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Characterization of 2-nitrophenol uptake system of *Pseudomonas putida* B2

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Uptake of substituted nitrophenols from the bulk solution into the cytoplasm limited reaction rates by *Pseudomonas putida* B2. Initial enzymatic conversion of 2-nitrophenol (ONP) to catechol is by an intracellular soluble enzyme, nitrophenol oxygenase [Zeyer J and Kearney PC. 1984. J Agric Food Chem 32: 238–242]. Addition of *N*-ethylmaleimide (NEM) to cell suspensions led to a decrease in specific reaction rates for ONP, dependent on the ratio of NEM to cellular protein. Maximal NEM inhibition resulted in an 80–90% decrease in the ONP reaction rate which could not be reversed following dilution. Cell-free enzyme extract isolated from NEM-inactivated cells demonstrated less than 20% loss of the specific ONP reaction rates. NEM apparently acted by inhibiting a protein which facilitated uptake of nitrophenol into the cytoplasm, prior to the first catabolic enzyme. Both intact organisms and protoplasts exhibited the same 80–90% decrease in reaction rate which established that NEM inhibition was localized in the plasma membrane. NEM elicited variable effects on reaction rates for a series of ring substituted 2-nitrophenols. The data indicated that uptake of substituted 2-nitrophenols involved at least two transport systems, one sensitive to NEM inactivation and a second insensitive uptake process.

Keywords: nitrophenol; uptake; transport; *Pseudomonas putida*; *N*-ethylmaleimide

Introduction

The cellular structures surrounding the cytoplasm perform a number of essential functions including the control of chemical movement in and out of the cell. The outermost cell layer in some Gram-negative bacteria is a negatively charged, capsule or sheath typically composed of polysaccharide. This charged surface is thought to influence the entry of charged molecules into the cell [6]. The next layer, the cell wall, is a rigid structure composed of an outer membrane peptidoglycan complex containing proteinaceous channels or porins, which act as molecular sieves allowing small hydrophilic molecules to pass [15]. Porins are generally believed to be passive channels with a molecular weight cut-off of about 600 daltons [6,15]. Diffusion across the outer membrane has been shown to limit uptake at low substrate concentrations [20]. The greatest selectivity in controlling uptake of chemicals into the cells resides primarily with the plasma membrane which contains numerous specific transport proteins. Non-specific diffusion of chemicals across the lipid bilayer also occurs. This innermost barrier controls the movement of inorganic ions and charged and uncharged organic molecules. All of these layers can act to facilitate or restrict uptake of chemicals into the bacterial cell. Understanding the mechanisms and energetics by which substituted aromatic compounds are transported into the cytoplasm is growing but still not very well understood for many compounds.

Solute transport across cell boundaries has been demonstrated to be by one of three main mechanisms: (1) passive diffusion; (2) passive facilitated uptake; or (3) energy-dependent facilitated uptake. Research suggests that mandelate can enter a cell by passive diffusion [17], facilitated diffusion [7], and active transport [18]. Though accumulation of benzoate by *Pseudomonas putida* has been interpreted as being an active transport process [27,28], uptake by *Rhodopseudomonas palustris* was found to be linked to formation of benzoyl CoA, with no evidence of energy dependence or protein involvement in the transport process [16]. Uptake of 4-chlorobenzoate has been linked to both pH and electrochemical gradients [17].

These processes have been characterized using a variety of experimental methods including manipulation of genes involved in transport [28], use of antibiotics which collapse pH and electrochemical gradients [14,23], use of radiolabeled substrates, nutrient-limited growth [5,25], and use of substrate analogs or uptake inhibitors. Hydrophobic compounds tend to partition into membranes and other cellular structures [1,13], leading to difficulty in separating sorption rates from actual increases in cytoplasmic solute concentrations. Sulfhydryl-directed protein modifying agents have also been successfully used to characterize uptake of compounds, such as lactose [12,24] by *E. coli*, tryptophane by *Brevibacterium linens* [4], and succinate [9], methylamine, and methanol [2] by *Pseudomonas*. Measurement of substrate disappearance rates for enzymes residing in the cytoplasm have also been a valuable tool for studying uptake. This method has been effectively employed in studying lactose uptake by monitoring β-galactosidase activity [24]. Potentially, these methods are more accurate in assessing the uptake of hydrophobic substrate because only the substrate available for degradation will be assessed and not the substrate bound to cellular components.

In this paper, we characterize the effects of uptake on

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the biodegradation of nitrophenol in *Pseudomonas putida* B2. Reaction rates were determined for a wide range of substituted nitrophenols. Both substrate analogs and chemical modifying agents as possible inhibitors were utilized in order to characterize the nitrophenol uptake process. The results of these experiments are reported here.

Materials and methods

Chemicals

The sources of most nitrophenols (Table 1) used in this study and of some other chemicals were reported earlier [26,29]. Additional chemicals were obtained from: Aldrich Chemical Company, Steinheim, Germany (chloramphenicol and *N*-ethylmaleimide (NEM)); Boehringer Mannheim AG, Rotkreuz, Switzerland (NADPH and bovine serum albumin, fraction V). 6-Cl was a generous gift of Dr Knackmuss, Institut für Mikrobiologie, Göttingen, Germany and 6-Me was donated by Dr Braun, Wella, Cosmital SA, Marly, Switzerland.

Microorganism, media and culture conditions

Isolation and characterization of the ONP-degrading strain of *Pseudomonas putida* B2 was described previously [29]. Batch cultures of organisms induced for ONP degradation were grown in a basal salts medium (pH 7.5) with 1 mM ONP and 0.02% yeast extract as described earlier [29–31].

Preparation of cell suspensions and enzyme isolation

The procedures for preparing resting cells and enzyme extracts with induced catabolic enzymes were as reported earlier [29–31]. Washed resting cells were stored at 4°C as a suspension in 20 mM phosphate buffer, pH 7.5, with the addition of 1 mM chloramphenicol and 1 mM succinate for use in cellular experiments. Cells were harvested and suspended fresh on the day of the experiment. Nitrophenol oxygenase was purified through the Sephadex G-150 chromatographic step as outlined previously [31]. The enzyme was eluted and stored at 4°C in 20 mM phosphate buffer, pH 7.5 until needed. Enzyme preparations were used for up to 4 days following elution from the gel filtration column with less than 15% loss in specific activity. Protein content of cell suspensions and enzyme extracts was determined by a modified method of Lowry with bovine serum albumin as a standard as described previously [30].

Resting cell and enzyme assays

The nitrophenol oxygenase activity was measured in 20 mM phosphate buffer, pH 7.5 with 4 mM $MgSO_4$, 100 μM NADPH, 0.1 mg ml^{-1} BSA with 100 μM nitrophenol unless otherwise indicated [31]. Resting cell assays for nitrophenol turnover were carried out in 20 mM phosphate buffer, pH 7.5 with 100 μM nitrophenol with no other additions unless stated. All assays monitored change in absorbance at 410 nm in quartz cuvettes, either 1 or 5 cm path length, in a total volume of 1 or 10 ml respectively. Absorbance was monitored using a Uvikon Model 810 spectrophotometer, Kontron, Zürich, Switzerland. The contents of the cuvette were maintained at 20 ± 1°C using a water jacketed cuvette holder unless otherwise indicated. Extinction coefficients for the substrates tested are listed in Table 1. Individual rates were routinely performed in triplicate and all tables and figures generally represent data collected on a single batch of cells or cell-free extracts to minimize batch to batch variability issues. Specific activities for conversion of nitrophenol to catechol were calculated and expressed in μmol min^{-1} g protein^{-1} as outlined previously [30]. No significant change in pH was recorded at these buffer concentrations and was constant throughout the experiment.

Rates of oxygen consumption were determined with a 2-ml suspension of resting cells in the same buffer used for monitoring nitrophenol reaction rates. Cell suspensions were placed in a Clark-type oxygen electrode calibrated with air-saturated water at 20 ± 1°C. Specific activities for

Table 1 Chemical and kinetic values for selected substituted nitrophenols

Name	Abb.	ϵ[a]	pK$_a$[b]	K_m[c] enzyme (μM)	V_{max}[d] enzyme	K_s[c] cell (μM)	V_{max}[d] cell	Inhibition by NEM[e]
2-Nitrophenol	ONP	3490	7.23	0.6	283	6	160	92
5-Fluoro-2-nitrophenol	5-F	5000	6.30	8.7	142	20	61	74
4-Chloro-2-nitrophenol	4-Cl	3980	6.43	0.1	85	2	26	72
4-Methoxy-2-nitrophenol	4-OMe	2830	7.40	0.6	43	3	13	45
4-Sec-butyl-2-nitrophenol	4-sBu	2540	7.59	4.7	65	nd	8	44
4-Phenyl-2-nitrophenol	4-Ph	2470	6.69	4.9	223	19	35	42
4-Methyl-2-nitrophenol	4-Me	2820	7.63	0.4	188	3	64	40
3-Methyl-2-nitrophenol	3-Me	990	7.00	2.9	222	41	83	37
5-Methyl-2-nitrophenol	5-Me	3560	7.34	7.6	238	6	32	23
6-Chloro-2-nitrophenol	6-Cl	4560	5.35	0.9	174	21	74	22
6-Methyl-2-nitrophenol	6-Me	1980	7.65	2.7	263	9	74	1
2,4-Dinitrophenol	DNP	10300	3.94	0.04	$<<$0.1	24	$<$0.1	ND

[a]Molar extinction coefficient at 410 nm in 20 mM phosphate buffer pH 7.5.
[b]Values from Reference 26.
[c]Values from Reference 10.
[d]Values from Reference 10 expressed in units μmol min^{-1} g protein^{-1}.
[e]% Inhibition calculated from data in Figure 2.
ND, value not determined.

rates of oxygen consumption were calculated by subtracting basal cellular respiration rates from the respiration rate observed after addition of the chemicals to be tested at the concentrations indicated and expressed in units of μmol min^{-1} g $protein^{-1}$.

Chemical modification with N-ethylmaleimide

A 10-mM stock solution of *N*-ethylmaleimide (NEM), in phosphate buffer, was prepared daily as required. Aliquots of this solution were added to a suspension of resting cells in the buffer as described above and allowed to incubate for 10 min at 20–23°C before assaying for activity. Cell suspensions were diluted into phosphate buffer then the indicated activities were monitored. Cell-free extracts were prepared by first treating intact cells with NEM then preparation of the extract by sonication and centrifugation.

Mureinoplast and protoplast formation

Protoplasts were formed using a procedure modified from a method outlined for a marine *Pseudomonad* [8,11]. Cells were grown and harvested as above but the final cell suspension was made in an osmotically balanced buffer (GP) containing: 0.2 M glycerol, 50 mM phosphate, 10 mM NaCl, 10 mM $MgCl_2$, 1 mM succinate, 1 mM chloramphenicol, pH 7.5. Mureinoplasts were prepared from resting cells by removal of the layers external to the peptidoglycan (polysaccharide sheath and outer membrane) by addition of a 1-M sucrose solution in GP buffer to give a final composition of 1 g wet weight cells ml^{-1} and 0.5 M sucrose. This suspension was incubated with mixing for 5 min at 28°C then diluted about 100-fold with GP buffer and centrifuged. Extended exposure of cells to 0.5 M sucrose resulted in loss in both nitrophenol turnover and oxygen consumption activity, possibly by cell lysis, so exposure was minimized. Mureinoplasts were then treated with lysozyme (1 mg ml^{-1}) in GP buffer with shaking at 100 rpm at ambient temperature for about 45 min then placed at 4°C until assayed for activity. The extent of protoplast formation was monitored by measuring the extent of cell lysis after dilution into distilled water, by following the change in absorbance at 540 nm and by visual inspection under the microscope. Assays for nitrophenol turnover were performed in GP buffer to maintain protoplast stability and minimize activity differences resulting from changes in ionic or osmotic strength. A 2-ml aliquot was placed directly into a Clark-type oxygen electrode for monitoring oxygen consumption.

Results

Direct monitoring of nitrophenol uptake

Nitrophenol is taken up by intact cells then converted to catechol by the nitrophenol oxygenase [29–31]. Further degradation is via the *ortho* cleavage pathway by the catabolic enzymes found in the cytoplasm. Reaction rates increased over the first 15–30 s after addition of 100 μM ONP to cell suspensions, then maintained a linear rate of reaction for nitrophenol concentrations below about 10 μM. Conversion of substrates to products was stoichiometric, generating the same results whether using a spectrophotometric or an HPLC-based method [10]. One explanation for the observed initial lag in rate is that nitrophenol uptake rates are slower than degradation rates resulting in a submaximal nitrophenol reaction rate. If the initial catabolic enzyme limits the rate of reaction in whole cells, the measured kinetic constants describing nitrophenol transformation by whole cells and by enzyme extracts may be proportional. However, it was observed previously that apparent K_s values for cellular nitrophenol reaction rates were significantly different than apparent K_m values for enzyme extracts [10]. Two common methods for determining uptake of radioactively labeled ONP were attempted, membrane filtration and centrifugation through silicone oil. Several membrane types were unsuccessfully tested, either demonstrating high sorption or poor recovery. Chasing the suspension into a higher concentration of cold ONP also failed to produce meaningful results. Neither method was able to allow differentiation of sorption or partitioning of ONP to hydrophobic cell components from translocation into the cytoplasm. Indirect methods were then pursued in order to establish the effects of uptake on degradation rates.

Characterization of NEM inhibition

Sulfhydryl-directed protein modifying agents have been successfully used to label and inhibit membrane-bound transport proteins. To determine the effects on ONP reaction rates, increasing amounts of NEM were added to intact cells and changes in ONP reaction rates were monitored for both cells and cell-free enzyme extracts prepared from treated cells. NEM exhibited a concentration-dependent inhibition of ONP degradation (Figure 1). The extent of NEM inhibition per gram of cellular protein was independent of starting protein concentration. Inhibition was irreversible following dilution into phosphate buffer which lowered both cell and NEM concentration. NEM inhibition apparently resulted from a covalent protein modification

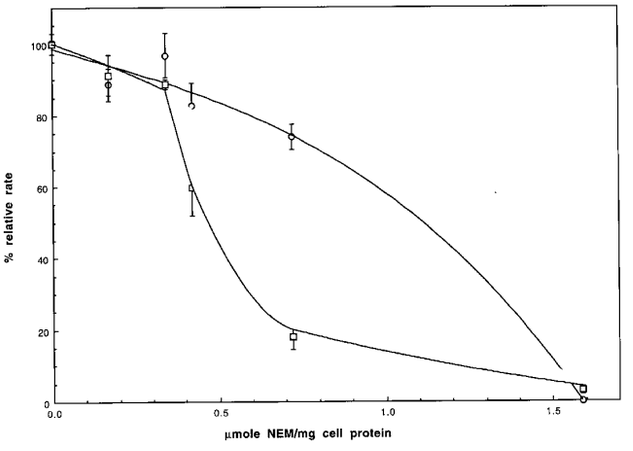

Figure 1 Effect of NEM on ONP reaction rate and on oxygen consumption rates. NEM in phosphate buffer was added to a resting cell suspension in phosphate buffer, incubated for 10 min at 20°C, then both ONP reaction rate and oxygen consumption rates were monitored on the same pool of treated cells. Relative rates of ONP reaction rate (100 μM) were determined on dilution of the cell suspension treated with the indicated amount of NEM (□). Relative rates of oxygen consumption were monitored on undiluted suspensions with 1 mM succinate present (○). The resulting relative rates (inhibited rate/uninhibited rate) were plotted as a function of amount of NEM added expressed as a ratio, μmol NEM mg^{-1} protein.

and not just binding to a sensitive site. Maximal inhibition resulted in greater than 80% loss in the reaction rate for ONP.

To determine whether NEM was affecting general cell physiology, succinate-stimulated oxygen consumption was monitored on the same batch of NEM-treated cells. There was less than 20% loss in oxygen consumption rates after addition of about 0.7 μmol NEM mg^{-1} protein whereas these same conditions resulted in greater than 80% loss of ONP reaction rates (Figure 1). This indicated that cells retained significant capacity to oxidize succinate and generate the requisite chemical energy, NADPH, even though much of their ability to convert ONP to catechol was inhibited.

One mechanism of NEM inhibition could be by entering the cell and directly affecting the nitrophenol oxygenase, inhibiting the cytoplasmic enzyme required for initial oxidation. To test this possibility, cell-free enzyme extracts were prepared from cells treated with 0.8 μmol NEM mg^{-1} cell protein. Under conditions where the NEM-treated cells lost greater than 80% activity, enzyme extracts from those same cells retained greater than 90% of the nitrophenol oxygenase activity when compared to untreated controls. Since NEM did not appear to directly affect nitrophenol oxygenase activity, it was concluded that NEM acted at the cell surface, presumably by retarding translocation of the substrate into the cytoplasm.

To further characterize the uptake process, reaction rates for a set of substituted nitrophenols were determined using untreated and NEM-treated cells (Figure 2). Variable degrees of inhibition for reaction rates were observed for this set of 11 compounds ranging from about 90% inhibition of ONP to negligible inhibition of 6-Me (Table 1). These results further support the conclusion that NEM was acting specifically on substrate uptake and not affecting general cell physiology. NEM demonstrated a substrate-dependent inhibition suggesting that there could be more than one mechanism for uptake of substituted nitrophenols into the cell.

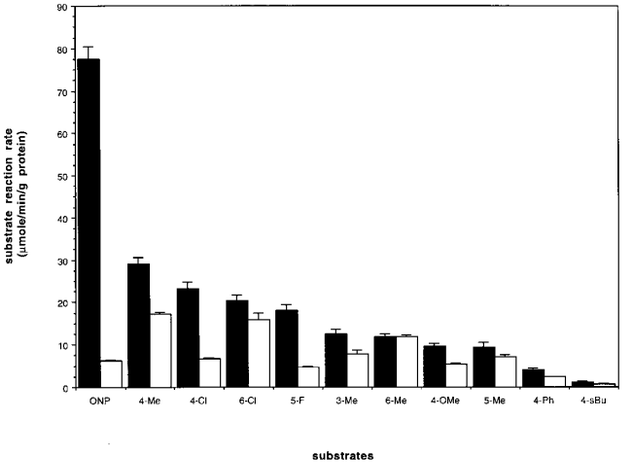

Figure 2 Inhibition of nitrophenol reaction rate by NEM for a set of substituted nitrophenols. To resting cells, 8 μmol NEM g^{-1} protein was added in phosphate buffer and allowed to react at 20°C for 10 min. Rates of substrate disappearance were measured in triplicate for each substituted nitrophenol with 100 μM initial substrate concentration. Rates were reported as μmol min^{-1} g protein^{-1} and were determined from a single batch of cells assayed on the same day; rate of reaction rate for untreated cells (▩) and for cells treated with NEM (□).

Localization of the NEM-sensitive uptake system

To determine whether membrane composition affected nitrophenol uptake, organisms were grown at either 20° or 30°C then harvested and assayed at five separate temperatures between 4° and 25°C. Cell-free extracts were also prepared from cells grown at 20°C for comparison. The resulting rates were plotted in the form of Arrhenius plots (ln (rate) *vs* l/T (°K^{-1})), from which activation energies (E_a) for the rate limiting step were calculated using a least-squares linear regression (Table 2). Since the nitrophenol oxygenase was the only kinetic step involved in the *in vitro* measurement, the calculated activation energy was this reaction. For the *in vivo* activities, E_a values were consistently lower for cells grown at 20°C than for cells grown at 30°C. E_a values were also lower for cells grown at 20°C than for enzyme extracts prepared from those same cells. Since the activation energy was different for *in vitro* enzyme activity, and for cells grown at the two different growth temperatures, it was concluded that there was a shift in reaction rate limitation from the cardinal reaction control of the single enzyme step to a different reaction control mechanism, involving processes prior to the nitrophenol oxygenase. This shift in reaction rate limitation could be either directly related to substrate transport or by partial control by each member of the process including transport and catabolism. Since each value for activation energy was determined from a single batch of cells, other general physiological responses which affect overall rate would be lost since E_a was determined from the slope and not the absolute position on the graph. Changes in membrane composition and fluidity are well-documented physiological responses to growth at different temperatures. This observed shift in E_a was fully consistent with a change in rate of substrate uptake prior to reaction by the nitrophenol oxygenase.

To more directly establish the location of the uptake systems, cells were systematically stripped of their polysac-

Table 2 Activation energies for turnover of several nitrophenols

Compound	E_a (kcal/°K)		
	Cells grown at 20°C	Cells grown at 30°C	Enzyme extracts
ONP	14	19	19
4-Me	13	17	18
4-Ph	12	14	20
5-F	14	15	15
4-Cl	14	ND	14

Cells were grown in batch culture at either 20 or 30°C. Enzyme extracts were prepared from cells grown at 20°C. Cells were harvested and stored on ice then incubated at the various assay temperatures for 5 min before assay initiation. Nitrophenol (100 μM) was added and reaction rates determined at five temperatures between 4° and 25°C. Least-squares linear regression analysis was used to determine E_a values from Arrhenius plots. Correlation coefficients from Arrhenius plots gave an $r^2>0.99$ for all values reported. ND, value not determined.

charide layer, outer membranes, and peptidoglycan layers to form mureinoplasts and protoplasts. Reaction rates for ONP and five substituted nitrophenols were determined for intact cells, mureinoplasts, and protoplasts in the absence of NEM (Figure 3). Overall rates were lower in this experiment due to the different buffer used to osmotically stabilize the cells. Although there was some decrease in specific activity for one or two compounds, in general, all demonstrated equal or greater reaction rates as either mureinoplasts or protoplasts were generated, despite the likelihood that some of the cells lost viability as a result of the treatment. The polysaccharide sheath, outer membrane, and peptidoglycan layers all apparently limit reaction rates for substituted 2-nitrophenols by restricting uptake.

The final experiment combined the observations from NEM inhibition and cell layer removal to localize the effect of NEM. Intact cells were treated with NEM then one batch was further treated to remove the murein layer and outer membrane to form protoplasts. Both sets of NEM-treated cells were then evaluated for activity. NEM-treated intact cells demonstrated an ONP reaction rate of 7.0 ± 0.6 μmol min^{-1} g protein^{-1}, whereas those same NEM-treated cells formed into protoplasts demonstrated a rate of 6.9 ± 2.1 μmol min^{-1} g protein^{-1}. NEM remained an effective inhibitor of ONP reaction rates for protoplasts which established that the NEM-sensitive system was located in the plasma membrane.

Discussion

Both bioavailability and uptake of chemicals influence the kinetics of substrate degradation and of cell growth [3,5,19,25]. An implicit assumption in determining apparent K_s or K_m values is that uptake rates limit growth or degradation. Previously, we reported a compilation of kinetic constants for the reaction rates of a series of substituted 2-nitrophenols [10]. Differences observed between cellular

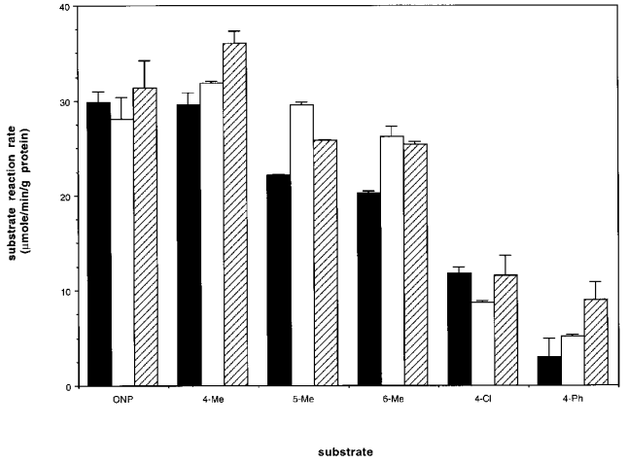

Figure 3 Reaction rates for various substituted nitrophenols using untreated cells, mureinoplasts and protoplasts. The three cell suspensions were prepared as described in the Materials and methods. Aliquots of each set of treatments were used to determine rates of reaction for various substituted nitrophenol as indicated (100 μM) in GP buffer. Reaction rates for each compound were determined in triplicate and reported μmol min^{-1} g protein^{-1} for intact cells (▩), mureinoplasts (□) and protoplasts (▨).

and enzymatic values for K_s, K_m and V_{max} for intact cells and purified nitrophenol oxygenase suggested the potential for an uptake limitation. Higher V_{max} values determined for enzyme extracts could arise from higher NADPH, oxygen, or nitrophenol concentrations used *in vitro* than available naturally when the enzyme is located within the cell. Lower oxygen or nitrophenol concentrations *in vivo* could be due to uptake limitations. Lower NADPH concentrations could be due to the naturally lower redox potential within the cell. This study focused on evaluating uptake limitations for nitrophenol.

In support of the theory that nitrophenol uptake limited reaction rates, it was also observed that reaction rates steadily increased over the first 30 s after the addition of ONP to a cell suspension. This suggested that intracellular concentration of ONP increased during this time, eventually achieving a steady state balance between uptake rates and reaction rates. ONP uptake rates were estimated to be quite rapid, either greater than or equal to the rate of reaction for nitrophenol. During these experiments, catabolic intermediates did not accumulate to significant levels and would not inhibit the initial oxidation of nitrophenol. No product inhibition had been observed during the characterization of kinetic parameters [10]. ONP degradation was limited either by the initial catabolic enzyme, the nitrophenol oxygenase, or by uptake.

Direct methods for monitoring substrate uptake were pursued in order to determine whether uptake of nitrophenol into the cell limited biodegradation rates. These experiments were unsuccessful for two primary reasons (data not shown). First, uptake rates were apparently quite rapid. Secondly, ONP partitioned strongly into both cellular components and adsorbed onto the membrane filters which made it difficult to clearly determine changes in cytoplasmic ONP concentrations. Direct methods for assessing uptake have been difficult for many classes of compounds. Differentiating association of naphthalene to cell components and uptake with *Pseudomonas putida* led the authors to report a combined rate since they could not separate individual activities [1]. Phenol was non-specifically absorbed by active and inactive phenol degraders [13]. At best, uptake experiments for chemicals which adsorb to cellular components will be a combination of two rates, sorption and translocation into the cytoplasm.

Due to the difficulties encountered in directly monitoring uptake, indirect methods were pursued. Chemical modifying agents have been powerful tools in the study of enzyme structure and function. Under conditions where substrate turnover by cytoplasmic enzymes is much faster than uptake rates, reaction rate can be used to monitor uptake rates [24]. Two uptake mechanisms, facilitated and active uptake, are generally protein-mediated. Furthermore, enzymes often contain critical amino acids susceptible to chemical modification resulting in lowered activity. If uptake is protein-mediated, then chemical modification can lead to a loss of activity. If uptake is by a diffusion mechanism through the lipid bilayer, then chemical modification would not likely affect uptake rates. Sulfhydryl-directed protein modifying agents were selected to test this hypothesis due to the reported sensitivity of a number of transport proteins to these agents [2,4,9,12,24].

NEM was selected to test the possibility that ONP uptake was protein-mediated. NEM was shown to inhibit reaction rates for ONP by more than 80% yet succinate specific oxygen consumption rates were inhibited by less than 20%. Decreased oxygen consumption rates could have resulted from the inhibition of the succinate transport protein, shown previously to be sensitive to another sulfhydryl-directed chemical modifying agent, iodoacetamide [9]. However, this lowered activity was deemed too small to account for the 80–90% loss in ONP reaction rate. It was concluded that NEM interfered specifically with some aspect of ONP degradation. Cell-free enzyme extracts prepared from NEM-treated cells retained greater than 90% of its nitrophenol oxygenase activity, the first catabolic pathway enzyme. *In vitro* enzyme activity was not adversely affected when cells were treated with NEM because chemical action was apparently at the cell surface. Under the experimental conditions for establishing initial turnover kinetics for ONP, $<$20% conversion of substrate to product, there was no significant accumulation of pathway intermediates or unique catabolites inhibiting conversion of ONP to catechol, as indicated by linear rates of substrate disappearance. Consequently, NEM treatment of intact cells must inhibit degradation of ONP prior to the initial conversion of ONP to catechol by the nitrophenol oxygenase. Though we have not yet identified the specific protein linked to ONP uptake, NEM probably acts by inhibiting such a protein. This NEM-sensitive transport protein was located in the plasma membrane since NEM expressed the same degree of inhibition on intact cells and on protoplasts.

Results presented here also indirectly establish that uptake limited the rate of biodegradation. As the polysaccharide, outer membrane and peptidoglycan layers were stripped from the cell, reaction rates actually increased for certain substituted nitrophenols tested. When considering that formation of mureinoplasts and protoplasts can lead to cell lysis [11], the increase in measured reaction rates was probably less than if all of the cells had remained active. As cell layers were removed, uptake rates actually increased resulting in faster reaction rates for nitrophenol. The murein and outer membrane layers act as permeability barriers by limiting diffusion of chemicals into the cell as previously reported [6,15,22].

Thermal adaptation of organisms often leads to changes in the lipid and lipopolysaccharide composition of the various permeability barriers in the cell [21]. These changes in composition affect the fluidity of plasma membranes. Growth of cells at 20°C led to higher specific activities and lower activation energies than for cells grown at 30°C for conversion of ONP, 4-Me and 4-Ph to the corresponding substituted catechols. These results are consistent with an increased membrane fluidity for cells grown at 20°C than for cells grown at 30°C when both sets of cells were assayed at a common temperature. Since (1) E_a values for cells were either less than or equal to that for enzyme extracts, and (2) E_a determinations are for the rate limiting step, these results further support the conclusion that uptake limited nitrophenol reaction rates, not only for NEM-treated cells but also for intact cells as well.

Since cellular reaction rates for different substituted nitrophenols were inhibited to differing degrees by NEM inhibition, two conclusions could be drawn. First, there appeared to be at least two mechanisms for the uptake of substituted 2-nitrophenols into the cell, one sensitive and one insensitive to inhibition by NEM. The NEM-sensitive system demonstrated differential affinity for the various substituted nitrophenols. NEM treatment led to a 92% drop in ONP reaction rates whereas it inhibited reaction rates of 5-Me, 6-Me and 6-Cl by less than 30%. Secondly, following NEM treatment, residual activities were more uniform for all compounds tested which suggested that a second uptake component was by a more general mechanism, such as diffusion across a lipid bilayer. This second uptake pathway demonstrated a lower selectivity for size, electron withdrawal, or hydrophobicity of the substrate. There were no apparent correlations observed between uptake rates and these parameters for the nitrophenols tested which could be used to indicate selectivity towards either anion or neutral chemical species. Consequently, cellular K_s and V_{max} values reported previously [10] apparently describe attributes of this uptake system.

The data presented in this paper support the conclusion that uptake of nitrophenols into the cell limit reaction rates of those chemicals in *Pseudomonas putida* B2. Translocation of substrate from the bulk solution into the cytoplasm was shown to be retarded by the polysaccharide sheath, outer membrane, and plasma membrane. NEM inhibition results suggest the existence of a protein-mediated transport component located in the plasma membrane. The data also indicate a secondary, non-specific component, presumably diffusion across the plasma membrane lipid bilayer. Future work could incorporate the use of radiolabeled NEM to directly confirm whether transport is protein-mediated. In addition, certain antibiotics could be employed to investigate the energy dependence of this process as long as cytoplasmic NADH pools were not disrupted leading to loss of nitrophenol oxygenase activity.

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