nutrient broth	Extract of cells grown on nutrient broth/styrene	Extract of cells grown on benzyl alcohol
Catechol	3.5	58	34
Catechol + EDTA	n.d.	62	n.d.
3-Methylcatechol	0.7	35	n.d.
4-Methylcatechol	1.1	18	n.d.

Figure 5.5: The variation in A_{260} of a catechol 1,2-oxygenase reaction mixture

The reaction mixture contained: 2.8 ml of 50 mM phosphate buffer, pH 7.0, 100 µl of extract from cells grown on benzyl alcohol (2.6 mg protein/ml, 0.03 mg protein/ml in assay, grown as described in Methods 2.4.4h) and 100 µl of 3 mM catechol (giving a final concentration of 0.1 mM). The progress of the reaction was monitored by measuring the absorbance at 260 nm over a period of 30 minutes.

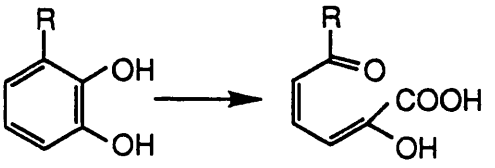
Figure 5.5: The variation in A_{260} of a catechol 1,2-oxygenase reaction mixture



grown on nutrient broth and styrene was followed for 30 minutes it was observed that the A_{260} increased rapidly at first, then very briefly decreased rapidly, and then decreased very slowly. The same was found with a reaction mixture containing extract from cells grown on benzyl alcohol with a catechol substrate (Figure 5.5). These results suggest that little, if any, cycloisomerase activity is present.

Unfortunately, the 3-vinylcatechol produced from styrene *cis*-glycol (Chapter 4.3.3) was not sufficiently pure to be used in assays of this type, because it contained several components which absorbed in the 260 nm region.

5.2.3 Catechol 2,3-oxygenase



Preliminary catechol 2,3-oxygenase assays were not reproducible. It was found that this problem was due to varying activities of the next enzyme, 2-hydroxymuconic acid semialdehyde hydrolase. Previous workers have found that this enzyme is inactivated by simple heat treatment (Bayly *et al.*, 1966), and it was found that an optimum catechol 2,3-oxygenase rate could be obtained by heat treatment of the extract at 55 °C for 5 minutes (Methods 2.4.7).

The assay was performed as in Methods 2.7.3, using catechol, 3-methylcatechol and 4-methylcatechol as substrates. Uninduced extract had no detectable activity (Table 5.3). Induced extract had a very low 4-methylcatechol 2,3-oxygenase activity, a higher catechol 2,3-oxygenase activity and with 3-methylcatechol 2,3-oxygenase giving the highest rate.

The 2,3-cleavage of 3-vinylcatechol was monitored using a modification of the above method. The reaction was performed at pH 7.6, and the absorbance spectrum of the reaction mixture was monitored, scanning every 2 minutes. The reaction mixture turned intense yellow during the assay, with two absorbance peaks accumulating, the

Table 5.3: Measurement of catechol 2,3-oxygenase activity with various substrates

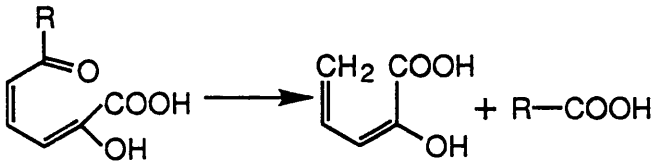
Assays were done as described in Methods 2.7.3, in a final volume of 3 ml of 50 mM phosphate buffer (pH 7.0). Assays were performed using heat treated extract, treated at 55 °C for 5 minutes, as in Methods 2.4.7. Uninduced extract contained 0.7 mg protein/ml (assay concentration 0.01 mg protein/ml), and induced extract was diluted with PEG buffer to give an identical protein concentration. All protein determinations were carried out prior to the heat treatment. The reaction was started by addition of the catechol, at a final concentration of 0.3 mM. Controls were performed without the catechol and with extract replaced with PEG buffer, both of which gave no measurable rate. All results are means of at least two determinations.

Absorption coefficients used for the 2,3-cleavage products of catechols: catechol, ε=48.4 $mM^{-1}cm^{-1}$ at 375 nm; 3-methylcatechol, ε=19.8 $mM^{-1}cm^{-1}$ at 390 nm; 4-methylcatechol, ε=33.2 $mM^{-1}cm^{-1}$ at 380 nm (Wallis & Chapman, 1990).

Substrate	Rate of product formation (nmol/min/mg protein)	
	Extract of cells grown on nutrient broth/styrene	Extract of cells grown on nutrient broth
Catechol	11	0
3-Methylcatechol	16	0
4-Methylcatechol	1	0

highest at 429 nm, the other at 345 nm (Figure 5.6), indicating that 3-vinylcatechol is susceptible to 2,3- cleavage.

5.2.4 2-Hydroxymuconic acid semialdehyde hydrolase



Heat treated extract was used to produce 2-hydroxymuconic acid semialdehyde from catechol (Methods 2.7.4a), and hydrolase activity was measured by monitoring the rate of decrease of A_{375} (Methods 2.7.4). No activity was found in extracts of uninduced cells (Table 5.4), while induced extract had a maximum activity of 6 nmol/min/mg protein.

5.3 Ion exchange separation of enzyme activities

In order to examine the enzymes of the pathway more closely, an ion exchange separation of the enzymes was performed. Cells which had been grown in the fermenter on nutrient broth/styrene were suspended in a buffer containing Tris pH 7.5, ethanol, glycerol and DTT (Methods 2.8), before being broken in a French press and centrifuged as usual to obtain the high-spin supernate (Methods 2.8). This extract was then separated on a column packed with DEAE sephacel super fine and, initially, a gradient of 0 - 1 M NaCl (Figure 5.7).

To enable better separation of enzyme activities, the gradient was made more shallow, 0.15 - 1 M NaCl (Methods 2.8). The assays below were all done on fractions from the same column (Methods 2.8.1).

5.3.1 Toluene *cis*-glycol dehydrogenase and styrene *cis*-glycol dehydrogenase

Fractions from the ion exchange column were assayed for toluene *cis*-glycol dehydrogenase activity (Methods 2.8.1a). The results (Figure 5.8) show that the activity

Figure 5.6: Absorbance spectrum of a 3-vinylcatechol 2,3-oxygenase reaction mixture

The substrate, 3-vinylcatechol, was produced by intact cells from styrene in the presence of 3-fluorocatechol and purified by t.l.c. (Methods 2.5.4). One-sixth of the 3-vinylcatechol produced from 3 g of cells was used for this assay. The reaction mixture contained 1.45 ml of 50 mM phosphate buffer, pH 7.6, 1.45 ml of 3-vinylcatechol in the same buffer, and was started with 100 µl of heat treated extract of cells grown on nutrient broth and styrene (4.7 mg protein/ml, 0.05 mg protein/ml in assay, heat treated as described in Methods 2.4.7).

The absorption spectrum was measured between 190 and 500 nm, with a new scan starting every two minutes. The baseline of the spectrophotometer was set on phosphate buffer.

Figure 5.6: Absorbance spectrum of a 3-vinylcatechol 2,3-oxygenase reaction mixture

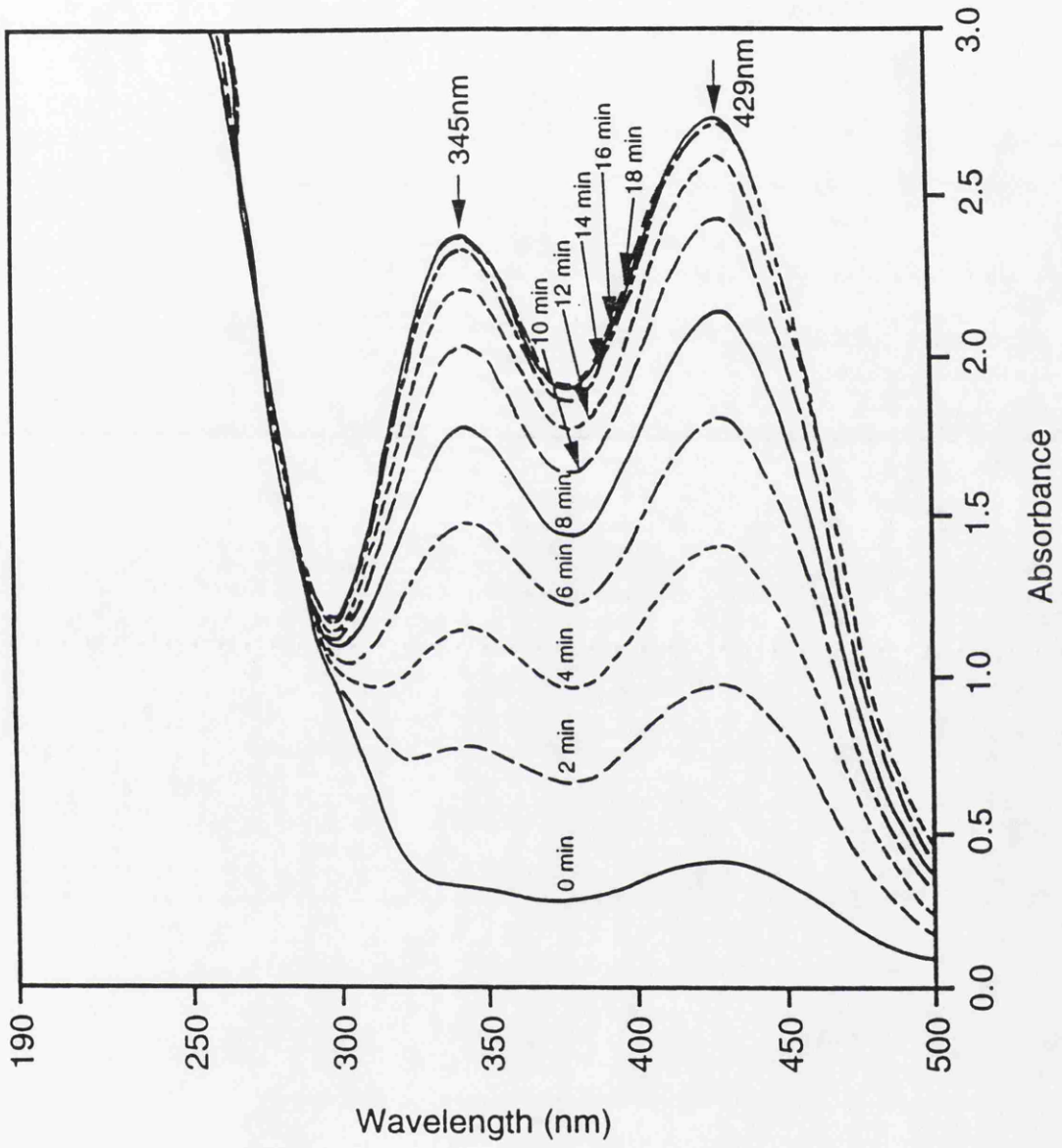


Table 5.4: Measurement of 2-hydroxymuconic acid semialdehyde hydrolase activity

Assays were done as described in Methods 2.7.4 in a final volume of 3 ml of 50 mM phosphate buffer (pH 7.0). The substrate, 2-hydroxymuconic acid semialdehyde, was prepared from heat treated extract and catechol, as described in Methods 2.7.4a, and was added at the concentrations specified. The assay was pre-incubated for 5 minutes before addition of the extract. The assay was started by addition of extract. Uninduced extract contained 0.7 mg protein/ml (assay concentration 0.01 mg protein/ml), and induced contained 4.0 mg protein/ml (assay concentration 0.07 mg protein/ml). Controls were performed without the substrate and with extract replaced by PEG buffer, both of which gave no measurable rate. All results are means of at least two determinations.

Absorption coefficient of 2-hydroxymuconic acid semialdehyde:
ε_{375}=48.4 $mM^{-1}cm^{-1}$ (Wallis & Chapman, 1990).

Approximate substrate concentration (mM), calculated from A_{375}	Rate of substrate removal, nmol/min/mg protein	
	Extract of cells grown on nutrient broth	Extract of cells grown on nutrient broth/styrene
0.009	0	4
0.015 [1]		6

[1] Further increases in substrate concentration did not further increase the recorded rate.

Figure 5.7: A typical ion exchange chromatography of an extract of *R. rhodochrous* 26

Cells which had been grown in the fermenter on nutrient broth/styrene were pressed in TEGD buffer (Methods 2.4.6) and 14 ml of this extract was applied at 20 ml/hr to a column packed with DEAE sephacel super fine (7 cm * 2.6 cm, volume 20 ml) which had been pre-equilibrated with TEGD buffer. The column was washed with approximately two column volumes of TEGD buffer at 20 ml/hr. A linear 0 - 1 M NaCl/TEGD gradient was used to elute enzyme activities (Methods 2.2.8). The gradient volume was 250 ml, and the flow rate was 20 ml/hr. When the gradient had finished, 2M NaCl/TEGD was used to remove any traces of bound material. Fractions of 10 ml were collected until fraction 9, when the fraction size was reduced to 2 ml.

The concentration of protein in the fractions was estimated by measuring their A_{280} (Methods 2.3.8). The ionic strength of fractions was estimated by measuring their conductivity and converting this value to M NaCl by comparison with the conductivity of standards (Methods 2.3.2).

Under these conditions, the catechol 2,3-oxygenase activity eluted between fractions 45 and 60 and the apparent catechol 1,2-oxygenase activity between fractions 45 and 70 (see following graphs for details of assay methods)

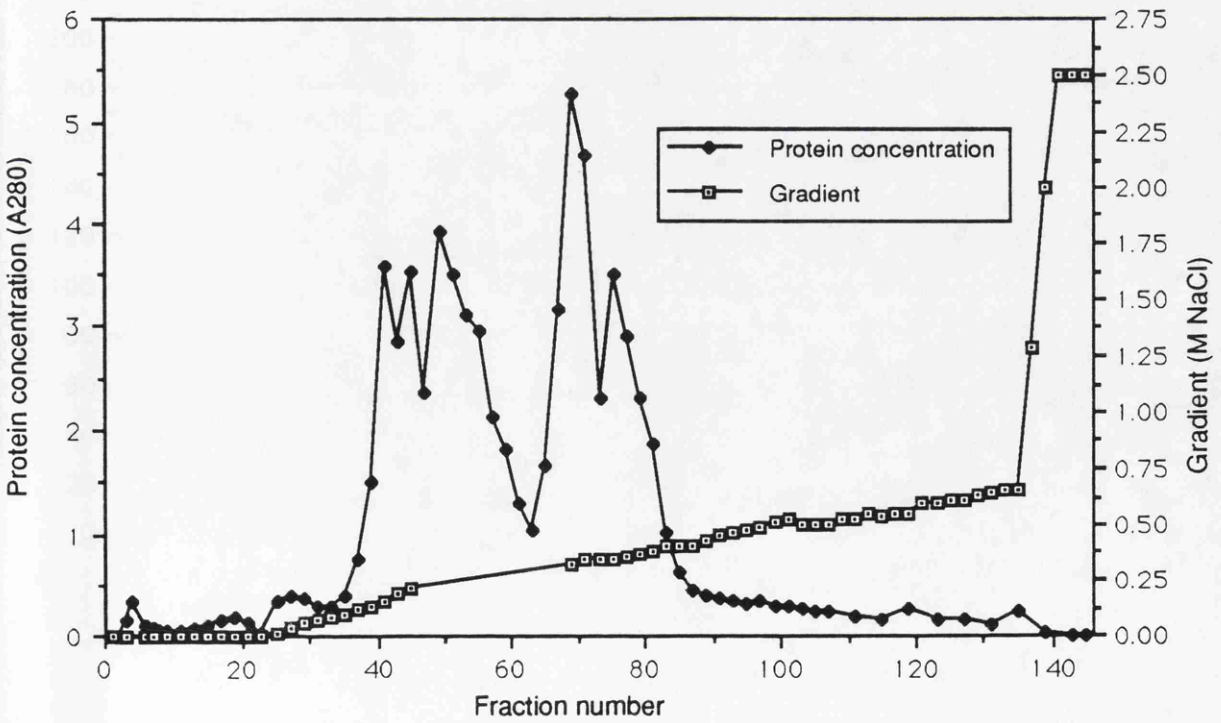
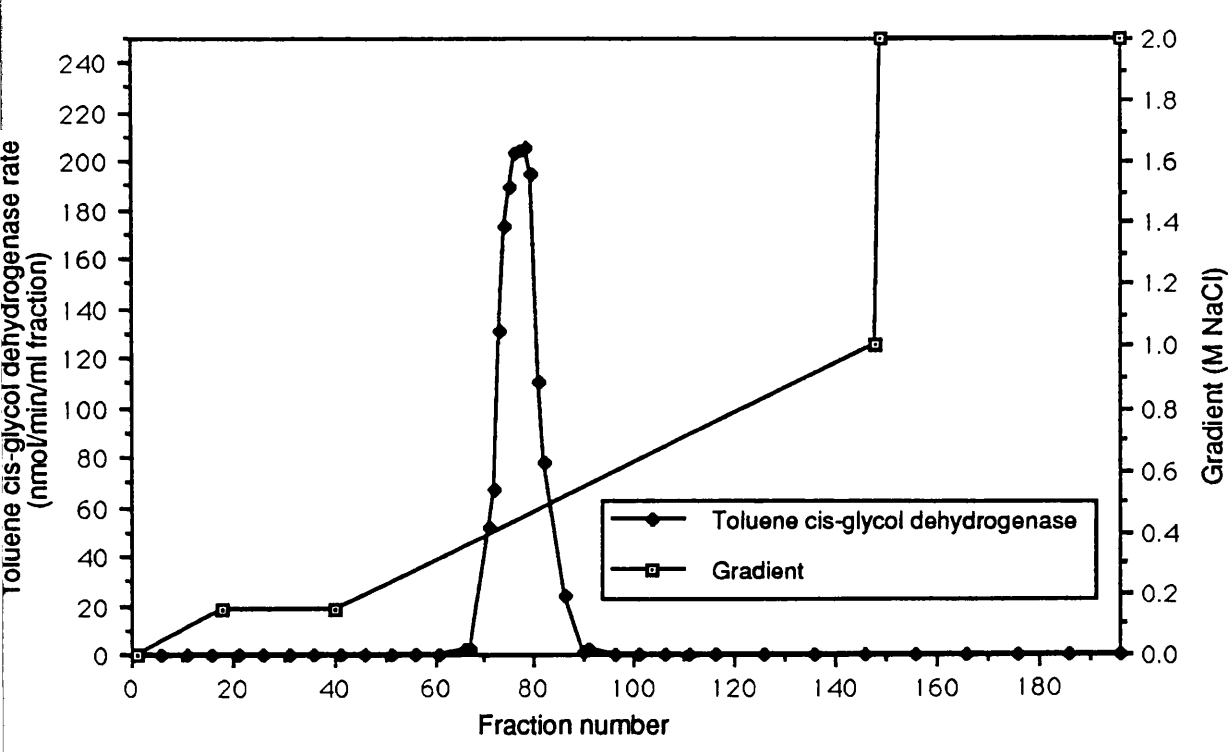


Figure 5.8: Measurement of toluene *cis*-glycol dehydrogenase activity in the fractions from an ion exchange separation

Cells which had been grown in the fermenter on nutrient broth/styrene were pressed in TEGD buffer (Methods 2.4.6) and 15 ml of this extract was applied at 20 ml/hr to a column packed with DEAE sephacel super fine (7 cm * 2.6 cm, volume 20 ml) which had been pre-equilibrated with TEGD buffer. The column was washed with approximately two column volumes of TEGD buffer at 20 ml/hr, and then with approximately two column volumes of 0.15 M NaCl/TEGD buffer. A linear 0.15 - 1 M NaCl/TEGD gradient was used to elute enzyme activities (Methods 2.8). The gradient volume was 300 ml, and the flow rate was 20 ml/hr. When the gradient had finished, 2M NaCl/TEGD was used to remove any traces of bound material. Fractions of 5 ml were collected until fraction 30, when the fraction size was reduced to 2 ml.

The ionic strength is calculated from the flow rate and the known buffer concentrations and gradients. Toluene *cis*-glycol dehydrogenase was assayed by measuring the rate of increase of NADH absorbance at 340 nm, using 100 µl of the fraction under test (Methods 2.8.1a).



eluted in a single, symmetrical peak, at a calculated buffer concentration of between 0.4 and 0.5 M NaCl.

It was considered likely that the styrene *cis*-glycol dehydrogenase activity (Methods 2.8.1b) would probably be due to the same enzyme as the toluene *cis*-glycol dehydrogenase activity. This hypothesis was tested using the ion exchange fractions.

a) Comparison of the elution of toluene *cis*-glycol dehydrogenase on ion exchange chromatography with that of styrene *cis*-glycol dehydrogenase

Both activities co-purified during ion-exchange chromatography (Figure 5.9).

b) Comparison of the effect of heat denaturation on the two *cis*-glycol dehydrogenase activities

Another method of distinguishing two enzymes is by heat denaturation, since usually different enzymes have different rates of denaturation. The reduction of the two activities, toluene *cis*-glycol dehydrogenase and styrene *cis*-glycol dehydrogenase, on heating at 34 °C was almost identical (Figure 5.10, Methods 2.8.2). Both activities were slightly stimulated by heating for about 30 seconds.

These results strongly indicate that toluene *cis*-glycol and styrene *cis*-glycol activities are the result of one enzyme with a flexible substrate specificity.

5.3.2 Catechol 2,3-oxygenase

There was a single peak of activity of catechol 2,3-oxygenase (Methods 2.8.1c), at an approximate buffer concentration of between 0.3 and 0.35 M NaCl (Figure 5.11).

5.3.3 Catechol 1,2-oxygenase

There appeared to be two peaks of catechol 1,2-oxygenase activity (Methods 2.8.1d), the first at an approximate buffer concentration of 0.28-0.35 M NaCl and the second at 0.35-0.45 M NaCl (Figure 5.12). It was noticed that the assays within the first peak appeared to show a sigmoidal increase of A_{260}, i.e. a slow initial rate, followed by

Figure 5.9: Comparison of the activities of toluene and styrene *cis*-glycol dehydrogenase on ion exchange chromatography

These assays were performed on the same chromatographic separation as that in Figure 5.8.

Styrene and toluene *cis*-glycol dehydrogenase were assayed by measuring the rate of increase of NADH absorbance at 340 nm, using 100 µl of the fraction under test (Methods 2.8.1a, b). In reaction mixtures with high rates of styrene *cis*-glycol dehydrogenase activity the cuvette contents became yellow.

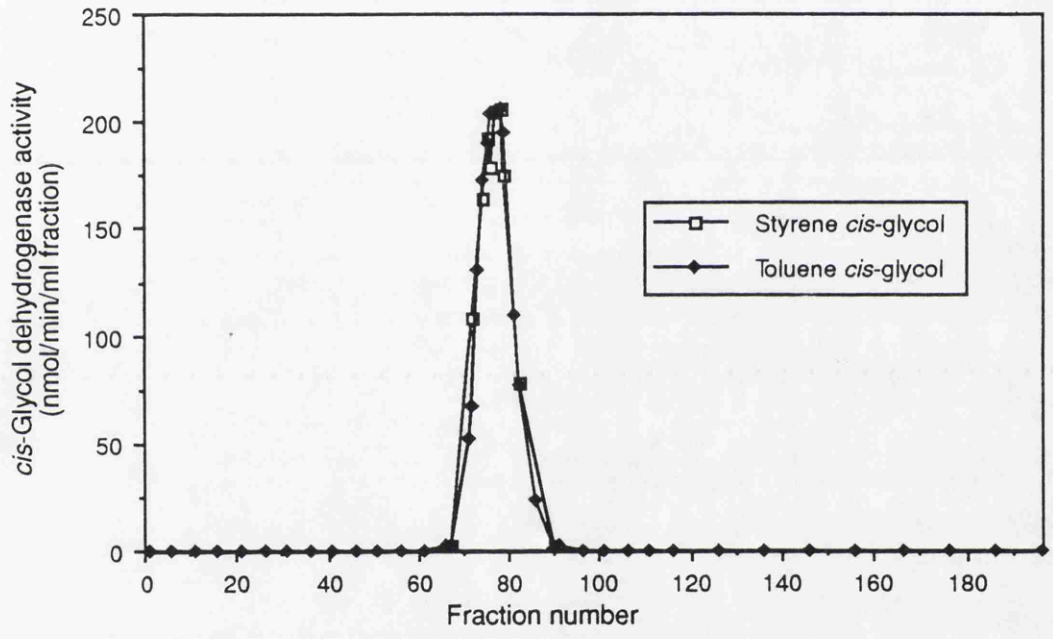


Figure 5.10: Comparison of heat denaturation of toluene and styrene *cis*-glycol dehydrogenase activities

Column fractions with high *cis*-glycol dehydrogenase activity, numbers 72 to 82 (Figure 5.9), were pooled. The pooled extract was incubated at 34 °C (Methods 2.8.2), then the styrene and toluene *cis*-glycol dehydrogenase activities were assayed by measuring the rate of increase of NADH absorbance at 340 nm, using 50 µl of the heat treated extract (Methods 2.8.2). All the assays containing styrene *cis*-glycol went yellow.

The graph shows the reaction rates as a percentage of the rate recorded for unheated extract, which were 15 nmol/min/1 ml extract for toluene *cis*-glycol and 18 nmol/min/1 ml extract for styrene *cis*-glycol.

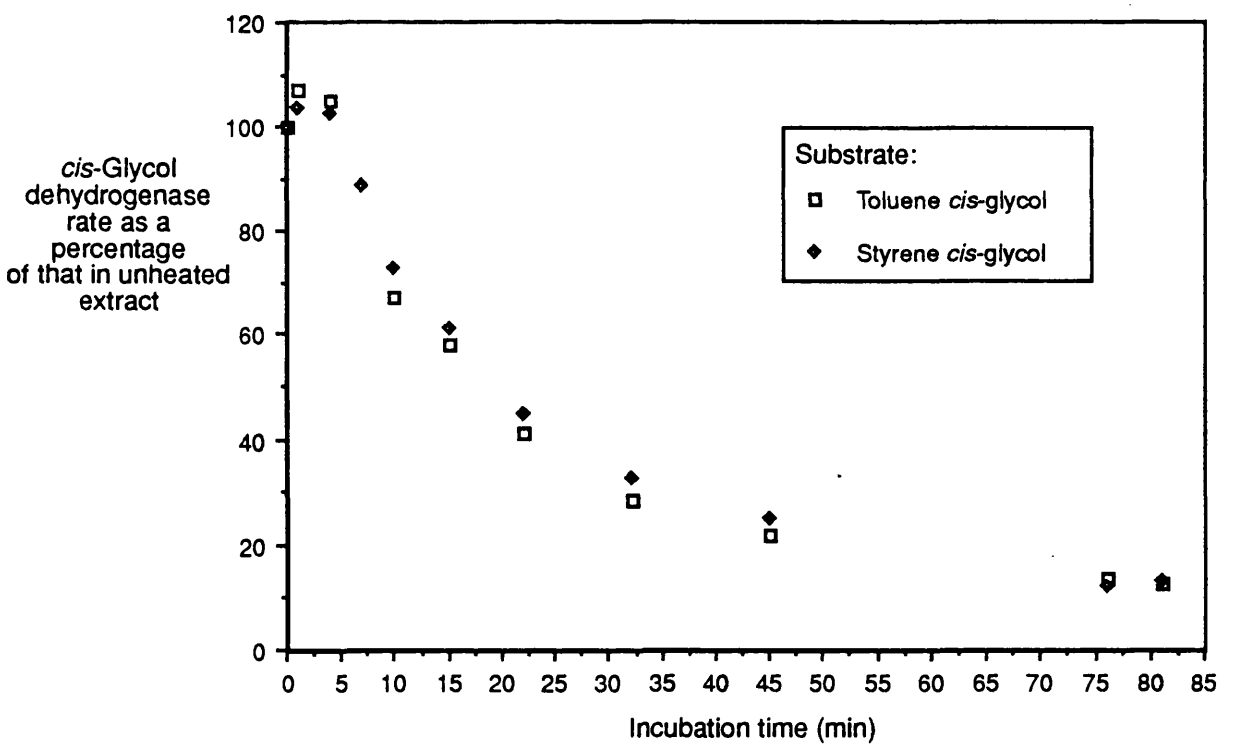


Figure 5.11: Measurements of catechol 2,3-oxygenase activity in fractions from an ion exchange separation

These assays were performed on the same chromatographic separation as that in Figure 5.8.

The ionic strength is calculated from the flow rate and the known buffer concentrations and gradients. Catechol 2,3-oxygenase activity was assayed by measuring the rate of increase of absorbance at 375 nm, using 50 µl of the fraction under test (Methods 2.8.1c). The reaction was started with catechol, at a final concentration of 0.3 mM.

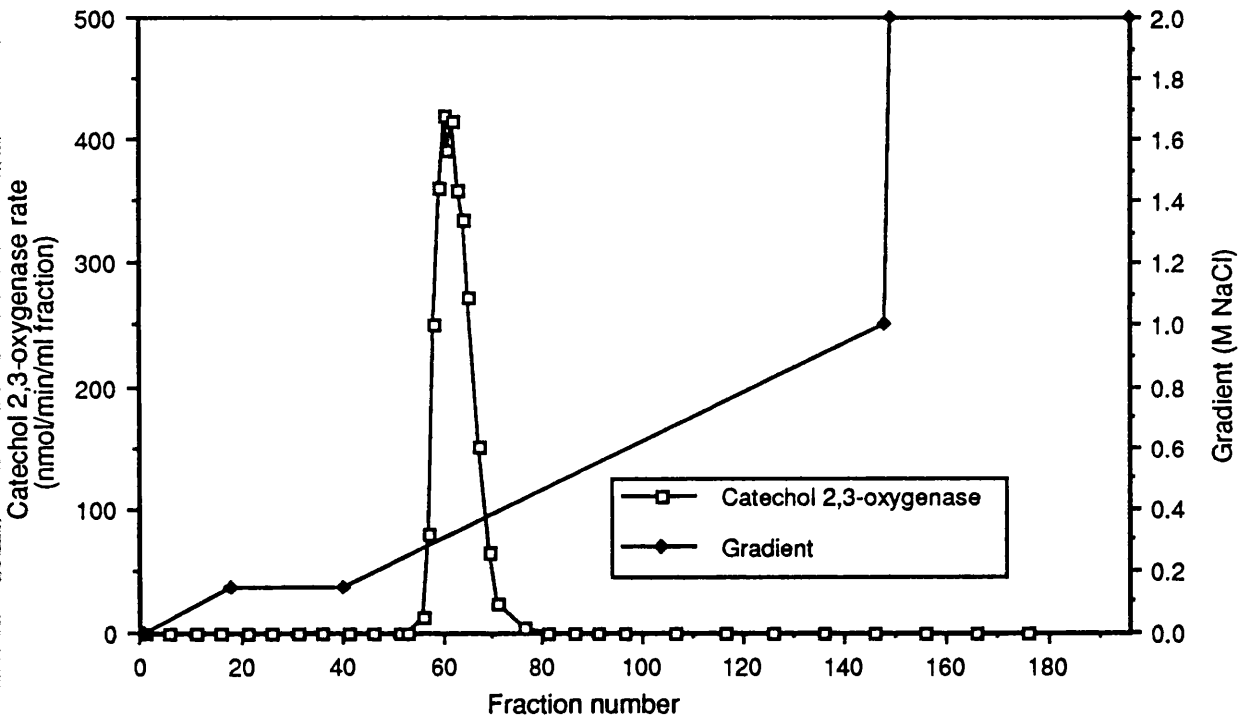
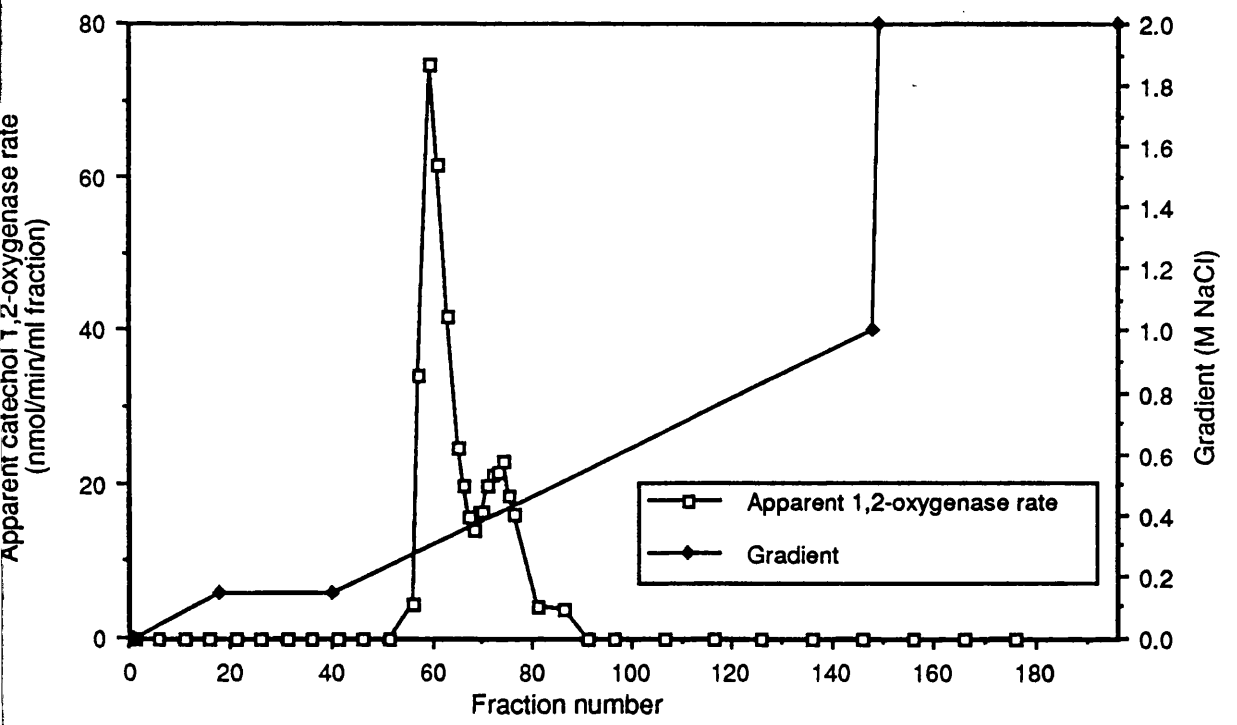


Figure 5.12: Apparent catechol 1,2-oxygenase activities in fractions from an ion exchange separation

These assays were performed on the same chromatographic separation as that in Figure 5.8.

The ionic strength is calculated from the flow rate and the known buffer concentrations and gradients. Apparent catechol 1,2-oxygenase activity was assayed by measuring the rate of increase of absorbance at 260 nm, using 100 µl of the fraction under test (Methods 2.8.1d). The reaction was started with catechol, at a final concentration of 0.1 mM.



an acceleration, and finally a plateau. The assays in the second peak showed no slower initial rate.

In order to explain this result, the assays were repeated, but instead of just measuring the A_{260}, the absorbance of the reaction mixture was measured between 220 nm and 400 nm, to observe the formation and removal of components.

If the assay was performed using a fraction from the centre of the second (later) activity peak there was initially an absorbance peak at 275.3 nm, the catechol, and when the fraction was added this peak increased and moved, until the absorbance peak was at 258.4 nm. This is consistent with the production of *cis, cis*-muconic acid, which would be expected to have a λ_{max} = 257 nm at pH 8.0 (Dorn & Knackmuss, 1978b), i.e. the activity present in these fractions is the 1,2-cleavage of catechol.

When a similar experiment was performed with a fraction from the centre of the first activity peak the initial absorbance peak at 275.3 nm was present due to the catechol, but after the fraction was added this peak increased in size and moved to 270.4 nm, before its size slowly reduced. In addition, there were smaller, transient absorbance peaks at around 323 nm and 376 nm, consistent with the acid and base form of 2-hydroxymuconic semialdehyde, the product of catechol 2,3 oxygenase cleavage.

From the pathway in Figure 5.1 it can be seen that 2-hydroxymuconic acid semialdehyde is usually cleaved by 2-hydroxymuconic acid semialdehyde hydrolase, producing a carboxylic acid and 2-hydroxypent-2,4-dienoate. The latter compound has an absorbance maximum of 265 nm at pH 7.0 (Collinsworth *et al.*, 1973). The substance accumulating in the reaction mixture giving an absorbance maximum at about 270 nm could well be this compound, with the absorbance peak slightly shifted because of the presence of residual catechol and of protein.

If this explanation was correct, then the fractions in this peak should contain both catechol 2,3-oxygenase activity and 2-hydroxymuconic acid semialdehyde hydrolase, and little or no catechol 1,2-oxygenase activity. The sigmoidal accumulation at 260 nm would then be explained by an initial delay to enable 2-hydroxymuconic acid

semialdehyde to accumulate from 2,3-cleavage of the catechol, followed by an accumulation of 2-hydroxypent-2,4-dienoate, whose absorbance peak at 265 nm is broad, so it is also measurable at 260 nm.

5.3.4 2-Hydroxymuconic acid semialdehyde hydrolase

In order to confirm the hypothesis in the previous paragraph, 2-hydroxymuconic acid semialdehyde hydrolase activity was assayed in the fractions (Methods 2.8.1e). The results (Figure 5.13) clearly showed a single peak of hydrolase activity at about 0.20 - 0.30 M NaCl, overlapping with, but eluting slightly earlier than, the catechol 2,3-oxygenase activity. When compared with the peaks of apparent catechol 1,2-oxygenase activity, the hydrolase activity overlaps with the first apparent 1,2-oxygenase activity peak, with no activity at the second (Figure 5.14). When Figures 5.13 and 5.14 are compared, it can be seen that the first apparent catechol 1,2-oxygenase peak matches perfectly the area of overlap between the catechol 2,3-oxygenase and the hydrolase activities (at about fraction 60).

The results support the idea that the first apparent peak in catechol 1,2-oxygenase activity in the column fractions is due to an accumulation of 2-hydroxypent-2,4-dienoate, produced by the consecutive action on catechol of catechol 2,3 oxygenase and 2-hydroxymuconic acid semialdehyde hydrolase (Figure 5.1).

The 2-hydroxymuconic acid semialdehyde hydrolase activity could be completely inactivated by heating the column fractions at 55 °C for 2 minutes.

5.4 Production and attempted identification of 2-hydroxy-6-oxo-octa-2,4,7-trienoic acid

The production, purification and n.m.r. identification of the 1,2-cleavage product of catechol, 2-vinylmuconic acid, was described in the previous chapter (Section 4.5.2). However, the structure of the 2,3-cleavage product had not been confirmed by n.m.r. There are many problems with the purification and characterisation of 2-hydroxy-muconic acid semialdehydes, for example:

Figure 5.13: A comparison of catechol 2,3-oxygenase activity and 2-hydroxymuconic acid semialdehyde hydrolase activity in fractions from an ion exchange separation

These assays were performed on the same chromatographic separation as that in Figure 5.8.

Catechol 2,3-oxygenase activity was assayed by measuring the rate of increase of absorbance at 375 nm, using 50 µl of the fraction under test (Methods 2.8.1c). The reaction was started with catechol, at a final concentration of 0.3 mM. 2-Hydroxy muconic acid semialdehyde hydrolase was assayed as described in Methods 2.8.1e, in a final volume of 3 ml of 50 mM phosphate buffer (pH 7.6). The substrate, 2-hydroxymuconic acid semialdehyde was prepared from heat treated extract and catechol, as described in Methods 2.7.4a. The reaction was started with 100 µl of the fraction under test, and the decrease in A_{375} was measured.

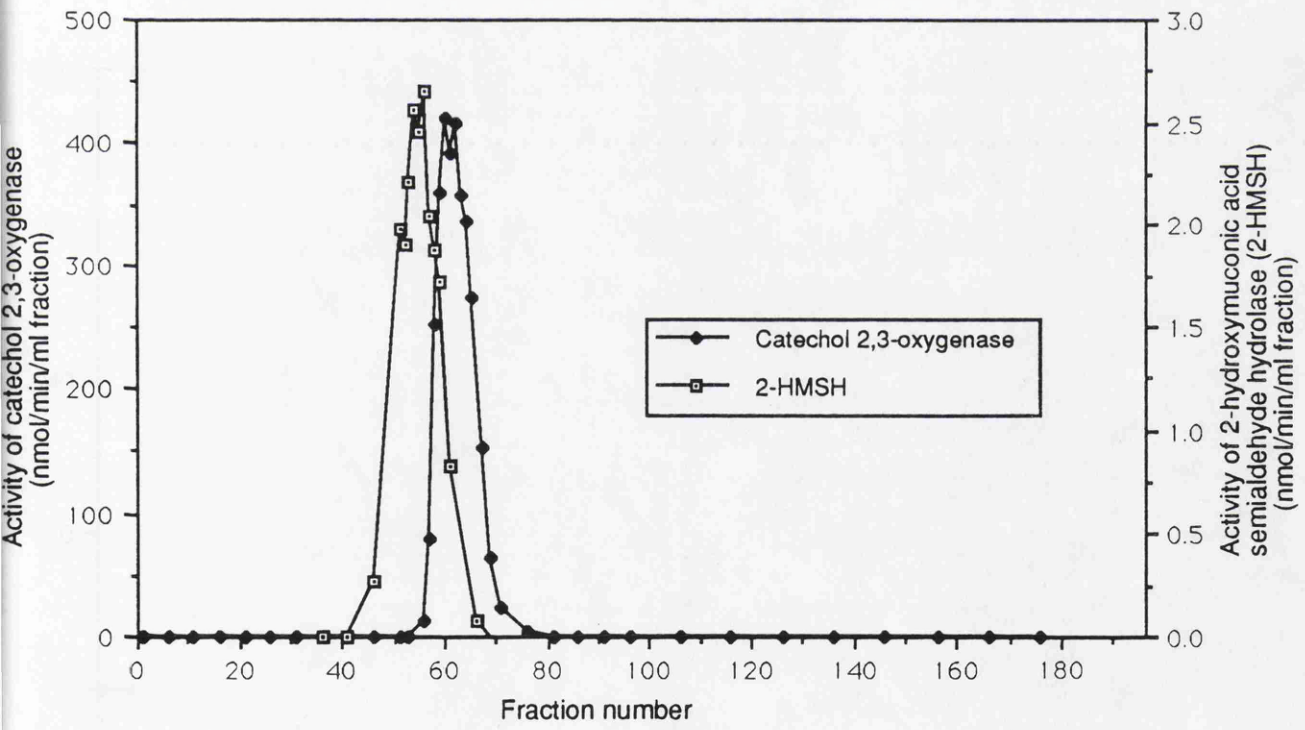
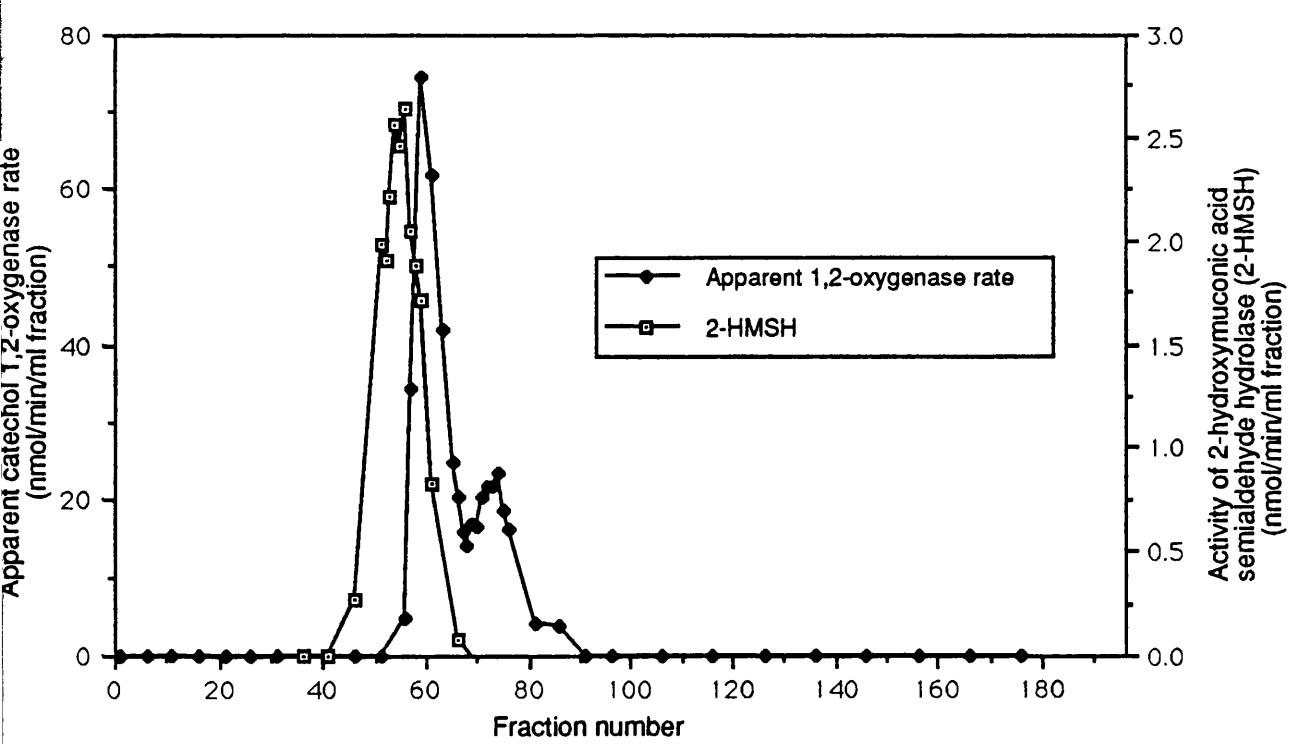


Figure 5.14: A comparison of apparent catechol 1,2-oxygenase activity and 2-hydroxymuconic acid semialdehyde hydrolase activity in fractions from an ion exchange separation

These assays were performed on the same chromatographic separation as that in Figure 5.8.

Catechol 1,2-oxygenase activity was assayed by measuring the rate of increase of absorbance at 260 nm, using 100 µl of the fraction under test (Methods 2.8.1d). The reaction was started with catechol, at a final concentration of 0.1 mM. 2-Hydroxy muconic acid semialdehyde hydrolase was assayed as described in Methods 2.8.1e, in a final volume of 3 ml of 50 mM phosphate buffer (pH 7.6). The substrate, 2-hydroxymuconic acid semialdehyde was prepared from heat treated extract and catechol, as described in Methods 2.7.4a. The reaction was started with 100 µl of the fraction under test, and the decrease in A_{375} was measured.



1) They are very unstable, so derivatisation to a picolinate (see below) is usually necessary before purification and identification.

2) Large quantities are needed for reliable identification, because of losses from breakdown and in the process of transformation to a picolinate.

Accumulation of a yellow material (λ_{max} = 401 nm, Chapter 3.3.2) had been noticed when *R rhodochrous* 26 was grown on styrene in a fermenter. Previous workers have used growth media as a source of 2,3-cleavage products for identification, e.g. Cripps *et al.* (1978). Large amounts of medium were generally required for these isolations, there were often stability problems, and in the case of *R. rhodochrous* 26 growing on styrene the medium would also contain large amounts of 2-vinylmuconic acid. It was therefore decided that isolation from growth medium would not be practical.

Experiments earlier in this chapter (Sections 5.2.1, 5.2.3) had shown the accumulation of the putative 2,3-cleavage product from both styrene *cis*-glycol and 3-vinylcatechol. This seemed to be a more promising route for the production of this intermediate. There were problems with reliably producing large quantities of 3-vinyl-catechol using the 3-fluorocatechol inhibition method (Chapter 4.4.2), so styrene *cis*-glycol transformation was used to produce the 3-vinylcatechol.

5.4.1 Production method

NAD-linked transformation of styrene *cis*-glycol to 3-vinylcatechol and then the transformation of this 3-vinylcatechol to its 2,3-cleavage product using ion exchange column fractions is described in Methods 2.5.8. Upon acidification the deep yellow 2,3-cleavage product changed to a deep red. This product was then extracted with ethyl acetate, dried, evaporated down and dried again. The product was then extracted with 50% concentrated NH_3, producing a red solution (Methods 2.5.8). A diluted absorbance scan on this material showed two peaks, one at 284 nm, the other at 412 nm. This extract was then left overnight at 30 °C, after which the colour was a golden yellow. An absorbance scan of this material, diluted as with the last sample, showed that the absorbance at 412 nm had vanished, and that there was a shoulder of absorbance at 260-

290 nm. Further extraction and purification of this sample was attempted, but there was not enough material for any further identification by n.m.r..

Although not enough material could be obtained for definitive identification of the picolinate derived from the 2,3-cleavage product of 3-vinylcatechol, the changes in absorbance spectrum of the NH_3 extract are consistent with the formation of a picolinate, and this in turn is consistent with the formation of a 2-hydroxymuconic acid semialdehyde from 3-vinylcatechol.

5.5 The metabolism of the 2,3-cleavage product of various catechols

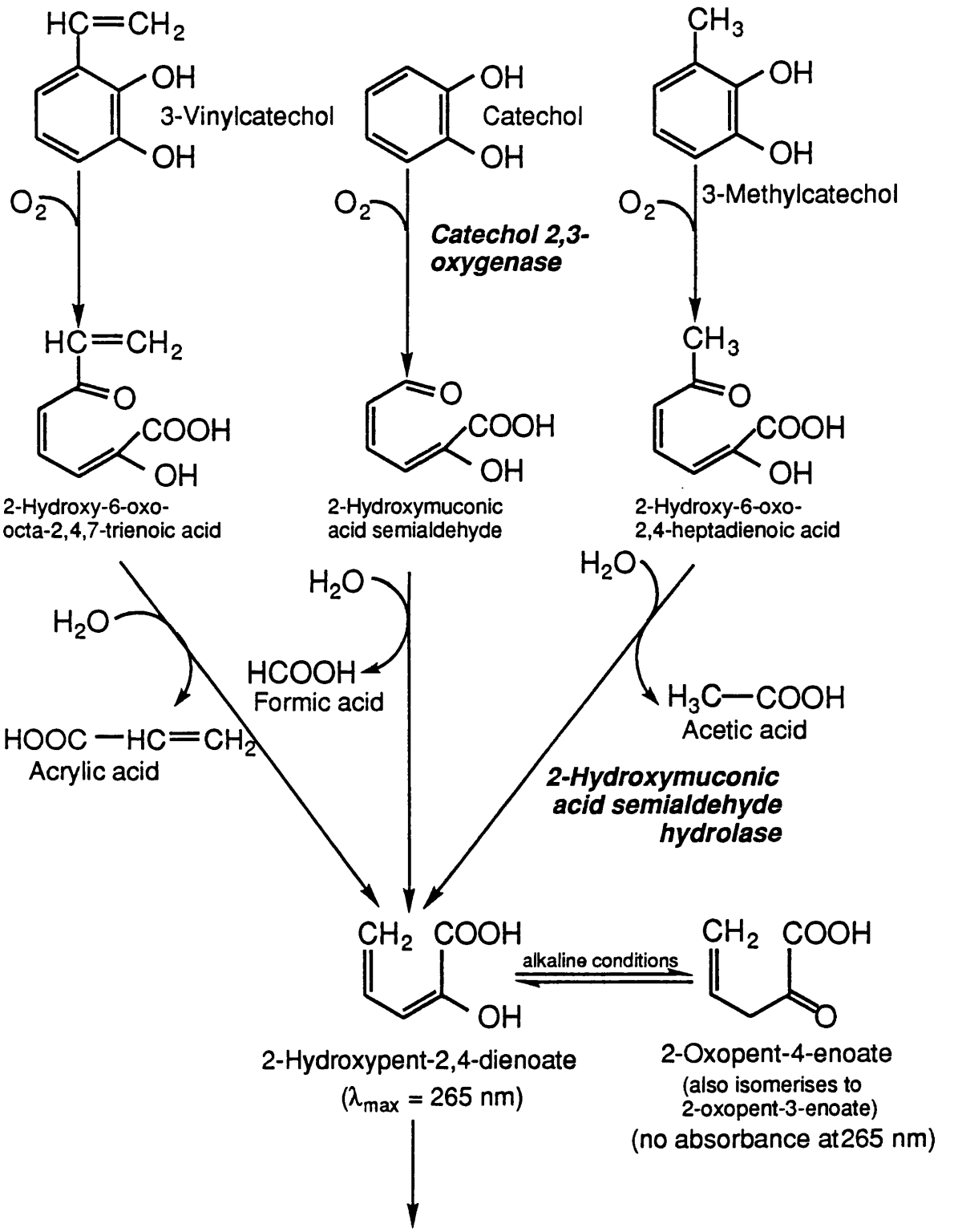
Earlier workers using other organisms have shown that catechol and 3-methylcatechol are both metabolised by catechol 2,3-oxygenase and 2-hydroxymuconic acid semialdehyde hydrolase to produce 2-hydroxypent-2,4-dienoate (Figure 1.31, Figure 5.15). The experiments on the apparent catechol 1,2-oxygenase activity peak from the ion exchange separation of extract (actually containing catechol 2,3-oxygenase and 2-hydroxymuconic acid semialdehyde hydrolase, Section 5.3.3) indicated that *R. rhodochrous* 26 might use the same pathway for the breakdown of catechol. It also seemed likely that this pathway would be used to metabolise 3-methylcatechol and 3-vinylcatechol. The partial separation of the catechol 2,3-oxygenase and 2-hydroxymuconic acid semialdehyde hydrolase by ion exchange chromatography enabled this hypothesis to be tested.

5.5.1 Tentative identification of 2-hydroxypent-2,4-dienoate as the product of catechol and 3-methylcatechol metabolism

a) Catechol

A reaction mixture containing catechol and 100 µl of a column fraction, which had a high level of catechol 2,3-oxygenase activity and a low level of 2-hydroxymuconic acid semialdehyde hydrolase activity, was monitored between 220 and 420 nm every 2 minutes (Figure 5.16a). Two peaks of absorbance accumulated, one at 325.6 nm, the other at 375.7 nm (with a spike at 368.3 nm), similar to the acid and alkaline absorbance

Figure 5.15: The conversion of catechol, 3-methylcatechol and 3-vinylcatechol to 2-hydroxypent-2,4-dienoate



Based on Harayama *et al.* (1989)

Figure 5.16: The production of putative 2-hydroxypent-2,4-dienoate from catechol using column fractions

a) The accumulation of 2-hydroxymuconic acid semialdehyde

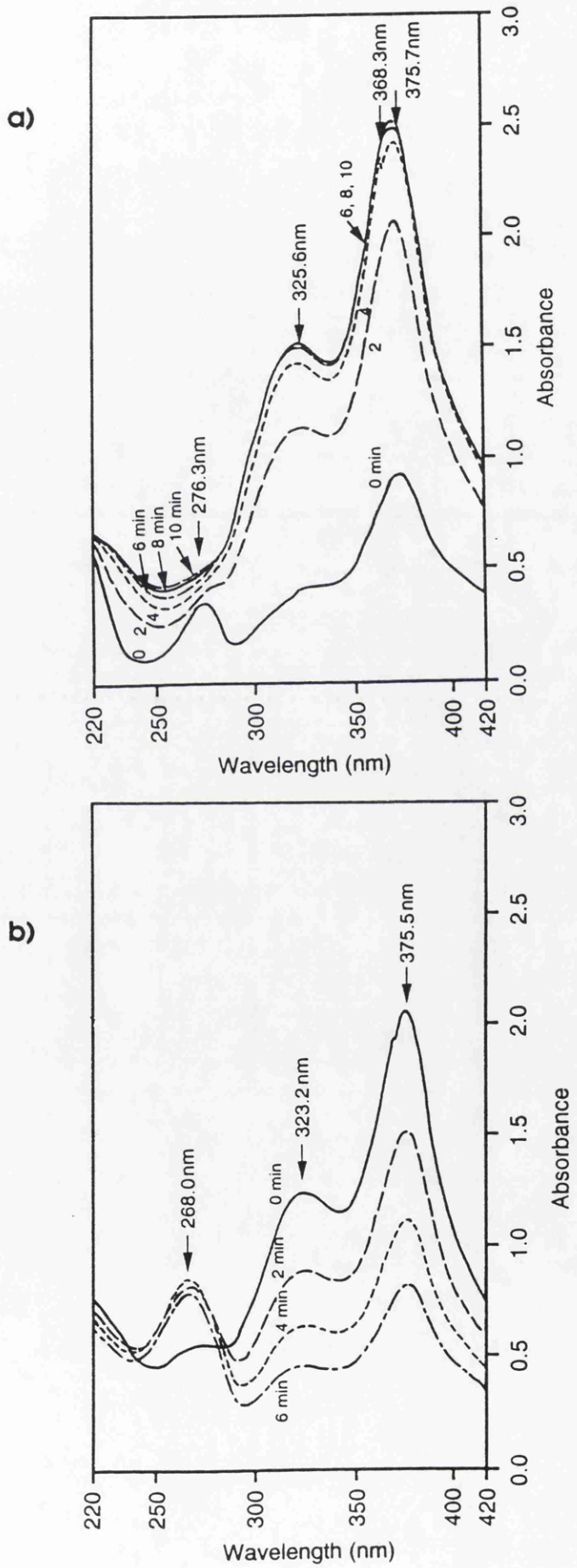
The baseline of the spectrophotometer was set between 220 and 420 nm on 2.8 ml of 50 mM phosphate buffer, pH 7.0, and 100 µl of fraction 64 (from the ion exchange column, containing high levels of catechol 2,3-oxygenase, see text). Catechol (100 µl of a 3 mM solution) was then added as substrate (final concentration 0.1 mM). The absorption spectrum of the reaction mixture was then repeatedly measured between 220 and 420 nm, with a new scan starting every two minutes. Once the height of the main peaks had stabilised, the absorbance scanning was stopped and the next stage of the reaction was undertaken, described in b) below.

b) The conversion of 2-hydroxymuconic acid semialdehyde to 2-hydroxypent-2,4-dienoate

This experiment was a continuation of that described in a) above, with the spectrophotometer still set up in the same way.

The above assay mixture had 100 µl of fraction 53 added to it (from the ion exchange column, containing high levels of 2-hydroxymuconic acid semialdehyde hydrolase activity, see text). The absorbance spectrum of the reaction mixture was then monitored every two minutes between 220 and 420 nm.

Figure 5.16: The production of putative 2-hydroxypent-2,4-dienoate from catechol using column fractions



peaks of 2-hydroxymuconic acid semialdehyde (Kojima *et al.*, 1961). These peaks had reached a maximum after 10 minutes, and then started to decrease slowly.

After 12 minutes, 100 µl of a fraction containing a high 2-hydroxymuconic acid semialdehyde hydrolase activity was added. The two peaks of 2-hydroxymuconic acid semialdehyde absorbance immediately diminished, while a new peak appeared at 268.0 nm (reaction mixture pH 6.3), which later started to diminish slowly (Figure 5.16b).

The reaction mixture was acidified to pH 1.8 with 5M HCl, and the protein precipitate was centrifuged off (Methods 2.3.7), and the acidified reaction mixture was scanned. Two absorbance peaks were visible, one at 274.4 nm and the other at 316.1 nm. The reaction mixture was then adjusted to pH 13 with 5M NaOH, and was then immediately scanned. Two peaks were visible on the first scan, one at 375.9 nm, the other at 298.4 nm. However, by the second scan, 2 minutes later, the peak at 298.4 nm had almost vanished, and there was subsequently only a single peak, at 375.9 nm.

b) 3-Methylcatechol

A similar experiment to that above was performed with 3-methylcatechol instead of catechol. Initial test showed that the cleavage of the 3-methylcatechol was much faster than that of the catechol, so only 50 µl of the catechol 2,3 oxygenase fraction was used. After the 3-methylcatechol had been added, an absorbance peak at 320 nm accumulated, reaching a maximum after about 2 minutes, with a very small peak at 386.4 nm (Figure 5.17a). These absorbance maxima are similar to those of the acid and alkaline absorbances of 2-hydroxy-6-oxo-2,4-heptadienoic acid, the 2,3- cleavage product of 3-methylcatechol (Duggleby & Williams, 1986).

The hydrolase fraction was then added, 100 µl of fraction 53, and the scan was re-started (Figure 5.17b). The peak at 320 nm rapidly disappeared, with nothing left after 6 minutes. At the same time a new peak rapidly formed at 267.0 nm (at pH 6.0).

The reaction was then acidified , and scanned again (Figure 5.18a), and a single peak was visible at 271.6 nm. Then the reaction mixture was made alkaline and scanned; a peak at 299.2 nm was visible on the first scan, which had vanished 2 minutes

Figure 5.17: The production of putative 2-hydroxypent-2,4-dienoate from 3-methylcatechol using column fractions

a) The accumulation of 6-methyl-2-hydroxymuconic acid semialdehyde

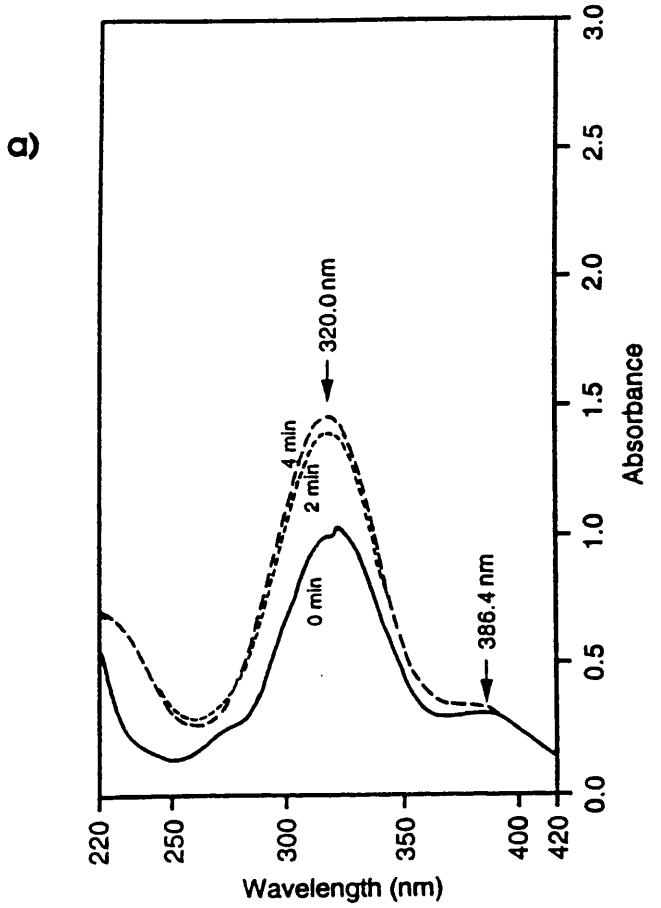
The baseline of the spectrophotometer was set between 220 and 420 nm on 2.8 ml of 50 mM phosphate buffer, pH 7.0, and 50 µl of fraction 64 (from the ion exchange column, containing high levels of catechol 2,3-oxygenase, see text). The substrate, 3-methylcatechol, was then added, 100 µl of 3 mM (final concentration 0.1 mM). The absorption spectrum of the reaction mixture was then repeatedly measured between 220 and 420 nm, with a new scan starting every two minutes. Once the height of the main peaks had stabilised, the absorbance scanning was stopped and the next stage of the reaction was undertaken, described in b) below.

b) The conversion of 6-methyl-2-hydroxymuconic acid semialdehyde to 2-hydroxypent-2,4-dienoate

This experiment was a continuation of that described in a) above, with the spectrophotometer still set up in the same way.

The above assay mixture had 100 µl of fraction 53 added to it (from the ion exchange column, containing high levels of 2-hydroxymuconic acid semialdehyde hydrolase activity, see text). The absorbance spectrum of the reaction mixture was then monitored every two minutes between 220 and 420 nm. After the final scan the pH of the reaction mixture was measured (Methods 2.3.1) and found to be 6.0. The reaction mixture was then acidified as described in Figure 5.18.

Figure 5.17: The production of putative 2-hydroxypent-2,4-dienoate from 3-methylcatechol using column fractions



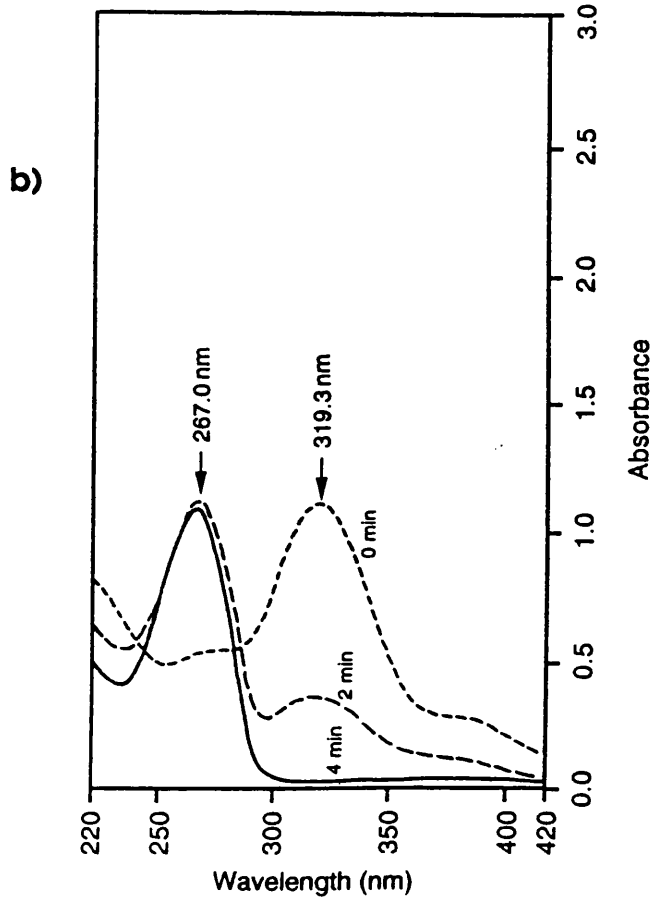


Figure 5.18: Acidic and alkaline scans of the putative 2-hydroxypent-2,4-dienoate produced from 3-methylcatechol using column fractions

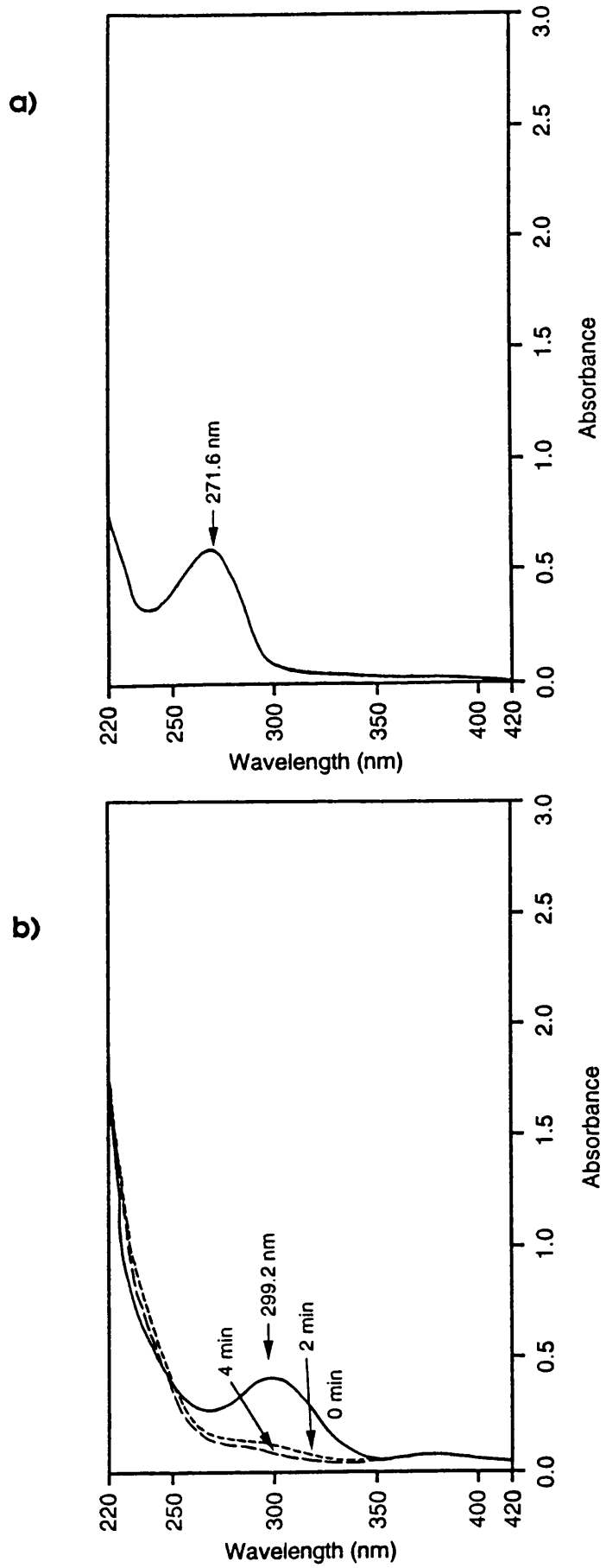
a) Acidic scan of 2-hydroxypent-2,4-dienoate

The assay mixture produced from the experiment described in Figure 5.17 was acidified to pH 1.6 with 3 drops of 5 M HCl. The baseline of the spectrophotometer was set on 50 mM phosphate buffer, pH 7.0, between 220 and 420 nm. The absorption spectrum of the acidified reaction spectrum was then measured.

b) Alkaline repeated scan of 2-hydroxypent-2,4-dienoate

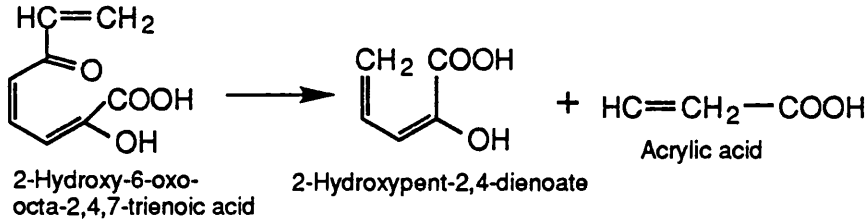
The acidified reaction mixture from a) above was adjusted to pH 13.1 with 6 drops of 5 M NaOH, and the absorbance spectrum was immediately measured, as described in a) above. This measurement was repeated every two minutes for 6 minutes.

Figure 5.18: Acidic and alkaiine scans of the putative 2-hydroxypent-2,4-dienoate produced from 3-methylcatechol using column fractions



later. There was also a very small peak at 379.2 nm, presumably due to some remaining 2,3-cleavage product.

5.5.2 Examination of the 2,3- cleavage pathway with 3-vinylcatechol - the identification of acryiic acid as a product using column fractions



The hydrolysis of 2-hydroxy-6-oxo-octa-2,4,7-trienoic acid, the 2,3-cleavage product of 3-vinylcatechol, would be expected to produce 2-hydroxypent-2,4-dienoate and acrylic acid. The dienoate could be detected by absorbance characteristics, as above, but the acrylic acid is not easily detectable by these means, however, it is easy to detect small quantities of acrylic acid using the h.p.l.c. (Methods 2.5.1c).

The 3-vinylcatechol was produced from approximately 2 mg styrene *cis*-glycol, extracted into diethyl ether and dissolved in 50 mM phosphate buffer (Methods 2.5.3), and was then split into various portions for the following reactions, as specified. The reaction was started by the addition of 100 µl of fraction 64 (containing catechol 2,3-oxygenase activity) to a cuvette containing buffer and the specified quantity of 3-vinylcatechol. The absorbance spectrum was scanned until the accumulated peaks reached a maximum, when 100 µl of fraction 52 (containing hydrolase activity) was added and the scanning restarted. When there was no further changes in absorbances the reactions were acidified with 3 drops of 5M HCl and any precipitate was spun off. Samples of 50 µl were removed where specified for h.p.l.c. analysis (Methods 2.5.1c), to determine concentrations of acrylic acid. Cuvettes were kept covered and on ice between sampling where possible. Three experiments based on this method were carried out to help elucidate this pathway

(i) Using the 3-vinylcatechol from approximately 0.6 mg of styrene *cis*-glycol it was found that the absorbance at wavelengths below 340 nm was too high for any peaks to

be distinguished, though peaks at 343.7-346.4 nm (maximum reading 1.93A)and 428.8-430.4 nm (maximum reading 2.05A)were visible after addition of fraction 64. These peaks then diminished to virtually nothing on addition of fraction 52, containing the 2-hydroxymuconic acid semialdehyde hydrolase activity.

Six minutes after addition of fraction 52 a sample was taken for h.p.l.c. analysis, which showed a peak consistent with a concentration of acrylic acid in the assay of 0.09 mM. Authentic acrylic acid had a retention time identical to that of the product of the reaction mixture (Figure 5.19). A duplicate sample of the reaction mixture also gave an acrylic acid peak equivalent to 0.09 mM.

(ii) This experiment was repeated, but with less 3-vinylcatechol than (i), using that produced from approximately 0.4 mg of styrene *cis*-glycol, which meant that absorbance peaks were visible below 340 nm. A mixture of buffer and 3-vinylcatechol showed peaks at 247.1 nm (2.9A), 255.5 nm (3.0A) and 292.8 nm (1.4A). After addition of fraction 64 the cuvette went yellow immediately. The repeat scan (Figure 5.20a) showed a rapid accumulation of absorbance at 345.8 nm (1.2A) and 424.9 nm (0.8A), with the peak noticed at 255.5 nm in the initial reaction mixture containing 3-vinylcatechol no longer distinguishable, while the other two original peaks were unchanged.

After 4 minutes, 100 µl of fraction 52 was added, and scanning resumed (Figure 5.20b), as the peaks at 345 and 419 nm disappeared. However, because of the absorbance of the other peaks in the UV it is not possible to clearly distinguish any new peaks. However, a shoulder is visible at around 275 nm, which initially increased while all other peaks were reducing or stable. A sample for h.p.l.c. was taken 8 minutes after the addition of fraction 52, and it showed an acrylic acid peak equivalent to 0.07 mM in the original reaction mixture.

(iii) This was performed identically to run (ii), and the same absorbance characteristics were noted. However, a sample was taken 2 minutes after fraction 64 was added, and before the addition of fraction 52, and it was tested in the h.p.l.c. for acrylic acid, and a small peak was found, equivalent to approximately 0.01 mM acrylic

Figure 5.19: Typical h.p.l.c. traces obtained in the identification of acryiic acid formed from 3-vinylcatechol

Samples of 50 µi were removed from reaction mixtures, and were then injected straight on to the h.p.l.c. as described in Methods 2.5.1c.

a) Acrylic acid standard

50 µl of a fresh standard of 0.05 mM acrylic acid in distilled water was injected to establish the location of the acrylic acid peak, and give an approximate conversion factor for peak area to concentration.

b) Sample of reaction mixture

In the first 3-vinylcatechol transformation experiment (see text) a sample was taken for h.p.l.c. six minutes after the addition of fraction 52. The area of the putative acrylic acid peak is consistent with a concentration in the assay of 0.09 mM.

c) Reaction mixture sample after the addition of acrylic acid

After acidification 500 µl of the reaction mixture from the first 3-vinylcatechol transformation reaction (see text) was mixed with 500 µl of 0.1 mM acrylic acid in buffer, and injected into the h.p.l.c.. The elution peak with the retention time expected for acrylic acid is shown. Note that this is a single, symmetrical peak, strongly indicating that the reaction mixture contains acrylic acid, rather than another component with a similar retention time. The area of this peak is consistent with approximately 0.1 mM acrylic acid, as would be expected if each of the solutions mixed contained approximately 0.1 mM acrylic acid.

Figure 5.19: Typical h.p.l.c. traces obtained in the identification of acrylic acid formed from 3-vinylcatechol

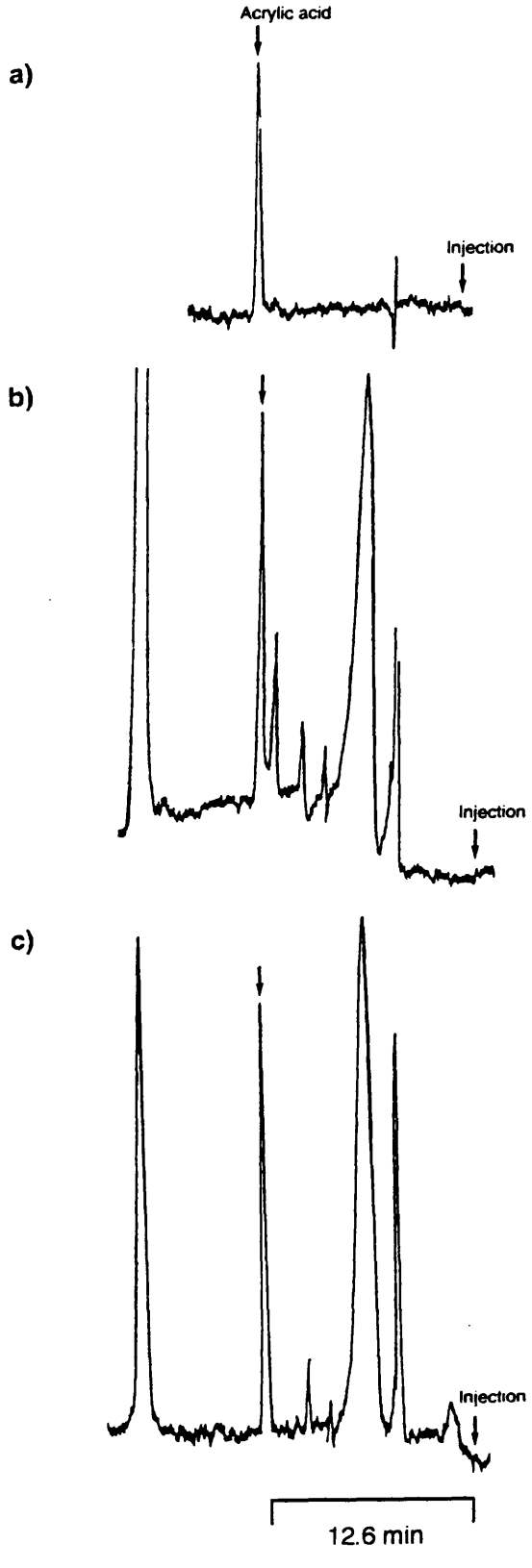


Figure 5.20: The production of 2-hydroxypent-2,4-dienoate from 3-vinylcatechol using column fractions

a) The production of 2-hydroxy-6-oxo-octa-2,4,7-trienoic acid

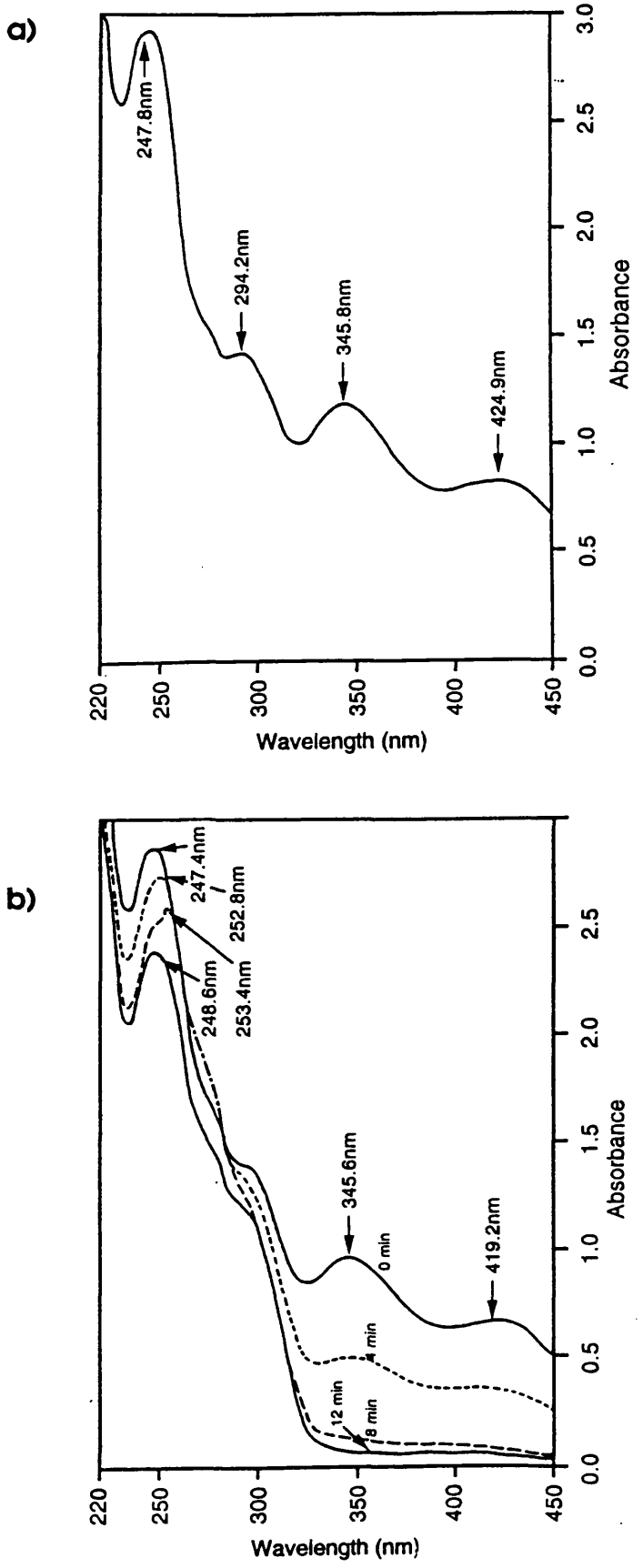
The baseline of the spectrophotometer was set between 220 and 450 nm on 2.9 ml of 50 mM phosphate buffer, pH 7.0, and 100 µl of fraction 63 (from the ion exchange column, see text). Another cuvette was used for the reaction mixture, which contained 2.4 ml of 50 mM phosphate buffer, pH 7.0, and 500 µl of a solution in the same buffer of 3-vinylcatechol produced from approximately 0.4 mg of styrene *cis*-glycol. The 3-vinylcatechol used had been extracted into diethyl ether before being dissolved in buffer (Methods 2.3.9). Catechol 2,3-oxygenase activity was then added, 100 µl of fraction 63 (see text). The absorption spectrum of the reaction mixture was then recorded between 220 and 450 nm. It was found that the concentration of 2-hydroxy-6-oxo-octa-2,4,7-trienoic acid reached a maximum before a second scan was started.

b) The conversion of 6-vinyl-2-hydroxymuconic acid semialdehyde to 2-hydroxypent-2,4-dienoate

This experiment was a continuation of that described in a) above, with the spectrophotometer still set up in the same way.

The above assay mixture had 100 µl of fraction 52 added to it (from the ion exchange column, containing high levels of 2-hydroxymuconic acid semialdehyde hydrolase activity, see text). The absorbance spectrum of the reaction mixture was then monitored every two minutes between 220 and 450 nm. After 8 minutes a sample of 50 µl was removed for h.p.l.c. analysis, see Table 5.5.

Figure 5.20: The production of 2-hydroxypent-2,4-dienoate from 3-vinylcatechol using column fractions



acid. Two further samples were taken at the end of the experiment, one 30 minutes after the addition of fraction 52, and the second after acidification. The first gave a peak consistent with 0.08 mM acrylic acid, the second 0.09 mM.

An additional control sample (2.9 ml of buffer plus 100 µl of fraction 52) was analysed by h.p.l.c., but no acrylic acid was detected. This evidence strongly suggests that the acrylic acid detected by h.p.l.c. is being produced from 3-vinylcatechol by the combined action of fraction 64 (catechol 2,3-oxygenase) and fraction 52 (2-hydroxymuconic acid semialdehyde hydrolase). The presence of a very small concentration of acrylic acid before the addition of fraction 52 can be explained by a small amount of hydrolase activity being present in this fraction (as can be seen in Figure 5.13).

5.5.3 Measurement of acrylic acid concentrations in culture medium of *R. rhodochrous* 26 growing on styrene.

It was now clear that acrylic acid was likely to be a metabolite of styrene, but it was not known if *R. rhodochrous* 26 could metabolise this substrate. It had earlier been found that the organism was unable to grow on acrylic acid under the conditions tested (Table 3.5), although a small increase in oxygen consumption was found when acrylic acid was added to intact cells in the oxygen electrode (Table 3.7). If acrylic acid is not metabolised by *R. rhodochrous* 26 then it would be expected to accumulate in the medium of cells growing on styrene.

Two 500 ml flasks fitted with mini-inert valves and containing 100 ml of Z1 buffer with Fe^{++} (Methods 2.5.7) were inoculated with 5 ml of a nutrient broth culture of *R. rhodochrous* 26, and 10 µl of styrene was placed in each flask (giving an effective concentration of approximately 1 mM). The flasks were incubated at 30 °C on the orbital shaker (Methods 2.4.4c).

After 43 hours, when growth had started, as indicated by measurements of optical density, two 50 µl samples were removed from one flask and injected into the h.p.l.c.. An elution peak was visible at the same retention time as that of authentic acrylic acid,

but it corresponded to an acrylic acid concentration in the flask of only about 0.01 mM. Further samples were taken from this flask after 65 and 70 hours, and the apparent acrylic acid concentration in both these samples was again only approximately 0.01 mM. When optical density measurements had indicated that growth had ceased, after 89 hours, the contents of the other flask - which had not been previously sampled - were subjected to h.p.l.c. but no peak was detectable at the retention time for acrylic acid.

Since the concentration of acrylic acid during or after growth never rose above 0.01 mM, compared with an initial addition of 1 mM styrene, it seems likely that the acrylic acid produced from 2-hydroxy-6-oxo-octa-2,4,7-trienoic acid was immediately metabolised.

5.6 Discussion

At the beginning of this chapter a pathway for the breakdown of styrene by *R. rhodochrous* 26 was proposed (Figure 5.1, with R = $HC{=}CH_2$). All the evidence produced in this chapter has supported the hypothesis that this is the pathway used by this organism for the breakdown of styrene, except that the *cis, cis*-muconate is a dead-end product:

(1) Using a $^{18}O_2$ / $^{16}O_2$ mixture to produce 3-vinylcatechol from styrene by intact cells it was proved that the initial ring attack was by a dioxygenase, not a monooxygenase (Section 5.1.1). However, it was not possible to measure this dioxygenase activity in extracts, presumably because of the instability of the enzyme involved.

(2) An NAD-dependent dehydrogenase that used both toluene and styrene *cis*-glycol as substrates was present in cells grown on nutrient broth and styrene, but not cells grown on nutrient broth only (Section 5.2.1). NADP was not a cofactor for this reaction. A yellow colour appeared (λ_{max} =334 nm and 423 nm) then vanished when styrene *cis*-glycol was the substrate; this is consistent with the formation of a 2-hydroxymuconic acid semialdehyde.

Partial purification of this dehydrogenase was accomplished using ion exchange chromatography (Section 5.3.1). It was found that toluene *cis*-glycol dehydrogenase and styrene *cis*-glycol dehydrogenase activities both eluted at the same ionic strength. The rate of heat denaturation of the two activities was identical. This strongly suggests that there is a single *cis*-glycol dehydrogenase, capable of oxidising both toluene *cis*-glycol and styrene *cis*-glycol. Studies in other organisms have shown that glycol dehydrogenases frequently have a wide substrate specificity, as long as the substrate is a *cis*-glycol not a *trans*-glycol (Axcell & Geary, 1973; Patel & Gibson, 1974; Rogers & Gibson, 1977). This provides further evidence that the styrene glycol produced by *P. putida* UV4 (Chapter 4.2.2) is a *cis*-glycol, as has been assumed.

(3) A catechol 1,2-oxygenase activity was found in extracts of induced cells, and it was present at a much lower level in extracts of non-induced cells (Section 5.2.2). Activity towards different catechols was in the order catechol > 3-methylcatechol > 4-methylcatechol. The level of this activity was found to be little affected by EDTA, an inhibitor of muconate cycloisomerase. By monitoring a reaction over a longer period of time at 260 nm the absorbance initially rose fast, then briefly decreased rapidly before levelling out to a slow decrease. Part of the increase and the brief rapid decrease could be explained by the production and breakdown of 2-hydroxypent-2,4-dienoate at 265 nm (see (6) below). The slowness of the decrease in A_{260} after this initial drop supports the hypothesis that this organism has no muconate cycloisomerase activity. Some decrease in A_{260} might be expected, due to chemical lactonisation of the *cis, cis*-muconate, as has previously been recorded for *cis, cis*-3-fluoromuconate (Pieken & Kozarich, 1990).

(4) A catechol 2,3-oxygenase activity was found in extracts of induced cells, but not in extracts of uninduced cells (Section 5.2.3). Activity towards different catechols was in the order 3-methylcatechol > catechol >> 4-methylcatechol. When 3-vinylcatechol was used as a substrate a yellow product accumulated (λ_{max} = 430 nm and 345 nm). The presence of two absorption peaks of this type is a characteristic of a 2-hydroxymuconic acid semialdehyde.

(5) Although attempts to conclusively identify the 2,3-cleavage product of 3-vinylcatechol (2-hydroxy-6-oxo-octa-2,4,7-trienoic acid) were unsuccessful due to lack of material, it does appear that a picolinate was formed, which strongly suggests a structure similar to 2-hydroxymuconic acid semialdehyde (Section 5.4).

(6) Induced extracts contained a 2-hydroxymuconic acid semialdehyde hydrolase activity, which was not present in uninduced extracts (Section 5.2.4). This activity was partially purified using ion exchange, and it eluted in a single peak, partially overlapping with the catechol 2,3-oxygenase activity (Section 5.3.4). The overlap of these two activities in column fractions produced a 'false' catechol 1,2-oxygenase peak; the catechol in this reaction mixture was first cleaved to 2-hydroxymuconic acid semialdehyde, then hydrolysed to 2-hydroxypent-2,4-dienoate, which accumulated with a λ_{max} = 265-270 nm (Section 5.5.1 and below).

(7) By using the ion exchange column fractions containing catechol 2,3-oxygenase activity and those containing 2-hydroxymuconic acid semialdehyde hydrolase activity it was possible to investigate the hydrolysis of the 2,3-cleavage products of catechols (Section 5.5.1). Both catechol and 3-methylcatechol were found to produce a component with a λ_{max} of 267-272 nm after the consecutive action of fractions containing the two activities. This had an acid λ_{max} of 272-274 nm, and an alkaline λ_{max} of 298-299 nm. Under alkaline conditions this absorbance rapidly disappeared. These results are consistent with the accumulated component being 2-hydroxypent-2,4-dienoate. Other workers (Collinsworth *et al.*, 1973) have found this compound to have a neutral λ_{max} of 265 nm, acid λ_{max} of 270 nm and alkaline λ_{max} of 305 nm. Under alkaline conditions this compound rapidly tautomerises to 2-oxopent-4-enoate (Figure 5.15), with no absorbance in this region.

(8) When 3-vinylcatechol was used as a substrate for the reactions described in (7) it was not possible to definitely identify the accumulation of 2-hydroxypent-2,4-dienoate, due to interfering absorbances, though the movement of a shoulder of a peak was noticed in the correct region (Section 5.5.2). However, the other expected product of the hydrolysis of 2-hydroxy-6-oxo-octa-2,4,7-trienoic acid, acrylic acid (Figure 5.15), was

detected by h.p.l.c. (Section 5.5.2). To test if this acrylic acid could be metabolised by *R. rhodochrous* 26 the concentration of acrylic acid while cells were growing on styrene was measured. The maximum accumulation found while growing was 0.01 mM, and no acrylic acid was detected when growth had finished (Section 5.5.3), strongly suggesting that acrylic acid is metabolised.

6. Discussion

The results described in the previous three chapters allow the pathway given in Figure 6.1 to be confidently proposed as a route for the metabolism of styrene in *Rhodococcus rhodochrous* 26. The basis of this statement has already been discussed in the preceding chapters, so this chapter will concentrate on the implications of this pathway and a comparison with similar systems. For ease of analysis, I will divide the pathway into two sections, an 'upper pathway' from styrene to vinylcatechol and the 'lower pathway' from catechol to the intermediates of central metabolism.

6.1 Upper pathway - from styrene to 3-vinylcatechol

The upper pathway of styrene metabolism in *R. rhodochrous* 26 consists of an initial dioxygenase attack to give styrene *cis*-glycol, followed by a dehydrogenation giving 3-vinylcatechol. This sequence is well established in the breakdown of other non-carboxylated aromatics such as benzene, toluene, ethylbenzene and naphthalene, and is known to occur in other rhodococci, notably *Rhodococcus* sp. strain C125 (Introduction 1.1.7a, Sikkema & de Bont, 1993).

The TOL pathway also incudes a similar sequence, in the transformation of benzoic acid to catechol (Figure 1.27). *R. rhodochrous* 26 is also able to grow on benzyl alcohol and other TOL pathway intermediates (Table 3.5). In addition, when cells are grown on benzyl alcohol they can oxidise TOL pathway intermediates, but not toluene or styrene (Table 3.8). Since catechol is oxidised by these cells and protocatechuic acid is not, it is likely that benzoic acid is metabolised by a dioxygenase followed by a *cis*-glycol dehydrogenase. This would mean that this bacterium has two dioxygenase/*cis*-glycol dehydrogenase systems, one for the metabolism of styrene and other non-carboxylated aromatics, and another for the metabolism of benzoic acid (Figure 6.2). This poses interesting questions as to the specificity and induction of these systems.

Figure 6.1: Proposed route for the breakdown of styrene by *R. rhodochrous* 26

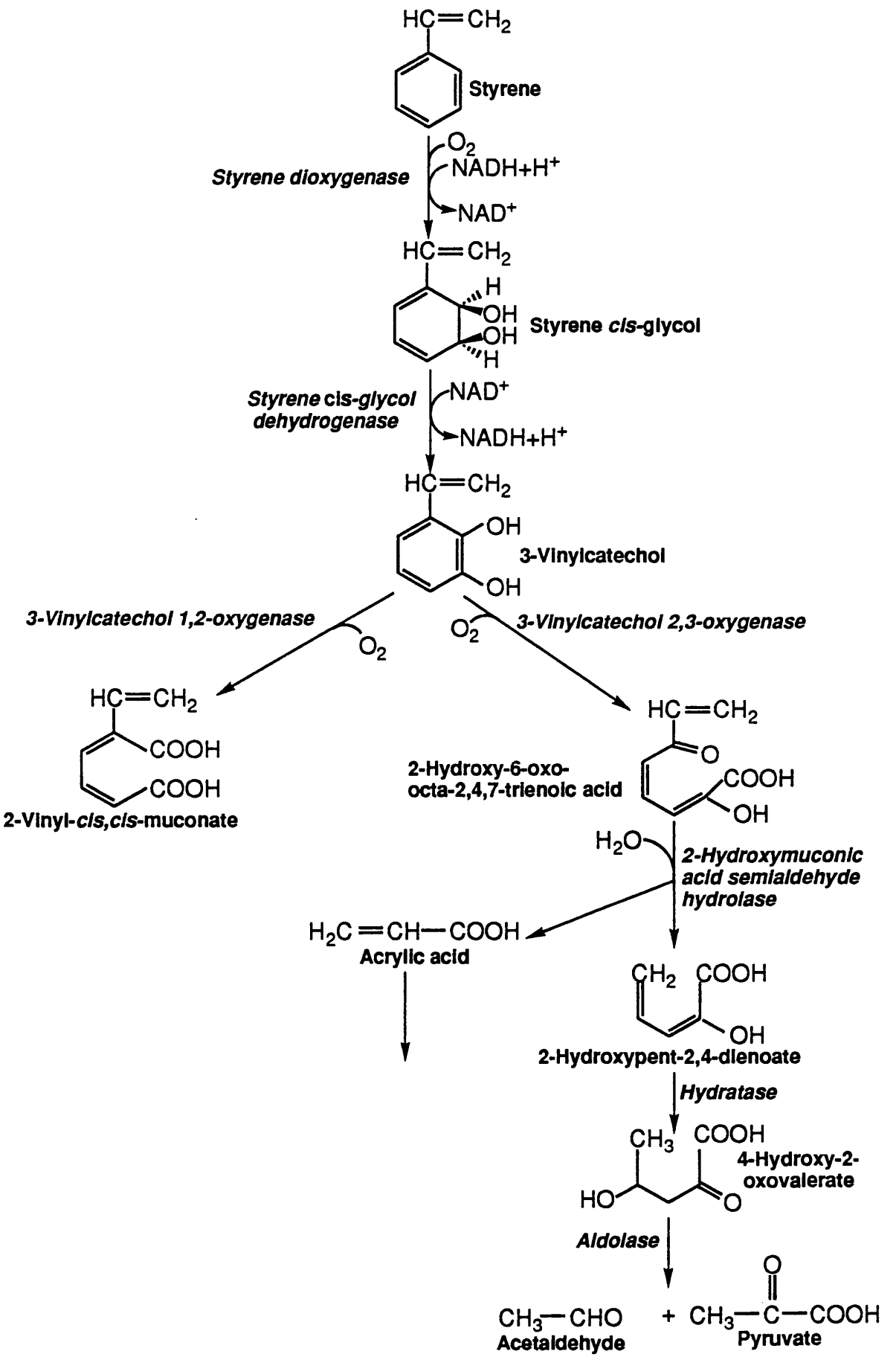
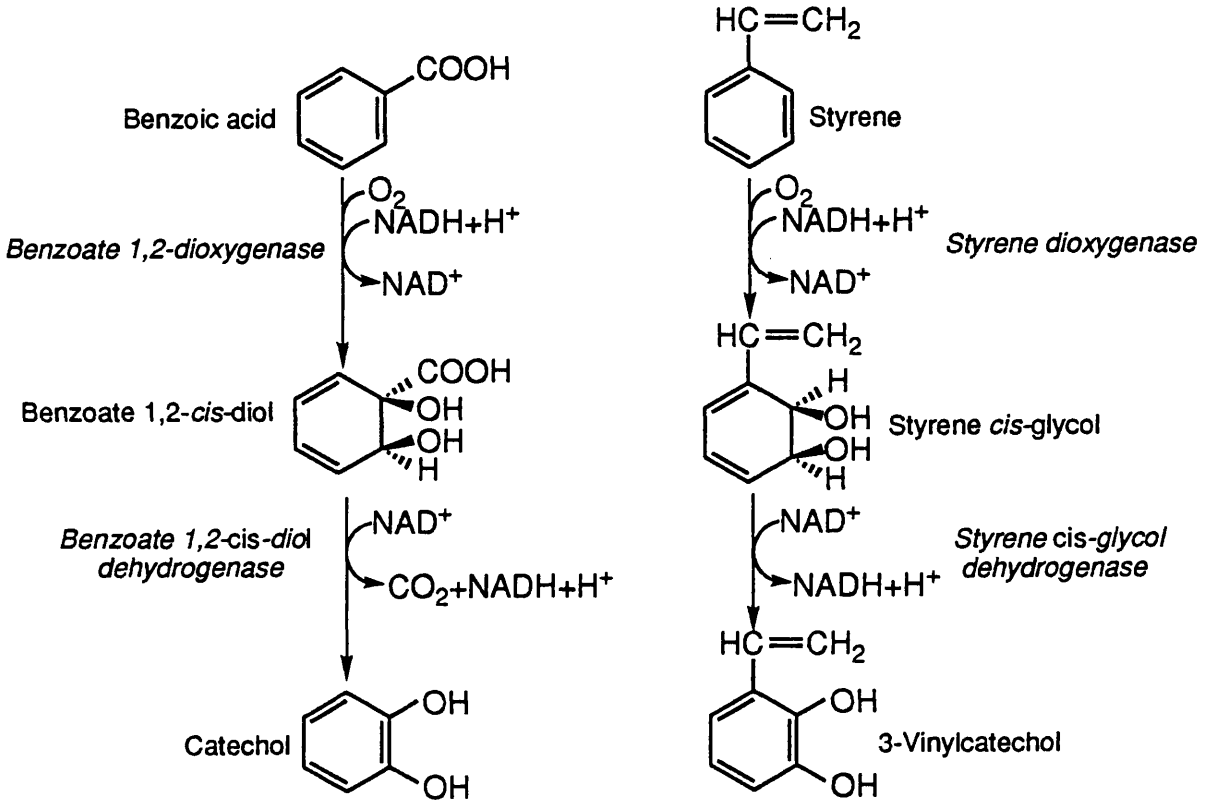


Figure 6.2: The two dioxygenase and *cis*-glycol dehydrogenase systems possibly present in *R. rhodochrous* 26



6.1.1 Dioxygenase systems

Unfortunately it was not possible to detect styrene dioxygenase activity in cell free extracts of *R. rhodochrous* 26. However, as discussed earlier (Chapter 5.6), the $^{18}O_2$ results and the presence of a styrene *cis*-glycol dehydrogenase very strongly indicate that this activity must exist.

The two main methods of examining the initial dioxygenase step of similar pathways have been the purification and characterisation of the dioxygenase and the production of mutants lacking *cis*-glycol dehydrogenase activity. The second method is usually easier, since the dioxygenase system is often very unstable, and is made up of several components.

a) The properties of purified dioxygenases

Some of the dioxygenase systems which have been purified and characterised are shown in Table 6.1. They seem to fall into two groups, three component oxygenases which are able to oxidise a wide range of aromatic substrates, but not those which have a carboxyl group on the ring, and two component oxygenases which can oxidise only carboxylated aromatics. However, this is not the case with phthalate dioxygenase, which attacks the ring opposite the two carboxyl groups, making the attack similar to that on toluene, though the enzyme has only two components.

The mechanisms of dioxygenases are complex and are still being elucidated. One well studied dioxygenase is the toluene dioxygenase from *P. putida* F1, and an outline of its mechanism is given in Figure 6.3. A detailed analysis of the mechanism of electron transfer in phthalate dioxygenase reductase has recently been published (Correll *et al.*, 1992).

b) Mutant studies

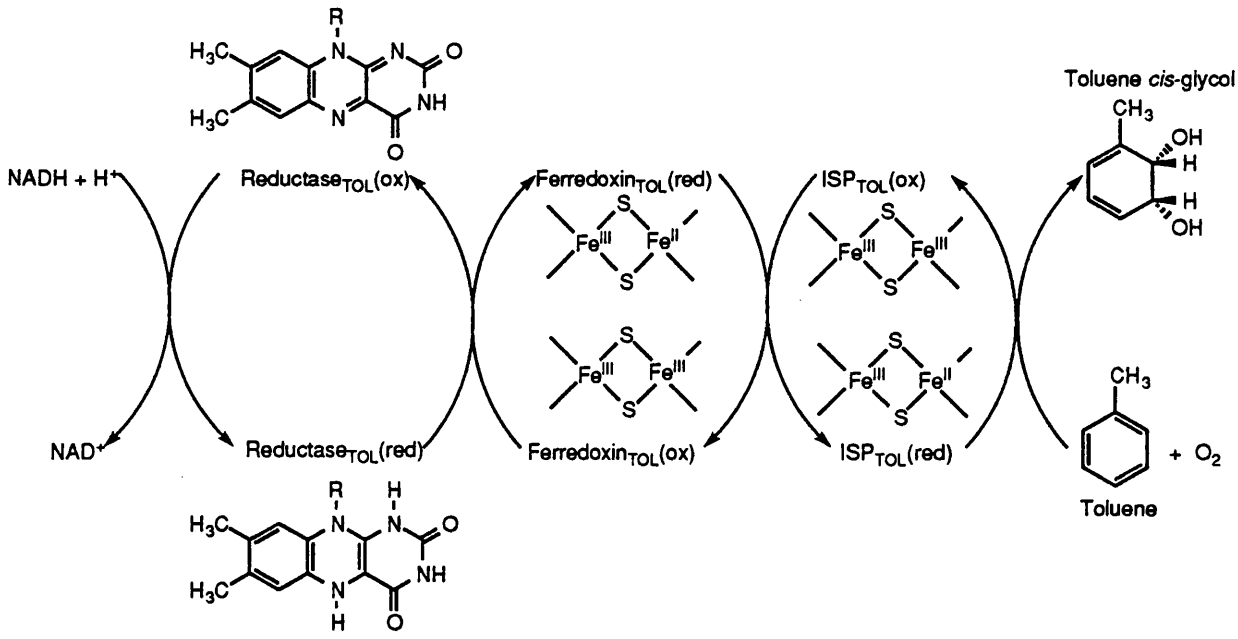
Several mutants lacking *cis*-glycol dehydrogenase activity have been isolated, including *P. putida* UV4 which was used in this study to produce styrene *cis*-glycol. Some of the transformations catalysed by these mutants are listed in Table 6.2, and these results again show the substrate flexibility of the dioxygenase reaction.

Table 6.1: Characteristics of some purified dioxygenases

Enzyme	Structure	Other substrates oxidised
Dioxygenase *Rhodococcus* sp. strain C125 (Sikkema & de Bont, 1993)	NAD or NADP-dependent (not purified)	*o*-Xylene Tetralin Naphthalene Toluene Benzene Ethylbenzene *o*-Diethylbenzene
Benzene dioxygenase *Pseudomonas putida* strain ML2 (Geary *et al.*, 1990)	3 component: 82 k NADH-specific reductase (ben) 12.3 k ferredoxin (ben) 168 k iron sulphur protein (ben), with four subunits, two each of 54.5 k and 23.5 k)	Lower alkyl-substituted benzenes Halogenated benzenes **Not:** Benzoic acid, Benzaldehyde
Toluene dioxygenase *P. putida* F1 (Wackett, 1990)	3 component: 46 k NADH-specific reductase (tol) 15.3 k ferredoxin (tol) 151 k iron sulphur protein (tol), with four subunits, two each of 52.5 k and 20.8 k	
Naphthalene dioxygenase *Pseudomonas* sp. str. NCIB 9816 (Ensley & Gibson, 1983; Haigler & Gibson, 1990a, b)	3 component: 36.3 k NADH-ferredoxin$_{NAP}$ reductase 13.6 k ferredoxin$_{NAP}$ 158 k iron sulphur protein, 2 each of 2 subunits, 55 k and 20 k	
Biphenyl dioxygenase *Pseudomonas* sp. str. LB400 (Haddock *et al.*, 1993)	3 component: reductase (NADH and NADPH dependent) ferredoxin iron sulphur protein with two types of subunits, 52 k and 27 k	
Phthalate dioxygenase *P. cepacia* (Batie & Ballou, 1990)	2 component 34 k NADH-dependent reductase dioxygenase, tetramer of 48 k subunits	Carboxyl groups necessary, but flexibility at other positions, e.g. 1,2-dicarboxypyridine
Benzoate 1,2-dioxygenase *P. arvilla* C1 (Yamaguchi & Fujisawa, 1978, 1980, 1982)	2 component: 37 k NADH-cytochrome *c* reductase (accepts NADPH) 201 k oxygenase, with 3 each of two components, 50 k and 20 k	Substituted benzoates **Not:** Benzene
4-Chlorophenylacetate 3,4-dioxygenase *Pseudomonas* sp. str. CBS (Markus *et al.*, 1984, 1986)	2 component: reductase (NADH most effective, but accepts NADPH) 140 k iron-sulphur protein, with 3 identical 46-52 k subunits	Halogenated phenylacetates Lower rate with phenylacetate **Not:** Benzoate

Figure 6.3: The toluene dioxygenase system in *P. putida* F1

(Wackett, 1990)



Reductase$_{TOL}$: 46 k flavoprotein, 1 mol noncovalently bound FAD per mole protein.

Ferredoxin$_{TOL}$: 15.3 k protein, with one [2Fe-2S] cluster, probably Rieske-type.

ISP$_{TOL}$: holoenzyme Mr = 151 k, two subunits ($\alpha_2\beta_2$) of 52.5 k and 20.8 k, when fully active contains 4-6 iron atoms per holoenzyme, details not yet clear.

Table 6.2: Substrate range of dioxygenase systems, using mutants lacking *cis*-glycol dehydrogenase

Organism	*cis*-Glycols produced (Underlined have had stereochemistry confirmed)
Pseudomonas putida 39D (TOD system) (Ziffer *et al.*, 1977; Hudlicky *et al.*, 1988)	Toluene Ethylbenzene Biphenyl Chlorobenzene *p*-Fluorotoluene Styrene Phenylacetylene
P. putida UV4 (Harrop *et al.*, 1992)	Benzene Toluene Naphthalene Styrene
P. putida BGXM1 (TOL plasmid system) (Whited *et al.*, 1986)	*p*-Toluate Benzoate *m*-Toluate 3-Ethylbenzoate 3,4-Dimethylbenzoate

Presumably it would be possible to generate a similar mutant of *R. rhodochrous* 26. These mutants are of commercial interest, due to the possible biosynthetic applications of *cis*-glycols (see below, 6.6.2).

6.1.2 Properties of *cis*-glycol dehydrogenases

This study has shown that *R. rhodochrous* 26 has a styrene *cis*-glycol dehydrogenase which can also oxidise toluene *cis*-glycol. Other, purified, dehydrogenases have also been found to have a flexible substrate specificity (Table 6.3), though the diol must be in the *cis*-form. None of the aromatic *cis*-diol dehydrogenases seem to have been tested with carboxylated substrates, but the *p*-toluate 1,2-*cis*-diol dehydrogenase from *P. putida* has no activity with toluene or naphthalene *cis*-glycols. More significantly, this enzyme cannot oxidise the methylated compound *p*-toluate 1,2-*cis*-diol methyl ester.

As discussed in chapter 3.4.3, the simultaneous induction results for *R. rhodochrous* 26 grown on benzyl alcohol (Table 3.8) show a high rate of oxygen consumption when toluene *cis*-glycol is added as substrate. At least one *cis*-glycol dehydrogenase activity must have been induced, either the same or similar to that induced by growth on styrene;alternatively a benzoate 1,2-*cis*-diol dehydrogenase had been induced which is able to oxidise toluene *cis*-glycol. However, when grown on cinnamic acid, cells are unable to oxidise toluene *cis*-glycol (Table 3.8). This is surprising, since it appears that cinnamic acid, like benzyl alcohol, is also metabolised through benzoic acid (see 4.6.2a).

Two *cis*-diol dehydrogenases from benzoate catabolising pathways have been sequenced and compared (Neidle *et al.*, 1992). The enzymes, from *Acinetobacter calcoaceticus* and the TOL plasmid of *P. putida*, have 58 % sequence identity, and are also related to the short-chain alcohol dehydrogenase superfamily.

6.1.3 The entire upper pathway

In this study, and others on the metabolism of a range of aromatics, the whole upper pathway was studied by blocking the activity of the catechol oxygenase system and

Table 6.3: Characteristics of some *cis*-glycol dehydrogenases

Enzyme	Structure	Other substrates oxidised
Styrene *cis*-glycol dehydrogenase *R. rhodochrous* 26 (This study, 5.3.1)	NAD-dependent	Toluene *cis*-glycol
cis-glycol dehydrogenase *Rhodococcus* sp. strain C125 (Sikkema & de Bont, 1993)	NAD-dependent	Benzene *cis*-glycol Toluene *cis*-glycol 1,2,5,6,7,8-Hexahydro-*cis*-1,2-naphthalene diol **Not:** Benzene *trans*-glycol
Toluene *cis*-glycol dehydrogenase *P. putida* (Rogers & Gibson, 1977)	Mr = 104 k four 27 k subunits NAD-dependent	Biphenyl *cis*-glycol Naphthalene *cis*-glycol Phenanthrene *cis*-glycol Anthracene *cis*-glycol **Not:** Naphthalene *trans*-glycol
Benzene *cis*-glycol dehydrogenase *Pseudomonas* (Axcell & Geary, 1973)	Mr = 440 k four 110 k subunits NAD-dependent	**Not:** Benzene *trans*-glycol
Naphthalene *cis*-glycol dehydrogenase *Pseudomonas putida* (Patel & Gibson, 1974)	Mr = 102 k four 25.5 k subunits NAD-dependent	Anthracene *cis*-glycol Phenanthrene *cis*-glycol Biphenyl *cis*-glycol Ethylbenzene *cis*-glycol Toluene *cis*-glycol Benzene *cis*-glycol **Not:** Naphthalene *trans*-glycol
Phenanthrene *cis*-glycol dehydrogenase *Alcaligenes faecalis* (Nagao *et al.*, 1988)	Mr = 100 k four 27.5 k subunits NAD or NADP-dependent, NAD preferred	
Benzoate 1,2-*cis*-diol dehydrogenase *Alcaligenes eutrophus* (Reiner, 1972)	Mr = 94.6 k four 24 k subunits NAD-dependent	**Not:** Benzene *cis*-glycol
p-Toluate 1,2-*cis*-diol dehydrogenase *P. putida* (Whited *et al.*, 1986)	Mr = 96 k NAD-dependent	Benzoate 1,2-*cis*-diol *m*-Toluate 1,2-*cis*-diol 3-ethylbenzoate 1,2-*cis*-diol 3,4-Dimethylbenzoate 1,2-*cis*-diol **Not:** Methyl ester of *p*-Toluate 1,2-*cis*-diol Toluene *cis*-glycol Naphthalene *cis*-glycol

identifying accumulated catechols. A comparison between the results obtained with *R. rhodochrous* 26 and some other organisms is given in Table 6.4. In addition to, again, indicating the flexibility of this system, the results from *Pseudomonas* sp. str. T-12 indicate the dichotomy between the carboxylated and non-carboxylated aromatic pathways. This organism is able to produce catechols from a wide range of substrates, including styrene, benzyl alcohol and benzaldehyde. It can also produce the catechol of benzoic acid methyl ester, but not that of benzoic acid itself, a reversal of the results for *p*-toluate 1,2-*cis*-diol dehydrogenase from *P. putida*, which is able to oxidise the acid but not the ester.

Comparing the catechols which can be produced by *Pseudomonas* sp. str. T-12 with the simultaneous induction results of styrene-grown *R. rhodochrous* (Table 3.7) indicates some possible parallels. Styrene grown cells can oxidise benzyl alcohol and to a lesser extent benzaldehyde, but not benzoic acid - this could mean that these substrates are being oxidised through the dioxygenase pathway to catechols or further. The catechol production results from *Pseudomonas* sp. str. T-12 (Table 6.4) show that catechols can be formed from benzyl alcohol and benzaldehyde but not from benzoic acid. These results indicate that these two organisms could have a dioxygenase pathway with very similar specificities.

Other work on rhodococci has indicated that the breakdown of aromatics through dioxygenase attack is common in this genus (e.g. Introduction, 1.1.7a, c). The metabolism of benzoate through a dioxygenase attack has been indicated in other work on rhodococci. Janke *et al.* (1988b) established that this reaction was likely in *Rhodococcus* sp. An 117 and 213, though they were not able to detect dioxygenase activity in extracts, probably due to instability. The metabolism of *p*-toluate by *R. rhodochrous* N75 and *R. corallinus* N657 is also believed to use this route (Introduction, 1.1.7b).

If *R. rhodochrous* 26 does possess two dioxygenase pathways, one for benzoic acid and one for non-carboxylated aromatics, then the induction of these pathways must be

Table 6.4: The range of catechols produced by various dioxygenase/*cis*-glycol dehydrogenase systems

Organism	Accumulation method	Catechols formed from
R. rhodochrous 26 (This study, Section 4.4)	3-Fluorocatechol	Styrene Toluene Ethylbenzene Acetophenone
Pseudomonas sp. NCIB 10643 (Smith & Ratledge, 1989b)	1,2,3-Trihydroxybenzene	Ethylbenzene Isopropylbenzene *n*-Amylbenzene Other alkylbenzenes
Pseudomonas sp. str. T-12 (Johnston & Renganathan, 1987; Renganathan & Johnston, 1989)	Benzonitrile or Fluorobenzene	Styrene β-Methylstyrene 1-Phenylethanol Acetophenone Benzyl alcohol Benzaldehyde Methyl ester of benzoic acid and many others... **Not:** Benzoic acid Benzamide Benzylamine

sensitively regulated, since cells grown on styrene cannot oxidise benzoic acid, and cells grown on benzyl alcohol cannot oxidise styrene.

6.1.4 Induction of the upper pathway

Robertson & Button (1987) have proposed that the toluene dioxygenase pathway in *P. putida* 39/D is induced by toluene *cis*-glycol, since this mutant organism cannot metabolise this compound any further. High concentrations of toluene are needed to induce the toluene dioxygenase, suggesting that toluene itself is not the inducer, but that induction occurs as a result of constitutive levels of toluene dioxygenase activity, producing toluene *cis*-glycol from toluene. This study also examined the induction kinetics of toluene breakdown in *Pseudomonas* sp. str. T2, another organism containing toluene dioxygenase. The hypothesis that pathway induction was at least partly due to residual levels of toluene dioxygenase activity was supported by the discovery that the longer the organism had been growing in the absence of toluene (up to a maximum tested of two years), the longer it took to fully induce the dioxygenase pathway when exposed to toluene, though it is difficult to determine whether any of this behaviour was due to the formation of mutants. The relevance of induction of the dioxygenase pathway to growth of *R. rhodochrous* on styrene is discussed in Section 6.4.4a.

The regulation of the benzoate dioxygenase in the TOL system is more complex, because in this system the pathway is split at the carboxylic acid into an upper portion, from toluene to benzoic acid, and a lower portion, from benzoic acid to central metabolism and benzoate dioxygenase is transcribed with the cleavage enzymes (Burlage *et al.*, 1989).

The upper pathway, from benzyl alcohol to benzoate, is regulated by the XylR protein, which has a wide effector specificity. Abril *et al.* (1989) have shown that effectors include toluene and benzyl alcohol, but benzaldehyde and benzene are not effective. When *R. rhodochrous* 26 was grown on toluene, the cells were able to oxidise benzyl alcohol and benzaldehyde at a higher rate than cells grown on styrene

(Table 3.6), suggesting that the benzyl alcohol metabolism pathway might be induced by toluene.

The control of the lower part of the TOL system, from benzoate, is rather complicated, regulated by the *xylS* gene (Burlage *et al.*, 1989). The effectors of this gene product are complex, and the XylR protein is also involved. Some organisms even express several homologous XylS proteins (Assinder *et al.*, 1993).

6.2 Lower pathway: *meta* cleavage of 3-vinylcatechol

One of the routes used by *R. rhodochrous* 26 to cleave 3-vinylcatechol is *meta* cleavage by 3-vinylcatechol 2,3-dioxygenase, followed by further metabolism of the 2-hydroxy-6-oxo-octa-2,4,7-trienoic acid to give acrylic acid and 2-hydroxypent-2,4-dienoate (Figure 6.1). This general pathway is known to occur in a wide range of organisms, and there is no evidence that the pathway in strain 26 differs from that established in other organisms, for example that encoded by the TOL plasmid (Figure 1.27). Although this study has not established the metabolic fate of the 2-hydroxypent-2,4-dienoate, it is likely that it is degraded by an aldolase and hydratase, as indicated in Figure 6.1. Further work on this organism has supported this hypothesis (see below, section 6.2.3).

6.2.1 Catechol 2,3-oxygenase

Catechol 2,3-oxygenases appear to be non-specific in all the organisms so far tested. However, there are significant variations in relative specificities towards different catechols. It is also common for organisms to have two catechol 2,3-oxygenases, usually with different specificities. This has been found among diverse TOL systems (Chatfield & Williams, 1986) and in the 2-methylaniline - degrader *R. rhodochrous* CTM (Introduction 1.1.7c; Schreiner *et al.*, 1991). Although only one activity peak eluted from ion exchange (Figure 5.11), this technique is not sensitive enough to guarantee separation of isozymes. It is therefore possible that more than one catechol 2,3-oxygenase is expressed in this organism.

Table 6.5: A comparison of catechol oxygenase relative activities with different substrates

(rate with catechol = 100)

a) Catechol 2,3-oxygenase

Substrate	*R. rhodochrous* 26 Catechol 2,3-oxygenase			*R. rhodochrous* CTM (Schreiner *et al.*, 1991)	
	Extract, this study (Table 5.3) grown on styrene	Partially purified grown on styrene (McIntosh, 1993)	Partially purified grown on benzyl alcohol (McIntosh, 1993)	Catechol 2,3-oxygenase I	Catechol 2,3-oxygenase II
Catechol	100	100	100	100	100
3-Methylcatechol	145	106	130	360	63
4-Methylcatechol	11	18	45	165	45

b) Catechol 1,2-oxygenase

Substrate	*R. rhodochrous* 26 Catechol 1,2-oxygenase			*R. erythropolis* 1CP (Mal'tseva *et al.*, 1991)	
	Extract, this study (Table 5.2) grown on styrene	Partially purified grown on styrene (McIntosh, 1993)	Partially purified grown on benzyl alcohol (McIntosh, 1993)	Catechol 1,2-oxygenase I	Catechol 1,2-oxygenase II
Catechol	100	100	100	100	100
3-Methylcatechol	60	50	20	99	200
4-Methylcatechol	30	20	50	88	225

The relative specificity of the catechol 2,3-oxygenase activity in extract of *R. rhodochrous* 26 grown on styrene and nutrient broth (Table 5.3) is shown in Table 6.5a, compared with those found for another *R. rhodochrous* strain. It was noted earlier (Table 3.8) that cells grown on benzyl alcohol appeared to have a higher oxygen uptake with catechol than 3-methylcatechol, suggesting that different catechol oxygenases might be induced by benzyl alcohol. However, as shown in Table 6.5, further work on this strain, using ion-exchange purified extract, suggests that there is probably no difference in the catechol 2,3-oxygenase specificity when induced by benzyl alcohol (McIntosh, 1993).

The catechol 2,3-oxygenases of the other *R. rhodochrous* strain shown in Table 6.5a do not have the same ratio of activity as that from strain 26. However, a 3-methylcatechol 2,3-dioxygenase has been purified from *P. putida* ATCC 23973, able to grow on toluidine, which does show a similar ratio of activities, with a k_{cat} ratio of catechol 100: 3-methylcatechol 175: 4-methylcatechol 40 (Wallis & Chapman, 1990). This enzyme is a tetramer of identical 33.5 k subunits, giving a holoenzyme Mr of 120 k. *Rhodococcus* sp. strain C125 also possesses a similar catechol 2,3 oxygenase activity when grown on *o*-xylene, with an activity ratio of catechol 100: 3-methyl-catechol 240: 4-methylcatechol 56 (Sikkema & de Bont, 1993).

6.2.2 2-Hydroxymuconic acid semialdehyde hydrolase

The *meta* cleavage products of 3-vinylcatechol, 3-methylcatechol and catechol are metabolised by 2-hydroxymuconic acid semialdehyde hydrolase activity of *R. rhodochrous* 26 to give short-chain carboxylic acids, respectively acrylic, acetic and formic acids, and 2-hydroxypent-2,4-dienoate. This enzyme is very unstable at 55°C, being completely inactivated in two minutes (5.3.4). The 2-hydroxymuconic acid semialdehyde hydrolase activity may be more rapid with the cleavage product of 3-methylcatechol than that of catechol (5.5.1b).

These characteristics fit in well with the those observed with the purified hydrolase enzymes from other organisms. Heat treatment at 55°C is a routine method used to

inactivate this enzyme (Bayly *et al.*, 1966). Duggleby & Williams (1986) purified the hydrolase encoded on the TOL plasmid pWW0 from *P. putida* mt-2, and found that it was a 65 k dimer of two equal sized subunits of 31-33 k. This enzyme displayed similar K_m values with the 2,3-cleavage products of catechol and 3-methylcatechol, but the V_{max} with the 3-methylcatechol cleavage product was fourteen fold higher than that with the catechol product. A similar ratio of activity was found by Bayly & Di Berardino (1978) for hydrolases purified from *P. putida* NCIB 10015 and *P. putida* NCIB 9865. The enzymes in this study had native Mr values of 118 k and 100 k respectively, each dissociating into four identical subunits.

6.2.3 The metabolism of 2-hydroxypent-2,4-dienoate

The final stage of the established *meta* cleavage pathway for catechols is the transformation of 2-hydroxypent-2,4-dienoate into acetaldehyde and pyruvate by the consecutive action of 2-hydroxypent-2,4-dienoate hydratase and 2-hydroxy-2-oxovalerate aldolase. Although this project has not investigated these enzymes, further work on *R. rhodochrous* 26 by Blunt (1993) has shown that extracts of cells grown on nutrient broth and styrene are able to transform both 2-hydroxypent-2,4-dienoate and 4-hydroxy-2-oxovalerate into acetaldehyde and pyruvate.

Collinsworth *et al.* (1973) purified the hydratase from *P. putida* NCIB 10015, and found it to consist of 28 k subunits combined in a 287 k holoenzyme. These workers also proposed that this enzyme could be closely related to the aldolase, since similar mechanisms could be proposed for both enzymes. Harayama *et al.* (1989) cloned a similar enzyme from the TOL plasmid of *P. putida* mt-2 into *E. coli*. This hydratase was a 27 k polypeptide, and it copurified with 4-oxalocrotonate decarboxylase, the preceding enzyme of the oxalocrotonate route of catechol breakdown (Introduction, Figure 1.31). This suggests that these enzymes may be associated, to enable rapid transformation of 2-hydroxypent-2,4-dienoate, which is unstable under some conditions. There was no evidence, however, of an association between 2-hydroxymuconic acid semialdehyde hydrolase and the hydratase in *P. putida* mt-2.

Recent research by Powlowski *et al.* (1993) has questioned the assumption that the aldolase is the final step of the *meta* cleavage pathway, at least in *Pseudomonas* sp. strain CF600. The aldolase activity was found to copurify with a aldehyde dehydrogenase (acylating), which formed acetyl CoA from the acetaldehyde product of the aldolase reaction. The aldolase had a Mr of 35 k, and the acetaldehyde dehydrogenase had an Mr of 40 k, and the purified complex contained two each of these subunits, giving a total Mr of 148 k. The two enzymes are encoded as consecutive genes on the *dmp* operon, which encodes the *meta* cleavage pathway for phenol degradation in this strain.

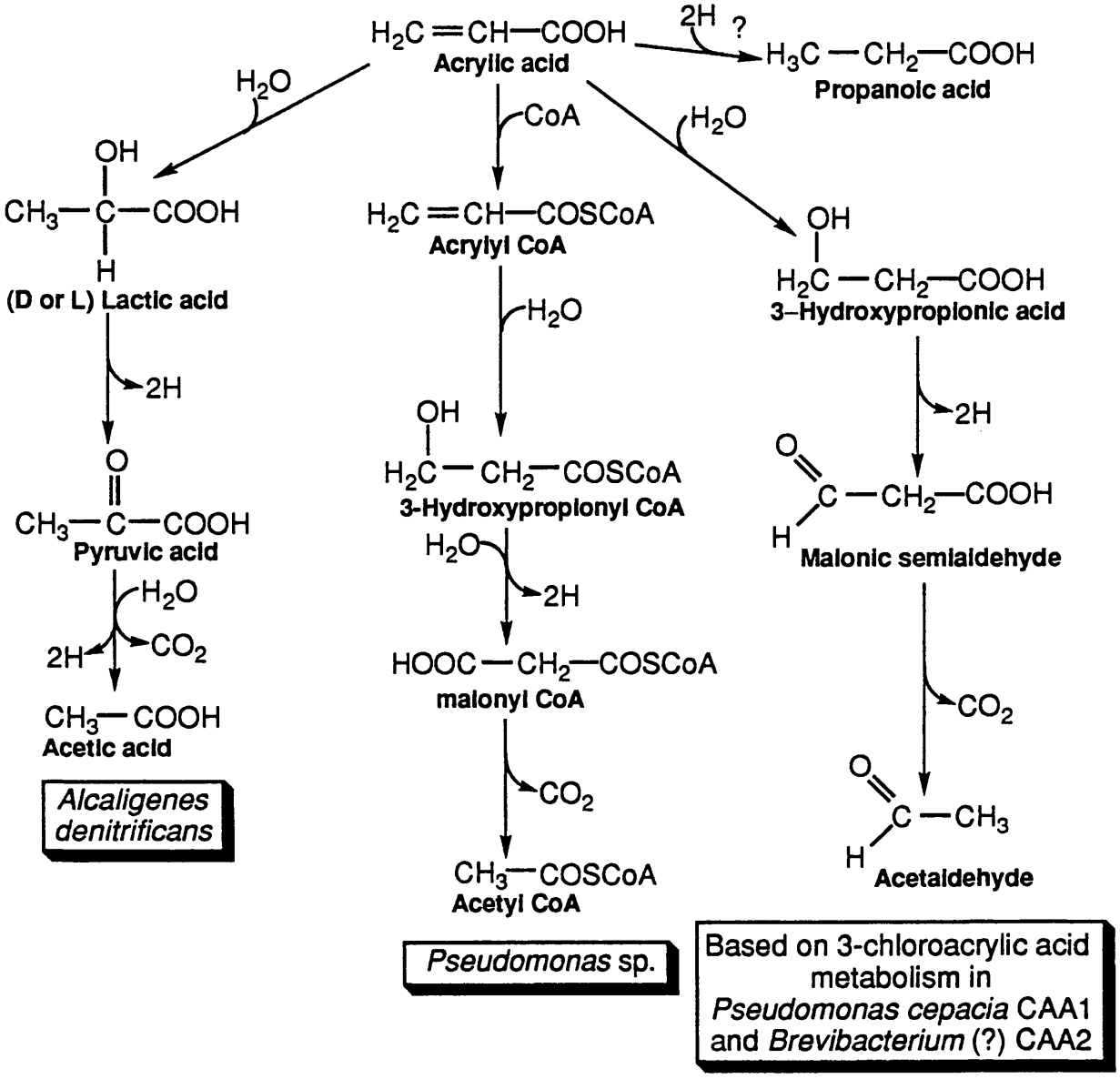
6.2.4 Acrylic acid metabolism

It is clear that acrylic acid is produced by the hydrolysis of 2-hydroxy-6-oxo-octa-2,4,7-trienoic acid by 2-hydroxymuconic acid semialdehyde hydrolase (Figure 6.1). Since there is no appreciable accumulation of acrylic acid in the growth medium (5.5.3), *R. rhodochrous* 26 must be able to metabolise this substrate. However, growth did not occur on acrylic acid at either 5 mM or 0.5 mM (Table 3.5), though 5 mM vinylacetic acid, which has one more methylene group than acrylic acid, did support growth. Simultaneous induction experiments showed a small oxygen consumption of 20 nmol O_2/min/mg protein when acrylic acid was added to cells grown on styrene or glucose (Table 3.7). Some other rhodococci are known not to degrade acrylic acid, for example *R. rhodochrous* J1 is used to produce acrylic acid from acrylonitrile (Introduction 1.1.9, Nagasawa *et al.*, 1990).

Acrylic acid is known to be toxic to many microorganisms, for example Andreoni *et al.* (1990) found that 500 mg acrylic acid /l (7 mM) inhibited the growth of about 99 % of the cells of *Bacillus subtilis* and *Escherichia coli* within six hours, though after twenty hours the surviving bacteria multiplied. Andreoni *et al.* (1990) also isolated an *Alcaligenes eutrophus* strain able to grow on at least 14 mM acrylic acid. Acetic acid and L-(+)-lactic acid were isolated from the growth medium as transient intermediates, and the breakdown pathway given in Figure 6.4 was proposed. Shanker *et al.* (1990) proposed a pathway through acrylyl CoA and β-hydroxypropionyl CoA for the

Figure 6.4: Possible routes for the metabolism of acrylic acid

(Andreoni *et al.*, 1990; Shanker *et al.*, 1990; Hartmans *et al.*, 1991)



breakdown of acrylamide through acrylic acid by a *Pseudomonas* sp.. *P. cepacia* CAA1 is able to metabolise 3-chloroacrylic acid through malonic semialdehyde, though it is unable to transform acrylic acid using the same enzymes (Hartmans *et al.*, 1991). Other bacteria able to grow on acrylic acid include an *Arthrobacter* sp. (Hayashi *et al.*, 1993).

It is also theoretically possible that acrylic acid could be reduced to propanoic acid before further metabolism (Figure 6.4), though there seem to be no examples of this.

6.3 Lower pathway: *ortho* cleavage of 3-vinylcatechol

The *ortho* cleavage pathway in *R. rhodochrous* 26 is rather a puzzle; it starts, but it doesn't seem to finish (a full *ortho* cleavage pathway is shown in Figure 1.30). A catechol 1,2-oxygenase is clearly present, but there is no sign of any further metabolism of even the *cis, cis*-muconic acid itself (5.2.2). This raises several questions - whether the pathway is present in the organism at all, why is the catechol 1,2-oxygenase expressed and is it possible theoretically for vinylmuconic acid to be metabolised by an *ortho* pathway?

6.3.1 Catechol 1,2-oxygenase

As with catechol 2,3-oxygenases, many organisms express several isozymes of catechol 1,2-oxygenase, with different specificities. These isozymes have been found frequently in organisms capable of metabolising chlorinated compounds; they frequently possess a catechol 1,2-oxygenase with a higher affinity for substituted catechols, such as methyl substituted or chlorinated catechols, than for catechol. These enzymes are often called chlorocatechol 1,2-dioxygenases. One example of this is the pair of catechol 1,2-oxygenases purified from the chlorophenol-degrader *R. erythropolis* (Introduction 1.1.7d, Mal'tseva *et al.*, 1992), whose relative activities are shown in Table 6.5b; catechol 1,2-oxygenase II could also be called a chlorocatechol 1,2-dioxygenase. The present study of *R. rhodochrous* 26 has shown that catechol is the preferred substrate for the 1,2-oxygenase, unlike the 2,3-oxygenase, for which the best substrate is 3-methylcatechol (Table 6.5b).

In another attempt to explain the anomalous benzyl alcohol simultaneous induction results (see above, 6.2.1), McIntosh (1993) tested the catechol 1,2-oxygenase specificities induced by strain 26 when growing on benzyl alcohol and styrene (Table 6.5b). The catechol 1,2-oxygenases co-eluted when purified by ion-exchange chromatography, and showed similar patterns of specificity whichever was the growth substrate. One possible explanation for the increase in catechol oxidation relative to 3-methylcatechol oxidation in simultaneous induction experiments could be a difference in the level of induction of the catechol 1,2- and 2,3-oxygenase activities; more 1,2-oxygenase would lead to an increase in intact cell specificity towards catechol.

6.3.2 Possible reasons for little or no metabolism of muconic acids

Four possible explanations for the accumulation of muconic acid are:

a) Lack of genes

R. rhodochrous 26 could have obtained the catechol 1,2-oxygenase enzyme without the rest of the pathway during some form of genetic transfer. Alternatively, it could have lost the genes during some form of genetic event, or they could have been severely mutated.

b) Only muconate cycloisomerase is missing or mutated

Since *cis, cis*-muconate was not metabolised, muconate cycloisomerase is either not expressed or is expressed at very low levels. However, the rest of the enzymes of the pathway could be present. The muconate isomerase may just be mutated, making it inactive or slightly active.

c) Regulatory problems

Ornston (1966) investigated the regulation of the *ortho* cleavage pathway in *P. putida* ATCC 12633, and found that the catechol 1,2-oxygenase was separately regulated from muconate cycloisomerase and muconolactone isomerase (the *cis, cis*-muconate block), and the enzymes below these in the pathway were regulated separately from this pair (the pathway is illustrated in Figure 1.30). The inducer for the

expression of both catechol 1,2-oxygenase and the *cis, cis*-muconate block was found to be *cis, cis*-muconate. The regulation of the *cis, cis*-muconate block (the *catBC* operon) is controlled by a regulatory protein, CatR, which can bind to *cis, cis*-muconate, increasing its affinity for the *catBC* promoter region 20 fold (Parsek *et al.*, 1992).

If *R. rhodochrous* 26 has a similar regulatory system, with all the genes of the *ortho* cleavage pathway present, the accumulation of muconic acids could occur because of failures of the induction mechanism for muconate cycloisomerase and muconolactone isomerase:

a) The promoter region of either the catabolic operon or the regulator operon could be mutated, preventing transcription.

b) The regulator protein could be damaged, and unable to bind to *cis, cis*-muconic acid.

c) Binding of the regulator to the operator of the catabolic operons could be ineffective, either due to a mutation of the regulator protein or the operator.

Any of these explanations could lead to a very low or non-existent level of *cis, cis*-muconate metabolism. If the catechol 1,2-oxygenase was separately regulated, then it could still be induced by *cis, cis*-muconic acid.

d) The true substrate is metabolised

It is possible that there is a 'true substrate' for the *ortho* pathway, which is converted into a muconate which can be metabolised.

6.3.3 Why is catechol 1,2-oxygenase still present and induced?

As mentioned above, there is a precedent for catechol 1,2-oxygenase being separately regulated from the rest of the *ortho* pathway, so there is no reason why catechol 1,2-oxygenase cannot be induced separately from the rest of the pathway. However, the synthesis of this enzyme requires cell resources, and its activity removes growth substrate from the bacterium's cell supply. It is hard to find a precedent of an

enzyme from an established catabolic pathway being expressed without the rest of the pathway to produce a dead end metabolite, apart from mutants made in the laboratory. Why hasn't natural selection stopped the expression of this enzyme?

a) Positive selection for production of muconic acids

a) There is no information on the chemicals which *R. rhodochrous* 26 was exposed to prior to its isolation from the environment. Since it was isolated from an industrial chemical dump (Methods 2.4.1), it could have been exposed to a complex cocktail of chemicals. It is possible that one or more of these chemicals produced toxic intermediates when metabolised through an *ortho* cleavage pathway, whereas their *cis*, *cis*-muconic acid might be less toxic. For example, in organisms without a modified *ortho* pathway, methyl-substituted 4-carboxymethyl-but-2-en-1,4-olides accumulate as deadend metabolites from 4-methylcatechols, though these are not known to be particularly toxic (Catelani *et al.*, 1971).

b) Vinylmuconic acid could have positive effects on the environment of the bacterium. It would reduce the pH, which could be useful in an alkaline environment. It might also affect the growth of competing organisms.

b) A lack of selective pressure

a) As mentioned previously, there is no information on the chemical environment that *R. rhodochrous* 26 was exposed to before isolation. It is possible that mutagens were present, damaging the DNA of this organism. There may not have been sufficient generations since this event to select for a more 'efficient' production of catechol cleavage enzymes. There would have been an element of selection in the isolation procedure, however.

b) In the conditions that *R. rhodochrous* 26 was growing in there may have been an excess of usable carbon sources, resulting in little selective pressure.

c) In the natural growth conditions the concentrations of aromatic growth substrate could have been very low. If the K_m of the catechol 1,2-oxygenase was substantially higher than that of catechol 2,3-oxygenase, then the catechol 1,2-oxygenase would be

almost inactive. However, preliminary studies by McIntosh (1993) have indicated that the opposite is the case.

6.3.4 Could vinylmuconic acid be metabolised by other organisms?

By analogy with the pathways of *ortho* breakdown of catechols given in Figure 1,30 a theoretical pathways for the breakdown of vinylmuconic acid can be produced (Figure 6.5). Whether either of these pathways could occur would have to be tested with organisms known to have non-specific *ortho* pathways, such as *R. rhodochrous* N75 (Introduction, 1.1.7b). The right hand pathway in Figure 6.5 would be most likely, by analogy with the breakdown of methylcatechols in *R. rhodochrous* N75.

6.4 Physiology of aromatic metabolism by *R. rhodochrous* 26

6.4.1 Versatility

R. rhodochrous 26 possesses a very versatile metabolic capability, due to three main factors:

1) The presence of several pathways for aromatic breakdown, including the benzyl alcohol pathway and the styrene pathway.

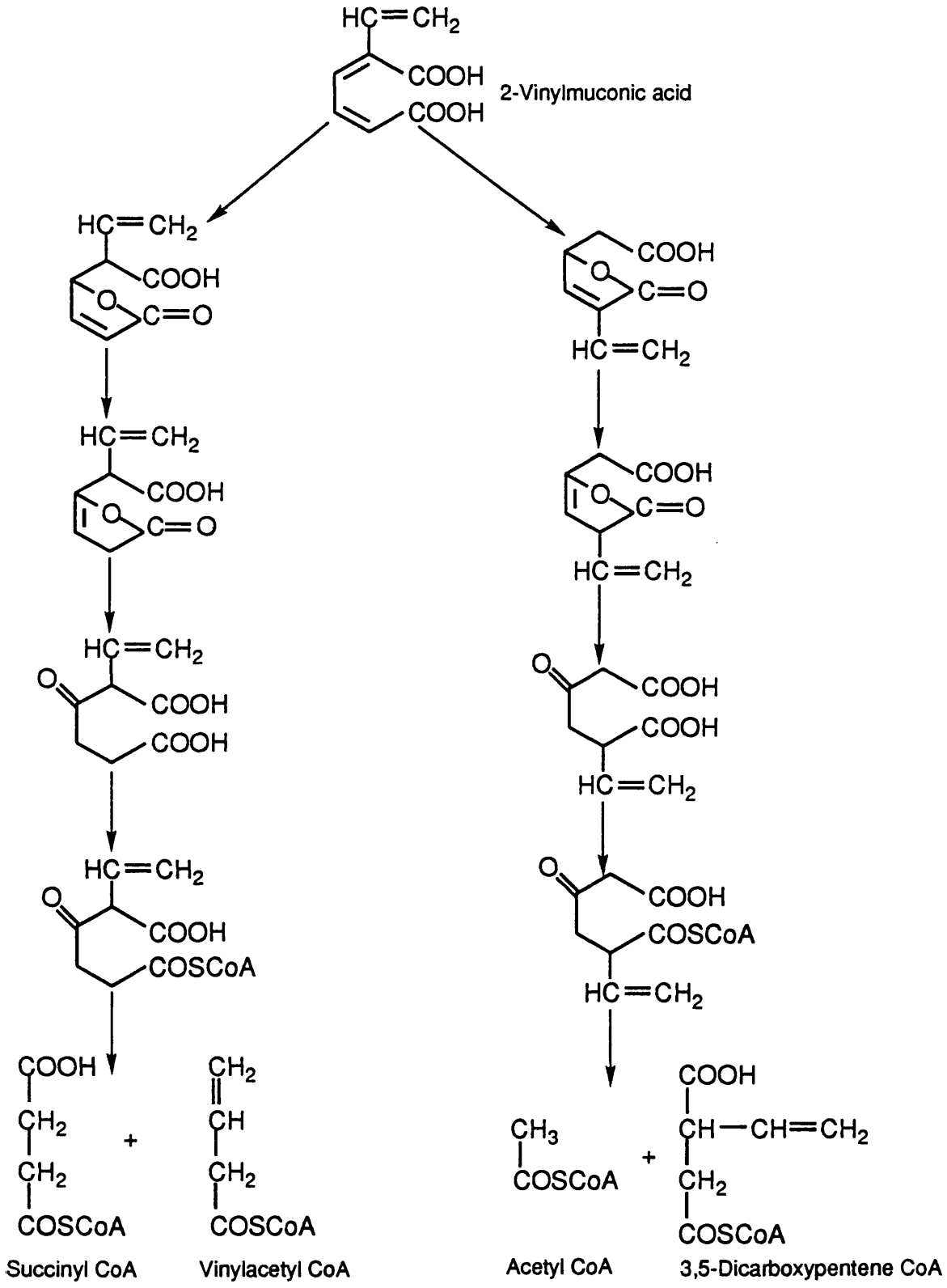
2) The non-specificity of the enzymes of these pathways, allowing a diverse range of substrates to use the same pathway.

3) The ability of *R. rhodochrous* 26 to continue to metabolise aromatics such as styrene in the presence of other growth substrates such as nutrient broth.

This last property seems to be common to many rhodococci (e.g. Introduction 1.1.7c, d) and is in contrast to the sophisticated methods of catabolite repression frequently present in pseudomonads.

It is worth noting here that one of the other strains investigated in this work, strain 80, was provisionally identified as another *Rhodococcus* sp (Table 3.1). As determined

Figure 6.5: Theoretically possible intermediates in the metabolism of vinylmuconic acid



earlier (3.2.4), this strain appears to degrade styrene through phenylacetic acid, rather than the *cis*-glycol route. This demonstrates the metabolic versatility of the genus *Rhodococcus*.

6.4.2 The growth rate

Like many rhodococci (Introduction 1.1.3), *R. rhodochrous* 26 grows slowly, with a doubling time of about 10 h on styrene in a fermenter (3.3.2b). An approximation of the rate of styrene metabolism necessary to support this growth rate can be made. The full calculation is given in Figure 6.6, giving the result that a rate of approximately 50 nmol/min/mg protein is necessary for each catabolic enzyme in order to support a doubling time of 10 h. This result is of the same order of magnitude as those found in extracts: styrene/toluene *cis*-glycol dehydrogenase, 50 nmol/min/mg protein; 3-methylcatechol 2,3-oxygenase, 20 nmol/min/mg protein and 2-hydroxymuconic acid semialdehyde hydrolase, 10 nmol/min/mg protein (Tables 5.1, 5.3 and 5.4).

It is important to note that only in the case of styrene *cis*-glycol dehydrogenase is the rate given above that of the enzyme with the intermediate from the styrene pathway, the other rates given come from the toluene or benzene pathways. A comparison of the intact cell oxygen consumption with styrene, 140 nmol O_2/min/mg protein, with that for benzene, 40 nmol O_2/min/mg protein (Table 3.7), suggests some enzymes of the pathway may have a higher affinity for styrene than for benzene. These enzyme assays were also not fully optimised.

Figure 6.6 also gives a maximum estimate of the oxygen consumption rate needed to support a doubling time of 10 h. This value, 500 nmol O_2/min/mg protein, compares with the actual measurement of 140 nmol O_2/min/mg protein (Table 3.7), which is rather lower. However, this value was found to vary amongst different fermenter batches.

6.4.3 How does styrene enter the cell?

In order to be oxidised by styrene dioxygenase, the styrene must either enter the membrane or the cytoplasm. Since the styrene dioxygenase has not yet been

Figure 6.6: An approximate calculation relating growth rate on styrene to metabolic capability of the degradative enzymes

Assumptions and constants:

Doubling time on styrene in fermenter is 10 h (Section 3.3.2b)
Mr (styrene, C_8H_8) = 104.14, of which 92 % is carbon
33 % of styrene substrate is completely oxidised, 33 % lost as muconic acid, and 33 % is assimilated.

For complete oxidation of styrene:

$$C_8H_8 + 10O_2 \longrightarrow 8\ CO_2 + 4\ H_2O$$

50 % of cell dry weight is carbon
50 % of cell dry weight is protein

Calculation

If cells double every 10 hours, then 1 mg dry weight (d.w.) of cells produces 1 mg dry weight of cells in 10 h
If 50 % d.w. is carbon, 1 mg d.w. cells makes 0.5 mg new cell C in 10 h
If 33 % styrene is assimilated, 1 mg d.w. cells oxidises 1.5 mg styrene C in 10 h
Since styrene is 92 % carbon, 1 mg d.w. cells oxidises 1.6 mg styrene in 10 h
If 50 % d.w. is cell protein, 1 mg protein oxidises 3.2 mg styrene in 10 h

$\therefore$ 1 mg protein oxidises 0.32 mg styrene per h

$\therefore$ 1 mg protein oxidises 3 µmol styrene per h

$\therefore$ 1 mg protein oxidises 50 nmol styrene/min

$\therefore$ Rate of styrene oxidation must be at least 50 nmol styrene/min/mg protein

10 moles of O_2 are required for the complete oxidation of 1 mole of styrene, so oxygen consumption rate needed for the complete oxidation of styrene is 500 nmol O_2/min/mg protein.

(this ignores the 2 moles O_2 used to produce vinylmuconic acid from 33 % of the styrene, and the oxygen needed to convert the remaining styrene into cell material)

characterised, it is not known whether it is cytoplasmic, membrane-associated or embedded in the membrane. Three methods of moving extracellular styrene into the cell are active transport, facilitated diffusion and passive transport.

a) Active transport

Active transport of substrates into the cell involves the expenditure of energy, frequently using proton symport or antiport mechanisms, taking advantage of the proton gradient across bacterial membranes. *Comamonas testosteroni* T-2 uses a inducible proton symport system to transport 4-toluene sulphonate and 4-chlorobenzene sulphonate (Locher *et al.*, 1993). The coryneform bacterium NTB-1 uses a similar mechanism in the uptake of 4-chlorobenzoate (Groenewegen *et al.*, 1990). These substrates are all lipophobic, because of their ionised groups, and so are rather different from styrene.

b) Facilitated diffusion

Facilitated diffusion involves a carrier molecule within the membrane which is able to transport the substrate from one side of the membrane to the other. There is no energy use in this process, the carrier only enables concentrations inside and outside the membrane to be equalised. This method is, again, only really necessary for lipophobic substrates.

c) Passive transport

Styrene is an aromatic hydrocarbon with no ionised substituents, and so it is very much more soluble in a non-polar environment, such as a lipid membrane, than in a polar environment such as a growth medium or the cell cytoplasm. This means that the cell membrane is likely to naturally concentrate the styrene from the surrounding medium, with no further intervention necessary from the cell. All that is required is that the styrene dioxygenase can remove the styrene from the membrane, or transform it within the membrane.

This mechanism has been proposed for other lipophilic aromatics, for example the breakdown of toluene by *Pseudomonas* sp. strain T2 (Robertson & Button, 1987).

6.4.4 The problems starting growth

One of the problems encountered when growing *R. rhodochrous* 26 on styrene was the frequency with which a flask or fermenter failed to grow. This seems to be a problem with many rhodococci and other Gram-positive bacteria. There are a variety of possible factors which could be causing these problems: the induction of degradative enzymes, the production of extracellular products and changes to the cell ultrastructure.

a) Pathway induction

When a bacterium is introduced to a growth substrate different from the one it has been metabolising, it must immediately induce the degradative pathway for this substrate. This is particularly important if this substrate is provided as the sole carbon and energy source, for instance when *R. rhodochrous* 26 is inoculated into a styrene fermenter or flask. As mentioned earlier (6.1.4), degradative pathways are frequently induced, not by the substrate, but by a product of the pathway's transformation of the substrate. If this is the case, the rapidity of pathway induction will depend on several factors:

1) The residual level of activity of the enzyme(s) involved in transforming the substrate to the inducer. This will depend on how long ago the organism was last exposed to the substrate, as well as any constitutive level of expression (Robertson & Button, 1987).

2) In the case of non-specific pathways like the *cis*-glycol pathway of *R. rhodochrous* 26, the activity of the enzyme(s) involved in producing the inducer from the growth substrate.

3) The specificity of the regulatory protein towards the inducer produced from this particular growth substrate.

It is likely that some growth substrates would result in failure of growth more often than others, both because of different levels of toxicity, and different enzyme and inducer specificities. In the case of *R. rhodochrous* 26, anecdotal evidence from growth

experiments during the present study suggests that growth on toluene is reliable, with growth on benzene and styrene less reliable.

If the pathway is induced by a pathway product, then the cell density of the culture will affect the ease of induction, since more cells with a low level of pathway induction will produce a higher medium concentration of the inducer. For growth in a fermenter on styrene, the *R. rhodochrous* 26 inoculum was grown in a small flask (so a high cell density) on styrene (Methods 2.4.4i), so cells added to the fermenter were already fully induced. Fully induced cells added to the fermenter would produce more of any inducer from styrene than the same number of uninduced cells.

Nishino & Spain (1993) established that *P. putida* JS444 displayed cell-density dependent adaptation to biodegradation of *p*-nitrophenol. These cultures displayed a lag period when exposed to *p*-nitrophenol, and the lag was inversely proportional to cell density. This lag period could be reduced by the addition of hydroquinone, an intermediate in *p*-nitrophenol degradation, though this did not completely abolish the lag, so was probably not the inducer itself. Induction was proposed to occur by accumulation of an inducer in the medium, which accounted for the lag period.

b) Alterations in cell ultrastructure

As styrene is lipid-soluble, it might be expected that its presence in the cell could have effects on the cell membranes. It is also possible that the cells might need to alter their membrane composition in order to cope with this incursion. Delays in induction of these systems, possibly affected by the nature of the previous cultivation of the cells - temperature and medium for example - could result in different toxicity to the cells. Initial attempts at the growth of *R. rhodochrous* 26 on concentrations of styrene above 1 mM have shown that styrene is toxic to this organism (3.3.2).

Other research has indicated that substantial changes do occur in bacteria exposed to lipophilic chemicals. Some of these changes are clearly caused by the incursion of the chemical into the membrane structure, but other effects may be responses from the cell.

Sikkema *et al.* (1992) have investigated the effects of the lipophilic compound tetralin (1,2,3,4-tetrahydronaphthalene) on both artificial and bacterial membranes. In liposomes prepared from *E. coli* phospholipids it was found that the lipid/buffer partition coefficient of tetralin was about 1,100 (by weight), significantly lowering its concentration in the water phase. The accumulation of tetralin in the phospholipid bilayer of these vesicles produced an expansion of the membrane. Beef heart cytochrome *c* oxidase was added into the liposomes to generate a proton motive force, and it was found that tetralin produced a decrease in both the pH gradient and the electrical potential. Intact bacterial cells of several strains were also studied, some of which were able to degrade tetralin, including *Rhodococcus* sp. strain C125 (Introduction, 1.1.7a). These studies show similar effects on the pH gradient and, to a lesser extent, on the electrical potential. The dissipation of membrane gradients was probably due to an increase in the proton permeability of the membrane, the effects of which could considerably reduce growth rates. The concentrations used in these experiments were very low, 200 µmol/l of tetralin (equivalent to 5 µmol tetralin/mg phospholipid at the cell densities used), but still led to a 50 % reduction in the pH gradient of *Rhodococcus* sp. strain C125 cells (Sikkema *et al.*, 1992).

Cell density clearly affects the membrane concentration of tetralin and similar lipophilic substrates and, though bacterial growth rates were initially suppressed by tetralin, growth could recover, presumably due to a dilution of tetralin because of the larger amount of membranes. The presence of an additional carbon source, such as succinate, accelerated this recovery (Sikkema *et al.*, 1992).

Three of the tested bacteria were capable of growth on tetralin, *Acinetobacter* strain T5, *Arthrobacter* strain T2 and *Rhodococcus* sp. strain 125, and the latter two organisms caused emulsification of the growth medium. *Arthrobacter* strain T2 was less susceptible to tetralin than other bacteria tested, possibly because of this emulsification.

Some bacteria are able to survive in the presence of high concentrations of aromatic compounds, for example *P. putida* Idaho, which can grow on *p*-xylene at concentrations of up to 50 % (vol/vol) (Cruden *et al.*, 1992). Even in this organism, there is substantial

cell death when first exposed to the substrate, followed by exponential growth. Stationary phase was characterised by an increase in cell dry weight and turbidity, while cell viability dramatically decreased. Cruden *et al.* (1992) suggest that the bacterium may survive either by producing large amounts of membranes to replace those damaged by solvents or by having some difference in the membrane making it more resistant. During growth on *p*-xylene the outer membrane becomes convoluted and fragments are shed into the medium, the cytoplasmic membrane invaginates, forming vesicles and electron-dense intracellular inclusions form, which are not present in cells grown on succinate. Similar effects have been observed in *R. rhodochrous* and *R. ruber* growing on gaseous *n*-alkanes (Glazacheva *et al.*, 1990). The bacteria displayed an increase in membranes, fragmentation of the cytoplasm and the formation of inclusions.

c) Production of extracellular components

Some bacteria are known to excrete extracellular components such as emulsifiers when they are exposed to certain compounds. This property is well established among the rhodococci, notably among the alkane degraders (Introduction, 1.1.6a). As mentioned in the previous section, emulsifiers may reduce the toxicity of lipophilic substrates by retaining more of the substrate in solution rather than dissolved in the membrane. They also increase the solubility of insoluble hydrocarbons, avoiding two-phase systems which are also damaging to bacteria.

There is no evidence whether or not *R. rhodochrous* 26 produces emulsifiers. If emulsifiers are necessary for growth on styrene, then induction of their production would need to be rapid on introduction to a styrene medium and their production would be expected to be cell density dependant. The effects of different inocula would be similar to those described above for the induction of pathway enzymes.

6.5 Future work

Since this study is the first on *R. rhodochrous* 26, there are many different avenues that would be useful to study. Some of these are described below:

6.5.1 Upper pathway

There are many issues to be resolved in the biochemistry and regulation of the upper pathway. If benzoic acid is metabolised through a *cis*-glycol, then one study would be to purify and characterise the *cis*-diol dehydrogenase associated with this pathway and compare it with the styrene *cis*-glycol dehydrogenase also present. In addition to purifying these enzymes (assuming that there is not just one diol dehydrogenase), sequencing them would enable comparisons to be made between putative substrate binding regions. In addition, sequencing could probably enable the genes for the dioxygenase activities to be found, since based on previous systems the dioxygenase is probably encoded within the same operon. An attempt could also be made at purifying the benzoate 1,2-dioxygenase, since its activity might be more stable than the styrene dioxygenase. A comparison of the sequences of the two enzymes from the two pathways could give a great deal of evolutionary information.

The regulation of the styrene dioxygenase pathway could be studied further, with the simplest initial test being whether toluene or styrene *cis*-glycol is able to induce the pathway. Sequencing of the styrene and benzyl alcohol/benzoate metabolising operons could also produce further information on the regulation of these two pathways, as a regulator protein may be encoded within each of the operons.

6.5.2 Lower pathway, *meta* route

Further studies on the 3-vinylcatechol 2,3-oxygenase could include purification, and determination of its substrate range. The gene could also be cloned, and compared with the substantial number of 2,3-oxygenases which have been sequenced already.

It is not clear how acrylic acid is metabolised by *R. rhodochrous* 26; the isolation of intermediates might help to discover its breakdown route. It might also be possible to detect some enzyme activities in extracts.

The other *meta* cleavage pathway enzymes could also be purified and characterised. If the catechol 2,3-oxygenase is present at the start of an operon encoding these

enzymes, then cloning the oxygenase gene could give the rest of the enzymes in the pathway.

6.5.3 Lower pathway, *ortho* route

Several catechol 1,2-oxygenases have already been purified, characterised, cloned and sequenced. The enzyme from *R. rhodochrous* 26 could be compared with these enzymes, providing information on possible evolutionary relationships between this enzyme and others. As mentioned with the catechol 2,3-oxygenase activity, it is possible that the catechol 1,2-oxygenase gene could be followed by genes for the rest of the *ortho* pathway. In view of the inactivity of this pathway, it would be interesting if genes encoding the enzymes of it were still present.

The environmental fate of vinylmuconic acid could also be investigated, possibly using an organism known to contain a modified *ortho* pathway, such as *R. rhodochrous* N75 (Introduction, 1.1.7b).

It would also be useful to see if it is possible to introduce an active *ortho* cleavage pathway into strain 26. Adams *et al.* (1992) used a continuous culture mechanism to create a 3-chlorobiphenyl-utilising bacterium. Two bacteria were grown within this system, *Pseudomonas* sp. strain HF1, which could grow on 3-chlorobenzoate, and *Acinetobacter* sp. strain P6, which could grow on biphenyl. Exchange of DNA between these species led to the creation of *Pseudomonas* sp. strain CB15, which was able to utilise 3-chlorobiphenyl, unlike either of the parent strains. It would be interesting to see if this method could work with *R. rhodochrous* 26, maybe using *R. rhodochrous* N75 (Introduction 1.1.7b), possibly with the help of a halogenated aromatic in the selection medium to inactivate the *meta* cleavage pathway.

'Natural' gene transfers of this kind are preferable to gene cloning because of the fact that the organism created is still considered to be natural, since it would have been created by natural processes of bacterial gene transfer, rather than by gene cloning. In the context of release to the environment, the regulatory requirements for man made organisms are, rightly, very costly and time consuming. The advantages of natural

selection and recombination are therefore very substantial if any environmental release is possible.

6.5.4 General research

Some particular areas of general research into *R. rhodochrous* 26 are:

1) How effectively can it degrade mixtures of aromatics and other substrates? Many actual biological cleanup applications require organisms to degrade a wide variety of substrates simultaneously.

2) Does *R. rhodochrous* 26 produce any extracellular components such as emulsifiers?

3) How well can *R. rhodochrous* 26 survive in a natural environment? How well does it cope with starvation?

6.6 Possible applications

6.6.1 Pollutant treatment

One application of the metabolic capability of *R. rhodochrous* 26 could be the removal of aromatics and other chemicals from land, wastewater or air. The wide range of substrates oxidised by *R. rhodochrous* 26, together with the persistence in the environment known for other rhodococci (Introduction, 1.1.3), suggest that this strain could make a useful contribution to a mixed culture degrading pollutants.

One of the main problems with the use of *R. rhodochrous* to remove aromatics might be the accumulation of the muconic acid. One method of avoiding this would to mutate the organism so as to prevent the expression of catechol 1,2-oxygenase activity. Alternatively, strain 26 could be used in mixed culture along with a bacterium that could metabolise muconic acids. The most elegant solution would be that proposed above (6.5.3), with a full *ortho* cleavage pathway naturally transferred to the organism.

6.6.2 The production of *cis*-glycols

An aromatic *cis*-glycol is an unusual compound, which is optically pure and can have a variety of functional groups. The production of *cis*-glycols by chemical means is virtually impossible, so there is much interest in the use of bacteria to produce these compounds. Mutant bacterial strains such as *P. putida* 39D, lacking *cis*-glycol dehydrogenase activity, have already been used to synthesise unusual compounds. One example of the use of this organism is the production of (–)-zeylena, a novel hydrocarbon present in the roots of *Uvaria zeylanica*, using styrene as a starting material (Figure 6.7a, Hudlicky *et al.*, 1989).

R. rhodochrous 26 has a dioxygenase activity producing a *cis*-glycol, and mutants could presumably be produced which did not express dihydrodiol dehydrogenase activity. The resulting mutant could produce *cis*-glycols, possibly with a different substrate range to the presently characterised mutants, and also maybe with different physiological requirements, which might make it a better organism than the existing ones.

6.6.3 The production of novel compounds

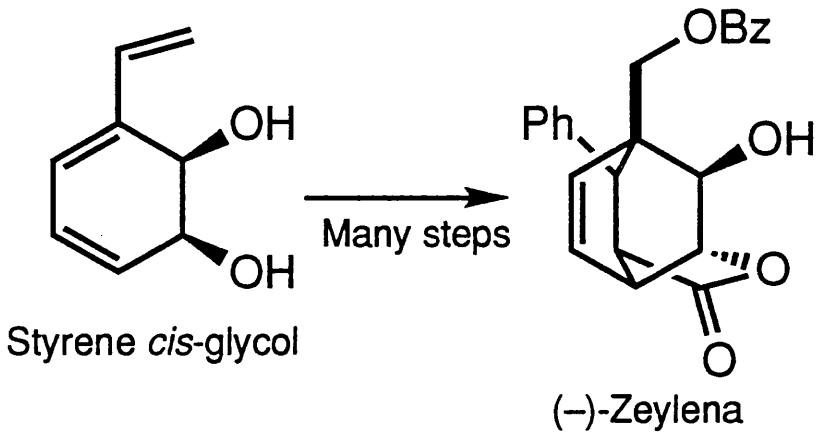
R. rhodochrous 26 is able to accumulate substituted muconic acids from substrates including styrene, toluene, ethylbenzene and 3-fluorocatechol (the structure of vinylmuconic acid is given in Figure 6.7b). It is always possible that there might be a synthetic application for one of these chemicals. These chemicals could be produced continuously from cells which were also using a percentage of the transformation substrate to grow on.

6.7 Conclusions

This is the first organism proven to metabolise styrene through a *cis*-glycol route during growth on styrene. Other organisms can metabolise styrene to vinylcatechol, and other workers have found evidence of aromatic ring attack in the breakdown of styrene.

Figure 6.7: Possible applications of biotransformations catalysed by *R. rhodochrous* 26

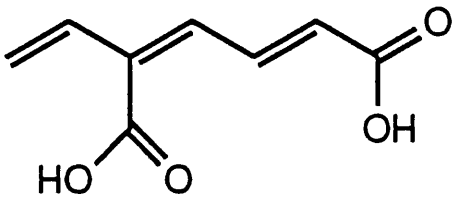
a) The production of (–)-zeylena from styrene *cis*-glycol by *P. putida* 39D (Hudlicky *et al.*, 1989)



Styrene *cis*-glycol

(–)-Zeylena

b) Another look at the structure of 2-vinylmuconic acid



It is possible that other characterised organisms with a toluene *cis*-glycol pathway could degrade styrene, but this has not yet been proven.

The *meta* cleavage of vinylcatechol proceeds through the well established pathway. However, the *ortho* cleavage pathway appears to be incomplete, with only the first enzyme, catechol 1,2-oxygenase, active. This results in the accumulation of vinylmuconic acid.

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