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Increased bacterial uptake of macromolecular substrates with fluid shear

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INCREASED BACTERIAL UPTAKE OF MACROMOLECULAR SUBSTRATES WITH FLUID SHEAR

by

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ABSTRACT

To investigate the effect of fluid shear on uptake rates of low diffusivity macromolecular substrates by suspended cultures, I measured the radiolabel and oxygen uptake of two model compounds, bovine serum albumin and dextran, in pure cultures of Zoogloea ramigera and E. coli, respectively. Oxygen utilization rates of stirred samples grown on BSA and dextran were 2.3 and 2.9 times higher, respectively, than undisturbed (still) samples. Uptake rates of [^{3}H]BSA and [^{3}H]dextran by stirred samples were 12.6 and 6.2 times higher, respectively, than still samples. Uptake rates of low-molecular-weight substrates with high diffusivities, such as leucine and glucose, were only slightly affected by fluid shear. Since macromolecules can comprise a major portion of bacterial substrate in engineered, laboratory, and natural systems, the demonstrated effect of fluid shear has wide implications for the evaluation of engineered bioreactors used for wastewater treatment as well as for kinetic studies performed in basic metabolic research.

1.0 INTRODUCTION

Macromolecular compounds comprise a major portion of dissolved organic carbon in both engineered and natural systems. In a study of domestic wastewater, sixty percent of dissolved organic carbon was in compounds greater than 1000 amu (Grady, et al., 1984). Eighty percent of the total amino acids in a North Pacific Ocean survey were found to be macromolecular, predominantly in the molecular weight range 1500 to 50,000 (Sugimura and Suzuki 1983).

Classical kinetic models, such as the Michaelis-Menton and Blackman models, assume uptake is only a function of enzyme and substrate concentration. They do not accurately reflect the physics and biology of macromolecular metabolism because they do not account for mass transfer limitations or the complexity of hydrolysis and transport. This inaccuracy is conspicuous when negative values for the kinetic parameters μ_{max} and K_s are regularly obtained in wastewater systems (Saunders and Dick 1981). The contribution of mass transfer and hydrolysis and transport to overall macromolecular metabolism needs to be determined to adequately predict the efficiency of wastewater treatment systems.

The rate of substrate transport to the cell (mass transfer) can be a limiting factor in nutrient uptake (Logan and Dettmer 1990, Logan and Kirchman 1991). Macromolecules have low diffusion coefficients, and, at low substrate

concentrations and high substrate utilization rates, diffusive transport due to Brownian motion may not be sufficient to prevent the development of concentration gradients around cells (Berg and Purcell 1977). The rate limiting step in macromolecule utilization may then become mass transfer across this boundary layer. The different fluid mechanical environments in which aquatic microorganisms exist- quiescence, advection and fluid shear- can affect this rate of mass transfer.

It has been calculated that fluid motion would increase substrate mass transfer (Logan & Hunt 1987). Advective flow past cells has been shown to increase substrate uptake. [^{3}H]Leucine uptake rates of Zoogloea ramigera fixed on filter membranes in a 1mm s^{-1} advective flow field were found to be 55-65% higher than leucine uptake rates by suspended cells (Logan and Dettmer 1990). In contrast, laminar fluid shear rates less than 50 s^{-1} were found to have no discernable effect on uptake of compounds with low molecular weight, and therefore, high diffusion coefficients, such as leucine and glucose (Logan and Dettmer 1990, Logan and Kirchman 1991). However, it has been predicted that fluid shear could increase bacterial uptake of low diffusivity, macromolecular substrates at similar shear rates (Purcell 1978, Logan and Hunt 1987, 1988).

Once macromolecules reach the cell surface they are metabolized much differently than small molecules. Bacteria do not assimilate intact macromolecules, but must first enzymatically hydrolyze them into subunits which can be transported into the cell (Ames 1986). Therefore, any compound that must be cleaved into subunits before uptake is defined here as a macromolecule.

The purpose of this investigation was to demonstrate that fluid shear can increase uptake rates of macromolecular substrates by suspended cultures. Using pure bacterial cultures, I examined uptake rates of two types of model macromolecules, a protein (bovine serum albumin) and a polysaccharide (dextran). Uptake rates were determined in mixed (shear) and undisturbed (still) reactors using both radiolabeled compounds and oxygen consumption. The effects of fluid shear on macromolecular uptake were contrasted to those obtained in identical studies using representative monomers of protein (leucine) and polysaccharides (glucose).

2.0 LITERATURE REVIEW

2.1 GENERAL

This section includes several topics relevant to fluid motion effects on bacterial nutrient uptake. First, the occurrence and importance of macromolecules in engineered and natural systems is reviewed. Second, information is presented on the mechanism of macromolecule metabolism. Sections are included on: (i) the extracellular enzymes necessary for macromolecule hydrolysis, (ii) the structure of the Gram-negative bacterial cell wall through which the hydrolyzed macromolecular fragments must pass, and (iii) a currently accepted model which describes a mechanism of solute transport across the Gram-negative cell wall. Gram-negative cells were used in this study and are more abundant than Gram-positive cells in both wastewater treatment systems and natural environments. Third, the effect of fluid motion on the mass transfer of macromolecules to cells is then described. Several studies are presented that address the location of macromolecular hydrolysis (whether it is cell-associated or occurs in the bulk solution) in the context of the effect of fluid motion on mass transfer. The literature review concludes with two studies that indicate macromolecules are preferred microbial substrates.

2.2 IMPORTANCE OF MACROMOLECULES

2.2.1 Wastewater treatment applications

Studies on the molecular weight distribution of soluble organic components in wastewater have emphasized the importance of macromolecules and indicate the size distribution is sample site specific. Levine et al. (1985) found substantially different mass distributions in primary effluent obtained from two different locations. In one instance, the soluble organics were determined to have an average molecular weight of 10,000, whereas samples from the second site had a bimodal distribution with peaks at average molecular weights of 10,000 and 200,000. In other studies of domestic wastewater, 50 to 60% of soluble organic carbon (SOC) was in compounds greater than 1000 amu (Grady et al. 1984; Logan and Jiang 1990). Compounds with molecular weights in excess of approximately 1000 can be considered macromolecular, because as discussed below, polypeptides and polysaccharides greater than six or seven monomeric units (approximately 1000 amu) cannot be assimilated directly but must be hydrolyzed into monomers or smaller polymers prior to assimilation.

In addition to the macromolecules present in wastewater, when activated sludge cultures are grown on low molecular weight substrates (<1000 amu) there can be microbial production of significant amounts of dissolved large molecular weight organics (>10,000 amu) (Grady et al. 1984). Many of

these large molecular weight organics are polysaccharides thought to be important mediators of cell attachment (Characklis 1981). In addition to attachment functions which are important for floc formation in activated sludge and biofilm formation in trickling filters, these microbially produced macromolecules can become degradable substrate (Obayashi and Gaudy 1973).

There exists the potential for the abundance of macromolecules in a wastewater to affect the efficiency of the treatment process. In activated sludge treatment, the flux of substrate to a floc, and diffusion within a floc, are directly proportional to the diffusivity of the substrate (Bailey and Ollis 1977). The lower diffusion rates of macromolecular substrate as compared to low molecular weight substrate may therefore result in lower removal rates for macromolecules. Similarly, in trickling filters substrate mass transfer (via diffusion) to and within the biofilm decreases as molecular weight increases which may result in lower removal rates for macromolecules (Logan et al. 1987a,b).

2.2.2 Natural systems

In natural systems, macromolecules are available as the products of primary production (large molecular weight algal proteins and polysaccharide exudates), dead biomass awaiting decomposition and discharges from anthropogenic sources.

Gjessing (1970) calculated 50% of aquatic humus had an apparent molecular weight greater than 20,000 amu. Eighty percent of the total amino acids in a North Pacific Ocean survey were found to be in combined form, predominantly in the molecular weight range 1500 to 50,000 (Sugimura and Suzuki 1983). Manka and Rebhun (1982) estimated 61% of a secondary wastewater treatment facility effluent (dischargeable to natural aquatic systems) contained organic compounds with apparent molecular weights greater than 20,000 amu. Amy (1987) found that of the SOC in trickling filter effluent, 43% in winter samples and 50% in spring samples was greater than 5000 amu. In conventional activated sludge effluent 69% of winter sample SOC and 38% of spring sample SOC was greater than 5000 amu.

2.3 EXTRACELLULAR ENZYMES

Bacteria are primarily responsible for the decomposition of dead biomass in aquatic environments, but it is generally accepted that they do not assimilate exogenous proteins intact as has been noted in some eukaryotes (Dautry-Varsat and Lodish 1984), but must first cleave them to their component amino acids or peptide fragments before transporting them through the cell membrane. The largest oligopeptide that can be transported into the cell intact is approximately seven amino acids (Law 1980). Entire proteins synthesized by bacteria are

excreted from cells, however, so an internal to external transmembrane protein transport system must exist.

Since bacteria must first hydrolyze macromolecules prior assimilation, they need a mechanism for this process. Extracellular enzymes accomplish this function. Enzymes may exist extracellularly due to two processes. First there are cytoplasmic enzymes that leak from intact cells or are released after cell lysis and may retain activity (Burns 1982). Second, there are extracellular enzymes which are excreted purposefully by normally metabolizing cells (Burns 1982). In this study, we are concerned with the second case. Since there seems to be no standard terminology that describes the functional locations of extracellular enzymes, several definitions are presented as they are used in this paper. An extracellular enzyme is any enzyme that contains an active site accessible to substrate in bulk solution. Extracellular enzymes can be categorized as either attached or exocellular. An attached extracellular enzyme is any extracellular enzyme that is in some manner bound to the cell. An exocellular enzyme is any extracellular enzyme that exists in bulk solution, attached neither to the cell nor to other colloidal or particulate matter.

According to the signal hypothesis (Priest 1984), extracellular enzymes are thought to possess a variable length (15- 32 amino acids) amino terminus segment which acts as a

signal to induce membrane binding. Membrane binding occurs and the nascent protein is secreted through the cell membrane as translation occurs. The signal sequence is then cleaved with an endopeptidase, releasing the protein. Often an enzyme is secreted as an inactive precursor which is subsequently modified to produce the active form (Priest 1984).

A notable characteristic of extracellular enzymes is that they have a very low frequency of cysteine in their amino acid compositions (Law 1980). A low incidence of disulfide bonds in a protein would confer upon it a high degree of flexibility which would allow it to be more easily extruded through the cytoplasmic membrane.

The production of some extracellular enzymes is under metabolic control (Law 1980, Burns 1982). Bacillus lichenformis produces maximal amounts of an extracellular protease under nitrogen-limited conditions (Wouters & Buysman 1977). One exocellular protease, produced by a Pseudomonas sp., causes proteolysis in fish skin lesions (Li & Flemming 1967). Group N streptococci, Lactobacillus bulgaricus and E. coli, among others, are reported to possess attached extracellular proteases (Law 1980). Except in specific instances in which bacteria remain in intimate contact with the substrate or exist in dense aqueous culture, cell-bound enzymes have an advantage over exocellular enzymes in that hydrolyzed peptides and amino acids can be directly exploited

by bacterial transport systems. The nutritional advantage is gained by the specific microbe which expends the energy to produce the protease.

In addition to a role in substrate uptake, numerous proteases located in the outer membrane appear to play a role in processing cellularly produced enzymes. MacGreggor, et al. (1979) presented evidence for the existence of an outer membrane protein in E. coli which cleaves one of the subunits of nitrate reductase, a cytoplasmic membrane protein, and possibly other cytoplasmic membrane and outer membrane proteins. Nitrate reductase is an electron-transport-chain enzyme used during anaerobic respiration when nitrate is used as a terminal electron acceptor. It is composed of three subunits and is synthesized as a pro-enzyme. The outer membrane protease apparently cleaves off a portion of the ß-subunit to produce the final, active form of the enzyme.

Sugimura and Higashi (1988) have isolated an outer membrane protease that degrades human gamma interferon produced by recombinant E. coli. The proteolytic activity is present in both the recombinant and other E. coli strains. This protease exhibited the ability to cleave other proteins and peptides, with strict specificity for cleavage between consecutive basic amino acids.

2.4 STRUCTURE OF GRAM NEGATIVE BACTERIAL CELL WALL

2.4.1 General

It is important to describe the structure of the component parts of the Gram-negative cell wall because nutrients and other substances which enter Gram-negative cells must traverse this complex structure. The Gram-negative cell wall is composed of three distinct layers, which from outside to inside are the outer membrane, peptidoglycan layer, and inner or cytoplasmic membrane. The space between the outer and inner membrane is referred to as the periplasmic space and contains the peptidoglycan layer. In addition, some bacteria secrete a polysaccharide slime layer or capsule which may encase the entire cell. The following sections describe in detail the chemical and structural makeup of these cell wall components. The structure and function of gram-negative bacteria have been studied most extensively in Escherichia coli and the following descriptions, although representative of Gram-negative bacteria in general, are specific to E. coli unless otherwise noted. A depiction of the Gram-negative cell outer membrane-peptidoglycan complex is presented in Figure 2.1 (Nikaido and Nakae 1979).

2.4.2 Outer membrane

The structural configuration of the outer membrane is an asymmetric bilayer of two major types of lipids,

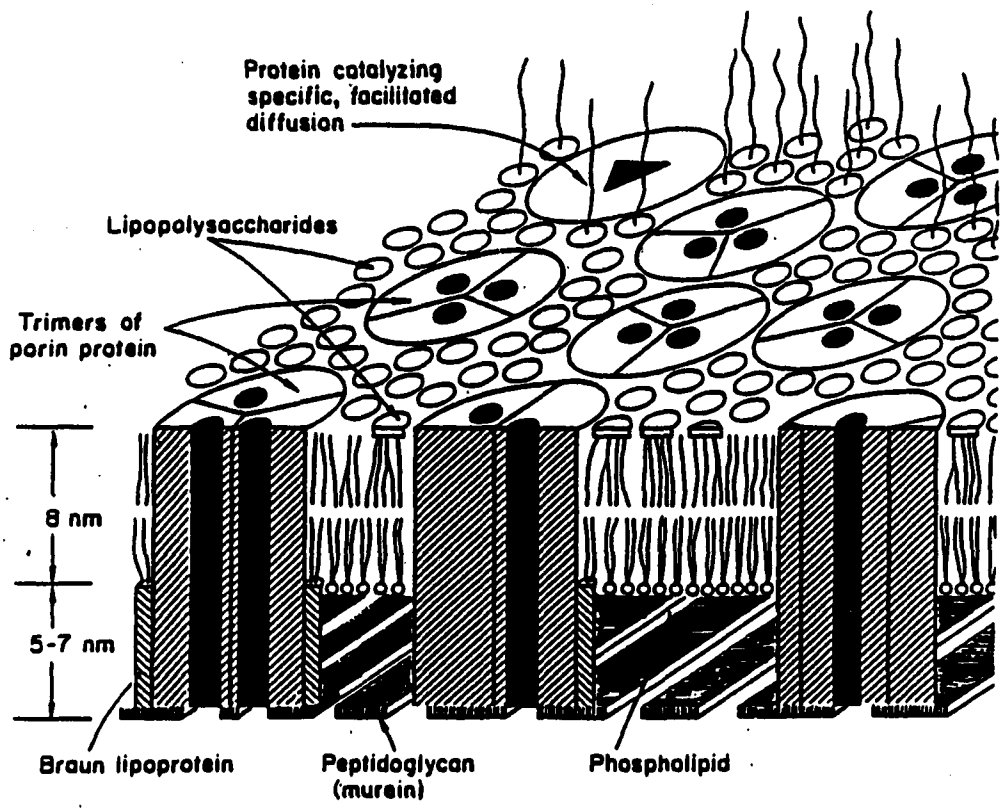

Figure 2.1: Schematic drawing of the outer membrane-peptidoglycan complex in E. coli (Nikaido and Nakae 1979).

lipopolysaccharides and phospholipids. The lipopolysaccharides are oriented to the exterior of the cell; the phospholipid portion of the bilayer faces the periplasmic space. Lipopolysaccharides are unique to the outer membrane. They are present elsewhere only as intermediates in biosynthetic pathways. Structurally, lipopolysaccharides are composed of a hydrophilic core oligosaccharide, a hydrophobic lipid region oriented towards the cell interior and composed of six or seven saturated fatty acids on a glucosaminyl-ß(1-6)glucosamine backbone, and a hydrophilic, O antigen polysaccharide oriented towards the cell exterior. The phospholipid composition of the outer membrane is slightly richer in phosphatidylethanolamine, but otherwise similar to the phospholipids of the cytoplasmic membrane (see below).

Contained within the lipid bilayer are numerous proteins. Many of these proteins span the outer membrane bilayer and are responsible for transport of substances through the outer membrane as discussed below. Another protein, thought to be involved in amino acid transport, is the OmpA protein (Omp stands for outer membrane protein.) Structurally, it consists of two relatively rigid domains separated by a flexible hinge region. The N-terminal domain is embedded in the outer membrane, the hinge region at the inner surface of the outer membrane, and the remainder of the protein projects into the

periplasmic space. There are approximately $2x10^5$ transport proteins per E. coli cell (Nikaido and Varra 1987).

Murein lipoproteins (approximately $7x10^5$ per cell) are covalently bonded to the peptidoglycan layer at the carboxyl terminus. The remainder of the protein portion spans the space between the peptidoglycan layer and the outer membrane. The lipid portion is bound to the n-terminus and extends into the outer membrane lipid bilayer, effectively anchoring the outer membrane to the peptidoglycan layer.

Phospholipase and protease enzymes have also been identified in the outer membrane (Fiss et al. 1979, MacGregor et al. 1979).

2.4.3 Peptidoglycan layer

The peptidoglycan (murein) layer of Gram-negative bacteria is thinner than that of Gram-positive bacteria. It confers rigidity to the cell and is considered to be permeable to both hydrophobic and hydrophilic compounds (Ames 1986). It is composed primarily of N-acetyl glucosamine and N-acetyl muramic acids cross linked with short peptides (Park 1987).

2.4.4 Cytoplasmic membrane

The cytoplasmic membrane is an effective barrier to virtually all hydrophilic molecules except water itself (Cronan et al. 1987). Also able to pass relatively unhindered are medium-chain fatty acids (Maloy et al. 1981). All other

types of hydrophilic molecules require cytoplasmic membrane transport proteins to enter the cell (Cronan et al. 1987).

Structurally, the cytoplasmic membrane is a phospholipid bilayer composed of three main phospholipid types, phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and diphosphatidylglycerol or cardiolipin (CL), as well as several minor phospholipids. PE accounts for approximately 75% of cytoplasmic membrane phospholipid content with the relative amounts of the other components dependent upon the growth phase of the cell. Lipid chains consist of the saturated fatty acids, hexadecanoic (palmitic) and tetradecanoic (myristic) and the monounsaturated fatty acids cis-9-hexadecanoic (palmitic) and cis-11-octadecanoic (cis-vaccenic)(Cronan and Rock 1987). The phospholipids are arranged in the bilayer with their hydrophilic phosphate "heads" toward the outside and the hydrophilic lipid "tails" toward the inside. This is the thermodynamically most stable configuration for phospholipids in aqueous solution. Purified phospholipids assume this configuration in aqueous solution to form synthetic bilayers.

The cytoplasmic membrane also contains at least 100 different types of proteins (Cronan et al. 1987). The proteins can be functionally divided into two major categories, those proteins which are components of the membrane bound electron transport system, and those proteins

involved in cytoplasmic membrane solute transport, which is discussed below. Also contained within the cytoplasmic membrane are quinones, non-protein components of the membrane bound electron transport system.

2.5 SOLUTE TRANSPORT SYSTEMS

In a recent article, Ames (1986) presented a theory of solute transport systems in Gram-negative bacteria. The individual components and mechanisms of the maltose and histidine transport systems are most completely understood, and evidence is accumulating that indicates that the same type of transport system also works for other substrates including oligopeptides (eg. leu-isl-val) and maltodextrins, glucose polymers up to six or seven glucose residues long.

Features of the transport models described by Ames (1986) are presented in Figures 2.2 and 2.3. Transport channels that are comprised of proteins span the outer membrane. These channels can either be specific, allowing the passage of nutrients based on chemical composition, or non-specific (in which case they are called porins) and allow transport based primarily on molecular size (Benz 1988). Since transport through these channels is passive, and driven by a concentration difference across the outer membrane, no energy is expended. Nutrients or antibiotics which are highly hydrophobic cannot diffuse through the lipid bilayer of the outer membrane and must be

transported through protein channels. There is, however, a degree of control which the cell exerts over these channels. For example, there exists a porin in Pseudomonas aeruginosa which allows passage of solutes with molecular weights up to 5000 daltons. However, P. aeruginosa is highly resistent to antibiotics which have much lower molecular weights. This indicates there is some mechanism by which porins can be selectively opened or closed (Benz 1988). Phosphate transport in Enterobacteriaceae is mediated by anion-selective protein channels. The synthesis of these proteins is inducible under low phosphate conditions as part of the pho regulon which concurrently induces periplasmic and cytoplasmic membrane proteins required for enhanced phosphate uptake (Benz 1988).

Once the solute is in the periplasmic space, it is reversibly bound to a specific transport protein. This binding lowers the effective periplasmic solute concentration and maintains a solute concentration gradient across the outer membrane. This promotes diffusive solute transport across the outer membrane. The binding of solute to the periplasmic protein causes a conformational change in the protein. This transport protein then delivers the bound solute to a cytoplasmic membrane transport system. The interaction between the periplasmic transport protein-solute complex and

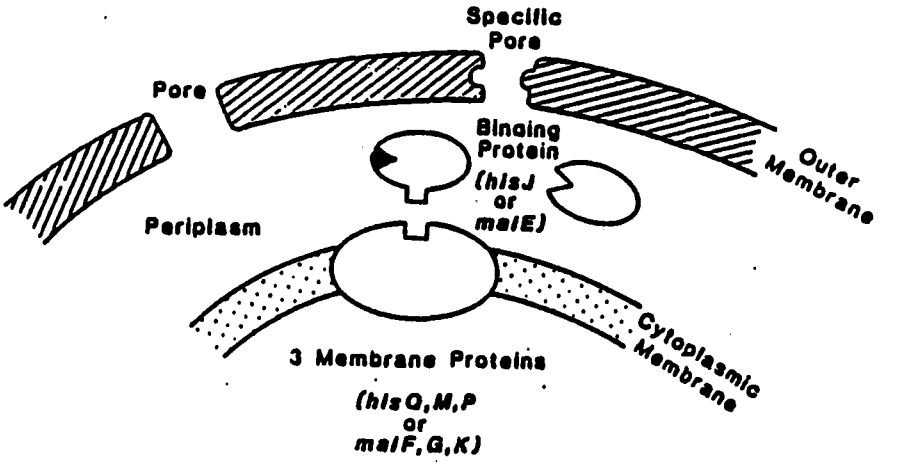

Figure 2.2: Schematic representation of the histidine and maltose permeases (Ames 1986).

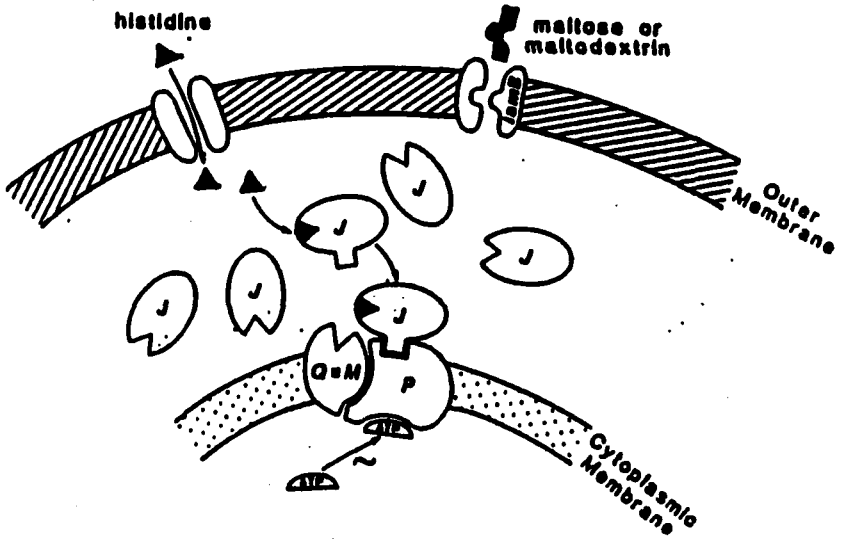

Figure 2.3: Schematic representation of the histidine periplasmic transport model (Ames 1986).

the cytoplasmic membrane transport system is thought to involve direct interaction between periplasmic and cytoplasmic proteins rather than only solute-cytoplasmic transport protein interaction. For example, mutants that produce incomplete histidine periplasmic transport proteins, but which do contain an intact histidine binding site, are unable to assimilate histidine (Kustu & Ames 1974).

All well characterized cytoplasmic membrane-bound transport systems consist of three proteins, two of which are extremely hydrophobic (Ames 1986). Membrane bound transport systems are difficult to study due to the difficulty in isolating, purifying and then reconstituting an operable enzyme complex. Energy input is required for the complex to function because cells concentrate substrate inside the cytoplasmic membrane, but the source of energy has not been conclusively determined. The involvement of ATP, acetyl-phosphate and proton motive force have all been suggested. Several studies indicate that rather than one energy mediator, a combination of factors such as high proton motive force and acetylphosphate, (or an acetylphosphate derivative) as well as the overall metabolic requirements of the cell are determinants of nutrient transport (Berger & Heppel 1974, Hong et al. 1979, Ames 1986).

2.6 EFFECT OF FLUID MOTION ON NUTRIENT UPTAKE

2.6.1 General

The effect of fluid motion on nutrient uptake has been studied most extensively in the context of natural aquatic systems. The two types of fluid motion, advection and shear, can be found in numerous natural environments. Attached bacteria and algae in rivers and streams experience an advective flow regime. Planktonic diatoms and particles of marine snow (aggregates of bacteria and/or detrital material) sink through the water column and also experience advective flow. Suspended cells can experience shear environments in turbulent rivers and streams and in the waves and Langmuir spiral currents of standing waters.

2.6.2 Advection

It has been found that advective flow can affect microbial kinetics. An increase in the uptake of phosphate by the planktonic diatoms Thalassiosira fluviatilis and Thalassiosira pseudonana in advective flow was reported by Canelli and Fuhs (1976). Cells were held on filters and subjected to advective flow of a solution containing ^{32}P labeled phosphate. Phosphate uptake was measured by liquid scintillation counting. Uptake rates increased with increasing flow rate up to a maximum uptake value. Further flow rates increases had no effect on uptake rates.

Diatom blooms commonly form aggregates of metabolizing cells and sink through the water column in response to nutrient-limited conditions (Alldredge and Silver 1988). One explanation for this phenomenon may be that there is a nutritional advantage for existing in aggregates. Diatom aggregates are highly porous, with porosities calculated to be in the range of 0.99931 to 0.99984 (Logan and Alldredge 1989). Because of this high porosity there is significant advective fluid flow within the aggregate as it sinks through the water column or encounters fluid shear (Logan and Hunt 1987). This advective flow can lead to the increased uptake of nutrients from the bulk fluid (Logan and Hunt 1987, Logan and Alldredge 1989). Logan and Alldredge (1989) calculated that a diatom aggregate undergoing gravitational settling takes up nutrients up to 2.1 times faster than dispersed individual diatoms.

Using a heterotrophic marine bacterial assemblage, Logan and Kirchman (1991) found [^{3}H]leucine uptake rates by fixed cells exposed to an advective flow field of 0.23-0.81 mm s^{-1} eight times higher than uptake rates of cells in a 0.035 mm s^{-1} field (Logan and Kirchman 1991). Therefore, it has been experimentally shown in numerous studies that advection does affect microbial uptake kinetics.

2.6.3 Fluid shear

Fluid shear has been predicted to affect substrate uptake by dispersed cells (Purcell 1978). A bacterial cell is

substantially smaller than the Kolmogorov microscale of turbulence encountered in natural aquatic habitats or mixed reactors and exists in a laminar, rather than turbulent, shear environment. More precisely, a bacterium in a mixed reactor encounters a variety of laminar shear intensities with some average value. This value of shear, G, can be defined as

$$G = \left(\frac{\varepsilon_t}{\nu}\right)^{1/2} \quad (1)$$

where ε_t is the turbulent energy dissipation rate per unit mass and ν is the kinematic viscosity. Shear rates of approximately $10^6 s^{-1}$ - much higher than those typical of the natural environments or engineered bioreactors- would be required to reduce the microscale of turbulence to the size of a bacterium (1 μm) (Purcell 1978). The size of the Kolmogorov microscale, λ, is

$$\lambda = \left(\frac{\nu}{\varepsilon_t}\right)^{1/4} \quad (2)$$

Pasciak and Gavis (1975) used a rotating shear device to study the effects of laminar shear on nitrate uptake by the diatom Ditylum brightwelli. They found that uptake was proportional to shear up to approximately $G = 4\ s^{-1}$ at which point nitrate uptake was approximately 1.1 times the non-mixed

uptake rate. Higher shear rates did not further increase uptake.

2.7 LOCATION OF HYDROLYTIC ACTIVITY

A fundamental assumption of the concept of a mass transfer limitation for macromolecular substrate utilization by bacteria is that the hydrolysis of substrate into membrane transportable subunits is cell-associated. If hydrolysis were to occur in bulk solution, it would be the small molecular weight, high diffusivity hydrolysis fragments which were transported to the cell, and it would be predicted that there would be no uptake enhancement with fluid shear. Numerous studies support the attached hydrolysis hypothesis. Two different approaches have been used by investigators to study this problem. In both approaches, samples are divided into cell-containing or cell-free subsamples. Hydrolysis rates in subsamples are compared. One approach measures the hydrolysis or uptake of radiolabeled macromolecular substrates. The other approach uses artificial fluorogenic substrate analogs of macromolecular substrates. In this technique a fluorogenic molecule, such as 4-methylumbelliferyl (MUF), is bound to a substrate monomer. For a carbohydrate analog, glucose is bound to MUF with a glycosidic linkage. For a protein analog the MUF is bound to an amino acid with a peptide-like bond. The bound MUF is non-fluorogenic, but upon hydrolysis of the

peptide or glycoside bond fluorogenic MUF is released and can be measured with high sensitivity in alkaline solution. The appearance of fluorescence allows measurement of hydrolysis rates.

Hollibaugh and Azam (1983) examined the mechanisms by which marine bacteria assemblages utilize dissolved protein and amino acids. They used hydrolysis rate experiments, competition experiments with amino acids and small peptides, and size fractionation experiments with protein degradation products to determine whether protein hydrolysis was cell associated and whether hydrolysis products were released back into the bulk water or retained in the pericellular environment. They used ^{125}I-BSA as a model protein to investigate whether protein hydrolysis occurred in bulk seawater or in the pericellular environment and found that cell-free subsamples had only 0.2% of the proteolytic activity observed in the cell-containing subsample. This is evidence for pericellular protein hydrolysis. They used amino acids and small peptides in competition experiments and found that they did not effectively inhibit BSA hydrolysis. If the hydrolyzed protein fragments were released freely into the bulk solution, effective amino acid and peptide competitive inhibition of labeled BSA uptake would be expected. They used Sephadex G-100 and G-10 gels to fractionate protein hydrolysis products and found a gradual decrease in the 68,000 amu BSA

peak and a gradual increase in the less than 700 amu peak. There was no accumulation in an intermediate size range. Considered together, evidence for pericellular protein hydrolysis, evidence that amino acids and small peptides do not effectively competitively inhibit BSA uptake, and the low molecular weight of hydrolysis products strongly support an uptake mechanism which involves close pericellular protein association from initial binding through protein hydrolysis and membrane translocation.

In a study of extracellular protease activity in an intertidal mudflat, Mayer (1989) demonstrated that 99.5% of the total proteolytic activity was associated with the particulate phase and that only 0.5% was associated with the pore water. Using MUF-alanine, proteolytic activity at various sediment depths was measured. Native substrate availability and bacterial numbers were also determined. There was a positive correlation between native substrate availability, bacterial numbers, and measured proteolytic activity. Enzyme inhibitor studies indicated that the extracellular proteases were metalloproteases and thiol proteases; serine proteases were not present. The experimental methods used did not distinguish between cell bound proteases and exocellular enzymes which may have been adsorbed to particulate matter.

Using 4-methylumbelliferyl-ß-D-glucopyranoside (MUFGlc) as a carbohydrate analog, Chróst (1989) analyzed the ß-glucosidase activity (ßGlcA) in the Plußsee, a naturally eutrophic lake in northern Germany. On average, 90% of the ßGlcA was found in the 0.2-3.0 µm size fraction. This fraction consisted predominately of bacteria with only an estimated 2.3 ± 0.5% of phytoplankton chlorophyll a indicated using chlorophyll a measurements. The ßGlcA of the cell free (< 0.2 µm) fraction contained an average of 3.4% of ßGlcA. These results indicate that in this natural system 90% of carbohydrate hydrolysis is cell-associated.

In a study of extracellular enzymatic activity in two Baltic Sea fjords, Hoppe (1983) found only 7% of extracellular proteolytic activity to be associated with the cell free (< 0.2 µm) fraction. Extracellular carbohydrate hydrolysis, however, was found predominately (61%) in the cell-free fraction.

Somville and Billen (1983) found that in an English Channel water sample approximately 80% of proteolytic activity was cell associated whereas in a eutrophic Belgian coastal zone of the North Sea, most proteolytic activity was in the cell-free fraction.

The location of extracellular hydrolysis is apparently a complex problem, which is to a large degree specific to the sampling site. Oligotrophic waters tend to have bound

hydrolytic enzymes. This makes sense from a teleological viewpoint as the cost to the bacterium in energy and carbon to make and export hydrolytic enzymes would outweigh the possible benefit received from assimilable hydrolyzed substrate. Under eutrophic conditions, however, the situation is more complex as there is the possibility of a net gain in carbon and energy from the release of hydrolytic enzymes. Therefore, results appear to be site specific and cannot be completely generalized.

2.8 PREFERENCE FOR MACROMOLECULES

Although bacterial assimilation of low molecular weight monomeric substrates is a less complex process than assimilation of macromolecules, Hollibaugh and Azam (1983) have indicated that heterotrophic seawater bacteria preferentially utilize large proteins with an affinity of proteins > large peptides > small peptides and amino acids. Similarly, Kirchman and Hodson (1984) demonstrated that bacteria from a freshwater lake (Lake Oglethorpe, Georgia) and an estuary at Andros Island, Bahamas preferentially utilize dipeptides as compared to amino acids. One explanation for the preference for larger substrate molecules is that the energy required for substrate transport is similar irrespective of the peptide length, but the metabolic benefit derived from peptide transport increases in direct proportion

to length. The de-novo synthesis of amino acids is an energy intensive process. For example, the synthesis of one molecule of arginine from glutamate, ammonia, and carbon dioxide requires five high energy phosphate bonds (Lehninger 1977, p. 726).

3.0 MATERIALS AND METHODS

3.1 General

To demonstrate that fluid shear can increase uptake rates of macromolecular substrates by suspended cultures I used axenic cultures metabolizing individual substrates. Using pure cultures and single substrates simplifies the biological system and eliminates interactions which occur in mixed systems. This permits more accurate analysis of how observed results relate to physical and biological processes. Zoogloea ramigera was used for protein and amino acid experiments because it is an important species in activated sludge wastewater treatment bioreactors. Z. ramigera would not grow on dextran as a sole substrate, therefore, dextran and glucose experiments utilized Escherichia coli because it would grow on dextran and it also occurs in wastewater treatment bioreactors.

To compare uptake rates in stirred and still reactors I measured radiolabled substrate uptake directly. Also, in similar, parallel experiments with non-radiolabeled substrates I measured O_2 uptake as a surrogate parameter for substrate uptake. Because uptake as measured using radiolabeled substrates is actually the difference between what is taken in by the cell and what is released or exported as metabolites, I used molecular size fractionation to monitor the accumulation of radiolabel in the small molecular weight

fraction (less than 10,000 amu) during the BSA radiolabel uptake experiments.

In this section I also present a mass transfer model which predicts the effect of fluid shear on substrate uptake for bacteria in suspended culture. Experimental observations will be compared with model predictions.

3.2 CULTURE CONDITIONS and CELL PREPARATION

Pure cultures of Zoogloea ramigera (ATCC 19623), a flocculent Gram-negative rod, were used in all protein and amino acid experiments. Long term cultures were maintained on nutrient agar (Difco) and transferred to sterile liquid media for each experiment. Liquid media consisted of 1.0 g/l BSA (U.S. Biochemical Corp.) in a mineral salt buffer (MSB) containing per liter of water: 0.57 g NH_4NO_3, 0.5 g KH_2PO_4, 0.2 g $MgSO_4$, 0.04 mg $FeCl_3$, 0.02 mg vitamin B_{12}. Cells used in experiments were either harvested from a 1.0 liter bench top fermentor (Virtis Co.) operated as a continuously stirred tank reactor (CSTR) maintained at an average detention time of 16 hours or from batch reactors (500 ml Erlenmeyer flasks) on a shaker table (120 rpm) in late exponential growth phase. Prior to all experiments cells were centrifuged (5200 x g for 25 minutes), rinsed twice, resuspended in fresh MSB, and then filtered through a pre-rinsed 5.0 μm cellulose acetate filter (Millipore Corp.) to remove flocs.

Pure cultures of Escherichia coli (ATCC 15597) were used in all dextran and glucose experiments. Cell growth, storage and preparation procedures were identical to those used for protein and amino acid experiments except that 1.0 g/l dextran (Sigma average MW 70,800) or glucose (American Drug and Chemical Co.) was used as substrate and only batch reactors were used.

3.3 RADIOLABEL UPTAKE EXPERIMENTS

Uptake is defined as the amount of radiolabel in bacteria retained on 0.2 μm filters after vacuum filtration at 250 mm Hg. Sixty-ml BOD bottles with magnetic stir bars were used as reactors except where indicated otherwise. Sheared environments were obtained by mixing the reactors on a magnetic stir plate. I determined BSA uptake by Z. ramigera by resuspending washed cells in MSB, adding 1.5 mg/l of [^{3}H]BSA (preparation described below) and immediately separating the culture into shear and still reactors. Uptake was measured by filtering samples through 0.2 μm polycarbonate filters (Poretics) in a ten place manifold (Hoefer Scientific), rinsing the filters with two 5 ml aliquots of MSB, adding 10 ml of scintillation cocktail, and analyzing them using a Beckman 3801 liquid scintillation counter with H-number correction for sample quenching. Cells were not killed because formaldehyde addition causes protein cross linking

which prevents unassimilated BSA from being washed through the filter.

To insure that observed increases in ^{3}H activity were due to biological uptake rather than biosorption, we compared our observed uptake of [^{3}H]BSA to uptake in two other reactors containing sodium cyanide (10^{-1} M) or sodium m-arsenite (10^{-1} M). Both of these compounds inhibit oxidative metabolism. Cyanide binds to cytochrome oxidase preventing the transfer of electrons to oxygen (Lehninger 1977, p. 495). Arsenate reacts with dithiol in lipoic acid thus inactivating oxidative enzymes requiring lipoic acid and causing the accumulation of pyruvate and other α-keto acids (Metzler 1977).

To determine that low uptake rates in still cultures were not due to oxygen depletion, oxygen consumption was monitored using an oxygen specific electrode in a parallel culture using non-radiolabeled BSA as a substrate at similar concentrations used in the radiolabeled uptake experiments.

Dextran uptake in E. coli was determined in a manner similar to leucine uptake in Z. ramigera. Dextran concentrations of approximately 10 mg/l were obtained from a combination of ^{3}H radiolabeled and non-radiolabeled dextrans.

Previous studies have shown that leucine uptake is unaffected by fluid shear (Logan and Dettmer 1990). Therefore I also examined leucine uptake to verify this was true for the experimental conditions used in this study for macromolecular

substrates. Leucine uptake by Z. ramigera was determined in a manner similar to that used for BSA except that formaldehyde (final concentration 3%) was used to kill cells and stop uptake prior to filtration. [^{3}H]leucine (ICN Biomedicals, Inc., 60 Ci/mmole) was used at a concentration 0.26 μg/l.

3.4 MOLECULAR SIZE FRACTIONATION

During macromolecule degradation, bacteria can release $^{3}H_2O$, hydrolyzed [^{3}H]BSA fragments, and low-molecular weight metabolites into solution. The accumulation of small molecular weight compounds can be used as a measure of the rate of released label, while filterable activity is a measure of uptake and incorporation. I monitored the accumulation of radiolabel in the small molecular weight size fraction (less than 10,000 amu) during the course of an uptake experiment. I separated samples using low protein binding ultrafiltration units (Millipore Ultrafree - MC 10,000 NMWL) by centrifuging at 2040 x g for 15 minutes and measured the ^{3}H activity of the filtrate using liquid scintillation counting.

3.5 OXYGEN UPTAKE

Oxygen uptake is defined as the amount of oxygen used as measured by oxygen specific electrodes. I used oxygen uptake as a surrogate parameter for substrate uptake. In BSA and glucose experiments the oxygen uptake of still suspensions was

compared to uptake in two shear environments: a Yellow Springs Instruments model 5300 Biological Oxygen Monitor with integral mixing cells and 60 ml B.O.D. bottles with a Yellow Springs Instruments Model 57 Oxygen Meter and mixing electrode. Oxygen levels were continuously monitored in the shear environments. For still conditions, replicate 60 ml B.O.D. bottles were periodically sacrificed and oxygen measured using the YSI Model 57 electrode. In the dextran experiment the shear environment was provided by the YSI 5300; the still environment by quiescent 60 ml B.O.D. bottles continuously monitored by a Wheaton 60 Second B.O.D. System electrode which allows accurate oxygen measurement without mixing. Cultures were prepared as described for radiolabel uptake experiments. Dextran was prepared using ultrafiltration cells as described below and used at a concentration of 10 mg l^{-1}. BSA and glucose were used as received.

3.6 PREPARATION of RADIOLABELED MACROMOLECULES

[^{3}H]BSA was prepared from non-radiolabeled BSA as the [^{3}H]methyl derivative in a reaction using [^{3}H]formaldehyde (DuPont), sodium cyanoborohydride (Sigma) and BSA using modified procedures of (Jentoft & Dearborn 1979). This procedure adds [^{3}H]methyl groups to ϵ-amino groups of lysyl residues and the α-NH_2 terminus. To prepare [^{3}H]BSA I combined 150 μl of 4.2 mg BSA in 100 mM hepes buffer, 50 μl 4.0 mM

[^{3}H]formaldehyde, and 50 µl 100 nM cyanoborohydride in a Millipore Ultrafree - MC 10,000 NMWL low protein binding ultrafiltration unit and allowed them to react at room temperature for two hours. The ultrafiltration unit was centrifuged with its contents at 2040 x g for 15 minutes. The retentate was washed three times by resuspension in 400 µl hepes buffer and centrifuged (2040 x g) for 15 minutes. The retentate was resuspended in 400 µl hepes buffer with 0.02% NaN_3 added and stored refrigerated at 4°C.

Preliminary data from ultrafiltration experiments indicated that the [^{3}H]dextran (Amersham, 138 mCi/g, average molecular weight 70,000), as purchased, contained a significant fraction less than 10,000 amu. This small molecular weight fraction was removed by placing 0.5 ml [^{3}H]dextran solution in disposable Millipore Ultrafree-MC 10,000 NMWL ultrafiltration units (10,000 amu cutoff) and centrifuging at 2040 x g for 15 minutes. The retentate was diluted with 0.5 ml ultrapure (Millipore Milli-Q system) water and ultrafilterd three times, 0.2 µm sterile filtered using polycarbonate membrane filter units (Poretics Corp.) and stored at 4°C until use. The final activity of the dextran was 0.046 mCi ml^{-1} and the concentration was 0.33 mg ml^{-1}.

Non-radiolabeled dextran, when dissolved in distilled water, also contained a significant fraction of less than 10,000 amu material. This low molecular weight material was

removed by ultrafiltration using 200 ml stirred cells (Amicon 8200 series) and Amicon YM-10 (10,000 amu cutoff) ultrafiltration membranes. The retentate was diluted with ultrapure water, ultrafiltered three times and sterile filtered (0.2 μm Gelman Acrodisc syringe filters). The concentration of the nonradiolabeled dextran stock solution was determined to be 1.5 g l^{-1} using the anthrone method (Ramanathan, et al. 1968).

3.7 MASS TRANSFER MODEL

The effect of fluid shear on substrate uptake by dispersed cells under unsaturated conditions can be determined using a mass transfer analysis. The rate of mass transfer, Q, to a cell is

$$Q = 4\pi a D Sh E_g C \qquad (3)$$

where a is the cell radius, D is the substrate molecular diffusivity, Sh is the Sherwood number (equal to unity for a cell in a stagnant fluid), E_g is the collector efficiency, and C is the bulk solution substrate concentration (Logan and Dettmer 1990). The collector efficiency is a function of collision efficiency, cell shape and enzyme coverage of the cell, and varies between 0 and 1. The rate substrate disappears from solution, r_s, is

$$r_s = QN \quad (4)$$

where N is cell number. Likewise, the rate of oxygen disappearance, r_{02}, is

$$r_{O_2} = \frac{1}{Y} QN \quad (5)$$

where Y is the yield coefficient with respect to oxygen (mg substrate consumed / mg O_2 consumed).

Several empirical Sherwood number correlations have been proposed to account for the effect of shear on mass transfer of substrate to spheres in fluid shear environments. The applicability of a particular correlation is dependent upon the value of the dimensionless Peclet number,

$$Pe_a = \frac{a^2 G}{D} \quad (6)$$

which is a function of cell radius, shear rate, G, and substrate diffusivity. The Peclet number is an indication of the relative importance of shear and diffusive forces. High Peclet numbers describe shear dominated systems whereas low

Peclet numbers describe diffusion dominated systems. Among the correlations which have been proposed are

$$Sh_G = 1 + 0.28\, Pe_G^{1/2} \qquad [Pe<1] \tag{7}$$

developed analytically by Frankel and Acrivos (1968), and

$$Sh_G = 1 + 0.55\, Pe_G^{1/2} \qquad [Pe>10] \tag{8}$$

derived by Batchlor (1980). The values of Peclet numbers used in this study were typically on the order of unity, a region poorly defined by either Equation 7 or 8. Logan and Hunt (1987), by interpolation between $Pe_G = 0.1$ in Equation 7 and $Pe_G = 10$ in Equation 8 have proposed

$$Sh_G = 1.54\, Pe_G^{0.163} \qquad [0.1 \le Pe \le 10] \tag{9}$$

The correlations in Equations 7 through 9 therefore describe the ratio of mass transfer to a cell in a shear field, Q_G, to that of a cell in a stagnant fluid, Q, which can also be written as

$$\frac{Q_G}{Q} = Sh_G \tag{10}$$

The diffusion coefficients of substrates were either

calculated from available correlations or obtained from the literature. For proteins such as BSA, with molecular weights in the range of 10^3 - 10^7 amu, Polson (1950) provided the empirical correlation:

$$D = 2.74 \times 10^{-6} M^{-1/6} \quad (11)$$

where D is in $cm^2\ s^{-1}$, and M is the molecular weight. An empirical correlation for the diffusion coefficients of dextrans in water was obtained by Frigon (1983) as:

$$D = 7.04 \times 10^{-6} M^{-0.47} \quad (12)$$

The diffusivity of leucine in water was calculated using the Wilke-Chang correlation (Welty et al. 1976), which is valid for compounds in water with molecular weights less than approximately 1000 amu, using:

$$D = \frac{5.1 \times 10^{-8} T}{\mu V_b} \quad (13)$$

where T is the temperature [K], μ is the dynamic viscosity of water [cp], and V_b is the compound molal volume at normal boiling point (Welty et al. 1976), which is estimated from standard calculations as V_b=171 g cm^3 for leucine. The diffusivity of glucose at 20°C is 0.6 x 10^{-5} $cm^2\ s^{-1}$ (Thibodeaux 1979).

3.8 KINETICS

In order to test the mass transfer model, it is necessary to insure that substrate concentrations of BSA, dextran, leucine, and glucose used are in the range expected for unsaturated enzyme kinetics. I determined the Michaelis-Menton half saturation constant, K_s, for all substrates. I measured uptake rates in shaken flasks, and calculated K_s using double reciprocal (Lineweaver - Burke) plots and normalizing rates to cell concentrations using:

$$\frac{1}{V} = \frac{K_s}{\mu_{max}}\left(\frac{1}{s}\right) + \frac{1}{\mu_{max}} \qquad (14)$$

Bacterial numbers were determined using acridine orange epifluorescense direct counts (Hobbie et al. 1977). Protein concentration was measured using a Coomassie protein assay kit (Pierce). Carbohydrate concentration was determined using the anthrone method (Ramanathan, et al. 1968).

4.0 RESULTS

4.1 BSA UPTAKE

Fluid shear increases bacterial uptake of macromolecular substrates. Net BSA uptake was significantly higher in the stirred reactor, A, than in the still reactor, B (Fig. 4.1). This trend is in agreement with the mass transfer model for low diffusivity, high molecular weight substrates, but the uptake rate in the stirred reactor, $4.3 \pm 0.2 \times 10^{-19}$ g-BSA min^{-1} $cell^{-1}$ for the first eight hours, is 12.6 times the still reactor rate, $3.4 \pm 0.9 \times 10^{-20}$ g-BSA min^{-1} $cell^{-1}$ (Table 4.1). The mass transfer model predicts the shear uptake rate will be approximately 1.6 times the still rate (Table 4.2). After 25 hours the reactor A was taken off the stirrer and reactor B was stirred. When reactor B was stirred, the amount of radiolabel in the cells increased within the first 8 hr at a rate of 3.6×10^{-19} g-BSA min^{-1} $cell^{-1}$, which is similar to the rate observed for reactor A when it was stirred. When reactor A was no longer stirred there was a decline in the amount of radiolabel in cells. This decline, which suggests there was a substantial release of radiolabel back into solution, is likely a result of microbial utilization of BSA for energy production.

Addition of inhibitors sodium cyanide and sodium m-arsenite to stirred reactors prevented cellular accumulation of radiolabel (Fig. 4.2). In the non-inhibited control

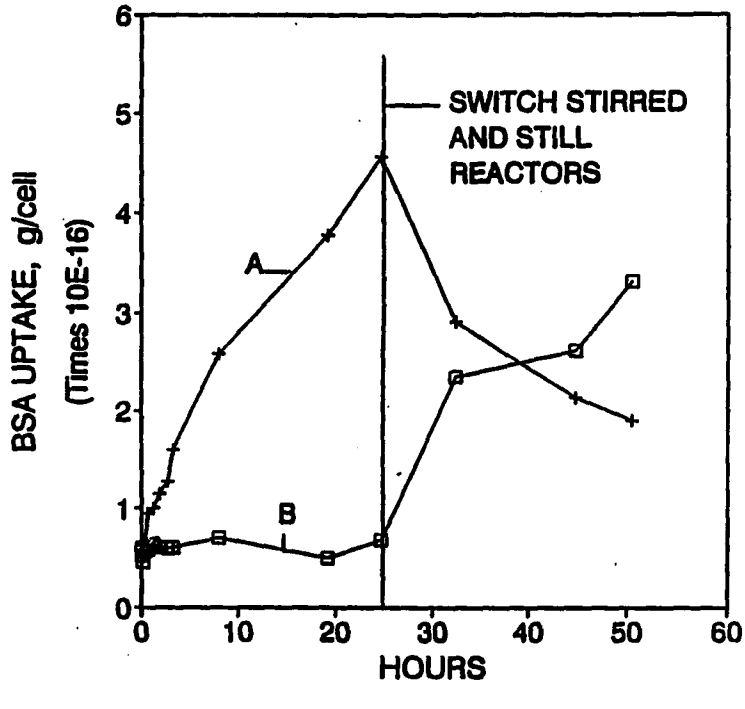

Figure 4.1: Uptake of [^{3}H]BSA by Z. ramigera cultures in BOD bottles. Initially, A is stirred and B is still (0 to 24 h); thereafter, B is stirred and A is still.

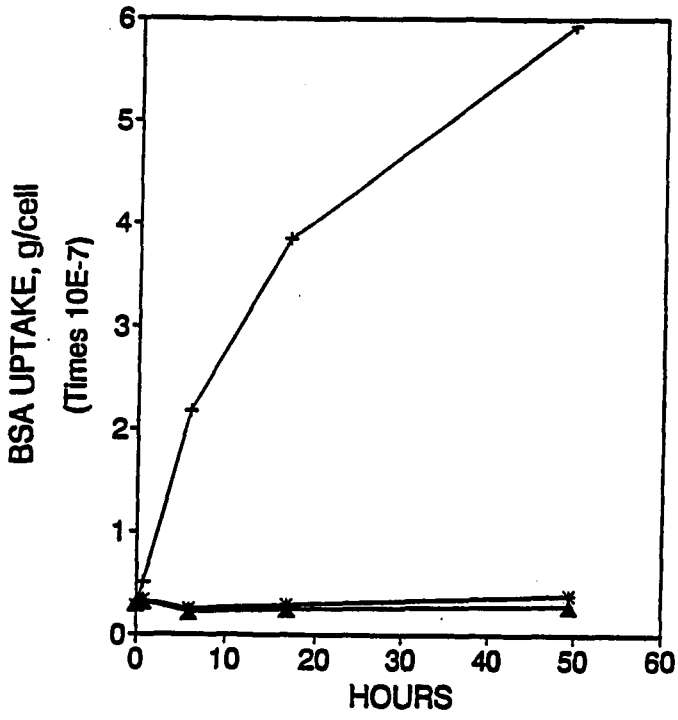

Figure 4.2: The addition of two inhibitors, 0.1 M arsenite (*) and cyanide (▲), greatly reduced [^{3}H]BSA uptake by stirred cultures of Z. ramigera compared to controls (+), indicating that increased uptake in stirred samples was not due to biosorption.

Substrate		Bacteria		Measured Variable	Uptake rate [g min^{-1} $cell^{-1}$]	
Type	[mg l^{-1}]	Type	[l^{-1} x 10^{-11}]		Stirred	Still
BSA	1.5	*Z. ramigera*	4	^3H	4.3 x 10^{-19}	3.4 x 10^{-20}
BSA	40	*Z. ramigera*	0.1	O_2	1.5 x 10^{-15}	6.4 x 10^{-16}
Dextran	10.3	*E. coli*	2	^3H	4.7 x 10^{-15}	7.6 x 10^{-16}
Dextran	10.3	*E. coli*	2	O_2	1.2 x 10^{-16}	4.2 x 10^{-17}
Glucose	50	*E. coli*	1	O_2	1.6 x 10^{-15}	1.6 x 10^{-15}
Leucine	0.00027	*Z. ramigera*	2	^3H	7.2 x 10^{-23}	6.3 x 10^{-23}

Table 4.1: Comparison of uptake rates of stirred and still reactors.

Substrate	K_s [mg l^{-1}]	Diffusivity [cm^2 s^{-1} x 10^{-6}]	Ratio of Stirred/Still Uptake		
			Predicted	Observed-^3H	Observed-O_2
BSA	50	68	1.6	12.6	2.3
Dextran	60	37	1.8	6.2	2.9
Glucose	40	600	1.16	—[a]	1.0
Leucine	<100	874	1.12	1.15	—[a]

[a]-Not determined

Table 4.2: Comparison of predicted and observed ratios of uptake by stirred samples to still samples.

culture ^{3}H accumulation is comparable to that in Fig. 4.1. This indicates that the observed radiolabel increases are due to increased metabolic activity and not biosorption.

Since the still samples were not mixed, oxygen depletion could have resulted in a decreased rate of BSA uptake by still samples. However, during a 36 h period, oxygen concentrations decreased from 7.6 mg l^{-1} to 7.4 mg l^{-1} in a separate, undisturbed reactor containing similar concentrations of BSA (2.0 mg l^{-1}). Since K_s values for O_2 are less than 1 mg l^{-1} (Grady and Lim 1980), oxygen uptake by bacteria is saturated at concentrations used in these experiments, and low O_2 concentration cannot account for the low observed BSA uptake rate in the still reactor.

4.2 SIZE FRACTIONATION

The ^{3}H activity of the filtrate in the ultrafiltration separations is indicative of all radiolabeled species less than 10,000 amu. Since the molecular weight of BSA is approximately 68,000 this small molecular weight fraction is composed of either hydrolyzed protein fragments or exported cellular metabolites. Filtrate activity was observed to increase more rapidly in shear than still reactors indicating more active metabolic processes (Fig. 4.3a). When stirred, A, and still, B, reactors were switched, the rate of increase in filtrate activity of reactor B increased, while that of

reactor A decreased (Fig. 4.3b). This is consistent with an overall increase in Z. ramigera metabolic activity in stirred reactors.

4.3 DEXTRAN UPTAKE

The effect of shear on [^{3}H]dextran uptake in E. coli is comparable to the effect of shear on BSA uptake in Z. ramigera. Shear increases the uptake rate more than that predicted by the mass transfer model. The uptake rate in the stirred reactor, 4.7 ±1.3 x 10^{-15} g-dextran min^{-1} $cell^{-1}$, was 6.2 times the uptake of 7.6 ±5.6 x 10^{-16} g-dextran min^{-1} $cell^{-1}$ determined in the still reactor over a 90 min period (Fig. 8 and Table 1). Initial mixing needed to disperse cells and radiolabeled substrate may cause a very rapid initial increase in activity as substrate is bound to cell receptor sites. The discrepancy in initial activity between shear and still conditions may be due to sampling the stirred reactor slightly after stirring began.

4.4 OXYGEN UPTAKE

Oxygen uptake proved a reliable surrogate parameter for substrate uptake in all experiments for which there was both oxygen uptake and radiolabel substrate uptake data. In stirred reactors, oxygen uptake rate as well as radiolabel substrate was greater than in still reactors for the large

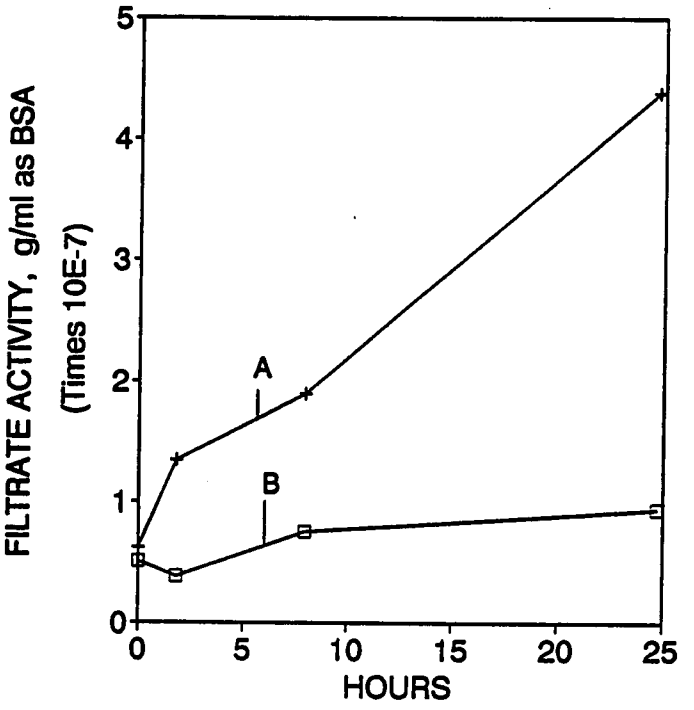

Figure 4.3a: The amount of ^{3}H-label (converted to g-BSA) appearing in filtrate (<10,000 amu, collected during the experiment reported in Fig. 4.1) during utilization of [^{3}H]BSA by Z. ramigera in stirred (A) and still (B) reactors.

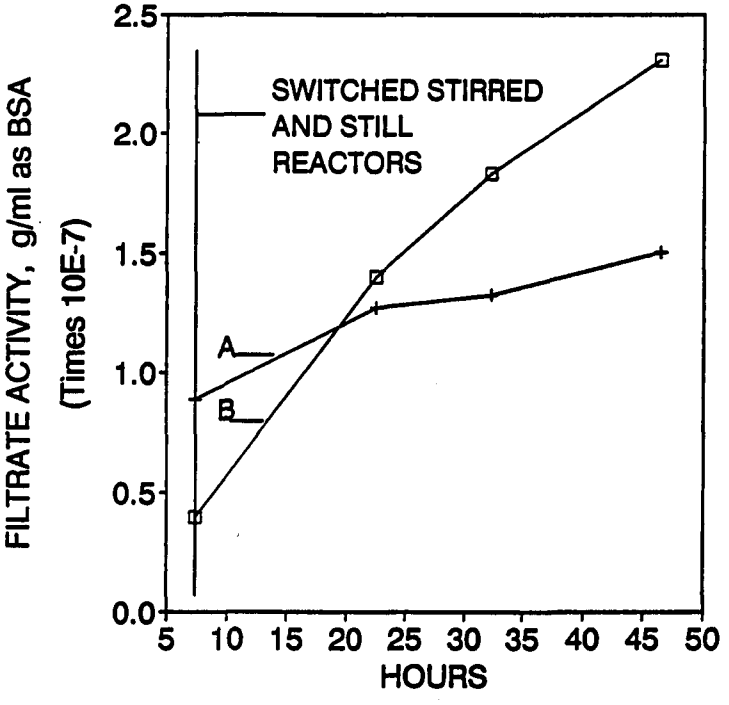

Figure 4.3b: Same as Fig. 4.3a, but after reactor A switched at 7 h from stirred to still, and reactor B switched from still to stirred.

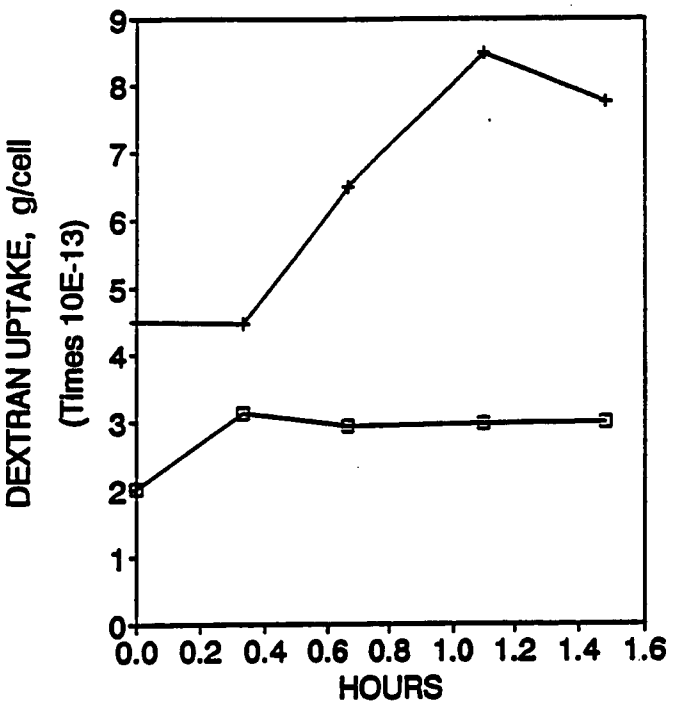

Figure 4.4: Uptake of [^{3}H]dextran by *E. coli* cultures in stirred (+) and still (□) bottles.

molecular weight substrates BSA and dextran (Figs. 4.5, 4.6). I added 40 mg l^{-1} of BSA to a washed suspension of Z. ramigera, and separated the suspension into several BOD bottles, half of which were mixed on stir plates. I also placed 4 ml of this suspension into a DO chamber. Bottles were periodically sacrificed and analyzed for DO over a period of 240 min. The rate of oxygen utilization in the stirred BOD bottles and the DO chamber was 2.7 and 2.3 times faster, respectively, than the rate in the still bottles (Fig. 4.9) during the 80-240 min interval. The steady state oxygen uptake rate in the stirred reactors was 1.7 $\pm$0.2 x 10^{-15} g-O_2 min^{-1} $cell^{-1}$ in the BOD bottle and 1.5 $\pm$0.2 x 10^{-15} g-O_2 min^{-1} $cell^{-1}$ in the DO chamber, versus 6.4 $\pm$0.8 x 10^{-16} g-O_2 min^{-1} $cell^{-1}$ for the still reactor (Table 4.1). During the initial 80 min, the oxygen utilization rate in the two samples was similar. If we assume that oxygen utilization reflects cell growth, this might imply that initial cell activity was not affected by the fluid mechanical environment. However, it more likely that O_2 consumption was initially uncoupled from substrate uptake. After 80 min, the rate of oxygen utilization by all samples increased, with the greatest rate noted for the sample in the stirred (high shear) DO chamber. This suggests that substrate transport to the microorganisms in mixed reactors permitted higher oxygen utilization, and therefore, higher growth rates.

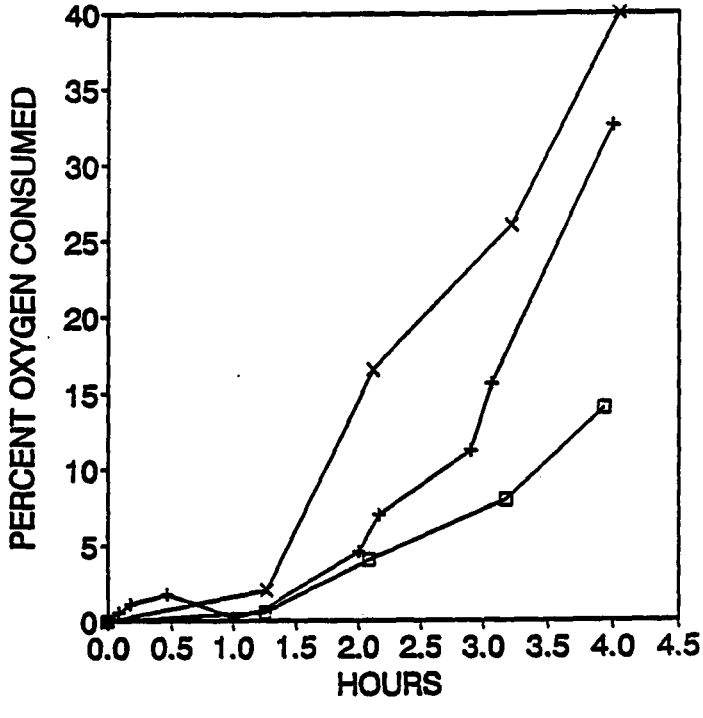

Figure 4.5: Oxygen consumption by *Z. ramigera* growing on BSA in stirred (+, DO chamber; X, BOD bottle) and still (□, BOD bottle) samples.

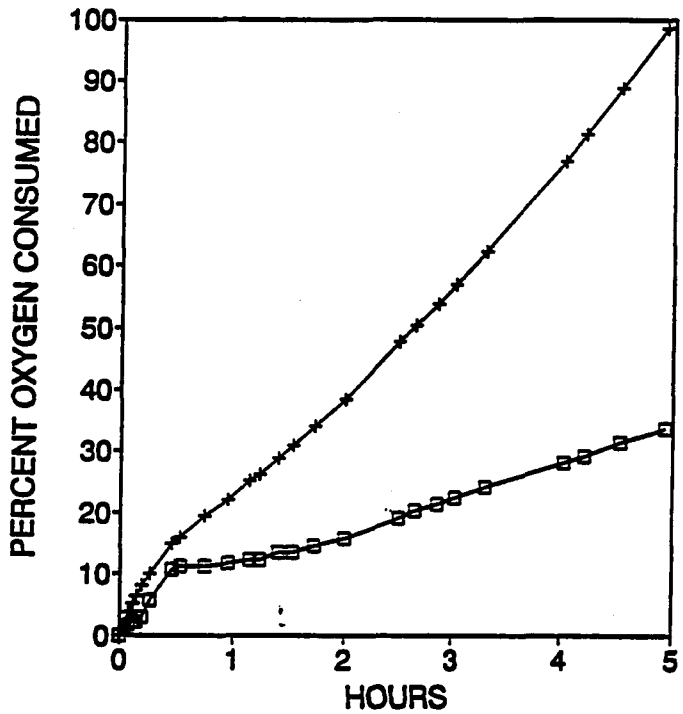

Figure 4.6: Oxygen consumption by <u>E. coli</u> growing on dextran in stirred (+) and still (□) samples.

I conducted a similar oxygen utilization experiment using E. coli grown on dextran, except I continuously monitored the still bottle using the non-consumptive DO probe, and I used only the DO chamber for the stirred sample. The rate of oxygen utilization of the stirred sample was 2.9 times faster than the still sample (Fig. 4.6). Oxygen concentrations decreased in the stirred bottle to 38% saturation (1.23 ±0.02 x 10^{-16} g-O_2 min^{-1} $cell^{-1}$) versus 75% saturation in the still sample after 200 min (4.2 ±0.2 x 10^{-17} g-O_2 min^{-1} $cell^{-1}$). During the initial 20 min, the oxygen utilization rate in the two samples was similar, suggesting that oxygen transport to the cells was not affected by the fluid mechanical environment. After 20 min, the rate of oxygen utilization by the still sample slowed down, suggesting that substrate (dextran) transport to the microorganisms was limiting oxygen utilization in still samples.

4.5 UPTAKE of LOW MOLECULAR WEIGHT COMPOUNDS (leucine and glucose)

It has previously been shown, using radiolabeled substrates, that fluid mixing in a laminar shear device (Logan and Dettmer 1990, Logan and Kirchman 1991) did not affect the uptake of small molecular weight substrates such as glucose and leucine. However, I was concerned that the potentially higher shear rates in the BOD bottles, particularly near the

stirring bar, might injure microbes resulting in higher respiration rates. To test this hypothesis, I examined oxygen utilization by E. coli using glucose as the sole carbon and energy source. I found that oxygen uptake rates were the same in all three mixing environments (still and stirred BOD bottles and DO chamber) at a rate of 1.6 ±0.1 x 10^{-15} g-O_2 min^{-1} $cell^{-1}$ (Fig. 4.7). This indicates that the effects we measured due to stirring were due to macromolecular substrates used, and that increased cell respiration rates were not an artifact of the experimental set up.

I also examined [^{3}H]leucine uptake by cultures of Z. ramigera grown on BSA and resuspended in MSB with no substrate. I found that [^{3}H]leucine uptake in the stirred reactor was 7.2 ±0.2 x 10^{-23} g $cell^{-1}$ min^{-1}, 1.15 times the still reactor rate of 6.3 ±0.2 x 10^{-23} g $cell^{-1}$ min^{-1} in the still reactor (Fig. 4.8). This result indicates that shear slightly increased uptake of the leucine and compares favorably to the 1.12 times the still reactor rate predicted by the mass transfer model (calculated below).

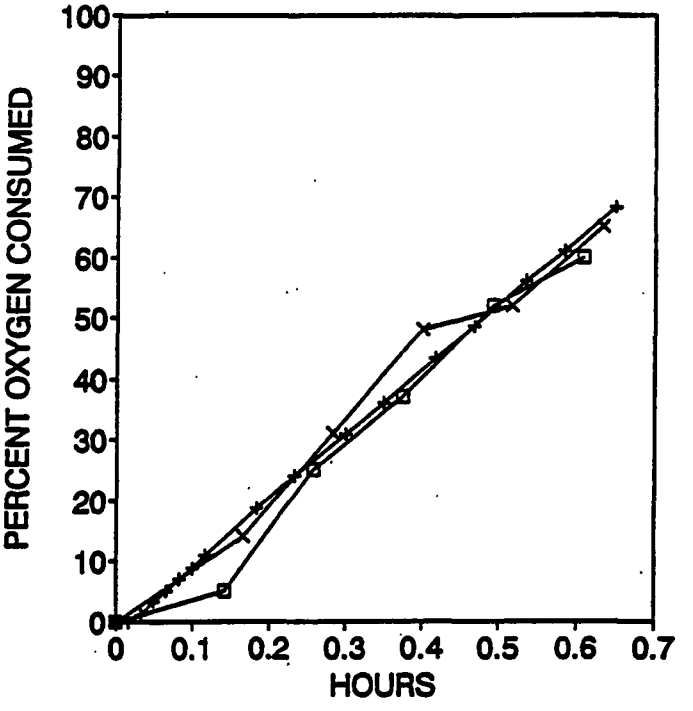

Figure 4.7: Oxygen consumption by *E. coli* growing on glucose in stirred (+, DO chamber; X, BOD bottle) and still (□, BOD bottle) samples.

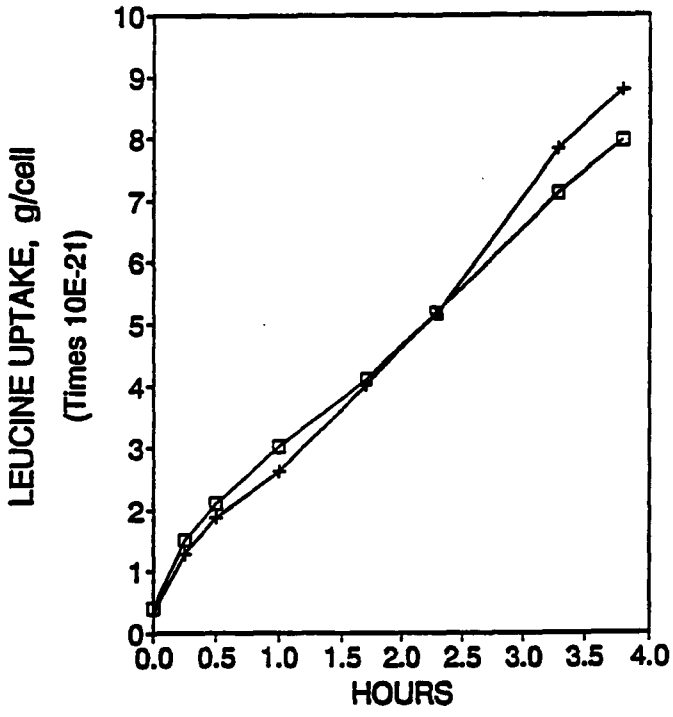

Figure 4.8: Uptake of [^{3}H]leucine by *Z. ramigera* cultures in stirred (+) and still (□) bottles.

4.6 MASS TRANSFER MODELS

4.6.1 Predicted uptake rates

The effect of shear on substrate uptake rates can be predicted by the mass transfer model using Equations 7-10. The effective radius, determined during cell counting, of either Z. ramigera or E. coli was approximately 0.7 μm. The calculated diffusion coefficients for the substrates used range from 37 - 874 x 10^{-8} cm^2 sec^{-1} and are shown in Table 4.2. The shear rate of the reactors used could not be directly measured. I estimated it to be on the order of 200 s^{-1}, based on other reported shear rates for an unbaffled, cylindrical reactor mixed at approximately 200 rpm (Camp 1968). In Fig. 4.9, I show the effect of fluid shear rate on the rate of mass transfer to microorganisms over a shear rate range of 10-1000 s^{-1} for the different substrates used in this study. For BSA, I calculate uptake would increase by a factor of 1.07, 1.63, and 2.08 at shear rates of 10, 200 and 1000 s^{-1}, respectively. For leucine, fluid shear over this range would only be expected to increase uptake by a factor of 1.02 to 1.49.

4.6.2 Observed uptake rates

Assuming that 200 s^{-1} is a reasonable estimate for shear in the stirred BOD bottles, we can compare the experimentally observed increases in uptake with those predicted by the mass

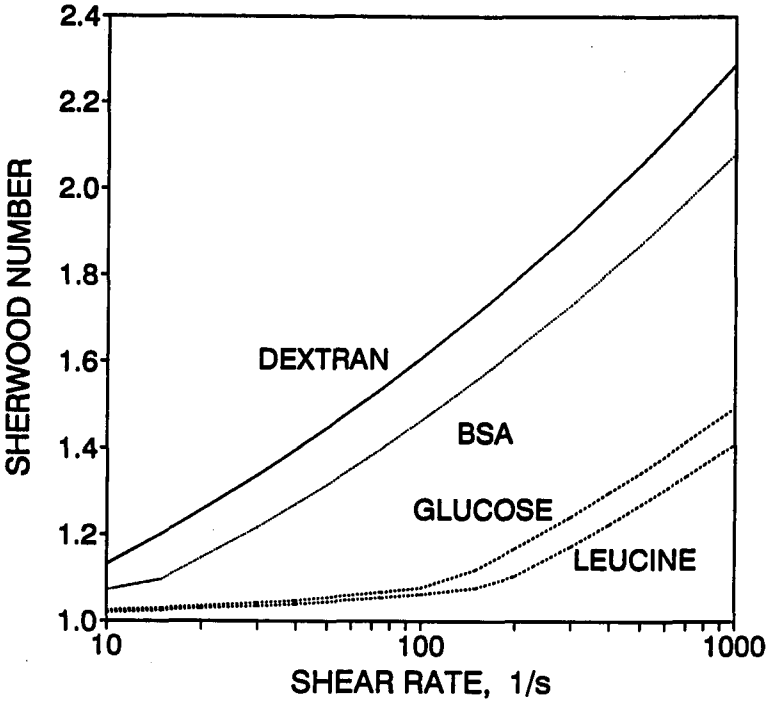

Figure 4.9: Predicted effect of fluid shear of bacterial uptake of different compounds when uptake is limited by mass transfer to the cell surface. According to the model, the ratio of bacterial uptake in a sheared fluid to uptake in an undisturbed fluid is equal to the dimensionless Sherwood number (Eq. 10).

transfer model. For BSA, mass transfer calculations indicate uptake should be 1.6 times greater in the stirred bottle than in the still bottle. However, the measured rate of stirred samples was 35 times the still reactor rate when radiolabeled substrates were used. The oxygen utilization rates indicated an overall increase of 2.3 times, which is much closer to the model value. For dextran, I calculated an increase of a factor of 1.8 for stirred samples, versus observed increases by a factor of 6.2 using radiolabeled compounds and 2.9 using oxygen.

For the small molecular weight substrates, I calculated increases of 1.12 and 1.16 for leucine and glucose as a result of stirring. This result for leucine compares favorably to the factor of 1.15 observed using radiolabeled substrates. I was not able to measure any increases in glucose uptake as a result of fluid shear using oxygen measurements.

4.7 Kinetics

K_s was determined to be 48 mg/l and U_{max} 0.0014/min (t_d = 8 hrs) for Z. ramigera growing on BSA as a sole carbon source. The K_s value for glucose uptake by E. coli (ATCC 15597) used in these experiments was similarly determined in our lab to be 40 mg/l (Greg Haldane, unpublished data). The concentration of glucose used in oxygen uptake experiments, 50 mg/l, is within the unsaturated range. The dextran concentration used

experimentally, 10 mg/l, should also be within the unsaturated range. Leucine uptake in Z. ramigera is unsaturated for values below 100 mg/l (Logan and Dettmer 1990), eight orders of magnitude higher than the higher than the 2.0 ng/l used in this experiment.

5.0 DISCUSSION

5.1 UPTAKE EXPERIMENTS

The uptake of macromolecules, measured using radiolabeled chemicals and oxygen consumption, are higher in stirred environments than still environments. When oxygen consumption was measured using BOD bottles, BSA and dextran uptake rates of stirred samples were 2.3 and 2.9 times higher, respectively, than undisturbed samples. When radiolabeled chemicals were used, the amount of radiolabel retained in the cells in stirred bottles increased 12.6 and 6.2 times faster for BSA or dextran, respectively, than in still samples. For the low molecular weight substrates there was no significant difference in uptake rates between fluid shear and still environments for glucose and only a slight difference for leucine.

An increase in the rate of BSA uptake by a factor of approximately 1.6 in the shear reactor over the still reactor is predicted by the mass transfer model. The observed increase was significantly higher than predicted. Similarly, the observed increase in BSA uptake in the still reactor after stirring is begun is predicted, but the release of radiolabel from the shear reactor cells after stirring was stopped was not. These two phenomena are possibly related. One interpretation is that when Z. ramigera is grown on BSA as a sole substrate it must be utilized for both cell growth and as

an energy source. Under shear conditions both anabolic and catabolic metabolism are augmented. Anabolism predominates, however, and the observed result is an increase in cellular radiolabel activity or uptake. When mixing is stopped, effective substrate availability is decreased. Anabolic metabolism decreases, catabolism predominates and previously assimilated substrate is released to the bulk solution as metabolite ^3H_2O. This hypothesis is supported by the data from size fractionation experiments where there was an increase in the release of small molecular weight components when stirring was stopped. A contributing factor to the higher than expected increase in apparent uptake rate with shear is likely due to uptake in the still reactor being underestimated because of the high proportion of catabolic activity in the still reactor as compared to the shear reactor. This same effect is observed to a lesser degree for dextran uptake where apparent shear uptake rate is 6.2 times the still value.

Other factors which may contribute to the discrepancy between predicted and observed uptake rates are the assumptions made in the development of the mass transfer model as well as the correlations used to calculate diffusivities and Sherwood numbers. Bacterial uptake and metabolism of macromolecular substrates is a multi-step process involving transport to the cell, binding to the hydrolytic enzymes at the cell surface, hydrolysis of the macromolecules to

transportable sized fragments, transport across the cell membrane structures, and anabolic and catabolic metabolic processes. The mass transfer model assumes that mass transfer to the cell is the rate limiting step. The possibility that another process is rate limiting is not addressed. Also not addressed in the model is the effect of possible feedback of metabolic processes on hydrolysis and transport functions at the level of genomic expression. It has been suggested that environmental factors can directly effect the bacterial genome by influencing tertiary DNA structure, thus controlling such factors as gene expression and resultant phenotypic expression (Kraweic and Riley, 1990). It is likewise a reasonable suggestion that the metabolic state of the bacteria will directly affect the internal cell environment and genomic expression. The number or activity of the enzymes that mediate macromolecule hydrolysis or hydrolysis fragment transport could be under environmentally influenced genetic control.

Oxygen consumption appears to more accurately correlate with predictions of the mass transfer model for macromolecular substrates. A reason for this might be that there would be no underestimation of the still reactor rate as a result of not counting metabolite $^{3}H_2O$ as is possible in radiolabel uptake experiments. The experimental values are approximately 50% higher than predicted values. Again, this amount of

discrepancy between experimental and predicted values could be accounted for by inaccuracies in mass transfer calculations or in effective diffusivities being much lower than calculated values. Both BSA and dextran have extensive opportunities for hydrogen bonding with themselves and with water and the average effective molecular size may be much larger than 68,000 or 70,000. For dextran, although we filtered out molecules less than 10,000 amu, macromolecules greater than 70,000 amu may have been a significant portion of the overall composition. These situations would result in lower effective diffusivities and increased mass transfer due to the effect of fluid shear.

5.2 SUPPORT for ATTACHED HYDROLYSIS

This mass transfer model for substrate uptake is predicated on the concept that the macromolecular substrates arrive intact at the cell surface. Macromolecules such as BSA cannot be assimilated intact, but must first be hydrolyzed to individual amino acids or small peptides. The maximum size of a transportable peptide is approximately seven amino acids (Law 1980). The increase in BSA uptake under shear conditions supports a protein uptake model in which protein hydrolysis occurs in the pericellular environment with preferential assimilation of hydrolyzed amino acids or peptides. If hydrolysis were to occur in the bulk phase, the assimilated

species would be high diffusivity peptides or individual amino acids which, like leucine, would not demonstrate enhanced uptake with shear due to high diffusion coefficients. The experimental evidence does not preclude pericellular hydrolysis followed by subsequent release of hydrolysis products to the bulk solution as the increased bulk phase concentrations of peptides and amino acids would lead to increased uptake. This model seems unlikely on a teleological basis, however, as it would be energetically unfavorable for a cell to possess the apparatus necessary for pericellular hydrolysis and then release the assimilable products to the bulk phase rather than take direct advantage of existing membrane transport systems for amino acids.

5.3 IMPLICATIONS for MACROMOLECULAR KINETICS RESEARCH

Fluid shear has been demonstrated to significantly increase the uptake of macromolecules, and kinetic models should account for reactor hydrodynamic conditions. This effect has profound implications for macromolecular metabolic research. Common reactor configurations such as flasks on shaker tables or magnetic stir plates and bench-top reactors can have significantly different shear conditions depending on fluid volumes and mixing speeds. Results obtained in one reactor configuration may not be repeatable in another. Reaction vessel shear conditions should therefore be considered in

analysis of kinetic data. Also, it should become standard practice to report these conditions in kinetic studies.

5.4 IMPLICATIONS for WASTEWATER TREATMENT REACTORS

The biochemical oxygen (BOD) test is the standardized test used to evaluate the oxygen requirements of all wastewaters, and the efficacy of wastewater treatment bioreactors such as trickling filters and activated sludge. The results of BOD tests are also used to predict treatment process performance. Models such as the modified Velz equation and the Logan model (Logan et al. 1987[a,b]) are used for trickling filter predictions. In the BOD test, wastewater is combined with a phosphate buffer solution and diluted in a series of bottles so that the oxygen demand is within the range of oxygen solubility for the duration of the test (typically 5 days). Bottles are incubated in the dark at 20°C, and are not mixed during the incubation period.

In contrast to the quiescent hydrodynamic conditions of a BOD test, wastewater reactors such as the activated sludge reactor are characterized by high shear environments of 90-220 s^{-1} (Parker et al. 1970). From the results obtained in this test, we expect that oxygen utilization rates obtained in BOD studies would underestimate rates occurring in wastewater treatment bioreactors, particularly since approximately half of the oxygen demand of wastewaters can be associated with the

macromolecular size fraction (Miller 1988, Logan and Jiang 1990). This suggests that we may need to alter the conditions of the BOD test to more accurately reflect the biological hydrodynamic conditions of wastewater treatment bioreactors.

A BOD test which more accurately reflects treatment process hydrodynamics would be a significantly better predictor of wastewater treatment system performance than the currently used test. A complication, however is that the molecular size distribution of a particular wastewater is not constant. Logan and Jiang (1990) have shown that in the wastewater received by a single treatment plant, the fraction of dissolved organic carbon less than 1000 amu can vary from 45% to 95% throughout the year. Also, the removal of small molecular weight organics in trickling filters is predicted to be faster than the removal of large molecular weight organics (Logan, 1986). More complete understanding of these factors- the dependence of macromolecular kinetics on hydrodynamics, the kinetics of wastewater treatment as a function of molecular size distribution, and the variability of molecular size distribution in a particular wastewater with time- should contribute to the development of a model that will be able to predict wastewater treatability under various reactor conditions based on an evaluation of wastewater composition.

6.0 INDICATIONS FOR FUTURE EXPERIMENTS

The question of whether the increased release in less than 10,000 amu material is due to metabolite water or possibly low molecular weight hydrolysis products could be answered by using ^{14}C labeled substrates. If the increase were still observed it would indicate the release of hydrolysis products. If there were no increase it would be further evidence for the anabolism / catabolism rate change argument proposed in the discussion. Alternatively, CO_2 evolution could be measured during radiolabel substrate uptake experiments. Ratios of CO_2 evolved to radiolabel incorporated would be an indication of the relative importance of catabolism and anabolism.

The use of artificial fluorogenic substrates (which release an easily measured fluorogenic molecule upon hydrolysis) can help further elucidate the location of hydrolysis. By monitoring hydrolysis rates in fractions containing cells as well as in cell-free fractions, direct evidence for hydrolysis can be obtained. Furthermore, the manipulation of culture conditions such as relative oligotrophy and copiotrophy, when combined with fluorogenic substrate hydrolysis could provide information on the environmental factors which may affect macromolecular metabolism, such as enzyme induction. In light of the conflicting reports of hydrolysis location in copiotrophic environments, pure culture and assemblage culture experiments

might provide insight into whether a single species can hydrolyze macromolecules using both attached and unattached mechanisms or whether the data obtained from environmental samples which signify an increase in exocellular hydrolysis in copiotrophic environments is due to a shift to dominance of microbial species toward those with constitutive exocellular hydrolysis enzymes.

Other studies that would provide valuable information would entail monitoring the concentrations of combined and free amino acids using acid hydrolysis and/or fluorgenic amino acid derivitization followed by HPLC separation during the course of stirred and still uptake experiments. This would show whether hydrolyzed fragments of macromolecules during attached extracellular hydrolysis are released back into solution or are tightly held for subsequent uptake. It would also yield information as to whether any specific amino acids are preferentially used or accumulate in solution.

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