THE BIODEGRADATION OF PHENOLS
# BY A EUKARYOTIC ALGA

BY

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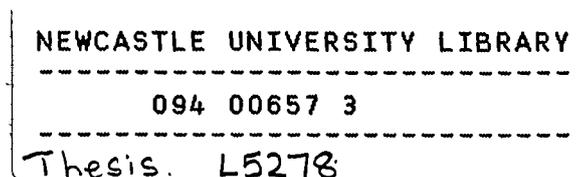
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To my Family

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The University of Newcastle upon Tyne

Biodegradation of phenols by a eukaryotic alga

Kirk T. Semple

Axenic cultures of *Chlamydomonas ulvaensis* [CCAP 11/58], *Scenedesmus brasiliensis* [CCAP 276/1B] and *Ochromonas danica* [CCAP933/28] were screened for their ability to grow on and remove phenol from their incubation media. Neither *C. ulvaensis* nor *S. brasiliensis* removed the phenol substrate as rapidly as *O. danica* which is a nutritionally versatile chrysophyte. *O. danica* was found to grow on phenol and *p*-cresol as the sole C-source at concentrations up to 4mM in cultures grown in both photoheterotrophic and heterotrophic conditions. The alga would not grow on cresols or xylenols unless phenol was present.

Oxygen uptake and turnover studies confirmed that the enzymes involved in phenolic catabolism were inducible and that the organism showed a decrease in activity resulting from the position and number of the methyl substituents on the aromatic ring.

[*U*-^{14}C]Phenol was completely mineralised with some 65% of the ^{14}C-label appearing as ^{14}CO$_2$, approximately 12% remaining in the aqueous medium and the rest accounted for in the biomass. Analysis of the biomass showed that ^{14}C-label had been incorporated into the protein, nucleic acid and lipid fractions; phenol carbon is thus unequivocally assimilated by the alga.

Phenol-grown cultures of *O. danica* converted phenol to catechol which was further metabolised by the *meta* cleavage pathway. 2-Hydroxymuconic semialdehyde and pyruvate, characteristic products of *meta* cleavage, were found in incubations of catechol with cell-free extracts of phenol-grown cells together with the appropriate enzyme activities. This is , as far as I am aware, the first definitive identification of the *meta* cleavage pathway for aromatic ring degradation in a eukaryotic microorganism.

1. INTRODUCTION

1:1   Xenobiotics in the Environment.

Before the beginning of man's industrial activities, concentrations of organic

compounds on the surface of the planet remained fairly constant with biosynthesis

and biodegradation kept in equilibrium by the activities of animals, plants and

microorganisms. With the escalation of industrial processes, however, there has

been a vast increase in the amount of potentially toxic, carcinogenic and/or

mutagenic compounds released into the environment. Natural ecosystems are thus

faced with the problem of dealing with compounds which either do not fit naturally

into the global cycles of carbon, nitrogen, sulphur or other elements, or have

deleterious effects on the environment. In relation to the total volume of organic

compounds involved in the carbon cycle, the formation of anthropogenic organic

compounds is modest. Total turnover of organic matter in the carbon cycle by

photosynthesis and biodegradation is approximately 2×10^{11} tons per year. Total

oil production is estimated at approximately 3×10^9 tons per year; and total

production of synthetic chemicals, pollutants and others equals about 2×10^8 tons

per year (Leisinger, 1983). Thus just over 1% of organic material is derived from

Man's activities.

Environmental pollutants, whether they are chemicals of natural or synthetic

origin, are released either by man's activities into the environment or on man by

bioaccumulation from environmental sources. Such organic compounds are

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released into the environment by four major routes (Leisinger, 1983). The first

results from the direct use of specific products, e.g. aerosol propellants. The

second route is through the release of recalcitrant effluents present in municipal

sewage treatment systems, e.g. solvents, hard detergents. The penultimate

pathway occurs with chemicals resistant to biological attack in industrial waste

treatment systems e.g. polychlorinated aromatic compounds. The final route is by

direct discharge, deliberate or by accidental spillage, leading to the entry of

chemicals into the environment.

Chemicals exhibiting transitory or prolonged accumulation and which express

undesirable effects on the environment are termed pollutants. To assess a

compound's pollution potential, three major factors have to be considered in relation

to its putative removal from the environment:

Chemical structure: Mineralisation of organic compounds is primarily due to

microorganisms. In their evolution, microorganisms have been exposed to a wide

range of compounds, and over time, have evolved the enzymic apparatus

necessary to degrade a wide variety of compounds. Man-made compounds are

more likely to be susceptible to microbial degradation if their chemical structures

are similar to those of naturally occurring (biogenic) compounds. Consequently,

structural dissimilarities may lead to retardation of microbial attack and, thus, to

their persistence and accumulation.

Concentration: Hutzinger (1981) stated that the term 'xenobiotic compound' should

not be reserved only for chemicals with structural features foreign to life, but should

be used for 'all compounds that are released in any compartment of the

environment by the action of man and thereby occur in a concentration in this or in

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another compartment of the environment that is higher than 'natural". This definition can of course be extended to include the release of compounds which are liberated through the action of natural processes, also.

Toxicity: Because there are over 65,000 chemicals in everyday use, it is impossible to monitor them all. But it is important to identify and control potentially hazardous compounds. This involves the amalgamation of a broad range of disciplines including toxicology, epidemiology, analytical chemistry, microbiology and ecology; toxicity, mutagenicity and carcinogenicity being the most important criteria in evaluating the harmful effects of the pollutant. In the early 1970's, millions of pounds of potentially toxic compounds were discharged in treated or partially-treated industrial effluents (Guthrie and Davis, 1985). In response to this, a list of 65 classes of toxic compound emanating from 21 different industrial categories was drawn up by the USEPA in 1976; by 1979 there were 34 different industrial categories. The 65 classes of toxic compound contained 129 chemicals of which 114 were organic.

1:2 Phenols as Sources of Pollution.

Phenol and its derivatives are widely used in the chemical industry to manufacture synthetic resins and plastics, plasticizers, disinfectants, wood preservatives, insecticides, herbicides, fungicides, dyes, antioxidants, surface active agents, medicinals, flavours and perfumes. Phenols are also important in explosives and photographic chemicals.

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Major producers of effluents containing phenols are the coal carbonisation, petroleum and petrochemical industries. In the U.S.A., the principle source of phenol is benzene obtained from the afore mentioned industries, with cresols and xylenols coming directly from high temperature carbonization of coal (Beychok, 1967). Phenols have also been reported in effluents from dyestuffs, wood distillation plants, and the paper making industry. Now, nearly all phenol is synthesised. One process uses the fusion of sodium benzenesulphonate with alkali (Figure 1.1); another is the Dow process in which chlorobenzene is allowed to react with aqueous sodium hydroxide at a temperature of 360°C (Figure 1.2). Nearly all phenol is made at present, however, by a newer process that starts with cumene (isopropylbenzene). Cumene is converted by air oxidation into cumene hydroperoxide, which is converted by aqueous acid into phenol and acetone (Figure 1.3).

Coal carbonisation produces approximately $0.23m^3$ of contaminated water per tonne of coal processed. In the early 1970's when the United Kingdom was at the height of its coal production, 30 million tonnes of coal were carbonised per annum (Catchpole and Stafford, 1977). Wheatstone (1977) and Catchpole and Stafford (1977) showed that the large quantities of effluents from coking plants contained phenol, catechol, resorcinol, *m-*, *o-*, and *p*-cresols, all six isomers of xylenol, three ethylphenols and various other alkyl-substituted diphenols. Effluents produced from the coal gasification process were found to contain high concentrations of phenol (up to $3.8g.l^{-1}$), with ammoniacal liquor from the coke ovens containing up to a further $2.3g.l^{-1}$ of phenol (Wheatstone, 1977).
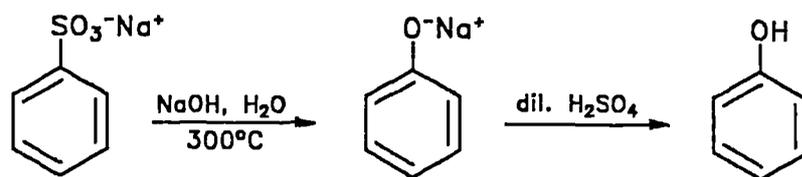
Figure 1.1: Production of Phenol from Benzenesulphonate.
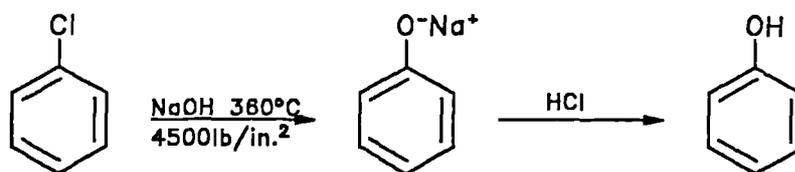
Figure 1.2: Production of Phenol from Chlorobenzene.
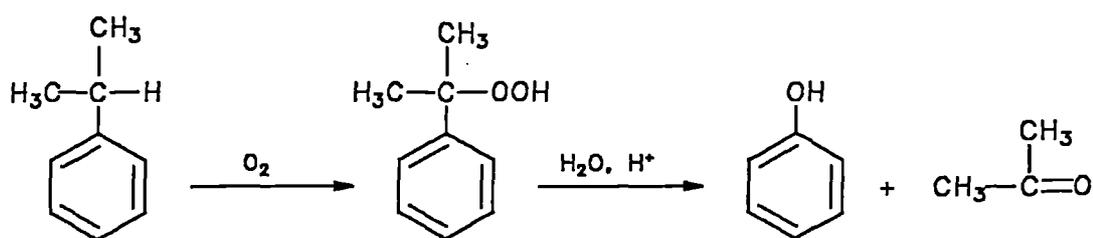
Figure 1.3: Production of Phenol and Acetone from Cumene.

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One set of analyses of coke-oven ammoniacal liquors showed (range, $mg.l^{-1}$):

phenol (714-886); o-cresol (13-78); m-cresol (123-194); p-cresol (63-114); 2,3-

xylenol (1-9); 2,4- and 3,5-xylenols (13-48); 2,5-xylenol (7-11); 2,6-xylenol (3-4);

3,4-xylenol (4-11); catechol (2-14) (Wheatstone, 1977; Figure 1.4).

Before discharge into the environment, most phenolic effluents are treated by

aerobic biological processes involving activated sludge, oxidation in waste

stabilisation and high rate algal ponds, in trickling filters, or in reed bed systems,

often with efficiencies as high as 90-95% reduction in Biological Oxygen Demand

and phenolic content (Ashmore, et al., 1970; McCarty, 1987; Fallowfield et al.,

1992). However, where the concentration of phenols from ammoniacal liquor, in

oxidation lagoons exceeded $1g.l^{-1}$, the lagoon was found to be entirely free of

microbial life (Stafford and Callely, 1973), which suggests that passive methods of

purification are not effective against such highly concentrated effluents and risk

percolation into natural waters.

Petroleum refining and petrochemical industries are another main source of

phenolic pollutants. Catalytic "cracking" of hydrocarbons can produce aqueous

scrubbing effluents containing a broad spectrum of phenols, the concentration of

which may be as high as $8g.l^{-1}$. The content of the effluent is very similar to those

found in coal-carbonisation and gasification effluents, but most phenols from this

process are reclaimed. McKinney (1963) calculated, from a survey of twelve

refineries in the United States, that the average effluent production was $5000l.min^{-1}$

which contained $27mg.l^{-1}$ of total phenols. After treating the effluent with gravity

separators, digestion and oxidation ponds, the concentration of the phenols was

reduced to the order of $10mg.l^{-1}$. Davis
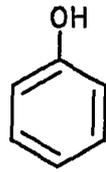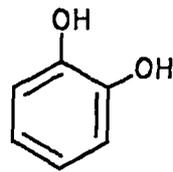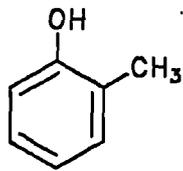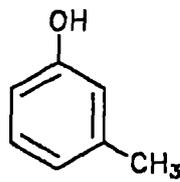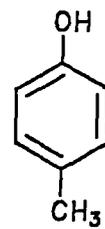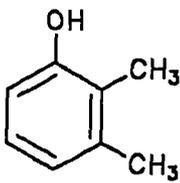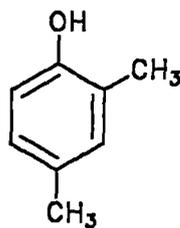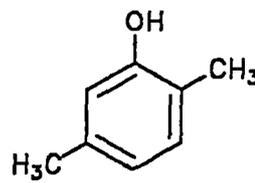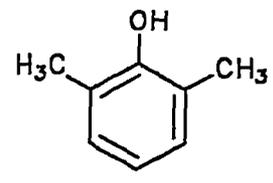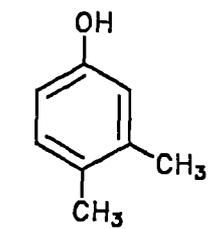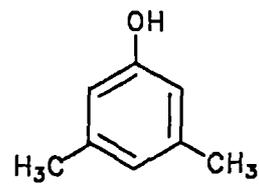
Figure 1.4: Phenols in the Coal Gasification Ammoniacal Liquor.

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(1967) described how aqueous effluent, from the petroleum industry, was treated to reduce the phenolic content by as much as 99.5%.

It appears that the major phenol-producing industrial processes discharge large volumes of low-level wastes, which may still be toxic, and are highly undesirable in sources used for potable water. Phenols can be tasted in water at levels of 50-100μg.l^{-1}, and if chlorinated, at about 5μg.l^{-1} (Beychok, 1967). The World Health Organisation (1961, 1963) and the United States Public Health Service (1963) suggested that the phenolic concentration in drinking water should, at most, be 1-2μg.l^{-1}.

Natural waters generally contain low phenol concentrations; in two examples from Essex the River Mole contains less than 3ng.l^{-1} and the River Cherwell contains 3μg.l^{-1} (Water Research Centre, 1980). However, severe phenol pollution is occasionally recorded, particularly in the leachate from landfill sites which contained 2-6mg.l^{-1} of phenol, with other phenols such as catechol, quinol and resorcinol being detected at similar concentrations (Water Research Centre, 1980).

A wide variety of phenols are found in nature as minor constituents of essential and ethereal oils: eugenol, *oil of cloves*; isoeugenol, *oil of nutmeg*; anethole, *oil of aniseed*; vanillin, *oil of vanilla bean*; thymol, *oil of thyme and mint*; and safrole, *oil of sassafras*. Many phenolics ranging from simple monocyclics to polycyclic materials such as flavanoids, alkaloids, coumarins, tannins and lignins, with the phenolic groups being free acids or bonded in ester, ether or acetal linkages, are also found in plants. Phenols are found in animals, being involved in amino acid and hormone syntheses. Phenols may also arise as intermediates in catabolic pathways by direct hydroxylation of benzenoid compounds or released

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from the breakdown of hormones such as thyroxin in mammalian systems.

1:2:1 *Toxicity of Phenol.*

The germicidal efficacy of phenol was dramatically demonstrated by Lister in 1867. Phenol exerts its bactericidal and fungicidal action by denaturing protein and destroying membranes. For this basic reason phenol solutions have been used extensively as antiseptics, and have thus found their way into the environment. Together with phenolic effluents from industrial and natural sources, they can generate problems in the aquatic environment. The toxicity of phenol increases with the number and type of substituents on the aromatic ring; cresols and xylenols become more lipophilic than phenol interfering with the integrity and function of cell membranes and the uptake of phosphate (Glass, 1973; 1975). Uncoupling of the oxidative phosphorylation mechanism can occur which may result in an increase in oxygen consumption in the presence of phenols (van Sumere *et al.*, 1972; Demos *et al.*, 1975). Phenols can also inhibit enzymes involved in protein synthesis, RNA metabolism and sugar transport (van Sumere *et al.*, 1972). Williams *et al.* (1984) carried out 96h LC_{50} tests for phenol (mg.l^{-1}) on a variety of freshwater invertebrates: worm, *Limnodrilus hoffmeisteri* (780); snail, *Physa fontinalis* (70); shrimp, *Gammarus pulex* (69); hoglouse, *Asellus aquaticus* (180); Mayfly, *Baetis rhodani* (15.5); and midge larva, *Chironomus riparius* (240). Milleman *et al.* (1984) determined the toxicities of various oil derivatives, including two phenols, for several algae, invertebrates and fish. In this study, phenol and β-naphthol were compared

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and it was found that the dicyclic phenol was up to forty-five times more toxic, especially to the macroinvertebrates.

There are a number of factors affecting the toxicity of phenols, the most important of which is the way environmental influences may modify the toxic effects. Temperature is important because it not only influences the metabolic activity and behaviour of organisms, which may effect their exposure to a pollutant, but it may also alter the physical and chemical state of the pollutant. The effects of temperature on toxicity have been reviewed by Cairns *et al.* (1975). In general, toxicity increases with temperature, as is the case with phenol causing paralysis and cardiovascular congestion which leads to suffocation in fresh-water fish (Smith and Heath, 1979). There are, however, many exceptions to an increase in toxicity with temperature. Brown *et al.* (1967) observed that the time of death of rainbow trout increased as temperature increased, but the LC_{50} decreased. The internal concentration of phenol is influenced by the relative rates of absorption and detoxification, both of which are directly proportional to temperature, but it is considered that temperature influences the rate of detoxification to a greater extent than the rate of absorption, at least at lower temperatures. Phenol, therefore, probably accumulates to higher levels at low temperatures, accounting for greater toxicity in the cold (Mason, 1981).

With such an array of natural and synthetic phenols in the soil and water - resulting from discharge of industrial waste and/or from natural decomposition - it is vital that there is a greater understanding of the demands and the limitations of the processes involved in biodegradation.

1:3   The Fate of Xenobiotics in the Environment.

When xenobiotics are released into the environment, initially one of two

things happens; either the chemical(s) disappear or they remain in the environment.

There are two basic processes involved in the removal of the pollutant: chemical

degradation or biological (microbial) transformation. Microbial transformation can

occur in one of five ways which include co-metabolic degradation, detoxification,

polymerisation and binding to naturally-occurring compounds, accumulation on or

within microorganisms and mineralisation. It is the actions of microorganisms on

organic compounds which will be discussed, with particular reference to

mineralisation.

Although many aromatic compounds are subjected to microbial

biogedradation, the introduction of certain kinds of substituents into the molecules

results in an increased resistance of the benzene ring to biodegradation (Alexander

and Lustigman, 1966). The resulting compounds frequently fail to support the

proliferation of microorganisms, yet they are metabolised to the same extent

(Hovarth and Alexander, 1970). This apparently widespread phenomenon, termed

co-metabolism, appears to account for the microbial transformation of many

xenobiotics in nature. These compounds do not supply the responsible populations

with a source of energy, carbon, nitrogen, or phosphorus (Alexander, 1965;

Horvath, 1970). More recently, Kohler et al. (1988) studying Acinetobacter sp.

strain P6 showed the cometabolism of polychlorinated biphenols while using

biphenyl as the substrate. The ability of this microorganism to transfom the

components of 25 of the 40 largest peaks of Arochlor 1254 makes it one the most

versatile PCB-transforming organisms yet reported.

Microorganisms are capable of *detoxifying* compounds which they cannot

mineralise. Detoxification in phenols involves a chemical change to the molecule,

but the carbon skeleton remains unaltered. One of the most common mechanisms

is O-methylation, which involves the conversion of chlorinated phenols, guaiacols,

syringols and hydroquinones (Allard *et al.*, 1985, 1987; Häggblom *et al.*, 1988;

Neilson *et al.*, 1984). The resulting methoxybenzenes may resist aerobic attack

(Allard *et al.*, 1985, 1987) and have a high potential for bioaccumulation. O-

Methylation of chlorophenols increases their lipophilicity and these compounds also

have a higher potential to bioaccumulate. The formation and subsequent

bioaccumulation of such chloroanisoles in fish has for example been observed after

an accidental discharge of a chlorophenol wood-preserving solution into a river-lake

ecosystem (Renberg *et al.*, 1983). O-Methylation of chlorophenols into anisoles

and guaiacols into veratroles have been reported for several bacteria (Allard *et al.*,

1985, 1987) and fungi (Cserjesi and Johnson, 1972; Gee and Peel, 1974) and may

thus be a significant alternative to degradation.

Polymerisation is another important biotransformation carried out by

microorganisms which involves the oxidative coupling of phenols leading to the

formation of polymers (Häggblom, 1992). This process is catalysed by phenol

oxidases or peroxidases in which phenols are oxidised to reactive phenoxy-radicals

that then react by self-coupling with other molecules (Bollag and Liu, 1985).

Chlorophenols may also cross-couple with other phenolics, which may explain how

humus-bound pesticide residues are formed in soil (Häggblom, 1992). Laccases

and peroxidases have been evaluated for their possible use to polymerise and

detoxify phenolic contaminants (Bollag *et al.*, 1988; Shannon and Bartha, 1988; Dec and Bollag, 1990). However, the process of biotransformation can occasionally result in the production of a metabolite more toxic than the parent compound: eg, the lignin peroxidase from *Phanerochaete chrysosporium* was found to produce a toxic product 3,3',4,4'-tetrachloroazobenzene from the dimerisation of 3,4-dichloroaniline (Pieper *et al.*, 1992).

When an organic compound is described as being "biodegradable", this is generally understood to mean that it is capable of being decomposed by microorganisms or other biological agencies. The process of biodegradation results ideally in the biotransformation or *mineralisation* of the compound resulting in the original characteristic properties of the chemical compound being completely lost or changed (Guthrie and Davis, 1985). Mineralisation of a compound involves the utilisation of the chemical as a source of energy or nutrient (Bull, 1980).

Biodegradative processes may be carried out by a single species in pure culture or, more often, may require the concerted efforts of a considerable mixture of organisms in the transforming population (Slater and Bull, 1982). It may also occur in either oxic or anoxic environments, depending upon the populations of degrading organisms, the structure of the compound(s) and the co-existence of methanogenic consortia.

Ultimate biodegradation was defined by Scow (1982) to be the mineralisation of the compound; *acceptable biodegradation* implies breakdown to the extent that the products are minimally toxic or below toxic concentrations; *primary biodegradation* may involve only a minor structural change in the parent molecule as long as its physical or chemical properties have been altered. The

environments in which a certain compound(s) is to be degraded are sometimes

considered severe, and include extremes of temperature, pH, salinity and ionic

composition, low dissolved oxygen and Eh values, inadequate nutrient

concentrations, variability of the supplementary organic content, presence of toxic

substances and the presence of recalcitrant anthropogenic compounds

(Kobayashi,1984).

1:4 Biodegradation of Aromatic Compounds.

One of the most widely distributed structural units in nature is the benzene

nucleus. Aromatic compounds are those with delocalised π electron systems with

benzene being the parent structure. Benzene and its derivatives take the form of a

ring structure which was first proposed by August Kekulé in 1865 and is known as

the Kekulé structure. This structure is extremely stable because of the

delocalisation of the six π electrons and, therefore, to degrade this compound and

its derivatives requires energetically powerful and specialised forms of attack. This

process of attacking and degrading the aromatic nucleus in Nature is achieved

almost exclusively by microorganisms utilising one of two enzymic processes; either

aerobically using oxidative mechanisms or anaerobically employing reductive

mechanisms.

Before going on to discuss aerobic degradation of aromatics, it would be

pertinent to briefly discuss the anaerobic mechanisms involved in the catabolism of

the aromatic nucleus. Higher plants and the vast majority of algae photosynthesise

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to reduce carbon dioxide as a source of carbon and energy. This same reducing power could be used to catabolise the aromatic nucleus. A variety of anoxic environments exist in Nature: the intestinal tract of animals, soils, marine sediments, bodies of water overloaded with organic matter and sewage treatment plants. In all these systems a whole host of both facultative and obligate anaerobic microorganisms are to be found.

The realisation that microorganisms could metabolise the aromatic nucleus (generally benzoate) occurred approximately 25 years ago. This was found to happen under a number of anaerobic conditions such as nitrate reduction (Taylor et al., 1970; Williams and Evans, 1975), methanogenesis (Ferry and Wolfe, 1976) and by photosynthetic bacteria (Dutton and Evans, 1969). Characterisation of the anaerobic metabolic pathway by various investigators (Guyer and Hegeman, 1969; Dutton and Evans, 1969; Williams and Evans, 1975; Ferry and Wolfe, 1976) was summarised in a review article by Evans (1977) in which he outlines the similarities among the probable pathways of benzoate degradation. Basically, the benzene ring is reduced to cyclohexane and cleaved with the aliphatic intermediates being catabolised to carbon dioxide and methane.

Phenol and catechol were demonstrated to be stoichiometrically metabolised to methane and carbon dioxide (Healy and Young, 1979) and an anaerobic pathway was proposed for their metabolism and ring fission by Balba and Evans (1980). More recently, the degradation of several substituted phenols (Boyd et al., 1983) and chlorinated phenols (Boyd and Shelton, 1984) has been reported. The mechanisms for phenol and catechol catabolism involve catechol being converted to phenol which is then reduced to cyclohexanol. The alicyclic alcohol is then

converted to cyclohexanone which is catabolised to adipate and then to carbon dioxide and methane (Young and Rivera, 1985).

The most important process in the degradation of the aromatic nucleus is the incorporation of oxygen into the molecule, which is achieved enzymically by oxygenases. The importance of oxygenases and thus "oxygen fixation" in the environmental fate of organic molecules cannot be over-emphasised because microbial oxygenases have been shown to participate in the metabolism of many natural and xenobiotic compounds (Dagley 1977; 1978; 1981). Dagley suggested that this type of reaction, frequently encountered in biodegradation, involves the incorporation of one or both atoms of molecular oxygen into an organic molecule. This does not happen spontaneously - the large quantities of both molecular oxygen and organic matter around us are evidence of that. However, if oxygen is combined with the iron-centre of an iron-containing enzyme, it becomes 'activated' and therefore capable of being incorporated into an organic molecule. The frequency with which this reaction takes place is reflected in the wide range of specific oxygenases found in bacteria. Since biological oxygenation was discovered, many similar reactions have been described and analysed; the first, described by Mason et al. (1955) for a fungal hydroxylase (Figure 1.5), showed the incorporation of one atom of $^{18}O_2$ into 3,4-xylenol to give 4,5-dimethyl benzoquinone with the other atom being reduced to water. Shortly afterwards Hayaishi and his collaborators (1955) (Figure 1.6) showed that the intradiol cleavage of catechol by the bacterial enzyme pyrocatechase (catechol 1,2-dioxygenase) involved the utilisation of both atoms of molecular oxygen, with no contribution from the oxygen of water. Ring-openings characteristically consume two atoms of oxygen, both of

which appear in the product. Two subclasses of ring-cleaving oxygenases have been defined (Hayaishi and Nozaki, 1969): monooxygenases and dioxygenases. Monooxygenases are responsible for the incorporation of a single atom of molecular oxygen into the substrate in the form of a hydroxyl group (Figure 1.7). Dioxygenases catalyse the incorporation of both atoms of molecular oxygen into the substrate, in the form of two hydroxyl groups if it is a ring hydroxylating dioxygenase or, in the case of ring-cleaving dioxygenases are responsible for the scission of an aromatic double bond (Figure 1.7).
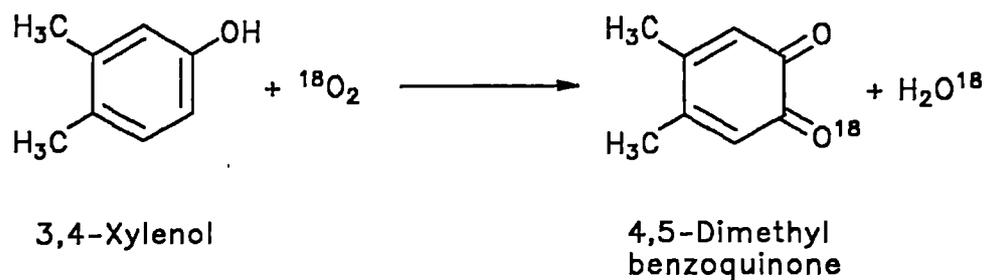
Figure 1.5:   Fungal Hydroxylation of 3,4-Xylenol.
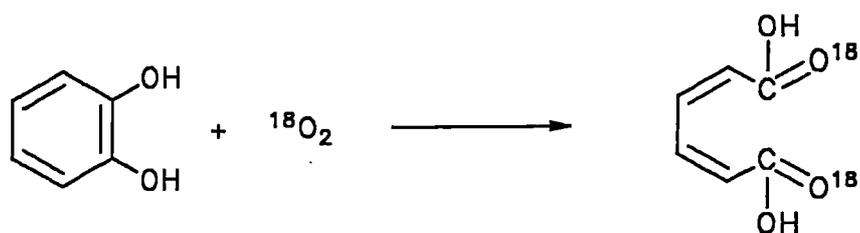
Figure 1.6:   Bacterial Oxidation of Catechol.

1:4:1 *Microbial Degradation of Aromatic Compounds.*

Numerous studies have shown that both bacteria and fungi possess the ability to modify and degrade aromatic compounds, and this body of information is used as a source of reference when considering aromatic degradation in other organisms. The unsubstituted aromatic ring, and, to a lesser extent, alkyl-substituted aromatic structures, are stable resonance forms, inert to attack by fission-catalysing enzymes (Dagley, 1971). Phenols are likewise electronically unsuitable for most enzymically catalysed fission reactions.

The essential first step in aromatic metabolism is thus the generation of a dihydroxylated aromatic compound which can occur in two ways (Figure 1.7). Dihydroxylation is a form of hydroxylation which occurs prior to ring fission carried out by dioxygenase enzymes incorporating both atoms of molecular oxygen simultaneously into the aromatic nucleus of the organic substrate to give two hydroxyl molecules in the *ortho*-position thus forming the appropriate catechol. Benzene, for instance, is converted by a ring hydroxylating dioxygenase to benzene *cis*-dihydrodiol; dehydrogenation of this yields catechol. Phenol is attacked by a phenol hydroxylase (monooxygenase) which introduces a second hydroxyl group into the *ortho*-position in the substrate molecule. Aromatic hydroxylases have been found in both bacteria (phenol hydroxylase, [Ribbons, 1970; Shingler, *et al.*, 1989]; orcinol hydroxylase, [Ohta and Ribbons, 1970; Ohta *et al.*, 1975]; salicylate hydroxylase, [Takemori *et al.*, 1969; White-Stevens and Kamin, 1972]) and yeast (phenol hydroxylase, [Neujahr and Gaal, 1973; Kalin, *et al.*, 1992]). The bacterial enzymes
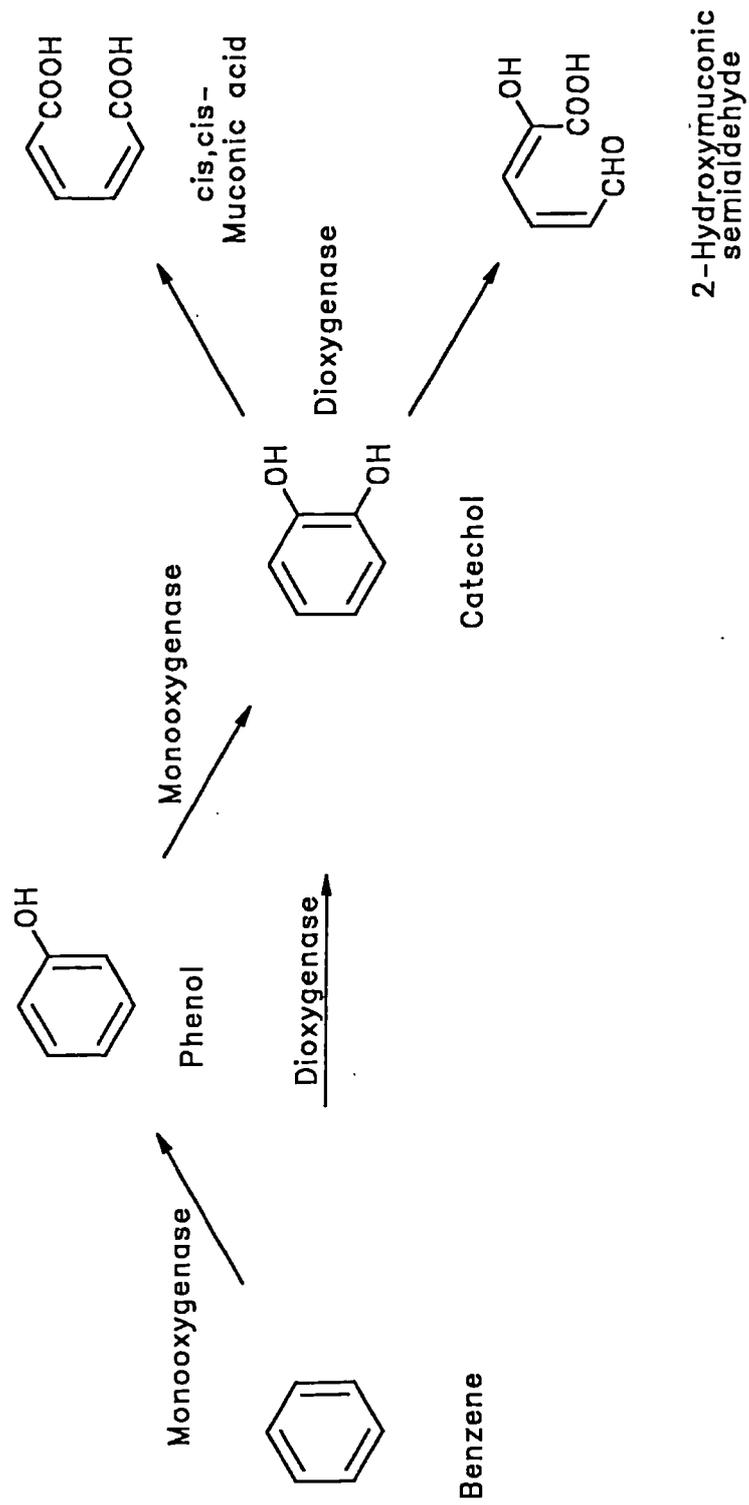
Figure 1.7: Reactions involving Monooxygenases and Dioxygenases.

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responsible for the *o*-hydroxylation of phenol are often relatively non-specific,

accommodating other monophenols like cresols and xylenols (Chapman, 1972;

Ribbons, 1970). The fungal phenol hydroxylase found in *Trichosporon cutaneum*

(Neujar and Gaal, 1973) was also found to be non-specific.

The next important step in the degradation of the aromatic nucleus is ring

fission. The ring-fission of *o*- and *p*-dihydroxylated aromatics is catalysed by either

intradiol or extradiol dioxygenases: catechol 1,2-dioxygenase and catechol 2,3-

dioxygenase promote, respectively, *ortho* and *meta*-fission of catechol (Figure 1.8).

Ring fission can also occur in aromatic compounds which contain two hydroxyl

groups which are not bound to adjacent carbon atoms. This is applicable to the

degradation of quinol and resorcinol where the aromatic ring is further hydroxylated

to form 1,2,4-trihydroxybenzene. This ring-cleavage precursor is then *ortho* cleaved

to maleylacetate which is then incorporated into the microorganism's central

metabolism. Detailed reports of these catabolic pathways have been published in

reviews by Dagley (1971), Gibson (1968) and Stanier and Ornston (1973). In

bacteria which degrade phenol, either pathway may operate, but in general a

particular microbial strain or species employs only one of the pathways for

dissimilation of the primary substrate (Stanier and Ornston, 1973). More recently,

however, it has been found that some microorganisms can utilise both pathways.

Wittich *et al.* (1990) showed that both catechol 1,2-dioxygenase and catechol 2,3-

dioxygenase were both active in *Sphingomonas* sp. strain RW1, grown on

polychlorinated dibenzo-*p*-dioxin. Catechol, gentisic acid and protocatechuic acid

which are involved as key catabolic intermediates in the degradation of many

aromatic compounds are the immediate precursors to ring-fission. In *ortho*

cleavage, orthodiols (catechol and protocatechuic acid) follow the same type of biochemical conversions, even though the two substrates are slightly different in structure. These *ortho*-diols are cleaved between the two carbon atoms to which the hydroxyl groups are bound, resulting in the formation of unsaturated dicarboxylic acid; *cis,cis*-muconic acid and β-carboxy-*cis,cis*-muconic acid, respectively.

In the *ortho*-cleavage of catechol (Figure 1.8), *cis,cis*-muconate is converted by a cycloisomerase into muconolactone, which is further isomerised to form the enol lactone of 3-oxoadipate. 3-Oxoadipate, produced from its enol lactone by a hydrolase-catalysed opening of the lactone ring, is first thio-esterified with succinyl-CoA and then cleaved at the 3-carbonyl group into acetyl-CoA and succinate in a thiolase-catalysed reaction. More recent examples have been discovered showing *ortho*-cleavage of halo-, nitro-, amino-, and sulpho-substituted phenols. *Pseudomonas* strain B13 was found to use two separate *ortho*-pathways to degrade catechol and chlorocatechol. The pseudomonad was able to achieve this because of the presence of two isofunctional enzymes: pyrocatechase I which was highly specific for catechol and showed little turnover of the haloderivatives and pyrocatechase II which had a relaxed substrate specificity with a high activity for chlorocatechols (Kashabek and Reineke, 1992).

Ortho-diols may also be cleaved between a carbon atom to which a hydroxyl group is attached and an adjacent carbon atom which does not carry a hydroxyl group. This is known as *meta* cleavage and may be either proximal or distal in relation to the non-hydroxyl substituents of the ring: it results in the

ORTHO PATHWAY

cis, cis-
Muconate

(+)-Muconlactone

B-Oxoadipate
enol-lactone

Acetyl-CoA
+
Succinate

3-Oxoadipyl CoA

3-Oxoadipate

META PATHWAY

2-Oxalocrotonate
(enol)

NADH

NAD⁺

2-Hydroxymuconic
semialdehyde

2-Oxalo-
crotonate
(keto)

HCOOH

CO_2

Pyruvate
+
Acetaldehyde

2-Oxo-4-hydroxyvalerate

2-Oxopent-4-enoate
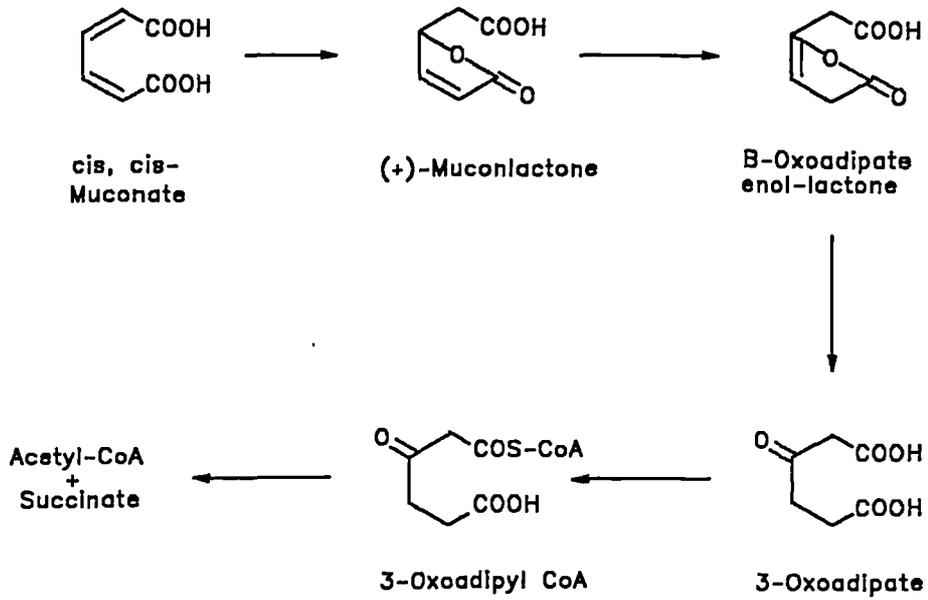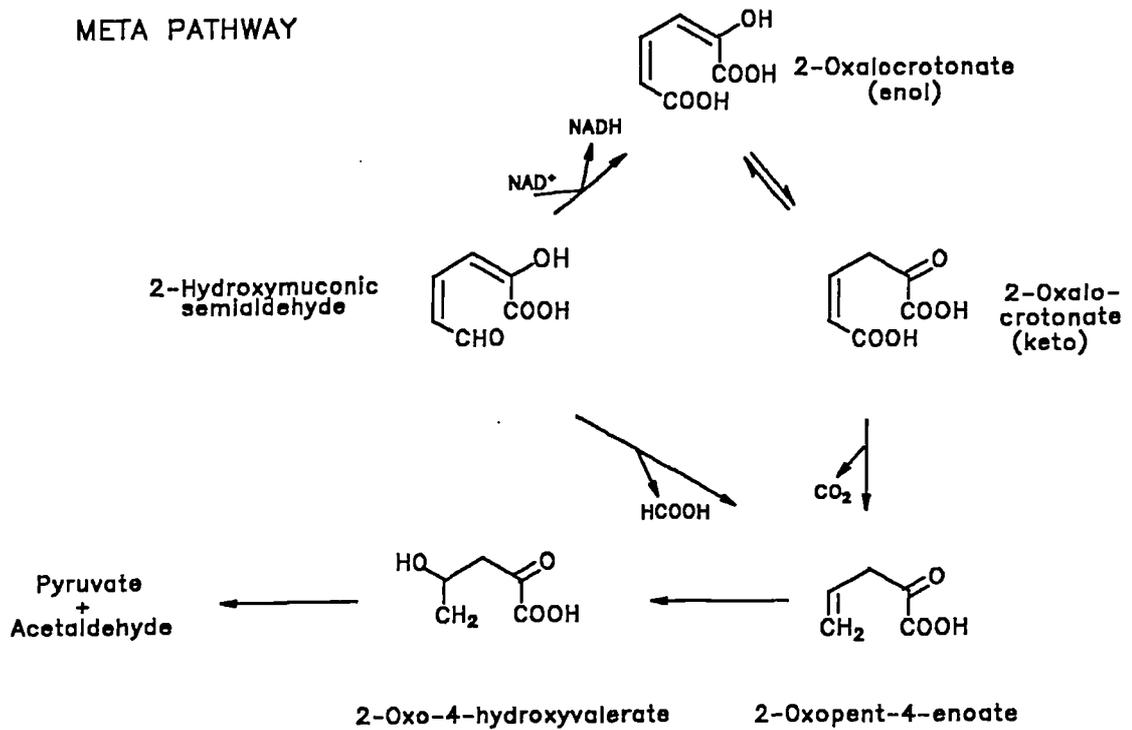
Figure 1.8: Degradation of Ring Cleavage Products of Catechol.

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production of 2-hydroxymuconic semialdehydes. The 2-hydroxymuconic semialdehyde from the *meta*-cleavage can react further by either of two alternative routes (Figure 1.8). This compound may undergo either hydrolysis to formate and 2-oxopent-4-enoic acid (Bayly and Dagley, 1969), or an NAD^+-dependant dehydrogenation to oxalocrotonate. The latter route is apparently the major one in *Azotobacter* and *Pseudomonas* species (Sala-Trepat and Evans, 1971; Catterall *et al.*, 1971). Oxalocrotonate gives rise to 2-oxo-4-hydroxyvalerate (and ultimately pyruvate and acetaldehyde) first by decarboxylation to 2-oxopent-4-enoate (Sala-Trepat and Evans, 1971; Catterall *et al.*, 1971) followed by the hydration of the double bond. The reactions from 2-oxopent-4-enoate are common to both pathways, with 2-oxo-4-hydroxyvalerate subjected to an aldolase cleavage to form pyruvate and acetaldehyde. The presence of alkyl substituents in the 3- and 4-positions of the catechol ring lead to the higher homologues of formate or acetaldehyde respectively (Bayly and Barbour, 1984).

Unlike those of the *ortho*-fission pathway, the enzymes of the *meta*-fission pathway are relatively tolerant of alkyl-substituted compounds and are often induced by alkyl-substituted compounds which led Ornston (1971) to suggest that the *meta*-fission pathway was a generalised one for the dissimilation of a variety of substituted aromatic compounds. Phenol itself induced the enzymes of *meta*-fission in a few strains of *Ps. putida* (Feist and Hegeman, 1969a; 1969b) and was also degraded by *meta*-fission in *Alcaligenes eutrophus* (Johnson and Stanier, 1971), *Micrococcus varians* (Jayasankar and Bhat, 1966) and *Ps. aeroginosa* (Ribbons, 1970). The great majority of the *Ps. putida* strains examined by Feist and Hegeman (1969a; 1969b) dissimilated phenol via the *ortho* route, and of other

pseudomonads, Stanier and Ornston (1973) listed nine species in addition to *Ps. putida* which would dissimilate aromatic compounds in this way.

In eukaryotic fungi too, aromatic catabolism is widespread though many fungi tend to detoxify or biotransform the more complex aromatics rather than catabolise them to CO_2. Cain *et al.* (1968) found that extracts of *Fusarium, Aspergillus, Penicillium, Cylindrocephalum, Phoma, Cephalosporium, Polystictus, Mucor, Debaromyces, Aureobasidium,* and *Candida* species all exhibited catechol 1,2-oxygenase activity when grown on appropriate inducer substrates. Representatives of many genera utilise several monophenolic compounds as growth substrates, those related to lignin constituents often giving particularly high growth yields. *Fusarium oxysporum* utilised phenol as the sole carbon and energy source. Utilisation of phenols was generally more restricted than that of benzoic acids, probably because of their toxicity.

A wide range of monophenols was oxidised by the soil yeast *Trichosporon cutaneum* (Neujahr and Gaal, 1973; Gaal and Neujahr, 1981). Phenol itself was degraded by *ortho*-cleavage in this species. However, *Candida tropicalis* was found to use extradiolic cleavage of 4-methylcatechol in the 1,6 position of the aromatic ring (Hashimoto, 1970; 1973). Henderson (1961a; 1961b) with several Hyphomycetes and Gross *et al.* (1956) with *Neurospora crassa* established that catechol and protocatechuate, derived from lignin-related compounds were also dissimilated by *ortho* cleavage. It thus seems that *meta*-cleavage of aromatics is rare in fungi (Cain *et al.*, 1968; 1980; Jones *et al.*, 1993; Middelhoven, 1993).

1:4:2 *The Effects of Substituents on Degradation.*

An important limiting factor in the oxidation of aromatics seems to be the number and position of substituents on the aromatic ring. Chapman (1972) described the different probable fates of the substituents, according to their chemical nature. For instance, methyl groups might remain intact or undergo oxidation, whereas larger alkyl chains were frequently not degraded unless they were susceptible to β-oxidation. The mechanisms of aerobic degradation differ amongst chlorophenols depending on the degree of chlorination, and there is a clear division of the bacterial isolates into two groups: (i) strains that degrade mono- and dichlorophenols (Figure 1.9), but will not attack more highly halogenated phenols, and (ii) strains that degrade pentachlorophenol and other highly chlorinated phenols (Figure 1.9), but will not degrade mono- and dichlorinated phenols (Haggblom, 1992). Mono- and dichlorinated phenols are degraded through chlorinated catechols which are, generally, the result of the action of a phenol hydroxylase on the appropriate chlorophenol, and then cleaved by a modified *ortho* pathway, mentioned earlier. Dehalogenation takes place as a fortuitous reaction, only after cleavage of the aromatic ring, during cycloisomerisation into 4-carboxymethylenebut-2-en-4-olide (Dorn *et al.*, 1974; Schmidt and Knackmuss, 1980). If there is a second chlorine atom, it persists through the lactone (2-chloro-4-carboxymethylenebut-2-en-4-olide) step to undergo hydrolysis to 2-chloromaleylacetate which eventually yields 2-chloro-4-oxoadipate. This is converted to maleylacetate with the release of the halide and then 3-oxoadipate is formed (Figure 1.9) (Kashabek and Reineke, 1992; Vollmer *et al.*,1993).

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In the catabolism of polychlorinated phenols, for example pentachlorophenol, the *para*-chlorine atom is first removed through a hydrolase enzyme producing 2,3,4,6-tetrachlorohydroquinone, then the remaining chlorine atoms are removed through successive reductive reactions (Apajalahti *et al.*, 1986). Schenk *et al.* (1989) suggested that the production of the tetrachlorohydroquinone may be an oxidative mechanism in their catabolic pathway. These reductions produce 1,2,4-trihydroxybenzene, which can be mineralised to CO_2 (Figure 1.10).

The aptitudes of microorganisms - what they can and cannot do - are a legacy of their evolutionary history (Dagley, 1978). Knackmuss (1981) postulated that one of the reasons for the major effects of substituents on the biodegradation of the aromatic ring was primarily due to enzyme specificity. The substituted compound must not only be a structural analogue of the normal enzyme substrate if it is to be converted, it must be of comparative reactivity, and must have functional groups of similar polarity and size to those of the parent compound. The enzyme must also catalyse the same reaction on the substituted analogue as on its parent compound, as the reaction product is specific to a particular enzyme reaction. Phenol and the isomeric cresols, for instance, can be degraded via catechol, 3-methyl-, or 4-methylcatechol through homologous reaction pathways to pyruvate (Sala-Trepat and Evans, 1971). The removal of a non-carbon substituent at some point is essential for the complete catabolism of the compound. This may determine the amount of energy available from a substituted compound, in that the more difficult a bond-cleavage, the more energy the microorganism has to expend to achieve it.

The presence of substituents on the aromatic ring is known to affect its
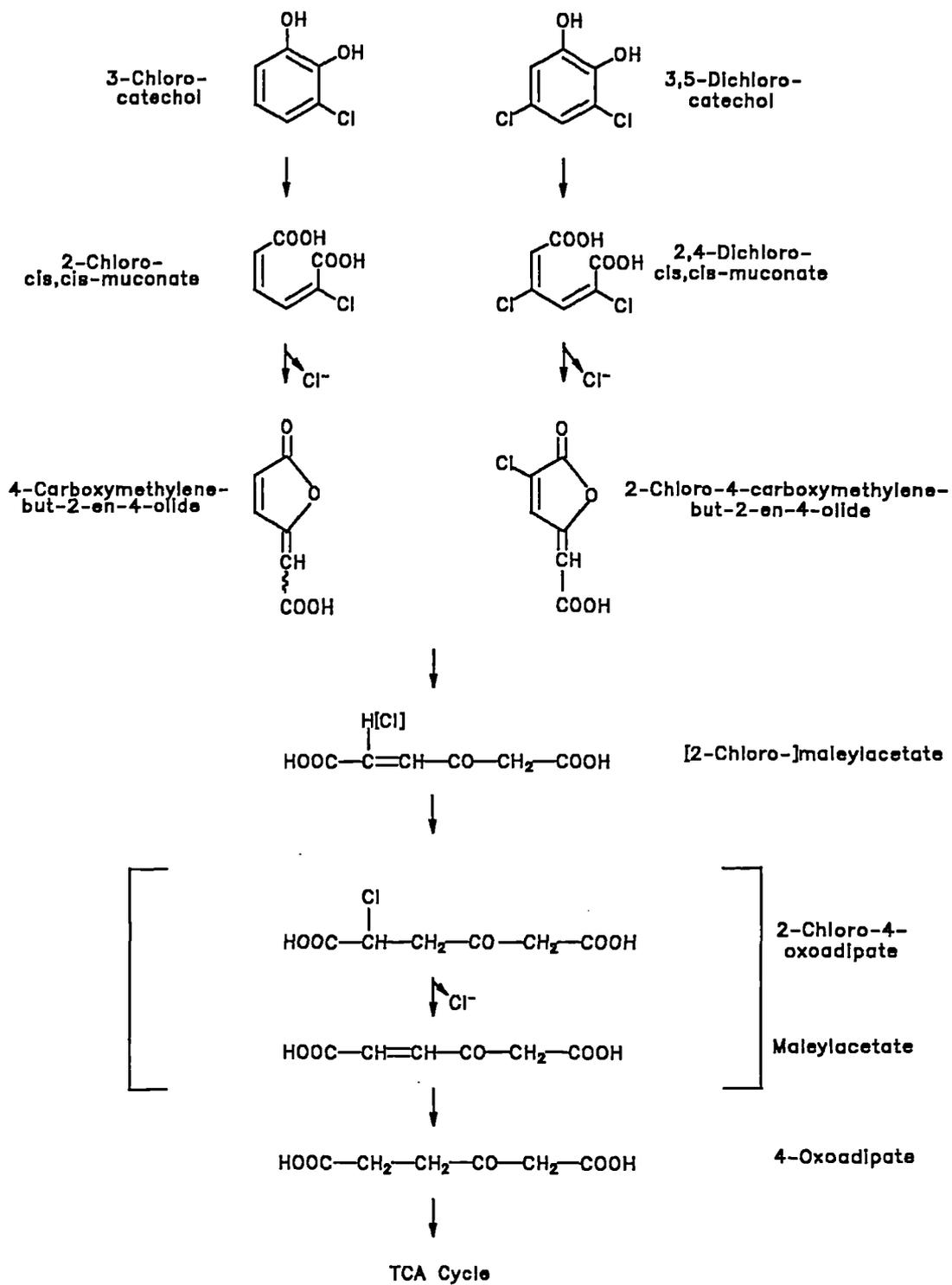
Figure 1.9: Degradation of Mono- and Dichlorophenols via a modified Ortho Pathway.

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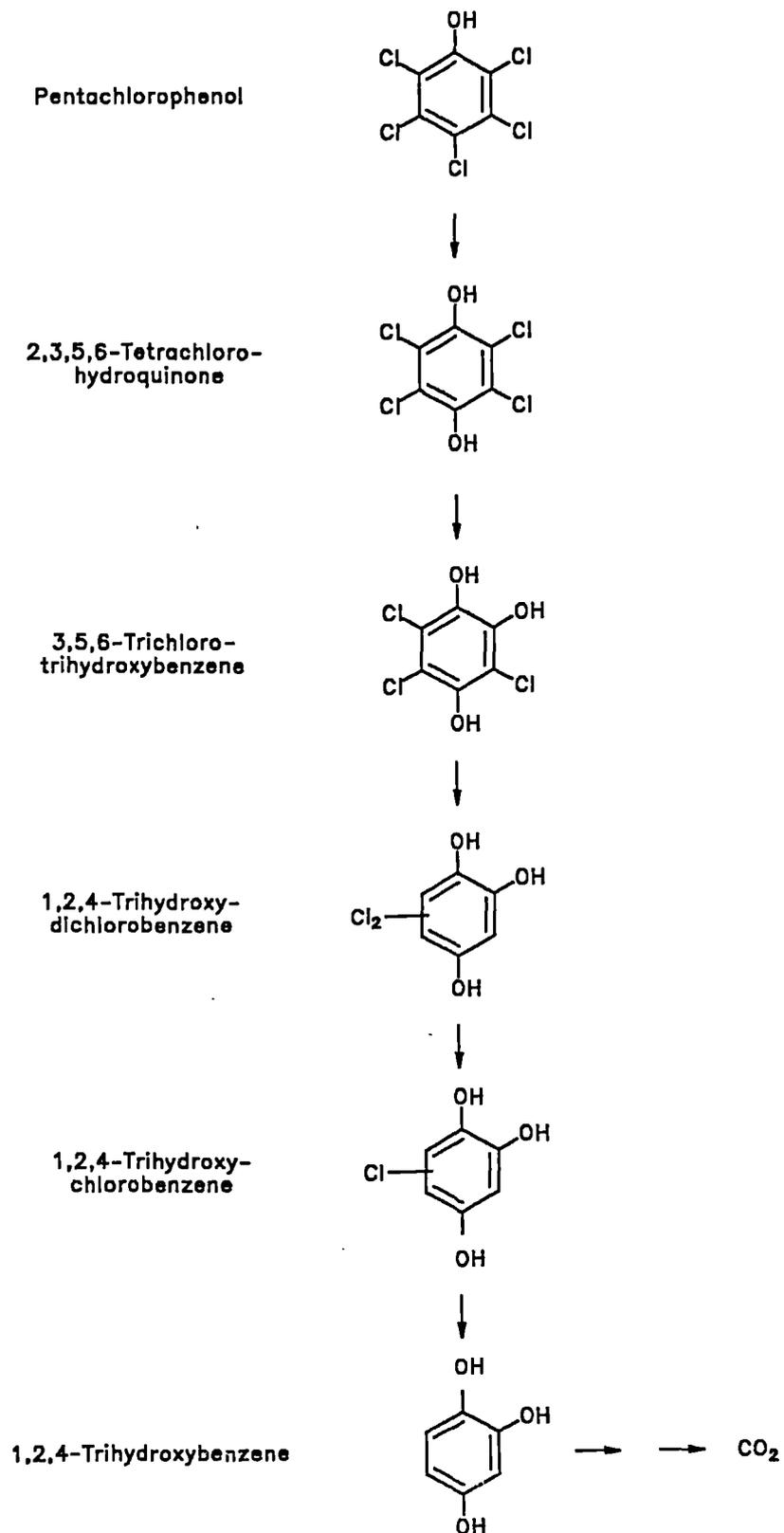
Figure 1.10: Degradation of Pentachlorophenol.

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degradation, and in some cases to prevent any oxidation. Both the position and the nature of such substituents are important in determining the degree of degradation possible. Chapman (1972) for instance was able to isolate degraders of all the xylenol isomers except 2,6-xylenol, which was the result of the methyl groups filling both the positions adjacent to the hydroxyl group, which would normally be hydroxylated; the formation of the appropriate catechol was thus prevented. However, it was found, later, that 2,6-xylenol was degraded by a much more complex route in which it was converted to 2,6-dimethylhydroquinone and then 2,6-dimethyl-3-hydroxyhydroquinone which was cleaved to citraconate and propionate (Ewers et al., 1989). Some of the enzymes in the breakdown pathways have very relaxed substrate specificities, and are almost unaffected by the presence of substituents. Nozaki (1970) found that purified catechol 2,3-oxygenase from Pseudomonas arvilla catalysed the cleavage of catechol, 3-methylcatechol, and 4-methylcatechol at similar rates, and it is thought the other enzymes of these pathways may have a similar broad functional specificity (Ribbons, 1970; Sagoo, 1979). Hopper and Taylor (1975) demonstrated that m- and p-cresols were degraded via meta cleavage, but the former could be degraded via the gentisate pathway also. Hopper and Chapman (1970) showed that m-cresol, 2,5- and 3,5-xylenols were catabolised by the gentisate pathway after oxidation of one of the methyl groups to a carboxyl. Hughes et al. (1984) reported that phenol, p-cresol, m- and p-toluate were degraded via the meta pathway, with the ring cleavage products, except phenol, being attacked by the NAD$^+$-dependant semialdehyde dehydrogenase. Sagoo (1979), working with Pseudomonas testosteroni on the degradation of 1-phenylalkane sulphonates, found that catechol, 3-methylcatechol,

4-methylcatechol, 4-ethylcatechol, 4-butylcatechol, 4-hexylcatechol, 4-octylcatechol

and 4-dodecylcatechol were cleaved via the *meta* pathway. The ring fission

products of the higher alkylcatechols were degraded only by the NAD^+-dependant

2-hydroxymuconic semialdehyde dehydrogenase. More recently, research has

continued with the *meta* cleavage of substituted monoaromatics, incuding phenols.

Higson and Focht (1992) described how *Pseudomonas cepacia* MB2 was able to

grow on 3-chloro-2-methylbenzoate as the sole carbon source by metabolism

through the *meta* fission pathway with susequent liberation of chloride. Olson *et al.*

(1992) elucidated the degradation of 4-hydroxyphenylacetate, which is derived from

aromatic amino acids and lignin decomposition. The ring cleavage precursor is

3,4-dihydroxyphenylacetate which undergoes a *meta* ring-cleavage catalysed by a

2,3-dioxygenase. Investigations into the more recalcitrant biphenyl and

dibenzofuran molecules have shown the existance of *meta* cleavage in their

degradation (Higson and Focht, 1989; Kohler *et al.*, 1988; Strubel *et al.*, 1991). In

fact, the same ring cleaving mechanism for 2-hydroxybiphenyl reported by Kohler *et*

al. (1988) in a pseudomonad strain HBP1 was found in a mutant of this strain

selected to grow on 2-propylphenol (Kohler *et al.*, 1993). The product of

monooxygenase activity was 3-propylcatechol which was *meta* cleaved to form 2-

hydroxy-6-oxo-nona-2,4-dienoate which was catabolised to give butyrate and 2-

hydroxypenta-2,4-dienoate.

In the recent past, it was thought that in bacteria, the types of reactions used

in the *meta*-fission pathway (Dagley, 1987) were more suited than those of *ortho*-

fission to degrading substituted aromatics. Thus, extracts of catechol-adapted *P.*

putida readily and extensively degrade methyl-substituted catechols, whereas

bacteria that used *ortho*-fission were more handicapped for degrading 3- and 4-methylcatechol because they accumulated a methyl-substituted lactone as a dead-end metabolite. However, with the discovery of a modified *ortho*-pathway, certain strains of bacteria were found to degrade methyl-substituted mononuclear aromatics (Pieper *et al.*, 1985; Bruce *et al.*, 1989). Chapman and Hopper (1968) found that 2,4-xylenol was catabolised via the *ortho* route after the oxidation of both the methyl groups and the formation of protocatechuic acid. A methyl substituent is no obstacle to the soil yeast *Trichosporon cutaneum*, which used the modified *ortho* pathway for 4-methylcatechol which is metabolised to β-methylmuconolactone ([-]-2,5-dihydro-3-methyloxofuran-2-acetate). This is degraded via 4-methyl-3-oxoadipate and citramalate without any problem to acetate and pyruvate and so used for growth (Powlowski and Dagley, 1985).

For some substrates the lactonising step assists the removal of critical substituents. This is the case when a chlorine or a fluorine substituent is eliminated by lactonisation that follows *ortho*-cleavage of 3,5-dichlorocatechol. On the other hand, *meta*-cleavage can prove to be a handicap. Thus, the ring-fission products from 3-chlorocatechols by catechol 2,3-dioxygenase are acyl-halides; they are suicide inhibitors that acylate the enzyme (Klečka and Gibson, 1981; Bartels, *et al.*, 1984) resulting in degradation being blocked.

1:4:3 Metabolism of Phenols by Higher Plants.

Seidel (1963; 1966), Seidel and Kickuth (1965) and Kickuth (1970) have shown that phenol penetrates the tissues of whole plants; indeed, it seems improbable that

such a compound can be excluded from plant cells. Having been absorbed by the plant cells, three major metabolic routes might accommodate exogenously-supplied phenol. Oxidative metabolism of phenolic compounds can readily be demonstrated in many plant tissues; the activity of polyphenol oxidase enzymes hydroxylates monophenolic compounds and further oxidises the resulting diphenols, forming reactive quinones. Ring-fission is the second possibility for the metabolism of monophenols in plant tissues. Finally, simple phenols in plant tissues are detoxified to glycosidic conjugates, in a process analogous to animal detoxification processes. These three systems can result in the eventual destruction, immobilisation, or detoxification of phenolic compounds.

Higher plant aromatic ring-fission reactions have been sought in many species, using as models the well-characterised aromatic metabolic sequences in microorganisms. The probable similarity of the products of plant and microbial aromatic biodegradation, and the comparative ease with which competent microbial populations can be selected make it important to determine that any observed activity in plants is due unequivocally to the plant tissues and not to contaminating microorganisms. Ellis and Towers (1970) expressed similar reservations concerning the interpretation of results purporting to show the degradation of aromatic compounds in whole plant tissue preparations which were not totally axenic.

Degradation of the aromatic ring by plants has, however, been successfully shown in axenic tissue and cell cultures. It should be borne in mind that the responses of a plant species in comparatively disorganised tissue-cultures need not reflect identical metabolism in the differentiated tissues of a whole plant subject to

normal physiological control, so studies with intact plants or undamaged but excised tissues are still required.

Surveys on aromatic-fission ability in plants have often been made without extensive characterisation of the pathways, relying for the most part on the detection of $^{14}CO_2$ production by experimental plant materials supplied with the radiolabelled aromatic substrates. As a result of a survey of twenty species from eleven families of plants, Ellis (1971) concluded that catechol ring-fission activity was widely spread in the Plant Kingdom. All his examples released $^{14}CO_2$ from [U-^{14}C]catechol when the substrate was applied to tissue cultures; fifteen cultures possessed the ability to decarboxylate [carboxyl-^{14}C]salicylate, and traces of $^{14}CO_2$ were released as a result of the fission of ring-labelled salicylate by thirteen species. The species most active against catechol (in tissue culture) were Malus coronaria, Phaseolus angularis, and Phaseolus aureus. Catechol degradation by P. aureus was also reported by Berlin et al. (1971), who also found that Glycine max possessed the same activity. P. aureus tissue was analysed further by Ellis (1971), when substantial conversion of catechol to its β-D-glucoside was found, together with a large (50%) fraction not recoverable in ethanolic extracts, suggesting that not only did the tissue degrade catechol, but also conjugated it, and rendered it immobile as the result of phenolase activity.

Studies of the aromatic metabolism of the tropical Tecoma stans, using compounds other than catechol, have provided some firm evidence for the existence of enzymes capable of degrading the aromatic ring through cleavage. Sharma et al. (1972) reported the oxygenative ring-cleavage of the catecholic 2,3-dihydroxybenzoic (pyrocatechuic) acid in this species, the isolated product being

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the bislactone 2,6-dioxa-3,7-dioxobicyclo(3:3:0)octane-8-carboxylic acid. Such a compound could have been formed by the rearrangement through one of the two isomeric muconolactones of the initial product of a pyrocatechuate 2,3-dioxygenase, 3-carboxy-*cis,cis*-muconate. The enzyme responsible for pyrocatechuate ring-fission in *Tecoma stans* was subsequently isolated from soluble and chloroplast fractions of leaves by Sharma and Vaidyanathan (1975a; 1975b). The enzyme was comparatively specific for pyrocatechuate, but showed activity with 2,3-dihydroxy-4-methylbenzoate and 2,3-dihydroxy-4-isopropylbenzoate, but was inactive against catechol, 4-methylcatechol and protocatechuate.

Subsequent examination of *T. stans* has shown that the species also possesses protocatechuate 3,4-dioxygenase. This enzyme, isolated only from the soluble fractions of tissue by Mohan *et al.* (1979), was purified almost to homogeneity, and was absolutely specific for protocatechuate. The product, 3-carboxy-*cis,cis*-muconate, was identified and isolated, but the enzyme, although performing a function similar to the enzymes from microbial sources, showed several differences from highly characterised fungal and bacterial preparations.

The likelihood of *meta* cleavage in plants was first suggested by Senoh and Sakan (1966) to explain the formation of naturally-occurring pyrone derivatives, stizolobic and stizolobinic acids, from 3,4-dihydroxyphenylalanine in *Stizolobium hassjoo*. The enzyme responsible for this was found by Saito and Komamine (1976).

1:5 Biodegradation of Xenobiotic Compounds by Eukaryotic Algae.

In general, algae are, like the higher green plants discusseded in 1.4.3, considered photoautotrophic organisms which reduce CO_2 in the presence of light to synthesise the complex organic molecules they need for survival. In reality algae, as a whole, possess a wide variety of nutritional capabilities which they employ to maintain their position in a particular ecosystem. Before attempting to show the capabilities of algae in attacking xenobiotics, it is important to understand just how versatile various species of algae are when considering their nutritional capabilities.

Heterotrophy has been adopted by algae to various degrees so they can be classified as either obligate photoautotrophs, obligate heterotrophs, or facultative heterotrophs. Obligate photoautotrophs are unable to utilise any organic carbon source for growth, although it is possible that suitable conditions for partial heterotrophy have not yet been found (Droop, 1974). Heterotrophy in algae implies the capacity for sustained growth in the dark with energy and cell carbon both being obtained from the catabolism of an organic substrate. This appears to be entirely aerobic in algae (Neilson and Lewin, 1974). Obligate heterotrophs are apochlorotic (lack photosynthetic pigment) and therefore *must* use organic carbon sources to survive, but many are clearly morphologically and biochemically related to the pigmented algae. These colourless forms occur naturally and have been used in many studies on algal growth on more complex carbon sources (Neilson and Lewin, 1974). *Prototheca zopfii*, an achlorophyllous alga, has been shown to degrade motor and crude oils (Meyer, 1975; Walker *et al.*, 1975).

Compared to bacteria and fungi, heterotrophic algae can utilise only a limited range of substrates. A wide range of algae is capable of growing on simple carbon sources including acetate, glucose, amino acids and organic acids. Cramer and Myers (1952) grew *Euglena gracilis* var *bacillaris* on acetate, pyruvate, butyrate, succinate, fumarate or malate in darkness. *Bumilleriopsis brevis* grew on sugars, such as glucose, mannose, sucrose and fructose (Belcher and Miller 1960), while *Ochromonas malhamensis* grew better with sugars than acetate (Pringsheim, 1952). Samejima and Myers (1958) showed that *Chlorella pyrenoidosa* also grew on glucose, galactose and acetate in darkness.

Algae which are capable of heterotrophy and maintain a fully functional photosynthetic apparatus are known as facultative heterotrophs. Further nutritional diversity is found in the photoheterotrophic algae which can use carbon sources for growth such, as glucose, if they are simultaneously provided with light (Neilson and Lewin, 1974). However, these photoheterotrophic algae are normally unable to incorporate the carbon sources into the cellular material when grown in the dark. In addition, a few algae can ingest particulate food by phagocytosis (Aaronson, 1973; 1974; Fenchel and Finlay, 1983; Andersson, *et al.*, 1989).

In the light, organic compounds are able to elicit a variety of responses from algae, and often involve photosynthetic metabolism. In general, phototrophs do not promote complete degradation of xenobiotic compounds, but only the transformation (Kobayashi and Rittmann, 1982). Photoassimilation involves the oxidation of the compound, but the carbon is not used for the growth of the organism. Zepp and Schlotzhauer (1983) studying green (*Chlamydomonas* sp., *Chlorogonium spp., Chlorococcum hypnosporum* and *Selenestrum capricornutum*)

and blue-green (*Anabaena cylindrica, Nostoc muscorum*) algae found that at cellular densities, measured as concentrations of their chlorophyll *a*, of 1-10mg.l^{-1}, they produced an increase in the photolysis rates of certain polyaromatic hydrocarbons, organophosphorous compounds and anilines as compared to freshwater controls. Many algae are capable of biotransforming anthropogenic compounds, even transforming certain compounds to the same extent as do bacteria. An example is the transformation of DDT, by *Dunaliella* sp., to TDE (trichlorodiphenyl dichloroethane) by dechlorination (Kobayashi and Rittmann, 1982). Patil *et al.*, (1972) showed that *Dunaliella* sp. and *Agmenellum quadraplicatum* converted dieldrin to photodieldrin. In addition to their ability to biotransform organic compounds, algae are potentially valuable because of their ability to bioaccumulate hydrophobic compounds (Table 1.1). Karydis (1980) showed the accumulation in *Cyclotella cryptica* of n-alkanes (containing C_{13}-C_{16} carbon atoms) with C_{16}-alkanes accumulating to twice the concentration of the original oil.

As with studies on photoheterotrophic algae, research on heterotrophic algae has been restricted to a small number of laboratory-cultured strains provided with simple organic acids, sugars or amino acids (Samejima and Myers, 1958; Droop, 1974; Hellebust, 1971a; 1971b; Vincent and Goldman, 1980; Andersson *et al.*, 1989).

Information is scarce on the relationship between algal heterotrophy and the biodegradation of xenobiotic compounds, far less than the information accumulated concerning bacteria and fungi. Studies have been carried out on the effect of crude oil and fractions of crude oil, particularly water-soluble fractions, on the

Table 1.1: Bioaccumulation of Anthropogenic Compounds by Eukaryotic Algae.

Algae	Compound
Chlorococcum sp., Dunaliella teriolecta, Chlamydomonas sp., Thalassiosira pseudonana, Porphyridium cruentum.	Mirex
Chlorella	Toxaphene, Methoxychlor
Nitzchia	Mirex, Methoxychlor, 2,4-DBE
Monoraphidium	Methoxychlor
Euglena gracilis, Scenedesmus obliquis	Parathion, DDT
Cylindrotheca closterium	DDT
Selenestrum capricornutum	Benzene, Toluene, Chlorobenzene, 1,2-Dichlorobenzene, Nitrobenzene, Naphthalene, 2,6-Dinitrotoluene, Phenanthrene, Di-*n*-butylphthalate, pyrene

growth of algae. The effect of oil on algae has been the subject of several reviews (O'Brien and Dixon, 1976) with the general consensus being that the effects vary with the type of oil and the species of alga. Both inhibition (Hilmer and Bate, 1983; Pulich *et al.*, 1974; Bate and Crafford, 1985; Winters *et al.*, 1976; Singh and Gaur, 1990) and stimulation of growth (Dunstan *et al.*, 1975; Masters and Zajic, 1970; Walker *et al.*, 1975; Meyer, 1975) have been observed at low concentrations of petroleum and its more water-soluble fractions.

1:5:1 *Algal Degradation of Aromatic Compounds.*

To date, no pathways for the oxidative fission of the aromatic ring by algae have been elucidated, although there is evidence to suggest that some algae are capable of this process. Craigie *et al.* (1965) showed that several eukaryotic algae produced $^{14}CO_2$ from $[^{14}C]$-phloroglucinol after preconditioning the algae with non-radiolabelled phloroglucinol which led to the enhanced destruction of the aromatic ring. Vose *et al.* (1971) incubated twenty-two species of unicellular marine algae with [*ring*-^{14}C]*DL*-phenylalanine and found nine species to produce $^{14}CO_2$. Ellis (1977) incubated six species of fresh water algae with [*U*-^{14}C]catechol and [*U*-^{14}C]phenol and found that all six produced $^{14}CO_2$ from the former but only four of the algae produced $^{14}CO_2$ from the latter. An interesting observation was made by Jacobson and Alexander (1981) in which non-axenic cultures, grown in the light and in the dark on acetate, were found to dehalogenate 4-chloro-3,5-dinitrobenzoic acid and produce a metabolite which was identified as 2-hydroxymuconic

semialdehyde, indicating *meta* cleavage. Kobayashi and Rittmann (1982) collated

a list of microorganisms which could attack anthropogenic compounds, including

phenols, polycyclic aromatics, and pesticides, and found that eukaryotic algae were

included along with bacteria and fungi. Jinqi and Houtian (1992) investigated the

degradation of azo dyes by *Chlorella* sp. and found that certain dyes could be

decolourised and actually used as carbon and nitrogen sources, depending on the

chemical structure. They also found that the algae could utilise aniline, a

degradation product of azo dye breakdown (Figure 1.11).

Polycyclic aromatic hydrocarbons are known to produce carcinogenic and

mutagenic effects and, therefore, are considered to be priority pollutants in the

environment, and as such, their modes of degradation have been studied in some

detail. Naphthalene has proved interesting to algal researchers, who found that this

compound was toxic to *Chlamydomonas angulosa*: 61% of cells were killed in open

flasks containing saturated aqueous solutions of naphthalene (Soto, *et al.* 1975a;

1975b). Cerniglia *et al.* (1979; 1980a,b; 1982) showed that both cyanobacteria

(blue-green algae) and eukaryotic microalgae were capable of biotransforming

naphthalene to the more water-soluble phenol, 1-naphthol. Cerniglia *et al.* (1980)

also showed that the cyanobacterium, *Oscillatoria* sp. strain JCM, could oxidise

biphenyl to 4-hydroxybiphenyl. More recently, Luther and Soeder (1987), Luther

and Shaaban (1990) and Luther (1990) were able to confirm that the alga,

Scenedesmus obliquus, was able to utilise naphthalenesulphonic acids as a source

of sulphur for their biomass, releasing the carbon ring into the medium.

Furthermore, Luther found that the alga could use nitro and amino substituents

from aminonaphthalenes and amino- and nitrobenzoates as nitrogen sources and

Azo Compound

Azo Reductase

Aromatic Amine

Simple Compounds or CO_2
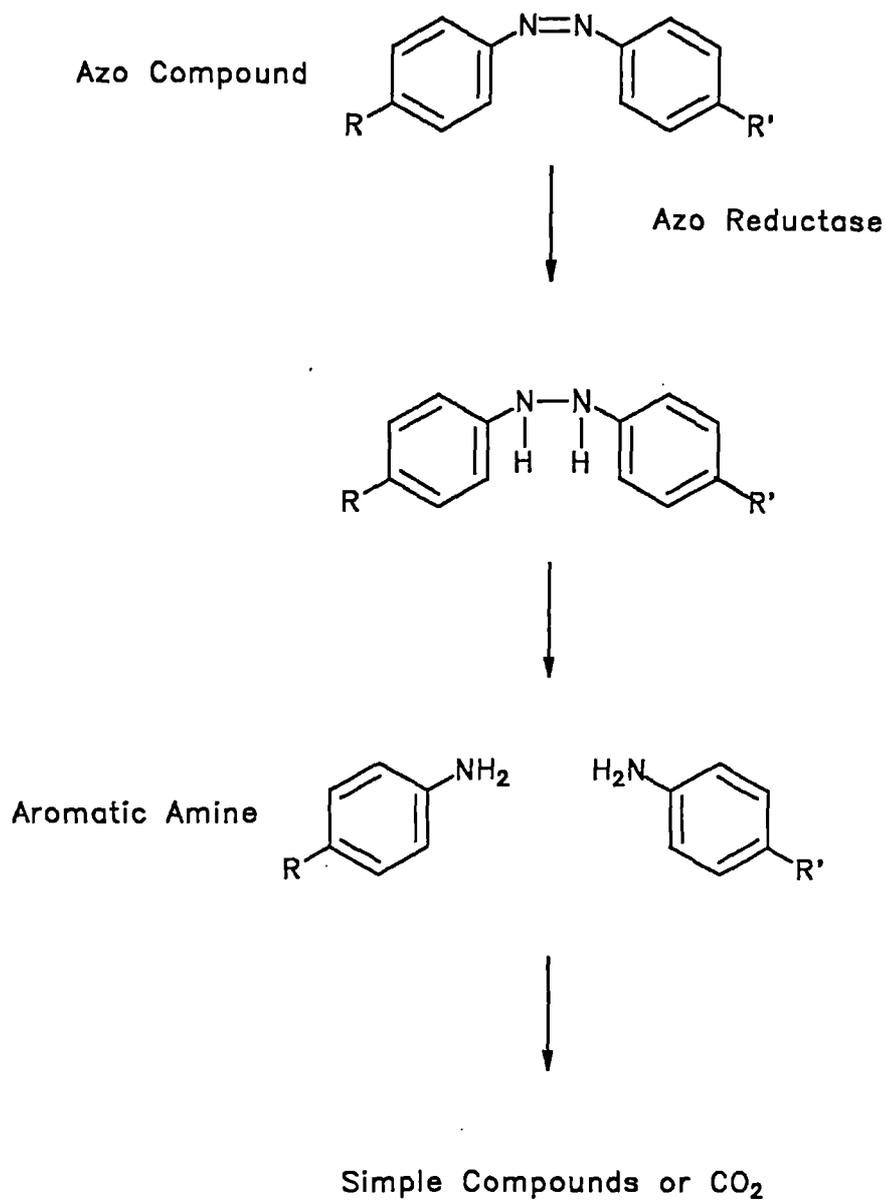
Figure 1.11: Degradation of Azo Dyes by Eukaryotic Algae.

chlorobenzoates could be dehalogenated and the chloride accumulated by the cells.

Lindquist and Warshawsky (1985; 1985), Warshawsky *et al.* (1983; 1988; 1990) and Schoeny *et al.*, (1988) examined the effects of the green alga, *Selenestrum capricornutum*, on benzo[a]pyrene. They found that the alga used a dioxygenase system to oxidise the compound to *cis*-dihydrodiols which were then converted to sulphate ester and α- and β-glucoside conjugates. The presence of this ring hydroxylating dioxygenase system is of particular importance as this mechanism is typically found only in bacteria and not in eukaryotes, where *trans*-dihydrodiols originate from epoxidation by the action of cytochrome P-450 monooxygenases and epoxide hydrolases on the polycyclic aromatic hydrocarbon. Liebe and Fock (1992) found that the green alga, *Chlamydomonas reinhardtii*, was able to remove some of the polycyclic aromatic hydrocarbons extracted into iso-octane from diesel particulate exhaust, after having been suitably adapted. *Ochromonas danica* is also capable of growing at the expense of novel polyol ester lubricants (Croce, 1992).

1:5:2 *Selective Use of Algae in the Treatment of Organic Effluents.*

Although research to date suggests that algae are not as versatile biodegradatively as bacteria or fungi, they are nevertheless capable of making a substantial contribution to the remediation of organic effluents. These algal processes, generally, require the presence of light (600-700nm) as the primary

energy source and aerobic or microaerophilic conditions producing self-sustaining populations which can partially degrade certain complex compounds by photochemical reactions. This process oxygenates the effluent and supports growth of other microbes which may be able to degrade the more complex pollutants further (Figure 1.12). This system has been used in the photosynthetic treatment of wastewater described by Fallowfield *et al.* (1992) and is used by two systems of waste treatment: waste stabilisation ponds and high rate algal ponds. Waste treatment is effected by the respiration of organic matter by the aerobic heterotrophic microbial population. Oxygen for microbial respiration originates from atmospheric diffusion and microalgal photosynthesis. Microalgal wastewater treatment systems aim to optimise the photosynthetic oxygen production. A major advantage of biogenic oxygen production is that soluble nitrogen and phosphorus present within wastewater is incorporated into the pond biomass during growth and is not discharged to cause eutrophication in natural waters. Jinqi and Houtian (1992) suggested that algae have a more dynamic role to play in oxidation ponds, related to their ability to breakdown xenobiotics such as azo dyes. Although algae are considered primarily photosynthetic, these authors suggested that the algae play a role in the actual degradation of these aromatic compounds. Oxidation ponds not only purify wastes, but can also be used to provide energy particularly in tropical climates - algae have a solar conversion efficiency of 3-5% and average production is $70t\ ha^{-1}yr^{-1}$ ($35t\ ha^{-1}\ yr^{-1}$ algal protein). When harvested they may be fed directly to animals, used as fertiliser or as a source of chemicals, fermented to produce methane or burnt to produce electricity (Polprasert, 1989).

In addition to the degradation of organic compounds, algae are potentially
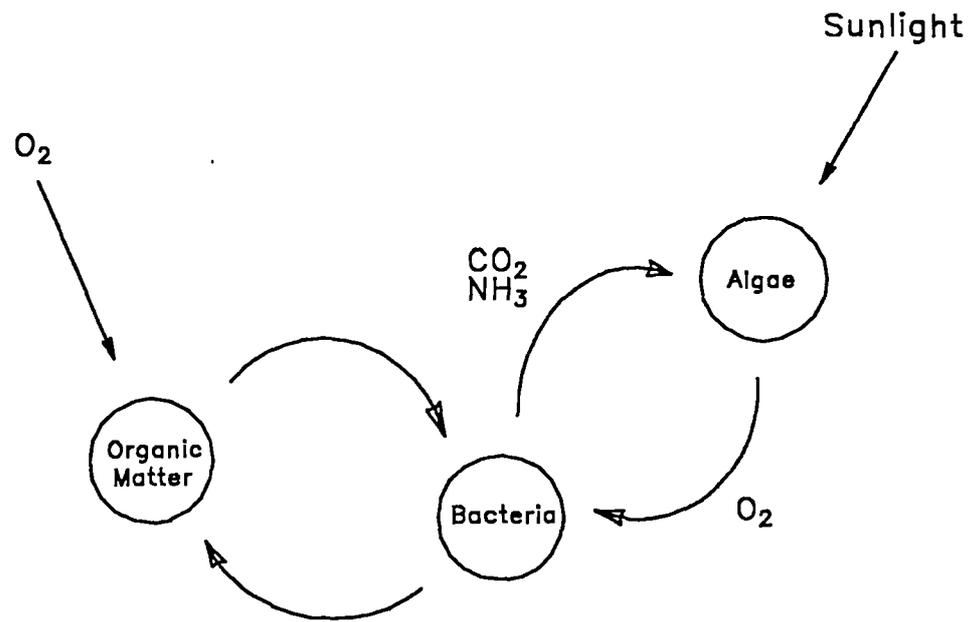
Figure 1.12: Summary of the Involvement of Algae in an Oxidation Pond

valuable because of their ability to bioaccumulate hydrophobic compounds.

Bioaccumulation is different from other environmental processes because it

concentrates rather than diffuses the chemical in question. There are two major

factors which influence the rate of bioaccumulation: high partition coefficient, i.e.,

lipophily, and recalcitrance towards all types of degradation. Although

bioaccumulation is a characteristic shared by many different microorganisms,

bioaccumulation by phototrophs offers the greatest potential for exploitation in

treatment processes, because phototrophs can self-sustain relatively large

populations without the presence of organic matter in concentrations high enough

to serve as carbon and electron donors. In addition, some algae (especially

Chlorella and certain diatoms) can be manipulated physiologically to develop large

lipid stores into which hydrophobic compounds may be sequestered (Lewin, 1962;

Lewin and Guillard, 1963; Miller, 1962).

1:6   Research Aims.

A previous study from this laboratory had shown that algae were major contributors to the degradation of polyol ester lubricants in freshwater ecosystems and stimulated a wish to establish how generally significant was the algal contribution to the degradation of xenobiotic pollutants. Phenols were chosen as model pollutants because (a) a large number of homologous and substituted phenols are commercially available and many are in any case major industrial pollutants; (b) they are readily analysed and identified by t.l.c., h.p.l.c. and g.c.; (c) they are water-soluble and therefore readily presentable to algae and (d) there is a fund of knowledge of their catabolism in other microorganisms for comparison and contrast.

To date, most of the research on phenol degradation has involved bacteria and, to a lesser extent, fungi and yeasts. Research involving higher eukaryotes is limited to a few studies on plants, plant cell cultures and green algae with radioisotopes; but, to date, no pathways have been properly characterised, especially at the enzyme level.

The main aims of this study were to find eukaryotic algae which were capable of removing, principally, phenol and its methyl-substituted homologues from their liquid growth media. Studies of the growth of the algae and their turnover of these compounds were carried out using h.p.l.c., T.O.C., respirometric (including the oxygen electrode and Warburg apparatus) and radioisotopic techniques. When a good phenol-degrader was obtained, attempts were made to isolate and identify

catabolic intermediates and then elucidate the degradative pathway using established enzymic techniques with the aim of establishing similarities with known fungal (eukaryotic) and bacterial (prokaryotic) pathways.

2. MATERIALS AND METHODS

2:1 Selection of Algae.

The algae employed in this study were obtained from the Culture Collection for Algae and Protozoa at the Institute of Freshwater Ecology, Ambleside, Cumbria. The three species of algae initially included two green algae: *Chlamydomonas ulvaensis* [CCAP 11/58] and *Scenedesmus brasiliensis* [CCAP 276/1B] and a golden brown alga: *Ochromonas danica* [CCAP 933/28] (Plate 2.1). These algae were selected on the basis of previous research carried out by Ellis (1977) who showed that the two green algae were able to liberate $^{14}CO_2$ from [U-^{14}C]phenol and catechol and by Croce (1992) who studied *O. danica*.

2:2 Growth Conditions.

Jaworski's medium (Beakes *et al.*, 1988) was used, containing the following ingredients per 100ml as stock solutions. From each stock solution 1ml was added to 1l of double-distilled water: *Solution I*, $Ca(NO_3)_2$. (2g); *Solution II*, KH_2PO_4. (1.24g); *Solution III*, $MgSO_4$. H_2O. (2g); *Solution IV*, EDTA-Na_2. (0.225g) and EDTA-NaFe. (0.225g); *Solution V*, H_3BO_5. (0.248g), $MnCl_24H_2O$. (0.15g) and $(NH_4)_6Mo_7O_{24}$. H_2O. (0.1g); *Solution VI*, $NaNO_3$. (8g); *Solution VII*, Na_2HPO_4. $12H_2O$. (3.6g); *Solution VIII*, $NaHCO_3$. (1.59g). The pH of the final solution was 6.6. (this
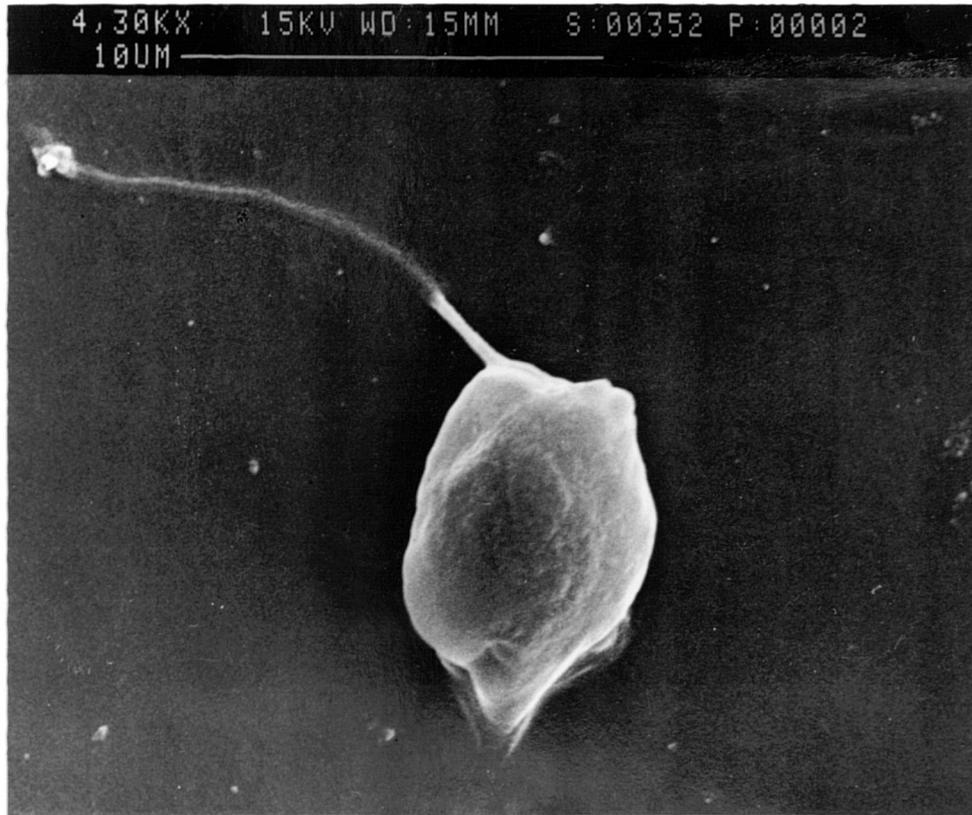
Plate 2.1: *Ochromonas danica* grown phototrophically at 25°.

formula was provided by the Institute of Freshwater Ecology). After the solutions

were combined, they were sterilised at 121°C for 20min, the following filter-

sterilised vitamin supplement was then added at $1ml.l^{-1}$ combined medium:

cyanocobalamin, (0.004g); thiamine HCl, (0.004g) and biotin, (0.004g) in 100ml

distilled water.

Stock cultures were maintained in an illuminated incubator at a light intensity

of 60μmol photons $m^{-2} s^{-1}$ at 25°C. Heterotrophic cultures were grown from an

inoculum which provided a final cell density of 1×10^3 cells.ml^{-1}, in dark or

illuminated (150μmol photons. $m^{-2}. s^{-1}$.) orbital incubators (Gallenkamp), shaken at

100 rev/min at 25°C. A variety of carbon sources, including acetate, glucose,

pyruvate and succinate (1mM) were examined, these were filter-sterilised through a

0.2μm membrane (the growth medium did not contain $NaHCO_3$ when other carbon

sources were present).

Glassware was washed in 10% (v/v) HCl and distilled water because the

phosphate content of detergent-washed glassware was found to be unsuitable for

algal growth .

2:3 Determination of Heterotrophic Ability.

2:3:1 *Growth of Organisms.*

Growth was measured by making direct counts of cells in samples (1ml) of

growing cultures. Cells were fixed using 15μl Lugol's iodine (100g of potassium

iodide (BDH), 50g of crystalline iodine (BDH) and 100ml glacial acetic acid (BDH) per l of distilled water) then enumerated microscopically using an Improved Neubauer haemocytometer (Guillard, 1973). Each culture was counted twice and the average determined. Every growth experiment was done in triplicate, unless otherwise stated.

2:3:2 Non-Aromatic Substrates for Growth.

Jaworski's medium was supplemented with 1mM acetate, glucose, pyruvate or succinate and the cultures were grown in the dark at 25°C. Growth was determined by direct counts.

2:3:3 Aromatic Substrates for Growth.

Initially cells were grown on mixtures of glucose (5mM) and phenol (500μM) in the light and the dark to establish whether or not the algae were capable of removing the phenol. This process allowed the tentative selection of a putative heterotroph, *O. danica*, for more detailed studies of aromatic degradation.

Ochromonas danica was then taken directly from the illuminated stock cultures and incubated in the dark with either phenol, *o*-cresol or *p*-cresol at a range of concentrations from 500μM to 4mM. Growth was monitored by direct cell counts. Simultaneously the disappearance of the substrates was monitored using

high pressure liquid chromatography (h.p.l.c.). Such heterotrophic experiments included measurements on no-substrate and boiled cell controls.

Standard microbiological techniques, together with light microscopy, were employed routinely to confirm that the algal cultures remained axenic. At the end of each heterotrophic experiment, samples of the incubation medium were plated out, aseptically, on Oxoid nutrient agar and on minimal salts agar containing phenol. When counting the test cultures, light microscopy was used to detect contamination during an experiment. Large volumes used to grow *O. danica* for whole cell and enzyme studies were tested for purity by removing a sample (1ml) containing approximately 2×10^5 cells ml^{-1}, prior to harvesting, and filtering it, asceptically, through a 0.2µm membrane and then placing the membrane on nutrient agar and on minimal salts agar containing phenol. Light microscopy was also used to check for contamination, when estimating cell numbers.

2:4 Harvesting of Cells.

Cultures were harvested in the late exponential growth phase by centrifugation at 5,000rpm (4000g) for 5min in a Beckman J2-21 centrifuge using a JA14 (6 x 250ml) rotor at 4°C. The cells were washed twice with cold (0-4°C) 0.2M sodium phosphate buffer, pH 7.2, by centrifugation in a Beckman J2-21 centrifuge using a JA21 (8 x 50ml) rotor at 5,000rpm (3,500g) for 5min. When whole cells were required, the washed cell pellet was resuspended in 5-10ml of washing buffer to a cell density of 1×10^8 cells ml^{-1}, and the cell suspension kept on crushed ice.

2:5 Preparation of Cell-Free Extracts.

Harvested cells were resuspended in 3ml of 0.2M sodium phosphate buffer, pH 7.2, containing 10% (v/v) acetone and lysed by sonication. To assess the success of the cell rupturing procedure, the activity of catechol 2,3-dioxygenase was used as the indicator. Initially, cell suspensions were given 5 x 15s bursts of ultrasound (100w MSE Sonicator) as adopted by Croce (1988) using the same alga, but this was found to produce low enzyme activity in the cell-free extracts. When the length of time for each burst of sonication was reduced to 5s intervals, higher rates of enzyme activity were detected in the extracts; this activity was maximal when 7 x 5s bursts (amplitude, 6μm peak to peak) were used, after which the rate of activity started to decrease again. From this point, all subsequent cell suspensions were pre-cooled in an ice-water bath and given 7 x 5s bursts of ultrasonic exposure. The cell debris was removed by centrifugation at 12,000 rpm (16,500g) for 10min in a Beckman J2-21 centrifuge using a JA21 (8 x 50ml) rotor at 4°C. This cell-free extract was then centrifuged in a Contron ultracentrifuge at 40,000 rpm (100,000g) for 1h, in a TFT 65.13 rotor. The resulting cell-free extract had a protein concentration of approximately 3mg ml^{-1}. Protein content was estimated using Serva Blue G-250 protein reagent (Bradford, 1976), with the calibration curve being prepared using known concentrations of bovine serum albumin dried to a constant weight in a dessicator.

2:6 Substrate Determinations.

2:6:1 *Glucose.*

During heterotrophic growth, samples (1ml) of cultures were taken aseptically and centrifuged (12,000 rpm) in a microfuge (Beckman) for 10min. The pellet was discarded and 0.2ml of supernatant was mixed with 0.2ml of water and the residual glucose in the growth medium was measured by the glucose oxidase method. The reaction involves the conversion of glucose to gluconic acid and H_2O_2 by glucose oxidase, then the peroxide oxidizes *o*-dianisidine by horse radish peroxidase to give a brown oxidation product, the colour of which is proportional to the concentration of glucose (Glucose Determination Kit, Sigma Chemical Co., Poole, Dorset, U.K.). The reaction mixture was developed over a 30min period at room temperature. The resulting solutions were read at 436nm on a DB-7 Beckman spectrophotometer. The test solutions were measured against a reference cell containing double-distilled H_2O in place of the glucose but taken through the same analytical procedure. The calibration curve for the standard glucose solutions was linear over the range of 0-1$\mu mol.ml^{-1}$.

2:6:2 *Phenolic Compounds.*

Two methods were employed to monitor the disappearance of the phenols: (a) colorimetric and (b) chromatographic.

(a) The Folin-Ciocalteau phenol reagent method was used in the analysis of culture media. The culture media was centrifuged at 10,000rpm for 20min at 4°C in a Beckman J2-21 centrifuge using a JA20 (18 x 10ml) rotor. The pellet was discarded and 5ml of supernatant was made alkaline by the addition of 0.75ml of NaHCO$_3$ (200g.l^{-1}). To this solution, 0.25ml of the Folin-Ciocalteau phenol reagent was added and the mixture left to develop at room temperature for 60min (Box, 1983). Solutions were measured at 750nm to determine the amount of phenolic compound present in the medium. Separate calibration curves of standard phenolic solutions were employed for each phenol tested and were linear over the range 0-1μmol.ml^{-1}.

(b) High pressure liquid chromatography (h.p.l.c.) was used both to resolve and determine phenols. Samples of the medium (1ml) were centrifuged in a microfuge (Beckman) at 12,000 rpm for 10min. The pellet was discarded and 0.5ml of the clear supernatant was mixed with 0.5ml of acetonitrile giving a 1ml test sample, of which 20μl was injected into the instrument. The LDC/Milton Roy HPLC system (LDC/Milton Roy, Stone, Staffs, U.K.) comprised a Constametric 3000 delivery system, equipped with a Gilson model 231 autosampler, spectromonitor 3000 variable wavelength u.v. detector set at 270nm and a CI-10B computing integrator. Resolution of the phenolics was effected with a Merck-Hibar Lichrosorb RP-18 column (250 x 4mm i.d.). The isocratic solvent system used in the h.p.l.c. was

normally 60:40 acetonitrile : water, but the ratio was varied when necessary.

2:6:3 *Total Organic Carbon.*

Total organic carbon (T.O.C.) measurements were made to establish that the

progressive disappearance of the substrate measured by h.p.l.c. gave rise to no

accumulating metabolites. Samples (1ml) were removed at intervals, filtered

through 0.2µm membrane to remove any particulate matter, and frozen at   -

20°C. Prior to analysis, the samples were acidified with a drop of concentrated

(85%) phosphoric acid, and sparged with oxygen to remove any dissolved CO_2.

Total organic carbon was measured using a Dohrmann DC-90 Total Organic

Carbon Analyser in combination with the uv-persulphate oxidation method

(Chaussod *et al.*, 1988; Wu *et al.*, 1990). The instrument gave values of 0.43µg C

ml^{-1} of glass double-distilled water and known amounts of phenol were measured

with an accuracy of 98%. The analyser was calibrated with 10ppm C standard

solutions, and phenol standards were analysed to compare the actual against the

theoretical amounts of carbon.

2:7 Studies with Whole Cells.

2:7:1 *Oxygen Uptake Studies.*

(a) Oxygen Electrode Studies:

The rate of oxygen uptake by whole cell suspensions was measured using a Clark-type oxygen electrode (Rank Bros, Bottisham, Cambridge) maintained at 25°C with a circulating water bath and linked to a chart recorder (Kipp and Zonen DB 40).

A suspension of washed cells (prepared according to Method 2:4) was diluted in 0.2M sodium phosphate buffer, pH 7.2, to give a cell density of 3×10^6 cells. ml^{-1}. A 1ml aliquot was removed and filtered through a pre-weighed cellulose-nitrate membrane (0.2μm), the filter was then washed, dried at 37°C for 48h (or until a constant weight) and weighed for calculation of biomass dry weight. The oxygen electrode and recorder were calibrated for full-scale deflection by using air-saturated buffer for setting the 100% air-saturation level and adding a few crystals of sodium dithionite to the solution to record the anoxic (zero) scale reading. A sample of the diluted cell suspension (2.9ml) was placed in the oxygen electrode cell and allowed to equilibrate to the working temperature. The substrate (0.1ml of a 1mM concentration in a neutralised aqueous solution) was then added using a Hamilton glass syringe to give a final concentration in the electrode cell of 33.33μM and the rate of oxygen consumption measured. All results were corrected

for endogenous rates of oxygen uptake by the cells (Newbould, 1987).

(b) Manometric Studies:

The rate of O_2 uptake and the total amount of oxygen used by the whole cell

suspensions was recorded using Warburg respirometric apparatus (Braun,

Melsungen, Germany) by the methods described by Umbreit *et al* (1949) and

Wilson and Goulding (1991). Flask constants were calculated using a nomogram

for manometer constants (Dixon, 1945).

In the main well of the flask, 2.3ml of cell suspension was added, 0.1ml of

20% KOH and a filter paper to increase the CO_2 absorbing area was placed in the

centre well and 0.1ml of the substrate in the side-arm giving a total volume of

2.5ml. The washed cell suspension had been diluted in 0.2M sodium phosphate

buffer, pH 7.2, to give a cell concentration of 3×10^6 cells.ml^{-1}. A cell dry weight

determination was obtained by using the same method as described for the oxygen

electrode studies. The Warburg flasks were attached to the manometer, with taps

open, and allowed to equilibrate to the experimental temperature of 25°C. The taps

were then closed, the manometers adjusted and endogenous respiration briefly

monitored to establish whether any set was leaking. If not, the initial manometer

reading was taken, the substrate then tipped into the main well and the rate of

oxygen utilisation was recorded at 5 or 10min intervals.

2:7:2 *Radioisotopic Studies.*

(a) Radiorespirometry:

The method employed in the manometric studies was also utilised in

experiments involving the mineralisation of [U-^{14}C]phenol (Aldrich) to $^{14}CO_2$. To trap

the $^{14}CO_2$, 0.1ml of 20% KOH was added to the fluted filter paper in the centre well

of the flask (Amy *et al.*, 1985). At timed intervals, individual flasks of an identical

series were sacrificed and samples of the KOH, growth medium and cells were

taken for analysis:

(i) The filter paper containing the KOH was removed and placed into a scintillation

vial. The centre well was then flushed with 2 x 0.2ml of methanol which was added

to the same vial together with 10ml of scintillant, to determine the radioactivity

released as CO_2.

(ii) A sample (0.3ml) of the culture medium was taken and centrifuged in a

Beckman microfuge at 12,000 rpm for 10min to remove cells. 0.2ml of the

supernatant was added to 10ml of scintillant to measure residual dissolved labelled

materials in the culture medium.

(iii) An aliquot (0.5ml) of the incubation medium was taken and filtered through a

cellulose-nitrate membrane (0.2μm) thus trapping the cells. These cells were then

washed by passing 5 x 10ml of cold (0-4°C) double-distilled H_2O through the

membrane filter to remove any external residual soluble substrate. The membrane

was then dried at 37°C for 48h and put into 10ml of scintillant to determine the

amount of label remaining within the cells. All the scintillation vials were then

analysed in a Beckman LS 3801 scintillation counter using Optiphase 'Hisafe' II as the scintillation fluid.

Controls consisted of incubations with boiled cells of the radiolabel to account for any non-specific adhesion of the label to the filter or the cells, and cell suspensions containing no [U-^{14}C]phenol to give the appropriate background readings. Corrections for chemical quenching were made automatically after the scintillation counter was programmed using quenched ^{14}C-standards. Controls for the effect of membranes on counting were carried out, which involved the counting of a known amount of ^{14}C on the membrane, then spiking the same membrane with a ^{14}C-standard ([U-^{14}C]hexadecane) and recounting. Any decrease in the number of counts was due to the quenching caused by the membrane; this was, however, found to be negligible. Each radiorespirometric study was repeated at least three times to ensure the efficacy of the results.

(b) Incorporation of [U-^{14}C]Phenol into Biomass Fractions:

Ochromonas danica was grown in 1l volumes on 1mM [U-^{14}C]phenol containing 1 x 10^6 dpm.l^{-1}. Cells were harvested in the late exponential phase (Method 2:4) and the pellet washed twice. The cell paste was divided into three portions on which different analyses was carried out, as explained by Wills and Phelps (1978), to demonstrate the incorporation of the label into the major constituents of the algal cell:

(i) *Chloroform-Methanol Extraction*: This procedure enabled the extraction of total lipid from the cells (Folch *et al.*, 1951; Robyt and White, 1987). To each 1ml of cell

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paste 9ml of a chloroform-methanol (2:1 v/v) mixture was added and the suspension was then sonicated (10 x 5s; 100w, at an amplitude of 6μm peak to peak) at 0-4°C. The resulting suspension was then stirred at room temperature for 3h and then centrifuged. From the clear organic layer, 0.1ml was taken and mixed with 10ml of scintillation fluid to count the amount of ^{14}C incorporated into the cellular lipid.

(ii) *Protamine Sulphate Precipitation*: This technique was used to extract nucleic acids and measure the amount of ^{14}C incorporated into this cellular fraction (Englardt and Seifter, 1990). Crude cell free extract was obtained from 1ml of cell paste by sonicating (10 x 5s) at 0-4°C. To 1ml of the crude cell free extract 0.1ml of a 10% (w/v) protamine sulphate solution was added and the solution was mixed for 3h at 4°C. The resulting precipitate was collected by centrifugation, washed twice, resuspended and then filtered through a cellulose-nitrate membrane (0.2μm) to collect the precipitate. The trapped precipitate was then washed by passing 5 x 10ml double-distilled water across the membrane which was dried at 37°C for 48h and then added to 10ml of scintillant.

(iii) *Trichloroacetic Acid (TCA) Precipitation*: TCA was used to isolate soluble protein from the algal suspension to measure the label in this cellular component (Ozols, 1990). Crude cell free extract (1ml) was obtained as in (b) above, and to the solution was added 0.2ml of a 20% (w/v) TCA solution and the mixture left to stand on ice for 1h. The resulting precipitate was then collected by centrifugation, washed with cold 0.2M sodium phosphate buffer, pH 7.2, resuspended and filtered through a cellulose-nitrate membrane (0.2μm) which retained the precipitate. The trapped precipitate was then washed by passing 5 x 10ml of double-distilled water

across the membrane which was dried at 37°C for 48h and then placed in 10ml of scintillant.

Controls involved the extraction of the same fractions from cells which had been grown on unlabelled phenol, to give appropriate background values. The samples were analysed in a scintillation counter (Beckman LS 3801) with appropriate adjustments for quenching.

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2:7:3 *Turnover Experiments and Substrate Utilisation.*

Volumes of 20ml of cell suspension (approximately 5×10^6 cells. ml^{-1}) in 0.2M sodium phosphate buffer, pH 7.2, were incubated in a 50ml conical flask at 25°C and shaken at 150 rev/min in a shaking water bath. One flask was used per substrate or combination of substrates to be tested which were added as 0.1ml or 0.2ml of a 50mM stock to give a starting concentration(s) of 250µM or 500µM in each flask, respectively (Newbould, 1987). An aliquot of the working cell suspension was boiled for 15min and used in control incubations in the turnover studies to eliminate the effect of biomass on the substrate(s) concentration or the possible unspecific binding of the phenols to the biomass.

2:7:4 *Anaerobic Turnover Studies.*

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Ochromonas danica was grown aerobically in the dark on 1mM phenol and

harvested in late exponential growth phase to give active cell suspensions. From this point, all solutions with which the cells were brought into contact had been bubbled with oxygen-free nitrogen gas (BOC) to remove O_2 from the solutions.

As with normal turnover experiments (Method 2:7:3), the cell suspension was resuspended in O_2-free sodium phosphate buffer, pH7.2, and diluted to 20ml to give the appropriate cell concentration, but incubated in an anaerobic cabinet (Gallenkamp) at 25°C for 1h. The anaerobic gas (BOC) consisted of 80% N_2, 10% H_2 and 10% CO_2. The cell suspensions were stirred every 15min to consume any residual O_2 before the addition, after one hour, of an O_2-free solution of phenol giving a final concentration of 500µM. A positive aerobic control, which was sampled every 20min, was run simultaneously with the anaerobic incubation to confirm the activity of the cells. The anaerobic flasks were incubated for 2h, stirred every 15min and sampled at 0min and 120min for analysis by h.p.l.c.. These anaerobic cultures were then made aerobic by bubbling air through the cell suspension; they were subsequently incubated at 25°C in air at 150 rpm for a further 2h. A sample of the media was taken every 20min and spun in a microfuge at 12,000 rpm to remove the cells; the supernatant was then analysed for phenol by h.p.l.c..

2:7:5 *Determination of Phenol Hydroxylase Activity.*

The procedure used in the turnover studies (Method 2:7:3) was used to determine putative phenol hydroxylase activity in *O. danica* grown, initially, on

phenol (Pieper *et al.*, 1988; Schmidt *et al.*, 1992). To obtain specific rates, the rate of phenolic disappearance in washed cell incubations was related to the amount of protein present in the biomass used per incubation. The protein content of whole cells was estimated by the method of Spector (1978) after cell lysis in the presence of NaOH (0.3M) for 5min at 95°C (Sander, 1991). The protein concentration of extracts was also measured using the Bradford method (Method 2:5). Cell suspensions were also obtained after growth on succinate and incubated with the phenol and cresols (Method 2:7:3) to show whether or not the enzyme activity was inducible.

2:7:6 *Production of Phenol Hydroxylation Products.*

In this turnover experiment, active cell suspensions were incubated with phenol (250μM) or *p*-cresol (250μM) as described earlier (Method 2:7:3) but in addition 3-chlorocatechol (50-100μM) was added to inhibit the catechol 2,3-dioxygenase in the cells, thus causing the accumulation of the appropriate catechol in the medium (Bartels *et al.*, 1984; Klečka and Gibson, 1981). The disappearance of the phenol substrate and the accumulation of the respective catechol was measured by h.p.l.c. (Method 2:6:2 (b)) with an isocratic solvent system of water:acetonitrile (20:80) and detection at 210nm. The identity of the resulting catechol products was confirmed by h.p.l.c. using authentic catechols as standards. The incubations were terminated after 2-3h, when approximately 75% of the phenolic substrate had been removed.

The putative catechols were extracted from the medium with four 1/3

volumes of ice-cold ethyl acetate. The combined organic fractions were mixed with

anhydrous sodium sulphate to remove water and the dried solutions filtered. The

resulting filtrate was then evaporated to dryness under vacuum leaving yellow

crystals, which were resuspended in 1ml methanol. This solution was analysed by

h.p.l.c. (Method 2:6:2 (b)) and also by t.l.c. using a solvent system of diisopropyl

ether:formic acid:water (200:7:4) against the authentic compounds (Fortnagel *et al.*,

1990). The spots of the compounds were located under uv-light.

2:8 Enzyme Assays.

Enzyme activities were assayed using a Beckman DU-7 spectrophotometer

(Beckman Industries Inc., USA), using quartz cuvettes (1cm light path) at 25°C.

One unit of enzyme activity was defined as the amount of enzyme required to

convert 1μmol of substrate to product in 1min at 25°C, at the pH specified.

2:8:1 *Phenol hydroxylase* (EC 1.14.13.7).

The putative hydroxylase activity was assayed spectrophotometrically by

measuring the decrease in absorbance at 340nm, due to the oxidation of NADH

(Sigma) or NADPH (Sigma) (ϵ_{340} = 6,220), in the presence of phenol. The reaction

mixture contained, in a total volume of 1ml: 870μl of 0.2M sodium phosphate buffer,

pH 7.2; 100µl of cell free extract; 0.15µmol of NAD(P)H; 0.15µmol of phenol. The reaction was started by adding phenol after the endogenous oxidation of NAD(P)H had first been established. The reference cell contained all components except NAD(P)H and phenol.

2:8:2 *Catechol 2,3-dioxygenase*: (Catechol: Oxygen 2,3-oxidoreductase, EC 1.13.11.2).

Activity was determined spectrophotometrically (Sala-Trepat and Evans, 1971) by following the increase in absorbance at 375nm, due to the formation of 2-hydroxymuconic semialdehyde (ε_{375} = 33,500). The reaction mixture contained 895µl of 200mM sodium phosphate buffer, pH 7.2, cell-free extract and water to 1ml. The reaction was started by the addition of 0.15µmol of catechol. The reference cell contained all the components except catechol.

2:8:3 *Catechol 1,2-dioxygenase*: (Catechol: Oxygen 1,2-oxidoreductase, EC 1.13.11.1).

Activity was determined after catechol 2,3-dioxygenase had been destroyed by incubating the cell-free extract with 0.01% (v/v) H_2O_2 for 15min (Nakazawa and Yokota, 1973) excess of which was removed with the addition of 1 unit of catalase. The enzyme was then assayed by following the increase in absorbance at 260nm,

due to the formation of *cis,cis*-muconate (ϵ_{260} = 17,500). Catechol absorbs only weakly at this wavelength (ϵ_{260} = 2000). The reaction mixture contained 665µl of 200mM sodium phosphate buffer, pH 7.2; 1.35µmol of Na_2EDTA, which completely inhibits *cis,cis*-muconate cycloisomerase in the extract (Hegeman, 1966); cell-free extract and water to 1ml. The reaction was initiated by the addition of 0.15µmol of catechol. The reference cell contained all components except catechol.

2:8:4 *Enzymes Metabolising the Ring-Fission Products of Catechol.*

Enzyme activity was determined by measuring the rate of decrease in absorbance at 375nm, due to the utilisation of the keto form of 2-hydroxymuconic semialdehyde, the yellow meta cleavage product of catechol. Two enzymes have been shown to degrade 2-hydroxymuconic semialdehyde: a hydrolase and a NAD^+-dependant dehydrogenase (Catterall *et al.*, 1971; Sala-Trepat and Evans, 1971).

The activities of the dehydrogenase and hydrolase were determined as follows:

(a) *2-Hydroxymuconic semialdehyde dehydrogenase* (2-hydroxymuconic semialdehyde: NAD^+ oxidoreductase; EC 1.2.1 -) was measured by following the decrease in absorbance at 375nm due to the disappearance of this ring-fission product, in the presence of NAD^+ (Sigma). The reaction mixture contained, in a total volume of 1ml; 870µl of 0.2M sodium phosphate buffer, pH 7.2; 50-100µl of cell free extract; 0.1µmol catechol. Catechol was first allowed to react with the cell-free extract until the reaction was complete, (i.e. until the increase in absorbance at

375nm ceased) once the ring-fission product began to slowly decrease (Δ A_{375} $\approx$ 0.0047min^{-1}. determined over 5min), 0.2μmol of NAD$^+$ was added to the reaction mixture and the accelerated decrease in absorbance at 375nm followed. The reference cuvette contained all components except catechol.

(b) *2-Hydroxymuconic semialdehyde hydrolase* (EC 3.7.1 -) was measured by following the decrease in absorbance at 375nm due to the keto form of the substrate, but using extracts which were preincubated with an NADase preparation (from *Neurospora crassa* (Sigma)). In these extracts, endogenous NAD$^+$ in the cell-free extract was destroyed by incubation at room temperature for 1h with an equal volume of NADase preparation containing 0.5 units of enzyme. Control extracts, without the NADase preparation, were similarly incubated in 0.2M sodium phosphate buffer, pH 7.2, showed no significant change in their ability to degrade the ring-fission product. Assay cuvettes contained 890μl of 0.2M sodium phosphate buffer, pH 7.2, 0.1μmol catechol and 25-100μl of NADase-treated extracts in a final volume of 1ml.

2:8:5 *Production of Pyruvate from Catechol by Cell-Free Extracts.*

Active cell-free extracts were prepared (Method 2:5) and incubated with catechol (0.05μmol) until all the yellow coloration, due to the 2-hydroxymuconic semialdehyde, had disappeared. The reaction mixture was then analysed in two ways for the presence of pyruvate:

(a) *L-Lactate dehydrogenase* assay: this enzyme specifically reduces pyruvate to L-

lactate in the presence of NADH (ϵ_{340} = 6,220). The original reaction mixture contained 750µl of 0.2M sodium phosphate buffer, 0.05µmol of catechol and 100µl of extract. When all the semialdehyde had been removed (followed by measuring the absorbance at 375nm), 0.2µmol of $MgSO_4$ and 0.2µmol of NADH were added to the cuvette. When the endogenous oxidation rate of NADH had been established over a 5min period (Δ A_{340} ≈ 0.021min^{-1}.), 0.2 units of LDH was added to the cuvette resulting in a sharp increase in the rate of NADH oxidation. When the rate returned to the endogenous rate, the net overall change in A_{340} was measured as it is correlated with the amount of pyruvate originally present. Catechol (0.05µmol) would generate 0.05µmol of pyruvate equivalent to a change in A_{340} of 0.311 in this assay.

(b) The colorimetric assay developed by Friedemann and Haugen (1942) was also used to determine the production of pyruvate quantitatively. This was achieved by mixing 1ml of sample with 0.33ml of acidic 2,4-dinitrophenylhydrazine and extracting the pyruvate dinitrophenylhydrazone into 1ml of xylene, which is specific for pyruvate 2,4-dinitrophenylhydrazone. THe xylene is then mixed with 2ml of Na_2CO_3 which is then mixed with 1.66ml of NaOH and the resulting colour is measured spectrophotometrically. The calibration curve derived from standard pyruvate solutions was linear over the range of 0-1µmol.ml^{-1}.

2:9 Chemicals

All chemicals used in this research program were of GPR quality and were obtained from BDH unless otherwise stated.

3. RESULTS

3:1 Screening for Putative Heterotrophy in Axenic Cultures of Eukaryotic Algae.

Physiological and biochemical studies of microalgae generally require axenic cultures. When a culture of an alga becomes contaminated, classical isolation techniques may not always yield axenic cultures. Streak and spray plating may yield axenic cultures directly but even algal units isolated with a capillary pipette or a micromanipulator may have associated contaminants. Repeated washing and transfer of algal units with a capillary pipette may yield axenic cultures but if the contaminants adhere to the algal cells, these procedures will not give axenic cultures unequivocally. For those contaminants which cling tenaciously to the algal cells, it may be necessary to kill or dislodge the contaminants *in situ* by chemical or physical methods.

In this study, *O. danica* was found to be contaminated on arrival from the culture collection after bacteria were observed microscopically. This contamination was confirmed by plating the algal culture on nutrient agar and so three methods were used, in combination, to obtain axenic cultures. These were centrifugation, ultrasonic irradiation and antibiotic treatments. To test that an algal culture was axenic, standard microbiological techniques involving the plating out onto nutrient agar and light microscopy were employed. When trying to obtain axenic cultures, it was imperative that all the equipment and chemicals, with which the cultures were

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brought into contact, were sterile.

Ochromonas danica is a naked, motile flagellate, which means that repeated micromanipulation and washing were extremely difficult. Further, the alga was extremely difficult to grow on minimal salts agar and under photoautotrophic, photoheterotrophic and heterotrophic conditions was readily outgrown by the bacterial contaminants. For these reasons the following methods for purifying the alga had to be used.

Before attemping to purify the alga, it was beneficial to understand as much about the contaminant as possible. Using Gram's stain and the hanging-drop method, the contaminant was identified as being a motile, Gram-negative rod/coccus, therefore possibly a pseudomonad. The next step was to find the antibiotics to which the contaminant was susceptible, which was achieved by placing commercial antibiotic 'Multodiscs' onto lawns of the contaminated algal culture plated out on nutrient agar. The bacteria were found to be susceptible to tetracycline, streptomycin and penicillin.

The first step in purification was accomplished by repeated centrifugation and washing of the alga. Algal suspensions were placed in centrifuge tubes and spun until they were only loosely packed after which the supernatant was discarded. The time and speed of the centrifugation were determined by counting the algal cells before and after centrifuging, with the aim of finding some of the algal cells still in the supernatant; this meant that most of the much smaller bacteria would be still suspended. After removing the supernatant the algal cells were resuspended in fresh medium and re-centrifuged; this process was repeated at least three times.

The second stage involved taking the washed algal suspension and treating the suspensions with ultrasonic irradiation. This treatment used a low intensity ultrasonic water bath by which the contaminants were physically displaced from the algal cells. The algal suspensions were then centrifuged, washed and re-sonicated; the process was repeated at least three times. The crucial factor in this treatment was the length of time of the ultrasonic treatment which was determined by microscopic examination of the algal cultures. Since *O. danica* is motile, this factor was used as an indicator of viability; no movement was held to indicate cell damage. It was found that after a 6min burst of the ultrasonic treatment, 20% of the cells were inactive but after 8min, almost 50% of the cells were inactive.

It was unlikely that the algal cells were completely freed of the bacterial contaminants by repeated washings and ultrasonic treatments. To increase the chance of success, a chemical method was incorporated into the protocol. Droop (1967) used antibiotic treatments over 24h to produce axenic cultures. In the present procedure, the above two physical methods, already carried out, were used together with a combination of the antibiotics already mentioned. A range of antibiotic concentrations were prepared from a stock solution containing 100mg of penicillin G and 50mg of streptomycin sulphate dissolved in 10ml of double-distilled water. To 1ml of 95% ethanol, 50mg of tetracycline was added and both antibiotic solutions were mixed and filter sterilised. To 19ml of minimal salts medium containing 10mg of yeast extract, the following volumes of antibiotic solutions were mixed with 1ml of the washed and ultrasonicated cultures: 0, 0.125, 0.25, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4ml. After 12h, 24h, 36h, and 48h samples of each were re-inoculated into sterile, antibiotic-free medium and incubated in a lit incubator at

25°C. The tubes were incubated for 2-3 weeks, when they were tested for axenicity by plating samples onto nutrient agar and minimal salts agar containing glucose, succinate or pyruvate and subcultured into fresh sterile-phototrophic medium containing carbonate. Viable cultures were acquired from the flasks which contained 1-2.5ml of antibiotics. These axenic algal cultures were ready to begin biochemical experimentation.

Cultures of *C. ulvaensis*, *S. brasiliensis* and *O. danica* were dark-grown on the following carbon sources (1mM): acetate, glucose, pyruvate and succinate (Figures 3.1-3.3). Growth was monitored over 10d in an orbital incubator at 25°C and run continuously at 100rpm. All three cultures exhibited heterotrophic growth on all of the carbon sources. However, *O. danica* grew more vigorously and achieved higher cell concentrations than *C. ulvaensis* and *S. brasiliensis*. This phenomenon may be because *O. danica* is a more nutritionally versatile organism and not as dependant upon photosynthesis as a source of energy, as are most green algae. In all three cases when a no substrate control was monitored, some growth was recorded initially but this ceased after the second day of the incubation. To continue the screening procedure, the three algae were introduced to phenol (500μM) under two different growth conditions. In the first study, phototrophic growth and phenolic disappearance were measured (Figures 3.4-3.6). Photosynthetic growth with no phenol was the control against which phototrophic growth with phenol was compared. *C. ulvaensis* and *S. brasiliensis* showed little difference in growth between the photosynthetic and the putative photoheterotrophic cultures. This corresponded with the negligible decrease of the phenol from the media (Figures 3.4-3.5). However, *O. danica* exhibited a marked difference

Figure 3.1: Heterotrophic Growth of *Chlamydomonas ulvaensis* on Non-Aromatic Substrates.

C. ulvaensis was incubated with no substrate (o), 1mM acetate (■), 1mM glucose (□), 1mM pyruvate (▲), and 1mM succinate (▲) at 25°C and 100rpm in the dark. Cell numbers were enumerated microscopically. Log N measured as cells ml⁻¹.

Figure 3.2: Heterotrophic Growth of *Scenedesmus brasiliensis* on Non-Aromatic Substrates.

S. brasiliensis was incubated with no substrate (o), 1mM acetate (■), 1mM glucose (□), 1mM pyruvate (▲), and 1mM succinate (▲) at 25°C and 100rpm in the dark. Cells were enumerated microscopically. Log N measured as cells ml⁻¹.
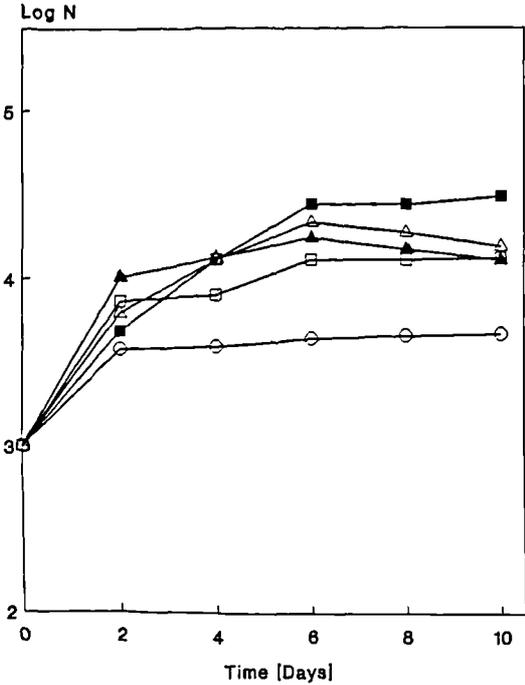
Figure 3.1

Figure 3.2
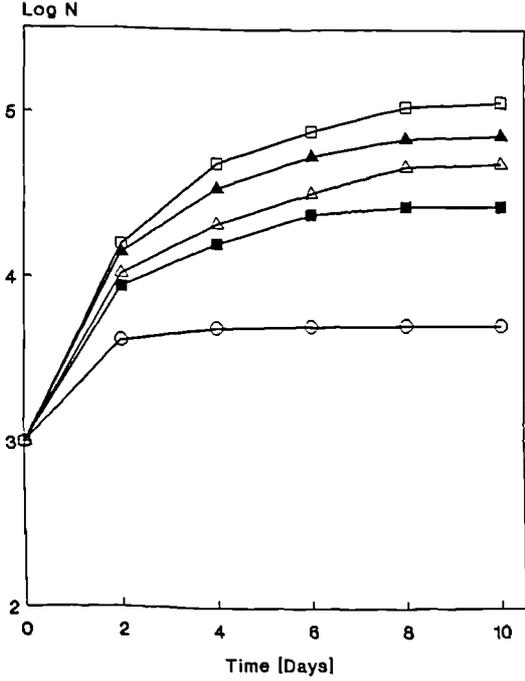
Figure 3.3: Heterotrophic Growth of *Ochromonas danica* on Non-Aromatic Substrates.

O. danica was incubated with no substrate (o), 1mM acetate (■), 1mM glucose (□), 1mM pyruvate (▵), and 1mM succinate (▴) at 25°C and 100rpm in the dark. Cells were enumerated microscopically. Log N measured as cells ml^{-1}.

Figure 3.3
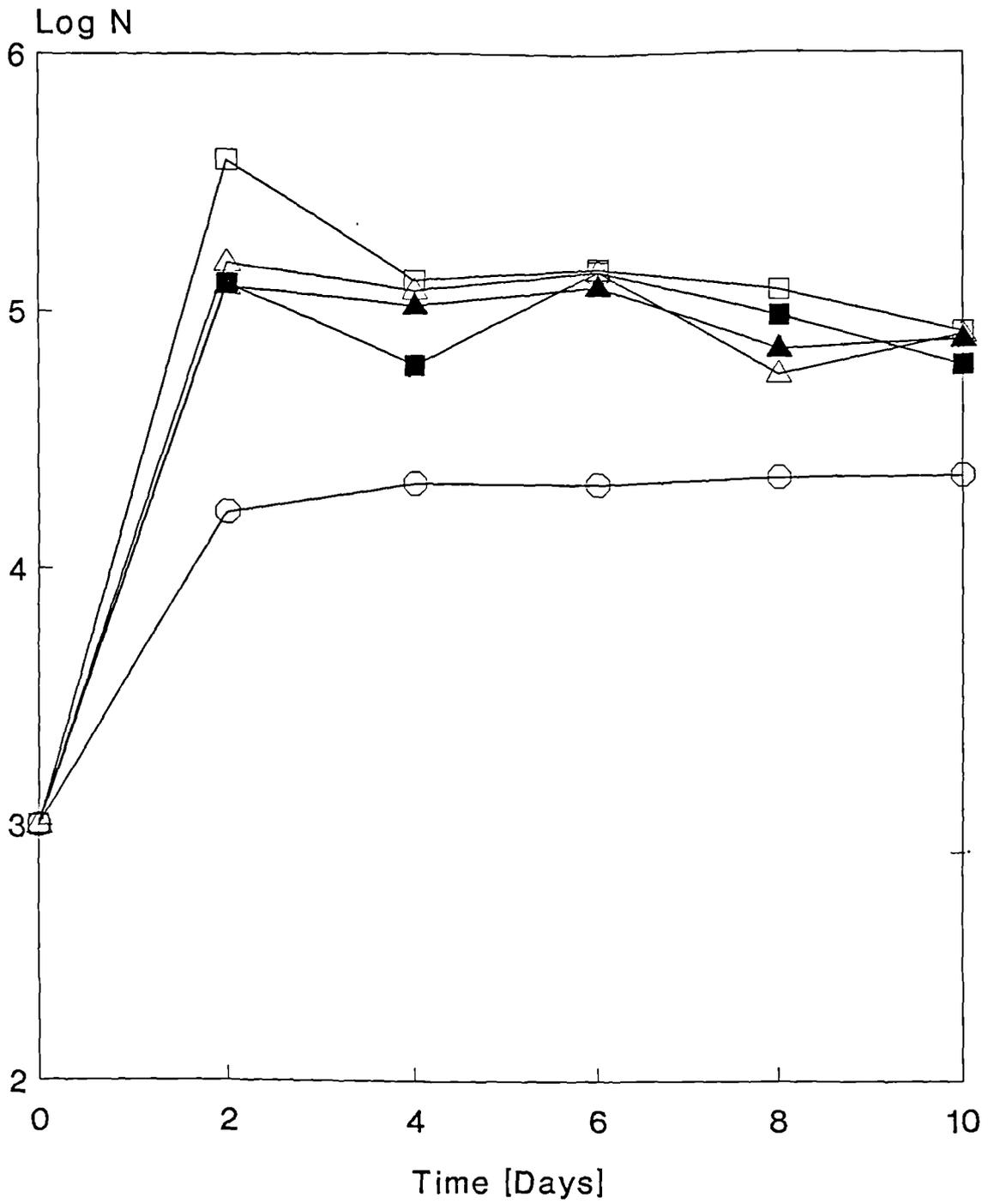
Figure 3.4: Photoheterotrophic Growth of *Chlamydomonas ulvaensis* in the Presence of Phenol.

C. ulvaensis was incubated in the light in the absence (△) and presence of 500µM phenol (▲) at 25°C and 100rpm. The removal of the phenol was monitored with boiled cultures (□) and vital cultures (■). Log N measured as cells ml^{-1}.

Figure 3.5: Photoheterotrophic Growth of *Scenedesmus brasiliensis* in the Presence of Phenol.

S. brasiliensis was incubated in the light in the absence (△) and presence of 500µM phenol (▲) at 25°C and 100rpm. The removal of the phenol was monitored with boiled cultures (□) and vital cultures (■). Log N measured as cells ml^{-1}.

Figure 3.4

Figure 3.5

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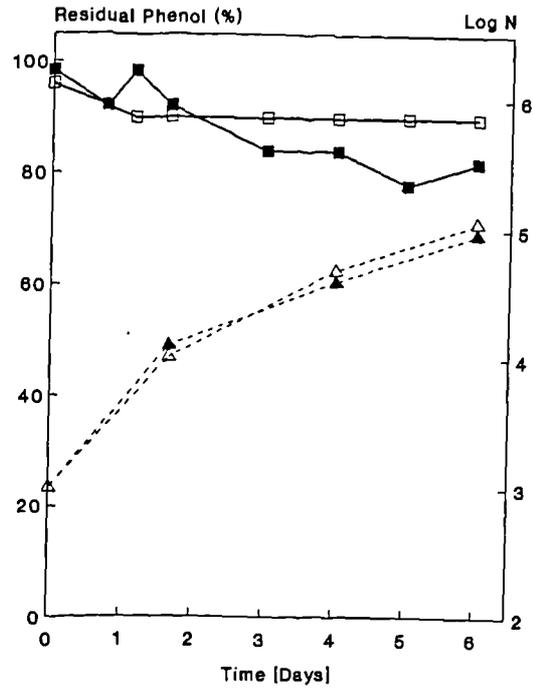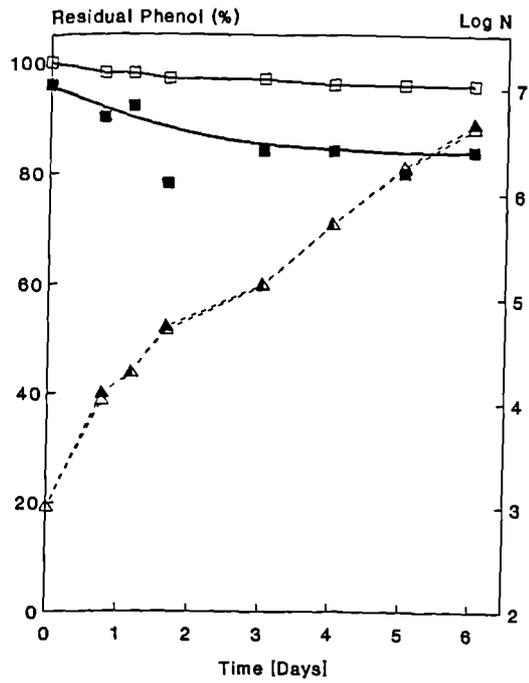
Figure 3.6: Photoheterotrophic Growth of *Ochromonas danica* in the Presence of Phenol.

O. danica was incubated in the light in the absence (▵) and presence of 500µM phenol (▴) at 25°C and 100rpm. The removal of the phenol was monitored with boiled cultures (□) and vital cultures (■). Log N measured as cells ml^{-1}.

Figure 3.6
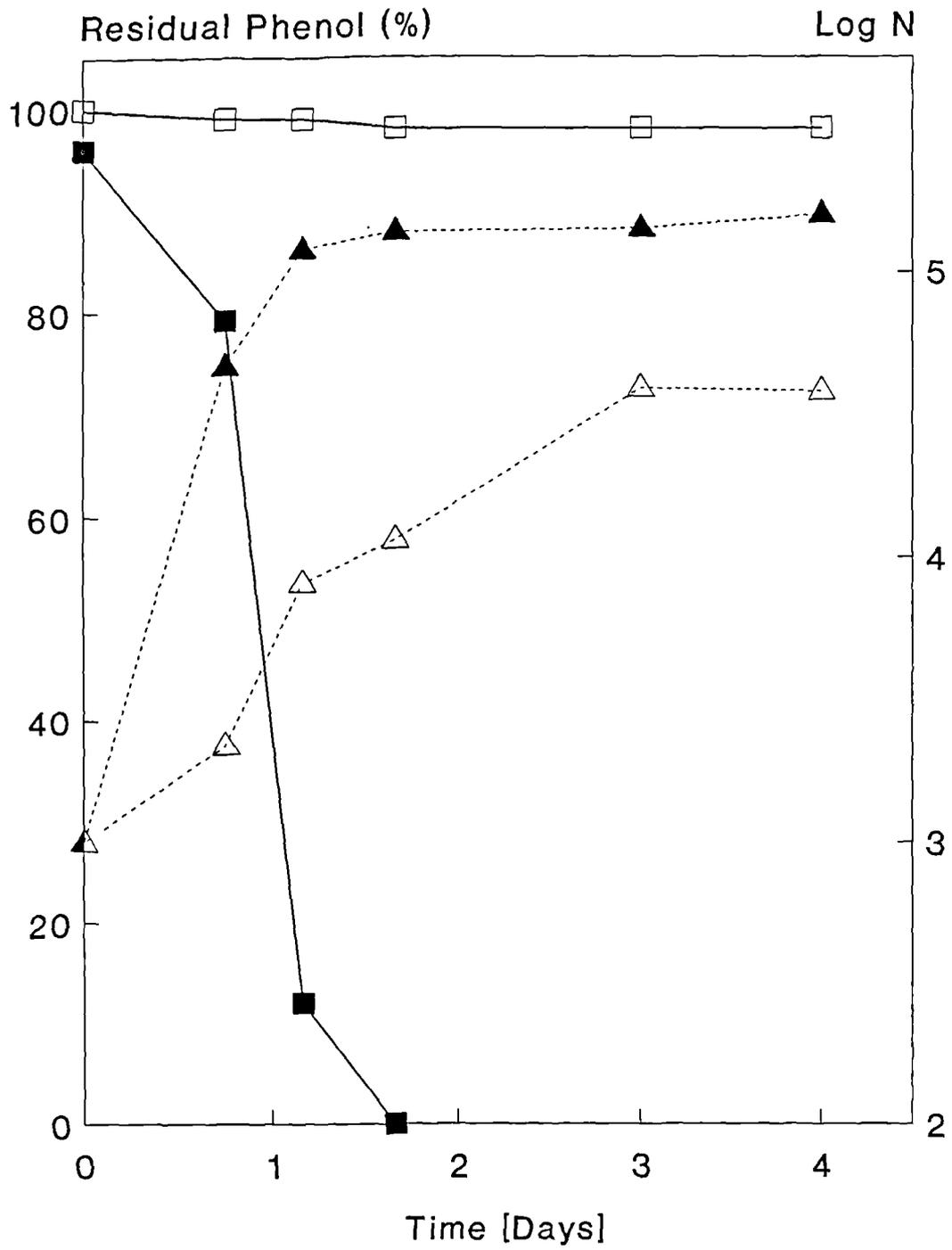
between the photosynthetic and photoheterotrophic cultures, both in the growth rate

and cell density (Figure 3.6). Growth in the presence of phenol corresponded to

the complete disappearance of the substrate after two days. The second growth

condition was carried out in the dark on 1mM glucose in the presence of phenol

(500µM) with growth and phenolic removal recorded. All three species grew

heterotrophically on glucose with the phenol at the same rates as the controls

which were grown on glucose alone (Figures 3.7-3.9). Both *C. ulvaensis* and *S.*

brasiliensis failed to remove the phenol from the media to any great extent. Only

O. danica exhibited the ability to remove the phenol from the growth medium

(Figure 3.9). When the phenol had disappeared, the cultures were reinoculated

with Jaworski's medium constituents and phenol but no glucose was added. The

result was a renewed removal of phenol, but this time the process occurred more

rapidly. On this second infusion of phenol, an increase in cell numbers was

recorded, but nowhere as great as that initially. This lack of cell biomass can be

attributed to the absence of glucose. Through this screening procedure, *O. danica*

appeared to be a competent heterotroph with putative aromatic degradative

capabilities. For these reasons, *O.danica* was selected for the further investigation

of aromatic biodegradation.

Figure 3.7: Heterotrophic Growth of *Chlamydomonas ulvaensis* on Glucose and Phenol.

C. ulvaensis was incubated in the dark with no carbon source (▲), on 1mM glucose (▵), and 1mM glucose plus 500μM phenol (○) at 25°C and 100rpm. The removal of the phenol was monitored with boiled (□) and vital cultures (■). Log N measured as cells ml⁻¹.

Figure 3.8: Heterotrophic Growth of *Scenedesmus brasiliensis* on Glucose and Phenol.

S. brasiliensis was incubated in the dark with no carbon source (▲), on 1mM glucose (▵), and 1mM glucose plus 500μM phenol (○) at 25°C and 100rpm. The removal of the phenol was monitored with boiled (□) and vital cultures (■). Log N measured as cells ml⁻¹.

Figure 3.7
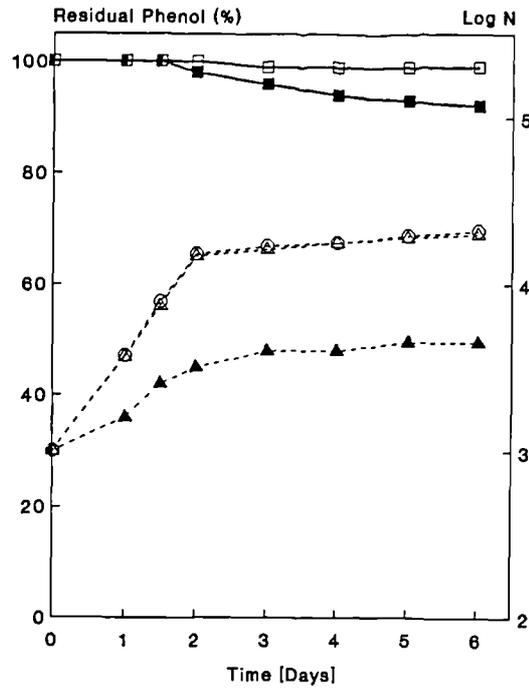
Figure 3.8
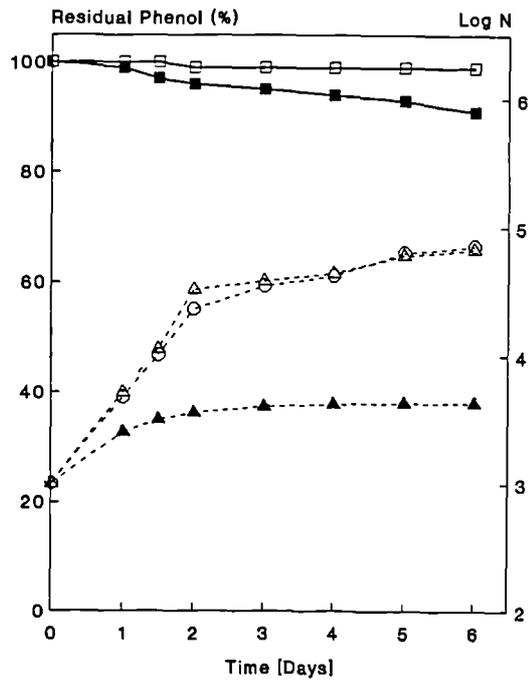
Figure 3.9: Heterotrophic Growth of *Ochromonas danica* on Glucose and Phenol.

O. danica was incubated in the dark with no carbon source (▲), 1mM glucose (△), and 1mM glucose plus 500µM phenol (○) at 25°C and 100rpm. The removal of the phenol was monitored with boiled cultures (□) and vital cultures (◊). After all the phenol had been removed, the cultures were reinoculated with fresh substrates (↓), and growth and the removal of the phenol were measured. Log N measured as cells ml^{-1}.

Figure 3.9
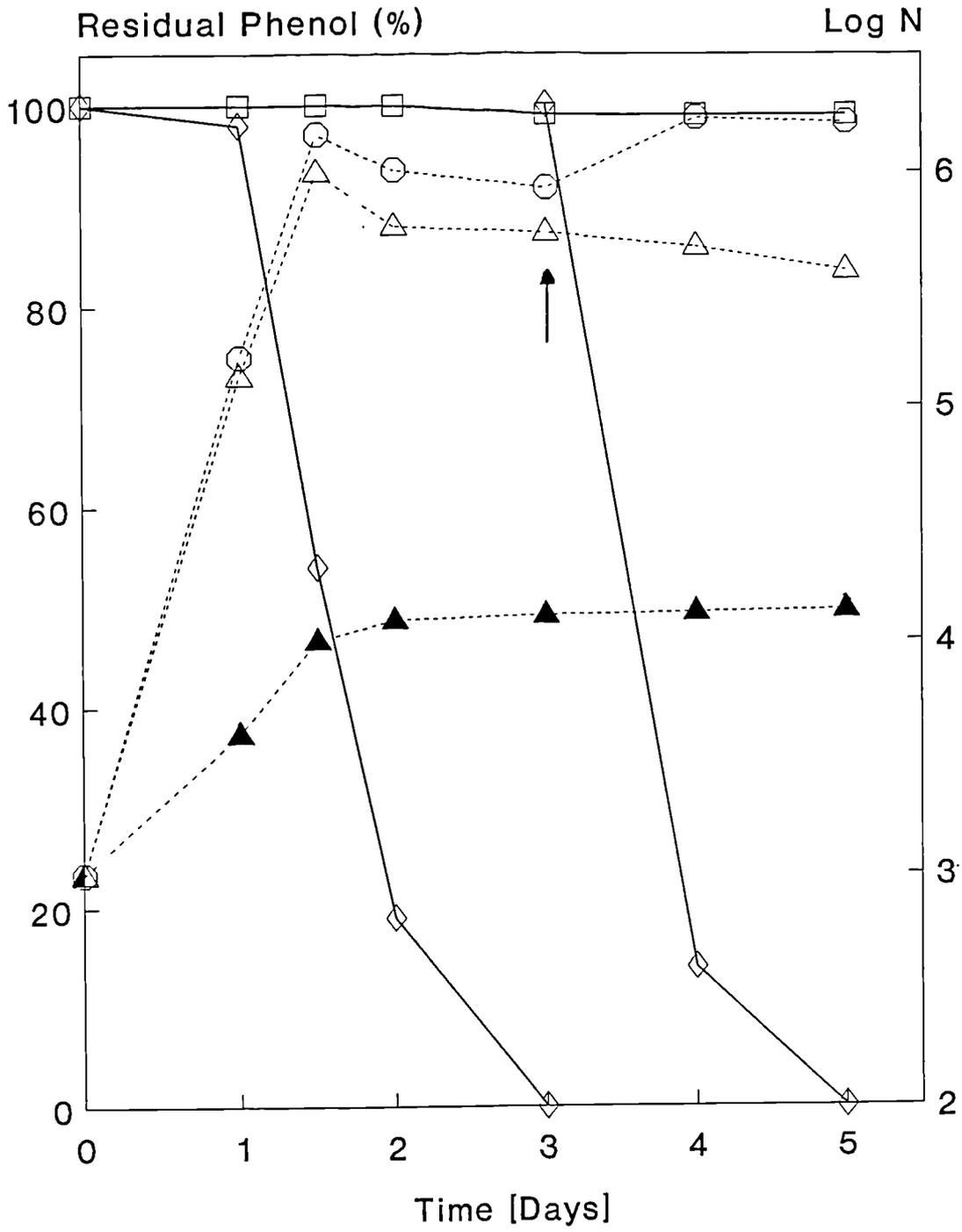
3:2 Growth and Metabolism Studies on *Ochromonas danica*.

3:2:1 *Effect of Temperature on Heterotrophic Growth.*

Having found a heterotrophically competent alga, *O. danica*, it was important

to find its optimum temperature for heterotrophic growth. A temperature range from

10°C to 35°C at 5°C intervals was adopted, with 2mM glucose as the carbon

source. Algal growth was monitored until stationary phase was achieved and all

the glucose utilised (Figure 3.10). Growth and the corresponding substrate

utilisation was recorded at all temperatures, with the most rapid occuring at 25°C

and the slowest at 10°C. From this experiment, it was decided that 25°C would be

the temperature best suited for further heterotrophic studies involving *O. danica*.

3:2:2 *Effect of Light Intensity on Photoheterotrophic Growth.*

Results from previous heterotrophic studies have shown that *O. danica* is

capable of growing on a carbon source in both light and dark regimes. To examine

whether there was a difference in the rates of growth in the light and the dark, and

the effect of varying light intensity on the growth rate of the alga, *O. danica* was

incubated at 25°C on 1mM glucose in an illuminated orbital incubator with multiple

bulbs, developing 0, 21, 64, 107, and 150μmol photons m^{-2}.

Figure 3.10: Effect of Temperature on the Growth of *O. danica*.

(a) *O. danica* was incubated in the dark on 2mM glucose at 100rpm at different temperatures: 10°C (□); 15°C (▲); 20°C (■); 25°C (●); 30°C (▵) and 35°C (o). Log N measured as cells ml^{-1}.

(b) The removal of the glucose (2mM) was monitored at 10°C (□); 15°C (▲); 20°C (■); 25°C (●); 30°C (▵) and 35°C (o).
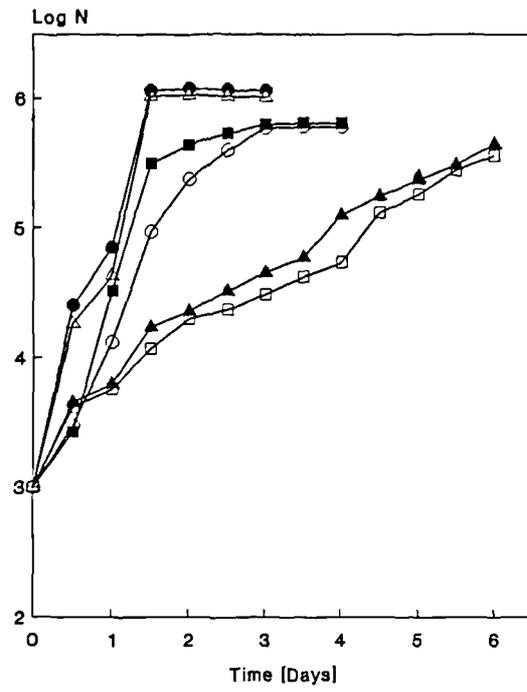
Figure 3.10a

Figure 3.10b
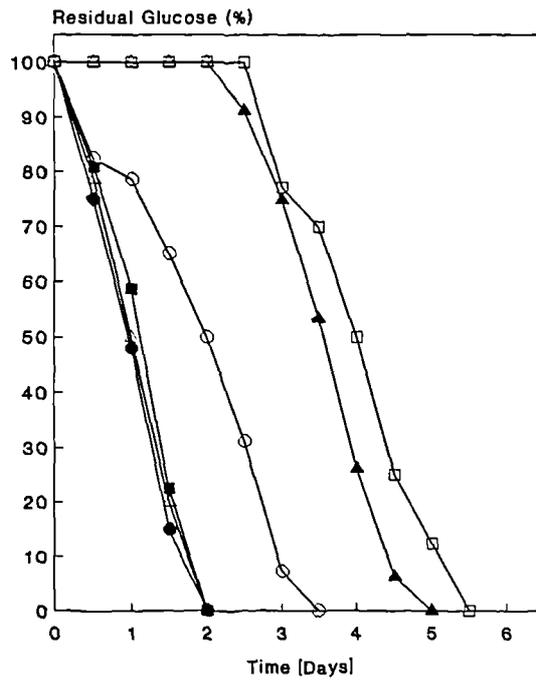
s^{-1}. Growth was monitored until stationary phase was achieved by the alga and was found to be similar in all cases including dark growth (Figure 3.11).

3:2:3 *Heterotrophy on Glucose in the Presence of Phenols.*

In the screening procedure described previously (3:1), *O. danica* was subjected to photoheterotrophic and dark heterotrophic conditions in the presence of phenol. In a second experiment, the alga was grown in the presence of phenol (500μM) or *p*-cresol (500μM) under three conditions: light, light with supplementary glucose (1mM) and dark with supplementary glucose (1mM), where growth and the removal of the phenol were recorded (Figures 3.12-3.14). The findings corroborated those obtained in the screening procedure but the results from the photoheterotrophic study with glucose and phenols were found to be similar to those found in the dark regime. In all three conditions additional phenol was reintroduced after it had disappeared and once again the phenol was removed at a faster rate on the second addition. The difference in the rates of removal of phenol suggested that, initially, the mechanism for the phenol removal was inducible and that on the addition of the second dose the enzymes were already active thus reducing the half-life of the aromatic substrate. Having established that *O. danica* can grow on glucose and remove phenol from the growth medium, it was important to know if this removal was carried out simultaneously with that of the glucose or whether the alga removed the co-substrates sequentially. In an experiment in which the growth and the removal of both the substrates were recorded

Figure 3.11: Photoheterotrophic Growth of *Ochromonas danica* at Different Light Intensities.

O. danica was incubated with 1mM glucose in the dark (■), with 21 (□), 64 (▲), 107 (▵), and 150 μmol photons $m^{-2}. s^{-1}$. (○) at 25°C and 100rpm. Log N measured as cells ml^{-1}. Log N measured as cells ml^{-1}.

Figure 3.11
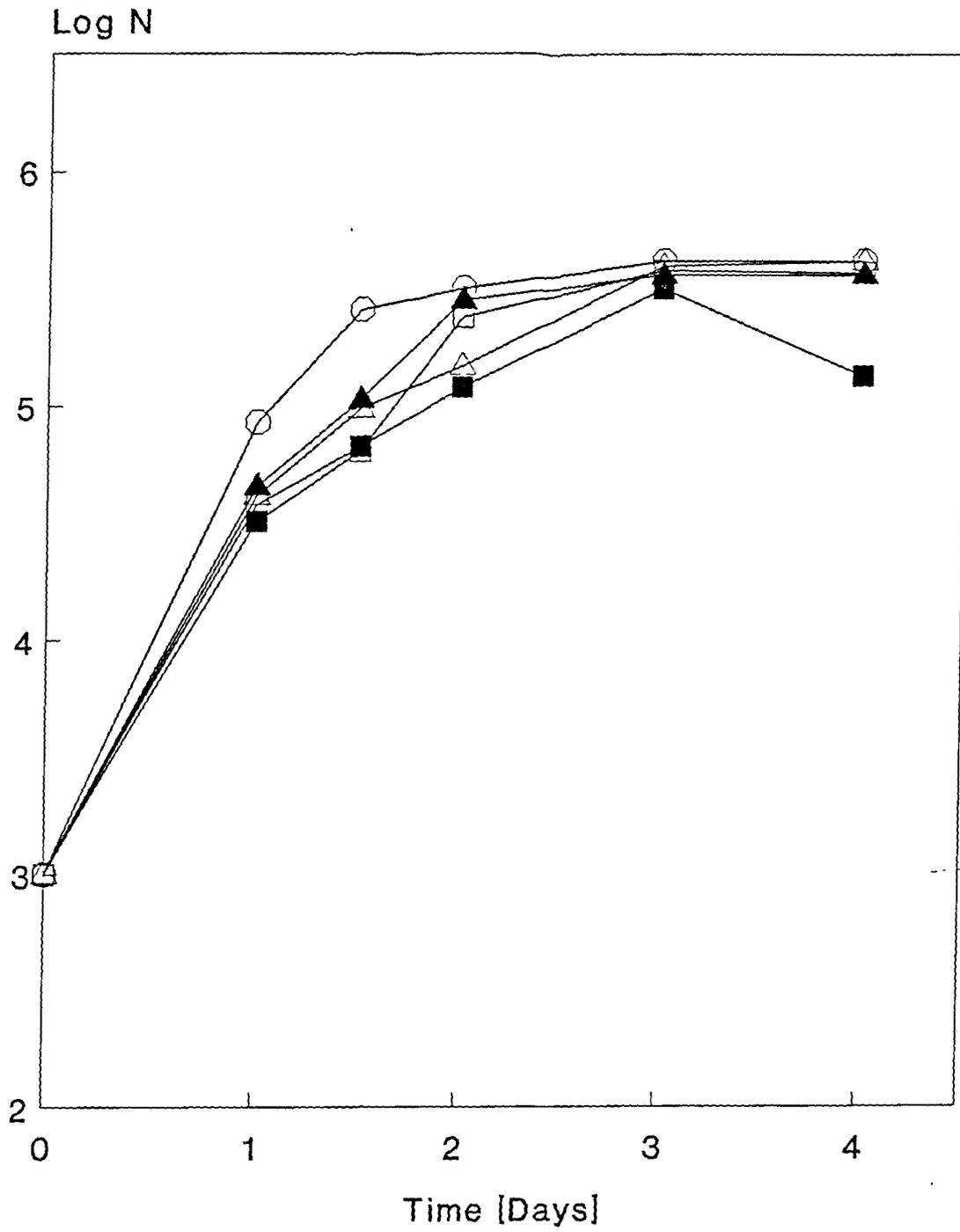
Figure 3.12: Photoheterotrophic Growth of *Ochromonas danica* on Phenol and *p*-Cresol.

O. danica was incubated in the light with no organic carbon source (▲), on 500μM phenol (▵), and 500μM *p*-cresol (o) at 25°C and 100rpm. The removal of the phenol (□) and the *p*-cresol (◊) was monitored. When all the substrates had been removed, the cultures were reinoculated with fresh phenolics (↓). Log N measured as cells ml^{-1}.

Figure 3.12
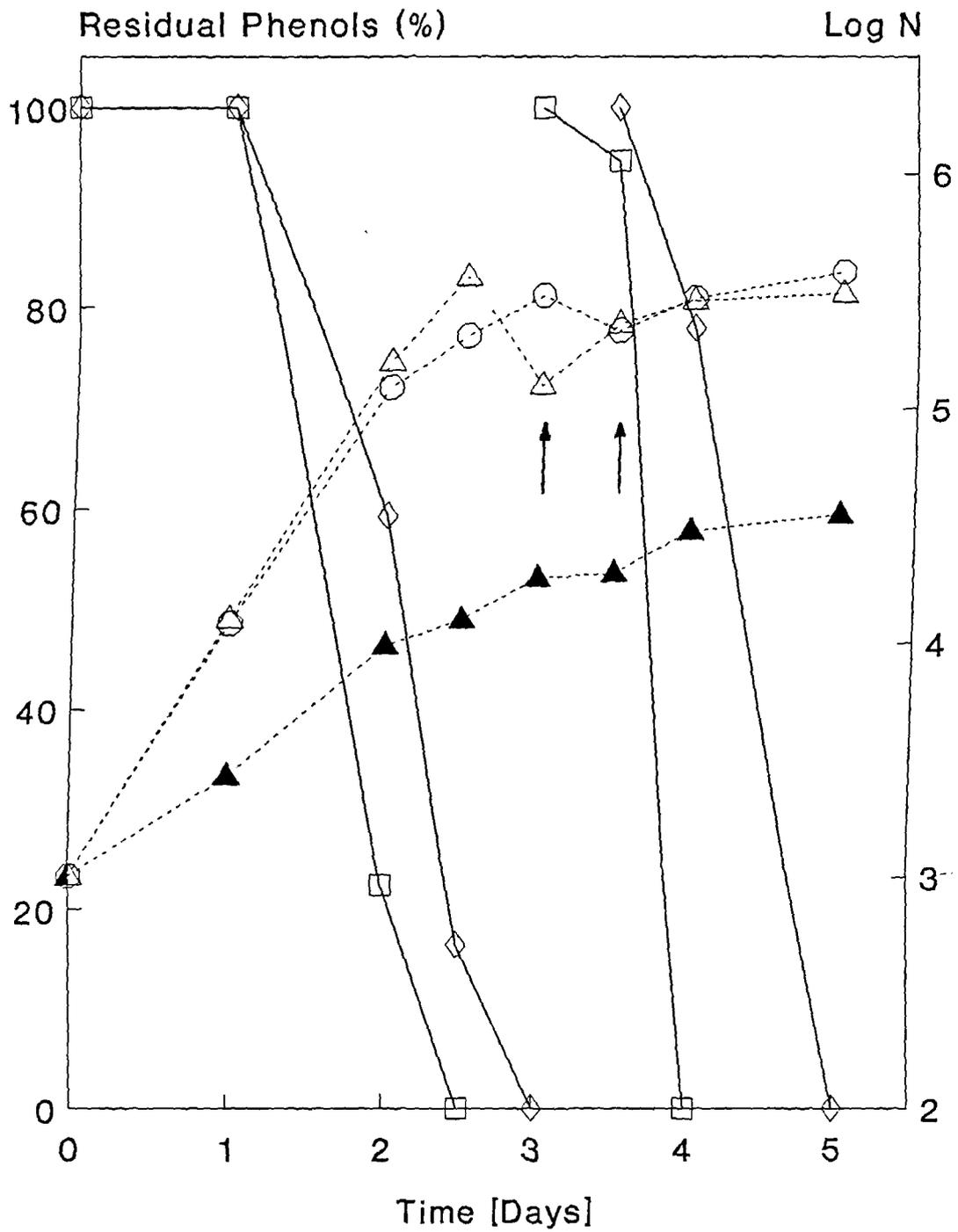
Figure 3.13: Photoheterotrophic Growth of *Ochromonas danica* on Glucose and Phenol or *p*-Cresol.

O. danica was incubated in the light with 1mM glucose (▲), 1mM glucose plus 500μM phenol (▵), and 1mM glucose plus 500μM *p*-cresol (o) at 25°C and 100rpm. The removal of the phenol (□) and the *p*-cresol (◊) was monitored. When all the substrate had been removed, the cultures were reinoculated with fresh phenolics (↓). Log N measured as cells ml⁻¹.

Figure 3.14: Heterotrophic Growth of *Ochromonas danica* on Glucose and Phenol or *p*-Cresol.

O. danica was incubated in the dark on 1mM glucose (▲), 1mM glucose plus 500μM phenol (▵), and 1mM glucose plus 500μM *p*-cresol (o) at 25°C and 100rpm. The removal of the phenol (□) and the *p*-cresol (◊) was monitored. When all the substrate had been removed, the cultures were reinoculated with fresh phenolics (↓). Log N measured as cells ml⁻¹.

Figure 3.13
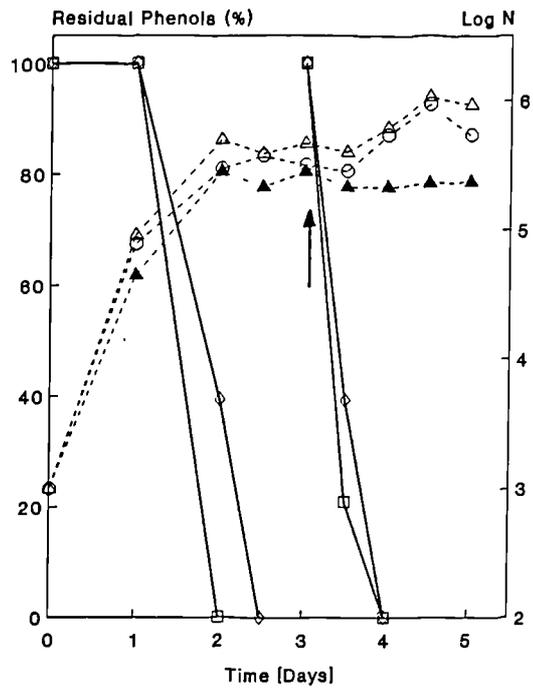
Figure 3.14
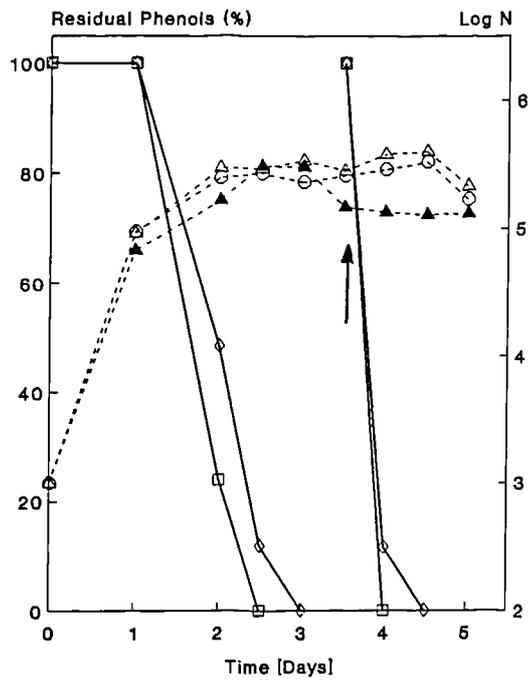
(Figure 3.15), *O. danica* removed glucose more rapidly than the phenol, with the rate of phenol removal accelerating when most of the glucose was exhausted.

3:2:4 *Heterotrophy on Phenols as the Sole Carbon Source.*

Having established that *O. danica* could remove phenol while growing on glucose in both light and dark conditions, the next stage was to establish whether the alga could grow on phenol as the sole carbon source. In previous studies, the alga was found to show some initial growth in the absence of any carbon. In this experiment the alga was incubated in the dark with no carbon. The aim was to reduce this endogenous growth and to see if this had any effect on the growth of the alga on phenol. The alga was incubated with 1mM phenol only (Figure 3.16) and was found to grow on and remove the substrate.

Verification of the alga's ability to metabolise phenol was provided by a combined experiment using T.O.C. and h.p.l.c. to monitor the disappearance of 1mM phenol (Figure 3.17). This experiment showed that not only was there removal of phenol, but there was also a corresponding decrease in the total organic carbon (ppm C) of the growth medium confirming the absence of accumulating metabolites. However, although the h.p.l.c. measured phenol as totally disappeared, the T.O.C. value plateaued at approximately 10ppm C. The T.O.C. values recorded for the no substrate control, however, showed an increase which reached 10 - 15ppm C, suggesting that the alga was excreting carbon products from lysis or its metabolic processes, which would account for the residual T.O.C.

Figure 3.15: Heterotrophic Growth of *Ochromonas danica* on Glucose and Phenol.

O. danica was grown in the dark on 2mM glucose (o) and 2mM glucose plus 500μM phenol (●) at 25°C and 100rpm. The removal of both glucose (□); glucose (▵), mixed with phenol; and phenol (▴), mixed with glucose was monitored. Log N measured as cells ml^{-1}.

Figure 3.15

Residual Substrate (%)   Log N
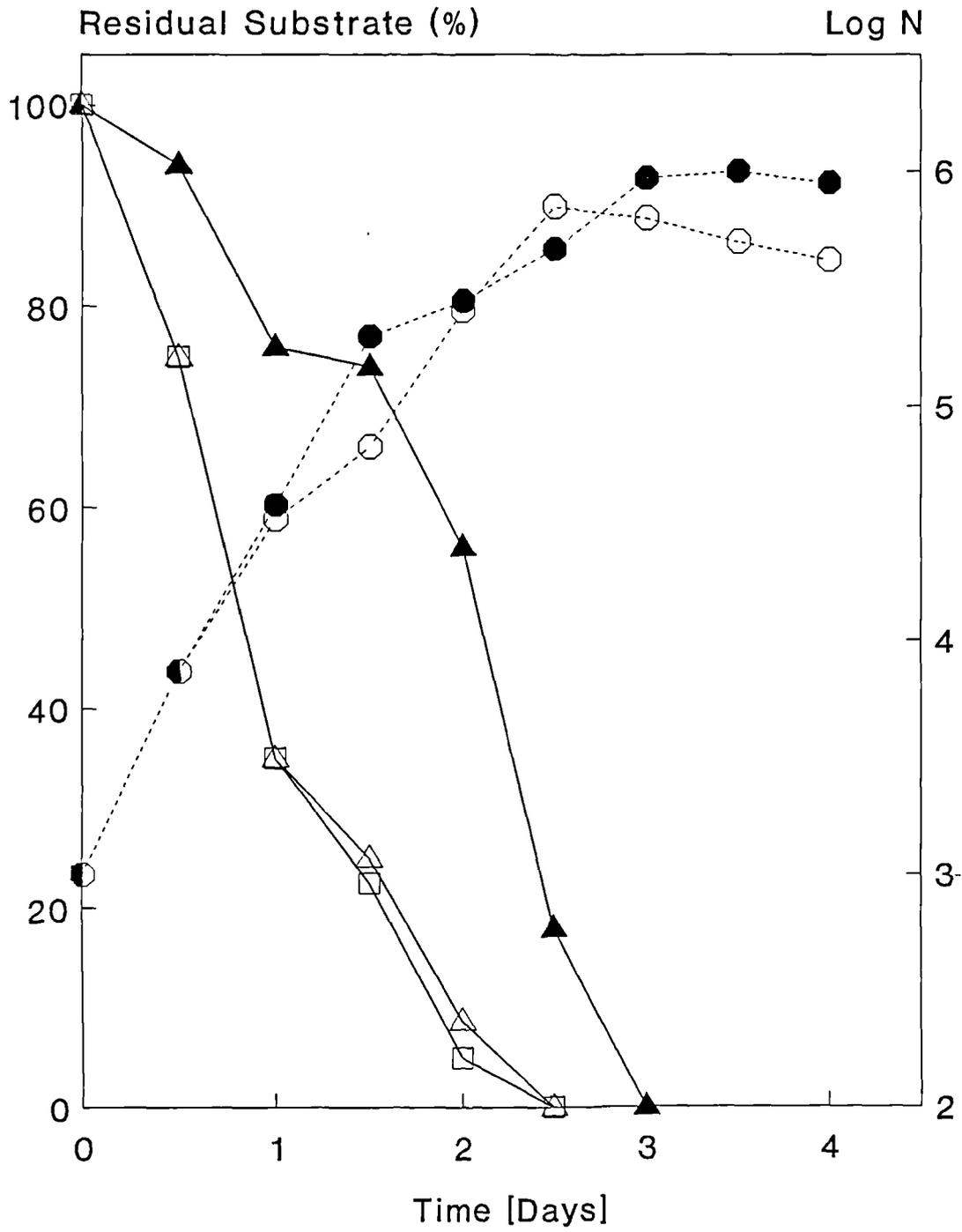
Figure 3.16: Heterotrophic Growth of *Ochromonas danica* on Phenol as the sole carbon source.

Starved cultures of *O. danica* were incubated in the dark at 25°C in the presence (o) and absence (☐) of 1mM phenol, with the phenolic disappearance being monitored by h.p.l.c (●). Log N measured as cells ml^{-1}.

Figure 3.16
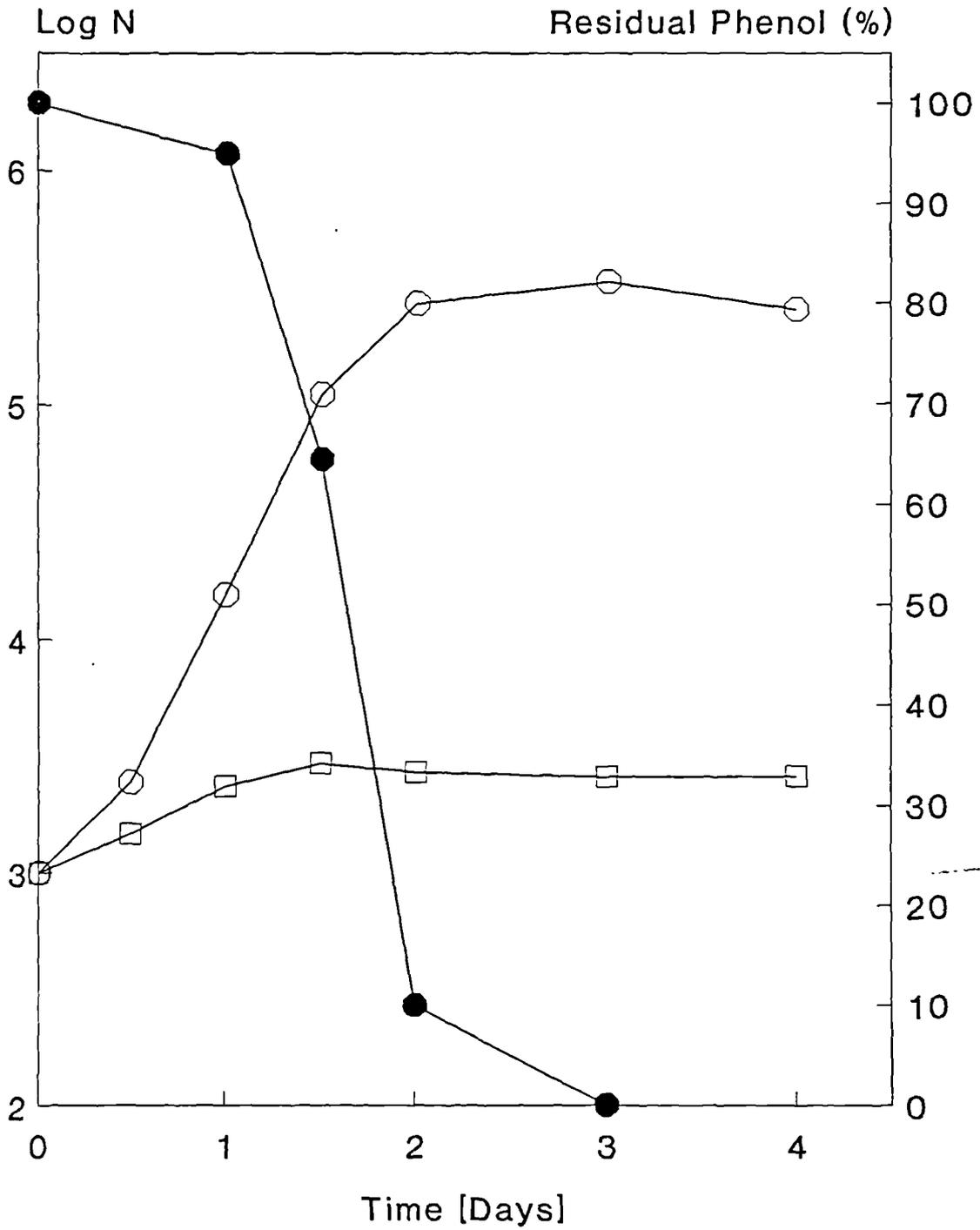
Figure 3.17: Removal of Phenol as the Sole Carbon Source by *Ochromonas danica*.

O. danica was incubated in the dark at 25°C and 100rpm in the presence and absence of phenol as the sole carbon source. The removal of the carbon source was monitored from two aspects: TOC[*] (●), and h.p.l.c. (▲). The no substrate control was sampled and tested with TOC (□).

[*] Total Organic Carbon (ppm C).

Figure 3.17
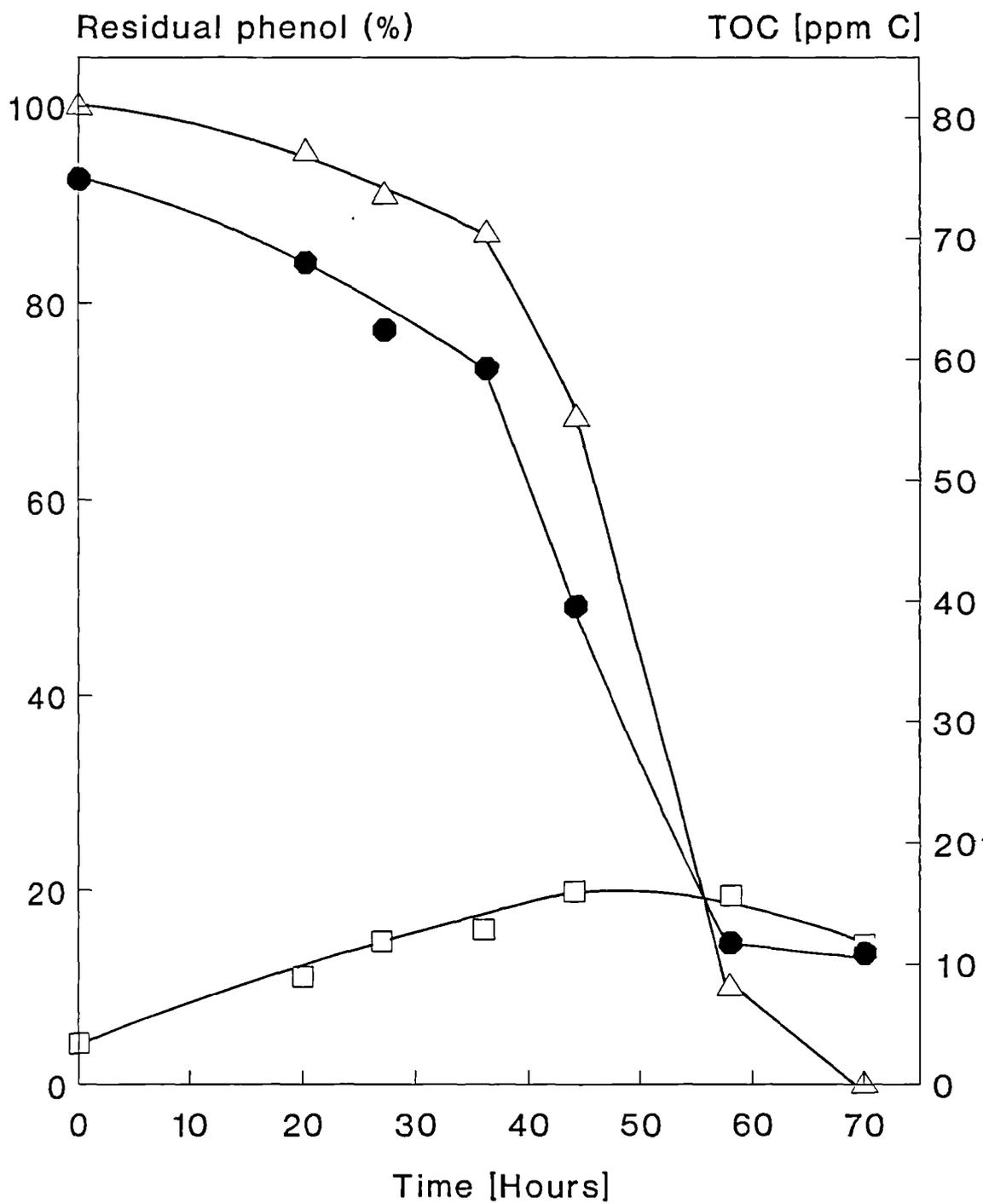
carbon remaining in the phenol flask.

The next stage in this investigation was to elucidate the biodegradative capabilities of this organism against various concentrations and mixtures of phenols in both light and dark conditions. From data already accumulated, it appeared that the alga grew and utilised organic substrates with a high degree of similarity in both the light and dark. Therefore, data will only be presented concerning dark, heterotrophic studies unless differences were found between light and dark incubations.

The primary objective was to establish whether or not the alga could grow on a range of phenol concentrations as the sole carbon source. The alga was incubated in light and dark conditions on a range of phenol concentrations (500μM- 4mM) with growth and the removal of the substrate being monitored. Figure 3.18 shows the growth of the alga and the removal of phenol in the dark. The growth of the alga corresponded to the disappearance of the phenol; the higher the concentration, the longer it took the alga to remove the substrate and the subsequent removal rate were slower. In both light and dark conditions growth patterns of the alga were found to be similar resulting in a rough doubling (above the control) for 0.5mM - 1mM, but growth on 2, 3 and 4mM were the same as for 1mM giving approximately 3.2×10^5 cells ml^{-1}. The reason for this phenomenon was never discovered, but with the increase in concentration the alga seemed to be able to maintain its cell numbers for a longer time.

Ochromonas danica was also exposed to other more recalcitrant phenolics: o-cresol and p-cresol, as the sole carbon source. The alga was incubated on a range of concentrations of the two isomers of cresol (500μM- 4mM) in the dark,

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Figure 3.18: Heterotrophic Growth of *Ochromonas danica* on a Range of Concentrations of Phenol.

(a) *O. danica* was incubated in the dark on no carbon source (□), and on phenol: 500µM (■), 1mM (▵), 2mM (▴), 3mM (o), and 4mM (●) at 25°C and 100rpm. Log N measured as cells ml⁻¹.

(b) The removal of the substrate was monitored: 500µM (■), 1mM (▵), 2mM (▴), 3mM (o), and 4mM (●).

Figure 3.18a
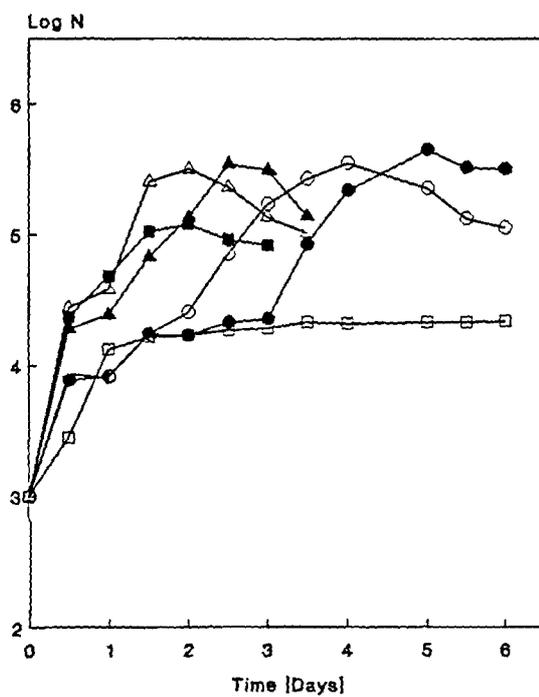
Figure 3.18b
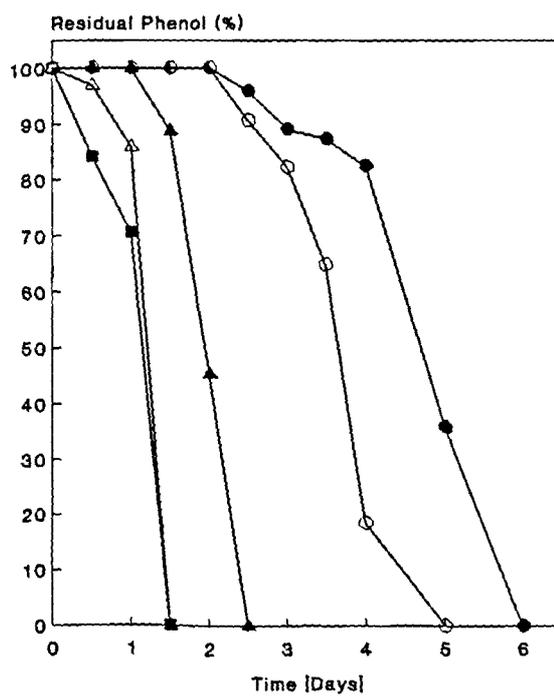
with growth and the removal of the substrates being recorded. In the case of *o*-cresol, growth was not achieved after a 14d incubation, whereas the alga was able to grow on and remove the *p*-cresol at all the concentrations after 13d (Figure 3.19). As with the growth on phenol at a range of concentrations (Figure 3.18), the alga exhibited the same growth patterns on 2, 3 and 4mM producing similar biomass yields. To give a more complete picture, the alga was also incubated with the 2,5-; 2,6-; 3,4- or 3,5-xylenols (500μM). These substrates were not used by the alga for growth and were not removed from the growth medium.

3:2:5 *Heterotrophy on Phenol with a Secondary Substrate.*

Ochromonas danica was found to grow readily on phenol, with growth on *p*-cresol and *o*-cresol becoming increasingly more difficult and the xylenols resistant to degradation. The next stage was to discover if the alga could increase its ability to remove the more recalcitrant phenols by incubating them together with phenol. To achieve this, the growth of the alga and its rate of removal of the individual substrates in mixtures were monitored. In the first incubations in both light and dark conditions, the alga was grown on phenol (1mM) plus *o*-cresol (500μM) or *p*-cresol (500μM) and its activity compared against the phenol-only control. In these experiments, only the dark data are shown (Figure 3.20). Phenol was removed most rapidly in all the incubations, but the rate of removal of phenol was reduced in the presence of *p*-cresol and even more severely affected with *o*-cresol. The cresols were removed after the phenol with *p*-cresol being removed faster than

Figure 3.19: Heterotrophic Growth of *Ochromonas danica* on a Range of Concentrations of *p*-Cresol.

(a) *O. danica* was incubated on a range of concentrations of *p*-cresol: 500µM (□), 1mM (■), 2mM (▵), 3mM (▴), and 4mM (○) at 25°C and 100rpm. Log N measured as cells ml^{-1}.

(b) The removal of the substrate was monitored: 500µM (□), 1mM (■), 2mM (▵), 3mM (▴), and 4mM (○).

Figure 3.19a
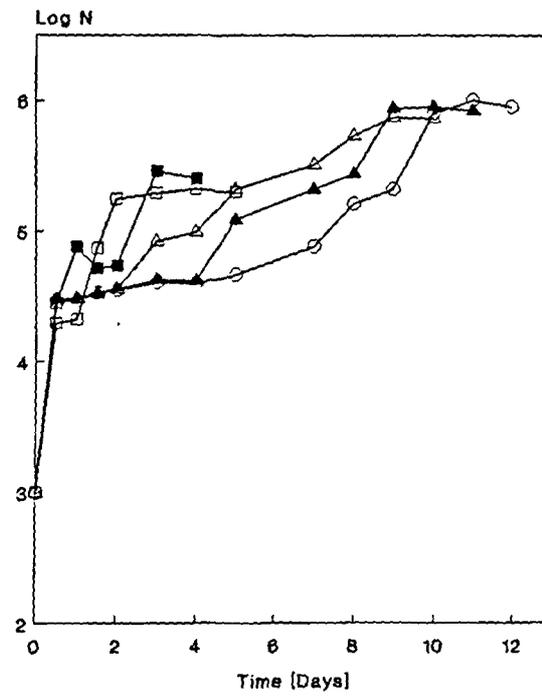
Figure 3.19b
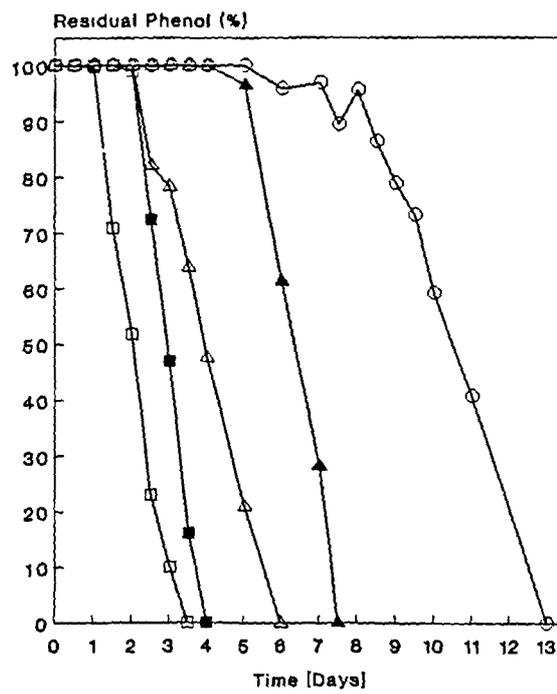
o-cresol. An identical experiment was carried out with phenol-cresol mixes, but with 1mM cresols. The alga was reluctant to remove the cresols at this concentration and the phenol was removed more slowly than in phenol-only controls. The above experiments were repeated with phenol (1mM) and 2,5-; 2,6-; 3,4- or 3,5-xylenols (500µM), both in the light and the dark (Figure 3.21). The phenol was removed most rapidly in all the incubations, but this was slower when xylenols were present, compared to the phenol-only control. The xylenols proved to be more recalcitrant than the cresols, with 2,6-xylenol being the most resistant and 3,4-xylenol removed fastest. The same experiment was repeated with both phenol and the xylenols at 1mM, but the alga was only able to remove the phenol very slowly in these conditions and unable to attack the xylenols.

Having established that *O. danica* was able to remove the cresols in the presence of phenol, the next step in this investigation was to elucidate whether or not these compounds could be removed after the phenol had been removed from the growth medium. The alga was grown in light and dark regimes, with growth and phenol removal being monitored (only the dark results are shown). In this study, the alga was first grown on phenol (1mM), then *o-* or *p*-cresol (500µM), administered aseptically, after all the phenol had been removed (Figure 3.22). Both cresols were removed with *p*-cresol being removed the most rapidly, the growth pattern coincided with the removal of these substrates.

Figure 3.20: Heterotrophic Growth of *Ochromonas danica* on Phenol in the Presence of *o*-Cresol or *p*-Cresol.

(a) *O. danica* was incubated in the dark with 1mM phenol (■), 1mM phenol plus 500µM *o*-cresol (▲), and 1mM phenol plus 500µM *p*-cresol (o) at 25°C and 100rpm. Log N measured as cells ml^{-1}.

(b) The removal of the substrates was monitored: 1mM phenol alone (■); 1mM phenol (▲), mixed with 500µM *o*-cresol (▲); 1mM phenol (o), mixed with 500µM *p*-cresol (☐).

Figure 3.20a
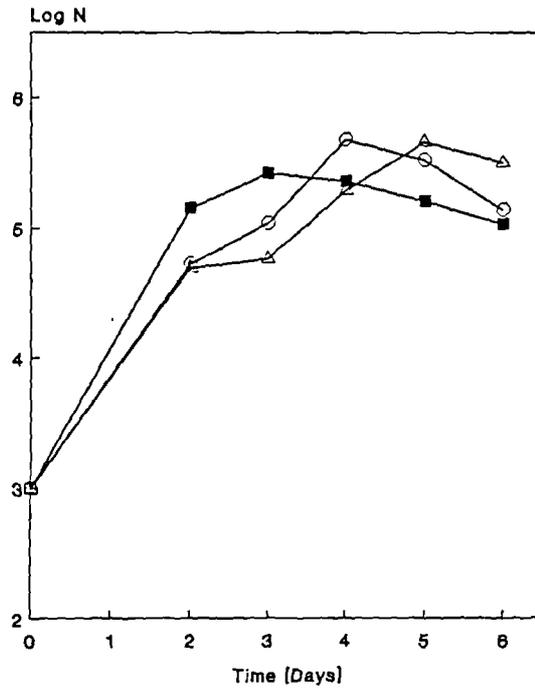
Figure 3.20b
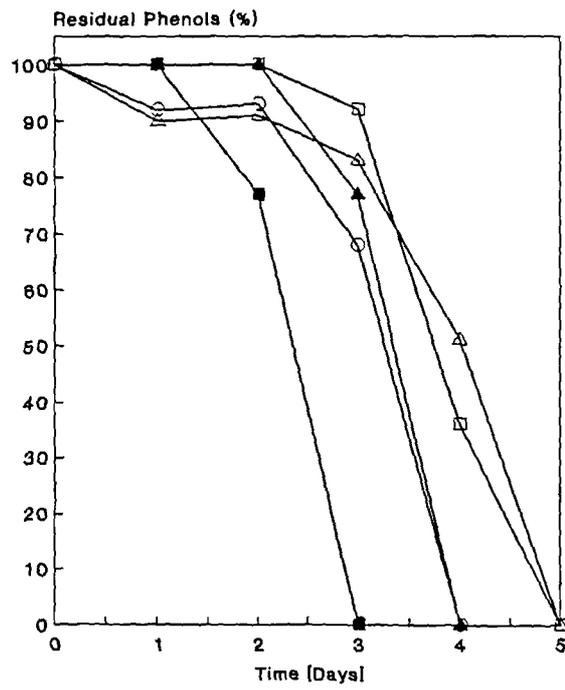
Figure 3.21: Heterotrophic Growth of *Ochromonas danica* on Phenol in the Presence of Xylenols.

(a) *O. danica* was incubated in the dark with 1mM phenol (■), 1mM phenol plus 500μM 2,5-xylenol (□), 1mM phenol plus 500μM 2,6-xylenol (▲), 1mM phenol plus 500μM 3,4-xylenol (▲), and 1mM phenol plus 3,5-xylenol (○) at 25°C and 100rpm. Log N measured as cells ml^{-1}.

(b) The removal of the substrates was monitored: 1mM phenol alone (■); 1mM phenol (□), mixed with 500μM 2,5-xylenol (▲); 1mM phenol (▲), mixed with 500μM 2,6-xylenol (○).

(c) The removal of the substrates was monitored: 1mM phenol alone (■); 1mM phenol (□), mixed with 500μM 3,4-xylenol (▲); 1mM phenol (▲), mixed with 500μM 3,5-xylenol (○).

Figure 3.21b

Residual Phenols (%)

Time [Days]

Figure 3.21c

Residual Phenols (%)

Time (Days)

Figure 3.21a

Log N

Time [Days]
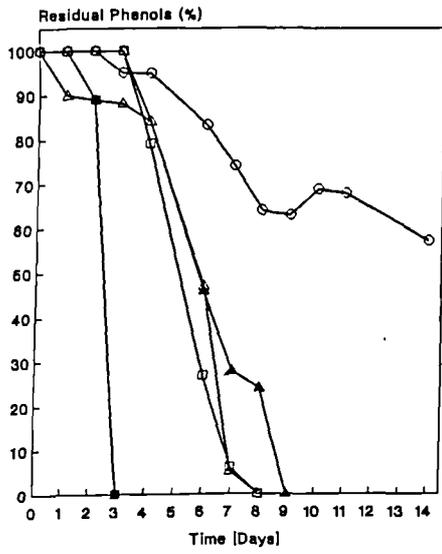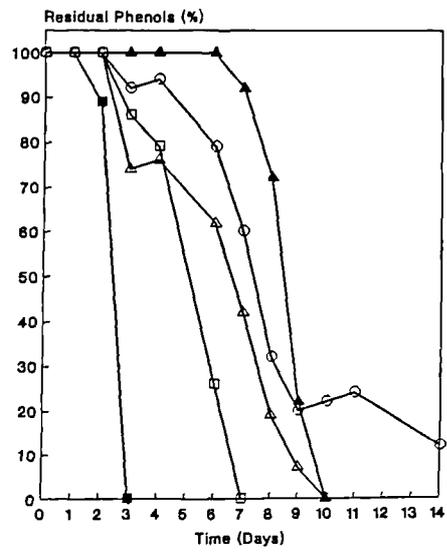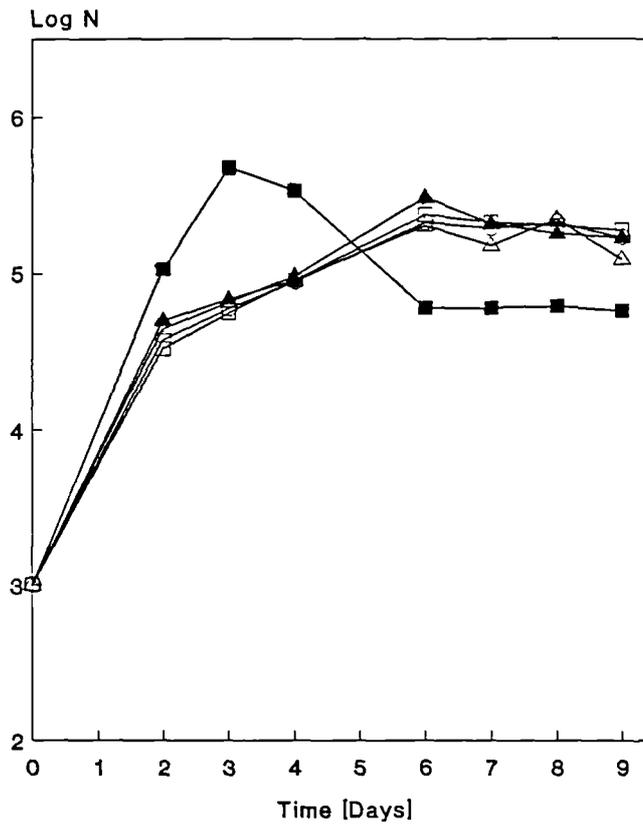
Figure 3.22: Heterotrophic Growth of *Ochromonas danica* on Phenol then *o*-Cresol and *p*-Cresol.

Growth of *O. danica* was measured (□) in the dark with 1mM phenol until all the phenol was metabolised; the cultures were then reinoculated (↓) with 500µM *o*-cresol (▵), or 500µM *p*-cresol (○) at 25°C and 100rpm. Log N measured as cells ml^{-1}. The disappearance of the substrates was monitored: 1mM phenol (■), 500µM *o*-cresol (▲), and 500µM *p*-cresol (●).

Figure 3.22
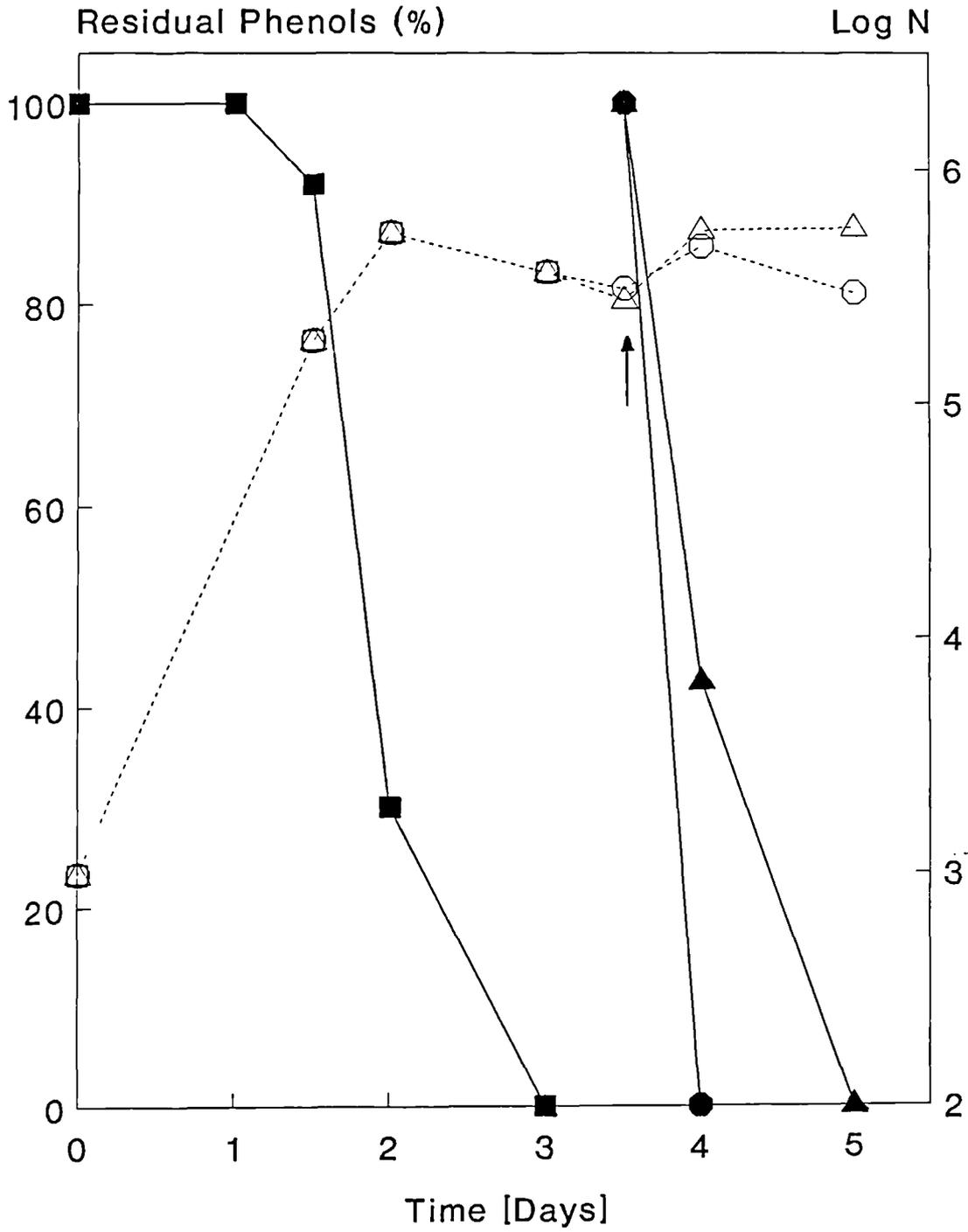
A similar experiment was carried out using phenol (1mM) as a carbon source; the alga was then exposed to the 2,5-; 2,6-; 3,4- or 3,5- xylenols (500µM), after the phenol was utilised (Figure 3.23). As in the previous experiment involving xylenols, 3,4-xylenol was removed the most rapidly, where as the other xylenols proved more recalcitrant to the alga.

3:3 Whole Cell Studies concerning Oxygen Utilisation.

The aim of this study was to elucidate whether or not the alga utilised oxygen to attack the phenolic substrates and in doing so, quantify the rates and amounts of oxygen used per mol of substrate. This was achieved using the oxygen electrode and the Warburg respirometric apparatus.

3:3:1 *Oxygen Electrode Studies.*

Ochromonas danica was grown on phenol (1mM), in light and dark regimes and the rate of oxygen utilisation by washed cells was measured against a range of different substrate analogues (Figures 3.24-3.26). As in the growth studies, only the results for dark-grown cells are shown due to the similarities between the data. The washed cell suspensions used for subsequent experiments were diluted to around 3×10^6 cells ml^{-1} and 1ml aliquots of this suspension were filtered and dried

Figure 3.23: Heterotrophic Growth of *Ochromonas* on Phenol then a variety of Xylenols.

(a) *O. danica* was incubated in the dark with 1mM phenol (■), until all the phenol had been metabolised; the cultures were then reinoculated (↓) with 500μM: 2,5- (□); 2,6- (○); 3,4- (▲); and 3,5- (△) xylenols at 25°C and 100rpm. Log N measured as cells ml⁻¹.

(b) The removal of phenol (1mM) and xylenols (500μM) was monitored: phenol alone (■); 2,5- (□); 2,6- (○); 3,4- (▲); and 3,5- (△) xylenols.

Figure 3.23a
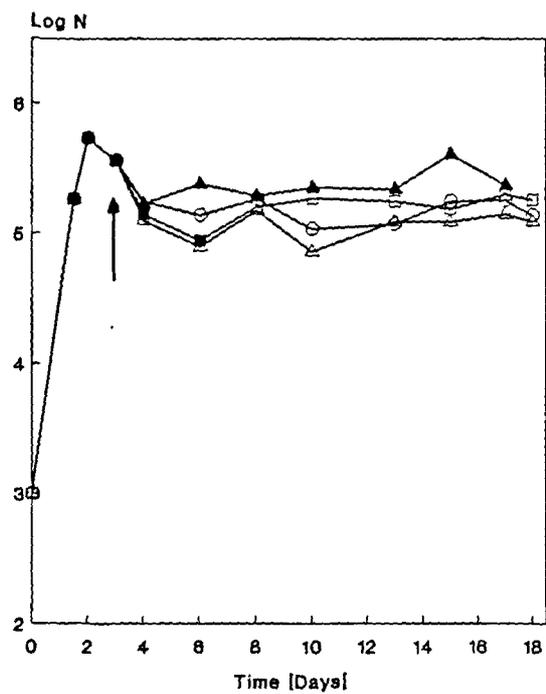
Figure 3.23b
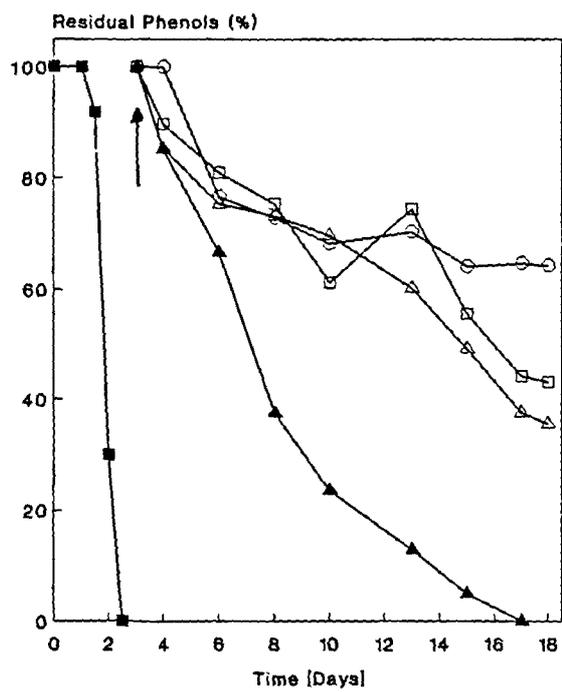
Figures 3.24: Net Rates of Oxygen Uptake by *Ochromonas danica* grown in the Dark.

Washed cells of *O. danica* which had been either grown on 1.5mM succinate (uninduced) or 1mM phenol (induced) (approx. 1mg dry wt. ml^{-1} 0.2M sodium phosphate buffer, pH7.2) were equilibrated in the electrode cell and 0.1µmol of substrate was added. Results are corrected for endogenous oxygen uptake and dry wt.

Figure 3.24

nmol O_2/min/mg dry wt

	Phenol	o-Cresol	p-Cresol
Uninduced	10.5	6.7	7.5
Induced	60.9	14.1	46

Substrates
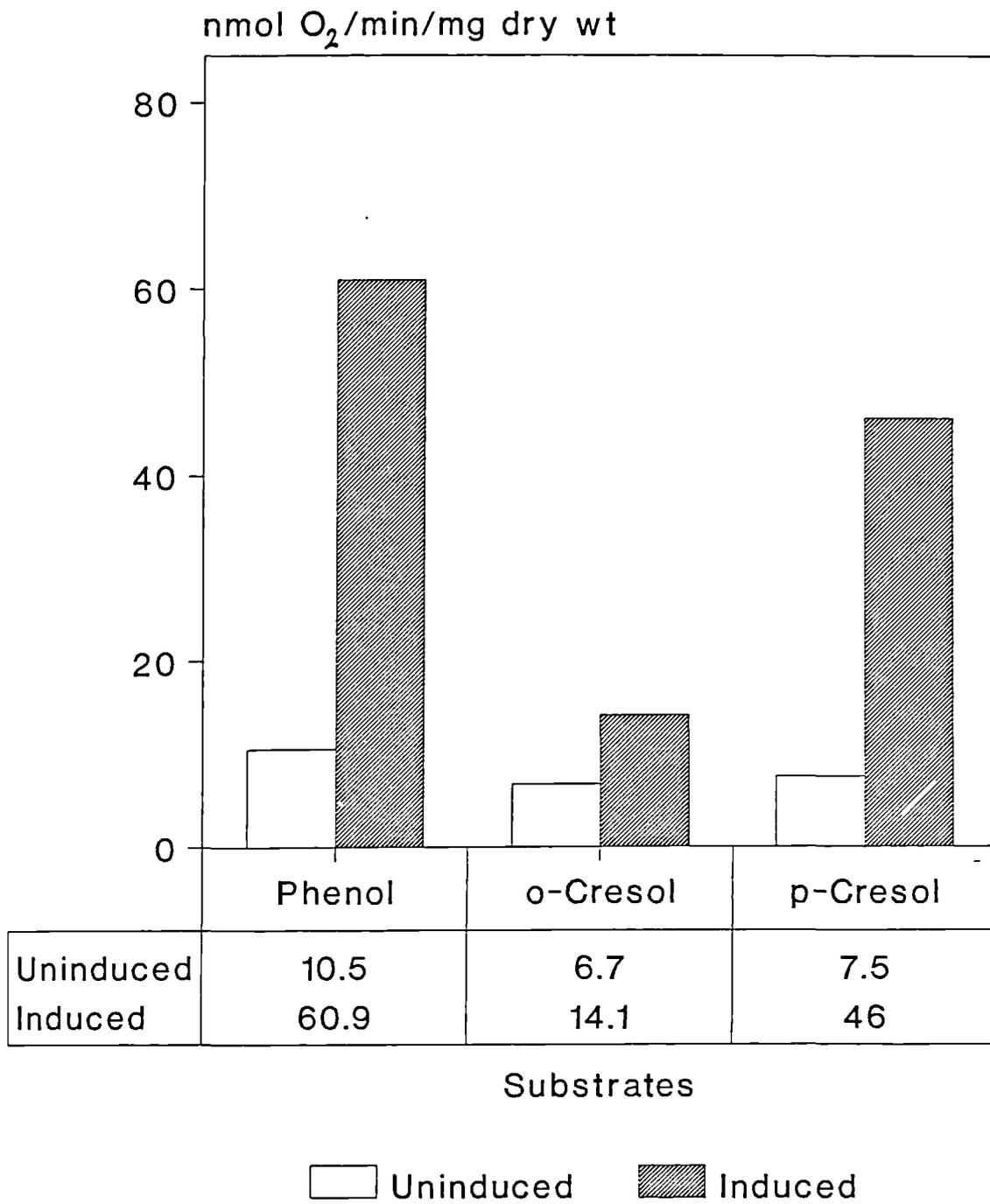
☐ Uninduced   ▨ Induced

Figures 3.25: Net Rates of Oxygen Uptake by *Ochromonas danica* grown in the Dark.

Washed cells of *O. danica* grown on 1mM phenol (induced) (approx. 1mg dry wt. ml^{-1} in 0.2M sodium phosphate buffer pH7.2) were equilibrated in the electrode cell and 0.1µmol of substrate was added. Results are corrected for endogenous oxygen uptake and dry wt.

Figure 3.25

nmol O_2/min/mg dry wt

	2,5	2,6	3,4	3,5
Induced	11.29	2.6	26.85	16.67

Substrates
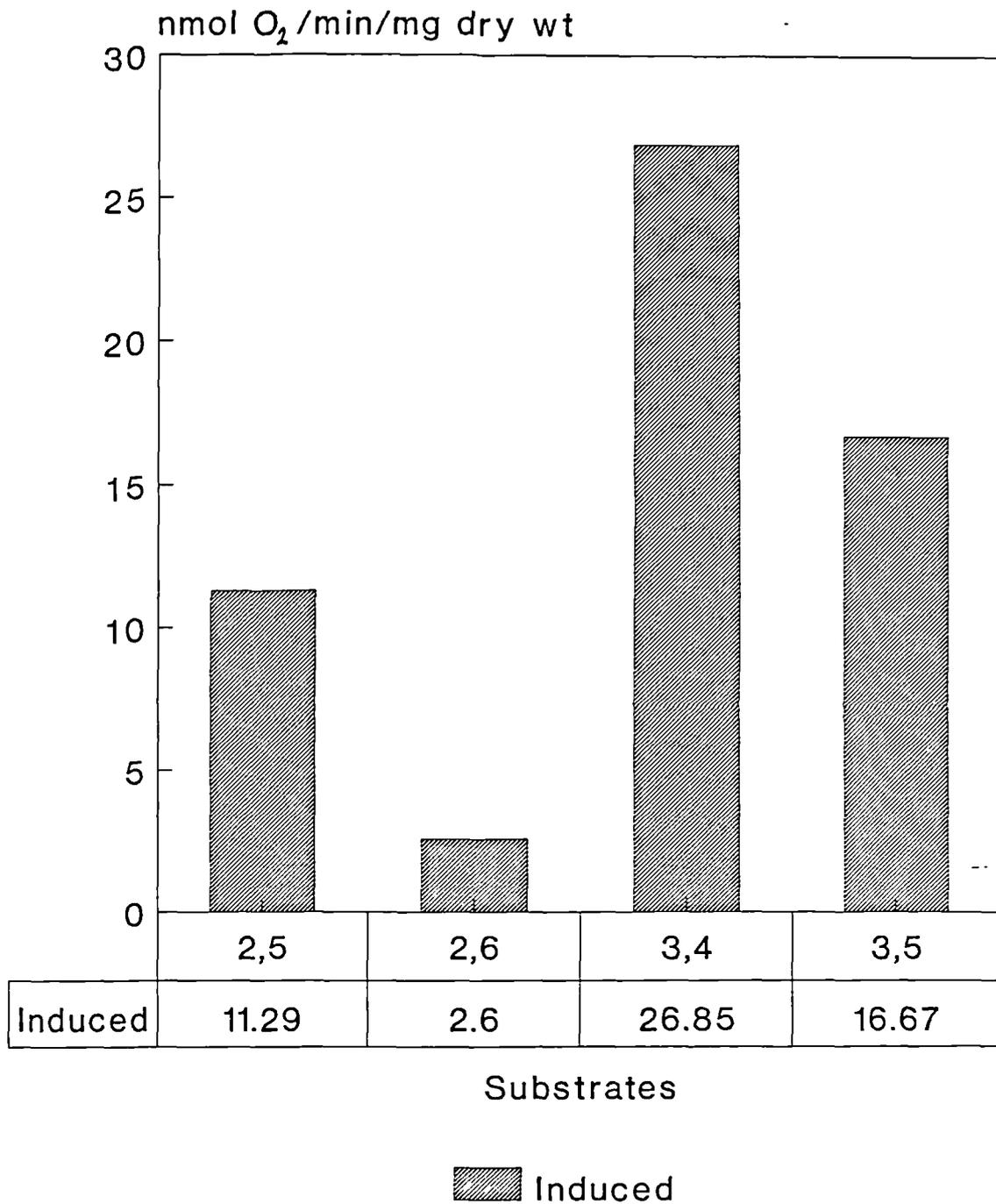
Induced

Figure 3.26: Net Rates of Oxygen Uptake by *Ochromonas danica* grown in the Dark.

Washed cell suspensions of *O. danica* either grown on 1.5mM succinate (uninduced) or 1mM phenol (induced) (approx. 1mg dry wt. ml^{-1} 0.2M sodium phosphate buffer, pH7.2) were equilibrated in the electrode cell and 0.1µmol of substrate was added. Results were corrected for endogenous oxygen uptake and dry wt.

Figure 3.26

nmol O_2/min/mg dry wt

	Catechol	3-Me-catechol	4-Me-catechol
Uninduced	6.2	5.8	6.2
Induced	69.1	23.8	36.5

Subtrates

☐ Uninduced   ▨ Induced
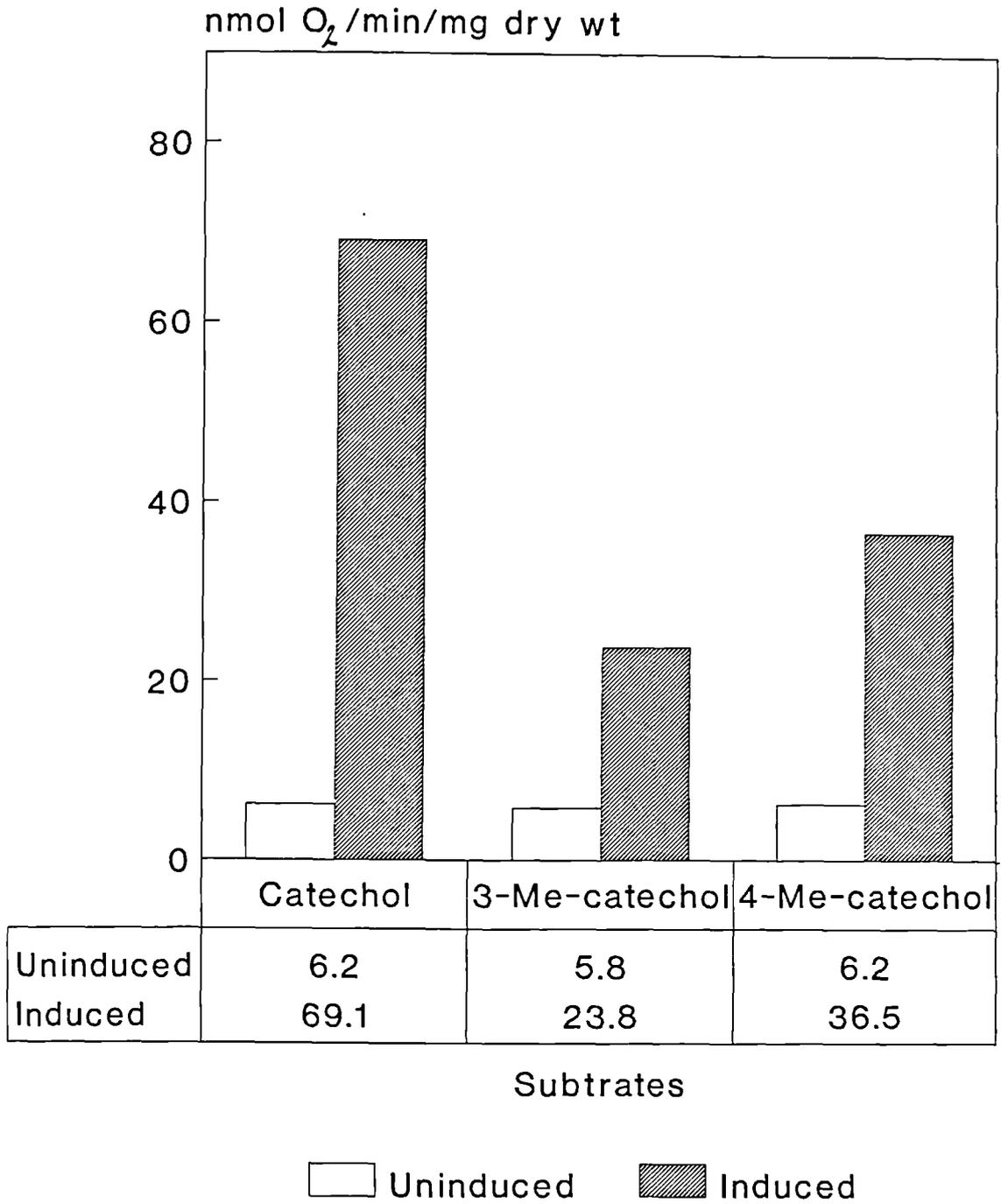
for estimation of dry weight. This concentration of cells was found to give conveniently measurable rates of oxidation with most substrates. The cells, in both regimes, oxidised a wide variety of substituted phenols and the rate of oxygen uptake was clearly influenced by the position of the substituents on the aromatic ring. In both growth conditions, the patterns for oxygen uptake were similar with catechol and then phenol giving the highest rates of oxidation. The next most reactive was *p*-cresol and 4-methylcatechol, with *o*-cresol being the least reactive of the mono-methylated phenols. Of the catechols, 3-methylcatechol was the most difficult to oxidise. Oxidation of the xylenols was less than that of *o*-cresol with the exception of the 3,4-isomer, which gave the highest rate of oxidation of the xylenols.

3:3:2 *Manometry.*

Ochromonas danica was grown on phenol in the dark and washed cell suspensions were used to measure the rates of oxygen uptake and the amounts of oxygen consumed when incubated with different phenols at 25°C, using the Warburg manometric apparatus. The alga was first incubated with 0.3μmol, 0.5μmol, 0.7μmol and 1.0μmol of phenol until the substrate was completely oxidised (Figure 3.27). In a subsequent experiment, the alga was exposed to phenol (0.5μmol), catechol (0.5μmol), *p*-cresol (0.5μmol), 4-methylcatechol (0.5μmol) and 3,4-xylenol (0.5μmol) all of which the alga was able to oxidise

Figure 3.27: Oxidation of Phenol by Washed Suspensions of *Ochromonas danica* grown on Phenol.

Warburg flasks contained, in a total volume of 2.5ml: 1.3ml 0.2M sodium phosphate buffer, pH7.2; 0.3μmol (■), 0.5μmol (□), 0.7μmol (▲), 1.0μmol phenol (△); 1ml suspension of washed cells (2.11mg dry wt. of cells); 0.1ml KOH (20% v/v) in the centre well. Incubation was at 25°C and 100rpm, with the endogenous respiration (1.4μl O_2/min/mg dry wt) being subtracted. The amounts of oxygen consumed per μmol of phenol were found to be proportional from 0 - 1μmol (□).
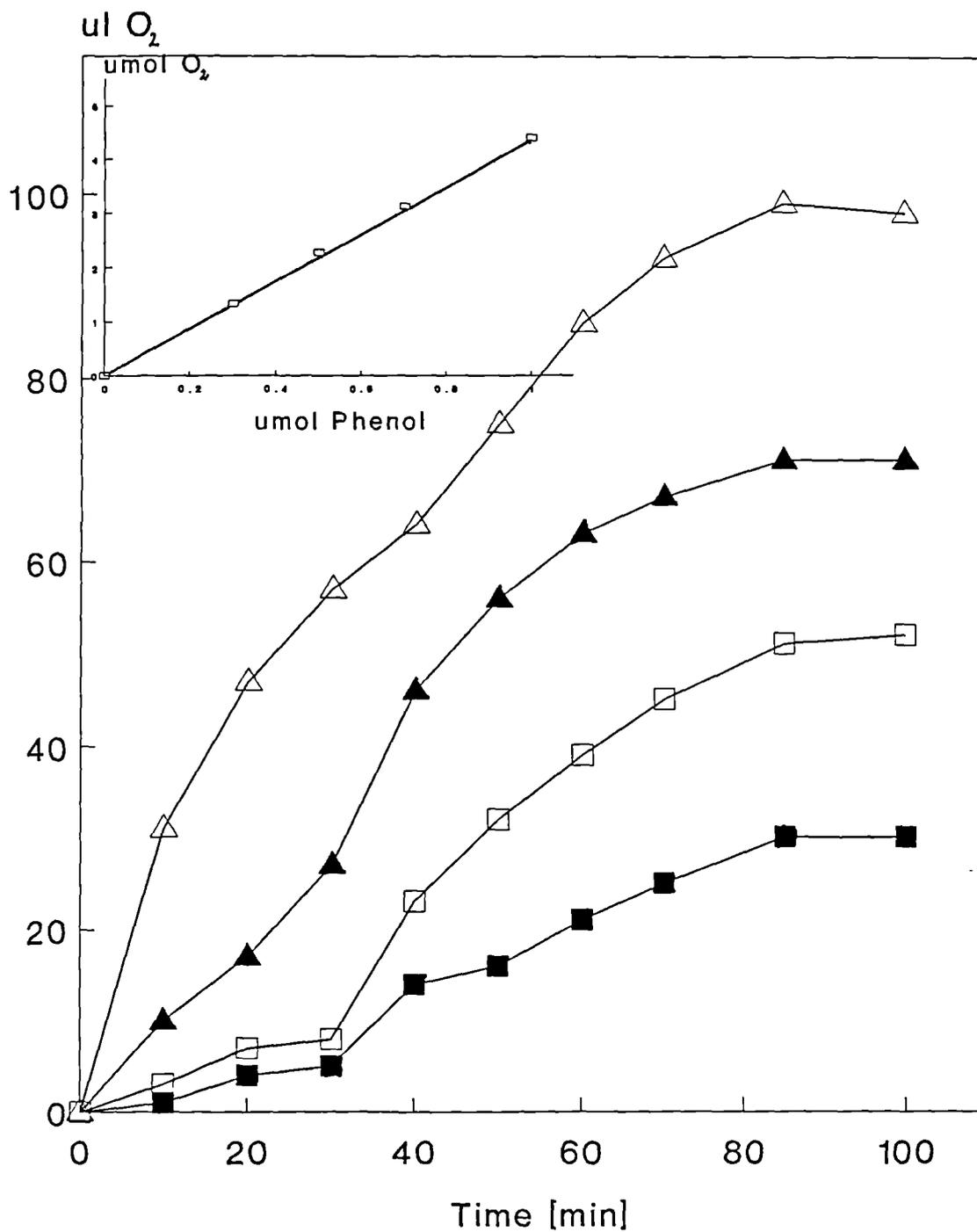
Figure 3.27

(Figure 3.28). In each case, the phenols were oxidised and the amount of oxygen

consumed per μmole of the substrate was approximately 65% of the theoretical

(Table 3.1).

3:4 Turnover Studies. .

3:4:1 *Studies with Single Substrates.*

To confirm the patterns of oxygen uptake measured in the oxygen electrode,

a series of 'turnover' experiments was made with washed cell suspensions, grown

under light and dark conditions on phenol (1mM), incubated with different

substrates for a 6h period. Samples from these incubations were analysed by

h.p.l.c., to accurately monitor the disappearance of the substrates. Unlike the more

limited oxygen electrode studies, the turnover experiments gave a direct measure

of the removal of substrate. On the whole, the turnover of single substrates on

cells grown in light and dark conditions followed the trends already observed in the

oxygen electrode studies, therefore only the dark incubations are shown (Figures

3.29 and 3.30). The washed cell suspensions were exposed to phenol (500μM),

m-, *o*- and *p*-cresols (500μM) and 2,3-; 2,4-; 2,5-; 2,6-; 3,4- and 3,5-xylenols

(500μM). The alga removed 500μM phenol from the incubation in 2h, and

metabolised *p*-cresol, *o*-cresol and 3,4-xylenol at respectively slower rates. The

Figure 3.28: Oxidation of Phenols and Catechols by washed suspensions of *Ochromonas danica* grown on Phenol.

Warburg flasks contained, in a total volume of 2.5ml: 1.3ml 0.2M sodium phosphate buffer, pH7.2; 0.5μmol substrate; 1ml suspension of washed cells (2.11mg dry wt. of cells); 0.1ml KOH (20% v/v) in the centre well. Incubation was at 25°C and 100rpm, with the endogenous respiration (1.4μl O_2/min/mg dry wt) being subtracted. Catechol (▲), 4-methylcatechol (○), phenol (□), *p*-cresol (■), and 3,4-xylenol (△).

Figure 3.28
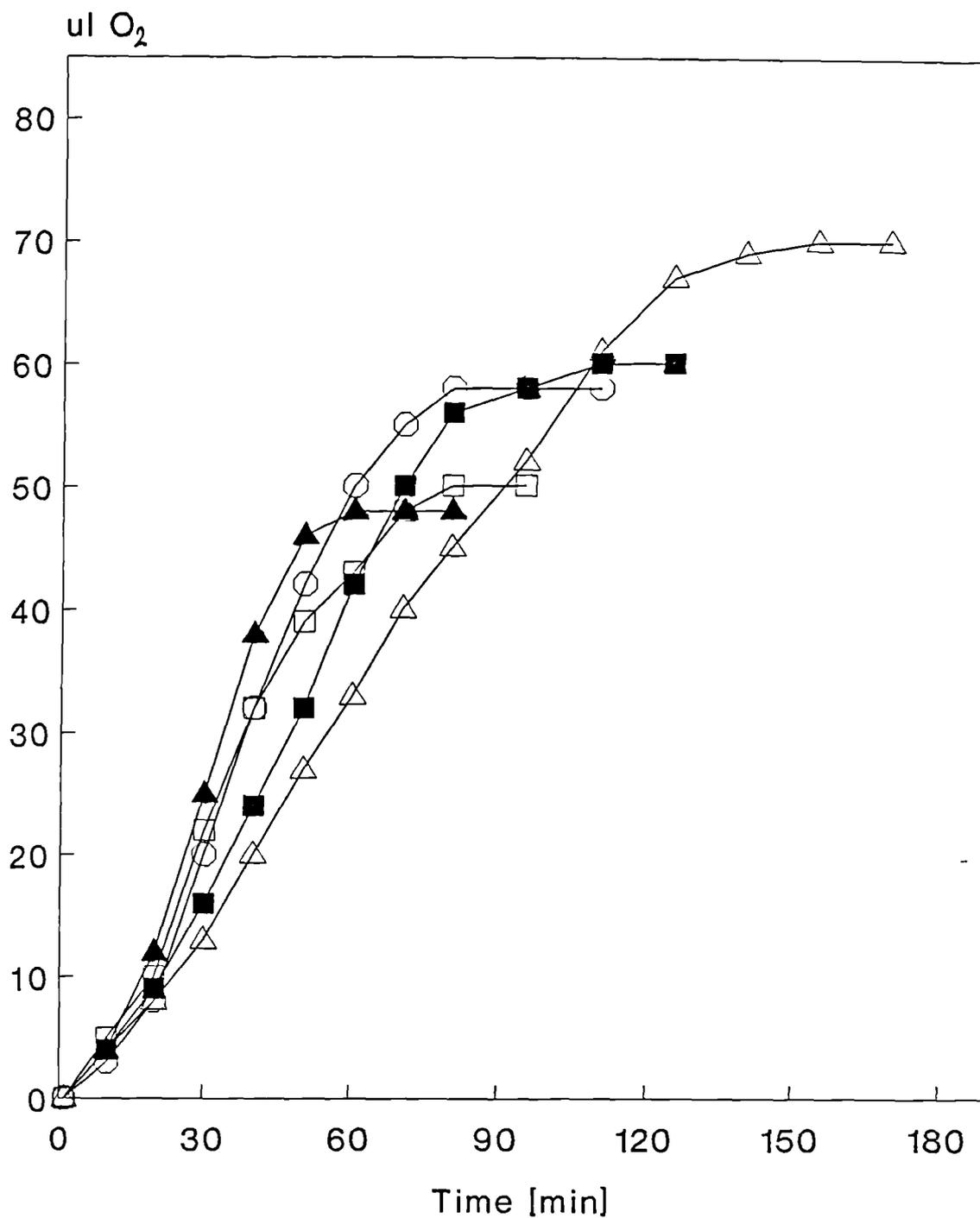
Table 3.1: Oxidation of Alkylphenols and Catechols by washed suspensions of _Ochromonas danica_ grown in the dark on Phenol.

Warburg flasks contained, in a total volume of 2.5ml: 1.3ml of 0.2M sodium phosphate buffer, pH7.2; 0.5μmol substrate; 1ml suspension of washed cells (2.11mg dry wt. of cells); 0.1ml KOH (20% v/v) in the centre well. Incubation was at 25°C and 100rpm, with the endogenous respiration rate (1.4μl of O_2/min/dry wt. of flask) subtracted. Values for total O_2 uptake were measured until the test respiration rate was equal to that of the endogenous rate.

Respiratory Substrate	Initial Rate of O_2 Uptake (μl/hr/mg dry wt. of cells)	Total O_2 Uptake (μmol O_2/μmol substrate)	Total O_2 Uptake (% of theoretical)
Phenol	28.4	4.5	64.3
Catechol	36.9	4.3	66.2
_p_-Cresol	25.6	5.4	63.5
4-Methylcatechol	34.1	5.2	65
3,4-Xylenol	19.9	6.3	62.5

Figure 3.29: Turnover of Phenol and Monosubstituted Phenols by washed cell suspensions of *Ochromonas danica* grown in the Dark on Phenol.

Each incubation flask contained 20ml of cell suspension (2.1mg dry wt.ml^{-1} in 0.2M sodium phosphate buffer, pH7.2) with 500µM substrate, and was incubated at 25°C and 100rpm in a water bath. Aliquots of 1ml were removed for h.p.l.c. measurement of phenol (■), *m*-cresol (●), *o*-cresol (▲), *p*-cresol (○), *p*-bromophenol (□), *p*-chlorophenol (◊) *p*-methoxyphenol (◆), 2,4-dichlorophenol (□).

Figure 3.29

Residual Phenols (%) vs Time [Days]
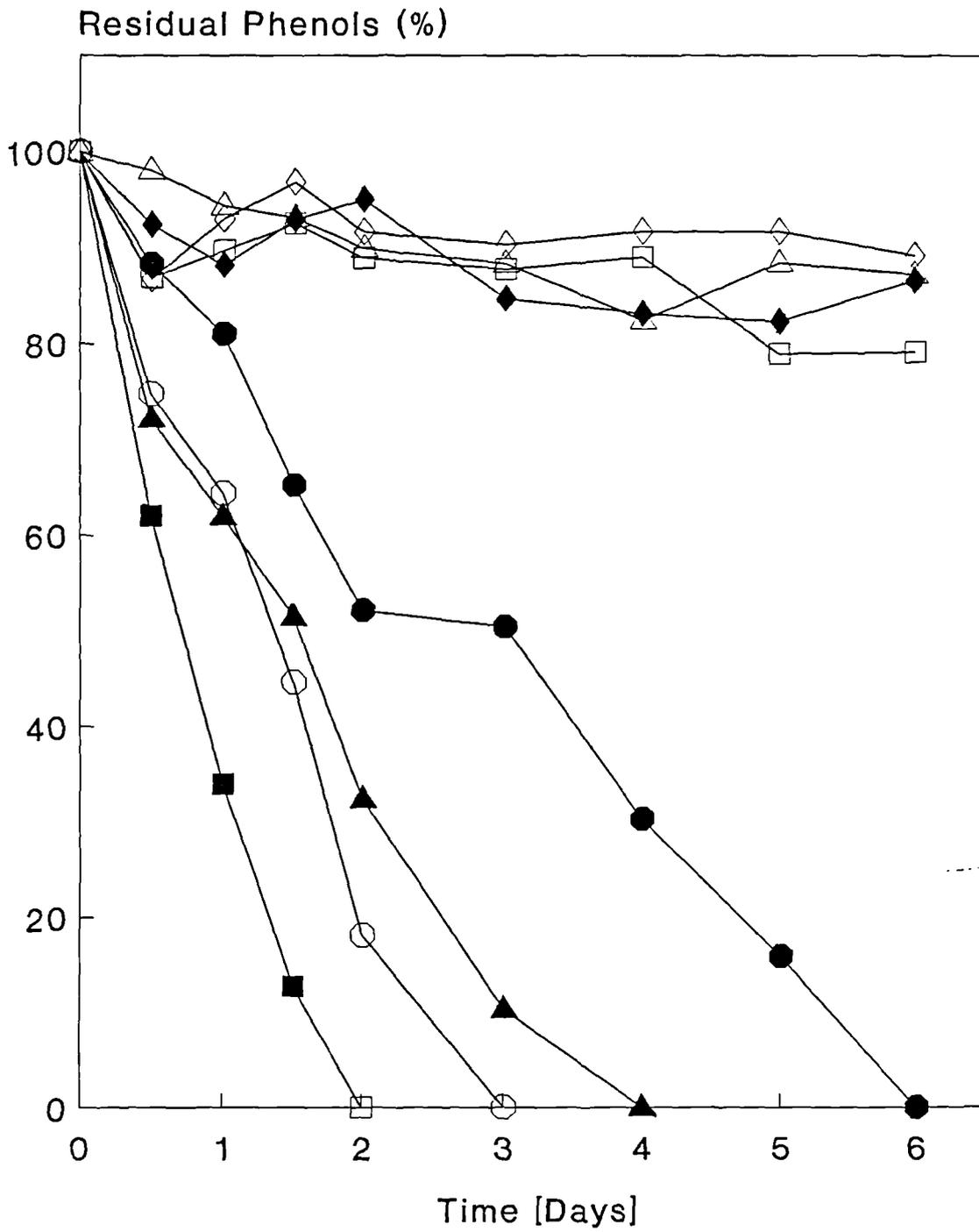
Figure 3.30: Turnover of Xylenols by washed suspensions of *Ochromonas danica* grown in the Dark on Phenol.

Each incubation flask contained 20ml of cell suspension (2.3mg dry wt.ml^{-1} in 0.2M sodium phosphate buffer, pH7.2) with 500µM xylenol, and was incubated at 25°C and 100rpm in a water bath. Aliquots of 1ml were removed for h.p.l.c. measurement of 2,3- (□), 2,4- (■), 2,5- (▵), 2,6- (▴), 3,4- (●), 3,5- (○) xylenols.

Figure 3.30

Residual Phenol (%)

Time [Hours]
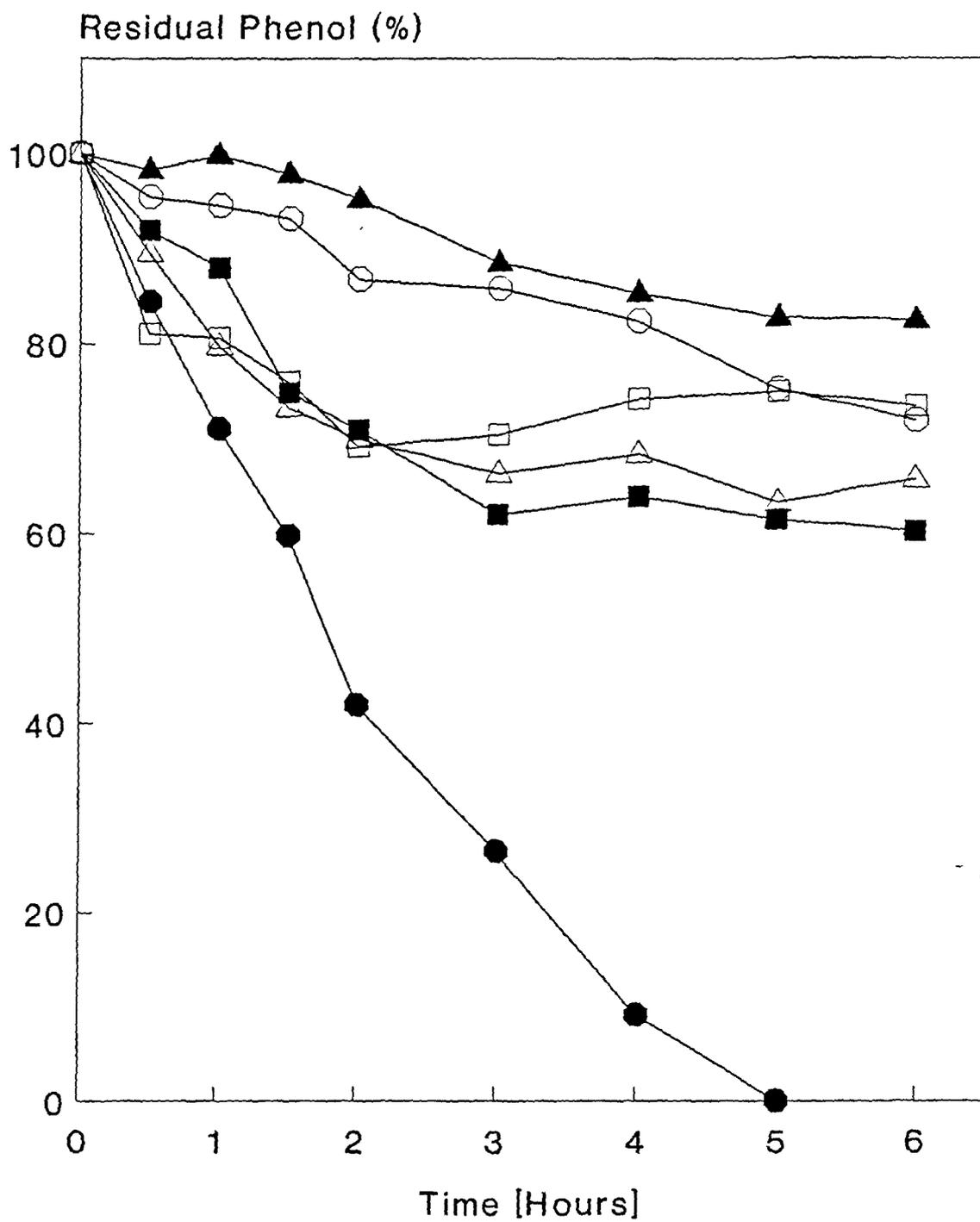
other xylenols proved to be more recalcitrant, with none of those tested being fully metabolised in 6h. The alga was also incubated after growth on phenol with a variety of other substituted phenols (500μM) such as *p*-bromophenol, *p*-chlorophenol, *p*-methoxyphenol and 2,4-dichlorophenol (Figure 3.29), but *O. danica* was unable to metabolise any of these substrates to any great effect over a period of 6h.

3:4:2 *Studies with Two Substrates.*

O. danica has been shown to grow on phenol and cresols or xylenols, with the resulting growth patterns and rates of substrate removal being very similar in both light and dark regimes. This pattern between light and dark cells has also been shown in the oxygen electrode and turnover studies on single substrates. Further experiments concentrated on cells grown in the dark on phenol (1mM) to produce active washed cell suspensions for examination of the effect on mixed substrates.

The alga was incubated with a mixture of phenol (500μM) and *o*- or *p*-cresol (250μM) and on phenol (500μM) only, as a positive control. Preliminary experiments with mixes of phenol (500μM) with cresols (500μM) or xylenols (500μM) showed that the alga had great difficulty in metabolising a mixture of phenols, the final concentration of which was >1mM. For this reason, a reduction in the concentration of the more recalcitrant compounds was implemented in these experiments. In every case, phenol was removed faster in the mixtures than were

the cresols, but not as quickly as in the control with the phenol alone (Figures 3.31

and 3.32). *p*-Cresol was metabolised more rapidly than the *ortho* isomer.

Ochromonas danica was incubated with phenol (500µM) plus one of 2,5-;

2,6-; 3,4-; or 3,5-xylenol (250µM); phenol (500µM) only was used as a positive

control. As recorded in the previous study, phenol in these mixtures was removed

at a faster rate than was any of the xylenols, but not as rapidly as the control

incubation (Figures 3.33-3.36). The only xylenol to be fully metabolised in the

incubation period was 3,4-xylenol, which corroborated the results recorded in the

earlier growth and metabolism studies. Active washed suspensions produced from

p-cresol-grown cultures of the alga were incubated with a mixture of *p*-cresol

(500µM) plus *o*-cresol (250µM) or 3,4-xylenol (250µM) mixes; an incubation with *p*-

cresol alone was used as the positive control (Figure 3.37). As the sole carbon

source, *p*-cresol was removed after 3h but when *p*-cresol was mixed with *o*-cresol

or 3,4-xylenol, it was removed only after 4h and 5h, respectively. The secondary

substrates were removed at a slower rate than the *p*-cresol, with *o*-cresol being

removed slightly faster than the xylenol.

The final experiment in this study was to investigate whether or not the

addition of phenol could influence the removal of more recalcitrant phenolic

compounds. *Ochromonas danica* was incubated with phenol (500µM) plus *o*-cresol

(250µM) or *p*-cresol (250µM) mixes and the removal of the individual substrates

was monitored. When all the phenol was removed, but the remaining cresols were

still being utilised, more phenol (500µM) was inoculated into the test flasks

Figure 3.31: Turnover of Phenol with o-Cresol by washed suspensions of Ochromonas danica grown in the Dark on Phenol.

Each incubation flask contained 20ml of cell suspension (2.2mg dry wt.ml^{-1} in 0.2M sodium phosphate buffer, pH7.2) with the substrate(s), and was incubated at 25°C and 100rpm in a water bath. Aliquots of 1ml were removed for h.p.l.c. measurement of 500µM phenol (□), and 500µM phenol (▲) mixed with 250µM o-cresol (○).

Figure 3.32: Turnover of Phenol with p-Cresol by washed suspensions of Ochromonas danica grown in the Dark on Phenol.

Each incubation flask contained 20ml of cell suspension (2.2mg dry wt.ml^{-1} in 0.2M sodium phosphate buffer, pH7.2) with the substrate(s), and was incubated at 25°C and 100rpm in a water bath. Aliquots of 1ml were removed for h.p.l.c. measurement of 500µM phenol (□), and 500µM phenol (▲), mixed with 250µM p-cresol (○).

Figure 3.31

Residual Phenols (%)

Time [Hours]

Figure 3.32

Residual Phenols (%)

Time [Hours]
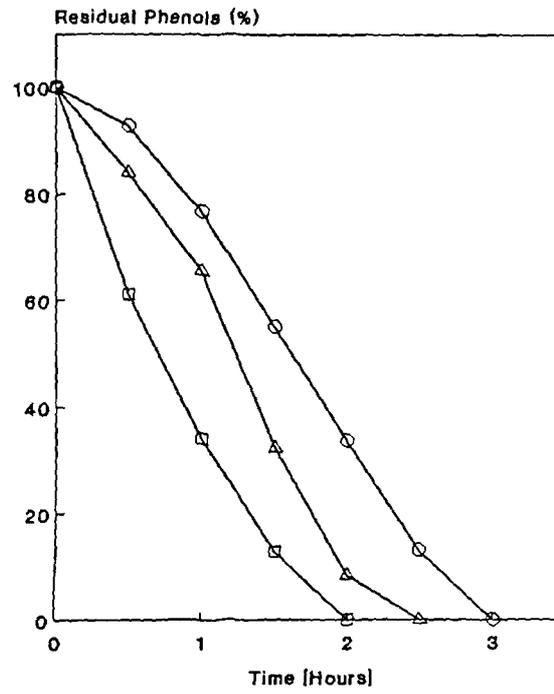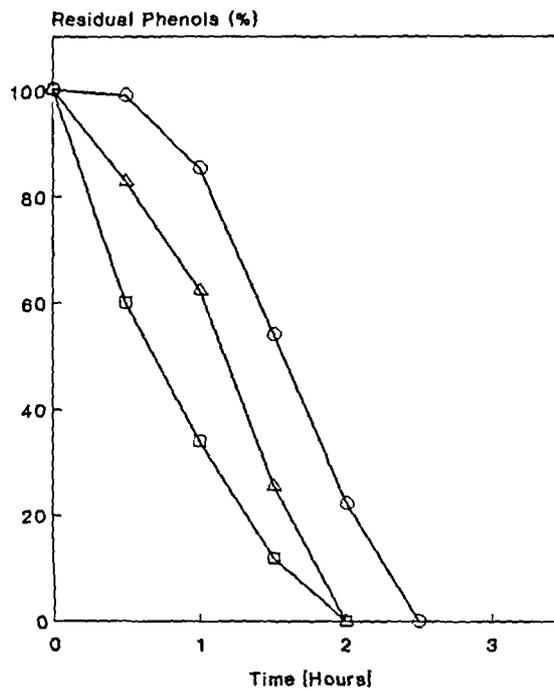
Figure 3.33: Turnover of Phenol with 2,5-Xylenol by washed suspensions of *Ochromonas danica* grown in the Dark on Phenol.

Each incubation flask contained 20ml of cell suspension (2.3mg dry wt.ml^{-1} in 0.2M sodium phosphate buffer, pH7.2) with the substrate(s), and was incubated at 25°C and 100rpm in a water bath. Aliquots of 1ml were removed for h.p.l.c. measurement of 500µM phenol (□), 500µM phenol (▲), mixed with 250µM 2,5-xylenol (o).

Figure 3.34: Turnover of Phenol with 2,6-Xylenol by washed suspensions of *Ochromonas danica* grown in the Dark on Phenol.

Each incubation contained 20ml of cell suspension (2.3mg dry wt.ml^{-1} in 0.2M sodium phosphate buffer, pH7.2) with the substrate(s), and was incubated at 25°C and 100rpm in a water bath. Aliquots of 1ml were removed for h.p.l.c. measurement of 500µM phenol (□), and 500µM phenol (▲), mixed with 250µM 2,6-xylenol (o).
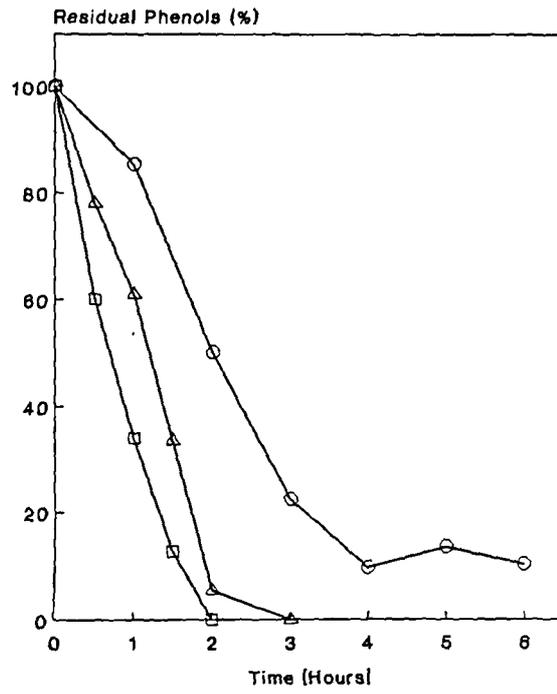
Figure 3.33

Figure 3.34
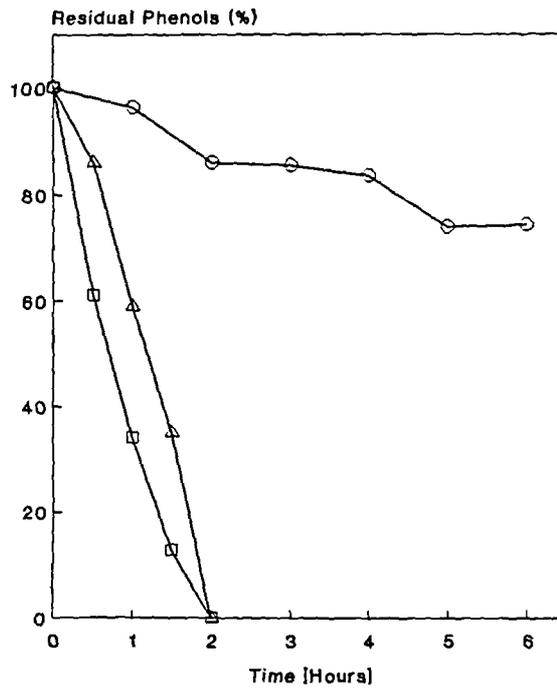
Figure 3.35: Turnover of Phenol with 3,4-Xylenol by washed suspensions of *Ochromonas danica* grown in the Dark on Phenol.

Each incubation flask contained 20ml of cell suspension (2.3mg dry wt.ml^{-1} in 0.2M sodium phosphate buffer, pH7.2) with the substrate(s), and was incubated at 25°C and 100rpm in a water bath. Aliquots of 1ml were removed for h.p.l.c. measurement of 500µM phenol (□), and 500µM phenol (▲), mixed with 250µM 3,4-xylenol (o).

Figure 3.36: Turnover of Phenol with 3,5-Xylenol by washed suspensions of *Ochromonas danica* grown in the Dark on Phenol.

Each incubation flask contained 20ml of cell suspension (2.3mg dry wt.ml^{-1} in 0.2M sodium phosphate buffer, pH7.2) with the substrate(s), and incubated at 25°C and 100rpm in a water bath. Aliquots of 1ml were removed for h.p.l.c. measurement of 500µM phenol (□), and 500µM phenol (▲), mixed with 250µM 3,5-xylenol (o).

Figure 3.35

Residual Phenols (%)

Time [Hours]

Figure 3.36

Residual Phenols (%)

Time [Hours]

118
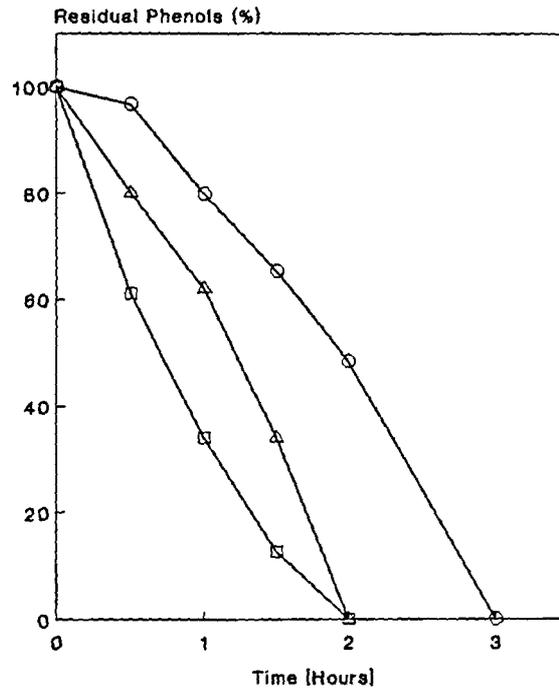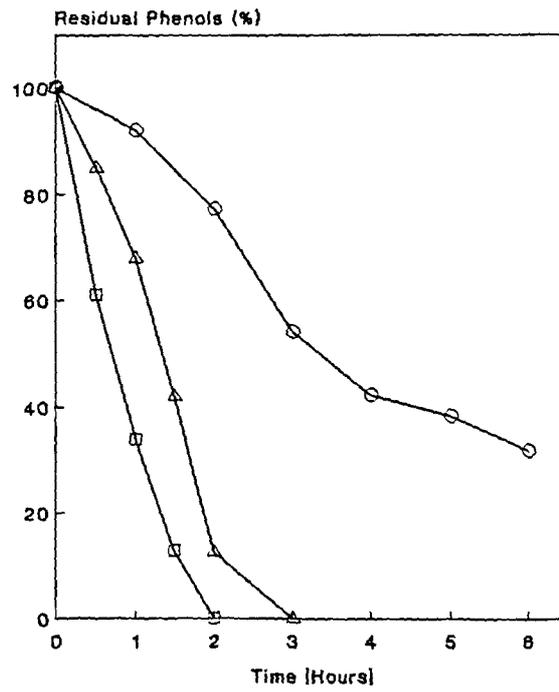
Figure 3.37: Turnover of *p*-Cresol with *o*-Cresol and 3,4-Xylenol by washed suspensions of *Ochromonas danica* grown in the Dark on *p*-Cresol.

Each incubation flask contained 20ml of cell suspension (2.3mg dry wt.ml^{-1} in 0.2M sodium phosphate buffer, pH7.2) with substrate(s), and incubated at 25°C and 100rpm in a water bath. Aliquots of 1ml were removed for h.p.l.c. measurement of 500μM *p*-cresol (■); 500μM *p*-cresol (□), mixed with 250μM *o*-cresol (▵); 500μM p-cresol (▴), mixed with 250μM 3,4-xylenol (○).

Figure 3.37

Residual Phenols (%)

Time [Hours]
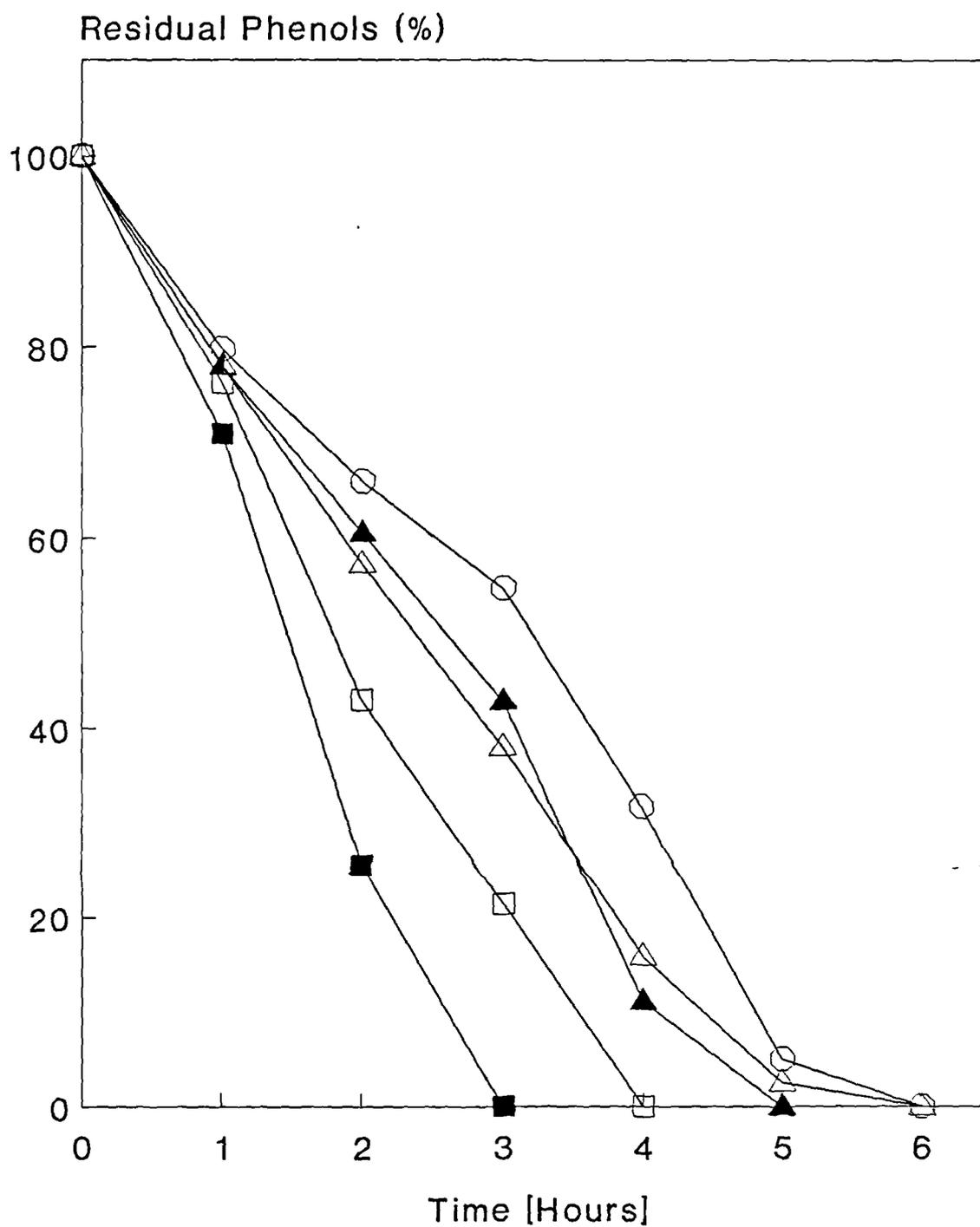
(Figures 3.38 and 3.39). The result was a pronounced reduction in the rate of removal of the cresols until all the second batch of phenol had been removed, at which point the rate of removal of the cresols increased to the original value. Phenol is thus the preferred substrate.

3:4:3   Anaerobic Turnover Studies.

Ochromonas danica was incubated anaerobically with phenol (500µM) for 2h at 25°C with the cell suspensions being shaken every 15min. Simultaneously, cells were incubated normally in air (Method 2:7:3) with phenol (500µM) and samples were taken every 20min and analysed on the h.p.l.c. (Figure 3.40). After the 2h . aerobic incubation all the phenol had been removed from the medium, whereas no phenol had been removed in the anaerobic cultures. At this point, the anaerobic cultures were made aerobic by gently bubbling air through the cell suspensions and then shaking the flasks at 150 rev/min in an shaking water bath at 25°C. When the medium from these original anaerobic incubations was sampled every 20min and analysed on the h.p.l.c. it showed that the phenol was now being steadily removed in the new aerobic conditions. The experiment thus establishes that phenol removal is an obligatory aerobic process, requiring molecular oxygen but that anaerobiosis does not have any permanently deleterious affect on the algal cells.

Figure 3.38: Turnover of *o*-Cresol with the addition of Phenol by washed suspensions of *Ochromonas danica* grown in the dark on Phenol.

Each incubation flask contained 20ml of cell suspension (2.2mg dry wt.ml^{-1} in 0.2M sodium phosphate buffer, pH7.2) with the substrate(s), and incubated at 25°C and 100rpm in a water bath. Aliquots of 1ml were removed for h.p.l.c. measurement of 500µM phenol (□) or 250µM *o*-cresol (▲). After 1.5h (↓) further 500µM phenol (○), was added to the suspension containing the *o*-cresol.

Figure 3.39: Turnover of *p*-Cresol with the addition of Phenol by washed suspensions of *Ochromonas danica* grown in the Dark on Phenol.

Each incubation flask contained 20ml of cell suspension (2.2mg dry wt.ml^{-1} in 0.2M sodium phosphate buffer, pH7.2) with the substrate(s), and incubated at 25°C and 100rpm in a water bath. Aliquots of 1ml were removed for h.p.l.c. measurement of 500µM phenol (□), 250µM *p*-cresol (▲). After 1.5h (↓) further 500µM phenol (○), was added to the suspension containing the *p*-cresol.

Figure 3.38

Residual Phenols (%)

Time (Hours)

Figure 3.39

Residual Phenols (%)

Time (Hours)

121
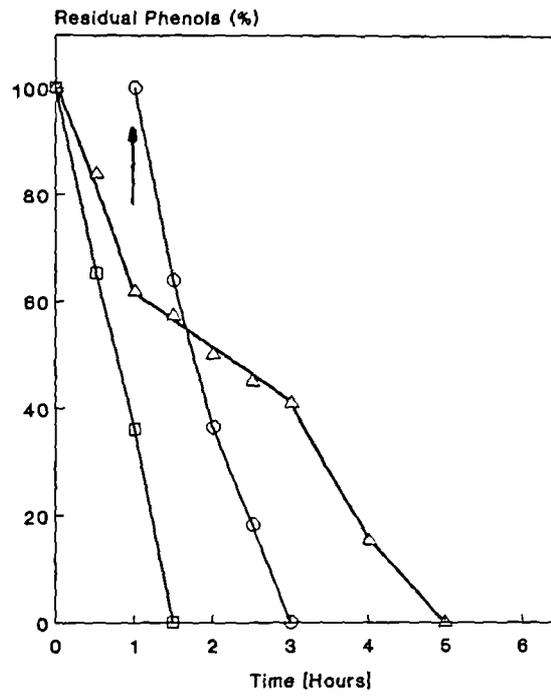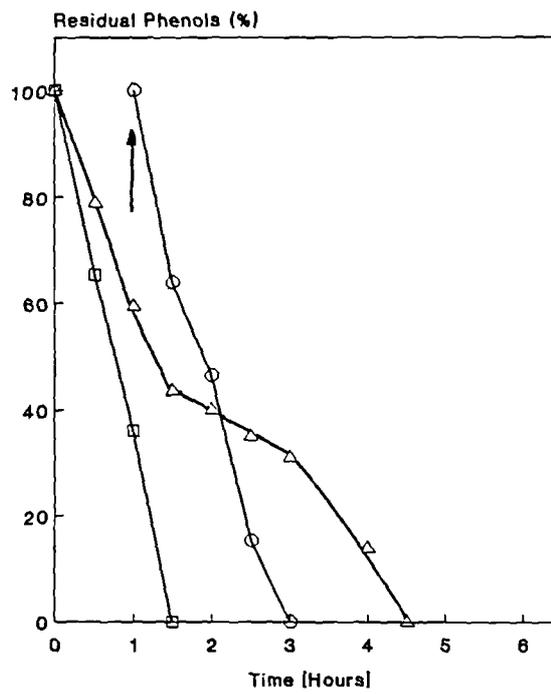
**Figure 3.40: Anaerobic Turnover of Phenol by cell suspensions of
Ochromonas danica grown Aerobically in the Dark on Phenol.**

Each incubation flask contained 20ml of cell suspension (2.3mg dry wt.ml^{-1} in O_2
free 0.2M sodium phosphate buffer, pH7.2) with O_2 free phenol, and incubated
anaerobically at 25°C and shaken every 15min. The aerobic control was incubated
at 25°C and 100rpm in a water bath. Aliquots of 1ml were removed for h.p.l.c.
measurement of aerobic control with 500μM phenol (□), anaerobic boiled cell
control with 500μM phenol (○), and anaerobic incubation with 500μM phenol (▲).
After 2h, the anaerobic incubations were made aerobic (↓) and incubated at 25°C
and 150rpm in a water bath.

Figure 3.40

Residual Phenol (%) vs Time [min]
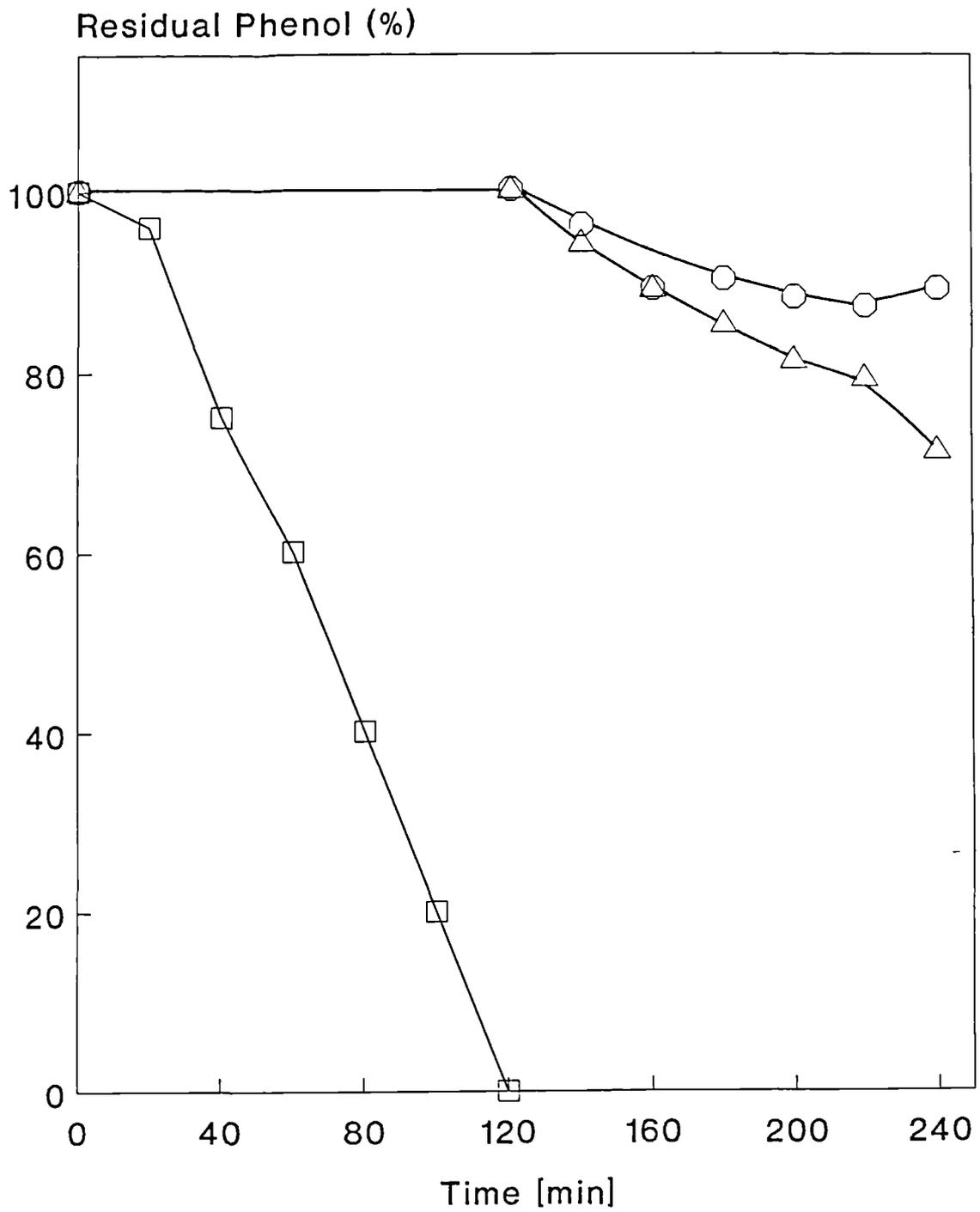
3:4:4 *Production of Phenol Hydroxylation Products.*

Active washed suspensions of *O. danica* were incubated with 500µM phenol

or 500µM *p*-cresol supplemented with 50-100µM 3-chlorocatechol to inhibit the

further metabolism of the catechol products of phenol oxidation (Bartels *et al.*,

1984; Harayama *et al.*, 1992; Klečka and Gibson, 1981). These cultures were

incubated for 3h with samples removed for h.p.l.c. measurement [acetonitrile:water

(80:20)] of the remaining phenolic substrate and accumulating catechol being

recorded (Figures 3.41 and 3.42). The identity of the catechols was made by

comparison of the retention times with those of the authentic materials. To confirm

that the appropriate catechol was being produced, the experimental products at pH

7.5 were extracted into ethyl acetate, the solution dried and evaporated to dryness

under vacuum and the resulting residues re-dissolved in methanol for analysis by

h.p.l.c. [methanol:water (80:20)]. The putative catechol peak (retention times for

catechol and 4-methylcatechol were 4.38min and 4.74min, respectively) was

confirmed by re-analysis of a sample spiked with the authentic compound which

produced an increase only in the sharp peak representing the putative catechol

(Figures 3.43 and 3.44). The catechol extractions from the alkylphenols were

reanalysed by h.p.l.c. [water:methanol (40:60)] and their retention times also

compared with those of the respective authentic alkylcatechols; the retention times

were identical (catechol, 2.47min; 4-methylcatechol, 3.02min).

The methanolic extracts containing the putative alkylcatechol were also

compared with authentic compounds by t.l.c. The solvent system used

Figure 3.41: Accumulation of Catechol from the oxidation of Phenol by washed suspensions of _Ochromonas danica_ grown in the dark on Phenol.

Each incubation flask contained 20ml of cell suspension (4mg dry wt.ml^{-1} in sodium phosphate buffer, pH7.2) with 250μM phenol and 50-100μM 3-chlorocatechol, and was incubated at 25°C and 100rpm in a water bath. Aliquots of 1ml were removed for h.p.l.c. measurement at time 0, 1; time 90min, 2; time 180min, 3.

3

2

1
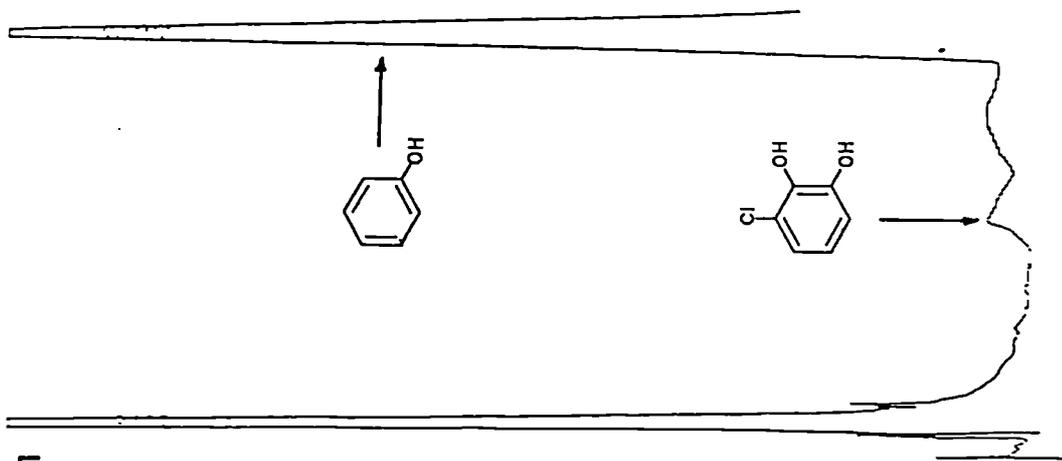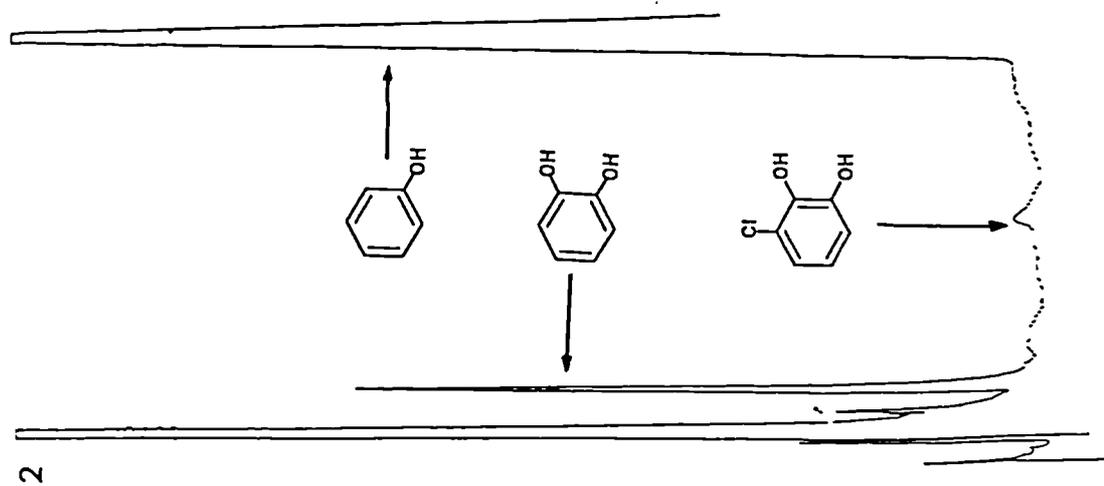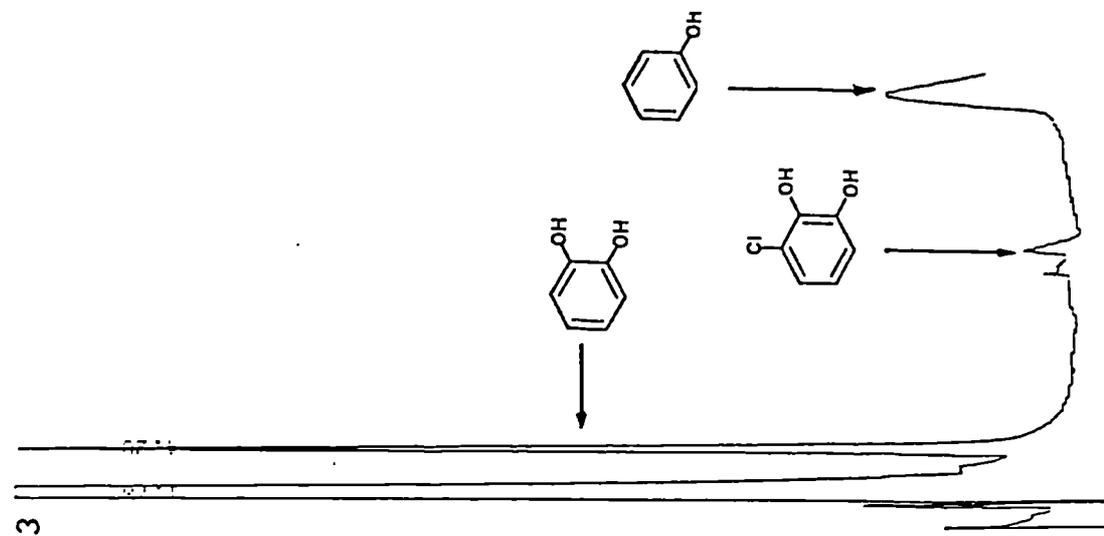
Figure 3.42: Accumulation of 4-Methylcatechol from the oxidation of *p*-Cresol by washed suspensions of *Ochromonas danica* grown in the dark on Phenol.

Each incubation contained 20ml of cell suspension (4mg dry wt.ml^{-1} in sodium phosphate buffer, pH7.2) with 250µM *p*-cresol and 50-100µM 3-chlorocatechol, and was incubated at 25°C and 100rpm in a water bath. Aliquots of 1ml were removed for h.p.l.c. measurement at time 0, 1; time 90min, 2; time 180min, 3.

125
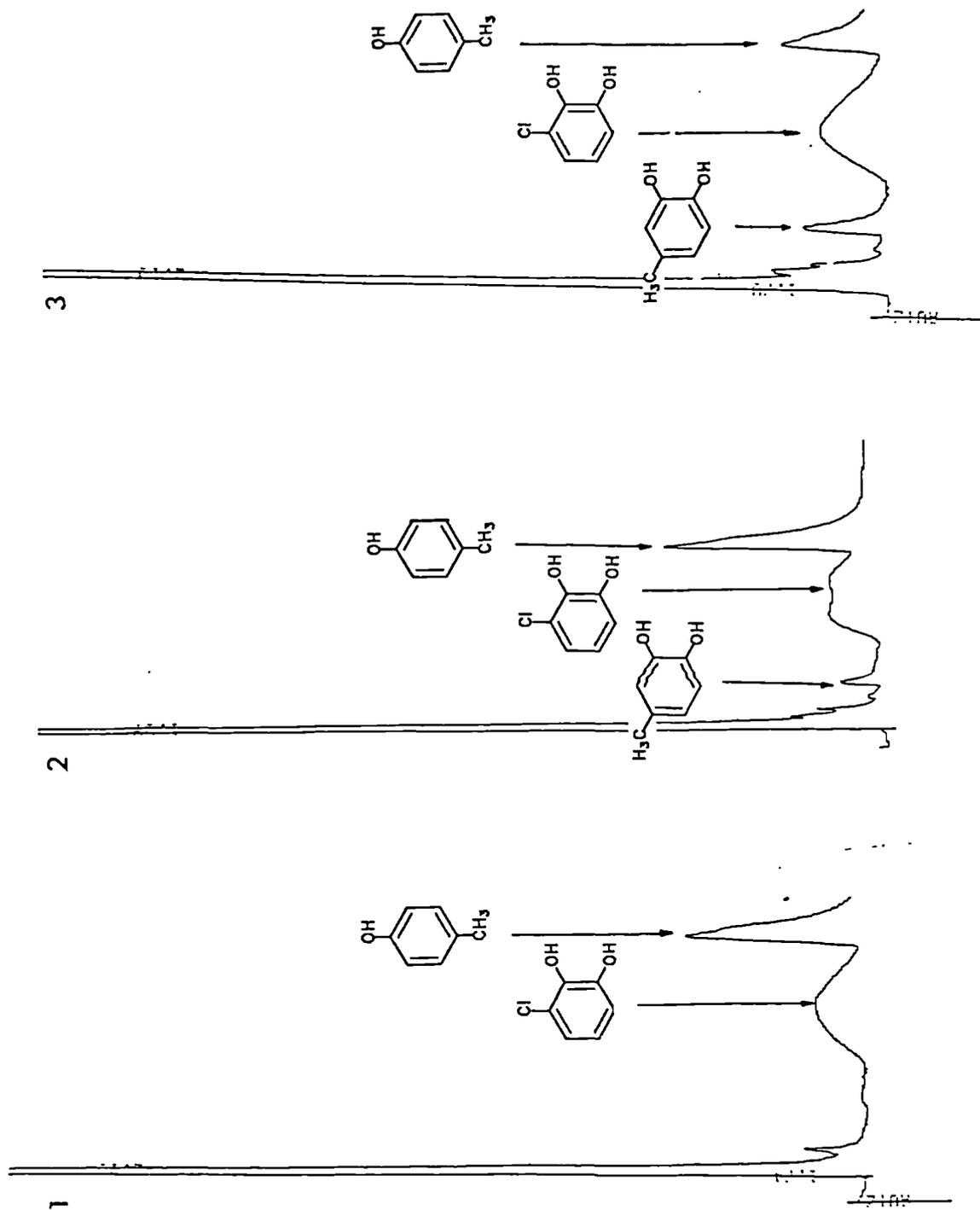
Figure 3.43: Comparison of authentic Catechol with the extracted product of Phenol oxidation.

The putative catechol (Figure 3.42) was extracted into ethyl acetate, the solvent evaporated to dryness and the residue then resuspended in 1 ml of methanol, samples of which were analysed using h.p.l.c.: authentic catechol, 1; extracted intermediate, 2; extracted intermediate spiked with authentic catechol, 3.
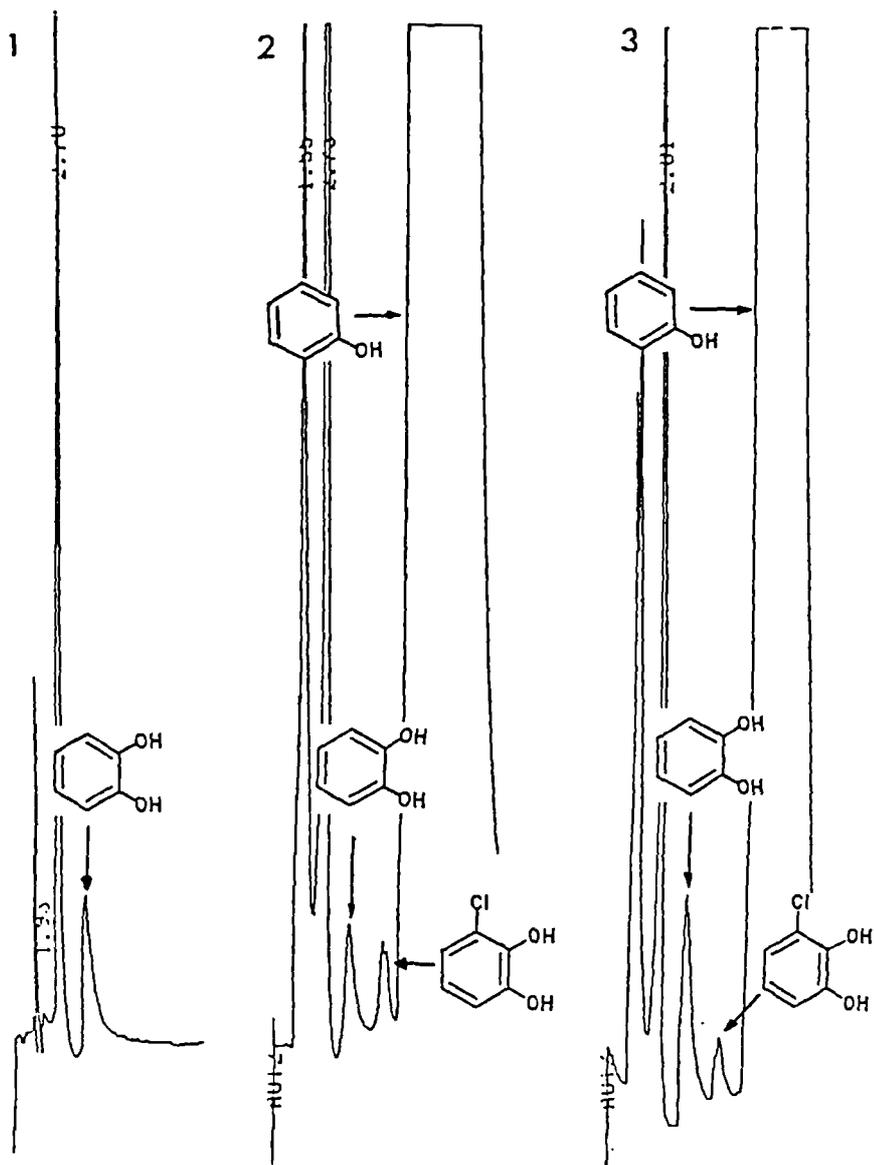
Figure 3.44: Comparison of authentic 4-Methylcatechol with the extracted product of *p*-Cresol oxidation.

The putative 4-methylcatechol (Figure 3.43) was extracted into ethyl acetate, the solvent evaporated and the residue then resuspended in 1ml of methanol, samples of which were analysed using h.p.l.c.: authentic 4-methylcatechol, 1; extracted intermediate, 2; extracted intermediate spiked with authentic 4-methylcatechol, 3.
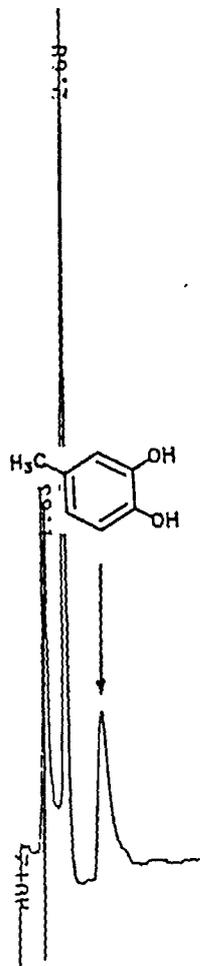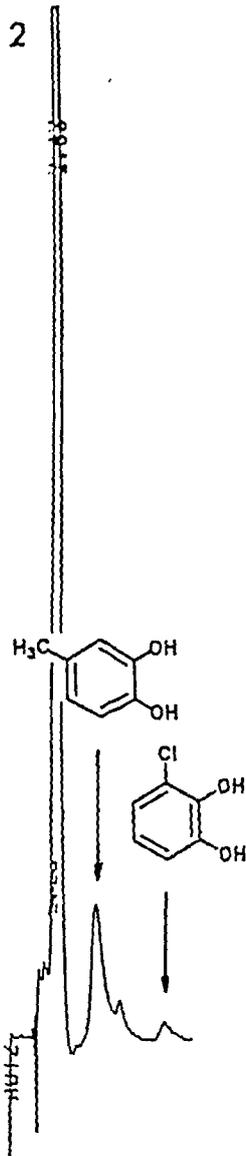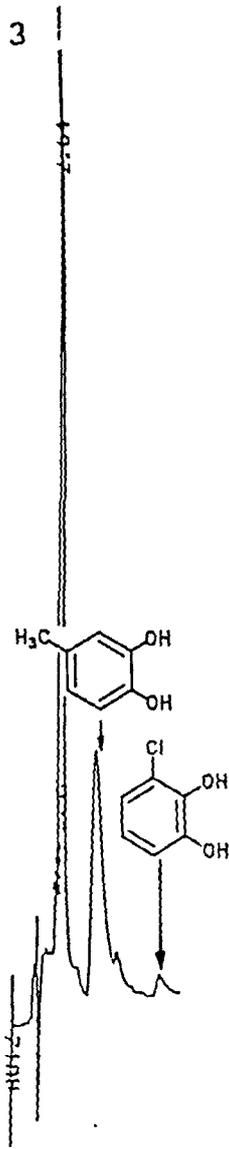
(Method 2:7:6) gave R_f values for authentic catechol and 4-methylcatechol of 0.78 and 0.75, respectively, which were identical to those of the experimental products when located by their absorbance under uv-light.

3:5 Radioisotopic Studies.

3:5:1 *Radiorespirometry.*

Ochromonas danica was grown on phenol (1mM) and harvested to give active washed cell suspensions which were equilibrated at 25°C in Warburg flasks in the dark. Then 0.3μmol, 0.5μmol and 0.7μmol [U-^{14}C]phenol was incubated with separate aliquots of cells until oxygen uptake had returned to the endogenous rate. At the end of the incubation period, samples were taken of the cells, the supernatant and the KOH containing the trapped $^{14}CO_2$ (Method 2:7:2a). In this study, the algal suspensions were able to remove 85-90% of the label from the medium, with 60-65% of the total label being oxidised to $^{14}CO_2$. The remaining ^{14}C was found in the cellular fraction which accounted for 20% of the label. The proportions did not vary significantly with the quantity of label supplied (Table 3.2).

The next stage of this study was to show the time course production of $^{14}CO_2$, the uptake of [U-^{14}C]phenol and the removal of the label from the growth medium by the cell suspension (Figure 3.45). This was achieved using Warburg

Table 3.2: Utilisation of [U-^{14}C]Phenol by washed suspensions of *Ochromonas danica* grown in the Dark on Phenol.

Warburg flasks contained, in a total volume of 2.5ml: 1.3ml of 0.2M sodium phosphate buffer, pH7.2; 0.3μmol, 0.5μmol, 0.7μmol [U-^{14}C]phenol; 1ml suspension of washed cells (3.3mg dry wt. of cells); 0.1ml KOH (20% v/v) in the centre well. Incubation was at 25°C and 100 rev/min for 4h. Samples were removed for scintillation measurement of [U-^{14}C]phenol remaining in the medium; ^{14}C within the cells; and ^{14}CO$_2$ produced. Control flasks contained cold phenol. The figures in parentheses are the amounts of label recovered in these fractions as a proportion of the label originally supplied as [U-^{14}C]phenol.

Total Phenol Added (dpm)	^{14}CO$_2$ (dpm)	Cells (dpm)	Growth Medium (dpm)
17231 (0.3μmol)	11381 (66)	3388 (20)	2452 (14)
28725 (0.5μmol)	18332 (64)	6494 (23)	3868 (13)
36934 (0.7μmol)	21099 (57)	9638 (26)	6160 (17)

Figure 3.45: Turnover of [U-^{14}C]Phenol by washed suspensions of *Ochromonas danica* Grown in the Dark on Phenol.

Warburg flasks contained, in a total volume of 2.5ml: 1.3ml of 0.2M sodium phosphate buffer, pH7.2; 0.3µmol [U-^{14}C]phenol; 1ml suspension of washed cells (4mg dry wt.); 0.1ml KOH (20% v/v) in the centre well. Incubation was at 25°C and 100 rev/min. Samples were removed from each flask for scintillation measurement of [U-^{14}C]phenol remaining in the medium (o); ^{14}C within the cells (▲); and ^{14}CO$_2$ produced (□).

Figure 3.45

Residual Label (%) vs Time [min]
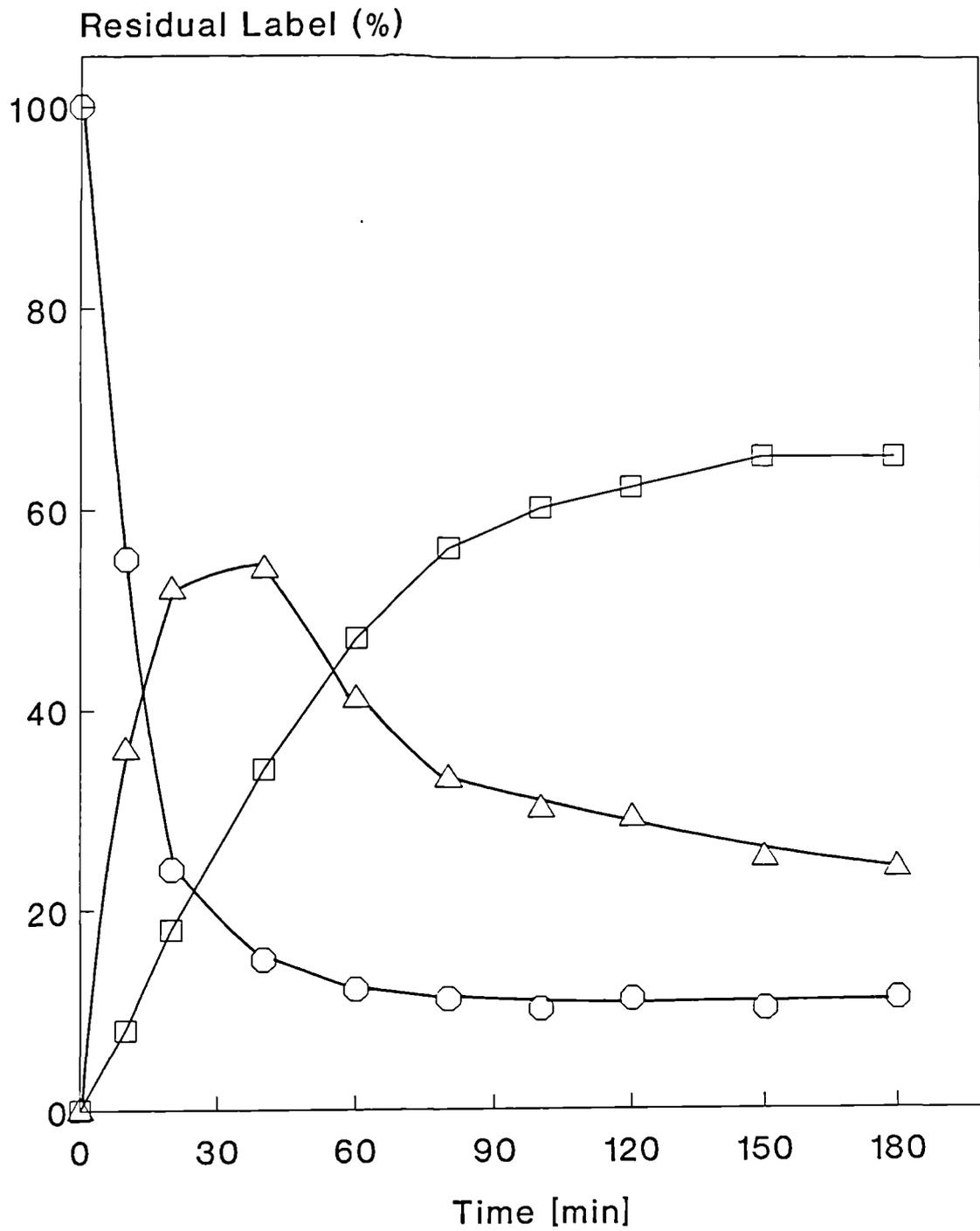
flasks, each of which contained 0.3μmol [U-^{14}C]phenol in the side arm and in the main well the active washed cell suspension. As in the previous experiment, the algal suspension was found to metabolise 60-65% of the label to $^{14}CO_2$ with 85-90% of the labelled phenol being taken up by the alga. Having established that $O.$ $danica$ was able to mineralise [U-^{14}C]phenol to $^{14}CO_2$ and also take up [U-^{14}C]phenol into the cells, it was decided to determine whether the alga incorporated the radiolabel into the components of the cellular biomass or if the label simply adsorbed to the cells. To answer this question, the cells were grown on 1mM [U-^{14}C]phenol in 1l volumes, which were harvested in late exponential growth phase. The resulting cell paste was then analysed for major cellular fractions and their content of ^{14}C (Table 3.3). In relation to whole cells, 86% of the label was found in the cell-free extract after sonication. Analysis of the label found in the lipid fraction comprised 23% of that found in whole cells. Measurement of ^{14}C found in the nucleic acid fraction accounted for 18% of total label found in the cell-free extract and 16% of the total found in whole cells. The TCA-precipitable protein fraction of the cell-free extract acccunted for 46% of the total label in extracts, equivalent to 40% of the total ^{14}C measured in the whole cells. The results obtained from these radioisotopic studies show, unequivocally, that phenol carbon is assimilated by this alga, as well as oxidised to CO_2.

Table 3.3: Incorporation of [U-^{14}C]Phenol into the Cell Biomass Fractions of *Ochromonas danica*.

O. danica was grown on 1mM [U-^{14}C]phenol. After counting a sample of the cell paste the resulting washed cell suspension was ruptured and certain fractions removed for scintillation measurement: lipid fraction was extracted with chloroform-methanol (2:1) mixture; cell-free extract; nucleic acids were precipitated with 10% (w/v) protamine sulphate (Protamine); soluble protein was precipitated with 20% (v/v) trichloroacetic acid (TCA). The amount of label is measured in dpm.g^{-1}.cells.

Total label in cells	CHCl$_3$-MeOH extractable material of cells (lipids)	Total label in cell-free extracts of cells	Protam. SO$_4$- precipt. material of extract (nucleic acids)	TCA- precipt. material of extract (protein)
152419	34810 (22.8)[a]	131788	24284 (18.4)[b]	60973 (46.3)[b]

[a] Label in lipids as a percentage of that in whole cells.
[b] Label in these fractions as a percentage of that in cell-free extracts.

3:6 Enzymology.

Studies on the degradation of phenol (Dagley, *et al.*, *1964*), phenol and cresols (Bayly *et al.*, 1966) in bacteria showed that catechol was an important intermediate in the degradation of the phenols. Having found that phenols were converted to their respective catechols by algal extracts, the next stage of the investigation was to show the presence of the enzyme responsible for this and then to demonstrate the enzymes responsible for the subsequent catabolism of the resulting catechol. To achieve this, *O. danica* was grown on phenol and crude-cell free extracts were prepared.

3:6:1 *Phenol Hydroxylase.*

Phenol hydroxylase is the enzyme responsible for the initial attack on phenol in bacteria (Bayly and Wigmore, 1973; Pieper *et al.*, 1988; Shingler *et al.*, 1989) and yeasts (Neujahr and Gaal, 1973; Neujahr and Kjellén, 1978; Kalin *et al.*, 1992) . Attempts to measure phenol hydroxylase activity in crude cell-free extracts failed to reveal any enzyme activity with either NADH or NADPH used as cofactor.

Because an enzyme capable of attacking phenol must be present in the whole cells, to permit their growth on phenol, it was concluded that either the enzyme must have been inactivated at some stage during the preparation of the cell free extract, or that this assay was failing to detect existing activity. To elucidate putative phenol hydroxylase activity (Figure 3.46), whole cells were

incubated with the substrate (500µM) and the rate of disappearance was related to the protein concentration of the cell suspension (Table 3.4). When the alga was grown on phenol, this substrate gave the highest specific activity, followed by *p*-cresol, *o*-cresol, and finally *m*-cresol.

The utilisation of the phenols only under aerobic conditions (Figure 3.40) and the identification of the catechol products (Figures 3.41-3.44) strongly suggest that a phenol hydroxylase (monooxygenase) was present in the cells.

3:6:2 *Metabolism of Catechol.*

The fate of catechols is largely dependent upon dioxygenase enzymes which lead to the cleavage of the ring. It is now well established that there are two major metabolic routes for the metabolism of catechol. In many species of bacteria and fungi, catechol undergoes an intradiol cleavage to form *cis,cis*-muconate - a mechanism known as *ortho* cleavage (Figure 3.47). Other species of bacteria perform an extradiol cleavage of the aromatic nucleus producing 2-hydroxymuconic semialdehyde - this mechanism is called *meta* cleavage (Figure 3.47).

Crude extracts from rigorously-checked axenic cultures of the alga which had been grown on phenol and *p*-cresol oxidised catechol, 3-methylcatechol, 4-methylcatechol, 4-bromocatechol and 4-fluorocatechol to produce at pH 7.2 an intense yellow colour, consistent with the meta cleavage of the catechols by a
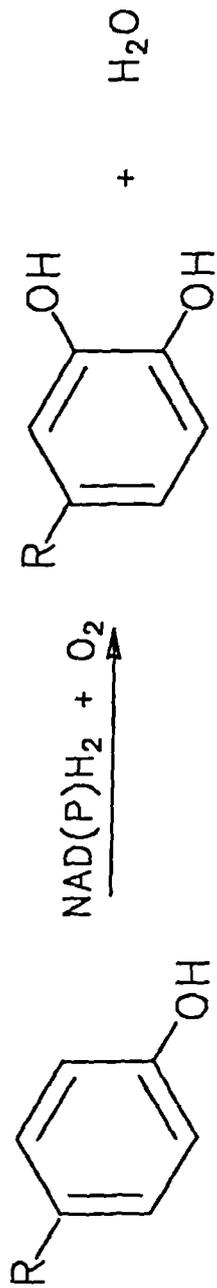
Figure 3.46: Oxidation of phenol to catechol.

R=CH$_3$ or H

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Table 3.4: Specific Activity of putative Phenol Hydroxylase in washed suspensions of *Ochromonas danica* Grown in the Dark on Phenol.

Each incubation flask contained 20ml of cell suspension (2.1mg dry wt.ml⁻¹ in 0.2M sodium phosphate buffer, pH7.2) with 500µM of substrate, and was incubated at 25°C and 100rpm in a water bath. Aliquots of 1ml were removed for h.p.l.c. measurement establishing a rate of substrate disappearance which was related to the cellular protein concentration. Specific rates were given as nmol/min/mg protein. Figures in brackets are the rates of phenolic disappearance relative to phenol (=100).

Growth Substrate	Specific Activity (nmol/min/mg protein) when tested with:			
	Phenol	*m*-Cresol	*o*-Cresol	*p*-Cresol
Phenol	17.66 (100)	4.33 (26)	8.66 (52)	12.2 (73)
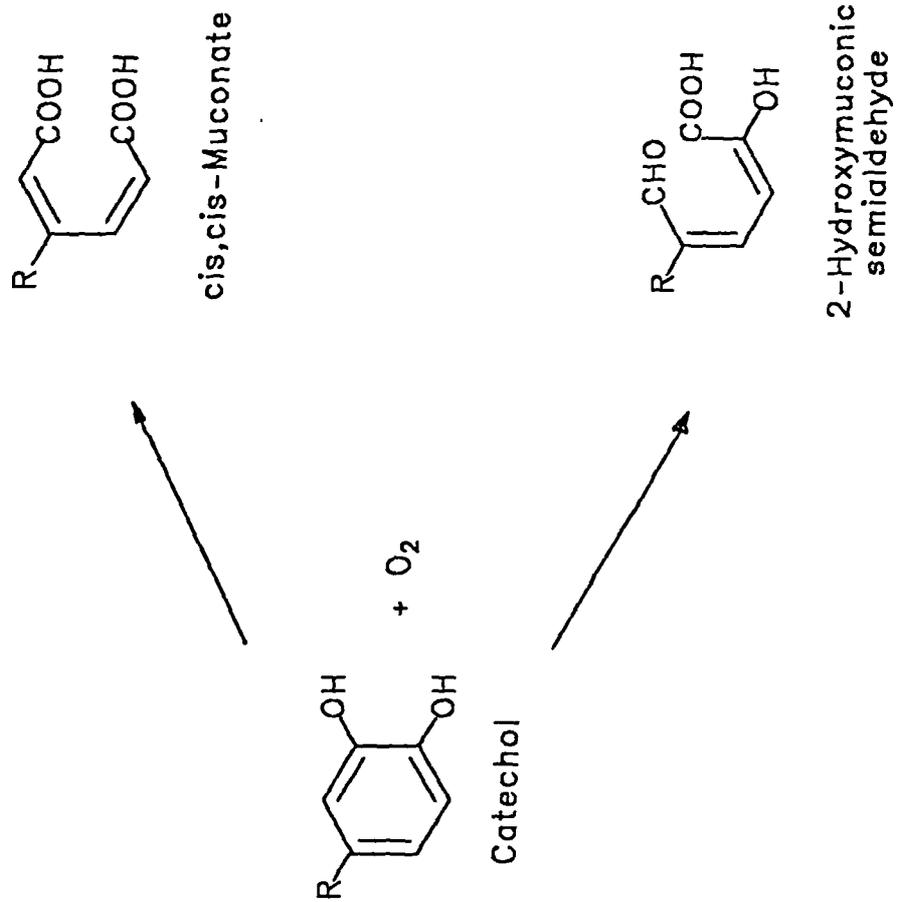
Figure 3.47:   The ring cleavage of catechol

R=H or CH₃

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catechol 2,3-dioxygenase (Tables 3.5 and 3.6). Succinate-grown cells oxidised

catechol at a much lower rate (Table 3.5).

3:6:3   *Properties of Catechol 2,3-Dioxygenase in Extracts of*

Ochromonas danica.

This inducible enzyme activity was present in extracts of *O. danica* grown in

the dark on phenol and *p*-cresol, in amounts of 44- and 34-fold, respectively,

greater than in succinate-grown cells (Table 3.5). Catechol 2,3-dioxygenase in

crude cell-free extracts of cells grown on phenol oxidised catechol and its 3- and 4-

alkyl- and 4-halo-substituted derivatives (Tables 3.5 and 3.6) at the following

approximate rates: catechol, 1; 3-methylcatechol, 0.12; 4-methylcatechol, 0.17.

Cell-free extracts obtained from *p*-cresol-grown cells oxidised catechol and its alkyl-

and halo-substituted derivatives at the following approximate rates: catechol, 1; 3-

methylcatechol, 0.13; 4-methylcatechol, 0.21. The appearance of the yellow

product formed from the oxidation of catechol is important in the identification of

meta cleavage (Table 3.7). The respective λ_{max} of the meta cleavage products of

catechol and the alkylcatechols (Table 3.7) were found to be identical to those

described by Bayly *et al.* (1966). Identification of this yellow product is vital to the

confirmation of meta cleavage and this was achieved by alternatively making

alkaline and acidifying the putative 2-hydroxymuconic semialdehyde, 2-hydroxy-6-

oxohepta-2,4-dienoate, 2-hydroxy-(4 or 5)-methylmuconic semialdehyde, 2-hydroxy-

Table 3.5: Specific Activities of Catechol 2,3-Dioxygenase in Cell-Free Extracts of *Ochromonas danica* grown in the Dark on Phenol.

Extracts were prepared from freshly harvested cells, and treated with 10% (v/v) acetone to preserve the dioxygenase activity and assayed immediately. Assay cuvettes contained, in a total volume of 1ml: 895μl of 0.2M sodium phosphate buffer, pH7.2; 25-100μl of extract; water and the reaction was started by the addition of 0.15μmol of alkylcatechol. Specific activities are in nmol/min/mg protein; the figures in brackets are the rates of meta-cleavage of the alkylcatechols relative to catechol (=100).

| Growth Substrate | Specific Activity (nmol/min/mg protein) when tested with: | | |
	Catechol	3-Methyl-Catechol	4-Methyl-Catechol
Phenol	219 (100)	27 (12.3)	37 (16.8)
p-Cresol	169 (100)	23 (13)	36 (21.3)
Succinate	4.9 (2.2)	N.D.	N.D.

Table 3.6: Specific Activities of Catechol 2,3-Dioxygenase in Cell-Free Extracts of *Ochromonas danica* grown in the Dark on Phenol.

Extracts were prepared from freshly harvested cells, and treated with 10% (v/v) acetone to preserve the dioxygenase activity and assayed immediately. Assay cuvettes contained, in a total volume of 1ml: 895μl of 0.2M sodium phosphate buffer, pH7.2; 25-100μl of extract; water and the reaction was started with the addition of 0.15μmol of catechol or halocatechol. Specific activities are given as $\Delta A_{\lambda max}$min/mg protein.

Growth Substrate	Specific Activity ($\Delta A_{\lambda max}$ min/mg protein) when tested with:		
	Catechol	4-Bromo-Catechol	4-Fluoro-Catechol
Phenol	7.3	0.8	4.2
p-Cresol	5.7	0.6	4.0

Table 3.7: Spectroscopic Properties of the Oxidation Products of Alkyl- and Halocatechols produced by the action of Catechol 2,3-Dioxygenase in crude extracts of *Ochromonas danica* grown in the Dark on Phenol.

Catechol	Meta Cleavage Product	λ_{max}	
		Found	Data of Bayly *et al.* (1966)
Catechol	2-Hydroxymuconic semialdehyde	375	375
3-Methylcatechol	2-Hydroxy-6-oxohepta-2,4-dienoic acid	388	388
4-Methylcatechol	2-Hydroxy-5-methylmuconic semialdehyde	382	382
4-Bromocatechol	2-Hydroxy-(4 or 5)-bromomuconic semialdehyde	378	-
4-Fluorocatechol	2-Hydroxy-(4 or 5)-fluoromuconic semialdehyde	386	-

(4 or 5)-bromomuconic semialdehyde and 2-hydroxy-(4 or 5)-fluoromuconic semialdehyde produced from catechol, 3- and 4-methylcatechol, 4-bromo- and 4-fluorocatechol, respectively (Table 3.8).

Webb (1963) and Bird and Cain (1974) showed that 4-isopropylcatechol irreversibly inhibited catechol 2,3-dioxygenase in a pseudomonad. To compare the catechol 2,3-dioxygenase found in *O. danica* with that found in bacteria, the algal enzyme was incubated with 4-isopropylcatechol under various conditions (Figure 3.48). Simultaneous addition of equal amounts (0.05μmol) of catechol and 4-isopropylcatechol to an assay mixture was accompanied by a decrease in the initial rate of enzyme activity (Figure 3.48b) compared with that to which catechol alone had been added (Figure 3.48a). Addition of further catechol, (either 0.05μmol or 0.2μmol) caused no resumption of activity once inhibition had occurred (Figure 3.48b). Simultaneous addition of catechol and 4-isopropylcatechol in the ratio 4:1 (0.2μmol:0.05μmol) to the enzyme preparation produced a similar reaction curve, but loss of enzyme activity was much reduced compared to that when equal amounts of inhibitor and catechol were added (Figure 3.48c). These observations suggested that catechol 2,3-dioxygenase was irreversibly (Bird and Cain, 1974) or pseudo-irreversibly (Webb, 1963) inhibited by 4-isopropylcatechol and showed further similarities between the algal and bacterial enzymes.

Table 3.8: Spectral Characteristics of the Oxidation Products of Alkyl- and Halocatechols produced by the Action of Catechol 2,3-Dioxygenase in crude extracts of _O. danica_ grown in the Dark on Phenol.

Ring-fission substrate	Ring-fission product	λ_{max} Acid	Base
Catechol	2-Hydroxymuconic semialdehyde	320	375
3-Methylcatechol	2-Hydroxy-6-oxohepta-2,4-dienoic acid	315	388
4-Methylcatechol	2-Hydroxy-(4 or 5)methyl-muconic semialdehyde	320	382
4-Bromocatechol	2-Hydroxy-(4 or 5)bromo-muconic semialdehyde	330	378
4-Fluorocatechol	2-Hydroxy-(4 or 5)-fluoro-muconic semialdehyde	315	386

Figure 3.48: Effect of 4-Isopropylcatechol on the Activity of Catechol 2,3-Dioxygenase.

Extracts were derived from phenol grown cells and treated with acetone to preserve activity. Cuvettes contained, in a total volume of 1ml: 175μmol of sodium phosphate buffer, pH7.2, and 100μl of extract added at time X. The reaction was started by adding 50nmol catechol to the reaction cuvette, at A; 50nmol catechol and 50nmol 4-isopropylcatechol (premixed) at B; 20nmol catechol at C; 100μl of extract at D; 20nmol catechol and 5nmol 4-isopropylcatechol at E.

(a) (b) (c)

Abs 375 nm

Time (min)
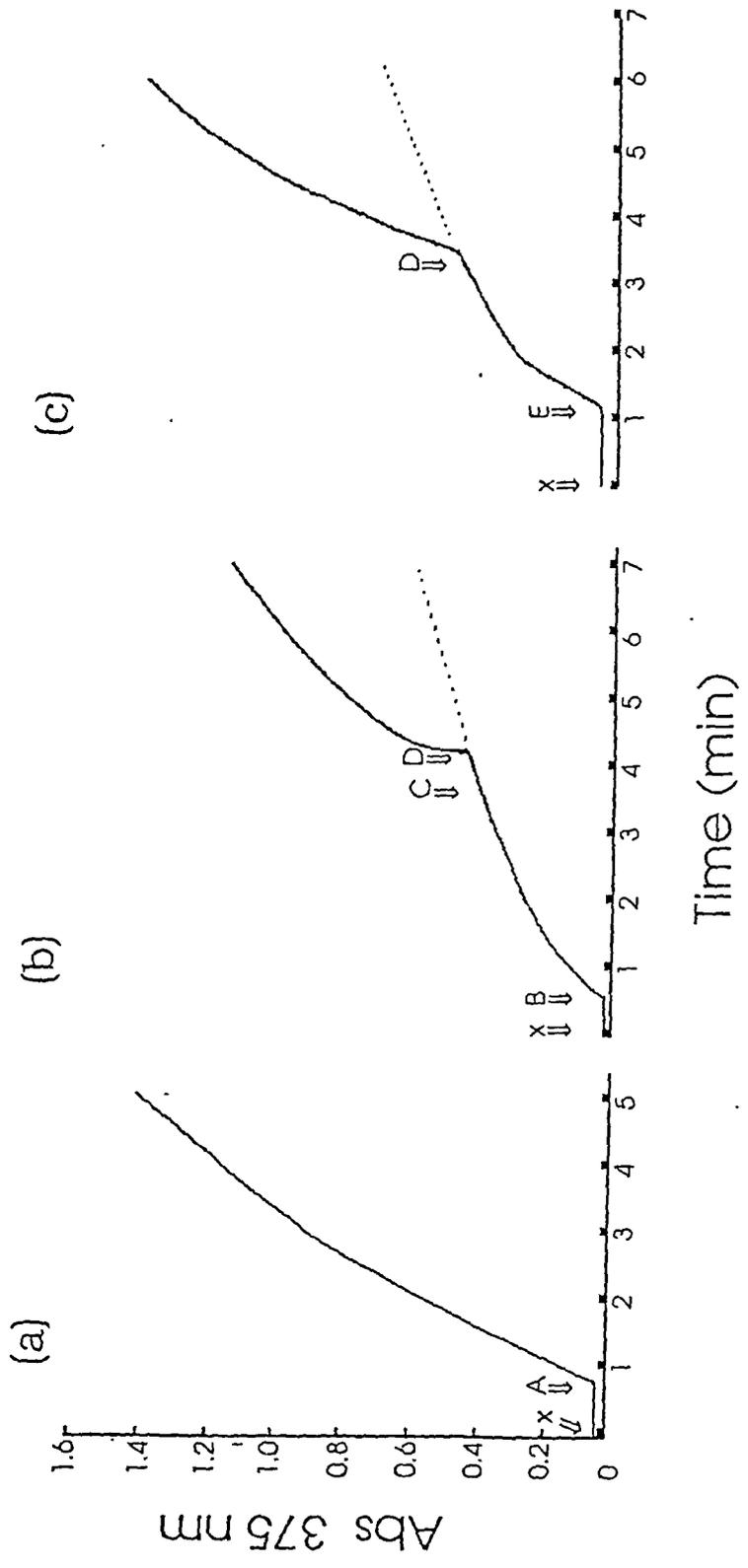
3:6:4 *Examination for Ortho Cleavage of Catechol by Algal Extracts.*

Extracts obtained from *O. danica* grown in the dark on phenol were assayed

for catechol 1,2-dioxygenase activity after extracts were treated with H_2O_2 to

inactivate the 2,3-dioxygenase activity. Activity was found to be 4.9nmol. min^{-1}. mg^{-1}

protein at 260nm. The activity of catechol 2,3-dioxygenase was also measured

after incubation with the inhibitor and was found to be 1.7nmol. min^{-1}. mg^{-1}protein

compared with 219nmol. min^{-1}. mg^{-1} protein found in fresh extracts (Table 3.5).

3:6:5 *Metabolism of the Ring-Fission Products of Catechol by Cell-Free*

Extracts of O. danica.

Studies in bacteria on the metabolism of 2-hydroxymuconic semialdehyde,

the ring fission product of catechol, have shown that there exist two different routes

for its further utilisation. Nishizuka *et al.* (1962) demonstrated the presence of an

NAD$^+$-dependant enzyme which converted 2-hydroxymuconic semialdehyde to 4-

oxalocrotonate (5-oxohex-2-ene-1,6-dioate); whereas Dagley and Gibson (1965)

and Bayly and Dagley (1969) showed that their strains employed a hydrolytic

enzyme which attacked 2-hydroxymuconic semialdehyde to yield formate and 2-

oxopent-4-enoic acid. These two pathways converged to a common intermediate,

4-hydroxy-2-oxovalerate but diverged again with the formation of different end

products: acetate and pyruvate (Nishizuka *et al.*, 1962); and acetaldehyde and

pyruvate (Dagley and Gibson, 1965). Caterall *et al.* (1971) and Sala-Trepat and

Evans (1971) showed the existence of both these pathways in an naphthalene-grown pseudomonad and a benzoate-grown *Azotobacter* sp. Both emphasised that the NAD^+-dependant pathway was of greater physiological importance, with only low activities of the hydrolase recorded and suggesting that this hydrolase played a negligible role in the metabolism of 2-hydroxymuconic semialdehyde. Subsequently, it was found that the hydrolase was important only in the metabolism of the 3-alkylcatechols and that catechol and its 4-alkyl analogues were catabolised principally via the NAD^+-mediated pathway (Bayly and Wigmore, 1973).

Cell-free extracts from phenol-grown *O. danica* metabolised the ring fission product of catechol more rapidly when NAD^+ was added to the reaction mixtures, with only low rates of hydrolytic activity being detected after treatment with NADase to remove any endogenous NAD^+ *(Table 3.9)*.

3:6:6 *Production of Pyruvate from Catechol by Cell-Free Extracts of O. danica grown in the Dark on Phenol.*

Nishizuka *et al* (1962), Dagley and Gibson (1965) and Bayly and Dagley (1969) all found pyruvate as the end-product of catechol metabolism by the *meta*-cleavage pathway. Having shown that both catechol and 2-hydroxymuconic semialdehyde were catabolised by enzymes in extracts of *O. danica*, it was important to show that the catechols, and therefore by implication the precursor phenols, were metabolised to a compound which would be integrated into the TCA cycle. Detection of pyruvate was achieved in three ways using long-term

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Table 3.9: Specific Activities of the Ring-Fission Product of Catechol in Cell-Free Extracts of *Ochromonas danica* grown in the Dark on Phenol.

Extracts were prepared from freshly harvested cells, and assayed immediately. Assay cuvettes contained, in a total volume of 1ml: 870µl of 0.2M sodium phosphate buffer, pH7.2; 50-100µl of extract; water and the reaction was started with the addition of 0.1µmol of catechol. The production from catechol of the ring cleavage product was followed until the rate began to decrease and eventually plateaued. At this point, both enzymes were assayed:

(a) *2-Hydroxymuconic semialdehyde dehyrogenase* was measured by following the decrease in absorbance at 375nm due to the disappearance of the ring-fission product, in the presence of NAD^+. Once the ring-fission product began to slowly decrease ($A_{375} \approx 0.0047min^{-1}$. determined over 5min), 0.2µmol of NAD^+ was added to the reaction mixture and the accelerated decrease in absorbance at 375nm followed.

(b) *2-Hydroxymuconic semialdehyde hydrolase* was measured following the decrease in absorbance at 375nm due to the keto form of the substrate, but using extracts which were preincubated with an NADase preparation. In these extracts, endogenous NAD^+ in the cell-free extract was destroyed by incubation at room temperature for 1hr with an equal volume of NADase preparation containing 0.5units of the the enzyme.

Table 3.9: Specific Activities of the Ring-Fission Product of Catechol in Cell-Free Extracts of *O. danica* grown in the Dark on Phenol.

Extracts assayed with:	Specific Activity (nmol/min/mg protein) of:	
	Dehydrogenase	Hydrolase
2-Hydroxymuconic Semialdehyde	6.3	0.87

incubations of catechol with cell-free extracts and only after all the 2-hydroxymuconic semialdehyde had disappeared. The first method was an enzymatic procedure which determined the pyruvate produced by its conversion to lactate with lactate dehydrogenase and excess NADH and measuring the net oxidation of excess NADH at 340nm. Addition of NADH and L-lactate dehydrogenase to the reaction mixtures resulted in an immediate drop in the extinction at 340nm due to the oxidation of the reduced nucleotide by the pyruvate. The product from the catabolism of 100nmol catechol was found to be equivalent to 37.5nmol of pyruvate. Control incubations which contained no catechol showed negligible NADH oxidation.

Pyruvate was also identified and quantified using the method developed by Friedemann and Haugen (1943) using xylene, which is specific for pyruvate dinitrophenylhydrazone, rather than ethyl acetate as the extraction solvent. From 50nmol of catechol, 22.45nmol of pyruvate was produced. Pyruvate was also confirmed by thin layer chromatography of its 2,4-dinitrophenylhydrazone derivatives extracted into xylene from a flask contents after 40h incubation of crude extracts with 100nmol catechol. Pyruvate 2,4-dinitrophenylhydrazone, produced from *authentic pyruvate and the same derivative of the enzymatically-formed* product co-chromatographed at R_f 0.57 and 0.69 (two figures are for the *syn* and *anti* stereoisomers) in a solvent containing *tert*-amyl alcohol: ethanol: water (5:1:4). Although the dinitrophenylhydrazones were evident in both uv- and visible light, the plates were sprayed with 0.1M-NaOH which gave a characteristic reddish-brown colour for both the authentic material and the experimental product. Dinitrophenylhydrozones of other common α-keto acids are dark brown after this

spray.

4. DISCUSSION

Phenolic pollution is characteristic of manufacturing and raw materials conversion processes, many of which are classed as heavy industries: the petroleum and coal industries in particular produce large volumes of phenol-contaminated waste (Stafford and Callely, 1973). While the effluent-treatment capabilities of some major industries are generally efficient, low-level contamination with phenolic materials in rivers and streams running through urban areas is widespread. Phenol, itself, is among the compounds most frequently found in rivers, industrial effluent outfalls, and landfill runoff waters in the United States, UK and many northern European countries (Water Research Centre, 1980).

The enormous turnover of carbon in Nature depends largely upon the catabolic reactions of microorganisms. After evolving the capacity to degrade naturally-occurring organic compounds over millions of years, microorganisms have only recently been challenged with synthetic chemicals released into the environment as pesticides, herbicides and a plethora of novel compounds contained in industrial effluents. The degradation of these organic compounds is not limited to the esoteric activity of a few microorganisms; it occurs rather frequently within bacteria (Chapman, 1972), fungi (Cain, 1980), yeasts (Anderson and Dagley, 1980), eukaryotic microalgae (Meyer, 1975) and higher plants (Prasad and Ellis, 1978). In Nature, it is possible but unlikely that any one organism will be responsible for the total degradation of a particular compound because any given ecosystem is a complex network involving the interaction of many organisms

(Slater and Bull, 1982). This important factor should be remembered when faced with the biodegradation of a given substrate by a particular species of microorganism in the laboratory. There are no certainties that this phenomenon will occur in the environment (Kaplan, 1976).

Before ascertaining the biodegradative role of algae in the environment, their putative catabolic processes must be identified under laboratory conditions. In this study of biodegradation by eukaryotic microalgae, it was imperative that the cultures under examination were axenic; otherwise, no assurance could be given that a particular alga was catabolising the compound in question. The importance of working with axenic cultures of algae is reinforced if the enzymology of their catabolic pathways is similar to that of the most likely contaminants, ie. bacteria and fungi. To achieve this a patient, methodical, step-by-step approach must be adopted in the purification of contaminated algal cultures as it is a very laborious and time-consuming aspect of phycological research.

Chlamydomonas ulvaensis and *Scenedesmus brasiliensis* were selected for screening of their heterotrophic abilities because Ellis (1977) had reported the production of $^{14}CO_2$ from $[U-^{14}C]$catechol and $[U-^{14}C]$phenol. *Ochromonas danica* was selected because of its reported heterotrophic versatility: phototrophy, heterotrophy, osmotrophy and phagotrophy (Pringsheim, 1952; Aaronson, 1973; 1974; Fenchel and Finlay, 1983; Andersson *et al.*, 1989; Croce, 1992) and is a ubiquitous species found in fresh, brackish and marine waters. These three eukaryotic algae grew heterotrophically on non-aromatic carbon sources (Results: Figures 3.1-3.3), an ability that is common in algae and has been reported frequently (Samejima and Myers, 1958; Droop, 1974; Neilson and Lewin, 1974;

Vincent and Goldman, 1980; Andersson *et al.*, 1989). In all three species some growth was recorded in control experiments in the absence of any carbon substrates. This was probably due to the utilisation of polysaccharide storage products (eg starch) in the Chlorophyta and chrysolaminarin (leucosin) in the Chrysophyta. Pringsheim (1952) found that *Ochromonas* also stored oil, volutin, and 'granules of unknown nature', with leucosin disappearing after external nutrient sources had been exhausted. The use of inocula which had been pre-starved, ie. grown through one culture without a nutrient source, led to a substantial reduction in this background growth.

Heterotrophy of aromatic compounds is less well documented in eukaryotic algae and for the purposes of this study it was important to find an algal species capable of this. The initial screening procedure showed that *O. danica* was a competent heterotroph with the putative capability to catabolise certain phenolic compounds. Therefore, further elucidation of the degradative capabilities concentrated on *O. danica*.

Optimum temperature and light intensities were elucidated before more detailed growth and metabolism studies were carried out on *O. danica*. Growth increased with temperature to an optimum at 25°C; higher temperatures, eg., 35°C, resulted in a decrease in the growth rate and final yield of cells (Results: Figure 3.10). Andersson *et al.* (1989) found a similar pattern with an *Ochromonas* sp. isolated from the Baltic Sea. The effects of light intensity on chemotrophic growth were negligible and the growth patterns of both photoheterotrophic and heterotrophic cultures were similar (Results: Figure 3.11). However, *O. danica* could survive at higher cell concentrations in the presence of light, after the

exhaustion of the substrate. Andersson *et al.* (1989) found similar phenomena with their Baltic Sea isolate, and extended the study by incubating the starved dark-grown cultures in the light to find that, initially, there was a slight increase in the cell concentration.

The utilisation of phenol as the sole carbon source was complete when measured by a specific phenol assay but when examined using T.O.C. analysis, organic carbon did not disappear completely from the culture supernatant, some 10% of the original carbon remaining (Results: Figure 3.17). The corresponding no-substrate controls, however, were observed to excrete soluble organic compounds to this concentration which may have been endogenous metabolites or products of cell lysis. This phenomenon is not uncommon in algae, which are known to excrete a variety of organic compounds including glycolate, various amino acids and hydrolysing enzymes (Pringsheim, 1952; Aaronson, 1973).

In both light and dark regimes, *O. danica* removed the phenol from the growth medium after three days. When the cultures were reinoculated with phenol, it was removed more rapidly than in non-adapted cultures, which suggested that the enzymes responsible for the removal of the phenol were inducible. This enzyme induction was later confirmed in oxygen electrode, turnover and enzymic studies. The capability of *O. danica* to grow on phenol and several of its methyl-substituted derivatives as the sole carbon sources was subsequently confirmed in many experiments (Results: Figures 3.16-3.21), but *o*-cresol and the xylenols were not utilised as growth substrates. This may be due to the substitution patterns of the methyl groups on the aromatic ring or to steric hinderance of a methyl group in the 2-position in these homologues, a point reinforced by the observation that 3,4-

xylenol was cometabolised.   The inability of the homologues to support growth, however, suggests that they may lead to dead-end metabolites.

Growth of the alga on phenol and cresols suggested that the carbon from these homologues was incorporated into the cellular biomass. But it was less clear if carbon obtained from the xylenols was being assimilated by the alga or simply converted to dead-end metabolites. To see if phenol was required by the alga only to induce the production of appropriate phenolic-degrading enzymes, the alga was first grown on phenol, with the cresols and xylenols introduced when the original phenol had been removed (Results: Figures 3.22 and 3.23). Similar results were obtained to those incubations conducted in the presence of phenol, suggesting that it was not the co-presence of phenol with the secondary substrate which was important, but the induction of the appropriate enzymes by the phenol itself.

Aerobic degradation of many organic compounds, especially aromatics, shows an obligatory need for molecular oxygen (Dagley *et al.*, 1960; Gibson *et al.*, 1970). The alga was shown to use oxygen when incubated with phenols and that non-adapted cells, grown on succinate, had lower activities on the phenols than phenol-adapted cells (Results: Figures 3.24-3.26). However, this was no assurance that the alga was actually oxidising the phenol because phenols can cause the uncoupling of the oxidative phosphorylation mechanism leading to an increase in the consumption of oxygen without utilisation of the substrate. The Warburg respirometer permitted quantification of the amount of oxygen consumed per mole of substrate supplied (Results: Table 3.1); this was found to be approximately 65% of the theoretical which correlates well with data produced by researchers working with bacteria mineralising aromatics (Farr, 1968; Bird, 1972).

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Further, anaerobic incubations showed unequivocally that the alga would not degrade the phenol without oxygen (Results: Figure 3.40).

The versatility of *O. danica* towards a range of alkylated phenols was tested in a series of turnover studies after the cells had been induced on phenol (Results: Figures 3.29 and 3.30). In mixed turnover incubations with phenol and a cresol or xylenol, the phenol was always removed more quickly than its co-substrate, suggesting that the enzymes involved had higher specificities, or lower K_m values, for phenol. This was further confirmed by incubating the cells initially with a cresol, then adding phenol while cresol utilisation was still underway; the result was the immediate and rapid removal of the newly added phenol while the rate of the removal of cresol was temporarily hindered until all the phenol had disappeared (Results: Figures 3.38 and 3.39).

In order to clarify the assimilatory role of phenol as a source of carbon for the alga, radioisotopic studies were carried out using $[U^{-14}C]$phenol (Results: Table 3.2). *O. danica* produced $^{14}CO_2$ when incubated with the radioisotope which confirmed that the alga was actually mineralising the phenol. The incorporation of phenol-carbon into biomass was confirmed by showing the actual presence of ^{14}C in different fractions of the cellular biomass (Results:Table 3.3). In particular, label appeared in lipid, protein and nucleic acid fractions. The information obtained from these radioisotopic studies showed that this alga unequivocally assimilated phenol. Having established that *O. danica* actually assimilated and mineralised phenol, a comparison of its specific growth rate (µ) was made against bacterial species also grown on phenol (Table 4.1). This showed that the bacterial growth rates were from twice to five-fold that of the alga.

Table 4.1: Comparison of the Specific Growth Rates (μ) of Microorganisms grown Heterotrophically on Phenol.

Organism	Growth Substrate	Specific Growth Rate (μ) h^{-1}	Author
P. aeruginosa	Phenol	0.3	Ribbons, 1970
P. putida B2	*o*-Nitrophenol	0.2	Zeyer *et al.*, 1986
Pseudomonas sp. strain HBP1 Prp	2-Propyl-phenol	0.17	Kohler *et al.*, 1993
Microbial population of activated sludge	Phenol	0.146	Rozich *et al.*, 1985
O. danica	Phenol	0.063	Semple

Although *O. danica* has been shown to mineralise some 65% of the added

phenol aerobically, the mechanisms by which this alga could achieve this were

unknown. To date, research in bacteria (Ribbons, 1970; Shingler *et al.*, 1989) and

yeasts (Neujahr and Gaal, 1973; 1978; Kalin *et al.*, 1992) has shown that a phenol

hydroxylase is responsible for the conversion of phenol to catechol; in fungi, it is

involved in the conversion of *p*-cresol to the ring-cleavage intermediate (Jones *et*

al., 1993). Further up the evolutionary ladder, in higher eukaryotes, there are

monooxygenases responsible for the hydroxylation of amino acids; for example,

phenylalanine 4-monooxygenase hydroxylates phenylalanine to form tyrosine

(Kaufman, 1966). Specific activities for the oxidation of phenol and its

monoalkylated homologues, elucidated using whole cells, showed phenol to have

the highest activity. Whole cells were necessary because the phenol

monooxygenase activity could not be measured in cell-free extracts. Higher

specific activities were obtained with phenol than with *p*-cresol, consistent with

findings in other microorganisms (Kalin *et al.*, 1992). The absolute specific

activities obtained were generally an order of magnitude less than those found in

cell-free extracts of bacteria and yeast, though when data from whole cell studies

with bacteria were recorded, these activities were found to be comparable to those

of the alga (Table 4.2).

O. danica had a ring-cleaving oxygenase which was 13 times more active

than the putative phenol hydroxlase. Jones *et al.* (1993) showed that the

protocatechuate dioxygenase was 19 times more active than the *p*-cresol

hydroxylase found in *Aspergillus fumigatus*. Kohler *et al.* (1993) found that

Pseudomonas sp. strain HBP1 Prp had a metapyrocatechase enzyme which was

Table 4.2: Comparison of the Specific Activities of Phenol Hydroxylase in Microorganisms.

Organism	Substrate	Specific Activity (nmol/min/mg)	Author
Trichosporon	Phenol	240[*]	Gaal and Neujahr, 1981
Pseudomonas	2-Propyl-phenol	195[*]	Kohler *et al.*, 1993
Sphingomonas[a]	Phenol	35[+]	Schmidt *et al.*, 1992
Aspergillus	*p*-Cresol	10[*]	Jones *et al.*, 1993
O. danica[a]	Phenol	16.7[+]	Semple

[a] Specific activities found using whole cells.

[*] Specific activities measured as nmol/min/mg protein

[+] Specific activities measured as nmol/min/mg dry wt of cells

30 times more active than the initial monooxygenase when attacking 2-propylphenol. This suggests that the NADH-dependant hydroxylase may often be the rate limiting step in the catabolism of phenols.

The demonstration of the appropriate alkylcatechols as intermediates in the catabolism of alkylbenzoic acids and alkylphenols has become easier following the observation of Klečka and Gibson (1981) that catechol 2,3-dioxygenase is specifically and irreversibly inhibited by 3-chlorocatechol thus permitting accumulation of the appropriate catechol from its precursors. The inactivation of catechol 2,3-dioxygenase by 3-chlorocatechol has attracted attention because of the resistance of halogenated compounds to microbial biodegradation via the *meta*-cleavage pathway. Two different mechanisms of enzyme inactivation have been proposed: (i) a "suicide" inactivation resulting from covalent modification of the enzyme by 3-chlorocatechol (Bartels *et al.*, 1984) and (ii) removal of the iron (II) cofactor from the active site of the enzyme by 3-chlorocatechol (Klečka and Gibson, 1981). Harayama *et al.* (1992), investigating the substrate specificity of catechol 2,3-dioxygenase encoded by TOL plasmid pWWO of *Pseudomonas putida*, supported the first mechanism (Figure 4.1), though in their model, it was concluded that the direct ring-cleavage product of 3-chlorocatechol, 5-chloroformyl-2-hydroxypent-2,4-dienoic acid was the acylating agent that inactivated the catechol 2,3-dioxygenase.

The next stage in the catabolism of phenol and its homologues is ring-cleavage, achieved in one of two ways: *ortho-* or *meta*-cleavage. To date, all eukaryotic microorganisms examined have degraded the aromatic ring via the *ortho* pathway (Cain, 1980; Gaal and Neujahr, 1979; Powlowski and Dagley, 1985). The

3-Chlorocatechol

5-Chloroformyl-2-
hydroxypent-2,4-dienoate

4-Oxalocrotonate
(enol)
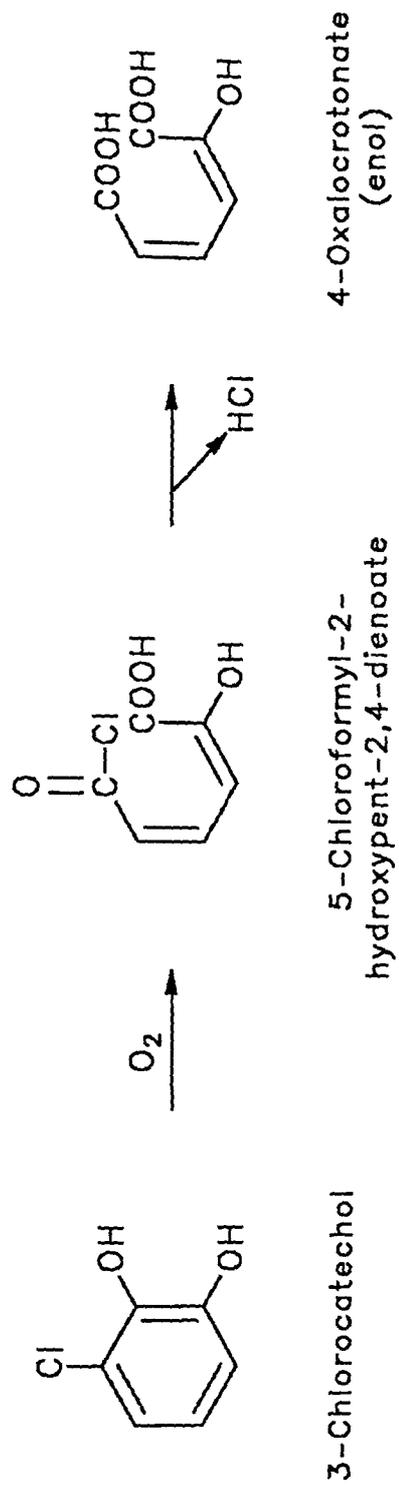
Figure 4.1: Oxidation of 3-Chlorocatechol by Catechol 2,3-Dioxygenase.

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only exception, to date, was observed with the catabolism of phenol by the eukaryotic microorganism, *Candida tropicalis* (Hashimoto, 1970; 1973). The yeast *meta*-cleaved catechol in the 1,6 position and from the extradiol-cleavage of 4-methylcatechol, 5-formyl-2-hydroxy-4-methyl-2,4-pentadienoate was isolated. *O. danica* was also, surprisingly, found to use the *meta* pathway. The *meta* pathway has been regarded as evolving for the degradation of alkyl aromatic compounds which when degraded by the *ortho* pathway often accumulate dead-end metabolites. For example, *P. putida* strain U can metabolise phenol, all three cresol isomers, 2,3- and 2,4-xylenols by the *meta* pathway; growth on any one of these compounds results in the induction of the enzymes that metabolise each of the other compounds and their respective metabolites (Bayly and Barbour, 1984). More recently, Higson and Focht (1992) showed the utilisation of 3-chloro-2-methylbenzoate by *Pseudomonas cepacia* MB2 through the *meta* cleavage of 4-chloro-3-methylcatechol. The catabolism of aromatic alkanoates by the *meta* pathway was presented by Olson *et al.* (1992) who found soil *Arthrobacter* strains to degrade 3,4-dihydroxyphenylacetate by this mechanism. Kohler *et al.* (1993) elucidated the *meta* cleavage of 2-propylphenol by a metapyrocatechase enzyme extracted from *Pseudomonas* sp. strain HBP1 Prp which had been originally isolated on 2-hydroxybiphenyl.

The presence of catechol 2,3-dioxygenase (metapyrocatechase) was found in crude cell-free extracts of *O. danica* with a high activities for catechol, 4-fluorocatechol and 4-methylcatechol. The good reactivity with 4-fluorocatechol could be due to the small atomic radius of the halide on carbon 4 thus mimicking the natural protonated analogue; this would explain why 4-bromocatechol was less

reactive. Schmidt *et al.* (1992) found this to be the case with *Sphingomonas* sp. strain SS3 grown on substituted diphenyl ethers with the corresponding 4-halocatechols decreasing in reactivity with increase of atomic radii (F > Cl > Br) and the position of the substituents on the phenol molecule (4 > 3 > 2). The position of the methyl substituents in relation to reactivity was also applicable to the algal enzyme where the order of activity was catechol > 4-methylcatechol > 3-methylcatechol. The properties of the 2-hydroxymuconic semialdehyde, a product of the the algal catechol 2,3-dioxygenase, was found to be identical to those of the bacterial type found in numerous studies (Bayly *et al.*, 1966; Bird, 1972; Crawford, 1975) (Results: Table 3.7). The similarities of the algal ring-cleaving enzyme with the corresponding bacterial enzyme also extended to their reaction with 4-isopropylcatechol. The data obtained with the algal enzyme was similar to that found by Bird and Cain (1974) where the *Pseudomonas* catechol 2,3-dioxygenase was irreversibly inhibited (Results: Figure 3.48) although the bacterial enzyme produces higher specific activities (Table 4.3). The oxygenase was inducible; when the specific activity for catechol 2,3-dioxygenase together with the specific activity of catechol 1,2-dioxygenase was assayed on extracts obtained from succinate-grown cells, both were found to be negligible.

Nishizuka *et al.* (1962) and Dagley and Gibson (1965) showed that 2-hydroxymuconic semialdehyde could undergo catabolism by two different routes, one involving a dehydrogenase and the other involving a hydrolase, respectively. Similar findings to that described above were found in cell-free extracts of benzoate-grown *Azotobacter* (Sala-Trepat and Evans, 1971), naphthalene-grown *Pseudomonas* (Catterall *et al.*, 1971) and benzoate- and toluene-grown

Table 4.3: Comparison of the Specific Activities of Catechol 2,3-Dioxygenase in Microorganisms.

Organism	Substrate	Specific Activity (nmol/min/mg protein)	Author
Pseudomonas	Catechol	4600	Sagoo, 1979
Azotobacter	Catechol	2000	Sala-Trepat and Evans, 1971
Alcaligenes	Catechol	460	Hughes *et al.*, 1984
Pseudomonas	Catechol	316	Hopper and Taylor, 1975
Pseudomonas	Catechol	177	Bayly and Wigmore, 1973
O. danica	Catechol	219	Semple

Pseudomonas arvilla mt-2 (Murray *et al.*, 1972). In this present study, only the

NAD$^+$-dependant dehydrogenase was active, with the hydrolase activity being

negligible. Activities of the hydrolase and the dehydrogenase in cell-free extracts of

O. danica grown on phenol suggested that, of these two routes for the dissimilation

of the ring-fission product of catechol, only the 4-oxalocrotonate pathway was of

significance. This was also the case with extracts of *Pseudomonas testosteroni*

PtS1 (Sagoo, 1979) grown on the homologous series of C_2-C_6 1-phenylalkane

sulphonates. The 4-oxalocrotonate pathway was also of greater physiological

significance in the metabolism of catechol by an *Azotobacter* sp. (Sala-Trepat and

Evans, 1971), where only low activities of uninduced hydrolase was observed. This

finding may explain the reluctance of the alga to grow on *m*- and *o*-cresols as sole

carbon sources and its failure to oxidise *o*-cresol extensively; both of these cresol

isomers are catabolised via 3-methylcatechol which is normally the substrate for the

hydrolase enzyme. The specific activities of the NAD$^+$-dependant dehydrogenase

appeared to be lower than the activities in many bacteria (Table 4.4).

One end-product of *meta* cleavage of catechol, whether via the hydrolase or

dehydrogenase route, is pyruvate. In the algal system, this was detected and

quantified, thus confirming the metabolism of catechol and its precursor phenol, to

normal intermediary metabolites. Approximately 40% of the theoretical pyruvate

yield was obtained when extracts of phenol-grown cells of *O. danica* were

incubated with catechol and $MgSO_4$ for 3h, whereas in bacterial systems, this figure

approached 100% (Sala-Trepat and Evans, 1971). Crawford (1975) investigating

the *meta*-fission of protocatechuate, recovered approximately 97% pyruvate from

extracts of *Bacillus circulans* incubated with protocatechuate.

Table 4.4: Comparison of the Specific Activities of the NAD$^+$-Dependant 2-Hydroxymuconic Semialdehyde Dehydrogenase in Microorganisms.

Organism	Substrate	Specific Activity (nmol/min/mg protein)	Author
Azotobacter	Catechol	350	Sala-Trepat and Evans, 1971
Pseudomonas	Catechol	160	Sagoo, 1979
Pseudomonas	Catechol	80	Hopper and Taylor, 1975
Pseudomonas	Catechol	36	Bayly and Wigmore, 1973
O. danica	Catechol	6.3	Semple

The enzymological data, discussed earlier in this study, showed a number of similarities between the algal and bacterial pathways. Firstly, in the regulation of the *meta* cleavage pathway, in order for the pathway to operate, it first has to be induced. Induction of *ortho* cleavage in most bacteria (Ornston and Stanier, 1966), in fungi (Jones *et al.*, 1993) and in yeasts (Gaal and Neujahr, 1981) and *meta* cleavage in bacteria (Ribbons, 1970; Kohler *et al.*, 1992) was initiated by the primary substrate. In the case of *O. danica*, phenol and *p*-cresol both induced the initial monooxygenase.

Further similarities were observed between the algal catechol 2,3-dioxygenase and the bacterial types. Both were more stable when incubated with acetone (Bird, 1972); both gave identical spectra for the 2-hydroxymuconic semialdehyde products and their alkylated homologues; both types showed a decrease in activity with the presence of the alkyl substituents in the 4- and 3-positions and a decrease in the enzyme activity when the 4-fluoro- analogue was replaced by 4-bromocatechol. Finally, the study carried out on the algal catechol 2,3-dioxygenase with 4-isopropylcatechol showed irreversible inhibition, consistent with that found with the corresponding dioxygenase in bacteria (Bird and Cain, 1974). The production of pyruvate as one of the end-products of *meta* cleavage in the algal system was comparable to that in the bacterial enzymes. However, the narrow specificity of the algal phenol hydroxylase compared to the bacterial systems may suggested differences.

Perhaps one of the most difficult questions which needs to be addressed, at this stage, is from where did the alga acquire this catabolic activity? The primary genetic mechanism for the adaption of a microorganism is the amplification of the

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gene pools which are involved in the metabolism of a chemical (Leahy and Colwell, 1990). The acquisition of additional genetic information can occur by means of genetic transfer, mutation, genetic recombination or transposition; for inducible catabolic pathways the appropriate regulatory genes also need to be acquired (Senior *et al.*, 1976; van der Meer *et al.*, 1992). The unusual - and unexpected - finding that *O. danica* employs *meta* cleavage for its dissimilation of aromatics raises the question of whether the *meta* cleavage pathway used by the eukaryotic *O. danica* and bacteria was the result of evolution from a common ancestor or from different ancestral lineages resulting in divergent or convergent evolution? To answer this question adequately, the genome of the alga would need to be compared with that of bacteria exhibiting similar catabolic attributes, but this information is not available at this present time.

There is now considerable evidence, however, for a close similarity between the respiratory mechanisms and cytochromes of eukaryotes and of purple non-sulphur photosynthetic bacteria (eg *Rhodopseudomonas sphaeroides*) and their non-photosynthetic relatives such as *Paracoccus* (Whatley *et al.*, 1979). Likewise there is strong evidence for the close homologies between photosynthetic apparatus of the eukaryotic red algae and of prokaryotic cyanophytes and between that of the eukaryotic green algae and of the prokaryotic prochlorophytes (Doolittle and Bonen, 1981).

O. danica's nutritional profile has already been discussed earlier in this chapter and it has been suggested that it is of a primitive nature, with some aspects of its metabolism comparable to that of prokaryotes (Pringsheim, 1952). It was suggested by Cavalier-Smith (1981) that the first eukaryote used either

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phagotrophy or osmotrophy as its mode of nutrition; *O. danica* was found to be capable of both (Aaronson, 1973). This phenomenon is not unique as links between eukaryotic algae and prokaryotic bacteria have been have been shown to exist. *Selenestrum capricornutum* was found to employ a bacterial-like dioxygenase system in its biotransformation of benzo[a]pyrene to *cis*-dihydrodiols (Warshawsky, 1988), instead of the typically eukaryotic *trans*-dihydrodiols (Jerina, 1974). Another pertinent example is the production, from 4-chloro-3,5-dinitrobenzoic acid by a non-axenic culture of *Chlamydomonas*, of 2-hydroxymuconic semialdehyde a typical product of *meta* cleavage. This intermediate was not found in the bacterial cultures of the same study and so was attributed to the alga. *O. danica* appeared to have a less well-developed photosynthetic system when compared to green algae in having chlorophylls *a, c*$_1$ and *c*$_2$ but no chlorophyll *b*. This lack of photosynthetic potential did not effect the heterotrophic ability in the light or the dark, a feature also confirmed by Fenchel and Finlay (1983) and Andersson *et al.* (1989) working with environmental isolates of *O. danica*.

There is very little information, to date, on how well *O. danica* would be able to compete for a common heterotrophic (aromatic) carbon source in a natural environment against microorganisms such as *Pseudomonas* or *Alcaligenes* which have been shown to be extremely efficient at degrading aromatic compounds. However, there has been some work carried out on nutritional competition between algae, which may shed some light on the competitiveness of *O. danica*. *Dinobryon* is a freshwater alga that is almost never found in waters with a high concentration of phosphorus which led some investigators to conclude that high concentrations of

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phosphorus were inhibitory to the growth of the alga. More detailed work (Lehman, 1976) showed that the alga in fact grew perfectly well at high concentrations of phosphorus, but in Nature other alga outcompeted *Dinobryon* in situations where there was abundant phosphorus. Only when the vernal blooms of diatoms or other phytoplankton had reduced phosphorus to a level that limited their own further growth could *Dinobryon* compete effectively. This demonstrates a basic rule (Eppley and Dyer, 1965) that among algae which have efficient uptake of nutrients (able to utilise low levels of a nutrient, low K_s) usually have lower maximum intrinsic growth rates (μ) than algae which are less efficient in taking up nutrients. These differences represent two variations of an adaptive scheme for nutrient utilisation. Some species exploit a resource-laden environment rapidly, whereas others display a measured efficiency in utilising an energy source (Lee, 1980). The ability to utilise a substrate effectively is also dependant on other environmental parameters, such as temperature. In laboratory studies, these "super degraders" are routinely incubated at pH and temperature optima and the threat of predation is removed; the result is the rapid assimilation of a plentiful growth substrate. Under these perfect conditions *O. danica* would not be able to compete at a biochemical level. In a natural environment, however, the temperature would not be at its optimum and substrate concentrations are usually very low, unless heavy contamination has occurred. Therefore, it is conceivable that the alga could compete and aid the removal of phenol from its environment. In the past, it was thought that in planktonic communities, it was unlikely that algae would be able to compete with bacteria for dilute organic substrates. Algal heterotrophy has been considered for the most part a laboratory artifact of axenic conditions of culture and artificially high

concentrations of organic compounds. Most attempts to examine algal

heterotrophy in the field relied solely upon short-term incubations with radiolabelled

organic substrates. Allen (1971) showed 20 years ago that planktonic flagellates

had active transport systems for glucose and acetate which permitted them to

compete with bacteria. Maeda and Ichimura (1973) demonstrated passive uptake

of labelled organic compounds in samples from an ice-covered lake. They

attributed this phenomenon to the algae and concluded that high algal densities

under the ice were sustained by a heterotrophic mode of nutrition. However, such

studies suffer both from restricted methodology and from the spurious assumption

that uptake of the isotopically labelled organic nutrients can be equated with

organic nutrient utilisation, hence heterotrophy (Vincent and Goldman, 1980).

Organic molecules may enter a cell but only support the synthesis of a very limited

range of biochemicals (Kornberg and Elsden, 1961) or they may fail to be

metabolised at all (Palmer and Togasaki, 1971). Vincent and Goldman (1980)

have listed the criteria which must be met to conclusively demonstrate algal

heterotrophy in Nature:

'1. Analytical description of the ambient concentrations and rate of supply of

utilisable substrates. 2. *In situ* evidence that the algae have high-affinity transport

systems which enable them to take up organic compounds effectively at ambient

concentrations. 3. Evidence that the algae are physiologically equipped to use

these substrates for growth. 4. An assessment of the relative contribution *in situ* of

dissolved organic substances versus light and carbon dioxide as carbon and

energy sources for phytoplanktonic growth.' Vincent and Goldmans' studies (1980)

in the oligotrophic Lake Tahoe (California and Nevada, USA) have provided some

of the best evidence that heterotrophy is significant to the carbon balance of

phytoplankton living near the bottom of the euphotic zone. They found significant

differences in the uptake of ^{14}C-organic compounds between light and dark bottle

incubations of water from the lake. The response to light did not occur *in situ*

below the maximum depth of inorganic photoassimilation and was completely

inhibited by a photosynthetic inhibitor. In the absence of light, acetate uptake in the

deep euphotic zone was stongly inhibited by a eukaryotic inhibitor.

Microautoradiographic analysis showed that two species of green algae were

capable of acetate transport of labelled substrate additions within previously

determined ambient limits; in axenic culture these two species grew

heterotrophically on acetate. Activities of key enzymes of the major inducible

pathway for acetate assimilation were high per unit ATP in the region of the water

column where acetate uptake was light stimulated. These data strongly support the

hypothesis of heterotrophically active phytoplankton populations at the bottom of

the euphotic zone.

On a more applied front, it is clear that algae have an important role to play

in the remediation of contaminated aquatic systems which is clearly shown in waste

stabilisation (Hawkes, 1983) and high rate algal ponds (Cromar and Fallowfield,

1992; Fallowfield *et al.*, 1992). The supposed main roles of algae in these

situations were to provide oxygen from photosynthesis and aid the removal of

nitrogen and phosphorus from influent wastes by incorporation into components of

the biomass or via pH-dependant physical and chemical processes of volatilisation

and precipitation (Cromar and Fallowfield, 1992). Chemical and Biological Oxygen

Demand in these ponds are reduced by bacterial and algal respiration of organic

matter, utilising oxygen derived from algal photosynthesis. Jinqi and Houtian (1992) suggested that algae may have a more active role in the degradation of organic compounds, than simply as the producers of oxygen from photosynthesis; these authors observed algae to be involved in the degradation of xenobiotics such as azo dyes.

The importance of carrying out biodegradation studies using pure cultures is often crucial to understanding the capabilities of particular organisms. Through this approach *O. danica* was found to degrade phenol *via* the *meta* pathway to pyruvate (Figure 4.2), but such approaches do not elucidate the contribution such algae make, if any, in the open environment when physiological, climatic and environmental stresses such as competition were placed on them. In studies of this type, various microorganisms are incubated with organic compounds, given in a single high level dose, the disappearances of which is monitored. This is invaluable as a means of screening pollutants and comparing the pollutant sensitivities of different species, but it does not mimic the field situation where pollution is often chronic and low-level. The mechanisms and rates of biodegradation of most compounds vary in response to mixed cultures, multi-substrate systems, different growth systems, a range of environments tending towards complete anaerobiosis, variable transient environmental conditions and culture and system heterogeneity (Slater and Bull, 1982). The problem with most studies is that they are concerned with the fate of organic compounds, particularly synthetic and xenobiotic compounds, and often fail to appreciate the diversity and heterogeneity of natural environments.

To continue this research, it would be beneficial to elucidate the enzymology
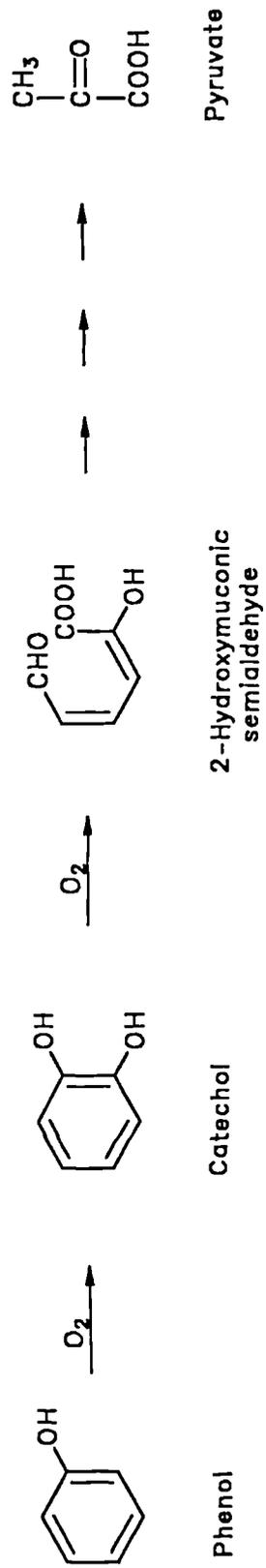
Figure 4.2   The Catabolism of Phenol by a Eukaryotic Alga.

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further; firstly, defining the putative phenol hydroxylase using $^{18}O_2$ labelling to clarify the mechanisms of the formation of catechol and also to optimise the activities of the enzyme in extracts to compare it realistically against bacterial and yeast enzymes. Secondly, the further elucidation and optimisation of the enzymes of the catabolic pathway between 2-hydroxymuconic semialdehyde and the formation of pyruvate. Another important aspect which should be investigated is, firstly, the effect that competition from other organisms, such as bacteria or yeasts, for phenolic substrates has on the alga. By recreating an environment where this alga is common, the alga's biodegradative potential could be examined using chemostats which have been used successfully to study the effects of low-level, long-term pollutants (Cloutier-Mantha and Harrison, 1980).

5. SUMMARY

(i) Phenol and its methylated homologues are commonly produced as by-products
of coal and petroleum industries and as such are found in aquatic effluent systems.
Phenol is already known to be degraded by bacteria, fungi and yeasts and was
chosen as a model substrate to investigate the ability of eukaryotic microalgae to
degrade organic pollutants.

(ii) Axenic cultures of *Chlamydomonas ulvaensis* [CCAP 11/58], *Scenedesmus
brasiliensis* [CCAP 276/1B] and *Ochromonas danica* [CCAP 933/28] were
examined for their abilities to remove phenol from the growth media in both light
and dark conditions. Of these algae, only *O. danica* could remove phenol rapidly
when incubated in light and dark conditions with glucose. Neither *C. ulvaensis* nor
S. brasiliensis removed the phenol substrate as rapidly.

(iii) As a putative phenol degrader, the metabolism of phenol by *O. danica* was
chosen for further investigation. The alga grew on phenol and *p*-cresol as the sole
carbon source up to concentrations of 4mM. Although the alga could not utilise *o*-
cresol or any of the xylenols as the sole carbon sources, the rate of their
degradation was significantly increased when the organism was incubated with
phenol; the addition of phenol was seen to facilitate the removal of the more
recalcitrant compounds.

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(iv) Whole cell studies involving the screening of the oxidative potential of the alga after growth on phenol, showed it to be an obligate aerobe. The position and number of methyl substituents on the aromatic ring of the phenols all affected their rate of oxidation by the phenol-grown cells. Turnover studies on the same phenolics by phenol-induced cells were consistent with their rates of oxidation.

(v) Radioisotopic studies using [U-^{14}C]phenol showed that some 60% of the isotope was recoverable in $^{14}CO_2$ after incubation with phenol-induced cells. Phenol is thus mineralised by the alga, which is the ultimate aim of biodegradation. Assimilation of the phenol-C into cellular biomass was confirmed by the discovery of ^{14}C incorporation into subcellular fractions of the algal biomass after incubation with [U-^{14}C]phenol. This is conclusive proof that the alga is able to metabolise phenol and use the available carbon to grow.

(vi) A survey of the enzymes of phenol metabolism was carried out using whole cells and cell-free extracts prepared from *O. danica* cultures grown in the dark on phenol or succinate. Activities for a putative phenol hydroxylase could not be found in cell-free extracts. However, this activity was determined by measuring phenol disappearance in whole cell incubations. The first oxidation product of phenol was catechol which was isolated by using 3-chlorocatechol to inhibit the catechol 2,3-dioxygenase. High specific activities were found for catechol 2,3-dioxygenase but not for catechol 1,2-dioxygenase in extracts, thus indicating the existence of *meta* cleavage in this eukaryotic microorganism. The degradation of the resulting ring-cleavage product, 2-hydroxymuconic semialdehyde, was found to be *via* an NAD$^+$-

dependant dehydrogenase enzyme. Prolonged incubation of catechol with extracts led to the formation of pyruvate, which is in accordance with the classical *meta* cleavage pathway found in bacteria.

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