

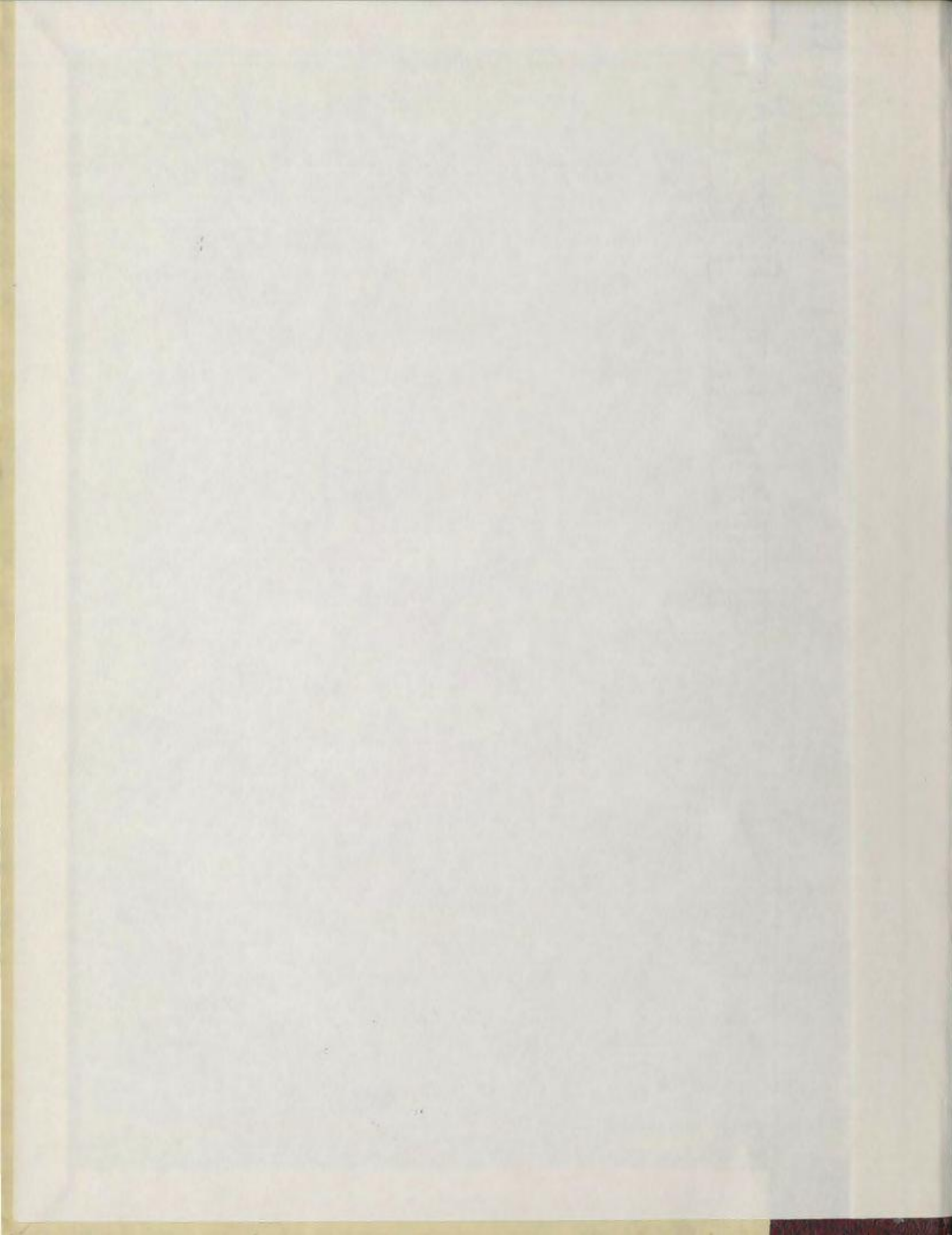
THE REGULATION OF
NAPHTHALENE METABOLISM IN PSEUDOMONADS

CENTRE FOR NEWFOUNDLAND STUDIES

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385586



THE REGULATION OF
NAPHTHALENE METABOLISM IN PSEUDOMONADS

A thesis

by

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Submitted in partial fulfillment of
the requirements for
the degree of Master of Science

Department of Biochemistry
Memorial University of Newfoundland

August, 1974

- 1 -

ACKNOWLEDGEMENTS

I express my thanks to Dr. E.A. Barnsley for his guidance and encouragement throughout the work, and to Dr. F.A. Aldrich, Dean of Graduate Studies and Dr. L.A.W. Feltham, former Head of the Department of Biochemistry, for their patient co-operation and help which enabled me to come to Memorial University.

The help offered by Memorial University by way of a fellowship and laboratory facilities is also gratefully acknowledged. Thanks are also due to Miss Judy Sheppard, Mr. D. Hall, Mr. M.G. Bray, and Mrs. Sonia Banfield for their co-operation and technical help.

ABSTRACT

Naphthalene oxygenase was induced in Pseudomonas NCIB 9816 when the organism was grown on salicylate, or on succinate and salicylate together. The enzyme was not induced when the organism was grown on catechol or on succinate and catechol. The levels of catechol 1,2-dioxygenase and of catechol 2,3-dioxygenase in extracts from cells that had been grown in the presence of salicylate were similar to those in extracts from cells that had been grown in the presence of catechol. These observations suggest that salicylate may be the inducer of naphthalene oxygenase. This is supported by the observations that when either 2-aminobenzoate or 2-hydroxybenzyl alcohol was added to cultures growing on succinate, naphthalene oxygenase was induced gratuitously.

Salicylaldehyde dehydrogenase and salicylate hydroxylase were also induced in the organism under the same conditions as caused the induction of naphthalene oxygenase, but the levels of these three enzymes were not constant relative to one another under the different growth conditions. In spite of this lack of proportionality which may be due to the limitations of the methods used for the determinations of these enzyme activities, the observation that all three enzymes may be induced by a single gratuitous inducer suggests that these enzymes, and possibly all involved in the degradation of naphthalene to catechol, are controlled coordinately.

Catechol 1,2-dioxygenase was induced in NCIB 9816 by catechol and salicylate, but not by the gratuitous inducers of naphthalene

Oxygenase. Catechol 2,3-dioxygenase levels in extracts from succinate grown organisms was comparable to that in extracts from cells induced with salicylate, catechol, 2-aminobenzoate or 2-hydroxybenzyl alcohol. The synthesis of this enzyme therefore seems to be constitutive in Pseudomonas NCIB 9816.

TABLE OF CONTENTS

	Page
ACKNOWLEDGEMENTS	i
ABSTRACT	ii
LIST OF TABLES	vii
LIST OF FIGURES	viii
LIST OF ABBREVIATIONS	x
INTRODUCTION	1
<u>Oxidation of aromatic hydrocarbons</u>	2
Benzene and its alkyl derivatives	2
Naphthalene	5
Phenanthrene and anthracene	8
<u>Oxidation of phenolic compounds</u>	11
Catechols	11
Gentisic acid	17
<u>Genetic basis of the degradation of aromatic compounds</u>	17
THE PRESENT WORK	19
MATERIALS AND METHODS	21
<u>Bacteria</u>	21
<u>Culture media</u>	21
Basal mineral media	21
Carbon sources and inducers	22
<u>Culture methods</u>	22
Growth on glucose, succinate, acetate and malate	23
Growth on naphthalene and salicylate	24
Growth on catechol	24

	Page
<u>Harvesting of the cells and the preparation of cell extract</u>	24
<u>Chemicals</u>	25
<u>Enzyme assays</u>	25
<u>Determination of the rate of oxygen uptake by cells of NCIB 9816 in the presence of naphthalene</u>	27
<u>Measurement of the concentration of inducers in culture media and the determination of the time-course of the induction of naphthalene oxygenase</u>	28
<u>Identification of salicylate produced by the incubation of NCIB 9816 with naphthalene</u>	29
<u>Initial rate of the accumulation of salicylate from naphthalene</u>	29
<u>Stoichiometry of the conversion of salicylaldehyde to salicylate and the identification of the product</u>	30
<u>Protein determination</u>	31
<u>Determination of the actual NAD⁺ content of a commercial preparation</u>	32
<u>RESULTS</u>	33
<u>Measurement of naphthalene oxygenase in whole cells of NCIB 9816</u>	33
<u>Measurement of naphthalene oxygenase in NCIB 9816 induced gratuitously</u>	40
<u>The time-course of the induction of naphthalene oxygenase by salicylate and gratuitous inducers in NCIB 9816</u>	40
<u>Salicylaldehyde dehydrogenase</u>	50
<u>Determination of the optimum pH</u>	50

	Page
Determination of K_m for NAD^+	53
Activity with other substrates and cofactors	60
<u>Measurement of the levels of some enzymes of naphthalene metabolism in whole cells and extracts of NCIB 9816</u>	64
<u>Induction of naphthalene oxygenase in NCIB 9816 grown on glucose, malate and acetate</u>	64
<u>The appropriateness of the method of preparation of cell extract</u>	67
DISCUSSION	69
<u>Measurement of naphthalene oxygenase</u>	70
<u>Measurement of other enzymes</u>	81
Salicylaldehyde dehydrogenase	81
Salicylate hydroxylase	81
Catechol dioxygenases	82
<u>Induction of some enzymes of naphthalene metabolism in NCIB 9816 under various growth conditions</u>	85
REFERENCES	93
APPENDIX - Enzymic reactions studied in this work	98

LIST OF TABLES

Page

I	Levels of naphthalene oxygenase in <u>Pseudomonas</u> NCIB 9816 grown on various carbon sources	41
II	Levels of naphthalene oxygenase in NCIB 9816 induced with 2-aminobenzoate and 2-hydroxybenzyl alcohol	42
III	Stoichiometry of the reaction between salicylaldehyde and NAD ⁺ catalyzed by the cell extract from induced cells of NCIB 9816	61
IV	Oxidation of some aldehydes by crude extracts from induced NCIB 9816	65
V	Levels of some enzymes of naphthalene metabolism in <u>Pseudomonas</u> NCIB 9816 grown under various conditions	66
VI	Effects of various carbon sources on the induction of naphthalene oxygenase in <u>Pseudomonas</u> NCIB 9816	68
VII	Effects of some inhibitors on the oxidation of naphthalene in whole cells of NCIB 9816	72
VIII	Activity of catechol 2,3-dioxygenase in extract of ATCC 17483 in the absence and in the presence of the extract of NCIB 9816	84

LIST OF FIGURES

	Page #
1. Initial steps in the oxidation of benzene and alkyl-benzenes by some microorganisms	3
2. Proposed pathway of naphthalene metabolism by pseudomonads	7
3. Proposed pathway for the metabolism of phenanthrene by soil pseudomonads	10
4. Proposed pathway for the degradation of anthracene by soil pseudomonads	12
5. <u>Ortho</u> and <u>meta</u> cleavage pathways in pseudomonads	13
6. Dependence of the initial rates of naphthalene disappearance on the concentration of bacteria	35
7. Initial rates of naphthalene oxidation by washed induced cells of NCIB 9816 at various concentrations of naphthalene	37
8. Lineweaver Burke plots of the initial rates of naphthalene oxidation at various concentrations of naphthalene	39
9. Time-course of the induction of naphthalene oxygenase by salicylate in <u>Pseudomonas</u> NCIB 9816 growing on succinate	45
10. Time-course of the induction of naphthalene oxygenase by 2-hydroxybenzyl alcohol in NCIB 9816 growing on succinate	47
11. Time-course of the induction of naphthalene oxygenase by 2-aminobenzoate in NCIB 9816 growing on succinate	49
12. Activity of salicylaldehyde dehydrogenase at different pH values in the crude extract of NCIB-9816 induced with 2-hydroxybenzyl alcohol	52
13. Activity of salicylaldehyde dehydrogenase at different concentrations of cell extracts	55
14. Activity of salicylaldehyde dehydrogenase in the crude extracts of NCIB 9816 at different NAD^+ concentrations	57

15. Determination of the K_m for NAD^+ in the oxidation of salicylaldehyde catalyzed by salicylaldehyde dehydrogenase 59
16. Absorption spectra of the solutions of authentic salicylate and 1,10-phenanthroline, and the spectrum of the product of reaction between salicylaldehyde and NAD^+ catalyzed by salicylaldehyde dehydrogenase in the presence of 1,10-phenanthroline 63
17. Initial rate of naphthalene disappearance compared with the initial rate of appearance of salicylate when a suspension of washed cells of NCIB 9816 is incubated with naphthalene 77
18. Initial rate of naphthalene disappearance compared with the initial rate of appearance of salicylate in a washed cell suspension of Pseudomonas NCIB 9816 that had been induced with 2-hydroxybenzyl alcohol 79

LIST OF ABBREVIATIONS

pCMB	p-Chloromercuribenzoate
CoA	Coenzyme A
EDTA	Ethylenediaminetetraacetic acid
FAD	Flavine-adenine dinucleotide
GSH	Glutathione (Reduced)
NAD ⁺	Nicotinamide-adenine dinucleotide
NADP ⁺	Nicotinamide-adenine dinucleotide phosphate
Tris	Tris (hydroxymethyl) aminomethane

INTRODUCTION

Aromatic hydrocarbons are natural products formed by higher and lower plants and microorganisms and they are also found in crude oils (see Zobell, 1971 for a review). These return to the soil along with their synthetic derivatives e.g. pesticides, dyes, drugs and other products of everyday use. Aromatic compounds are relatively inert but microorganisms degrade these compounds, probably completely to carbon dioxide and water. Studies on the microbial dissimilation of higher aromatic hydrocarbons have not progressed greatly. Van der Linden and Thijsse (1965) attributed this to the "non-physiological" nature of the compounds, difficulties involved in working with water-insoluble substances, and difficulty in obtaining the compounds in sufficiently pure form. The metabolism of the lower molecular weight compounds, e.g. benzene, naphthalene, phenanthrene and some of their alkyl derivatives has been investigated intensively, however, and pathways for their degradation are understood (see Gibson, 1971 for a review). Much progress has also been made in elucidating the microbial degradation of aromatic hydrocarbon derivatives, e.g. phenolic and carboxylic acid derivatives of benzene, some of which occur naturally in quantities much greater than those of the aromatic hydrocarbons themselves. Microbial enzymes incorporating oxygen atoms into the benzene ring are extremely labile (Marr and Stone, 1961), and this has made the acquisition of information about the oxidation of the parent hydrocarbons difficult.

The microbial degradation of aromatic compounds may be considered

conveniently under two main headings; the initial reactions incorporating oxygen into the benzene nucleus, and the oxidation of phenolic compounds. While these reactions occur aerobically, it seems that anaerobic dissimilation is also possible (Duttons and Evans, 1969 and Taylor, Campbell and Chinoy, 1970).

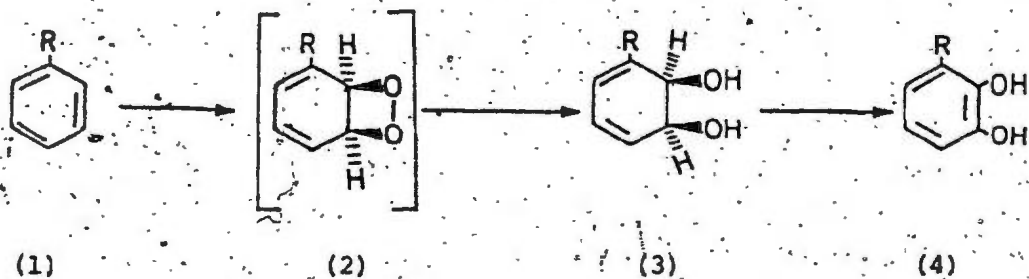
OXIDATION OF AROMATIC HYDROCARBONS

Benzene and its alkyl derivatives

Observations by various workers led to the elucidation of the pathways for the bacterial degradation of benzene and its alkyl derivatives. Degradation of benzene starts with the incorporation of two oxygen atoms into the benzene ring. With alkyl benzenes the initial attack may be on the alkyl side chain or on the benzene ring. When the initial attack involves the benzene ring the proposed degradative pathway is as shown in Figure 1. The first detectable intermediate is a cis-dihydroarenediol (3, Fig. 1) (Gibson, Koch and Kallio, 1968; Gibson, Hensley, Yoshioka and Marby, 1970; Gibson, Cardini, Maseles and Kallio, 1970 and Gibson et al., 1973). These authors also showed that the dihydroarenediol is oxidized by a NAD^+ dependent dehydrogenase to catechol or its alkyl derivatives (4, Fig. 1). Axcell and Geary (1973) made similar observations. The dioxetane (2, Fig. 1) has been suggested as precursor of the dihydroarenediol to account for the cis configuration of the latter (Gibson et al., 1968; Gibson, 1971). This is also in accord with

Figure 1

Initial steps in the oxidation of benzene
and alkylbenzenes by some microorganisms



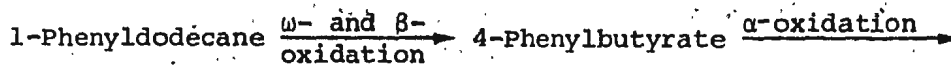
R = H, or an alkyl group

the observation that both atoms of oxygen in cis-benzenediol are derived from molecular oxygen (though both atoms may not come from the same molecule of oxygen) (Gibson, Cardini, Maseles and Kallio, 1970).

In the case of the degradation of alkylbenzenes with short-chain alkyl groups, the mode of initial attack depends on the type of organism. For example, toluene is degraded through 3-methylcatechol (4, Fig. 1, R = CH₃) by Pseudomonas putida (Gibson, Hensley, Yoshiok and Marby, 1970) and Pseudomonas mildenbergii (Nozaka and Kusonose, 1969). Similar observations were noted by Claus and Walker (1964) with a Pseudomonas species and an Achromobacter species. But Kitagawa (1956) and Nozaka and Kusonose (1968) demonstrated the dissimilation of toluene through the initial formation of benzyl alcohol by Pseudomonas aeruginosa. Similarly, Davis and Raymond (1961) found that when a Nocardia species was grown on a medium containing ethylbenzene together with either n-hexadecane or n-octadecane, the ethylbenzene was converted to phenylacetic acid. On the other hand six Pseudomonas species and a Nocardia species oxidized ethylbenzene through 3-ethylcatechol (4, Fig. 1, R = C₂H₅) (Gibson, 1971). The degradation of catechol and its derivatives will be dealt with later.

In the case of the degradation of alkylbenzenes with relatively longer side chains oxidation generally starts at the side chain (Davis and Raymond, 1961; Welby, Duff and Farmer, 1956; Sari-Aslani, Harper and Higgins, 1972 and Baggi, et al., 1972). The last named authors also demonstrated, however, the initial attack on the benzene ring of some higher alkylbenzenes. Sari-Aslani et al. (1972) proposed the following

pathway for an alkylbenzene with^a long side chain:



Naphthalene

Tattersfield (1928) demonstrated that naphthalene added to soil disappears due to bacterial degradation. Gray and Thornton (1928) isolated from soil several strains of naphthalene-utilizing bacteria. Walker and Wiltshire (1953) isolated a soil organism which could use naphthalene (5, Fig. 2) as the sole carbon source, and from its culture medium isolated salicylate (11, Fig. 2) and a compound which they tentatively identified as trans-1,2-dihydro-1,2-dihydroxynaphthalene. The trans configuration of the compound was suggested because it was found to have a specific rotation comparable to that of D-trans-1,2-dihydro-1,2-dihydroxynaphthalene isolated from the urine of rabbits that had been injected with naphthalene (Booth and Boyland, 1949). Using washed cell suspensions of naphthalene-grown cells they found immediate oxygen uptake in the presence of D-trans-1,2-dihydro-1,2-dihydroxynaphthalene, salicylate and catechol (12, Fig. 2). Similar results were obtained by Treccani, Walker and Wiltshire (1954) using this organism and four other organisms, two of which were Pseudomonas species.

Fernley and Evans (1958) demonstrated the oxidation of 1,2-dihy-

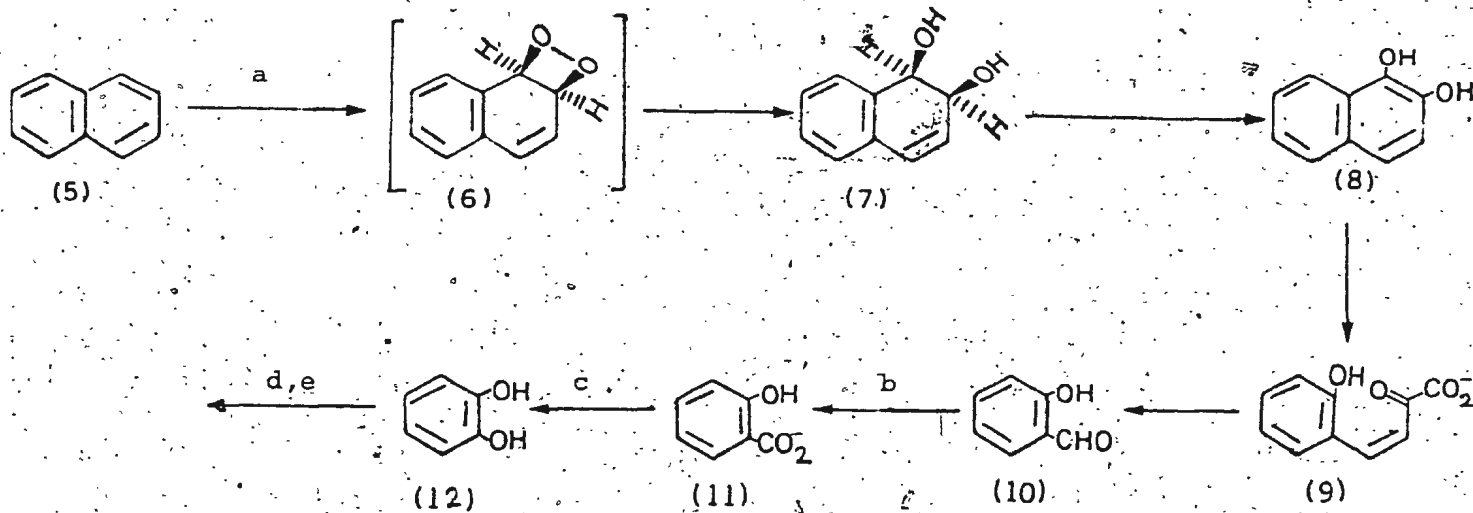
droxynaphthalene (8, Fig. 2) by a pseudomonad grown on naphthalene.

Davies and Evans (1964) demonstrated the formation of a new intermediate namely cis-o-hydroxybenzalpyruvate (9, Fig. 2) in naphthalene degradation by a fluorescent pseudomonad, which suggested that 1,2-dihydroxynaphthalene undergoes fission between the angular C and C-1 of the naphthalene nucleus. They proposed the pathway for bacterial degradation of naphthalene as shown in Figure 2.

The actual stereochemistry of 1,2-dihydro-1,2-dihydroxynaphthalene (7, Fig. 2) was not established until recently for lack of the microbially synthesized compound in quantities sufficient for thorough investigation. Jerina et al. (1971) (see also Gibson, 1971) obtained a mutant of a naphthalene-utilizing pseudomonad which was able to partially oxidize naphthalene. When grown on glucose in the presence of naphthalene, a compound accumulated which was identified as cis-1,2-dihydro-1,2-dihydroxynaphthalene. The compound was distinguished from the trans isomer by thin layer chromatography using multiple development with chloroform. Proton magnetic resonance spectra and the ease of formation of 1-naphthol by treatment with HCl indicated the metabolite to be the cis isomer. After [¹⁴C] naphthalene and cis or trans-1,2-dihydro-1,2-dihydroxynaphthalene were added to extracts of several strains of bacteria, including the parent strain of the mutant from which the cis isomer had been isolated, and the dihydrodiol was reisolated, radioactivity was predominantly in the cis isomer. These studies strongly suggested that cis-1,2-dihydro-1,2-dihydroxynaphthalene and a cyclic peroxide (dioxetane) (6, Fig. 2) are intermediates in the bacterial metabolism of naphthalene.

Figure 2

Proposed pathway of naphthalene metabolism by pseudomonads



Note: The letters a-e inclusive refer to reactions which are more fully presented in the appendix.

This stereochemistry is in accord with that reported for benzene degradation (Gibson, Cardini, Maseles and Kallio, 1970).

Catterall, Murray and Williams (1971) reinvestigated the oxidation of naphthalene by Pseudomonas NClB 9816 which was reported to metabolise naphthalene through trans-1,2-dihydro-1,2-dihydroxynaphthalene (Griffiths and Evans, 1965). Washed cell suspensions rapidly oxidized naphthalene, cis-1,2-dihydro-1,2-dihydroxynaphthalene and 1,2-dihydroxynaphthalene. trans-1,2-Dihydro-1,2-dihydroxynaphthalene was not detectably oxidized. Crude cell extract oxidized cis-1,2-dihydro-1,2-dihydroxynaphthalene nineteen times faster than the trans isomer, and cis-o-hydroxybenzalpyruvate accumulated. When [¹⁴C] naphthalene was metabolised by crude cell extracts in the presence of the cis and/or the trans isomer of 1,2-dihydro-1,2-dihydroxynaphthalene, the reisolated radioactivity was predominantly in the cis isomer.

Phenanthrene and anthracene

Microorganisms capable of degrading phenanthrene and anthracene occur widely in soils and marine sediments (Tausson, 1928 and Sisler and Zobell, 1947). trans-3,4-Dihydro-3,4-dihydroxyphenanthrene (14, Fig. 3) and 1-hydroxy-2-naphthoic acid (18, Fig. 3) were isolated as the products of bacterial degradation of phenanthrene (13, Fig. 3) by Rogoff and Wender (1957a) and Colla, Fieschi and Treccani (1959) respectively. Similarly, Rogoff and Wender (1957b) isolated 2-hydroxy-3-naphthoic acid (26, Fig. 4) and Colla et al. (1959) isolated a dihydroarenediol from bacterial cultures growing on anthracene (20, Fig. 4) and suggested

a 1,2 configuration for it. From these observations these authors suggested that the first ring to undergo cleavage in a polycyclic aromatic hydrocarbon is the end ring. Both groups of workers observed that salicylate and catechol are oxidized by suspensions of washed cells adapted to anthracene or phenanthrene.

Evans, Fernley and Griffiths (1965), using Pseudomonas aeruginosa strain P_R and Pseudomonas P_F and P_G (NCIB 9816) (Catterall and Williams, 1971), showed that phenanthrene-grown cells immediately oxidized 1-hydroxy-2-naphthoic acid, salicylate and catechol. This is in accord with the findings of Rogoff and Wender (1957a). In addition cells grown on phenanthrene oxidized without lag 3,4-dihydroxyphenanthrene (15, Fig. 3), 1-hydroxy-2-naphthaldehyde (17, Fig. 3) and 1,2-dihydroxynaphthalene (19, Fig. 3). Partially purified enzyme from cell extract oxidized 3,4-dihydroxyphenanthrene to cis-4-(1-hydroxynaphth-2-yl)-2-oxobut-3-enoic acid (16, Fig. 3) suggesting that ring cleavage had occurred between the angular carbon and C-4. Crude cell extracts in the presence of NAD⁺ oxidized 1-hydroxy-2-naphthaldehyde to 1-hydroxy-2-naphthoic acid. Based on these observations Evans et al. (1965) proposed the pathway for phenanthrene metabolism shown in Figure 3.

With anthracene adapted cells of Pseudomonas strain P_G, Evans et al. (1965) found immediate oxidation of 2-hydroxy-3-naphoic acid, salicylate and catechol. Similar results were obtained by Rogoff and Wender (1957b). Moreover, the organism also metabolised 1,2-dihydroxyanthracene (22, Fig. 4) and 2-hydroxy-3-naphthaldehyde (25, Fig. 4). Undiluted cell extracts in the presence of Fe²⁺ oxidized 1,2-dihydroxyanthracene to 2-hydroxy-

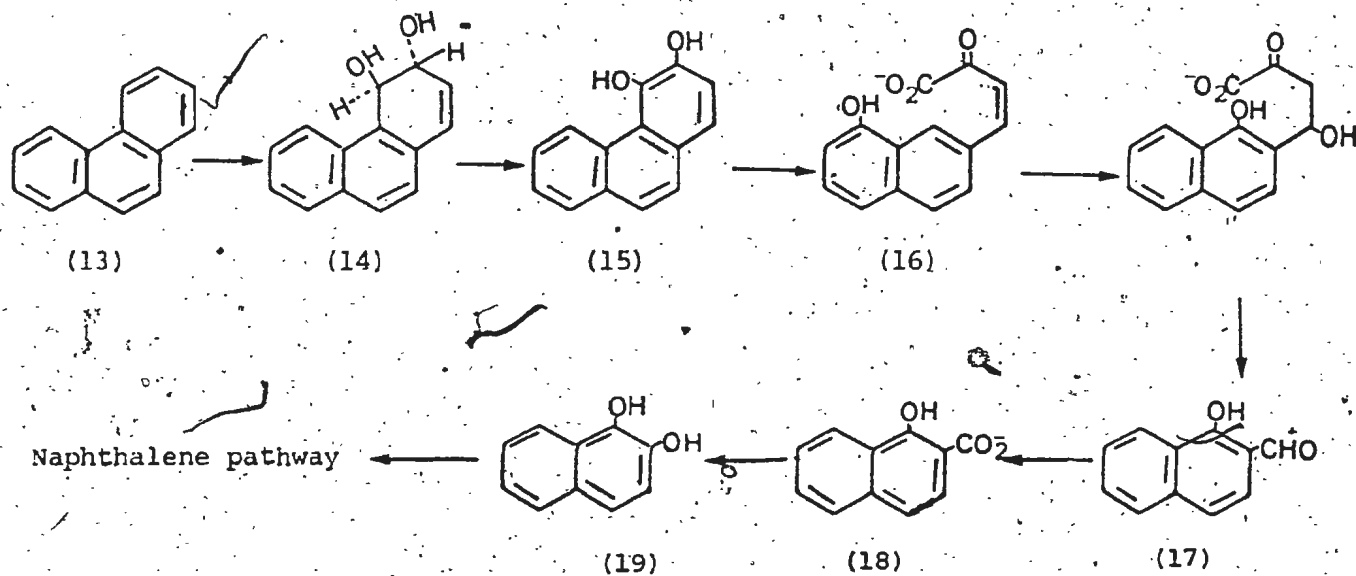


Figure 3

Proposed pathway for the metabolism of phenanthrene by soil pseudomonads.

3-naphthoic acid. cis-4-(2-Hydroxynaphth-3-yl)-2-oxobut-3-enoic acid (23, Fig. 4) was oxidized to 2-hydroxy-3-naphthoic acid in the presence of NAD^+ . Furthermore, with dilute cell extracts the degradation of 1,2-dihydroxyanthracene stopped after ring fission, and there was evidence for the formation of cis-4-(2-hydroxynaphth-3-yl)-2-oxobut-3-enoic acid. 2-Hydroxy-3-naphthaldehyde was oxidized by cell extracts to the corresponding acid. It was suggested that 2-hydroxy-3-naphthoic acid was converted to 2,3-dihydroxynaphthalene (27, Fig. 4) by oxidative decarboxylation. Compound 27 (Fig. 4) is metabolised to salicylate and catechol through unidentified steps. The pathway for degradation of anthracene as proposed by Evans et al. (1965) is shown in Figure 4.

OXIDATION OF PHENOLIC COMPOUNDS

Catechols

Catechol and its derivatives are common intermediates in the degradation of many aromatic hydrocarbons. In addition to the hydrocarbons mentioned above, phenol, benzoate, 2-aminobenzoate and several other compounds are converted to catechols (for a review see Dagley, 1971 and Ornston, 1971). These are metabolised through either of the two pathways shown in Figure 5. The benzene ring of the catechols (45, 28, Fig. 5) may be cleaved between the two hydroxyl groups producing cis, cis-muconic acids (33, 38, Fig. 5), which are further metabolised via β -ketoadipate (36, Fig. 5) to succinate and acetyl CoA (Ornston and Stanier, 1966). This pathway is often called the ortho

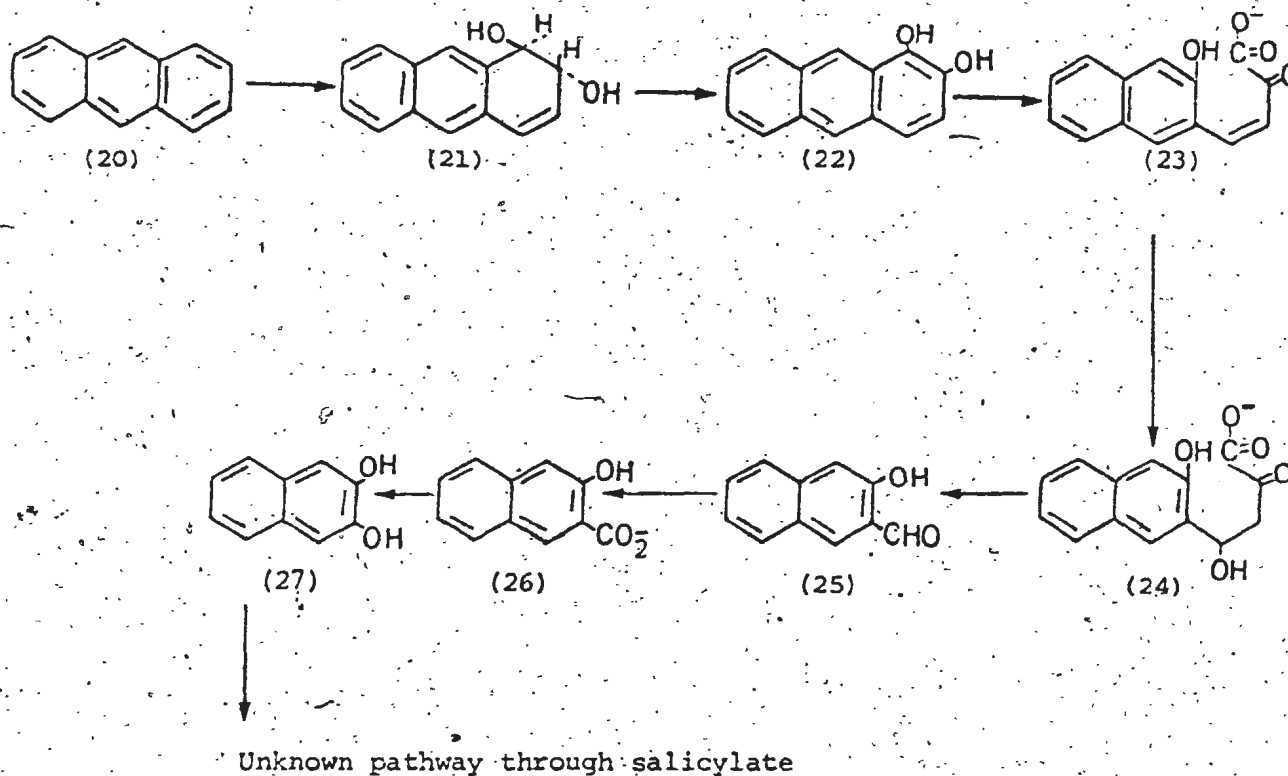
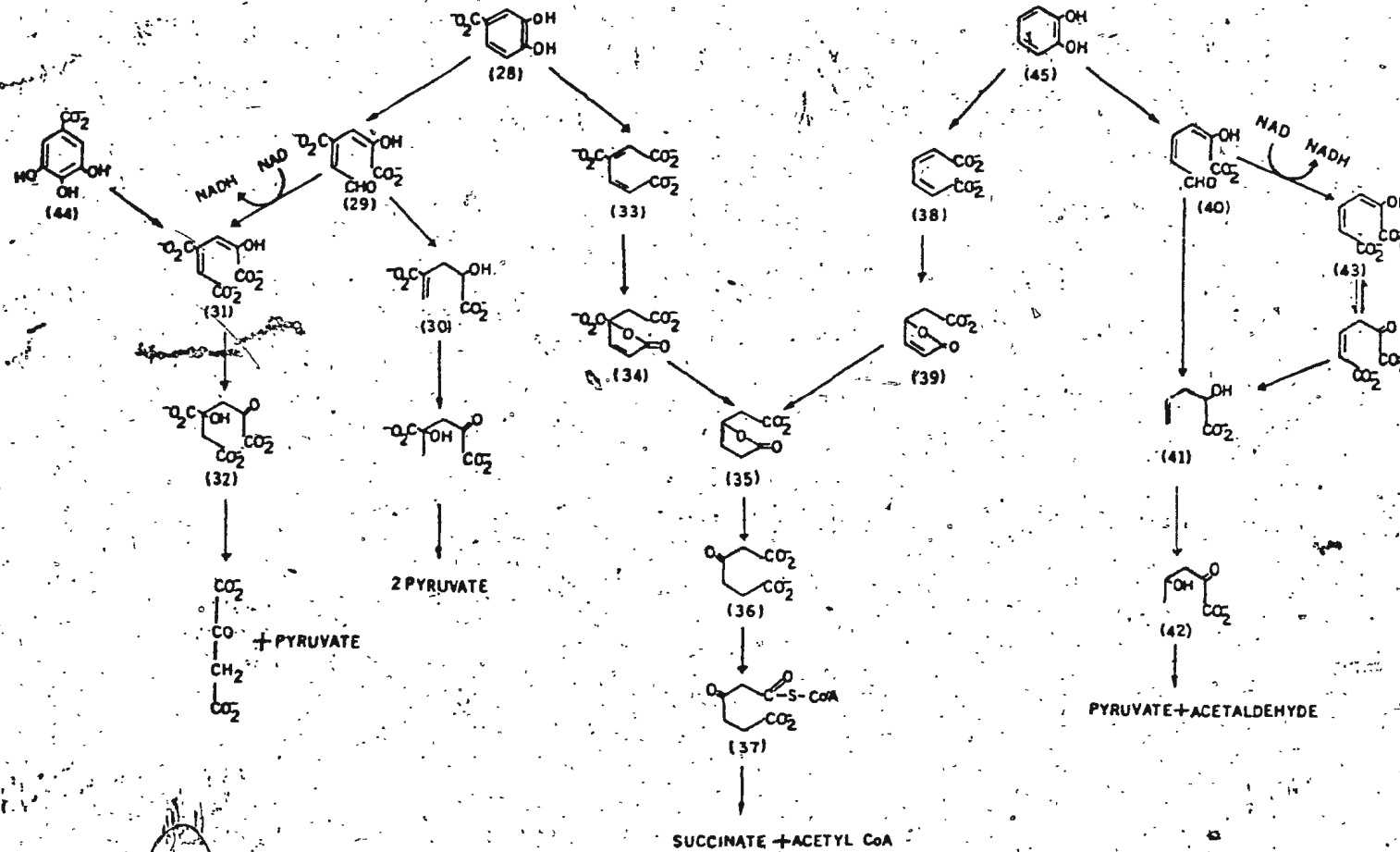


Figure 4

Proposed pathway for the degradation of anthracene by soil pseudomonads

Figure 5

Ortho and meta cleavage pathways in pseudomonads



or β -keto adipate pathway. Alternatively the ring cleavage may occur between a hydroxylated C atom and an adjacent non-hydroxylated C atom to produce 2-hydroxymuconic semialdehydes (29, 40, Fig. 5) (Dagley, Evans and Ribbons, 1960, Dagley, Geary and Wood, 1964). Ornston (1966a,b) purified the enzymes of the ortho pathway from Pseudomonas putida and studied their properties. He showed that the enzymes of the two branches of ^{the} ortho pathway are specific, i.e. enzymes of one branch are not active with intermediates of the other branch.

Dissimilation of catechols via the ortho pathway occur widely in bacteria. Thus benzoate and p-hydroxybenzoate are degraded through the ortho pathway in Nocardia opaca (Rann and Cain, 1969), Moraxella calcoacetica (Canovas and Stanier, 1967), Alcaligenes eutropha (Johnson and Stanier, 1971a) and a number of Pseudomonas species (Ornston, 1966c). Some organisms are endowed with the genetic capability to degrade catechol via both ^{the} meta and ^{the} ortho pathways, and the phenotypic expression of the pathway for catechol degradation depends on the primary growth substrate. For example in Pseudomonas putida the ortho pathway enzymes are induced if the growth substrate is benzoate, and the enzymes of the meta pathway are induced when the organism is grown in cresol or phenol (Feist and Hegeman, 1969). Similarly in Alcaligenes eutropha ^{the} ortho pathway is used in the degradation of benzoate and p-hydroxybenzoate, but phenol and cresol are degraded through the meta pathway (Johnson and Stanier, 1971b). Members of the acidovorans group of Pseudomonas use the meta pathway for the catabolism of protocatechuate

(Stanier, Palleroni and Dudoroff, 1966; Wheelis, Palleroni and Stanier, 1967). Four species of Azotobacter were found to oxidize benzoate via catechol through the meta pathway, but protocatechuate produced from p-hydroxybenzoate was oxidized via the ortho pathway by these species (Hardisson, Sala-Trepat and Stanier, 1969).

Enzymes of the meta pathway have broad specificity and oxidize methylcatechols (Bayley and Dagley, 1969; Ribbons, 1970; Murray et al., 1972). This is consistent with the suggestion of Dagley and associates (Dagley, 1965 and Dagley, Chapman, Gibson and Wood, 1964) who proposed that the meta pathway serves as a general reaction sequence for the dissimilation of methyl substituted aromatic compounds.

Ornston (1966c) studied the regulation of some enzymes of the ortho pathway in Pseudomonas putida. cis, cis-Muconate induces the synthesis of the muconate lactonizing enzyme, muconolactone (39, Fig. 5) isomerase and catechol 1,2 dioxygenase. The first two of these are co-ordinately controlled. β -Carboxy-cis, cis-muconate (33, Fig. 5) lactonizing enzyme, γ -carboxymuconolactone (34, Fig. 5) decarboxylase and β -keto adipate enol lactone (35, Fig. 5) hydrolase are coordinately induced by β -keto adipate or β -keto adipyl CoA. Kemp and Hegeman (1968) found the same regulatory mechanism in Pseudomonas aeruginosa. These authors also found that β -keto adipate succinyl CoA transferase is also induced co-ordinately with the enzymes of the protocatechuate branch.

On the other hand, Canovas and Stanier (1967) showed that in Moraxella calcoacetica protocatechuate coordinately induces all the enzymes catalysing the steps from protocatechuate to β -keto adipyl CoA.

(37, Fig. 5), and cis, cis-muconate co-ordinately induces the four enzymes responsible for the conversion of cis, cis-muconate to β -keto-adipyl CoA. Moreover, Moraxella calcoacetica synthesises two isofunctional sets of enzymes for the conversion of β -ketoadipate enol lactone to β -ketoadipyl CoA. One set is induced co-ordinately with the enzymes of the protocatechuate branch and the other with the enzymes of the catechol branch.

An alternative pathway for the degradation of 2-hydroxymuconic semialdehyde, the first product of the meta pathway, has been demonstrated (Nishizuka et al., 1962; Sala-Trepat and Evans, 1971). The 2-hydroxymuconic semialdehyde formed by the meta fission of catechol is oxidized by a NAD⁺-linked dehydrogenase to the corresponding acid, 4-oxalocrotonate (43, Fig. 5), which is decarboxylated to 2-oxo-pent-4-enoate (41, Fig. 5), an intermediate of the meta pathway. Both the hydrolase branch and the dehydrogenase branch of the meta pathway have been demonstrated in Azotobacter species (Sala-Trepat and Evans, 1971), Pseudomonas NCIB 9816 (Catterall, Sala-Trepat and Williams, 1971) Pseudomonas putida NCIB 10015 (Sala-Trepat, Murray and Williams, 1972) and Pseudomonas arvilla mt-2 (Murray et al., 1972).

Dennis, Chapman and Dagley (1973) demonstrated a divergent pathway for the metabolism of 4-carboxy-2-hydroxymuconic semialdehyde (29, Fig. 5) formed by the meta fission of protocatechuate in Pseudomonas testosteroni. The initial step in this pathway is similar to that in the metabolism of 2-hydroxymuconic semialdehyde: 4-Carboxy-2-hydroxymuconic semialdehyde is oxidized to the corresponding acid (31, Fig. 5) by a

NAD⁺-linked dehydrogenase. Gallic acid (44, Fig. 5) is also metabolised by this divergent pathway. Collinsworth, Chapman and Dagley (1973) found that two reactions in the meta pathway for catechol metabolism, namely the hydration of 2-oxo-pent-4-enoate and the fission of the hydrated product (42, Fig. 5) are catalysed by stereospecific enzymes.

Gentisic acid

Lack (1959, 1961) established the pathway for gentisic acid metabolism in a Pseudomonas species. Gentisic acid (2,5-dihydroxybenzoic acid) is oxidized to maleylpyruvate which is isomerised by maleylpyruvate isomerase to fumarylpyruvate. The latter undergoes cleavage to pyruvate and fumarate which in turn is converted to L-malate. Pseudomonas acidovorans has been found to degrade meta-hydroxybenzoate through this pathway (Wheelis et al., 1967). Methylgentisates, some xylenols, and cresols are metabolised through this sequence by some Pseudomonas species (for a review see Dagley, 1971).

GENETIC BASIS OF THE DEGRADATION OF AROMATIC COMPOUNDS

Though the regulation of the microbial degradation of the oxidation products of aromatic hydrocarbons is moderately well understood, the regulation of the oxidation of hydrocarbons themselves has only recently been studied, and these studies have been genetic in nature. Gunsalus et al. (1965) observed that some of the enzymes catalysing the early reactions in camphor degradation by Pseudomonas putida appeared

to be co-ordinately controlled suggesting they might be part of the same operon. Some of the genes coding for enzymes of camphor degradation were found to be linked and capable of transfer as a unit between strains (Chakrabarty, Gunsalus and Gunsalus, 1968). Recently Chakrabarty and Gunsalus (1971) found that the genes coding for enzymes involved in the degradation of camphor to isobutyrate are clustered in a transmissible plasmid. The ^{genes} coding for the enzymes of the entire salicylate degradative pathway (Chakrabarty, 1972) and those of the naphthalene degradative pathway upto and including salicylate hydroxylase may be plasmid borne in Pseudomonas putida (Dunn and Gunsalus, 1973).

THE PRESENT WORK

The purpose of the present work was to study the regulation of the metabolism of naphthalene in *Pseudomonas* NCIB 9816. There is little information on the regulation of the metabolism of aromatic compounds compared with that available on phenolic benzene derivatives.

The regulation of the bacterial degradation of naphthalene was selected because the pathway for its degradation has been elucidated. *Pseudomonas* NCIB 9816 was selected for this study as this organism has been used by other workers (Catterall, Murray and Williams, 1971; Catterall and Williams, 1971, and Catterall, Sala-Trepat and Williams, 1971), and as a result considerable information is available on its metabolic activities. Moreover, this organism has been found to be able to utilize aromatic hydrocarbons other than naphthalene (Evans et al. 1965).

The organism was grown under various conditions to determine the levels of different enzymes of naphthalene metabolism. In the course of the measurement of naphthalene oxygenase, the first enzyme of the pathway, washed cell suspensions metabolising naphthalene were found to accumulate salicylate. In order to determine what had limited the degradation of salicylate, the organism was grown on salicylate to induce the maximal oxidation of salicylate, and measurement of enzyme levels revealed that naphthalene oxygenase was also induced. This observation was contrary to those of Azoulay (1966) who found that washed cell suspensions of some soil *Pseudomonas* species oxidized naphthalene only if the organism had been adapted to naphthalene, and after growth on

salicylate or its metabolites the cells did not oxidize naphthalene.

The observation that growth on salicylate induces naphthalene oxidation suggested that salicylate or a metabolite was the inducer of naphthalene oxygenase, but growth on catechol did not induce naphthalene oxygenase. To confirm that salicylate might be the inducer, analogues of salicylate were sought that would induce naphthalene oxidation gratuitously. Two compounds, 2-hydroxybenzyl alcohol and 2-aminobenzoate were found to induce naphthalene oxygenase gratuitously.

Comparison of the levels of naphthalene oxygenase, salicylate hydroxylase and the catechol dioxygenases showed that all except catechol 2,3-dioxygenase were induced by salicylate, and that naphthalene oxygenase and salicylate hydroxylase were induced by the gratuitous inducers. Thus, barring the possibility that salicylate is converted to another compound with respect to which 2-hydroxybenzyl alcohol and 2-aminobenzoate are also analogues, it does appear that salicylate is the inducer of naphthalene oxygenase. Furthermore, salicylaldehyde dehydrogenase was also induced under conditions which induced naphthalene oxygenase.

MATERIALS AND METHODS

Bacteria

Pseudomonas species able to grow on a mineral medium on naphthalene as carbon and energy source were obtained from the National Collection of Industrial Bacteria (NCIB 9816) and from the American Type Culture Collection (ATCC 17483). NCIB 9816 was originally designated as Pseudomonas P_G by Evans et al. (1965) (Catterall and Williams, 1971).

Culture media

All media were sterilized in an autoclave at 121° for 10 minutes except glucose (115° for 15 minutes) and some carbon compounds specified below which were sterilized by filtration through sterile Millipore HAWP filters (pore size 0.22μ). Standard aseptic techniques were used throughout the work.

Basal mineral media

	(g/l)
A. NH ₄ Cl	1.00
K ₂ HPO ₄	4.36
NaH ₂ PO ₄ · H ₂ O	3.45
B. MgSO ₄ · 7H ₂ O	48.0
C. CaCl ₂ · 2H ₂ O	3.60
D. (NH ₄) ₆ Mo ₇ O ₂₄ · 4H ₂ O	0.02

$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	0.20
$\text{MnCl}_2 \cdot \text{H}_2\text{O}$	0.10
$\text{CoCl}_2 \cdot \text{H}_2\text{O}$	0.10
HCl (conc.)	0.5 ml

For use 1 volume of each of B, C, and D was added to 100 volumes of A.

The medium was at pH 6.8.

Carbon Sources and inducers

Glucose	(0.846 M)
L-Malic acid	(0.745 M adjusted to pH 6.8 with NaOH)
Succinic acid	(0.864 M adjusted to pH 6.8 with NaOH)
Salicylic acid	(0.363 M adjusted to pH 9.0 with NaOH, sterilized by filtration)
Catechol	(0.363 M sterilized by filtration)
2-Aminobenzoate	(0.036 M containing an equivalent of NaOH, sterilized by filtration)
2-Hydroxybenzyl alcohol	(0.349 M sterilized by filtration)
Naphthalene	(0.780 M in ether, sterilized by filtration)

For use the required volume of sterile solution was added to the basal mineral medium. When naphthalene was used, the ethereal solution was allowed to evaporate overnight in a sterile plugged flask and then the basal mineral medium was added.

Culture methods

Cultures were grown in conical flasks shaken in a gyrotory water

bath shaker (Model G 76 New Brunswick Scientific Co, Inc., New Brunswick, N.J., U.S.A.) at $25 \pm 1^{\circ}$. Growth was followed by measuring the absorbance of the culture at 600 nm in a Unicam SP800 spectrophotometer.

Maintenance of bacterial culture

NCIB 9816 and ATCC 17483 were replated to single colonies at fortnightly intervals on nutrient agar having the following composition:

	(g/l)
Oxoid yeast extract	3.0
Oxoid bacteriological peptone	6.0
Oxoid agar	20.0

The ability to degrade naphthalene was checked periodically by growing on basal mineral medium solidified with agar. Solutions B, C and D were added to solution A containing melted agar when the latter had cooled to about 50° , and the medium was then poured immediately into Petri dishes. After drying and inoculation, naphthalene was added to the lid of the Petri dish.

Growth on glucose, succinate, acetate and malate

Growth occurred rapidly on succinate after the organisms were inoculated from any growth media, but NCIB 9816 adapted slowly to growth on glucose, acetate and malate. It was therefore grown on basal mineral medium containing the carbon source and solidified with agar, and then transferred to the corresponding liquid culture.

Growth on naphthalene and salicylate

Growth of NCIB 9816 in cultures containing naphthalene or salicylate was usually preceded by a long lag period. Therefore, cultures growing in mid log phase on succinate (4.3 mM) medium containing either salicylate (3.5 mM) or naphthalene (0.1%) were diluted eleven times with fresh media containing naphthalene (0.1% W/V) or salicylate (3.5 mM). The diluted cultures had an initial absorbance of about 0.03 at 600 nm, and was used when the absorbance was 0.37 as a heavy inoculum for media containing salicylate (3.5 mM) or naphthalene (0.1% W/V) alone.

Growth on catechol

A portion of a culture of NCIB 9816 growing on succinate (8.5 mM) was transferred during the early exponential phase, to a fresh medium containing catechol (8.0 mM). The culture was incubated overnight without shaking. When growth resumed after 24 h the culture flask was shaken in a water bath shaker as usual.

Harvesting of cells and the preparation of cell extract

Cells were harvested by centrifugation at 5000 rpm at 2° in a Sorvall centrifuge (Model RC-3, Ivan Sorvall Inc., Newton, Connecticut, U.S.A.). Cells were washed by resuspending them in ice cold phosphate buffer (50 mM KH_2PO_4 adjusted with NaOH to pH 7.0) and centrifuging the suspension. Washing was generally repeated, and the cells were finally resuspended in the required volume of buffer.

Cell extracts were made by resuspending the cells at a concentration of 0.2 g wet weight/ml in phosphate buffer (pH 7.0) and disrupting the cells with an ultrasonic oscillator (Model W185, Heat systems-Ultrasonics, Inc., Plain View, New York, U.S.A.) at a nominal power of 70 W. Portions (2 ml) at 0° in ice-water were subjected to six 30 sec periods of sonication. Each period was followed by a 1 minute interval in which the oscillator probe and the suspension were cooled in ice-water. The disrupted cells were centrifuged at 105,000 g for 1 h at 2° in a Beckman ultracentrifuge (Model L3-50, Beckman Instruments, Inc., Palo Alto, California, U.S.A.).

Chemicals

Pyridine nucleotides, lactic acid dehydrogenase and L(+) lactic acid were obtained from the Sigma Chemical Company (St. Louis, Mo., U.S.A.). Naphthalene was purchased from Fisher Scientific Company (Fair Lawn, New Jersey, U.S.A.). Salicylic acid, salicylaldehyde and catechol were purchased from J.T. Baker Chemical Company (Phillipsburg, N.J., U.S.A.). 2-Hydroxybenzyl alcohol and 2-aminobenzoate were obtained from Eastman Organic Chemicals (Rochester, New York, U.S.A.). Salicylaldehyde was freshly redistilled and catechol, 2-aminobenzoate and 2-hydroxybenzyl alcohol were recrystallized before use. Other chemicals were obtained from various commercial sources and were the highest purity available.

Enzyme assays

Naphthalene oxygenase was determined using washed cells suspended

in phosphate buffer (pH 7.0) at a maximum concentration corresponding to an absorbance at 600 nm of 0.32. Twenty μ l of an ethanolic solution of naphthalene (10 mM) were added to 2.5 ml of cell suspension in a 1 cm cuvette, and the rate of decrease in absorbance due to naphthalene at 276 nm was measured in a Unicam SP800 spectrophotometer fitted with SP820 constant wavelength scan control, SP850 scale expansion accessory and a Perkin-Elmer 56 chart recorder. Naphthalene oxygenase activity was calculated using an experimentally determined extinction coefficient of $4.51 \text{ mM}^{-1} \text{ cm}^{-1}$. The appropriateness of the method is discussed later.

Salicylaldehyde dehydrogenase activity in cell extracts was determined by following spectrophotometrically at 340 nm the reduction of NAD^+ . The reaction medium contained 0.3 ml of salicylaldehyde (3 mM), 0.5 ml of NAD^+ (30 mM), 50 μ l of cell extract (diluted if necessary), and pyrophosphate buffer (50 mM tetrasodium pyrophosphate adjusted to pH 8.5 with HCl) to make 3.0 ml. The reaction was started by adding the cell extract. The solution of salicylaldehyde (3 mM) in water was prepared by diluting a 30 mM ethanolic solution of redistilled salicylaldehyde. The rate of increase in absorbance at 340 nm was measured with a Unicam SP800 spectrophotometer. Salicylaldehyde also absorbs at 340 nm, and so in order to calculate the rate of reduction of NAD^+ an extinction coefficient of $3.94 \text{ mM}^{-1} \text{ cm}^{-1}$ was used. The latter is the difference between the extinction coefficient of NADH ($6.22 \text{ mM}^{-1} \text{ cm}^{-1}$) and that of salicylaldehyde ($2.38 \text{ mM}^{-1} \text{ cm}^{-1}$) at pH 8.5. Tris at a concentration of 50 mM reacts with salicylaldehyde

and so is unsuitable for use as buffer.

Catechol 1,2-dioxygenase (EC 1.13.11.1) was measured spectrophotometrically at 25° by the method of Hegeman (1966) except that 30 mM tris (pH 7.6) was used instead of phosphate, and the concentration of catechol was 0.07 mM instead of 0.1 mM.

at 25°
Catechol 2,3-dioxygenase (EC 1.13.11.2) was measured by the method of Feist and Hegeman (1969) using 0.17 mM concentration of catechol instead of 0.06 mM.

Salicylate hydroxylase (EC 1.14.13.1) was measured spectrophotometrically at 25° by following the oxidation of NADH. The method is similar to that of Yamamoto et al. (1965). The reaction mixture contained 0.2 ml sodium salicylate (1.5 mM), 0.2 ml of NADH (1.5 mM), 0.1 ml FAD (0.3 mM), 50 µl cell extract, (diluted, if necessary) and 2.45 ml of phosphate buffer pH 7.0. The rate of the decrease in absorbance at 340 nm was linear with time, for rates up to about 0.1 absorbance change per minute, for about 1 min.

Determination of the rate of oxygen uptake by cells of NCIB 9816 in the presence of naphthalene

Naphthalene stimulated oxygen uptake by the induced whole cells of NCIB 9816 was measured polarographically. The reaction mixture contained 0.1-0.2 ml of a stock bacterial suspension ($E_{1\text{ cm}}^{600}$ about 1.0), 16 µl of naphthalene in ethanol (10 mM) and phosphate buffer (pH 7.0) to a final volume of 2 ml. A similar measurement was made with 16 µl ethanol instead of naphthalene solution as a control. The ratio of

the molar rate of oxygen uptake to the molar rate of the disappearance of naphthalene was determined by comparing the rate of oxygen uptake with that of the disappearance of naphthalene determined spectrophotometrically using the same concentration of bacteria.

Measurement of the concentration of inducers in culture media and determination of the timecourse of the induction of naphthalene oxygenase

Concentrations of the inducers (salicylate, 2-aminobenzoate or 2-hydroxybenzyl alcohol) were measured by taking samples of the culture medium immediately after the addition of the inducer, and at intervals thereafter. The samples were chilled on ice, and centrifuged at 2° to remove bacteria. The absorption spectrum of the clear supernatant was recorded. If necessary, the supernatants were accurately diluted. Samples of cultures without inducers were also treated in a similar way to determine whether or not interfering material was produced by the culture.

The bacterial pellets obtained in the above experiment were resuspended and used to determine naphthalene oxygenase at different time intervals. The volumes of the samples taken from the culture were such that after centrifugation and resuspension of the resulting pellet in 1 ml of phosphate buffer, the suspension had an absorbance at 600 nm of about 1.0. This suspension (0.2 ml) was used for the measurement of naphthalene stimulated oxygen uptake as described previously.

Identification of salicylate produced by the incubation of NCIB 9816
with naphthalene

To 20 ml of a suspension of washed cells ($E_{1\text{ cm}}^{600}$ 0.14-0.26) that had been induced with salicylate, 2-aminobenzoate or 2-hydroxybenzyl alcohol, 0.16 ml of 10 mM naphthalene in ethanol was added. After the reaction was complete, the mixture was extracted with two 10 ml portions of redistilled ether. The ether layer was discarded, and the reaction mixture was acidified with 0.2 ml of concentrated HCl and extracted again with two 10 ml portions of ether. The combined ether extracts were dried over anhydrous sodium sulphate, filtered, and the filtrate was evaporated to a small volume. A portion of this concentrated extract was applied to a thin layer chromatographic plate coated with a 0.25 mm thick layer of Silica gel-G. The plate was developed in a solvent system consisting of benzene, ether, acetic acid and ethanol in the ratio of 120:60:18:1 by volume. After allowing the plate to dry, salicylic acid was detected by its blue fluorescence in UV light. A second portion was dissolved in phosphate buffer and the absorption spectrum of the solution was scanned and compared with that of authentic salicylic acid.

Initial rate of the accumulation of salicylate from naphthalene

To a 2.5 ml suspension of bacteria ($E_{1\text{ cm}}^{600}$ 0.14-0.26) induced with salicylate, 2-aminobenzoate or 2-hydroxybenzyl alcohol, 20 μ l of an ethanolic solution of naphthalene (10 mM) was added and the ab-

sorbtion spectrum of the reaction mixture was recorded immediately after adding naphthalene and at timed intervals thereafter. From the absorbance values at 276 nm and at 295 nm and the known extinction coefficients of naphthalene and salicylate at these wavelengths, the concentrations of naphthalene and salicylate present at a given time were calculated (Dawes, 1969).

Stoichiometry of the conversion of salicylaldehyde to salicylate, and the identification of the product

To determine the stoichiometry of the reaction between salicylaldehyde and NAD^+ catalyzed by the crude extracts of NCIB 9816, a reaction mixture was made up containing 20.1 ml of sodium pyrophosphate buffer, pH 8.5 (50 mM tetrasodium pyrophosphate adjusted to pH 8.5 with HCl), 2.7 ml of salicylaldehyde (0.3 mM), 0.24 ml of 1,10-phenanthroline (0.5 M) to inhibit salicylate hydroxylase (Yamamoto et al., 1965), and 0.16 ml of extract prepared from NCIB 9816 induced with salicylate. After incubation for 10 minutes at 25° , the reaction was started by adding 0.8 ml of NAD^+ (0.15 M). The NADH produced was calculated from the increase in absorbance at 340 nm using an extinction coefficient of $3.84 \text{ mM}^{-1} \text{ cm}^{-1}$, the difference between that of NADH ($6.22 \text{ mM}^{-1} \text{ cm}^{-1}$) and of salicylaldehyde ($2.38 \text{ mM}^{-1} \text{ cm}^{-1}$) at pH 8.5. Twenty ml of the reaction mixture were extracted with five 4 ml portions of peroxide free ether, and the extracts were discarded. The residue was acidified

with 0.2 ml of concentrated HCl, and extracted with five 4 ml portions of ether. The combined ether extracts were evaporated on a warm water bath to remove ether, and the residue ^{was} dissolved in 5 ml of phosphate buffer, pH 7.0. After clarifying the solution by centrifugation, its absorption spectrum was recorded. There were present peaks at 263 nm and 295 nm consistent with the presence of 1,10-phenanthroline and salicylate. The absorbance at 295 nm was absent when salicylaldehyde was omitted from the reaction mixture. The absorbance values were measured as differences from the absorbance at 350 nm. The absorbance at 295 nm due mainly to salicylate contained a contribution due to 1,10-phenanthroline and was reduced by 11.9% of the absorbance at 263 nm to correct for this. The concentration of salicylate was then calculated by using the experimentally determined extinction coefficient for salicylate of $3.45 \text{ mM}^{-1} \text{ cm}^{-1}$. The identity of salicylate was confirmed by thin layer chromatography. The solution in buffer was acidified with 0.05 ml of concentrated HCl, extracted with three 3 ml portions of ether, and the ether was evaporated to a small volume. The solution was chromatographed as described previously.

Protein determination

The method of Lowry et al. (1951) was followed. To determine protein in intact bacteria, 2.5 ml of bacterial suspension (3 mg wet bacteria/ml) were added to 0.5 ml 30% (w/v) trichloroacetic acid. The precipitated protein was separated by centrifugation at 5000 rpm at 2°. For the determination of protein in bacterial extract, 0.1 ml

of the latter was added to 2.4 ml of 5% TCA and the precipitate sedimented as in the determination on intact cells. After discarding the supernatant, the residue was dissolved in 2.5 ml of reagent C. A sample of this solution (0.2 ml for cell extracts and 0.5 ml for whole cells) was diluted to 3 ml with reagent C. After 10 minutes 0.25 ml of Folin-Ciocalteu reagent was added. After half an hour the absorbance was measured at 700 nm in a Unicam SP800 spectrophotometer. For calibration, ^{samples} (0.1 ml to 0.8 ml) of a standard solution of bovine serum albumin (0.26-0.28 mg/ml in reagent C) were diluted to 3 ml with reagent C, 0.25 ml of Folin-Ciocalteu reagent was added after 10 minutes. After half an hour the absorbance was measured at 700 nm.

Determination of the actual NAD⁺ content of a commercial preparation

The actual NAD⁺ content of the commercial preparation used in the determination of salicylaldehyde dehydrogenase activity was measured by reduction with L(+) lactic acid in the presence of lactic dehydrogenase (Bergmeyer, 1965). The reaction mixture contained, 0.1 or 0.2 ml of NAD⁺, 2.9 or 2.8 ml of hydrazine-glycine /pH 9.5 ^{buffer} (0.4 M hydrazine, 1 M glycine), 0.1 ml of L(+) lactate (24 mM) and 30 µl of lactic dehydrogenase solution containing sufficient enzyme to complete the reaction in about 5 minutes. The NAD⁺ content was calculated from the increase in absorbance at 340 nm of the reaction mixture due to the reduction of NAD⁺ to NADH. A reaction mixture lacking NAD⁺, and two lacking all reagents except ^{appropriate amounts of} NAD⁺ served as controls, and the sum of ^{different controls} the absorbance values of these two at 340 nm was subtracted from the absorbance values at 340 nm of the reaction mixtures.

RESULTS

Measurement of naphthalene oxygenase in whole cells of NCIB 9816

Naphthalene oxygenase activity was measured in cells grown on naphthalene, salicylate or catechol as the sole carbon and energy sources, or cells grown on succinate to which any of these compounds had been added. To cultures growing on succinate sterile solutions of salicylate or catechol were added during the early exponential phase of growth (absorbance about 0.1 at 600 nm measured in a Unicam SP800 spectrophotometer). After the population had doubled twice the cells were harvested and naphthalene oxygenase activity was measured spectrophotometrically as described in Materials and Methods.

The initial rate of disappearance of naphthalene from washed bacterial suspension was directly proportional to the concentration of bacteria in the cuvette upto a maximum $E_{1\text{ cm}}^{600}$ due to bacteria of 0.32 (Figure 6). Above this concentration the reaction rate could not be measured satisfactorily as the expected increase in the absorbance at 276 nm due to the addition of naphthalene was not observed.

The initial rates of disappearance of naphthalene at different concentrations of the latter for a constant concentration of bacteria are shown in Figure 7. These data were fitted to a Lineweaver Burke plot by the method of least squares (Figure 8). The K_m value was calculated as 4 μM . The significance of this value and of rates measured with whole organism is dealt with in the Discussion.

Figure 6. Dependence of the initial rates of naphthalene disappearance on the concentration of bacteria

Initial rates of naphthalene oxidation were measured spectrophotometrically at various concentrations of bacterial suspension. The concentration of naphthalene was $40\mu\text{M}$.

Figure 6

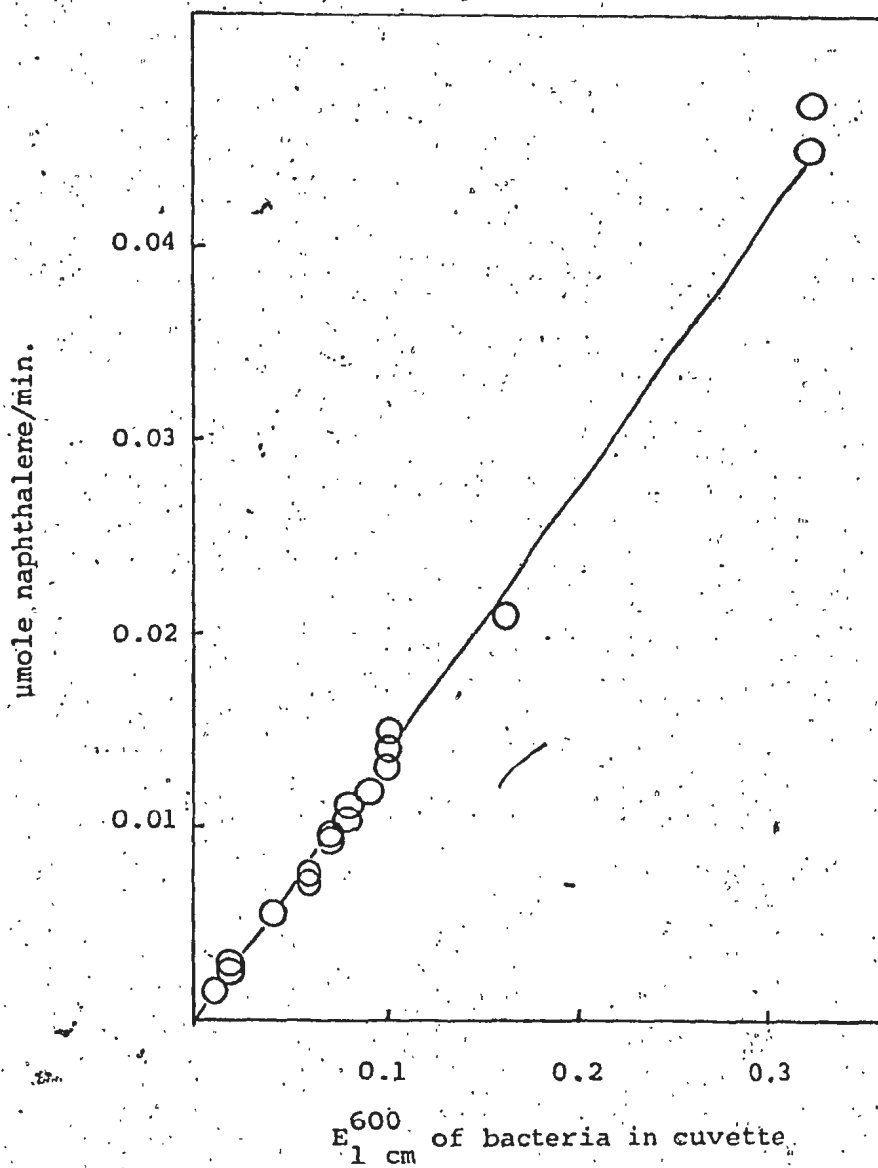


Figure 7. Initial rates of naphthalene oxidation by washed induced cells of NCIB 9816 at various concentrations of naphthalene

Initial rates of naphthalene disappearance from washed cell suspension were measured spectrophotometrically at the concentrations of naphthalene indicated. The concentration of cells in the suspension was 0.05 absorbance unit at 600 nm. The method of measurement is described in Materials and Methods. A unit of bacteria is defined as the quantity of bacteria in 1 ml with $E_{1\text{ cm}}^{600} = 1.0$.

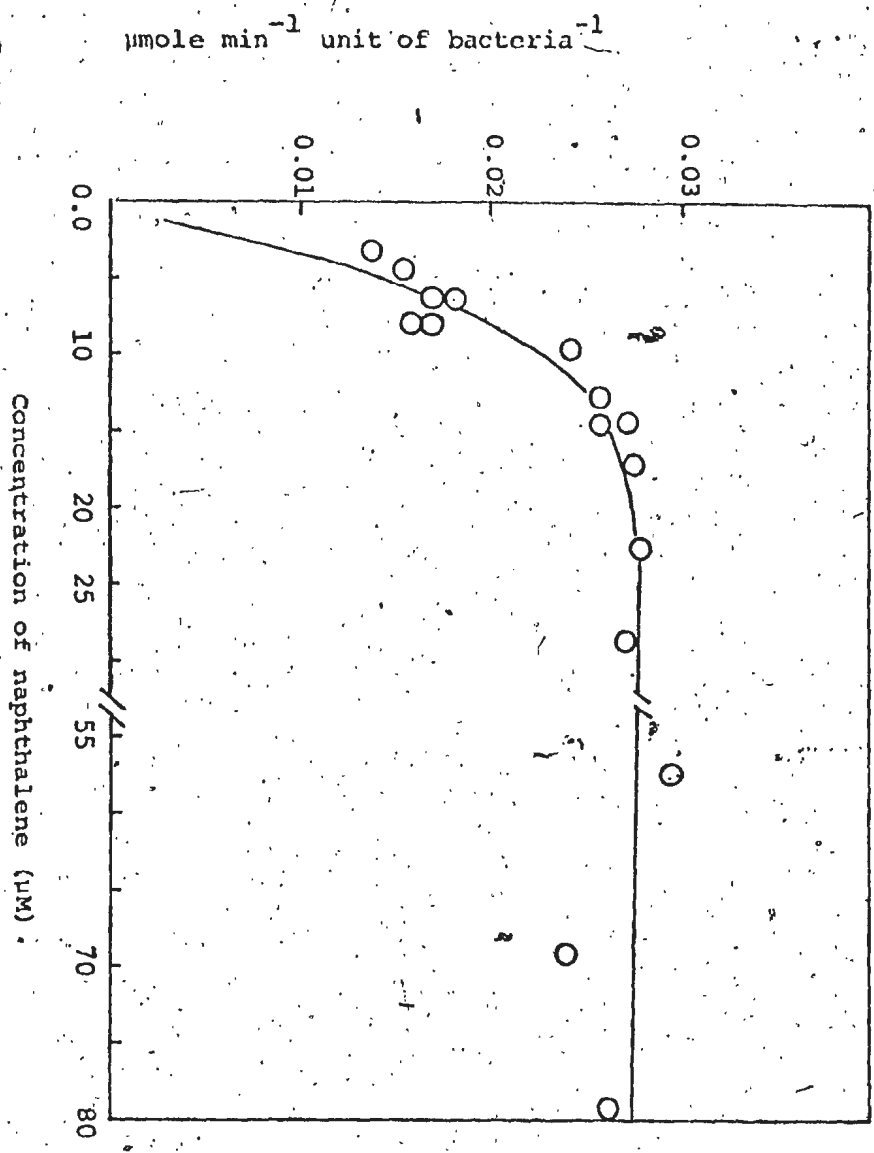


Figure 7

Figure 8. Lineweaver-Burke plots of the initial rates of naphthalene oxidation at various concentrations of naphthalene

Reaction conditions are as described in Figure 7. The velocities of the reaction are expressed as the change in absorbance at 276 nm per minute. The line is the best fit computed by the method of least squares and yields a K_m for naphthalene of $4\mu M$. A unit of bacteria is defined as that quantity in 1.0 ml with $E_{1\text{ cm}}^{600} = 1.0$.

Figure 8

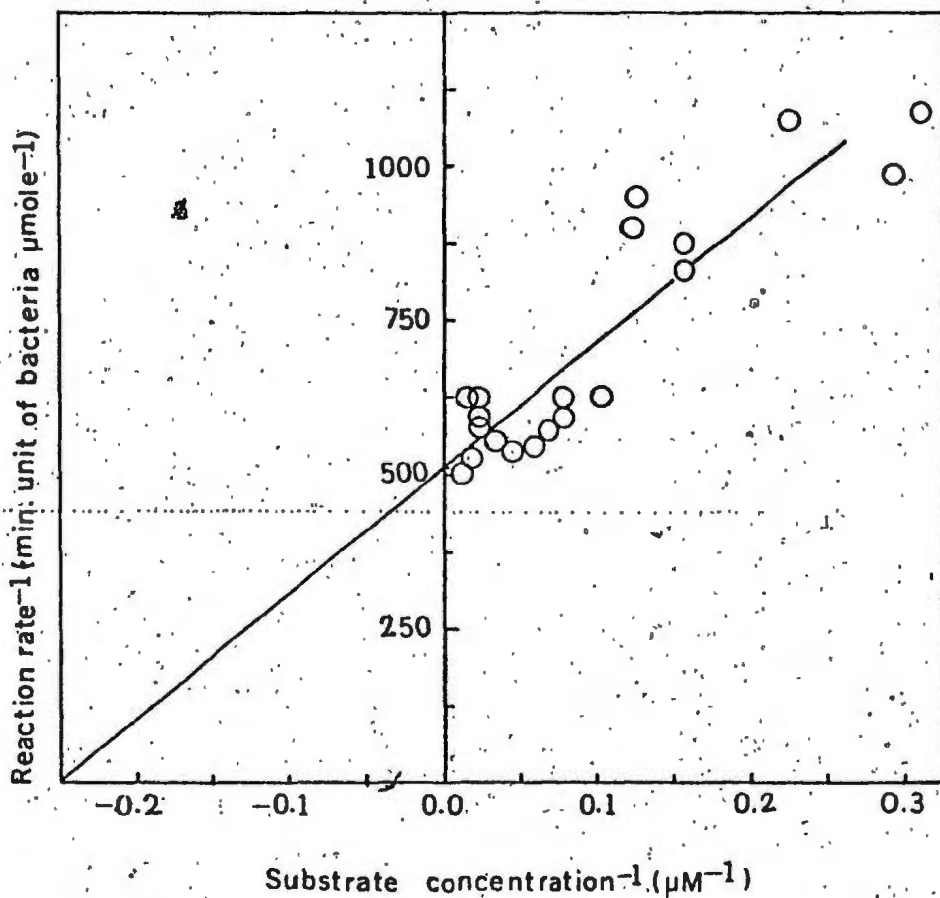


Table I shows the levels of naphthalene oxygenase in organisms grown on naphthalene, salicylate and catechol as the sole carbon and energy sources, and on succinate culture to which salicylate or catechol was added. The precise meaning of the numerical values in the table are not unambiguous in interpretation and will be discussed later.

Measurement of naphthalene oxygenase in NCIB 9816 induced gratuitously

Levels of naphthalene oxygenase in cells induced with 2-aminobenzoate and 2-hydroxybenzyl alcohol were measured. Sterile solutions of 2-aminobenzoate and 2-hydroxybenzyl alcohol were added to cultures growing on succinate when the absorbance at 600 nm was about 0.1. 2-Aminobenzoate was used at one tenth of the concentration of salicylate or 2-hydroxybenzyl alcohol. At this concentration 2-aminobenzoate caused the same lag in the growth of NCIB 9816 as did 3.5 mM salicylate. Higher concentration of 2-aminobenzoate inhibited growth for long periods. 2-Hydroxybenzyl alcohol at 3.5 mM concentration did not inhibit growth. Cells were harvested when the absorbance of the culture at 600 nm reached about 0.4.

The levels of naphthalene oxygenase in NCIB 9816 induced with the gratuitous inducers are shown in Table II.

The time-course of the induction of naphthalene oxygenase by salicylate and gratuitous inducers in NCIB 9816

The growth curve of NCIB 9816 growing on the basal mineral medium containing succinate (8.5 mM) to which salicylate was

Table I

Levels of naphthalene oxygenase in Pseudomonas NCIB 9816
grown on various carbon sources

Cells were grown on the carbon sources indicated and harvested during the exponential phase of growth. Alternatively they were grown on succinate, the second compound was added during the early exponential phase ($E_{1\text{ cm}}^{600}$ about 0.1), and the cells were harvested after doubling twice. The concentrations of the carbon compounds in the culture are given in parentheses. Naphthalene oxygenase was measured by the spectrophotometric method using a washed cell suspension.

<u>Carbon sources</u>	<u>Naphthalene oxygenase activity</u> <u>($\mu\text{mole}/\text{min}/\text{mg}$ protein)</u>
Succinate (8.5 mM)	0.003 \pm 0.006 ^a (n=4)
Naphthalene (0.1% w/v)	0.339
Salicylate (3.5 mM)	0.280
Catechol (8 mM)	0.008
Succinate (8.5 mM) + Salicylate (3.5 mM)	0.121 \pm 0.048 (n=6)
Succinate (8.5 mM) + Catechol (3.5 mM)	0.013

^a Mean \pm standard deviation where the figures accompany \pm sign. Others represent the average of duplicate measurements in an experiment.

Table II

Levels of naphthalene oxygenase in NCIB 9816 induced with 2-aminobenzoate and 2-hydroxybenzyl alcohol

Sterile solution of 2-aminobenzoate or 2-hydroxybenzyl alcohol was added during the early exponential phase to cultures of NCIB 9816 growing on basal mineral medium containing 8.5 mM succinate. Cells were harvested after doubling twice. Naphthalene oxygenase activity in whole cells was measured spectrophotometrically using whole cells. Concentrations of 2-aminobenzoate and 2-hydroxybenzyl alcohol in the culture are given in parenthesis.

<u>Inducer</u>	<u>Naphthalene oxygenase activity</u> <u>(μmole/min/mg protein)</u>
2-Aminobenzoate (0.35 mM)	0.165 \pm 0.057 ^a (n=4)
2-Hydroxybenzyl alcohol (3.5 mM)	0.151 \pm 0.039 (n=4)

^aActivities are reported as mean \pm standard deviation.

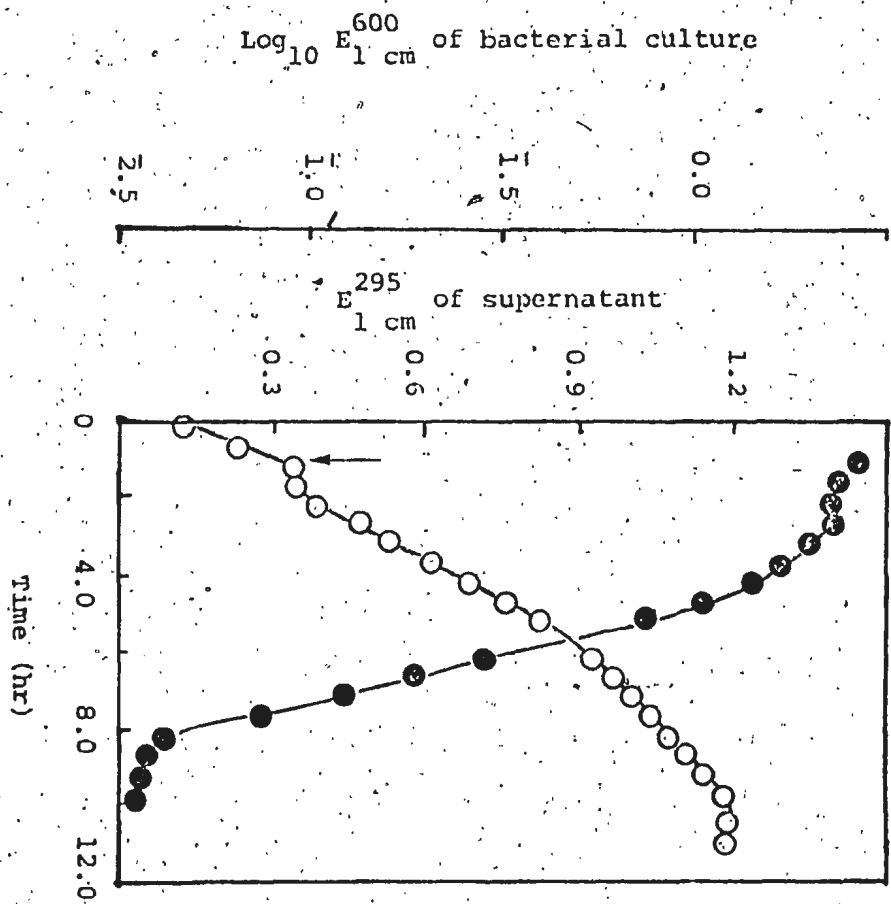
added (final concentration 3.3 mM) is shown in Figure 9, together with the levels of salicylate and the activities of naphthalene oxygenase induced throughout growth. The culture had initially a volume of 226 ml. At the intervals shown samples were taken out and centrifuged at 5000 rpm at 2°, and the absorption spectrum of the supernatant was scanned. The pellet was washed and used for measurement of naphthalene oxygenase polarographically as described in Materials and Methods.

The induction appeared to be complete within one doubling and but was maintained into the stationary phase of growth, /bacteria which had been grown under similar conditions and/entered the stationary phase sometimes appeared devoid of naphthalene oxygenase.

Figures 10 and 11 show the time-course of induction of naphthalene oxygenase, the concentrations of the gratuitous inducers during growth, and the growth curves of the cultures. The cultures growing on succinate were induced with 2-aminobenzoate (0.35 mM) or 2-hydroxybenzyl alcohol (3.5 mM) at the times indicated. Methods of measurements of the concentrations of inducers and naphthalene oxygenase were the same as followed in the case of the time-course of induction with salicylate. A small but steady rise in the absorbance at 309 nm of the supernatant derived from the culture was observed (Figure 11A). This is consistent with a rise in the pH of the culture from pH 6.8 to 7.2 (not shown) and a consequent rise in the extinction co-efficient of 2-aminobenzoate (from 2.73 mM⁻¹ cm⁻¹ at pH 6.8 to 2.83 mM⁻¹ cm⁻¹ at pH 7.2).

Figure 9. Time-course of the induction of naphthalene oxygenase by salicylate in Pseudomonas NCIB 9816

The conditions of the experiment have been described in Materials and Methods. (A) \bigcirc — \bigcirc \log_{10} of the absorbance of the culture at 600 nm, and \bullet — \bullet absorbance of the supernatant of the culture after eight fold dilution. (B) naphthalene-stimulated oxygen uptake by suspensions of washed cells harvested at different times after adding the salicylate. Unit bacteria is defined as the quantity of bacteria in 1 ml of suspension with $E_{1\text{ cm}}^{600} = 1.0$. The arrow indicates the time at which the inducer was added to the culture.



Naphthalene oxidation (μmole O₂/min/unit bacteria)

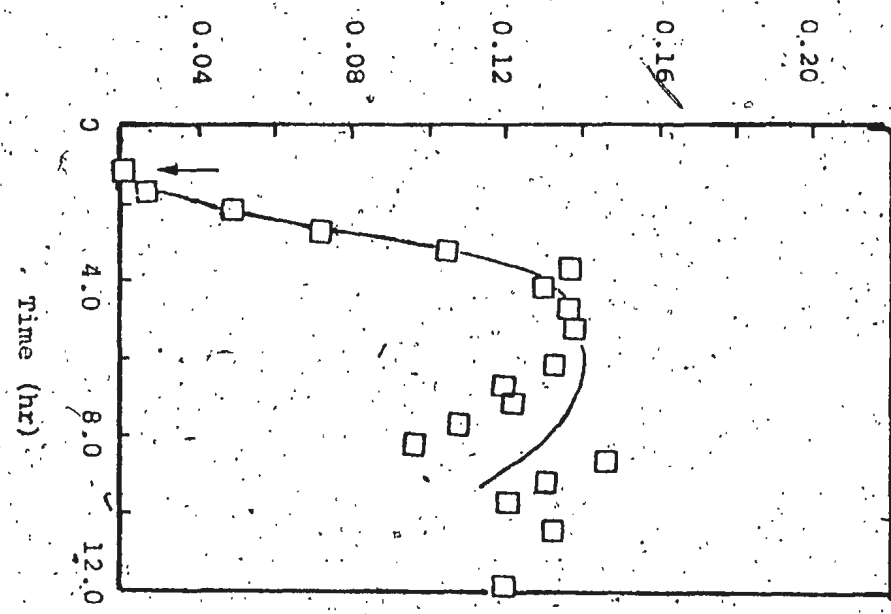


Figure 9

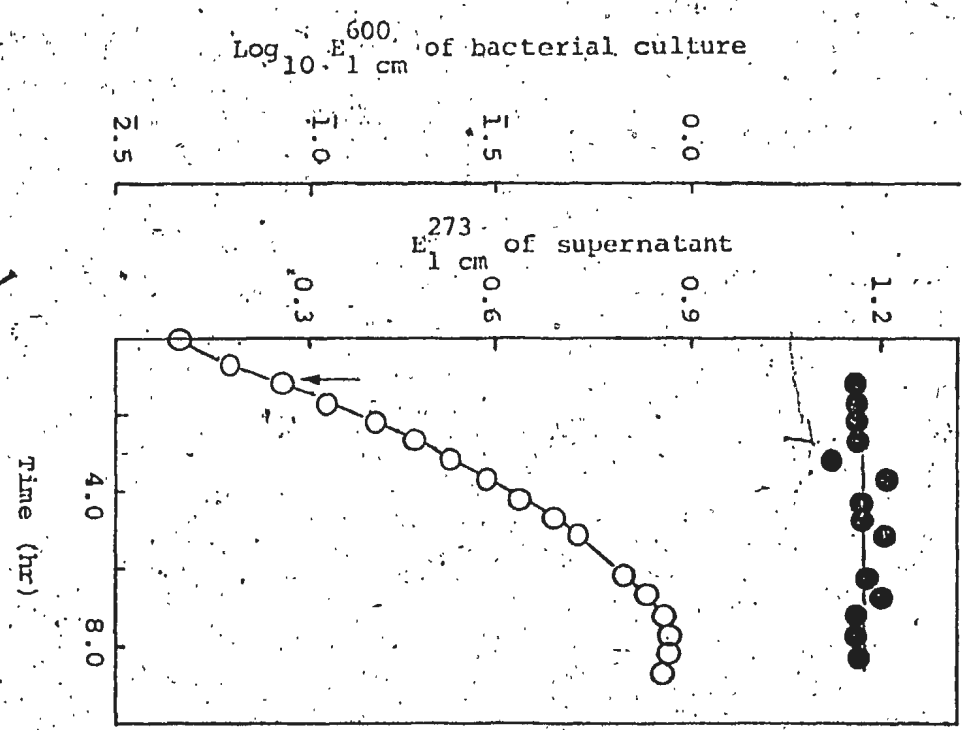
A

B

Figure 10. Time-course of the induction of naphthalene oxygenase by 2-hydroxybenzyl alcohol in NCIB 9816 growing on succinate

Experimental conditions are described in Materials and Methods.

(A) \bigcirc — \bigcirc $\log_{10} E_{1\text{ cm}}^{600}$ of the culture, \bullet — \bullet absorbance at 273 nm of the supernatant due to 2-hydroxybenzyl alcohol. (B) The naphthalene-stimulated oxygen uptake by the cells at different times. The arrow indicates the time at which 2-hydroxybenzyl alcohol was added to the culture. A unit of bacteria is defined as the quantity in 1 ml with $E_{1\text{ cm}}^{600} = 1.0$.



Naphthalene oxidation ($\mu\text{mole O}_2/\text{min}/\text{unit of bacteria}$)

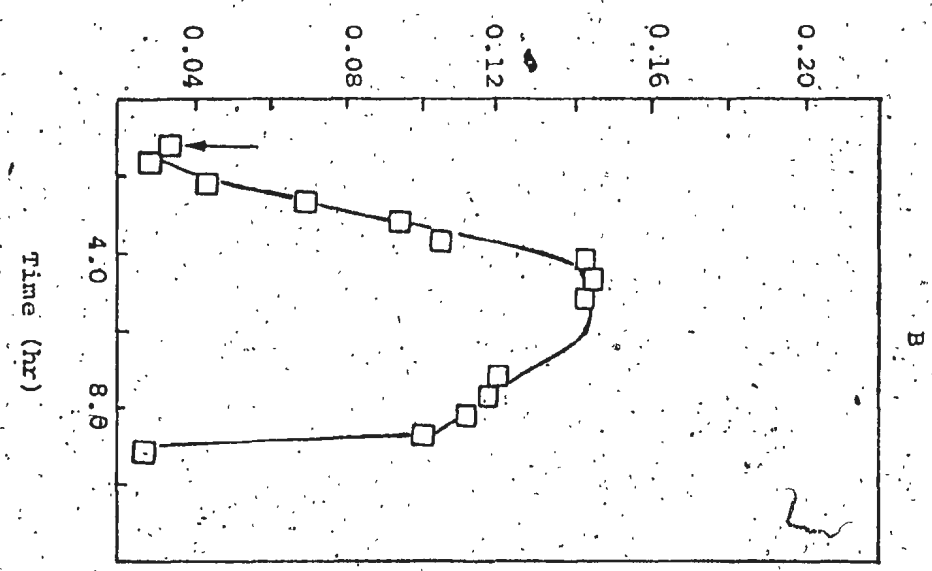
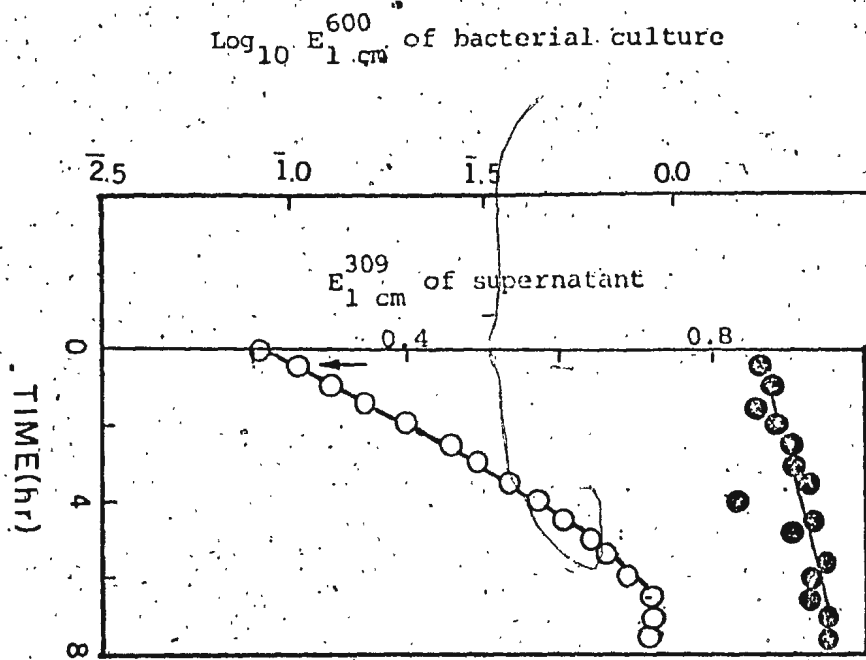


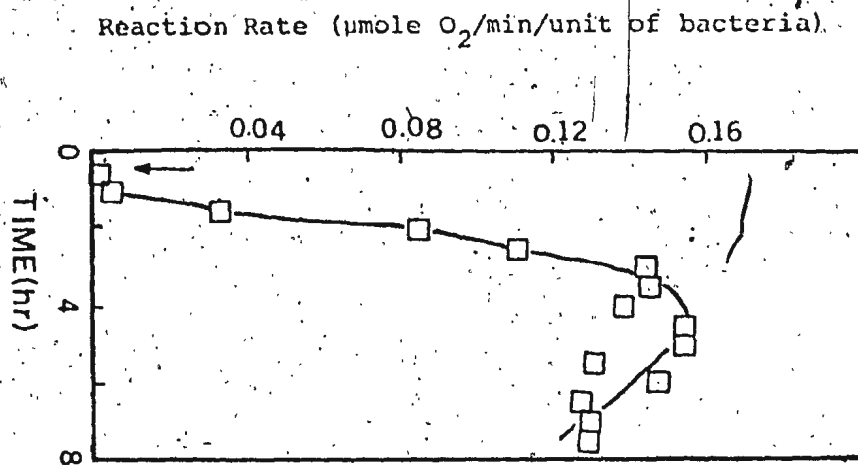
Figure 10

Figure 11. Time-course of the induction of naphthalene oxygenase by 2-aminobenzoate in NCIB 9816 growing on succinate.

Experimental conditions are as described in Materials and Methods. (A) \bigcirc — \bigcirc \log_{10} absorbance of culture at 600 nm; \bullet — \bullet absorbance at 309 nm of the supernatant due to 2-aminobenzoate. The rise in absorbance at 309nm is consistent with the rise in pH of the culture from 6.8 to 7.2. The extinction coefficients of 2-aminobenzoate were found to be $\frac{2.73}{\text{mM}^{-1}}$ at pH 6.8 and $\frac{2.83}{\text{mM}^{-1}}$ at pH 7.2. (B) naphthalene-stimulated oxygen uptake by cell suspensions harvested at different times. A unit of bacteria is the quantity in ml of suspension with $E_{1\text{ cm}}^{600} = 1.0$. The arrow indicates the time at which the inducer was added to the culture.



A



B

Figure 11

As shown in Figure 9A the concentration of salicylate decreased with the growth of bacteria. On the other hand, there was no detectable change in the concentration of 2-hydroxybenzyl alcohol and 2-amino-benzoate, as shown in Figures 10A and 11A respectively.

Salicylaldehyde dehydrogenase

Determination of the optimum pH

Salicylaldehyde dehydrogenase activity in the ~~crude~~ extract from NCIB 9816 induced with 2-hydroxybenzyl alcohol, was determined at different pH values. The buffers had a constant ionic strength ($\mu = 0.2$ M) and were prepared as follows: 25 ml of a solution of Na_2HPO_4 (133.3 mM) or $\text{Na}_4\text{P}_2\text{O}_7$ (40 mM) was adjusted with standard HCl solution and required volume of a standard NaCl solution was added and diluted to 50 ml with water (see Figure 12).

The reaction mixture contained 2.75 ml of buffer, 0.1 ml NAD^+ (150 mM), 50 μl salicylaldehyde (19.7 mM in ethanol) and 20 μl of crude extract. The rate of increase of the absorbance at 340 nm due to the production of NADH was measured in a Perkin-Elmer Coleman 124 spectrophotometer. The highest activity was observed at pH 8.5 with pyrophosphate buffer (Figure 12).

Dependence of initial reaction rates on the concentration of crude extract

The activity of salicylaldehyde dehydrogenase was measured using different concentrations of the crude extract from NCIB 9816.

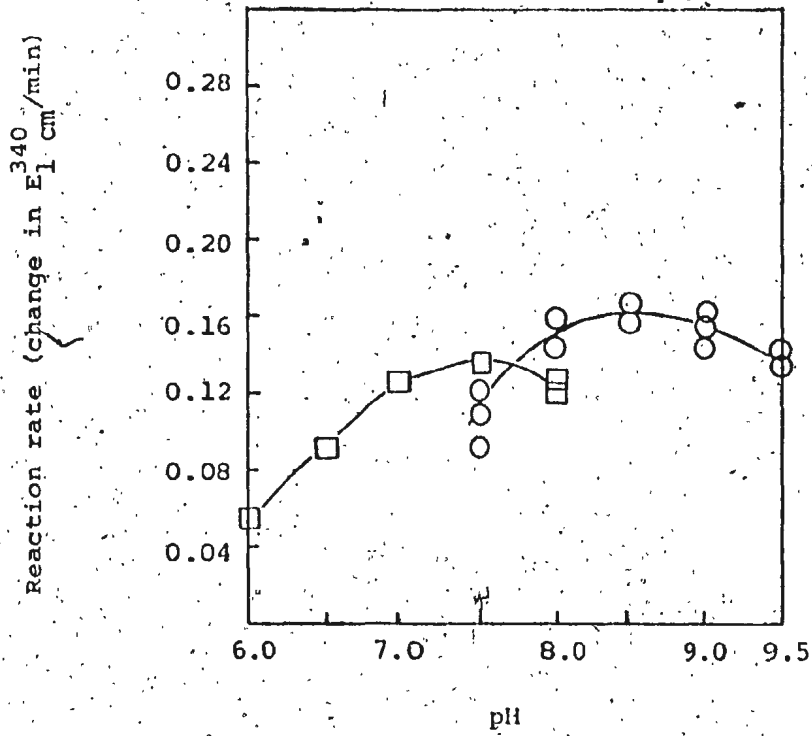
Figure 12. Activity of salicylaldehyde dehydrogenase at different pH values in the crude extract of NCIB 9816 induced with 2-hydroxybenzyl alcohol

The organism was induced with 2-hydroxybenzyl alcohol. The extract was prepared as described in Materials and Methods. The reaction mixture contained 2.75 ml buffer, 0.1 ml NAD^+ (150 mM), 50 μl salicylaldehyde (19.7 mM in ethanol) and 20 μl of crude extract. The activity was measured as the rate of increase in absorbance at 340 nm in a Perkin-Elmer spectrophotometer. \square — \square activity in phosphate buffer ($\mu=0.2\text{M}$), \circ — \circ activity in pyrophosphate buffer ($\mu=0.2\text{M}$).

The buffers were prepared as follows: 25 ml of a solution of Na_2HPO_4 (133.3 mM) or $\text{Na}_4\text{P}_2\text{O}_7$ (40 mM) was adjusted with 2N HCl to the required pH, the volume (given below) of a solution of 0.5 M NaCl was added, and the solution was diluted to 50 ml with water.

<u>Pyrophosphate</u>		<u>Phosphate</u>	
pH	Volume of 0.5 M NaCl ml	pH	Volume of 0.5 M NaCl ml
7.5	5.58	6.0	5.87
8.0	4.90	6.5	4.67
8.5	3.52	7.0	2.83
9.0	1.87	7.5	1.76
9.5	0.7	8.0	0.45

Figure 12



The concentrations of NAD^+ and salicylaldehyde were 5 mM and 0.3 mM respectively. The initial rates of increase in absorbance at 340 nm were found to be proportional to the concentration of crude extract when the latter was obtained from cells induced with 2-hydroxybenzyl alcohol. With extracts obtained from salicylate induced cells, the initial rates varied linearly with the enzyme concentration but were not directly proportional to the concentrations of extract (Figure 13).

Determination of K_m for NAD^+

The initial rates of the reaction of salicylaldehyde dehydrogenase in the extract were measured spectrophotometrically at different concentrations of NAD^+ . Sodium pyrophosphate (pH 8.5) was used as buffer, and the concentration of salicylaldehyde was 0.3 mM. The initial rates of reaction at different NAD^+ concentrations are shown in Figure 14. The data were fitted to a Lineweaver-Burke plot by the method of least squares (Figure 15), and the calculated K_m was 0.13 mM.

The K_m for salicylaldehyde was not determined as the rate of reaction was independent of substrate concentration down to concentration as low as 10 μM .

Identification of the product and stoichiometry of the reaction between salicylaldehyde and NAD^+ catalysed by the cell extract

The stoichiometry of the reaction was determined from the spectrophotometric measurement of NADH produced on completion of the

Figure 13. Activity of salicylaldehyde dehydrogenase in NCIB 9816 at different concentrations of cell extracts

The reaction mixtures contained, 0.2 ml of salicylaldehyde (4.5 mM in pyrophosphate buffer), 0.1 ml NAD^+ (150 mM), the volumes of cell extract indicated, and pyrophosphate buffer, pH 8.5 to a final volume of 3.0 ml. The activities were measured as the rates of increase in absorbance at 340 nm. \circ — \circ activity of extract from salicylate-induced cells and \square — \square activity of extract of cells induced with 2-hydroxybenzyl alcohol.

Figure 13

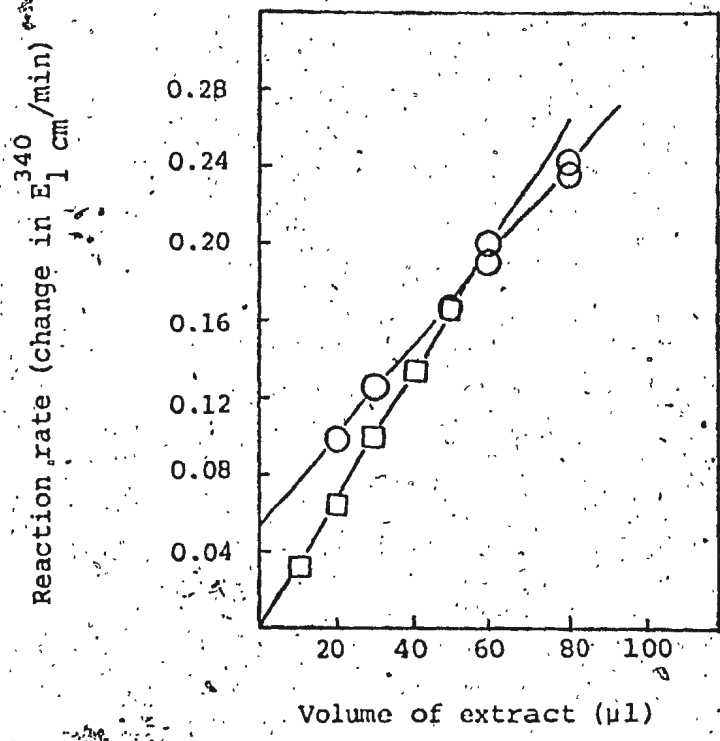


Figure 14. Activity of salicylaldehyde dehydrogenase in the crude extracts of NCIB 9816 at different NAD^+ concentrations

The rate of reduction of NAD^+ was followed spectrophotometrically at 340 nm as described in Materials and Methods, varying the NAD^+ concentrations as indicated. The crude extract was obtained from salicylate-induced cells. The rate is calculated as the increase in absorbance/min. at 340 nm.

Figure 14

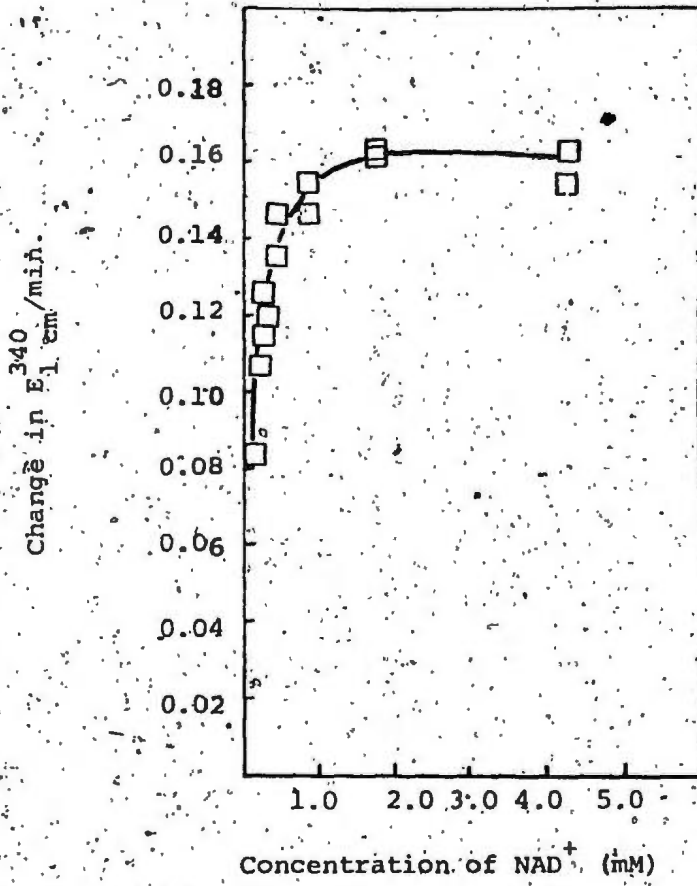


Figure 15. Determination of the K_m for NAD^+ in the oxidation of salicylaldehyde catalyzed by the salicylaldehyde dehydrogenase.

Reaction conditions are as described in Figure 14. The rates of the reaction are expressed as increase in $E_{340}^{1\text{ cm}}$ /min. The line is the best fit computed by the method of least squares and gives a K_m for NAD^+ of 0.13 mM.

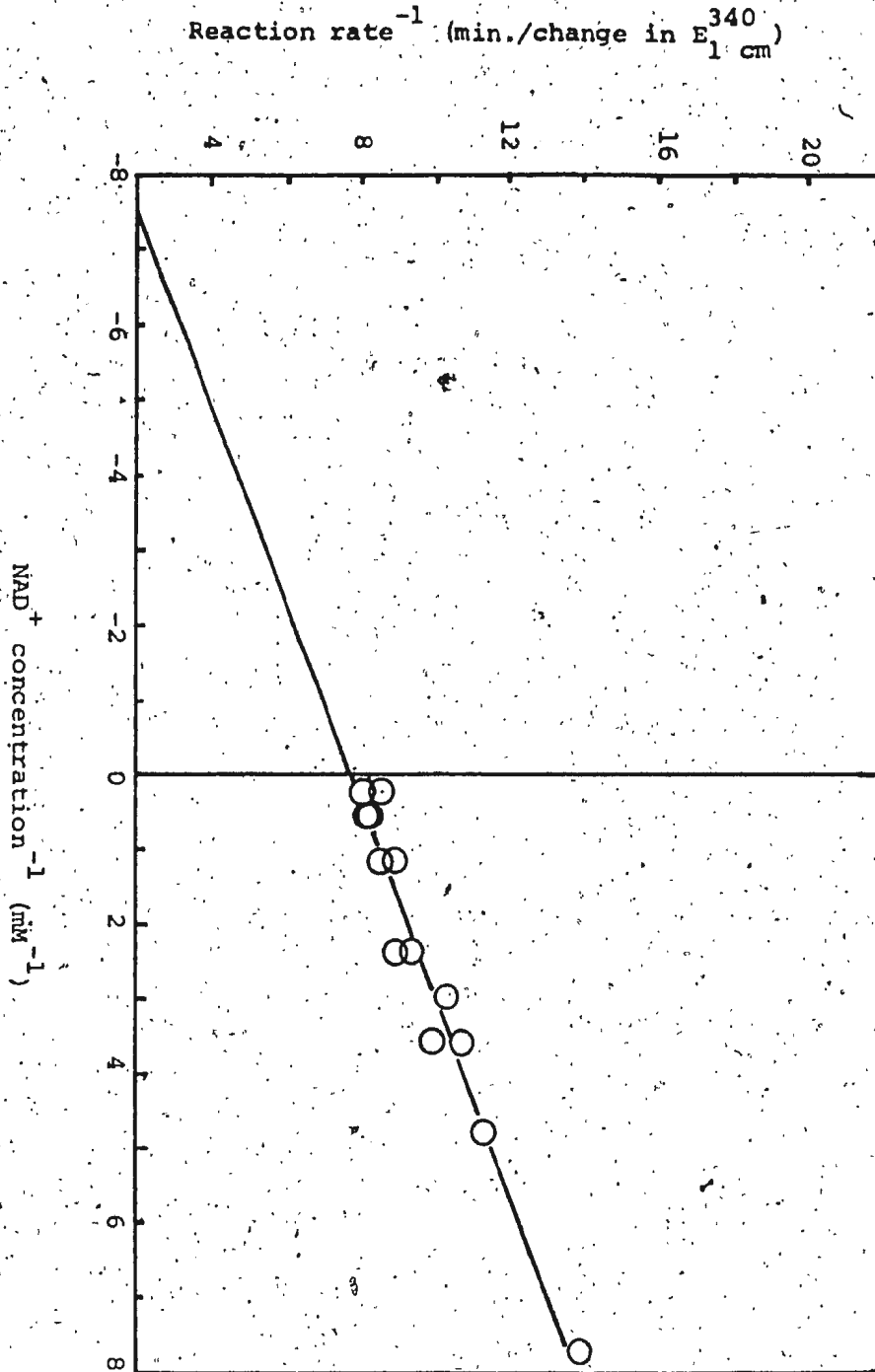


Figure 15

reaction carried out as described in Materials and Methods. The results are given in Table III. Figure 16A shows the absorption spectrum of the product isolated from reaction mixture 1 given in Table III, and the absorption spectra of authentic 1,10-phenanthroline and salicylate are shown in Figure 16B and 16C respectively. The absorbance of 1,10-phenanthroline at 295 nm is 11.9% of that at 263 nm. Applying this correction to the absorbance value at 295 nm of the product and subtracting the absorbance at 350 nm, the salicylate in the isolated product was found to be 0.645 and 0.641 μ mole in 20 ml of reaction mixture 1 and 2 respectively. This is equivalent to 0.96 and 0.95/mole respectively per mole of salicylaldehyde originally present in the reaction mixtures. The corresponding stoichiometry for NADH was 0.89 mole and 0.88 mole per mole of salicylaldehyde in reaction mixtures 1 and 2 respectively.

Identification of the product was confirmed by thin layer chromatography. A spot was observed which behaved identically with authentic salicylate. It gave a blue fluorescence in UV light and had an R_f value of 0.70. It was separate from 1,10-phenanthroline (R_f 0.00), but it was not well separated from salicylaldehyde (R_f 0.76). It was readily distinguishable from the latter, however, by its characteristic fluorescence.

Activity with other substrates and cofactors

The cell extract from NCIB 9816 induced with 2-hydroxybenzyl alcohol also oxidized benzaldehyde and acetaldehyde in the presence



Table III

Stoichiometry of the reaction between salicylaldehyde and NAD⁺ catalyzed by the extract from induced cells of NCIB 9816

The incubation of the cell extract with salicylaldehyde and NAD⁺ in the presence of 1,10-phenanthroline, and the isolation of the product was described in Materials and Methods.

	<u>Reaction mixture 1</u>	<u>Reaction mixture 2</u>
Absorbance at 340 nm of reaction mixture lacking NAD ⁺ (vol. 23.2 ml).	0.312	0.310
Absorbance at 340 nm after addition of NAD ⁺ and completion of reaction (total vol. 24 ml).	0.599	0.597
Absorbance at 340 nm of a reaction mixture lacking all reagents except NAD ⁺	0.184	
Absorbance of extracted solutes (from 20 ml reaction mixture) in 5 ml buffer at 295 nm.	0.642	0.614
Salicylaldehyde used μ mole (in 24 ml)	0.81	0.81
NADH formed μ mole (in 24 ml)	0.710	0.712
Salicylate formed μ mole (in 24 ml)	0.774	0.769

Figure 16. Absorption spectra of the solutions of authentic salicylate and 1,10-phenanthroline, and the spectrum of the product of reaction between salicylaldehyde and NAD^+ catalyzed by salicylaldehyde dehydrogenase in the presence of 1,10-phenanthroline

The experiment was carried out as described in Materials and Methods. (A) Product isolated from reaction mixture 1 (see Table III); (B) Authentic 1,10-phenanthroline; (C) Authentic salicylate.

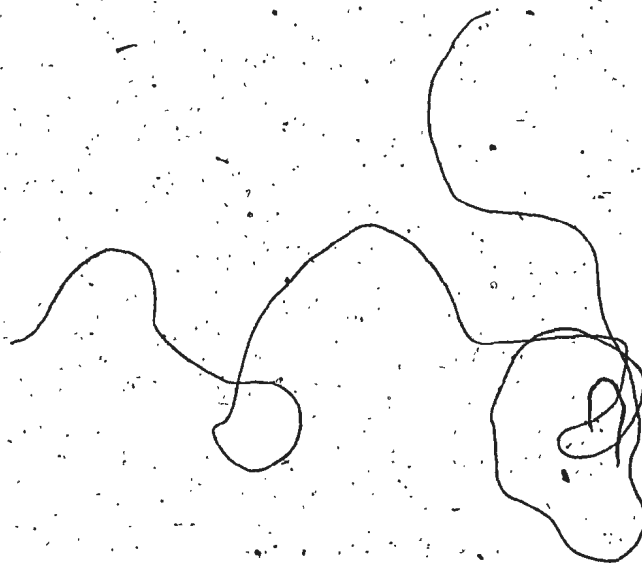
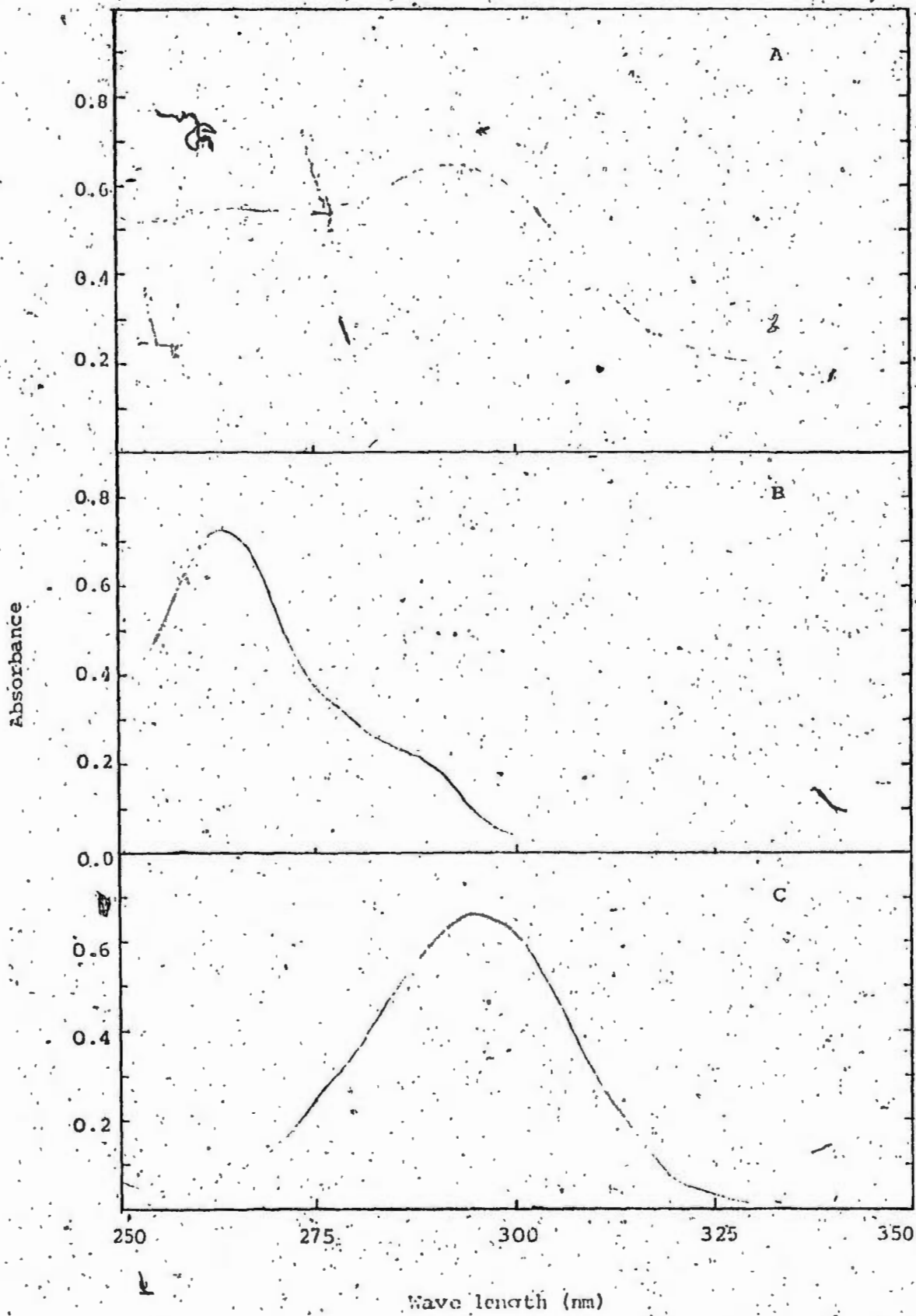


Figure 16



of 5 mM NAD⁺. Table IV shows the rates of oxidation of the three aldehydes.

The rate of oxidation of salicylaldehyde in the presence of 5 mM NADP⁺ was 7% of that given by 5 mM NAD⁺.

Measurement of the levels of some enzymes of naphthalene metabolism in whole cells and extracts of NCIB 9816

The levels of some enzymes of naphthalene metabolism were measured in whole cells and extracts of NCIB 9816. The organism was grown on naphthalene, salicylate or catechol as sole carbon source, or on succinate to which salicylate, catechol or the gratuitous inducers were added in the early log phase. Sterile solutions of these compounds were added when the cultures growing on succinate (8.5 mM) reached an absorbance at 600 nm of about 0.1. The cultures were harvested when the absorbance at 600 nm due to bacteria reached about 0.4.

The levels of naphthalene oxygenase measured in the whole cells and those of salicylaldehyde dehydrogenase, salicylate hydroxylase and catechol dioxygenases in extracts of the cells grown under the above conditions are shown in Table V.

Induction of naphthalene oxygenase in NCIB 9816 grown on glucose, malate and acetate

The possibility of the repression of naphthalene oxygenase by some substrates was examined using salicylate as the inducer.

Table IV

Oxidation of some aldehydes by crude
extracts from induced NCIB 9816

The crude extract was prepared from NCIB 9816 induced with 2-hydroxybenzyl alcohol as described in Materials and Methods. The dehydrogenase activity was measured by the reduction of NAD⁺ (5 mM) using salicylaldehyde (0.3 mM), benzaldehyde (0.52 mM), or acetaldehyde (0.46 mM).


<u>Substrate</u>	<u>Specific activity^a</u> <u>(μmole/min/mg protein)</u>
Salicylaldehyde	0.644, 0.553
Benzaldehyde	0.354, 0.307
Acetaldehyde	0.022, 0.016

^a Each figure indicates the average of at least two measurements in an experiment.

Table V

Levels of some enzymes of naphthalene metabolism in Pseudomonas NCIB 9816 grown under various conditions

Cells were grown under the conditions described in Tables I and II. Harvesting of the cells, preparation of the cell extracts and the measurement of enzyme levels have been described in Materials and Methods.

Specific Activity of Enzymes ^a					
Carbon source and additive	 Naphthalene oxygenase ^b	Salicylaldehyde dehydrogenase	Salicylate hydroxylase	Catechol 1,2-dioxygenase ^e	Catechol 2,3-dioxygenase ^f
Succinate	0.003±0.006 ^c (n=4)	0.071±0.028	0.000,0.003	0.003	0.059
Naphthalene	0.251	N.D. ^d	0.245	0.277	0.029
Salicylate	0.207	N.D.	0.386	0.400	0.025
Catechol	0.006	N.D.	0.001,0.001	N.D.	N.D.
Succinate+Salicylate	0.121±0.048(n=6)	0.536,0.413	0.358,0.255	0.363,0.304	0.015,0.015
Succinate+Catechol	0.013	N.D.	0.000	0.440	0.030
Succinate+2-aminobenzoate	0.165±0.057(n=4)	0.551,1.090	0.103	0.010,0.017	0.074,0.032
Succinate+2-hydroxybenzyl alcohol	0.151±0.039(n=4)	1.032,0.403	0.173	0.009±0.003(n=4)	0.024,0.056

^aCalculated per mg protein in whole cells for naphthalene oxygenase, and in extracts for other enzymes.

^bThe activities of naphthalene oxygenase shown here are reproduced from Tables I and II for the convenience of comparison between the levels of naphthalene oxygenase and those of the other enzymes.

^cWhere the values accompany + sign, figures represent mean ± standard deviation. Single figures represent the average of at least two measurements in an experiment.

^dN.D. indicates not determined.

Reaction mixture contained: (e) catechol (0.07 mM), EDTA (1,33 mM), cell extract (50 µl), tris HCl pH 7.6 (30 mM), final volume 3 ml. Absorbance was monitored at 260 nm. (f) catechol (0.17 mM), cell extract (50 µl), tris HCl pH 7.6 (30 mM) final volume of 3 ml. Absorbance was monitored at 375 nm.

Salicylate was added (final concentration 3.5 mM) during the early exponential phase to cultures growing on glucose (8.5 mM), succinate (8.5 mM), malate (7.5 mM) or acetate (7.4 mM), and the cells were harvested after doubling twice. See Table VI for details.

The level of the enzyme induced was significantly lower when the organism was grown on glucose plus salicylate (Table VD).

The appropriateness of the method of preparation of cell extract

Extracts were prepared by disrupting a suspension of cells with an ultrasonic oscillator as described in Materials and Methods. To find an optimum condition for sonication of the cells, 2 ml portions of a cell suspension were subjected to 1, 2, 4, 6 or 8 thirty second and periods of sonication, each period interspersed with an interval of 1 minute in which the suspension and the sonication probe were cooled in ice-water. After centrifugation of the disrupted cells, the levels of catechol 1,2 and 2,3-dioxygenases were determined in the extracts. Maximum activity was found in extracts prepared by six sonications of 30 seconds each.

Table VI

Effects of various carbon sources on the induction of naphthalene oxygenase in Pseudomonas NCIB 9816

Sterile solutions of salicylate (final concentration of 3.5 mM) were added during the early exponential phase to cultures of NCIB 9816 growing on basal mineral medium containing the carbon sources indicated, and the cells were harvested after doubling twice. Measurement of naphthalene oxygenase in washed whole cells was made spectrophotometrically as described in Materials and Methods. The concentrations of the carbon sources are given in bracket.

<u>Carbon source</u>	<u>Naphthalene oxygenase activity^a</u> <u>(μmole/min/mg protein in whole cells)</u>
Succinate (8.5 mM)	0.171, 0.220
Glucose (8.5 mM)	0.077, 0.068
Malate (7.5 mM)	0.185, 0.206
Acetate (7.4 mM)	0.170, 0.205

^a Each figure indicates an average of duplicate measurements.

DISCUSSION

The levels of some enzymes of naphthalene degradation in Pseudomonas NCIB 9816 were determined under different conditions of growth to examine the possible modes of regulation of these enzymes. The "coordinate" regulation of the enzymes catalyzing the degradation of naphthalene to salicylate appeared possible from the fact that salicylate accumulated in the incubation medium when NCIB 9816, that had been induced with salicylate or the gratuitous inducers, was incubated with naphthalene. The criteria for the coordinate regulation of a group of enzymes are the simultaneous induction of the enzymes by an inducer, and proportionality between the levels of the enzymes under the different conditions of induction. Simultaneous induction is not necessarily evidence for the coordinacy of induction (Hosakawa, 1970), but proportionality between the levels of a group of enzymes under different growth conditions is taken as an evidence for their coordinate regulation (Ornston, 1966c).

In the present work not only were naphthalene oxygenase and salicylaldehyde dehydrogenase found to be induced by the same inducers but in Pseudomonas NCIB 9816, but salicylate hydroxylase was also, the ratios of these three enzymes under different growth conditions were not the same. This may not, however, exclude the possibility that these enzymes are coordinately controlled because of the limitations of the methods used for the determination of the enzymes. The methods of determining enzyme activities will therefore be discussed first.

Measurement of naphthalene oxygenase

Naphthalene oxygenase activity was reported in extracts of naphthalene-grown *Pseudomonads* by Davies and Evans (1962), Catterall, Murray and Williams (1971), and Catterall and Williams, (1971). The latter authors measured oxygen uptake polarographically in the presence of NADH, reduced glutathione (GSH) and Fe^{2+} . In our hands the method proved unreliable and in any case recoveries were very low. The highest specific activity reported by Catterall and Williams (1971) was 0.031 $\mu\text{mole } O_2/\text{min}/\text{mg}$ protein, which was equivalent to 0.015 $\mu\text{mole naphthalene}/\text{min}/\text{mg}$ protein according to the stoichiometry reported by those authors. ($2 \pm 0.1 \mu\text{moles } O_2/\text{mole of naphthalene}$). With naphthalene-grown cells in our study the lowest activity calculated from the rate of disappearance of naphthalene in intact cells was 0.334 $\mu\text{mole naphthalene}/\text{min}/\text{mg}$ protein. A suspension of whole cells (protein content 0.25 mg/ml, $E_{1\text{ cm}}^{600} = 1.0$) after sonication and centrifugation gave a supernatant containing 0.15 mg protein/ml. Consequently the rate measured by Catterall and Williams (1971) was equivalent to 0.009 $\mu\text{mole naphthalene}/\text{min}/\text{mg}$ protein in intact cells, which is 2.8% of the lowest rate we obtained with whole cells.

Furthermore, Catterall and Williams (1971) did not describe how the correction for the oxidation of the cofactors was made. In our hands the initial rate of oxygen uptake due to NADH (0.5 mM), GSH (0.5 mM) and Fe^{2+} (0.2 mM) was 0.15 mM/min. This decreased to 0.025 mM/min after about 15 seconds. Catterall and Williams (1971)

used 0.1 ml of cell extract prepared from a suspension of cells containing 0.149 wet bacteria/ml. According to our results this is equivalent to 0.7 mg protein (0.14 g wet bacteria/ml has a calculated $E_{1\text{ cm}}^{600}$ of 46.7). Therefore, the maximum oxygen uptake due to naphthalene oxidation in 3 ml reaction mixture would be 0.022 $\mu\text{mole}/\text{min}$, i.e. 0.007 mM/min, which is less than one third of the lowest rate of oxygen uptake given by the cofactors in our study.

Some possible reasons for the poor recovery of naphthalene oxygenase in cell extracts were briefly examined. The superoxide anion has been found to be involved in many enzyme reactions (Fridovich, 1970, McCord and Fridovich, 1968, Misra and Fridovich, 1971). It is possible that a superoxide anion is involved in the oxidation of naphthalene, and the lack of activity in cell extracts may be due to removal of the superoxide anion by superoxide dismutase which is widely distributed in aerobic organisms (McCord and Fridovich, 1969). Attempts were made therefore to find an inhibitor which would selectively inhibit the dismutase. Cupferron, (ammonium salt of N-nitrosophenyl hydroxylamine), NaN_3 and KCNS at a concentration of 10 mM did not inhibit the superoxide dismutase present in the extract of NCIB 9816 assayed by the tetrazolium reduction method (McCord et al., 1973). KCN (3.3 mM) inhibited the dismutase; but was found to inhibit naphthalene oxygenase in intact cells (Table VII).

The separation of naphthalene oxygenase from the superoxide dismutase was attempted by Sephadex gel filtration. Cell extracts were passed through columns of G-25, G-75 and G-200 equilibrated with

Table VII

Effects of some inhibitors on the oxidation of
naphthalene in whole cells of NCIB 9816

Naphthalene-grown washed cells of NCIB 9816 were added to a polarographic cell to give a concentration of 0.05 absorbance unit at 600 nm. Sixteen μ l of an ethanolic solution of naphthalene and 50-100 μ l of the inhibitor indicated were added in steps. The effects of NaN_3 and KCN were studied by measuring the naphthalene oxygenase activity spectrophotometrically as described in Materials and Methods using the concentrations of the inhibitors indicated. The rate of oxygen uptake or of change in absorbance at 276 nm with the inhibitor was subtracted from the corresponding rate without the inhibitor to determine the amount of inhibition.

<u>Inhibitor</u>	<u>Concentration</u> <u>mM</u>	<u>% Inhibition</u>
KCN	1.1	86
NaN_3	1.3	93
pCMB	0.009	100
Rotenone	0.05	10.9
Antimycin A	0.09	10.0
1,10-phenanthroline	1.22	41

O₂ had been displaced phosphate buffer (pH 7.0) from which with N₂. The activity of superoxide dismutase was monitored in the column eluate, but no naphthalene oxygenase activity was detected in any of the fractions. Extracts prepared by the disruption of the cells in a French pressure cell (American Instruments Inc., Silver Spring, Maryland, U.S.A.) or by freezing and thawing also did not show any activity. Therefore the loss of activity is not probably due to the methods of disruption of the cells.

Consequently washed intact cell suspensions were used to measure the activity of naphthalene oxygenase spectrophotometrically. The rate of disappearance of naphthalene added to cell suspension was directly proportional to the concentration of bacteria in the cuvette upto a maximum $E_{1\text{ cm}}^{600}$ due to bacteria of 0.32 (Figure 6). Above this concentration the measurement was not satisfactory as the expected rise in the absorbance at 276 nm due to the addition of naphthalene was not observed. The rate of reaction was dependent on the concentration of naphthalene as shown in Figure 7. The plots of the reciprocals of the concentrations of naphthalene against those of the initial rates of reaction are shown in Figure 8. The best line fitting the data was computed by the method of least square and gave a Km of 4 μM . The results show the concentration of naphthalene used in the enzyme determination (80 μM) was saturating.

There are a number of objections to the measurement of naphthalene oxygenase using intact cells. The rate of uptake of naphthalene by the cells may be rate limiting, naphthalene oxygenase may be tightly coupled to a subsequent rate-limiting step, or the naphthalene

may be absorbed by the cells without being metabolized.

The absorption of naphthalene was examined by Rogoff (1962), who determined the uptake of naphthalene by "resting" cells of a pseudomonad strain grown on naphthalene. But it is not quite clear if the disappearance of naphthalene added to cell suspension was due to the absorption of naphthalene (as was claimed) or its actual metabolism. It is uncertain what was meant by "resting cells". In our studies it was observed that washed suspension of naphthalene-grown cells harvested during exponential phase of growth oxidized naphthalene rapidly even after a long period of storage on ice. The naphthalene-stimulated oxygen uptake reported by Rogoff (1962) was 612 $\mu\text{l/h/mg N}$. This according to our studies with NCIB 9816, represents about 0.014 $\mu\text{mole naphthalene/min/mg protein}$ (the molar ratio of the rate of oxygen uptake to that of naphthalene disappearance was found to be 5.2 in NCIB 9816). The reported naphthalene absorption was 2.18 $\mu\text{mole/mg N i.e. about 0.347 } \mu\text{mole/mg protein}$. Taking into consideration the 23 minute period for which the reaction mixture was incubated and centrifuged to remove the bacteria, the disappearance of naphthalene could well be accounted for by oxidation. However, the possibility of the absorption of some naphthalene by the cells can not be excluded entirely.

While the results of Rogoff (1962) do not unequivocally demonstrate whether the disappearance of naphthalene from a cell suspension is due to degradation or to absorption in the cell membrane, we observed a rapid and continuous disappearance of naphthalene measured spectrophotometrically which can not be explained as merely due to

absorption since it is accompanied by the accumulation of a large molar proportion of salicylate.

Accumulation of salicylate in the reaction medium also adds to the uncertainty in the numerical values obtained by the spectrophotometric method. In a suspension of cells induced with salicylate and containing 80 μM naphthalene the rate of disappearance of naphthalene was compared spectrophotometrically with that of the accumulation of salicylate and the results indicated that salicylate accumulates initially at 53% of the rate at which naphthalene disappears (Figure 17). Consequently, the extinction coefficient of naphthalene at 276 nm which was used for the computation of naphthalene oxygenase by the spectrophotometric method should be corrected, for salicylate also absorbs at 276 nm (extinction coefficient $1.44 \text{ mM}^{-1} \text{ cm}^{-1}$). Neglect of this correction in the example given above leads to an underestimation of the activity of naphthalene oxygenase in the salicylate-induced cells by 17%. The magnitude of this correction should be assessed in each individual case. For example, after NCIB 9816 was induced with 2-hydroxybenzyl alcohol, the ratio of the initial rate of accumulation of salicylate to the initial rate of disappearance of naphthalene was higher than after induction with salicylate. (Figure 18).

The correction for salicylate accumulation has not been applied to the results presented in Tables I, II, and VI, since the rate of salicylate accumulation by the organism under all these growth conditions was not obtained. Because of these limitations of the method used for the determination of naphthalene oxygenase, the numerical values of the levels

Figure 17. Initial rate of naphthalene disappearance compared with the initial rate of appearance of salicylate when a suspension of washed cells of NCIB 9816 is incubated with naphthalene

The cells were induced with salicylate. The initial rate of disappearance of naphthalene and the concomitant initial rate accumulation of salicylate were determined as described in Materials and Methods. ○—○ concentration of naphthalene, □—□ concentration of salicylate.

Figure 17

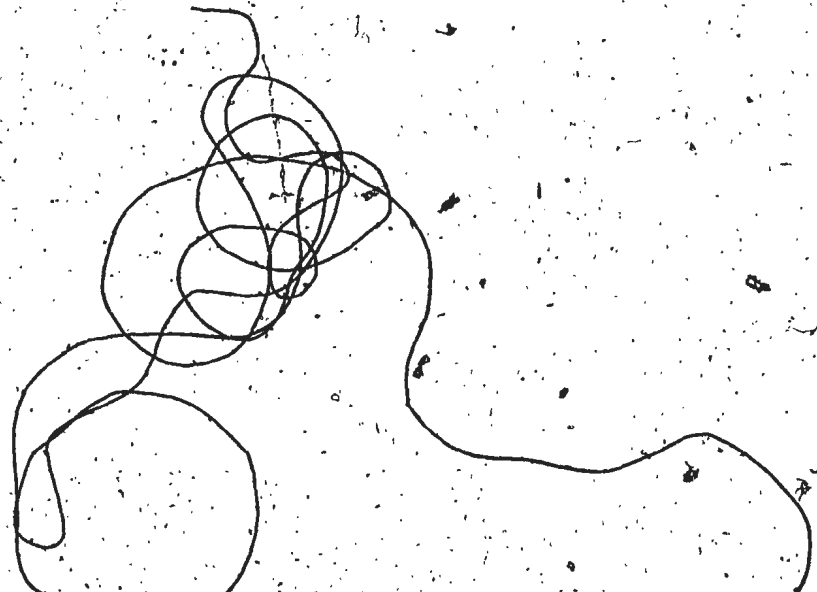
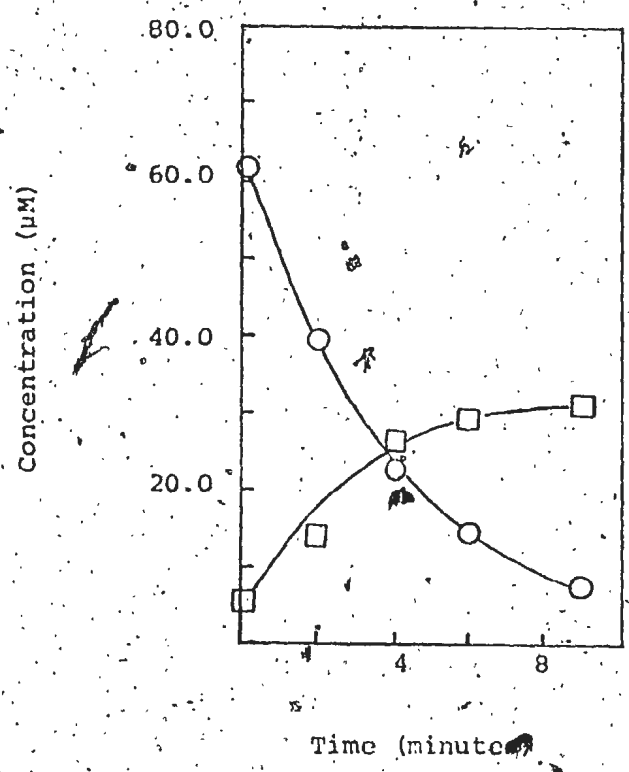
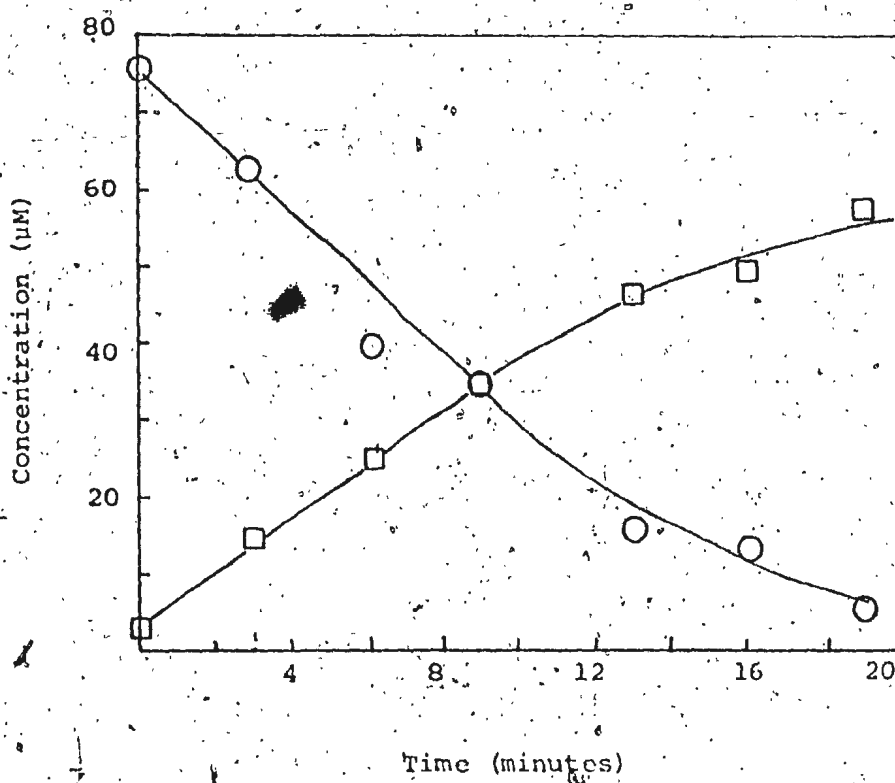


Figure 18: Initial rate of naphthalene disappearance compared with the initial rate of appearance of salicylate in washed cell suspension of Pseudomonas NCIB 9816 that had been induced with 2-hydroxybenzyl alcohol

See Materials and Methods for details of the measurement.

○—○ concentration of naphthalene (μM) □—□ concentration of salicylate (μM).

Figure 18



V,
of naphthalene oxygenase shown in Tables I, II and VI will be minimal. Even in the absence of these limitations the rates measured by this method may be minimal if naphthalene oxygenase is tightly coupled to a subsequent rate-limiting step.

A polarographic method for the measurement of naphthalene oxygenase in whole cells was adopted to determine the time-course of induction of naphthalene oxygenase and also in some experiments to determine the effects of inhibitors on naphthalene oxygenase. This method has the apparent advantage that it is more sensitive since the ratio of the rate of oxygen uptake to that of naphthalene disappearance was found to be 5.2 in an experiment with salicylate induced organisms. But this method also has limitations. Since the oxygen uptake is not only specifically due to the oxidation of naphthalene but also some subsequent reactions, the stoichiometry of oxygen uptake may vary in cells grown under different conditions. Therefore the rates of oxygen uptake shown in the determination of the time-course of induction (Figures 9B, 10B, 11B) must be taken as a qualitative demonstration of induction, at least up to the state of full induction.

Similarly, the compounds that inhibited the naphthalene stimulated oxygen uptake by the induced cells (Table VII) might have actually inhibited not only naphthalene oxygenase but some other steps to which naphthalene oxygenase might be tightly coupled.

Measurement of other enzymes

Salicylaldehyde dehydrogenase

The activity of salicylaldehyde dehydrogenase in the cell extract was determined spectrophotometrically by following the reduction of NAD^+ . Measurement of salicylaldehyde dehydrogenase under these conditions would probably be underestimated, since salicylate hydroxylase which is present in the crude extract will remove NADH . Oxidation of NADH by the crude extract in the absence of salicylate was very small, but in a reaction medium containing a limiting quantity of salicylaldehyde it was observed that the absorbance at 340 nm due to the production of NADH begins to decrease when the reaction was apparently only 66% complete. Probably this was due to the action of salicylate hydroxylase. 1,10-Phenanthroline (5 mM) completely inhibited the salicylate hydroxylase but there was considerable inhibition (30%) of salicylaldehyde dehydrogenase too. Therefore, no inhibitor was used, and hence the increase in the absorbance at 340 nm would be actually a balance between the NADH produced by the oxidation of salicylaldehyde and the oxidation of NADH by salicylate hydroxylase. The measured values of salicylaldehyde dehydrogenase by this method therefore represents minimal ones (Table V).

Salicylate hydroxylase

The activity of salicylate hydroxylase in crude extracts was measured by following spectrophotometrically the oxidation of NADH . This method is preferable to the method of oxygen uptake because in the latter case, oxygen uptake represents also the enzymatic oxidation of the

catechol formed, for the crude extract contained catechol dioxygenases also.

Catechol dioxygenases

The determination of the catechol dioxygenases in the cell extracts was based on the spectrophotometric measurements of the product formed. Since the crude extracts contain the enzymes to remove the product formed, these measurements may not be without fault. EDTA was used in the measurement of catechol 1,2-dioxygenase in crude extracts to inhibit the cis, cis-muconate lactonising enzyme (Sistrom and Stanier, 1954) which would otherwise remove cis, cis-muconate. Under the conditions of catechol 2,3-dioxygenase assay, 2-hydroxymuconic semialdehyde was stable. This is consistent with the observations of Feist and Hegeman (1969) and of Chakrabarty (1972).

Another factor which may add to the inaccuracy in the determination of the catechol dioxygenases is the competition of the two dioxygenases in the crude extract for the common substrates O_2 and catechol. To test this catechol 1,2-dioxygenase was determined in the crude extract of induced NCIB 9816 after inactivating the 2,3-dioxygenase with H_2O_2 and inactivating the excess H_2O_2 with catalase (Nakazawa and Yokota, 1973). Catechol 1,2-dioxygenase activity was not increased above the values found without this treatment. The results were similar to those of Nakazawa and Yokota (1973) and showed a decrease of 20% in the activity of catechol 1,2-dioxygenase accompanying total inactivation of catechol 2,3-dioxygenase.

Furthermore the K_m values for catechol and oxygen are $4 \mu\text{M}$ and $20 \mu\text{M}$ respectively in the case of catechol 1,2-dioxygenase (Nozaki, Kagamiyama and Hayaishi, 1963) and $3 \mu\text{M}$ and $7 \mu\text{M}$ respectively in the case of catechol 2,3-dioxygenase (Nozaki, 1970)². The concentrations of catechol used in the present study for the measurements of catechol 1,2 and 2,3-dioxygenases were $70 \mu\text{M}$ and $170 \mu\text{M}$ respectively and the solubility of oxygen at the reaction temperature (25°) is $250 \mu\text{M}$. Hence, both the substrates used in the assays had concentrations several times higher than their K_m values for the two enzymes and therefore on theoretical grounds the rate of reaction will not be limited by the substrate concentrations.

To confirm the validity of the determination of catechol 2,3-dioxygenase extracts from salicylate induced cells of ATCC 17483 which has undetectable level of catechol 1,2-dioxygenase were assayed for catechol 2,3-dioxygenase activity by the standard procedure, either alone or mixed with the extract from salicylate induced cells of NCIB 9816 which contained catechol 2,3-dioxygenase and a high level of catechol 1,2-dioxygenase. The activity of catechol 2,3-dioxygenase was additive when the two extracts were mixed/. This is further evidence which suggests that the catechol 2,3-dioxygenase activity measured under these conditions is not limited by the competition of catechol 1,2-dioxygenase for the substrates. It also suggests that there is no inhibitor of catechol 2,3-dioxygenase in extract of NCIB 9816, indicating that the apparent failure to induce catechol 2,3-dioxygenase in NCIB 9816 is real.

Table VIII

Activity of catechol 2,3-dioxygenase in extract of
in
ATCC 17483 in the absence and/the presence of the extract of NCIB 9816

Extracts were prepared from NCIB 9816 and ATCC 17483, after both the organisms had been induced with salicylate. Eighty μ l of the extract from NCIB 9816 and 10 μ l of that from 17483 were assayed for catechol 2,3-dioxygenase by the standard method, either alone or mixed together.

<u>Extract obtained from</u>	<u>Increase of absorbance at 375 nm/min.^a</u>
ATCC 17483	0.034
NCIB 9816	0.044
ATCC 17483 + NCIB 9816	0.079

^a Each figure represents the average of duplicate measurements.

Induction of some enzymes of naphthalene metabolism in NCIB 9816 under various growth conditions

Naphthalene oxygenase was induced in NCIB 9816 when grown on naphthalene or salicylate, or when the organism was growing on succinate to which salicylate was added. The level of the enzyme was found to be very low when the organism was grown on succinate or catechol, or succinate plus catechol (Tables, I and V). This suggests that salicylate, the immediate precursor of catechol, may be the inducer of naphthalene oxygenase. But it does not exclude the possibility that a metabolite of catechol is the inducer. Catechol formed as an intermediate from salicylate and that supplied in large amounts in the medium may be metabolized by different pathways. Indeed, there are similar instances. Chakrabarty (1972) observed that Pseudomonas putida strain R1 metabolized catechol through ^{the} ortho pathway when grown on benzoate but induced the enzymes of the meta pathway when grown on salicylate. Likewise, Farr and Cain (1968) found that a strain of Pseudomonas aeruginosa when grown on benzoate metabolized catechol through the ortho pathway, but catechol itself elicited a catechol 3,3-dioxygenase in non-induced cells.

In the present work, activities of catechol 1,2 and 2,3-dioxygenases in extracts of catechol-induced cells were similar to those of salicylate-induced cells (Table V). This reduces the probability that a metabolite of catechol is the inducer of naphthalene oxygenase. It does not of course exclude the possibility entirely because the presence of the first enzyme in a pathway does not necessarily mean that

the subsequent enzymes of the pathway are induced and consequently all the subsequent intermediates are formed.

The question arises as to which pathway is used by the strain NCIB 9816 for catechol degradation. Davies and Evans (1964) isolated 2-hydroxymuconic semialdehyde from reaction medium of some Pseudomonas species metabolizing naphthalene, salicylate, or catechol, and on the basis of this observation, suggested that these organisms used the meta pathway for the degradation of catechol. But this suggestion may not be valid since the presence of the first enzyme in a pathway does not necessarily mean that the whole pathway is active. Catterall, Sala-Trepat and Williams (1971) stated that high levels of the enzymes of both pathways of catechol metabolism (including catechol 2,3-dioxygenase) are induced in NCIB 9816. Evans et al. (1965) reported constitutive synthesis of catechol 2,3-dioxygenase in Pseudomonas NCIB 9816 (P_G) and also in Pseudomonas P_F. But unfortunately, neither of these groups presented any data. Our studies suggest that the synthesis of catechol 2,3-dioxygenase is constitutive in NCIB 9816.

As shown in Table V, the catechol 1,2-dioxygenase activity in extracts from succinate grown organism is 0.003 $\mu\text{mole}/\text{min}/\text{mg}$ protein. The level rose 133 fold during growth on succinate plus salicylate and 147 fold during growth on succinate plus catechol. With the gratuitous inducers of naphthalene oxygenase, the levels of catechol 1,2-dioxygenase varied between 0.006 to 0.017 $\mu\text{mole}/\text{min}/\text{mg}$ protein. On the other hand, the level of catechol 2,3-dioxygenase activity was 0.059 $\mu\text{mole}/\text{min}/\text{mg}$ protein in extracts from succinate grown cells, the levels are 0.015 and

0.030 $\mu\text{mole}/\text{min}/\text{mg}$ protein when the cells were grown in the presence of salicylate and catechol respectively, and after induction with the gratuitous inducers/the catechol 2,3-dioxygenase levels in the cell extract varied between 0.024 and 0.074 $\mu\text{mole}/\text{min}/\text{mg}$ protein. This indicates that catechol 2,3-dioxygenase is not inducible in NCIB 9816.

To confirm that salicylate is the inducer of naphthalene oxygenase a search was made among the structural analogues of salicylate to find a compound which would induce the synthesis of the enzyme gratuitously. 3-Nitrosalicylic acid, 5-nitrosalicylic acid, 3,5-dichlorosalicylic acid, *o*-anisic acid, 2-hydroxyacetophenone, methylsalicylic acid, veratrole and 2-hydroxy-5-methylbenzoic acid did not induce naphthalene oxygenase. 2-Acetylsalicylic acid did induce the synthesis of this enzyme but was itself metabolized. 2-Hydroxybenzyl alcohol and 2-aminobenzoate induced naphthalene oxygenase when added to culture growing on succinate, and were not themselves metabolized. The concentrations of these two compounds in culture were monitored spectrophotometrically along with the levels of naphthalene oxygenase which was determined polarographically at various intervals during growth of the organism. The concentrations of the gratuitous inducers 2-hydroxybenzyl alcohol and 2-aminobenzoate remained unchanged (Figures 10A and 11A), showing absence of metabolism of these compounds. However, there is still the possibility that a small undetected amount of these compounds was metabolized to some compounds which actually induced the synthesis of naphthalene oxygenase. Pseudomonas NCIB 9816 does slowly adapt to the gratuitous inducers, and can utilize them as

sole carbon and energy sources after a prolonged lag period of about 48 h.

In this connection it is relevant to ask if this rise in naphthalene oxygenase activity due to the addition of the inducers to the cultures growing on succinate is due to actual induction of the enzyme or the activation of a macromolecular precursor already present.

Sakaki, Kageyama and Egami (1969), using some inhibitors of protein synthesis, demonstrated that protocatechuate 3,4-dioxygenase in Pseudomonas aeruginosa was synthesized de novo, and not activated by the inducer. Our studies also indicate that the enzyme is induced, since the timecourse of induction is characteristic of induction phenomena, and unlikely to be due to, say, a slow penetration of the cell by an activator. Table II shows the levels of naphthalene oxygenase in NCIB 9816 fully induced with 2-hydroxybenzyl alcohol and 2-aminobenzoate. When either of these compounds was added to the culture growing on succinate and the cells were harvested after two doublings, the naphthalene oxygenase activity determined spectrophotometrically, was comparable with the levels obtained with salicylate induced cells (Tables I and V).

The level of naphthalene oxygenase in NCIB 9816 grown on salicylate plus glucose were probably significantly lower than that in the cells grown on succinate, acetate, or malate and induced, in each case, with salicylate (Table VI). This may be explained as due to possible repression of the synthesis of this enzyme by glucose.

From the above discussion it can be concluded that salicylate may be the inducer of naphthalene oxygenase. This is contrary to what one would expect from the observations of Azoulay (1966) who found that washed cell suspensions of some unidentified soil *Pseudomonads* oxidized naphthalene only if the organisms had been adapted to naphthalene. Adaptation to salicylate or the later metabolites did not induce naphthalene oxidation in the organisms.

The possibility that a precursor of salicylate is the actual inducer of naphthalene oxygenase cannot be entirely excluded. Although the NAD^+ dependent oxidation of salicylaldehyde to salicylate catalyzed by salicylaldehyde dehydrogenase would have a large negative standard free energy change at pH 7.0, formation of salicylaldehyde by an alternate pathway cannot be ruled out, and the gratuitous inducers could function as analogues of salicylaldehyde as well.

Davies and Evans (1964) stated that salicylaldehyde is an intermediate in naphthalene metabolism in *Pseudomonads*, but did not make any quantitative determination of the activity of salicylaldehyde dehydrogenase. It was interesting, therefore, to measure the activity of this enzyme under the different growth conditions to see if the induction of this enzyme paralleled that of naphthalene oxygenase. Significant levels of salicylaldehyde dehydrogenase were found in the non-induced cell extract as shown in Table V but the level did rise on induction with salicylate and with the gratuitous inducers.

The salicylaldehyde dehydrogenase in the extract from *Pseudomonas* NCIB 9816 is relatively specific for NAD^+ . This is consistent with the

observation of Davies and Evans (1964). There was some activity with NADP⁺, however, (7% of the activity with NAD⁺). This may have been due to reduction of NADP by transhydrogenase which may be present in the crude extract, or it may have been due to a different enzyme requiring NADP⁺. Gunsalus, Stanier and Gunsalus (1953) separated two benzaldehyde dehydrogenases with specific requirement for NAD⁺ and NADP⁺ from Pseudomonas fluorescens grown on mandelate. Purification of the enzyme would enable one to decide if the situation is similar in the organism of the present study.

The observation that the initial rate of accumulation of salicylate is 0.53 mole per mole of naphthalene metabolized by salicylate induced cells, whereas the corresponding rate of accumulation is 0.80 mole of salicylate per mole of naphthalene when cells are induced with 2-hydroxybenzyl alcohol metabolize naphthalene (Figures 17 and 18), may be in keeping with the observed lower levels of salicylate hydroxylase observed in extracts of cells that had been induced with the gratuitous inducers. Given the variability of the measured levels of enzymes it is, however, far from certain that the quantity of salicylate hydroxylase is limiting in gratuitously induced cells.

As can be seen in Table V, salicylaldehyde dehydrogenase and salicylate hydroxylase are induced under the same conditions as is naphthalene oxygenase. This may be true of other enzymes catalyzing the steps leading from naphthalene to catechol. Except for the cases reported here, substrates for the measurement of other reactions are not readily available. E.A. Barnsley (personal communication) has

observed the induction of 1,2-dihydroxynaphthalene oxygenase by the gratuitous inducers. It seems likely therefore, that a whole block of enzymes catalyzing the degradation of naphthalene to catechol, and not just to salicylate as was originally supposed, are induced co-ordinately.

Ornston (1966c) demonstrated the co-ordinate induction of some enzymes of the *ortho* pathway in *Pseudomonas putida*. The levels of some enzymes in cell extract were repressed or induced to the same magnitude under various growth conditions so that a strict proportionality was observed between the levels of these enzymes. This led him to conclude that the enzymes are in co-ordinate control. In our studies strict proportionality was not observed between the levels of naphthalene oxygenase, salicylaldehyde dehydrogenase and salicylate hydroxylase under different growth conditions (Table V). This casts doubt on the suggestion that the enzymes catalyzing the degradation of naphthalene to catechol are co-ordinately controlled. However, it is difficult to ascertain if the lack of proportionality is real because the factors limiting the accuracy of the determinations of the levels of enzymes may not contribute equally to all activities. For example, accumulation of salicylate in the reaction medium (which interferes with the determination of naphthalene oxygenase activity) is likely to be different for different growth conditions of the organism. Salicylate hydroxylase interferes with the measurement of salicylaldehyde

dehydrogenase. It is induced to somewhat different levels under different conditions of growth of the organism (Table V), and may interfere, therefore, to different extents with the determination of salicylaldehyde dehydrogenase.

Dunn and Gunsalus (1973) demonstrated that in Pseudomonas putida PpG7 the genes coding for the enzymes of naphthalene degradation, including that for catechol 2,3-dioxygenase are plasmid borne and Chakrabarty (1972) demonstrated a similar phenomenon for the genes determining salicylate degradation through the meta pathway. It is not known if all these enzymes are induced co-ordinately, but if they are the regulation would seem to be different from that in Pseudomonas NCIB 9816 in which catechol 2,3-dioxygenase is constitutive.

To summarize, 2-hydroxybenzyl alcohol and 2-aminobenzoate mimic salicylate in the induction of naphthalene oxygenase, salicylaldehyde dehydrogenase and salicylate hydroxylase. Though proportionality between the activities of the enzymes could not be found, possibly because of the limitations of the methods used for their determination, the induction of a whole series of enzymes by a single gratuitous inducer is striking, and does suggest that the enzymes catalyzing the steps from naphthalene to catechol are co-ordinately controlled in NCIB 9816. The extent of this co-ordinacy is less than in Pseudomonas putida PpG7 in which the genes determining enzymes for the degradation of naphthalene to 2-hydroxymuconic semialdehyde are plasmid borne (Dunn and Gunsalus, 1973), and in which 2-aminobenzoate appears to induce co-ordinately all these enzymes (E.A. Barnsley, personal communication).

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APPENDIX

Enzymic reactions studied in this work

- (a) Naphthalene + O₂ + 2H₂ → 1,2-dihydro-1,2-dihydroxynaphthalene
- (b) Salicylaldehyde + NAD⁺ + H₂O → salicylic acid + H⁺ + NADH
- (c) Salicylic acid + H⁺ + NADH + O₂ → Catechol + NAD⁺ + H₂O + CO₂
- (d) Catechol + O₂ → cis,cis-muconic acid
- (e) Catechol + O₂ → 2-hydroxymuconic semialdehyde

