To the Graduate Council:

I am submitting herewith a thesis written by Joyce Elaine Broyles entitled "Reduction of Naphthalene Uptake by Addition of Carbon Source in Pseudomonas putida." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Microbiology.

Thomas C. Montie

Thomas C. Montie, Major Professor

We have read this thesis
and recommend its acceptance:

Accepted for the Council:

L. Evans Roth

Vice Chancellor
Graduate Studies and Research

REDUCTION OF NAPHTHALENE UPTAKE BY ADDITION OF CARBON SOURCE IN *PSEUDOMONAS PUTIDA*

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Joyce Elaine Broyles

December 1981

ACKNOWLEDGMENTS

The author wishes to express her appreciation to her major professor, Dr. Thomas Montie, for the advice and encouragement that he has offered during the course of this investigation. The author also wishes to thank Dr. Jeffrey Becker and Dr. Gary Sayler for their suggestions and criticisms of this research. Finally, to David, the author wishes to express her appreciation for his wondrous art of insane sanity.

ABSTRACT

A model system using naphthalene-degrading strains of Pseudomonas putida and ^{14}C-1(4,5,8)-naphthalene was designed to study the mechanism of aromatic hydrocarbon uptake into bacterial cells. After incubation with C^{14}-naphthalene, cells were harvested by centrifugation through silicone oil and analyzed for naphthalent accumulation. Naphthalene uptake occurred in both naphthalene-grown and glucose-grown cells. Extraction and analysis of cell-associated label revealed the presence of a naphthalene metabolite, salicylate. Strains unable to metabolize naphthalene (NAH$^-$) did not exhibit uptake. Treatment with 200 µg/ml chloroamphenicol or rifampicin inhibited uptake in glucose-grown cells, but not in naphthalene-grown cells. Incubation with oxidizable carbon source (20 mM) blocked naphthalene uptake in succinate, glucose, and citrate-grown cells. Reversal of succinate inhibition of uptake was accomplished by treatment with malonate. Naphthalene-grown cells, although able to oxidize most carbon sources tested, were unaffected by the presence of carbon source. Incubation of cells with either catechol or salicylate, regardless of growth substrate, resulted in immediate cessation of uptake and loss of cell-associated lebel. Uptake was inhibited by cyanide or azide treatment of naphthalene- or glucose-grown cells; while treatment with dinitrophenol or arsenate inhibited glucose-grown cells only. Incubation with exogenous nucleotides did not affect uptake. Glucose-mediated reduction was found to be concentration dependent and not

affected by treatment with non-metabolizable glucose analogs. Levels of extracellular naphthalene metabolites and $^{14}CO_2$ production from labeled naphthalene were strongly reduced by the presence of an oxidizable carbon source in glucose-grown cells. Little reduction occurred in the presence of carbon source in naphthalene-grown cells. The presence of either catechol or salicylate also reduced levels strongly in both glucose-grown and naphthalene-grown cells. ^{14}C incorporation into protein from labeled naphthalene was strongly depressed by the presence of glucose in glucose-grown cells, but naphthalene-grown cells were little affected. The data, taken together, suggest that naphthalene uptake is a function of naphthalene metabolism. It also suggests that the presence of metabolizable carbon source may exert control upon uptake in uninduced cells. The mechanism of this control is unknown, although it probably is at the level of protein synthesis.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAH) are major constituents of fossil fuels and are constantly entering the environment through industrial processes. Many PAH's have been shown to have carcinogenic potential (14). PAH's, because of their extreme insolubility in water (μM range) and aromatic structure, are refractory to microbial degradation. It has been noted that a number of bacterial, fungal, and algal species are able to degrade smaller molecular weight PAH's (2). However, a wider range of PAH's may be metabolized through co-metabolism (51). Although the microbial metabolism of polycyclic aromatic hydrocarbons has been extensively studied, the uptake of PAH into the microbial cell prior to metabolism is ill defined.

The study was undertaken to investigate the uptake process of a model PAH compound, naphthalene, into strains of a naphthalene-degrading bacterium, Pseudomonas putida. In the initial course of the study, a strong relationship between naphthalene uptake and naphthalene metabolism was observed. An unexpected reduction of naphthalene uptake was also seen in cells incubated simultaneously with an oxidizable carbon source. The research discussed here is an effort to define and characterize this uptake and its inhibition. An understanding of the nature of this uptake and its regulation may

provide valuable information about the regulation of naphthalene metabolism in P. putida.

Literature Review

Aspects of Hydrocarbon Uptake

For the purposes of this review, uptake is defined as the initial binding of hydrocarbon to cellular components and any subsequent metabolism. Elucidation of uptake is extremely difficult owing to the lack of information on initial binding and the problem of separating initial binding and metabolism. Although a traditional transport system for an aromatic compound has been described (26), initial bonding is thought to occur by an interaction between hydrocarbon and hydrophobic cell components. This binding may be unfacilitated, or it may be enhanced by changes in membrane hydrophobicity, or by production of emulsifying agents (39, 55). It is unclear whether hydrocarbon metabolism occurs at the cell surface or whether the hydrocarbon must be transferred to the cell interior for metabolism to occur. Uptake of aromatic hydrocarbons has not been well characterized, but much information about aliphatic hydrocarbon systems has been discovered.

The uptake process for aliphatic hydrocarbons has been studied by Finnerty (33, 34), using an Acinetobacter species. Acinetobacter, when growing upon hexadecane as a sole source of carbon, developed large intracellular inclusions bounded by a monolayer membrane which were visible in electron micrographs. Examination of the inclusions by X-ray diffraction revealed the presence of unmetabolized hexadecane.

Upon growth in a medium of acetate and hexadecane, the inclusions did not appear until the acetate was exhausted and growth on hexadecane had begun. Proteins have been found in association with isolated inclusions (60), but no activities have been demonstrated. Acinetobacter was also found to produce extracellular vesicles during growth on hexadecane (31). The vesicles were characterized as a phospholipid and lipopolysaccaride rich particles which bound hexadecane in the form of a microemulsion. This was in addition to observed adherence of Acinetobacter to hexadecane droplets (33). The relationship between production of extracellular vesicles and the appearance of intracellular inclusions is not clear. Similar inclusions have been noted in marine organisms during growth on petroleum (3), as well as in a variety of organisms utilizing aliphatic hydrocarbons (60). Reports of inclusions occurring in organisms utilizing aromatic hydrocarbons have been few, but at least one report of naphthalene inclusions has been noted (60).

A possible reason for the slow growth of bacteria upon aromatic hydrocarbons is the problem of mass transfer between phases of the system, i.e., passage of sufficient carbon from the solid hydrocarbon into the liquid medium to support cellular growth. This transfer is complicated by the extremely low solubilities of most PAH. Unlike aliphatic hydrocarbons there have been few reports of direct contact between cells and aromatic hydrocarbon which would facilitate substrate transfer. Zilber and co-workers (79) have presented evidence that a portion of the pseudomonad population growing upon n-tetracosane remained attached to the solid substrate. Although initial growth only

occurred when attached to the substrate, log phase growth occurred in both attached and non-attached states with approximately 15% of the bacterial population remaining attached to substrate. Non-attached cells were shown to be growing upon n-tetracosane, rather than a metabolite. In the absence of direct contact, dissolved hydrocarbon would be the major source of cell carbon. Wodzinski and co-workers (77) have shown that generation times for organisms growing on different PAH's are inversely proportional to the water solubility of that compound. This suggested the hypothesis that bacteria utilize PAH only in the dissolved state. This hypothesis was supported by further experiments indicating that growth rates of bacteria on naphthalene and pheneanthrene were independent of the amount of solid hydrocarbon present in the medium (75, 76). Generation times in medium containing only dissolved hydrocarbon were identical to generation times in medium containing both solid and dissolved hydrocarbon.

Experiments with the aromatic hydrocarbon diphenyl methane (m.p. 26° C.) found that generation times of a Pseudomonas growing at 30° C. were decreased by the addition of liquid diphenyl methane to medium saturated with diphenyl methane (78). No corresponding decrease in generation time occurred with the addition of solid diphenyl methane at 20° C. Thus, at least in this special case, uptake from the liquid state was faster than uptake from the dissolved state, whereas uptake from the solid phase was slower or at the same rate as uptake from the dissolved state. If uptake of hydrocarbon occurs only from the dissolved state, extrapolation

suggests that higher weight PAH's would be unable to support bacterial growth because of their extremely low solubility in water. This is also indicated by the absence of strains able to metabolize PAH's of lower solubility than naphthacene (0.0066 μM) (77). However, such uptake could be enhanced by the action of secreted emulsifying agents or changes in membrane hydrophobicity, as mentioned earlier. Neither has been demonstrated for PAH degrading cells.

The interactions between the hydrophobic portion of the bacterial cell and hydrocarbon may be hypothesized to occur at the cell envelope. A major limiting factor for PAH uptake could be (1) the rate at which molecules are concentrated in the lipid portion from the aqueous phase; and (2) how rapidly these molecules enter the cell cytoplasm, possibly in the form of metabolites. The presence of the outer membrane in gram negative bacteria may serve as a second lipid phase for solubilization, or it may serve as a sieve to higher molecular weight PAH's. The effect of PAH upon bacterial membranes has not been well documented although Sanioto (58) has examined the effect of PAH on the organization of a model membrane composed of dipalmitoyl lecthin bilayers. He found that lower weight PAH's such as naphthalene (m.w. 128) did not disturb the ordering of the layers, while higher weight PAH's such as benzo (a) pyrene (m.w. 252) disturbed the ordering of the bilayers. The amount of disorder was proportional to the increase in molecular weight.
A thorough examination of the binding and uptake processes of

hydrocarbon in bacteria may contribute to the construction of new hydrocarbon degrading bacteria.

Naphthalene Degradative Pathway

Insights into the biochemical aspects of naphthalene degradation were first offered by Strawinski and Stone (69), who isolated salicylic acid from a culture of naphthalene-grown pseudomonads. Walker and Wiltshire (70) were able to isolate, in addition to salicylic acid, d-trans-1,2-dihydro-1,2 dihydroxynaphthalene. They were also able to show that both were possible intermediates in naphthalene metabolism. Murphy and Stone (45) noted that toxic amounts of 1,2-naphthaquinone accumulated in naphthalene-grown cultures of Pseudomonas lacking ferrous and magnesium ions. Attempts failed to show sequential induction to 1,2 dihydroxynaphthalene, the compound likely to be both the precursor of 1,2 naphthaquinone and the intermediate prior to initial ring fission. It was only after Fernley and Evans (21) took precautions to assure purity and to guard against non-enzymatic quinone formation that positive results with 1,2 dihydroxynaphthalene were obtained. They also isolated coumarin and detected o-coumaric acid and melilotic acid in the culture medium. These compounds were later found to be chemically oxidized by-products of major naphthalene metabolites (14).

The pathway for naphthalene metabolism was largely deduced by Davies and Evans (15). They found that naphthalene-grown cultures of Pseudomonas were simultaneously adapted to 1,2 dihydroxynaphthalene,

salicylaldehyde, salicylate, and catechol. They were also able to show oxidation of trans-1,2-dihydroxy-1,2 dihydronaphthalene, which was hypothesized to be the first stable intermediate of the pathway. It was later shown by Gibson in a series of elegant experiments that it was the cis rather than the trans form of the diol which was produced by bacterial action (29).

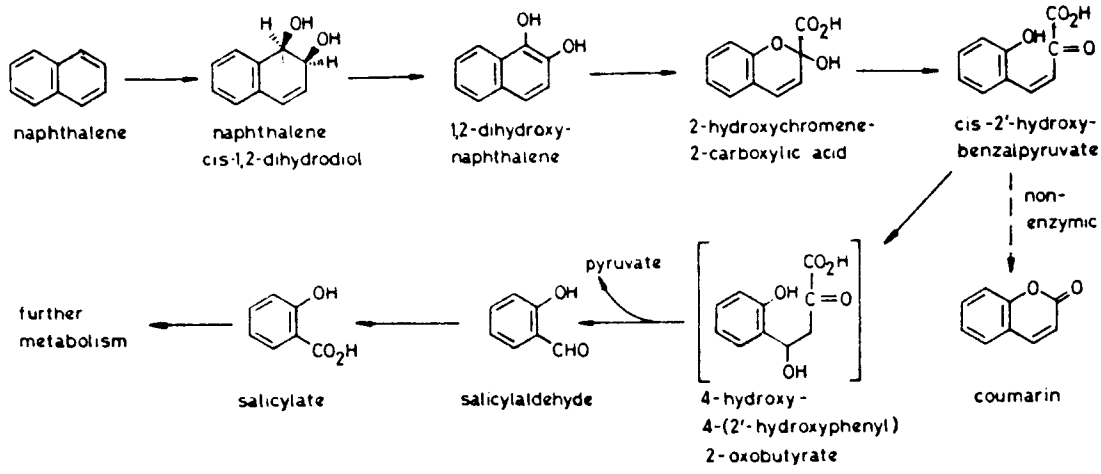
Evans proposed that the cis-1,2-dihydroxy-1,2-dihydronaphthalene was dehydrogenated producing cis-1,2 dihydroxynaphthalene, which was later cleaved by a dioxygenase to yield cis 2'-hydroxybenzalpyruvate (15). This reaction was later reexamined by Barnsley (6). He presented evidence that it was the direct oxidation of 1,2 dihydroxynapthalene which yielded 2-hydroxychromene-2-carboxylic acid. The latter compound was enzymatically converted into cis-2'-hydroxybenzalpyruvate. This evidence was based largely on the spectral characteristics of the accumulated reaction products. Non-enzymatic conversion of 2-hydroxychromene-2-carboxylate into cis-2'-hydroxybenzalpyruvate may occur at high pH, but an isomerase capable of catalysing the reaction was present in cell extracts and purified 20-fold (6).

Barnsley (6) has shown that cis-2'-hydroxybenzalpyruvate was further metabolized in crude extracts by an aldolase to yield pyruvate and salicylaldehyde. No intermediates of this reaction have been isolated. Salicylaldehyde has been shown to be oxidized to salicylate by a NAD^+ dependent dehydrogenase (62). Salicylate may be further metabolized in several ways. Salicylate may be metabolized to catechol,

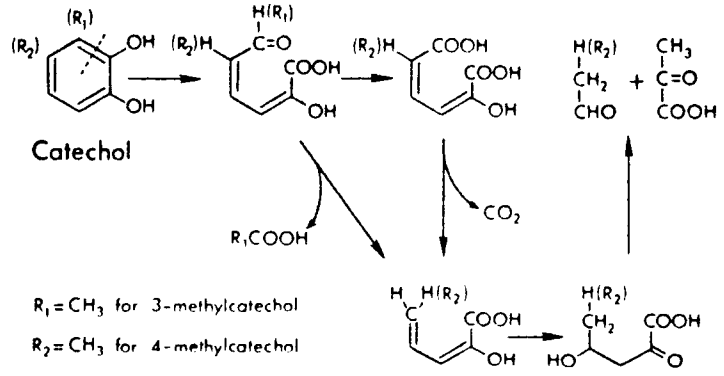
which may be metabolized by either ortho or meta cleavage. Meta cleavage is cleavage adjacent to the hydroxyl groups, which leads to the formation of pyruvate, formate, and acetaldehyde (27); ortho cleavage is between the hydroxyl groups, leading to succinate and acetyl-CoA (27). Both meta and ortho cleavage may be present in the same organism (7, 9, 15). It has been reported by Starovoitov and Skryabin (64) that gentisate is formed from napthalene by P. fluorescens. Since the bacterial metabolism of salicylate into gentisate has previously been reported (14), these results may not reflect the existence of a totally new pathway of naphthalene metabolism, but merely as an alternative mode of salicylate metabolism. It may also be possible that salicylate need not be metabolized, since Skryabin (65) has isolated a strain of Pseudomonas which did not metabolize salicylate, yet was able to grow vigorously on naphthalene. Salicylate accumulated extracellularly and was found to inhibit cellular growth at high concentration. The reactions of naphthalene metabolism are shown in Figure 1.

Genetic Basis of Naphthalene Metabolism

The genetic basis of naphthalene metabolism was first investigated by Dunn and Gunsalus (18) in strains of P. putida. They found that the ability to degrade naphthalene could be transmitted to other strains by conjugation and could be removed by treatment with mitomycin C. This led to the suggestion that such abilities were plasmid-born in many strains of Pseudomonas. The plasmid was isolated by Johnston and Gunsalus (30), and was found to be a 42



(A)



(B)

Figure 1. Pathway of naphthalene metabolism in Pseudomonas. A) Initial reactions. Source: Cripps and Watkinson (14). B) Meta cleavage of catechol. Source: Hopper (27).

kilobase plasmid. It was given the designation of NAH and was found to code for complete naphthalene degradation through a meta pathway. A second plasmid (SAL) which codes for the degradation of salicylate via the meta pathway was also discovered in P. putida (10). Restriction mapping of NAH and SAL revealed a similar pattern of fragments, suggesting a similar origin for both plasmids (19). Other degradative plasmids, such as TOL and OCT, are compatible with NAH (11). Recently two other NAH plasmids which appear to be compatible with the original NAH plasmid have been isolated by Dunn and Dunn (17).

A plasmid origin for naphthalene metabolism has not been found for all strains of P. putida. In many strains naphthalene metabolism seems to be chromosomally coded (18). Many, if not all, chromosomal strains are P. putida type B, which differs from type A in DNA homology (50). Further study of both plasmid-bearing and non-plasmid-bearing strains would give important clues to the evolution of degradative metabolism in Pseudomonas.

Aspects of Naphthalene Metabolism

The induction of naphthalene metabolism has been studied and at least two different mechanisms have been found. Williams (73) has shown that growth on naphthalene induced all degradative enzymes for naphthalene metabolism, including catechol 2,3 dioxygenase (meta pathway); while growth on salicylate induced catechol 1,2 dioxygenase (ortho pathway), but none of the enzymes for initial naphthalene metabolism. It was suggested from this data that either

naphthalene or some early naphthalene metabolite was the inducer of the naphthalene pathway. This is analogous to induction patterns found in some aromatic pathways. For example, Feist and Hegeman showed that growth on phenol induced the meta pathway, while growth on benzoate induced the ortho pathway (20). Barnsley (7) has shown in another strain of P. putida that growth on salicylate resulted in the appearance of a complete naphthalene pathway. He also found that these activities could be gratuitously induced by 2-aminobenzoate and 2-benzylalcohol in cultures grown on succinate (5, 7, 61, 62). Barnsley (7) found high levels of both ortho and meta enzymes in cultures of Pseudomonas P_G (Williams' strain) grown on naphthalene or salicylate.

Barnsley has suggested, from recent work with mutants, the possibility of salicylaldehyde serving as the inducer of the initial portion of the naphthalene pathway (13). Austen and Dunn (4) have recently presented genetic evidence which suggests that induction of complete naphthalene metabolism is controlled by two operons, one coding for metabolism from naphthalene through to catechol and the other coding for catechol metabolism.

There has also been some evidence which connects activities of naphthalene enzymes with levels of extracellular naphthalene metabolites. Skryabin (65) discovered an inhibition of naphthalene oxygenase when the extracellular salicylate concentration rose above 5g/L. Kitai (37) reported an increase in naphthalene oxidation in cells growing on naphthalene when extracellular salicylate

was removed by treatment with Amberlite IRA-40. These results have not been tested genetically, however, and such inhibition has not been reported with other naphthalene metabolites.

Extracellular metabolites may have other roles in naphthalene metabolism. Salicylate has been shown to act as a supplementary iron uptake system in Mycobacterium smegmatis. This effect only occurs in the absence of phosphate (56). Extracellular metabolites may also serve as a nutrient source for surrounding bacteria unable to metabolize the parent compound. A good example of this was shown by Brilon's (8) study of the metabolism of naphthalenesulfonates. Approximately 10% of the carbon present in the naphthalenesulfonates was secreted as extracellular metabolites, which were then readily utilized by other bacteria. Another possible function for extracellular metabolites may be their role as chemoattractants for other hydrocarbon degrading bacteria. At least one aromatic metabolite, β-ketoadipate, has been shown to be an attractant for P. putida (32), with an inducible uptake system possibly acting as a chemoreceptor (48).

CHAPTER II

MATERIAL AND METHODS

Organisms and Media

Parent strains of *Pseudomonas putida* used in this study were obtained from Dr. I. C. Gunsalus (18). Table 1 lists all strains used and their derivations. Stock cultures were maintained upon nutrient agar slants, with periodic passage on mineral salts-naphthalene agar for naphthalene utilizing strains. Strain 63^{+} was used for the majority of uptake assays.

The complete synthetic medium used in the study was a modification of the mineral salts (MS) medium used for the growth of *P. aeruginosa* (43). The medium consisted of NH_4Cl (1g/L), $MgSO_4 \cdot 7\ H_2O$ (0.5g/L), and $FeCl_3 \cdot 6\ H_2O$ (5.0 mg/L). Potassium phosphate buffer (pH 7.0) was added to a final concentration of 0.05 M. Carbon sources (glucose, citrate, succinate, and salicylate) were added to a concentration of 0.2% (w/v). Carbon source and buffer were sterilized by autoclaving separately. Naphthalene (0.2%), as a carbon source, was introduced into sterile flasks by dissolving it in 1 ml of acetone, placing the acetone into the flasks, allowing evaporation of the acetone and placing sterile medium into the flasks.

MS medium, minus carbon and nitrogen sources, was used as the medium for all uptake assays, unless otherwise noted. This medium will be referred to as uptake medium in further discussion.

Table 1. Strains of *P. putida* used in this study.

Strain[a]	Phenotype	Comments
7^+	NAH^+	Plasmid bearing (Type A)
7^-	NAH^-	Isolated from 7^+ after treatment with mitomycin C
63^+	NAH^+	Non-plasmid bearing (Type B)
63^-	NAH^-	Isolated from 63^+ (spontaneous mutant)
90	NAH^+	Type A
572	NAH^- CAM^+	Type A, able to acquire NAH by conjugation
1064	NAH^{-b}	

[a]All parent strains were the gift of I. C. Gunsalus. All strain numbers follow his designation (18).

[b]This strain was reported NAH^+ (18).

Growth Conditions

Approximately 1 x 10^4 cells were inoculated into 100 mls of MS medium with carbon source in a 250 ml Erlenmeyer flask. Cultures were grown at 26° C. in a rotary shaker. The cultures were monitored by an increase in absorbance at 590 nm. Late log cultures (6 x 10^8 viable cells/ml) were harvested for all assays. Naphthalene-grown cultures were first passed through filter paper (Whatman No. 41) to remove solid hydrocarbon before harvesting.

Uptake Assay

Cells were harvested by centrifugation, washed twice with 10 mls of uptake medium, and resuspended to an optical density of 0.70 at 590 nm with or without the presence of an additional carbon source (20 mM). This density of cells was equivalent to 3x10^8 viable cells per ml or 140 µg/ml cell protein. After a 15 minute incubation at room temperature the suspensions were transferred to a 26° C. reciprocal water bath. The assay was begun by the addition of 1(4,5,8)-^{14}C naphthalene (specific activity 5 mCi/mmol) to a final concentration of 40 µM. The naphthalene was dissolved in 10 µl of acetone and introduced into the reaction medium. Reaction flasks were kept tightly capped by rubber stoppers covered with aluminum foil to prevent volatization of the substrate. Duplicate 200 µl samples were taken at 10 minute intervals and layered over 100 µl of a silicone oil mixture in a 400 µl polyethylene microfuge tube. The mixture consisted of a 50:50 mixture (v/v) of 510 weight oil (50 cs) and 550 weight oil (Dow Corning Co.). The tubes were immediately capped and centrifuged for 90 seconds in a microfuge

(Coldman Instrument Co., Model 6-811) at 10,000 rpm (40, 68). Tips of the microfuge tubes were removed with a surgical scalpel, drained of excess oil, placed in Bray's solution, and counted in a Searle Isocap/300 liquid scintillation counter.

Protein Assay

Cell protein was assayed by the method of Oyama and Eagle (49), using bovine serum albumin as the protein standard.

Assay for Extracellular Naphthalene Metabolites

Cells were prepared as described above for the uptake assay and then incubated with 40 μM labeled naphthalene for 1 hour. Cell supernatants were collected by centrifugation and acidified to pH 4 with concentrated HCl to gassify aqueous carbonate species. The supernatants were incubated in a 30° C. water bath for 24 hours to volatize any unmetabolized naphthalene present. Portions (0.1 ml) of the supernatants were then counted in Bray's solution to determine the amount of radioactivity remaining. To prepare metabolites for analysis, supernatants were extracted with five volumes of ethyl acetate; the extracts were treated with anhydrous $CaSO_4$ and concentrated under a stream of nitrogen. Over 95% of the label present in the supernatants was extracted by this procedure.

$^{14}CO_2$ Mineralization Studies

The mineralization of $^{14}CO_2$ from labeled naphthalene was carried out in 25 ml sidearm flasks equipped with rubber serum stoppers (59). A plastic center well (Kontes) containing a 1x5 cm fluted paper wick was fitted in each stopper. Cells (10 mls) prepared for uptake assay were added to each flask with or without additional

carbon source. After a 10 minute incubation, the assay was begun by the addition of 40 μM ^{14}C-naphthalene to the cell suspension. The suspensions were incubated at 26° C. for 1 hour. To terminate the reaction and to gassify aqueous carbonate species, 1 ml of 2N H_2SO_4 was injected through the stopper into the reaction mixture. In the same manner, 0.4 ml of 0.2 N KOH was added to the center well to absorb gaseous $^{14}CO_2$. The flasks were shaken for 2 hours to allow complete absorption. After absorption, the serum stoppers and center wells were removed from the reaction vessels and the center wells were placed directly into Bray's solution for counting.

Extraction of Cell-Associated Label

Cells which had been pre-exposed to 40 μM ^{14}C naphthalene for 1 hour in uptake conditions were resuspended in 2 mls of uptake medium and disrupted by the addition of 0.180 ml of chloroform and 0.065 ml of 1.0% sodium dodecyl sulfate. The suspension was acidified to pH 4 and extracted with five volumes of anhydrous ether. The ether extracts were treated with anhydrous $CaSO_4$ and concentrated over a stream of nitrogen for analysis. Over 80% of the cell-associated label was extracted by this procedure.

Analysis of Naphthalene Metabolites

Extracts of naphthalene metabolites were spotted on a 5x7 inch cellulose thin layer chromatography plate with fluorescent indicator (Eastman No. 6065). A "fingerprint" of metabolites was obtained by running the chromatogram in two dimensions, the first dimension using a propanol:ammonium mixture (2:1, v/v) and the

second using a butanol:acetic acid:water mixture (12:3:4, v/v). The plates were allowed to dry for 24 hours to allow complete volatilization of unmetabolized naphthalene. Autoradiograms were made by exposing the plates to Kodak X-R5 film for 5-10 days at -70° C. Catechol and salicylate standards were run and detected by ultraviolet fluorescence and by a positive reaction with 5% $FeCl_3$ (1). Succinic acid and pyruvic acid standards were run and detected with bromocresol reagent. Spots also were counted directly by scraping them off the plate and placing them in Bray's solution.

Oxidation Studies

Determination of the rate of oxygen uptake of various substrates was conducted polarographically with a YSI (Yellow Springs Instrument) Clark-type electrode. The oxidations were conducted at 26° C. using 0.05 M phosphate buffer, pH 7.0, which was saturated with air before use. The reaction mixture, in a total volume of 1.8 ml, contained buffer and a portion of cell suspension. The reaction was initiated with the addition of substrate. Most substrates were added in 0.1 ml of water, with the exception of naphthalene, which was dissolved in 10 µl acetone. The organism was unable to utilize acetone and acetone did not cause any increase in oxygen uptake. Acetone did not interfere with naphthalene oxidation. Rates of oxygen uptake were calculated as µg atoms of oxygen consumed per minute per mg cell protein.

Inhibitor Studies

Cells were preincubated with inhibitor for 20 minutes before being assayed for uptake. Arsenate-treated cells and controls were incubated in 0.005 M phosphate buffer (42).

Incorporation of ^{14}C Naphthalene into Cellular Protein

Cells were incubated with labeled naphthalene in uptake medium for 2 hours at 26° C. Cells were incubated with or without carbon source (glucose or pyruvate). Control cells were treated with 200 µg/ml chloroamphenicol or 30 mM cyanide. Strain 572 (NAH^-) also served as a control. After incubation, aliquots of culture were filtered upon cellulose filters (Whatman No. 541). Filters were treated by the method of Kennell (35) to isolate cellular protein. The procedure was modified by three additional ether washes to remove cell-associated hydrocarbon. After treatment, the filters were dried and counted in Bray's solution.

Mitomycin C Curing

The method of Rheinwald was used (18) for curing of plasmid-bearing strains. Cured cells were isolated on nutrient agar and replica plated on mineral salts-naphthalene to test for loss of the NAH phenotype.

Chemicals

1 $(4,5,8)^{-14}C$ naphthalene (99% purity) was obtained from Amersham. 1-o-methyl-α-d-glucopyranoside (α-methyl-d-glucoside), 2 deoxy-d-glucose, d-glucuronic acid, chloroamphenicol, rifampicin,

mitomycin C, and sodium malonate were obtained from Sigma. All nucleotides were obtained as sodium salts (Grade II) from Sigma. All other chemicals used were reagent grade quality.

CHAPTER III

RESULTS

Development of PAH Uptake System

Previous observations with hydrocarbon uptake (W. R. Finnerty, personal communication) suggested that traditional uptake methods were ineffective because of high adherence of hydrocarbons to filters. It was believed that an uptake system utilizing centrifugation of cells through a silicone oil layer would trap most of the non-associated naphthalene and allow accurate measurement of cell-associated label. Sealed reaction flasks and capped microfuge tubes were used to reduce volatilization of the substrate.

Approximately 75-80% of the naphthalene introduced into the reaction flask at the beginning of the uptake assay remained after 1 hour of incubation. This percentage remained constant regardless of the prior evaporation of the solvent acetone before introduction of medium. Of the labeled naphthalene present in the total culture at the end of the uptake assay, 9.5% was associated with the cells, 13.5% was in the form of extracellular naphthalene metabolites, and 77% of the label was unmetabolized naphthalene. This distribution varied with the source of cells tested. Cells grown with naphthalene had higher cell-associated label and less extracellular metabolites than those grown with non-aromatic substrates.

While the purpose of the silicone oil mixture in the microfuge tube was to serve as a sink for non-associated naphthalene, it is

also possible that the presence of the oil could leach cell-associated label from the cells. By careful sectioning of each phase of the microfuge tube, a profile of label distribution was obtained (Table 2). The percentage of label present in the oil layer decreased by 16% in the presence of metabolizing cells. The apparent shift of label from the oil layer to the tube tip probably represents a binding of hydrocarbon to cellular components. A linear relationship between amounts of cell-associated label and cellular protein was found for all concentrations tested (70-200 μg/ml, data not shown). To determine if hydrocarbon binding were dependent upon the presence of metabolically active cells, cyanide-killed cells were used. Killed cells bound a greater percentage of naphthalene than did the blank controls but bound 3-4 fold less naphthalene than metabolically active cells.

As a further evaluation of the method, the silicone oil mixture was omitted from the assay and cells were centrifuged through the uptake medium. Centrifugation through medium resulted in an increase in cell-associated label (data not shown). However, extremely high and nonreproducible counts from control cells (cyanide-killed) were found. Since direct centrifugation was unacceptable, centrifugation with silicone oil was routinely used in this study.

Naphthalene Uptake by NAH^+ and NAH^- Strains

Results of naphthalene uptake by various strains of P. putida grown on glucose are shown in Figure 2. NAH^+ strains (7^+, 63^+, 90) utilized naphthalene as a sole source of carbon, showed vigorous

Table 2. Percentage distribution of labeled naphthalene between phases of microfuge assay tube.

Phase	Source of Cells[a] Added			
	Naphthalene-Grown	Glucose-Grown	Minus Cells	Cyanide[b] Killed
Water	85	85	75	75
Oil	5	8	24.5	22.5
Tube tip (cells)	10	7	0.5	2.5

[a]Cells were exposed to label for 1 hour before centrifugation, sectioning, and counting in Bray's solution.

[b]30 mM.

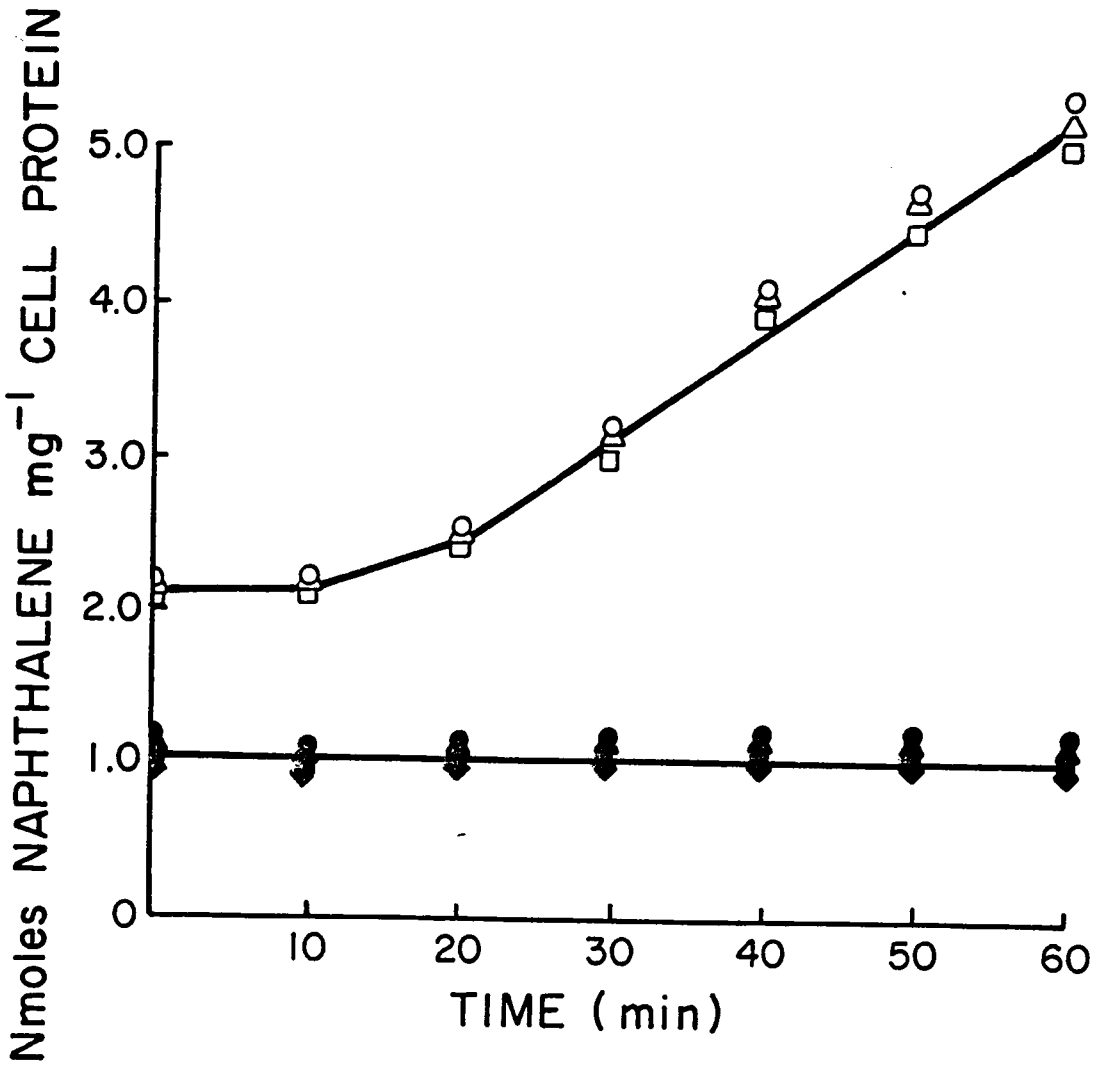


Figure 2. Uptake of naphthalene by naphthalene-utilizing (NAH^+) and non-utilizing strains of P. putida. Cultures were grown on 0.2% glucose. NAH^+ strains used were 7^+ (○), 63^+ (△), and 90 (□); and NAH^- strains were 7^- (●), 63^- (▲), 572 (■), and 1064 (◆).

naphthalene oxidation, and produced extracellular naphthalene metabolites. After a 10 minute lag, these strains accumulated label rapidly and reached a plateau after 70 minutes. NAH^- strains (7^-, 63^-, 572, 1064) were unable to utilize naphthalene as a carbon source or to produce detectable naphthalene metabolites after prolonged incubation. None of the NAH^- strains showed any naphthalene uptake. While some cell-associated label was detected in NAH^- strains ($\sim$ 1.3 nmoles/mg protein), the level of label detected did not differ from results obtained with Escherichia coli and P. aeruginosa (not shown). Each of the NAH^- strains tested either had been derived from a NAH^+ strain, or had previously been shown to accept and express the NAH genes. Growth curves of derived strains grown on non-aromatic substrates were identical to parent strains. Strain 63^+ was chosen for further experimentation because of the genetic instability of other strains tested.

Requirement for Protein Synthesis for Naphthalene Uptake

Naphthalene uptake was measured using glucose-grown cells preincubated with either chloroamphenicol or rifampicin (Figure 3). Cells were preincubated for 15 minutes with 200 µg/ml of inhibitor. In the presence of inhibitor, cells revealed little naphthalene uptake. Removal of inhibitor by washing restored uptake capacity (not shown). Cells grown on naphthalene were unaffected in uptake by the presence of inhibitor (Figure 3). It should be noted that naphthalene-grown cells exhibited no lag period in uptake compared to glucose-grown cells.

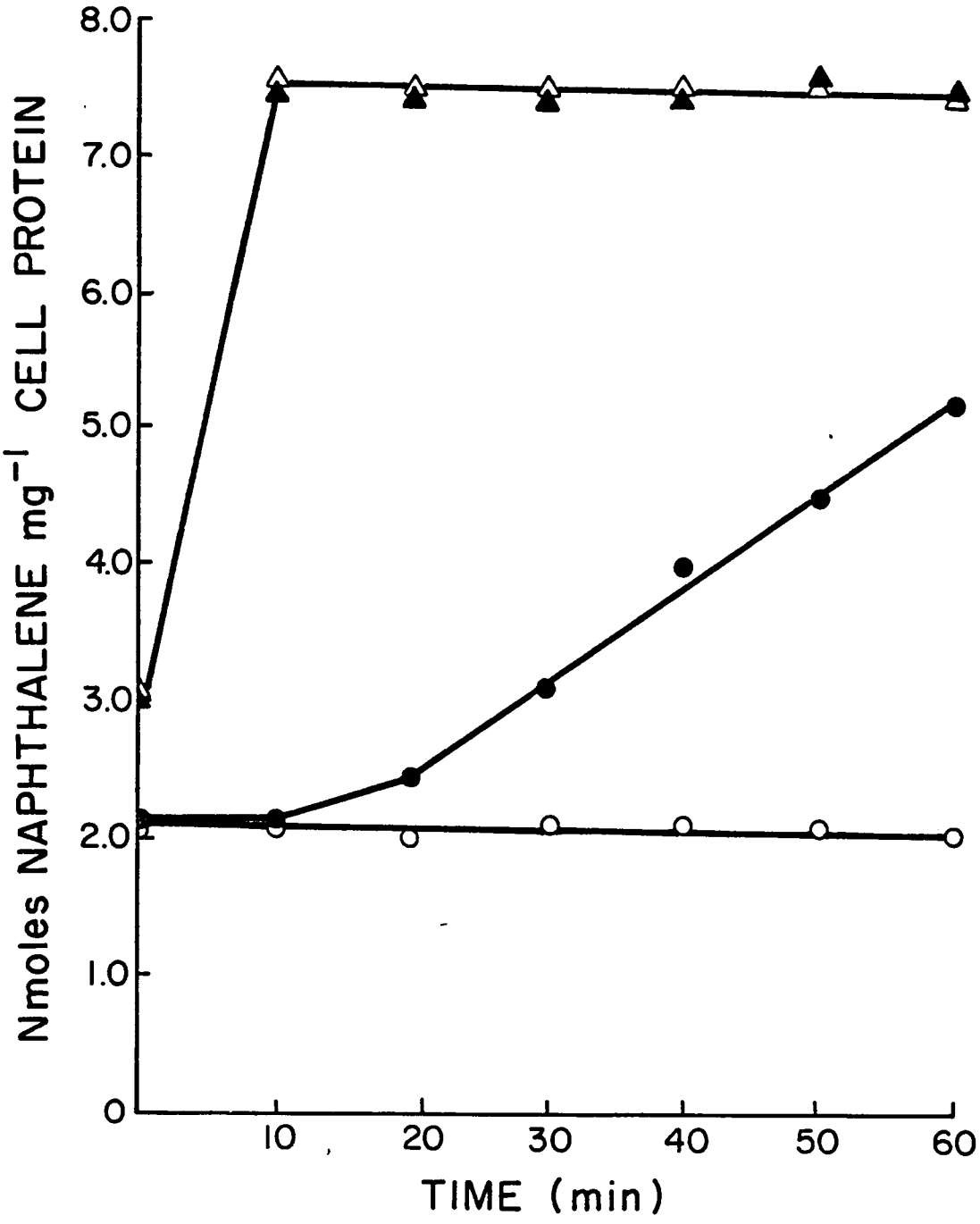


Figure 3. Uptake of naphthalene in presence or absence of protein synthesis inhibitor. Naphthalene-grown (triangles) and glucose-grown (circles) cells were incubated in presence (open) or absence (shaded) of 200 µg/ml chloroamphenicol. Identical results were obtained with 200 µg/ml rifampicin.

Naphthalene Uptake in Presence of Additional Carbon Sources

To test the effect of a second carbon source upon naphthalene uptake, cells were preincubated with 20 mM amounts of carbon source for 10 minutes and then assayed for uptake. The results for glucose-grown cells are shown in Figure 4A. Incubation with either glucose-6-phosphate, α-ketoglutarate, or citrate had little effect upon naphthalene uptake; while incubations with glucose, gluconate, fumarate, malate, succinate, or pyruvate completely eliminated uptake. Incubation with either catechol or salicylate, intermediates of the naphthalene degradative pathway, also eliminated uptake in glucose-grown cells. When the carbon source was added after the addition of naphthalene (Figure 4B), uptake was eliminated if the carbon source was added during the 10 minute lag period. Those carbon sources added prior to label addition which did not eliminate uptake also were ineffective if added any time during the assay. Addition of any carbon source after the first 10 minutes did not affect naphthalene uptake. Addition of either catechol or salicylate at any point in the assay caused immediate loss of cell-associated label.

Results from succinate-grown cells are shown in Figure 5. Incubation with either glucose or citrate did not affect naphthalene uptake, while the presence of catechol, salicylate, pyruvate, fumarate, malate, and succinate eliminated uptake. Cells treated with succinate plus dinitrophenol also eliminated uptake (not shown). Addition of citrate eliminated uptake with citrate-grown cells (Figure 6) but addition of glucose or succinate was ineffective.

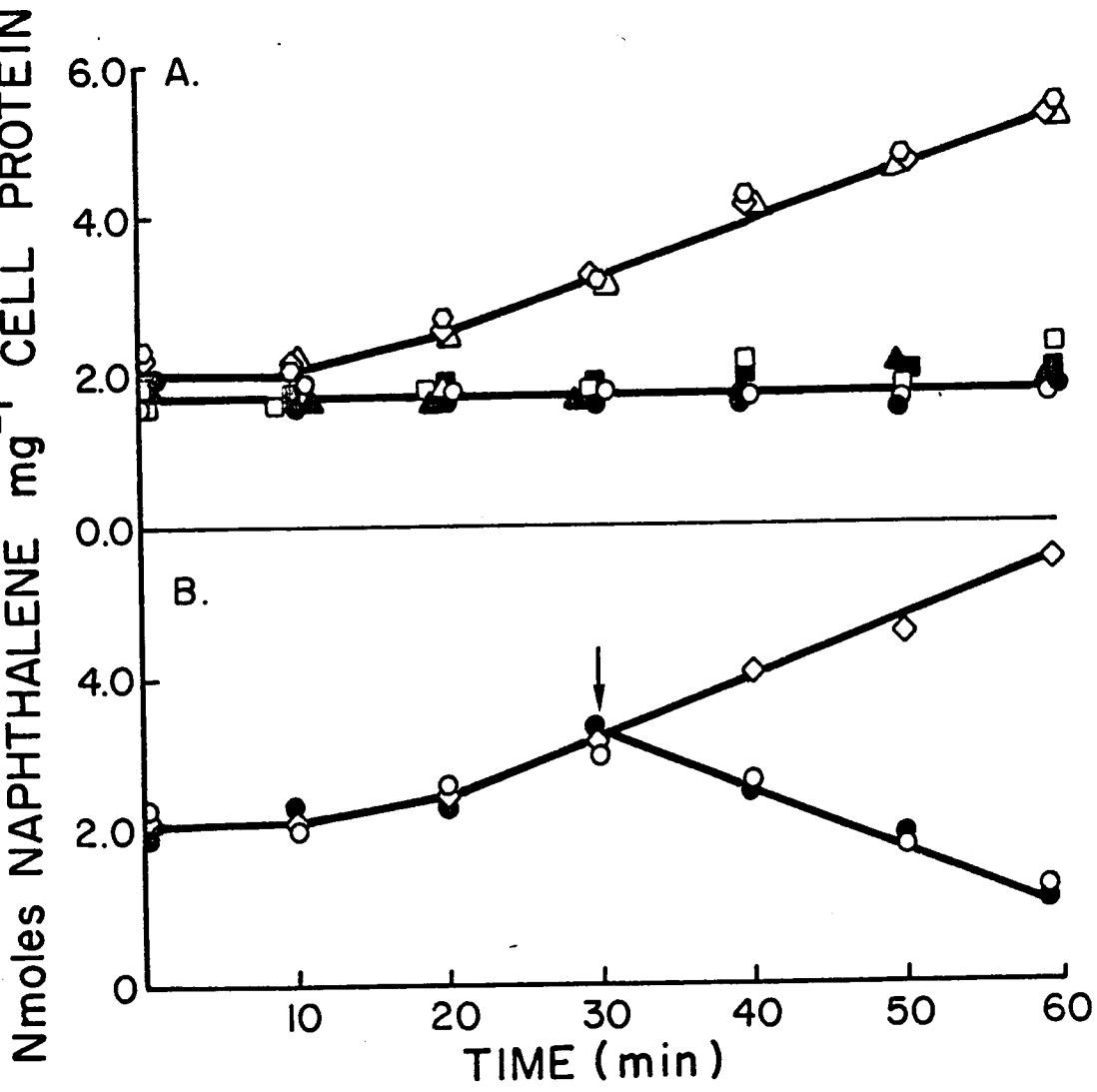


Figure 4. Uptake of naphthalene by glucose-grown cells in the presence of additional carbon source. (A). Addition before uptake assay. Additions were: none (◇), glucose (■), glucose-6-phosphate (⬡), pyruvate (□), citrate (△), succinate (▲), salicylate (○), and catechol (●). (B). Addition 30 minutes into assay. Additions are symbolized as above.

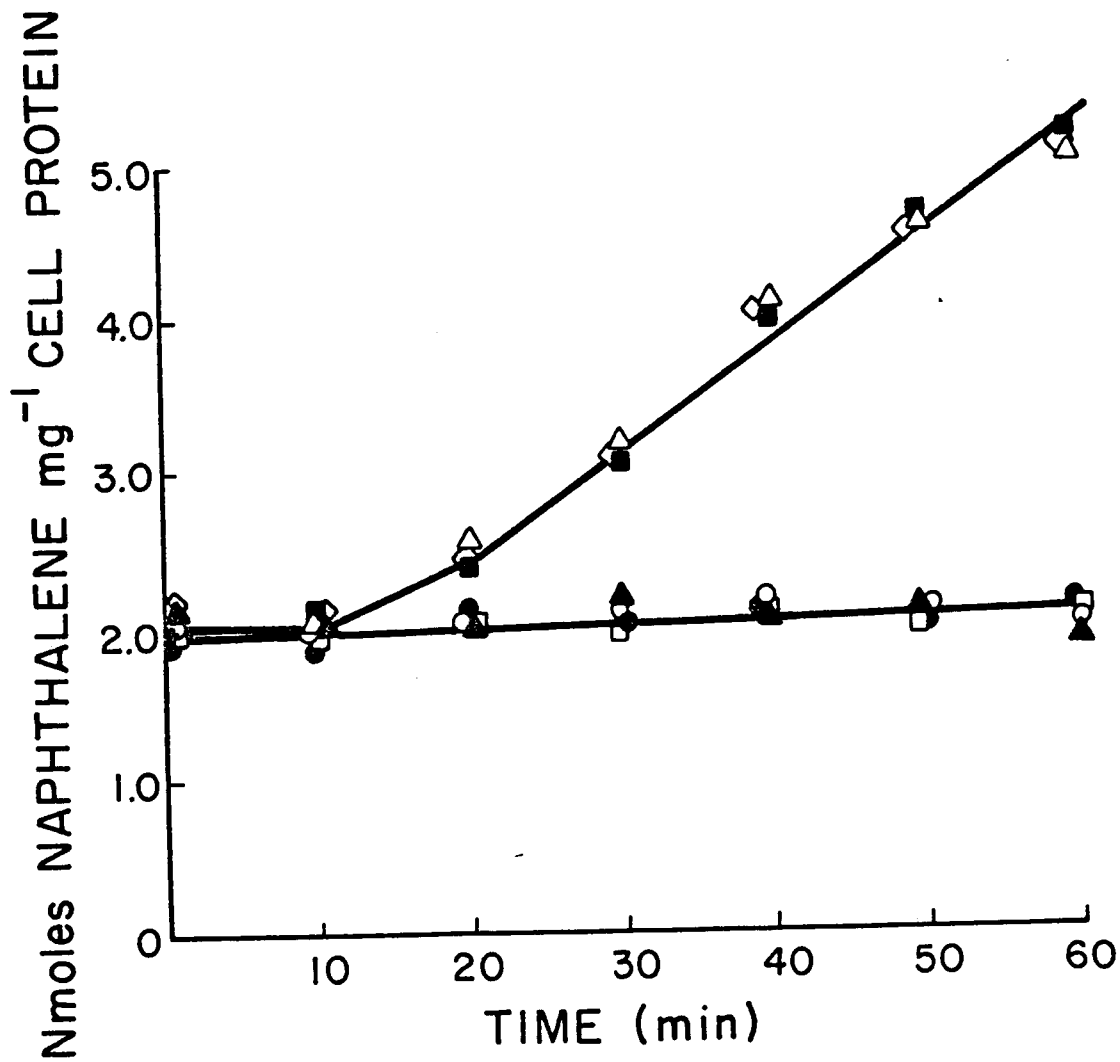


Figure 5. Uptake of naphthalene by succinate-grown cells in presence of additional carbon source. Additions were: none (◇), citrate (△), glucose (■), succinate (▲), pyruvate (□), catechol (●), and salicylate (○).

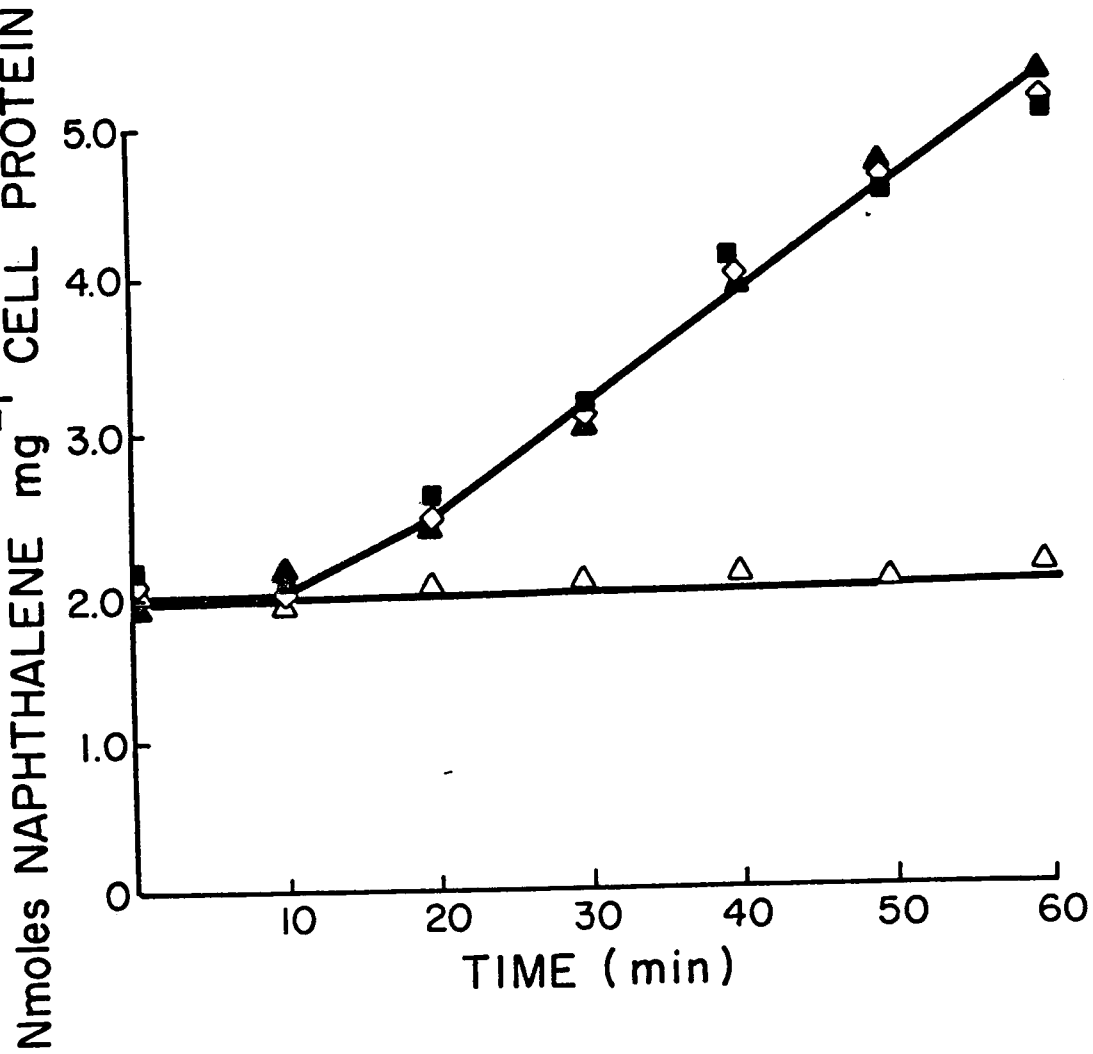


Figure 6. Uptake of naphthalene by citrate-grown cells in presence of additional carbon source. Additions were: none (◇), citrate (△), glucose (■), and succinate (▲).

Cells grown on naphthalene were not affected in naphthalene uptake when preincubated with citrate, glucose, succinate, pyruvate, fumarate, malate, and glucose-6-phosphate (Figure 7A). Addition of salicylate eliminated uptake, but did not cause a loss of cell-associated label. In contrast, catechol caused a rapid loss of cell-associated label. The addition of either catechol or salicylate to naphthalene-grown cells resulted in the accumulation in the medium of a yellow compound with an absorption maximum at 375 nm. This compound was tentatively identified as 2-hydroxymuconic semialdehyde (4), a metabolite of the meta pathway of catechol cleavage. Addition of catechol caused immediate production of the compound, while with salicylate addition, the compound was not detected for 35 minutes. Addition of either catechol or salicylate at any point during the assay resulted in rapid loss of cell-associated label (Figure 7B). Addition of other carbon sources during the assay period did not affect uptake. Cells grown on salicylate as a possible inducer of naphthalene metabolism revealed the same uptake pattern as naphthalene-grown cells (compare Figure 7A and Figure 8).

Concentration Dependence of Glucose-Mediated Reduction of Uptake

To determine more precisely the level of glucose needed to cause elimination of uptake, glucose-grown cells were incubated with various concentrations of glucose and assayed for uptake. The results are shown in Figure 9. Concentrations of glucose of 1.0 mM or more completely eliminated uptake. 0.5 mM glucose partially prevented uptake, increasing lag time to 20 minutes.

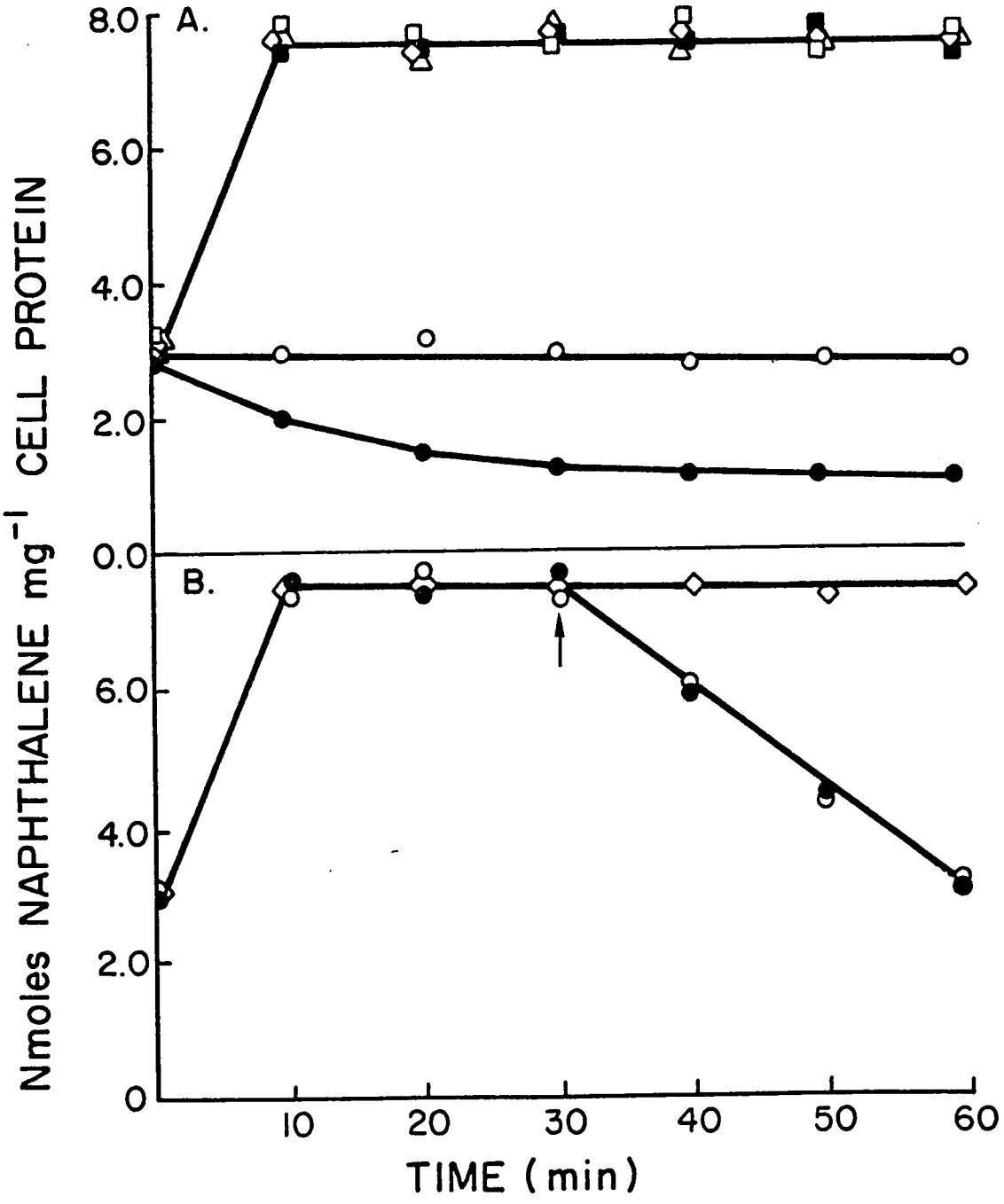


Figure 7. Uptake of naphthalene by naphthalene-grown cells in the presence of additional carbon source. (A). Addition before uptake assay. Additions were: none (◇), pyruvate (□), glucose (■), citrate (△), catechol (●), and salicylate (○). (B). Addition 30 minutes into assay. Additions are symbolized as above.

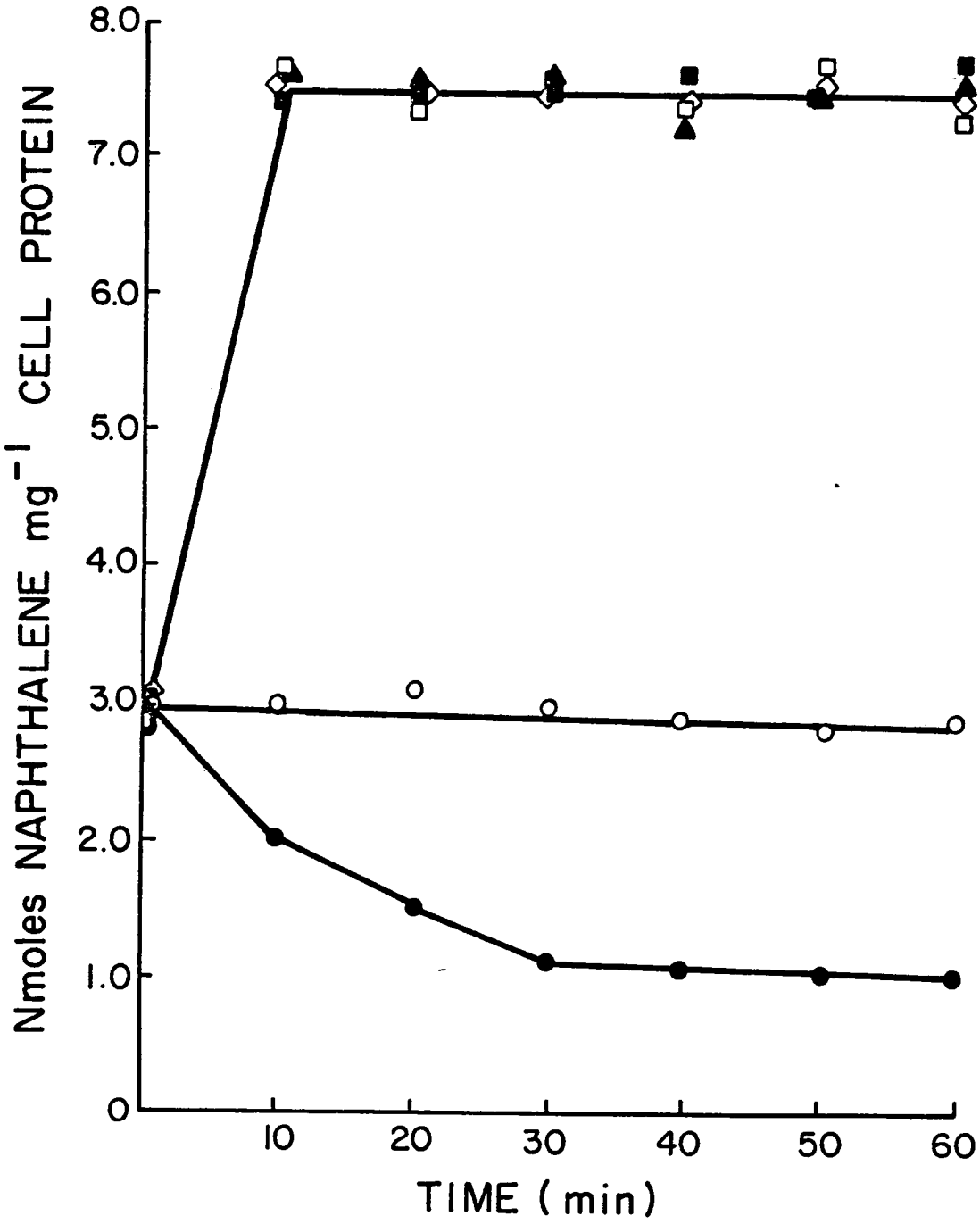


Figure 8. Uptake of naphthalene by salicylate-grown cells in the presence of additional carbon source. Additions were: none (◇), glucose (■), pyruvate (□), succinate (▲), salicylate (○), and catechol (●).

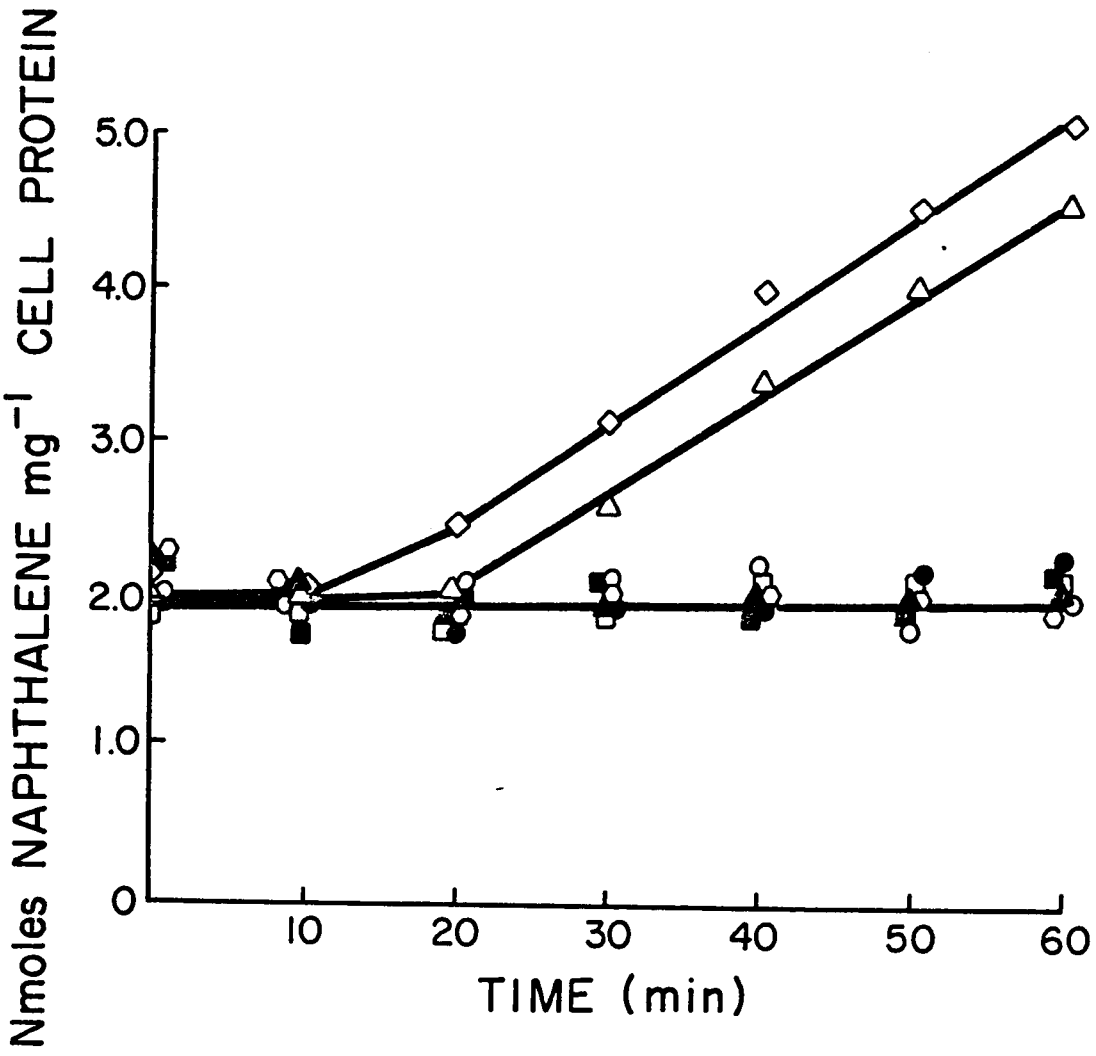


Figure 9. Concentration dependence of glucose-mediated reduction of uptake. Additions were: none (◇), 0.5 mM glucose (△), 1.0 mM glucose (■), 2 mM glucose (⬡), 2.5 mM glucose (●), 5 mM glucose (○), 10 mM glucose (▲), and 20 mM glucose (□).

Effect of Glucose Analogs Upon Uptake

The effect of various glucose analogs upon naphthalene uptake is shown in Figure 10. Glucose-grown cells were incubated with either 20 mM glucuronic acid, α-methyl glucoside, or 2-deoxy-d-glucose and assayed for naphthalene uptake. Cultures also were assayed for reversal of glucose mediated inhibition by incubating 20 mM glucose with each analog. Two of the analogs, α-methyl glucoside and glucuronic acid did not affect naphthalene uptake; but incubation with 2-deoxy-d-glucose eliminated uptake. None of the analogs was able to reverse glucose-mediated inhibition when incubated with 20 mM glucose. Further experiments with the analogs revealed that only one analog tested, 2-deoxy-d-glucose, was able to serve as a sole carbon source for growth in P. putida.

Reversal of Succinate-Mediated Reduction by Malonate

To reverse possible succinate-mediated inhibition of uptake, succinate-grown cells were incubated with either 20 mM succinate, 20 mM malonate, or a equimolar mixture of the two and assayed for uptake (Figure 11). Malonate alone did not eliminate naphthalene uptake. Naphthalene uptake did occur in the presence of the succinate-malonate mixture, while succinate alone eliminated uptake. It was concluded that malonate effectively reversed the effect of succinate upon uptake, and that metabolism is required for its effect on uptake.

Effect of Energy Inhibitors Upon Uptake

To examine the energy requirements for naphthalene uptake both glucose-grown cells and naphthalene-grown cells were preincubated

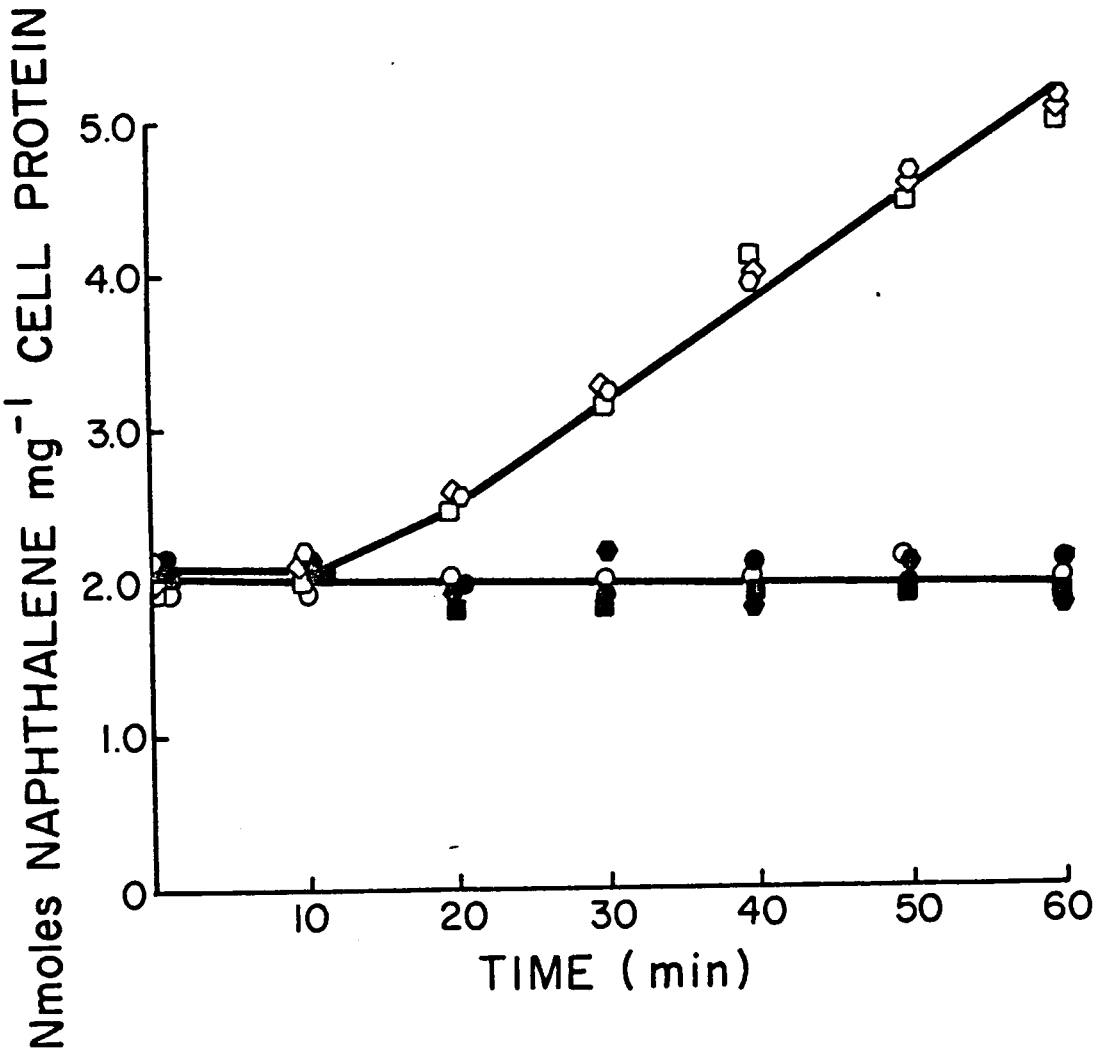


Figure 10. Effect of glucose analogs upon uptake. Additions were: none (◇), α-methyl glucoside (□), d-glucuronic acid (⬡), and 2-deoxy-d-glucose (○). Shaded symbol represents incubation with glucose plus analog.

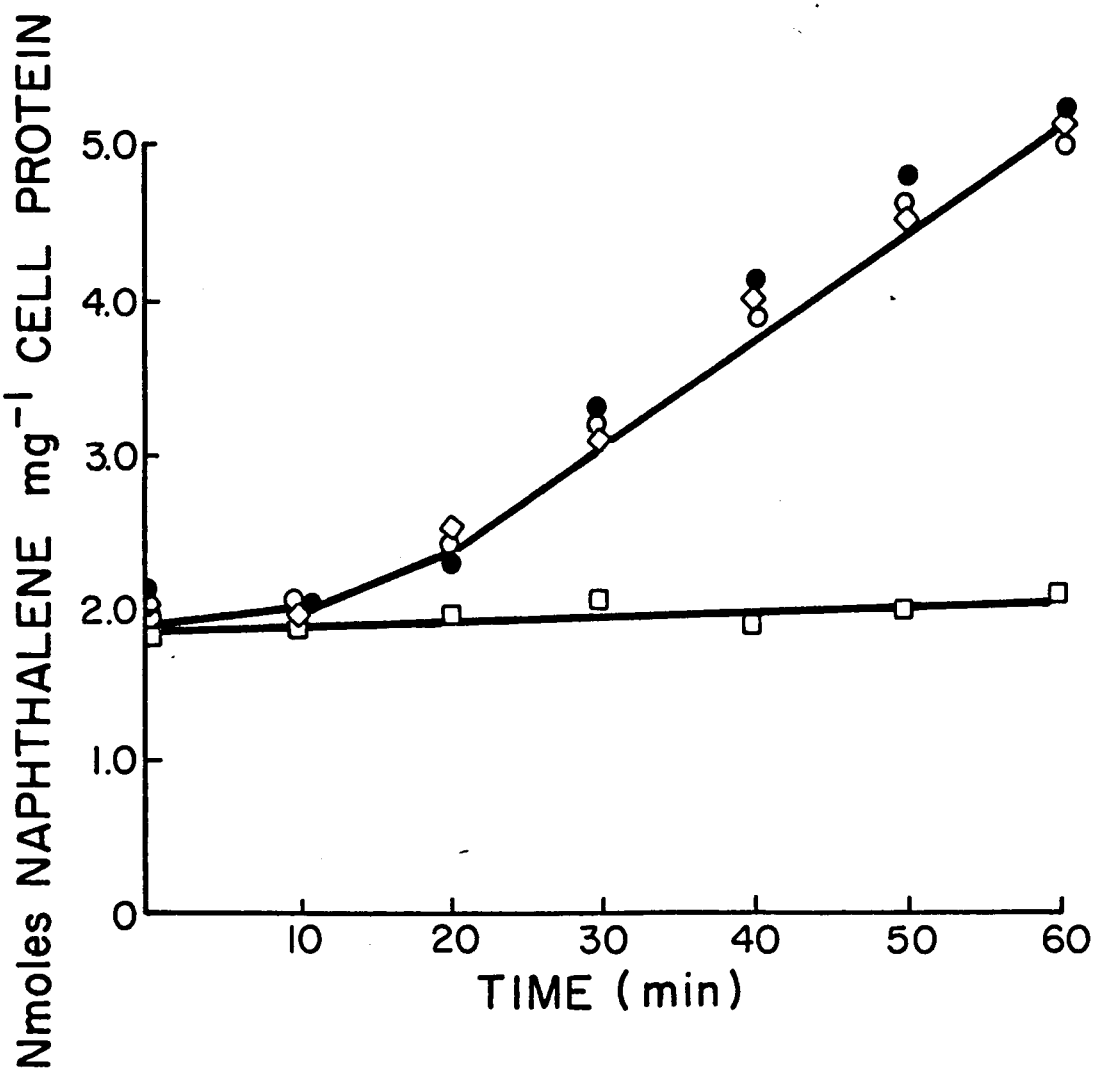


Figure 11. Reversal of succinate-mediated depression of uptake. Cells were preincubated for 10 minutes with malonate (○), succinate (□), a mixture of the two (●), or no addition (◇) prior to assay.

with various energy inhibitors and assayed for uptake. The inhibitors used were 30 mM sodium azide, 30 mM sodium cyanide, 0.01 M sodium arsenate, or 200 µg/ml dinitrophenol. The results for naphthalene-grown cells are shown in Figure 12. Uptake did not occur in the presence of either cyanide or azide. In other experiments, addition of either compound at any point in the assay resulted in a rapid loss of cell-associated label. In contrast, neither sodium arsenate nor 2,4 dinitrophenol had any effect upon uptake in naphthalene-grown cells. Results for glucose-grown cells are shown in Figure 13. All inhibitors tested eliminated naphthalene uptake.

Effect of Exogenous Nucleotide Upon Uptake

Fitzgerald (36) has demonstrated inhibition of alkylsulfatase synthesis by the presence of exogenous nucleotide in P. aeruginosa. Since inhibition of this enzyme has also been demonstrated in the presence of high amounts of carbon source (22), it was determined to test for inhibition of naphthalene uptake in the presence of exogenously added nucleotide. Glucose-grown, succinate-grown, and naphthalene-grown cells were incubated with 2, 5, and 10 mM concentrations of ATP, UTP, CTP, GTP, or cAMP and assayed for uptake. Nucleotide incubation times used were 10 minutes, 4 hours, or 8 hours. The presence of nucleotide did not affect uptake in any cell tested. In all experiments, duplicate cultures of glucose-grown cells were simultaneously incubated with 20 mM glucose and nucleotide and assayed for uptake. The presence of any nucleotide did not reverse glucose reduction of uptake.

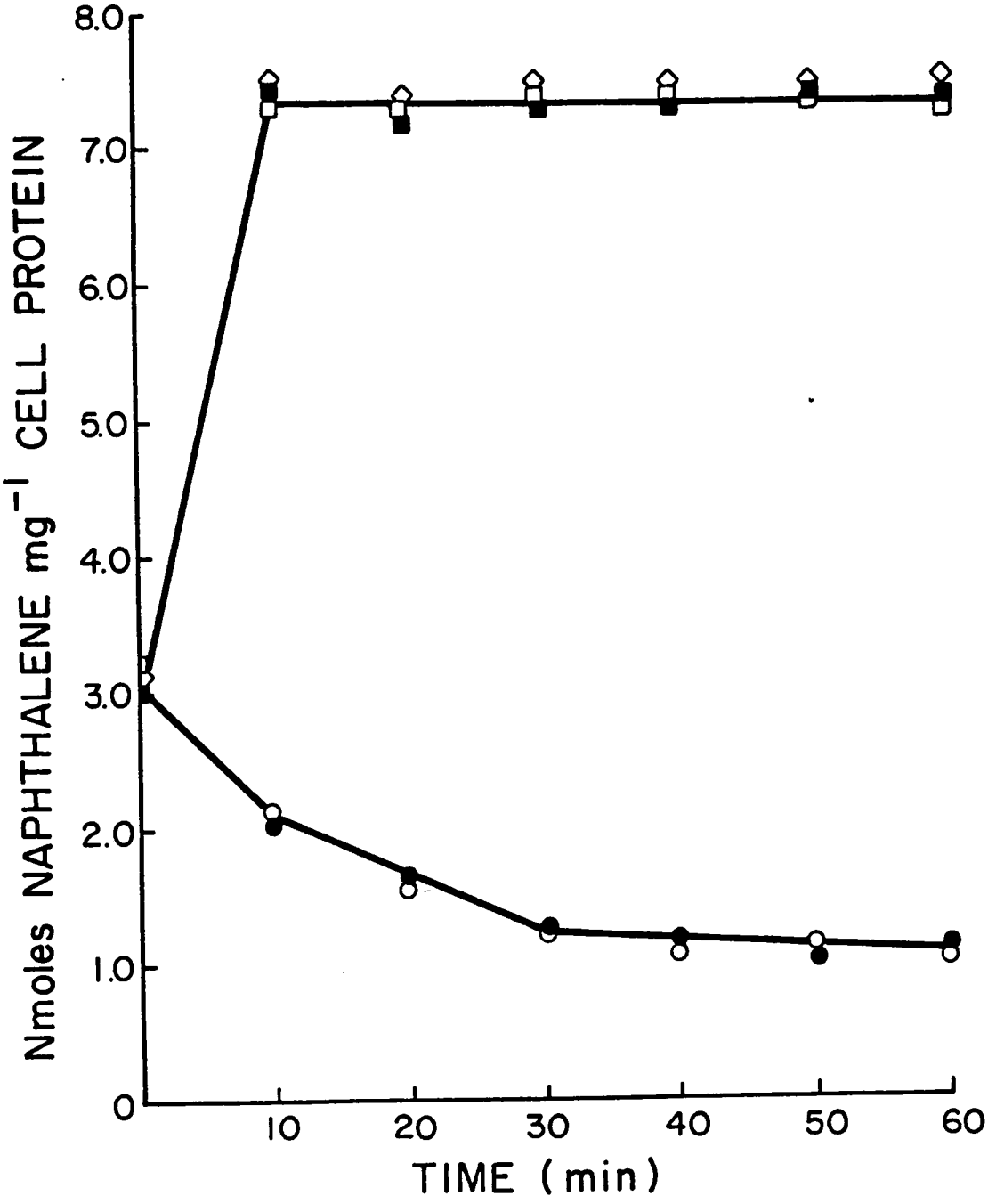


Figure 12. Effect of metabolic inhibitors on uptake in naphthalene-grown cells. Additions were: none (◇), sodium azide (○), sodium cyanide (●), 2,4 dinitrophenol (□), and arsenate (■).

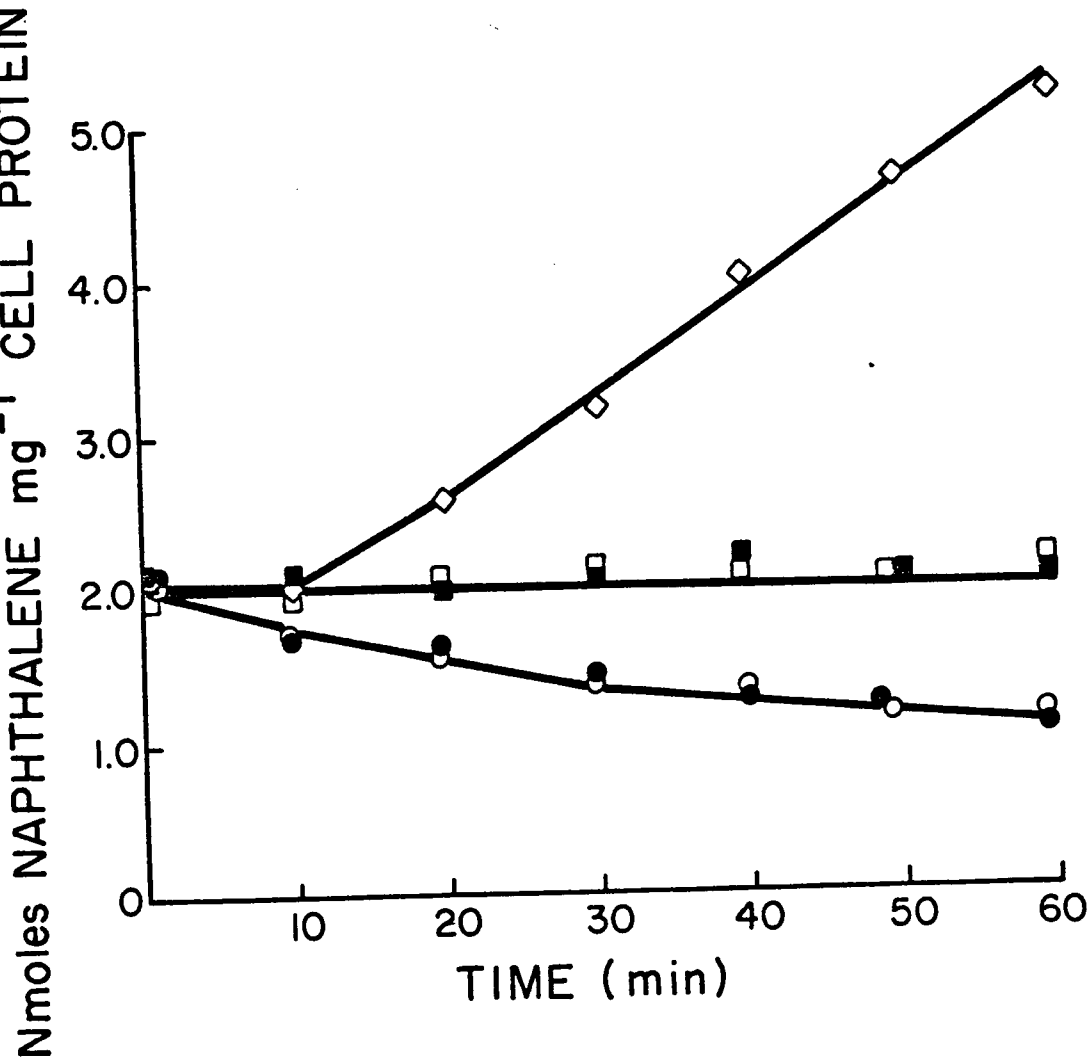


Figure 13. Effect of metabolic inhibitors on uptake in glucose-grown cells. Additions were: none (◇), sodium azide (○), sodium cyanide (●), 2,4 dinitrophenol (□), and arsenate (■).

Oxidation of Carbon Source by Glucose-Grown, Succinate-Grown, and Naphthalene-Grown Cells

To ascertain which carbon sources previously tested in naphthalene uptake were oxidized by cultures of P. putida, oxygen uptake using cells grown on different substrates were measured under similar conditions as those for naphthalene uptake. The results are summarized in Table 3. Glucose-grown cells were able to oxidize glucose, gluconate, succinate, pyruvate, malate, and fumarate but were unable to oxidize α-ketoglutarate, citrate, glucose-6-phosphate, salicylate, naphthalene, catechol, ATP, cAMP, UTP, CTP, and GTP. Succinate-grown cells were able to oxidize succinate, malate, pyruvate, glucose, and fumarate but were unable to oxidize α-ketoglutarate, citrate, glucose-6-phosphate, naphthalene, salicylate, catechol, and all nucleotides tested. Naphthalene-grown cells were able to oxidize pyruvate, malate, succinate, glucose, salicylate, catechol, and naphthalene but were unable to oxidize citrate and nucleotides.

Effect of Additional Carbon Source Upon CO_2 Production from Labeled Naphthalene

To test if the addition of a carbon source could affect levels of CO_2 produced from ^{14}C naphthalene, CO_2 trapping was carried out in similar conditions as naphthalene uptake. The results are summarized in Table 4. In glucose-grown cells, the addition of glucose, pyruvate, or succinate reduced CO_2 levels by approximately 50%; while the addition of catechol or salicylate reduced levels by 75%. Citrate did not affect CO_2 production by glucose-grown

Table 3. Rates of oxygen uptake by glucose-grown, succinate-grown, and naphthalene-grown cells.

Addition to Medium[a]	Oxygen Uptake (μg Atoms O_2 Consumed, min/mg protein) Glucose-Grown Cells	Succinate-Grown Cells	Naphthalene-Grown Cells
No addition	.055 (1.00)[b]	.071 (1.00)[b]	.041 (1.00)[b]
α-ketoglutarate	.055 (1.00)	.091 (1.28)	ND
Citrate	.069 (1.25)	.071 (1.00)	.040 (1.00)
Glucose	.336 (6.10)	.186 (2.61)	.347 (8.46)
Glucose-6-phosphate	.055 (1.00)	.071 (1.00)	ND
Gluconate	.110 (2.00)	ND	ND
Pyruvate	.146 (2.65)	.202 (2.84)	.092 (2.24)
Succinate	.117 (2.30)	.349 (4.92)	.163 (3.98)
Malate	.092 (1.67)	.369 (5.19)	.143 (3.48)
Fumarate	.093 (1.69)	.278 (3.91)	ND
Catechol	.054 (1.00)	.071 (1.00)	.133 (3.24)
Salicylate	.056 (1.00)	.070 (1.00)	.122 (2.98)
Naphthalene	.055 (1.00)	.071 (1.00)	.531 (12.95)
ATP	.054 (1.00)	.071 (1.00)	.041 (1.00)
CTP	.055 (1.00)	.070 (1.00)	.043 (1.00)
UTP	.053 (1.00)	.071 (1.00)	.041 (1.00)
GTP	.055 (1.00)	.070 (1.00)	.042 (1.00)
TTP	.055 (1.00)	.072 (1.00)	.041 (1.00)
cAMP	.055 (1.00)	.071 (1.00)	.040 (1.00)

ND = Not done.

[a]Concentration of carbon source added was 20 mM, with the exceptions of naphthalene (80 μM) and nucleotides (2 mM).

[b]Values in parentheses are the response relative to endogenous respiration.

Table 4. Effect of additional carbon source upon $^{14}CO_2$ production from 1(4,5,8)-^{14}C naphthalene.

Growth Substrate	Addition to Medium[a]	CO_2 Produced (10^5 cpm/mg protein)	Percent of Control
Glucose	None	1.00	-
	Glucose	0.69	52
	Pyruvate	0.76	63
	Succinate	0.78	66
	Citrate	0.98	98
	Catechol	0.53	28
	Salicylate	0.49	22
	Azide	0.37	0
	Cyanide	0.37	0
Naphthalene	None	2.75	-
	Glucose	2.20	78
	Pyruvate	2.10	72
	Succinate	2.35	83
	Citrate	2.60	93
	Catechol	0.96	25
	Salicylate	0.93	24
	Azide	0.75	16
	Cyanide	0.80	18
Blank (without cells)		0.35	

[a]Concentration of carbon source was 20 mM, with the exception of cyanide and azide (30 mM).

cells. Carbon dioxide production by naphthalene-grown cells was decreased from 7-28% in the presence of non-aromatic carbon sources. Addition of either catechol or salicylate to naphthalene-grown cells decreased production of CO_2 by 75%.

Effect of Additional Carbon Source Upon Accumulation of Extracellular Naphthalene Metabolites

Accumulation of extracellular metabolites was measured in the presence or absence of carbon source. Results for glucose-grown and naphthalene-grown cells are summarized in Table 5. Accumulation from glucose cells was reduced 74% by the presence of glucose or 85% by gluconate; while the addition of pyruvate, malate, and fumarate reduced metabolite levels by 46-56%. Addition of catechol or salicylate decreased metabolite levels 95% in glucose-grown cells. Accumulation from naphthalene-grown cells was decreased 46% by catechol and 77% by salicylate. Addition of other carbon sources or nucleotides did not affect metabolite levels in naphthalene-grown cells.

Analysis of Naphthalene Metabolites

Autoradiograms of two-dimensional thin layer chromatograms of extracellular naphthalene metabolites revealed the presence of 12 spots (Figure 14). Only one spot was found to co-migrate with the standards tested, salicylate. There were no differences in profiles between extracts from control cells, either naphthalene-grown or glucose-grown cells, and cells which were incubated with an additional carbon source. Extracts from cyanide-killed cells and

Table 5. Effect of additional carbon source upon accumulation of extracellular napthalene metabolites.

Addition to Medium[a]	Extracellular Metabolites (10^5 cpm/5 ml of culture)	
	Glucose-Grown Cells	Naphthalene-Grown Cells
None (control)	6.75 (100)[b]	3.04 (100)[b]
Glucose	1.75 (26)	2.83 (93)
Pyruvate	3.78 (56)	2.93 (96)
Gluconate	0.98 (15)	ND
α-Ketoglutorate	6.50 (96)	ND
Fumarate	3.04 (45)	3.00 (99)
Malate	3.36 (50)	2.86 (94)
Succinate	3.36 (50)	2.86 (94)
Citrate	6.40 (95)	2.87 (94)
Malonate	6.20 (92)	3.00 (99)
ATP	6.40 (95)	2.97 (98)
cAMP	6.60 (98)	2.78 (92)
UTP	6.54 (97)	3.02 (99)
CTP	6.65 (99)	3.02 (99)
Cyanide	0.38 (6)	0.98 (32)
Azide	0.31 (5)	0.98 (32)
Catechol	0.37 (6)	1.65 (54)
Salicylate	0.32 (5)	0.69 (23)
Blank (without cells)	0.10	

ND = Not done.

[a]Concentrations of carbon source added was 20 mM, with the exceptions of cyanide/azide (30 mM) and nucleotide (2 mM).

[b]Values in parentheses are the percentage of control.

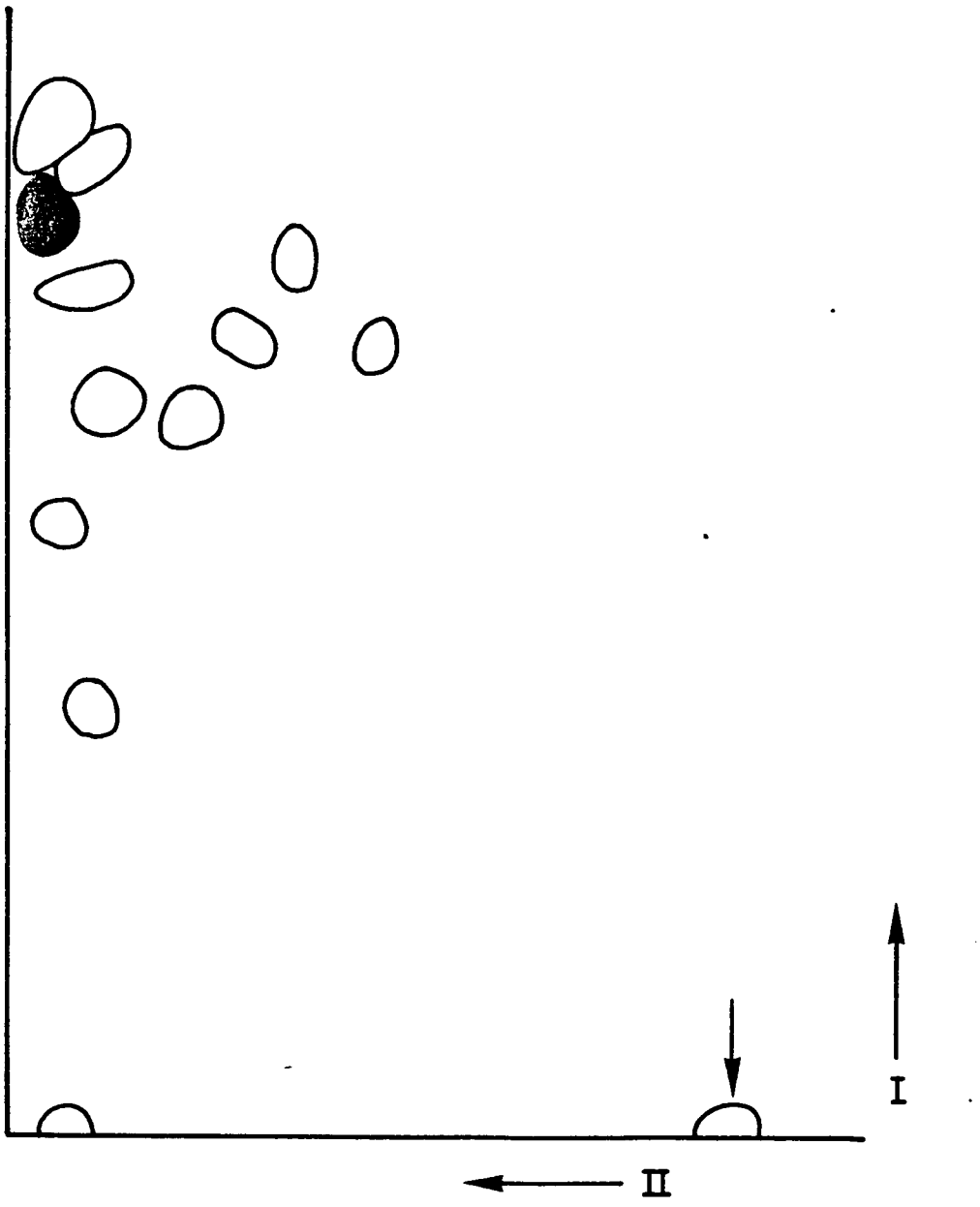


Figure 14. Tracing from autoradiogram of extracellular naphthalene metabolites. Shaded area is salicylate standard. Arrow points to origin.

cells preincubated with catechol or salicylate revealed no spots on the chromatogram. In contrast, extracted cell-associated label revealed only one spot on the chromatogram which co-migrated with salicylate. Identification of other possible spots was hampered by the smearing of the extract over much of the TLC plate. This result was common to extracts from cells incubated with or without carbon source, and absent with cyanide or salicylate treated cells.

Effect of Additional Carbon Source Upon Incorporation of ^{14}C Naphthalene Into Cellular Protein

To test what addition of carbon source would do to the incorporation of ^{14}C from naphthalene into protein, protein was isolated from both naphthalene-grown and glucose-grown cells in the presence or absence of carbon source. Cyanide-treated cells, chloroamphenicol-treated cells, and cells of a NAH^- strain were used as controls. The results are summarized in Table 6. The amount of ^{14}C present in glucose-grown cells decreased 98% with the addition of either glucose or pyruvate. This amount of incorporation was equal to the control cells. Label in naphthalene-grown cells decreased 23% with the addition of glucose and 15% with the addition of pyruvate.

Table 6. Effect of additional carbon source upon incorporation of ^{14}C naphthalene into P. putida protein.

Growth Conditions	Addition to Medium	^{14}C Incorporation (10^4 cpm/mg)	Percent of Control
Naphthalene	None (control)	2.84	-
	Glucose (20 mM)	2.20	78
	Pyruvate (20 mM)	2.39	84
	Chloroamphenicol (200 µg/ml)	0.72	25
	Cyanide (30 mM)	0.14	5
Glucose	None (control)	3.63	-
	Glucose (20 mM)	0.07	2
	Pyruvate (20 mM)	0.08	2
	Chloroamphenicol (200 µg/ml)	0.18	5
	Cyanide (30 mM)	0.02	1
P. putida Strain 572 (NAH^-) (Glucose-grown)	None	0.02	

CHAPTER IV

DISCUSSION

The results presented in Chapter III indicate that the assay devised from the uptake of PAH can be used in the study of naphthalene uptake in P. putida. Uptake was shown to occur in both naphthalene-grown cells and cells grown on non-aromatic substrates. Both groups of cells revealed different patterns of uptake. Cells grown with naphthalene or sodium salicylate exhibited a rapid uptake with no detectable lag (Figure 7, p. 32, and Figure 8, p. 33). This uptake was unaffected by the presence of protein synthesis inhibitors (Figure 3, p. 26). Growth with either naphthalene or salicylate has been shown to induce naphthalene metabolism in P. putida (5), and the absence of a lag period in uptake assays suggests that these cells are also induced for naphthalene uptake. This does not appear to be the case with cells grown upon non-aromatic substrates (Figures 4, 5, and 6, pp. 28, 29, 30). Uninduced cells show linear uptake only after a short lag period and, if treated with protein synthesis inhibitors (Figure 3, p. 26), little uptake occurred. Uptake is regained by removal of inhibitor. A similar pattern of inhibition was reported by Shapiro (24) for alkane metabolism in uninduced cells of P. putida. Mandelate uptake was also inhibited in uninduced cells by the presence of a protein synthesis inhibitor (26). This inhibition of uninduced cells suggests that uptake of naphthalene cannot occur by simple absorption of hydrocarbon to cellular

components, but that protein synthesis must occur before uptake begins. This is also supported by the lack of uptake in NAH^- strains discussed below. Even though naphthalene-grown cells could not be presumed to be free of intracellular reserves of naphthalene (60), or naphthalene metabolites, induced cells took up approximately 7 nmoles naphthalene per mg cell protein. This is compared to 5 nmoles/mg for uninduced cells. Salicylate-grown cells, which may be presumed to be free of intracellular naphthalene reserves, took up equivalent amounts of label as naphthalene-grown cells. This indicates that the greater uptake of induced cells is related to the expression of some activity of naphthalene metabolism, rather than a sequestering of naphthalene into intracellular naphthalene inclusions.

As another control to measure non-metabolic absorption of naphthalene to cellular components, NAH^+ and NAH^- strains were tested for naphthalene uptake (Figure 2, p. 24). Uptake assays for NAH^- strains of P. putida revealed no significant uptake, whereas in NAH^+ strains uptake did occur. Since all NAH^- strains used had either been derived from NAH^+ strains or had been shown capable of expressing NAH genes (Table 1, p. 14), uptake in NAH^- cells should be an accurate indication of naphthalene binding in the absence of metabolism. A possibility of an increase in binding which would be coded by the NAH locus is possible, but extensive membrane changes involved with hydrocarbon metabolism have only been shown in organisms actively growing on hydrocarbon, not as a prerequisite for hydrocarbon binding (55). In conjunction with the protein inhibitor work

described above, naphthalene uptake is coded as a function of the NAH locus and must be synthesized for uptake to occur. The possibility of the NAH^- strains having some physiological or structural defect which would not allow uptake is possible, but unlikely considering that the growth rates of the NAH^- strains are identical to the NAH^+ strains from which they were derived. The amount of cell-associated label present in NAH^- strains was also found to be equal to that found in strains of P. aeruginosa and E. coli tested. Neither species has been reported to metabolize naphthalene. Therefore the basal amount of label present in NAH^- cells ($\sim$ 1.3 nmole/mg) probably represents the amount of naphthalene absorbed to components of the bacterial cell surface. Since this amount is 17-20% of that present in NAH^+ strains and does not increase during the assay period, the remainder of the increase in NAH^+ strains is due to activity of the NAH genes.

That uptake is a direct consequence of naphthalene degradation is indicated by an analysis of cell-associated label. Approximately 80% of the label present in association with the cell were found to be naphthalene metabolites. The presence of metabolites indicates that naphthalene uptake is a function of metabolism, and the increase in cell-associated label is the accumulation of naphthalene metabolites. This accumulation is consistent with the loss of cell-associated label when cells are incubated with high amounts of salicylate or catechol (Figures 4 and 7, pp. 28 and 32). High amounts of catechol or salicylate were also shown to depress levels of extracellular naphthalene metabolites (Table 5, p. 45) and CO_2 production from labeled naphthalene (Table 4,

p. 43), which suggests a more complex effect than dilution of radioactive metabolites. It is also important that while induced cells can rapidly metabolize both catechol and salicylate as shown by the appearance of 2-hydroxymuconic semialdehyde, uninduced cells can only metabolize catechol and salicylate to a low degree (Table 3, p. 42). This indicates that the inhibitory effects are caused by salicylate or catechol itself rather than a metabolite of either substrate.

There have been several reports in the literature concerning control of naphthalene metabolism by naphthalene metabolites. Many naphthalene metabolites such as salicylate (69) and cis-1,2-diol (29) have been found to occur extracellularly in naphthalene culture. Skryabin (65) has shown an inhibitory effect upon naphthalene oxygenase by high levels of extracellular salicylate in one species of Pseudomonas. Kitai (37) noted a preference for salicylate metabolism over naphthalene metabolism in Pseudomonas when large amounts of salicylate accumulated in the medium. The levels of salicylate added externally in this present study are easily comparable to those which have been produced in culture (69) and may exert a similar inhibitory effect upon initial naphthalene metabolism which could result in loss of cell-associated label. There have been no reports of similar controls exerted by catechol, and it is unclear whether catechol and salicylate affect uptake similarly. In the absence of further data, it is suggested that catechol may exert a similar effect upon uptake as salicylate because of their structural similarity. More data is needed on this point. However, it is clear that the mechanism exerted by catechol and salicylate is definitely different from those exerted by other

carbon sources (discussed below), since both induced and non-induced cells are affected by their presence.

One of the more interesting aspects of hydrocarbon metabolism is "sparing." Sparing is the non-utilization of hydrocarbon in the presence of another carbon source. LePetit and Tagger (38) found that an intermediate product of hydrocarbon metabolism, acetate, reduced the utilization of hexadecane, a fact confirmed by Finnerty (34). A diauxic phenomenon has been reported for pristane degradation in the presence of hexadecane (53). A similar diauxic effect has been shown for camphor metabolism in the presence of succinate (25), a process that is, like naphthalene, plasmid-coded (71). Fitzgerald (36) has shown that alkylsulfate metabolism is reduced in the presence of many other carbon sources in P. aeruginosa. The exact relationship between sparing and classical catabolite repression is not clear as in most cases rigorous enzyme studies have not been done. Since metabolism of some hydrocarbons has been shown to be affected by carbon source, it was determined to measure naphthalene uptake in the presence of high amounts of an additional carbon source (Figures 4-8, pp. 28, 29, 30, 32, and 33).

In uninduced cells, uptake was strongly depressed by the addition of carbon source. If one makes a comparison between the ability of the uninduced cells to oxidize a compound (Table 3, p. 42) and that compound's ability to have an effect upon naphthalene uptake (Figures 4-6, pp. 28-30), it becomes evident that only oxidizable carbon sources eliminate naphthalene uptake. Similarly, only oxidizable carbon sources depressed naphthalene metabolism in uninduced cells (Tables 4 and 5,

pp. 43 and 45). The fact that the carbon source must be added before or during the lag period to eliminate uptake suggests that the carbon source acts at the level of protein synthesis, not allowing induction to occur. The pattern of oxidization of compounds by cells grown with different substrates was similar to those reported in the literature for Pseudomonas. Glucose transport and metabolism were shown to be induced by growth on glucose in P. aeruginosa (41, 47), but not by growth on organic acids. It was also shown that this induction was at the level of the glucose transport system (44, 67). Glucose-6-phosphate uptake was found not to be inducible by either glucose or gluconate in Pseudomonas (74). Clarke and Meadow (12) found that citrate transport but not the enzymes for metabolism were lacking in cultures grown with other organic acids. Citrate was oxidized without lag by cells which had been grown upon citrate or acetate, but not by cells grown with succinate. Succinate-grown cells oxidized succinate, malate, and several other organic acids without lag. However, the P. putida used in this study oxidized glucose when grown on succinate, without any effect upon uptake in the presence of glucose. Since oxygen uptake is not always equivalent to full metabolism of that compound, the discrepancy may be explained by the oxidation of glucose by an extracellular dehydrogenase which converts glucose to gluconate at high glucose levels (72). This enzyme is present in uninduced cells and it is the transport system for glucose and gluconate which are induced by growth with glucose. Thus, oxidation of glucose need not be equivalent to glucose metabolism

by succinate-grown cells. Although this evidence does not distinguish between metabolism of carbon source and its oxidation, it does suggest that the carbon source should be metabolized to have an effect upon naphthalene uptake.

In contrast, it was found, with the exception of salicylate and catechol previously discussed, that uptake in naphthalene grown cells was not affected by the presence of an additional carbon (Figure 7, p. 32). This cannot be attributed to a lack of capacity to utilize a given carbon source, since induced cells were able to oxidize many of the carbon sources tested without lag (Table 3, p. 42). Since none of the carbon sources had an effect upon naphthalene metabolism (Table 4 and 5, pp. 43 and 45), it would be expected that uptake would be unaffected in the presence of carbon source. This lack of inhibition in induced cells suggests that the carbon source effect must work at the level of induction of naphthalene metabolism. It also eliminates the possibility that the carbon source effect could occur by a dilution of cell associated metabolites (such as pyruvate) or that an excess of carbon source could directly inhibit enzymes of naphthalene metabolism.

One possible explanation for the carbon source effect on uptake would be catabolite repression. Such repression has been mentioned in conjuction with naphthalene metabolism in P. putida (5), and since uptake has been shown to be a function of naphthalene metabolism, it was determined to study the carbon source effect from the standpoint of catabolite repression. There has been at least one well documented case of catabolite repression with an aromatic substrate (25). At

present, the molecular basis for catabolite repression in Pseudomonas is unclear. Clarke and workers have shown indirect evidence that catabolite repression of the hut operon in P. aeruginosa is regulated by a cAMP-dependent protein (54, 66). However, measurements of intracellular cAMP levels in Pseudomonas (52, 63) did not correlate with catabolite repression conditions and repression could not be relieved by the addition of exogenous cAMP. Fitzgerald (23) has suggested that repression may be controlled as a function of intracellular ATP levels. He has also presented evidence that induction of alkyl-sulfatase could be inhibited by the addition of exogenous nucleotides, with the greatest inhibition accomplished by UTP and ATP (36). This type of control is not confined to Pseudomonas. Romano (57) proposed a similar mechanism for glucose transport in Thiobacillus, where the presence of a preferred energy source, thiosulfate, inhibits glucose transport. Since it had been shown that naphthalene uptake in induced cells was not affected by either dinitrophenol or arsenate (Figure 13, p. 40), it was felt that uptake was independent of ATP levels when fully induced. Incubation of nucleotides with uninduced cells would show if induction of uptake could be affected by nucleotide levels. Such incubation with high amounts of exogenous nucleotide was carried out, with no effect upon uptake. The data suggests that, unlike Fitzgerald's P. aeruginosa, P. putida was unable to take up sufficient amounts of nucleotides to affect induction of naphthalene uptake. Treatment to increase cellular permeability to nucleotides could not be attempted since an intact cell is essential to the uptake assay.

Fitzgerald (23) has also presented evidence that succinate-mediated repression in Pseudomonas may be relieved by treatment with dinitrophenol. It had been hypothesized that this reversal was accomplished because of lowered ATP levels in the cell. Another and more likely possibility is the documented effect of dinitrophenol upon succinate transport (16). Treatment of succinate-depressed cells showed no increase of naphthalene uptake. Reversal attempts with arsenate were also unsuccessful. Experiments with sodium azide and sodium cyanide, which can strongly bind and inactivate iron-based enzymes such as oxygenases (46), show rapid loss of cell-associated label and loss of uptake ability in both induced and non-induced cells (Figures 12 and 13, pp. 39, 40). This inhibition of uptake by cyanide and azide sheds no light upon the nature of the carbon source effect, but it does suggest that naphthalene uptake may be inhibited if naphthalene metabolism is inhibited. This is supported by the lack of metabolites, both intra- and extra-cellular, observed in cyanide-treated cells.

Succinate mediated catabolite repression in P. aeruginosa has also been reversed by malonate (23). Malonate is an analog of succinate and is a poor carbon source for P. putida (24). Malonate is a competitive inhibitor of succinate dehydrogenase and, in P. putida, does not inhibit succinate transport (16). The presence of malonate did cause uptake to occur in the presence of succinate (Figure 11, p. 37). Malonate alone did not affect uptake, which suggests that

succinate must be metabolized to exert an effect upon naphthalene uptake. This is supported by the demonstration of concentration dependence of glucose-mediated inhibition (Figure 9, p. 34). A value of 0.5 mM glucose was found to delay but not eliminate uptake in glucose-grown cells. This was lower than the 2.5 mM glucose required to inhibit alkylsulfatase synthesis in P. aeruginosa (22). Although concentration dependence is not complete proof that the carbon source must be metabolized, the lack of effect upon naphthalene uptake by non-metabolizable analogs of glucose strongly suggests that the carbon source must be metabolized to exert an effect upon uptake. This finding is in opposition to that reported by Fitzgerald (22), who reported some inhibition of alkylsulfatase synthesis with non-metabolizable analogs. However, he fails to explain how such analogs could provide sufficient ATP to cause inhibition. In our hands, only one analog, 2-deoxy-d-glucose, eliminated uptake in glucose-grown cells. Although 2-deoxy-d-glucose has been reported to be a non-metabolized analog of glucose in P. aeruginosa (44), glucose-grown cells of P. putida were able to grow slowly upon 2-deoxy-d-glucose as the sole carbon source. This indicates that P. putida is able to derive sufficient energy from 2-deoxy-d-glucose, or a contaminate of 2-deoxy-d-glucose, to affect naphthalene uptake. The other two analogs used, α-methyl-glucoside and glucuronic acid, appeared to be non-metabolized by P. putida. The data taken together suggest the carbon source must be metabolized to have an effect upon naphthalene uptake, although a mechanism for control remains to be demonstrated.

Since naphthalene uptake has been shown to be affected by the addition of an oxidizable carbon source and uptake has been shown to be an aspect of naphthalene metabolism, a study of other aspects of naphthalene metabolism in the presence of carbon source was carried out. The accumulation of extracellular naphthalene metabolites is an accurate indication of naphthalene metabolism, since naphthalene metabolites are less volatile and more soluble than the parent compound. Results for induced and non-induced cells are in Table 5, p. 45. Possibly because of dilution of labeled naphthalene with in-racellular naphthalene reserves (60), the levels of metabolites produced by induced cells is lower than non-induced cells. The presence of an oxidizable carbon source resulted in reduced levels of metabolites, as might be expected if little naphthalene uptake occurred. Little or no reduction was noted in induced cells, paralleling results obtained for uptake. It is interesting to note the extreme reduction of metabolite level in the presence of catechol or salicylate in both induced and non-induced cells. Metabolite levels paralleled cyanide controls, suggesting little naphthalene metabolism in the presence of either compound. Although an excess of either compound would be expected to dilute those metabolites formed from salicylate to acetaldehyde, it would not be expected to dilute those prior to salicylate. This brings up the possibility of most of the extracellular metabolites being salicylate or a metabolite of salicylate, which would seem unlikely. This again suggests a role in inhibition of naphthalene metabolism for catechol/salicylate.

Analysis of extracellular metabolites by two-dimensional thin layer chromatography revealed the presence of many compounds, forming a fingerprint. Only one compound, salicylate, was identified in both extracellular and intracellular extracts. While salicylate was a major portion of the extracts, especially in the cell-associated label, it is impossible to know if both initial and secondary stages of naphthalene metabolism were present. While the fingerprints of control and repressed cells are identical, it does not guarantee that identical pathways are operating in both normal and repressed conditions. More analysis is needed on this point. It is suggested from this preliminary evidence that the differences in naphthalene uptake in non-induced cells in presence or absence of carbon is not a reflection of a different pattern of induction of naphthalene metabolism.

Another assay for hydrocarbon metabolism is the production of radioactive carbon dioxide from labeled hydrocarbon (Table 4, p. 43). Because of the symmetry of the naphthalene molecule, the CO_2 produced may come from several places in the pathway. If each naphthalene molecule is completely metabolized to CO_2, approximately 25% would come from the metabolism of naphthalene to salicylate, with the remaining 75% coming from the metabolism of salicylate. The presence of an oxidizable carbon source in non-induced cells depressed CO_2 levels, while little depression occurred with induced cells. A dilution effect from excess catechol and salicylate would be expressed by a 75% decrease in CO_2 production, which did occur. This is, however, assuming a complete metabolism of naphthalene to

CO_2, which has been shown not to occur by the profusion of extracellular metabolites. From the uptake data studied, large amounts of naphthalene carbon are excreted as extracellular metabolites or accumulated in the cell, which suggests a greater metabolism of the initial steps of the naphthalene pathway. This again suggests an inhibitory effect for catechol/salicylate in naphthalene metabolism apart from any dilution effect.

In conclusion, naphthalene uptake has been shown to occur as a function of naphthalene metabolism. This has been shown by (1) uptake occurring only in NAH^+ strains, (2) the requirement for protein synthesis in uptake by non-induced cells, (3) extraction of naphthalene metabolites from both induced and non-induced cells, (4) elimination of uptake from cells incubated with large amounts of carbon source. The latter phenomena was more closely examined for the mechanism of action. The mechanism for uptake was proposed to be an initial adherence of naphthalene to the cell with immediate metabolism and accumulation of metabolites. Although this data does not rule out the possibility of facilitated binding of hydrocarbon (28) with immediate metabolism, it is felt that the first hypothesis is preferred until such components may be isolated. Further study is needed to search for such components, as well as to reconcile this present study with observation of intracellular naphthalene inclusions (60). It is not clear whether sustained growth can occur with the amounts of naphthalene used in this assay, so inclusion formation may be needed for growth. The mechanism of action of carbon source upon naphthalene uptake has not been elucidated. It is believed

that such control must involve the synthesis of enzymes, a hypothesis supported by reduction of metabolism in the presence of carbon source. Whether this control is mediated by a common metabolite of the carbon sources tested (i.e., the TCA cycle intermediates) or some common energy intermediate is unknown. It is suggested that catabolite repression may be involved in this control, but rigorous enzyme studies would be needed to establish the exact mechanism.

LITERATURE CITED

LITERATURE CITED

1. Aranha, H. G., and L. R. Brown. 1981. Effect of nitrogen source on end products of naphthalene degradation. Appl. Environ. Microbiol. 42: 74-78.

2. Atlas, R. M. 1981. Microbial degradation of petroleum hydrocarbons: an environmental perspective. Microbiol. Rev. 45: 180-209.

3. _____, and C. E. Heintz. 1973. Ultrastructure of two species of oil-degrading marine bacteria. Can. J. Microbiol. 19: 43-45.

4. Austen, R. A., and N. W. Dunn. 1980. Regulation of the plasmid-specified naphthalene catabolic pathway of Pseudomonas putida. J. Gen. Microbiol. 117: 521-528.

5. Barnsley, E. A. 1975. The induction of the enzymes of naphthalene metabolism in pseudomonads by salicylate and 2-aminobenzoate. J. Gen. Microbiol. 88: 193-196.

6. _____. 1976. Naphthalene metabolism by pseudomonads: the oxidation of 1,2-dihydroxynaphthalene to 2-hydroxychromene-2-carboxylic acid and the formation of 2'-hydroxybenzalpyruvate. Biochem. Biophys. Res. Comm. 72: 1116-1121.

7. _____. 1976. Role and regulation of the ortho and meta pathway of catechol metabolism in pseudomonads metabolizing naphthalene and salicylate. J. Bacteriol. 125: 404-408.

8. Brilon, C. W., W. Beckman, M. Hellwig, and H. J. Knackmuss. 1981. Enrichment and isolation of naphthalenesulfonic acid-utilizing pseudomonads. Appl. Environ. Microbiol. 42: 39-43.

9. Catterall, F. A., J. M. Sala-Trepat, and P. A. Williams. 1971. The coexistence of two pathways for the metabolism of 2-hydroxymuconic semialdehyde in a naphthalene-grown pseudomonad. Biochem. Biophys. Res. Comm. 43: 463-469.

10. Chakrabarty, A. M. 1972. Genetic basis of the biodegradation of salicylate in Pseudomonas. J. Bacteriol. 112: 815-823.

11. Chou, G., D. Katz, and I. C. Gunsalus. 1974. Fusion and compatibility of camphor and octane plasmid in Pseudomonas. Proc. Nat. Acad. Sci. U.S.A. 71: 2675-2678.

12. Clarke, P. H., and P. M. Meadow. 1959. Evidence for the occurrence of permeases for tricarboxylic acid cycle intermediates in Pseudomonas aeruginosa. J. Gen. Microbiol. 20: 144-155.

13. Conners, M. A., and E. A. Barnsley. 1980. Metabolism of naphthalene by pseudomonads: salicylaldehyde as the first possible inducer in the metabolic pathway. J. Bacteriol. 141: 1052-1054.

14. Cripps, R. E., and R. J. Watkinson. 1978. Polycyclic aromatic hydrocarbons: metabolism and environmental aspects. In: Developments in Biodegradation of Hydrocarbon-1. R. J. Watkinson (Ed.). Applied Science Publishers, London.

15. Davies, J. I., and W. C. Evans. 1964. Oxidative metabolism of naphthalene by soil pseudomonads: the ring-fission mechanism. Biochem. J. 91: 251-261.

16. Dubler, R. E., W. A. Toscano, and R. A. Hartline. 1974. Transport of succinate by Pseudomonas putida. Arch. Biochem. Biophys. 160: 422-429.

17. Dunn, N. W., H. M. Dunn, and R. A. Austen. 1980. Evidence for the existence of two catabolic plasmids coding for the degradation of naphthalene. J. Gen. Microbiol. 117: 529-533.

18. _____, and I. C. Gunsalus. 1973. Transmissable plasmid coding early enzymes of naphthalene oxidation in Pseudomonas putida. J. Bacteriol. 114: 974-979.

19. Farrell, R., I. C. Gunsalus, I. P. Crawford, J. B. Johnston, and J. Ito. 1978. Restriction endonuclase sites and aromatic metabolic plasmid structure. Biochem. Biophys. Res. Comm. 82: 411-416.

20. Feist, C. F., and G. D. Hegeman. 1969. Phenol and benzoate metabolism by Pseudomonas putida: regulation of tangential pathways. J. Bacteriol. 100: 869-877.

21. Fernley, H. N., and W. C. Evans. 1958. The oxidative metabolism of polycyclic hydrocarbons by soil pseudomonads. Nature (London) 182: 373-375.

22. Fitzgerald, J. W., and L. C. Kight. 1977. Physiological control of alkylsulfatase synthesis in Pseudomonas aeruginosa: effects of glucose, glucose analogs, and sulfur. Can. J. Microbiol. 23: 1456-1464.

23. _____, L. C. Kight-Olliff, G. J. Stewart, and N. F. Beauchamp. 1978. Reversal of succinate-mediated catabolite repression of alkylsulfatase in Pseudomonas aeruginosa by 2,4 dinitrophenol and by sodium malonate. Can. J. Microbiol. 28: 1567-1573.

24. Grund, A., J. Shapiro, M. Fennewald, P. Bacha, J. Leahy, K. Markbreiter, M. Nieder, and M. Toepfer. 1975. Regulation of alkane oxidation in Pseudomonas putida. J. Bacteriol. 123: 546-556.

25. Hartline, R. A., and I. C. Gunsalus. 1971. Induction specificity and catabolite repression of the early enzymes in camphor degradation by Pseudomonas putida. J. Bacteriol. 106: 468-478.

26. Higgins, S. J., and J. Mandelstam. 1972. Evidence for induced synthesis of an active transport factor for mandelate in Pseudomonas putida. Biochem. J. 126: 917-922.

27. Hopper, D. J. 1978. Microbial degradation of aromatic hydrocarbons. In: Developments in Biodegradation of Hydrocarbons-1, R. J. Watkinson (Ed.). Applied Science Publishers, London.

28. Jacoby, G. A., J. E. Rogers, H. E. Jacob, and R. W. Hedges. 1978. Transposition of Pseudomonas toluene-degrading genes and expression in E. coli. Nature 274: 179-180.

29. Jerina, D. M., J. W. Daly, A. M. Jeffery, and D. T. Gibson. 1971. Cis-1,2-dihydroxy-1,2-dihydronaphthalene: a bacterial metabolite from naphthalene. Arch. Biochem. Ciophys. 142: 394-396.

30. Johnston, J. B., and I. C. Gunsalus. 1977. Isolation of metabolic plasmid DNA from Pseudomonas putida. Biochem. Biophys. Res. Comm. 75: 13-19.

31. Kappeli, O., and W. R. Finnerty. 1979. Partition of alkane by an extracellular vesicle derived from hexadecane-grown Acinetobacter. J. Bacteriol. 140: 707-712.

32. Karimian, M., and L. N. Ornston. 1981. Participation of the β-ketoadipate transport system in chemotaxis. J. Gen. Microbiol. 124: 25-28.

33. Kennedy, R. S., W. R. Finnerty, K. Sudarsanan, and R. M. Young. 1975. Microbial assimilation of hydrocarbons. I. The fine-structure of a hydrocarbon oxidizing Acinetobacter species. Arch. Microbiol. 102: 75-84.

34. Kennedy, R. S., and W. R. Finnerty. 1975. Microbial assimilation of hydrocarbons. II. Intracytoplasmic membrane induction in Acinetobacter species. Arch. Microbiol. 102: 85-90.

35. Kennell, D. 1967. Use of filters to separate radioactivity in RNA, DNA, and protein. In: Methods in Enzymology, v. 12A. L. Grossman (Ed.). Academic Press, New York.

36. Kight-Olliff, L. C., and J. W. Fitzgerald. 1978. Inhibition of enzyme induction in Pseudomonas aeruginosa by exogenous nucleotides. Can. J. Microbiol. 24: 811-817.

37. Kitai, A., H. Tone, T. Ishikura, and A. Ozaki. 1968. Microbial production of salicylic acid from naphthalene. II. Product inhibitory kinetics and effect of product removal on the fermination. J. Ferment. Technol. 46: 442-451.

38. LePetit, J., and S. Tagger. 1976. Degradation des hydrocarbures en presence d'autre substances organiques par des bacteries isolees de l'eau de mer. Can. J. Microbiol. 22: 1654-1657.

39. MacDonald, C. R., D. G. Cooper, and J. E. Zajic. 1981. Surface-active lipids from Nocardia erythropolis grown on hydrocarbons. Appl. Environ. Microbiol. 41: 117-123.

40. Manno, J. H., and D. Schacter. 1970. Energy-coupled influx of thiomethyl galactoside into Escherichia coli. J. Biol. Chem. 245: 1217-1224.

41. Midgley, M., and E. A. Dawes. 1973. The regulation of transport of glucose and methyl α-glucoside in Pseudomonas aeruginosa. Biochem. J. 132: 141-154.

42. Montie, T. C., and D. B. Montie. 1979. Methionine transport in Pseudomonas aeruginosa. Can. J. Microbiol. 25: 1103-1107.

43. Moulton, R. C., and T. C. Montie. 1979. Chemotaxis by Pseudomonas aeruginosa. J. Bacteriol. 137: 274-280.

44. Mukkada, A. J., G. L. Long, and A. H. Romano. 1973. The uptake of 2- deoxy-d-glucose by Pseudomonas aeruginosa and its regulation. Biochem. J. 132: 155-162.

45. Murphy, J. F., and R. W. Stone. 1955. Bacterial dissimilation of naphthalene. Can. J. Microbiol. 1: 579-588.

46. Neilands, J. B. 1974. Iron and its role in microbial physiology. In: Microbial Iron Metabolism. J. B. Neilands (Ed.). Academic Press, New York.

47. Ng, F.M.W., and E. A. Dawes. 1973. Chemostat studies on the regulation of glucose metabolism by citrate. Biochem. J. 132: 129-140.

48. Ornston, L. N., and D. Parke. 1976. Properties of an inducible uptake system for β-ketoadipate in Pseudomonas putida. J. Bacteriol. 125: 475-488.

49. Oyama, V. I., and G. Eagle. 1956. Measurement of cell growth in tissue culture with a phenol reagent (Folin-Ciocalteau). Proc. Soc. Exp. Biol., N.Y. 91: 305-312.

50. Palleroni, N. J., R. W. Ballard, E. Ralston, and M. Doudoroff. 1972. Deoxyribonucleic acid homologies among some Pseudomonas species. J. Bacteriol. 110: 1-11.

51. Perry, J. J. 1979. Microbial cooxidations involving hydrocarbon. Microbiol. Rev. 43: 59-72.

52. Phillips, A. T., and L. M. Mulfinger. 1981. Cyclic adenosine 3',5'-monophosphate levels in Pseudomonas putida and Pseudomonas aeruginosa during induction and carbon catabolite repression of histidase synthesis. J. Bacteriol. 145: 1286-1292.

53. Pirnik, M. P., R. M. Atlas, and R. Bartha. 1974. Hydrocarbon metabolism by Brevibacterium erythrogenes: normal and branched alkanes. J. Bacteriol. 119: 869-878.

54. Potts, J. R., and P. H. Clarke. 1976. The effect of nitrogen limitation on catabolite repression of amidase, histidase and urocanase in Pseudomonas aeruginosa. J. Gen. Microbiol. 93: 377-387.

55. Ratledge, C. 1978. Degradation of aliphatic hydrocarbons. In: Developments in Biodegradation of Hydrocarbons-1. R. J. Watkinson (Ed.). Applied Science Publishers, London.

56. _____, L. P. Machan, K. A. Brown, and B. J. Marshall. 1974. Iron transport in Mycobacterium smegmatis: a restricted role for salicylic acid in the extracellular environment. Biochim. Biophys. Acta 372: 39-51.

57. Romano, A. H., N. J. Van Vranken, P. Preisand, and M. Brustolon. 1975. Regulation of the Thiobacillus intermedius glucose uptake system by thiosulfate. J. Bacteriol. 121: 577-582.

58. Sanioto, D. L., and S. Schreier. 1975. Phase transition in lipids induced by carcinogenic aromatic hydrocarbons. A spin label study. Biochem. Biophys. Res. Comm. 67: 530-537.

59. Saylor, G. S., L. C. Lund, M. P. Shiaris, T. W. Sherrill, and R. E. Perkins. 1979. Comparative effects of Aroclor 1254 (polychlorinated biphenyls) and pheneanthrene on glucose uptake by freshwater microbial populations. Appl. Environ. Microbiol. 37: 878-885.

60. Scott, C.C.L., and W. R. Finnerty. 1976. A comparative analysis of the ultrastructure of hydrocarbon oxidizing organisms. J. Gen. Microbiol. 94: 342-350.

61. Shamsuzzaman, K. M., and E. A. Barnsley. 1974. The regulation of naphthalene oxygenase in pseudomonads. J. Gen. Microbiol. 83: 165-170.

62. _____. 1975. The regulation of naphthalene metabolism in pseudomonads. Biochem. Biophys. Res. Comm. 60: 582-587.

63. Siegel, L. S., P. B. Hylemon, and P. V. Phibbs, Jr. 1977. Cyclic adenosine 3',5'-monophosphate levels and activities of adenylate cylase and cyclic adenosine 3',5'-monophosphate phosphodiesterase in Pseudomonas and Bacteroides. J. Bacteriol. 129: 87-96.

64. Skryabin, G. K., and I. I. Starovoitov. 1975. An alternative pathway of naphthalene catabolism in Pseudomonas fluorescens. Dokl. Akad, Nauk. SSSR 221: 141-143.

65. _____, A. N. Borisoglebskaya, and A. M. Boronin. 1978. Oxidation of naphthalene by a strain of Pseudomonas putida carrying a mutant plasmid. Mikrobiology 47: 222-225.

66. Smyth, P. F., and P. H. Clarke. 1975. Catabolite repression of Pseudomonas aeruginosa amidase: the effect of carbon source on amidase synthesis. J. Gen. Microbiol. 90: 81-90.

67. Stinson, M. W., M. A. Cohen, and J. M. Merrick. 1976. Isolation of dicarboxylic acid- and glucose-binding proteins from Pseudomonas aeruginosa. J. Bacteriol. 128: 573-579.

68. Strauss, P. R., J. M. Sheehan, and E. R. Kashket. 1976. Membrane transport by murine lymphocytes. I. A rapid sampling technique as applied to the adenosine and thymidine systems. J. Exp. Med. 144: 1009-1021.

69. Strawinski, R. J., and R. W. Stone. 1955. Biological oxidation of naphthalene. Can. J. Microbiol. 1: 206-210.

70. Walker, N., and G. H. Wiltshire. 1953. The breakdown of naphthalene by a soil bacterium. J. Gen. Microbiol. 8: 273-276.

71. Wheelis, M. L. 1975. The genetics of dissimilarity pathways in Pseudomonas. Ann. Rev. Microbiol. 29: 505-524.

72. Whiting, P. H., M. Midgley, and E. A. Dawes. 1976. The role of glucose limitation in the regulation of the transport of glucose, gluconate, and 2-oxogluconate, and of glucose metabolism in Pseudonomas aeruginosa. J. Gen. Microbiol. 92: 304-310.

73. Williams, P. A., F. A. Catterall, and K. Murray. 1975. Metabolism of naphthalene, 2-methylnaphthalene, salicylate, and benzoate by Pseudomonas P_G: regulation of tangential pathways. J. Bacteriol. 124: 679-685.

74. Winkler, H. 1974. Distribution of an inducible hexose transport system among various bacteria. J. Bacteriol. 116: 1079-1081.

75. Wodzinski, R. S., and D. Bertolini. 1972. Physical state in which naphthalene and biphenyl are utilized by bacteria. Appl. Microbiol. 23: 1077-1081.

76. _____, and J. E. Coyle. 1974. Physical state of pheneanthrene for utilization by bacteria. Appl. Microbiol. 27: 1081-1084.

77. _____, and M. J. Johnson. 1968. Yield of bacterial cells from hydrocarbons. Appl. Microbiol. 16: 1886-1891.

78. _____, and D. Larocca. 1977. Bacterial growth kinetics on diphenylmethane and naphthalene-heptamethynonane mixture. Appl. Microbiol. 33: 660-665.

79. Zilber, I. K., E. Rosenberg, and D. Gutnik. 1980. Incorporation of ^{32}P and growth of pseudomonad UP-2 on n-tetracosane. Appl. Environ. Microbiol. 40: 1086-1093.

VITA

Joyce Elaine Broyles was born in Sparta, Tennessee in 1955. She attended public school in Sparta, Tennessee and was graduated from White County High School in 1973. The same year, she entered Tennessee Technological University, Cookeville, Tennessee and in 1977 received the Bachelor of Science degree with a major in biology. Later that year, she entered The University of Tennessee, Knoxville, Department of Microbiology, where she held a Graduate Teaching Assistantship.

The author is a member of Phi Kappa Phi, Beta Beta Beta, Omicron Delta Kappa, Kappa Delta Pi, and Phi Alpha Theta.

She is the daughter of Mr. and Mrs. E. Rascoe Broyles of Sparta, Tennessee and is married to David C. Cullison, Jr.