Increased Bacterial Uptake of Macromolecular Substrates with Fluid Shear

DAVID R. CONFER and BRUCE E. LOGAN*
Environmental Engineering Program, Department of Civil Engineering,
206 Civil Engineering Bldg., University of Arizona,
Tucson, Arizona 85721

Received 31 May 1991/Accepted 20 August 1991

To investigate the effect of fluid shear on uptake rates of low-diffusivity macromolecular substrates by suspended cultures, we measured the uptake of two compounds as models of macromolecules, a protein (bovine serum albumin [BSA]) and a polysaccharide (dextran), using pure cultures of Zoogloea ramigera and Escherichia coli, respectively. Oxygen utilization rates of stirred samples grown on BSA and dextran were 2.3 and 2.9 times higher, respectively, than those of undisturbed (still) samples. Uptake rates of $[\text{H}]$-BSA and $[\text{H}]$-dextran by stirred samples were 12.6 and 6.2 times higher, respectively, than those by still samples. These experimentally obtained increases are larger than those predicted with a mass transfer model. Model results indicated that stirring would increase uptake by factors of 1.6 and 1.8 for BSA and dextran. As predicted by the model, we also found that 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 make up a major portion of bacterial substrate in natural, laboratory, and engineered systems, the demonstrated effect of fluid shear has wide implications for kinetic studies performed in basic metabolic research as well as in the evaluation of engineered bioreactors used for wastewater treatment.

Macromolecular compounds can make up a major portion of dissolved organic matter (DOM) in natural waters and wastewaters. Although molecular size distributions of DOM vary, 80% of the total amino acids in a North Pacific Ocean survey were in compounds found to have molecular weights of >1,500 atomic mass units (amu) (21). In typical domestic wastewaters, 50 to 60% of the dissolved organic carbon has a molecular weight of >1,000 amu (5, 14).

Classical enzyme kinetic models, such as the Michaelis-Menten and Blackman models, have significant limitations when applied to bacterial degradation of macromolecular substrates. With these kinetic models, uptake is only a function of the type and concentration of enzyme and substrate. However, bacterial uptake and utilization of macromolecules include several steps, and models should incorporate other factors. Bacteria must first enzymatically hydrolyze macromolecules into subunits which can be transported into the cell (1). In this context, any compound that must be cleaved into subunits before uptake is considered a macromolecule. In addition, mass transfer of substrate to the cell can limit uptake (11, 15). Macromolecules have low diffusion coefficients, and at low substrate concentrations and high substrate utilization rates, diffusive transport due to Brownian motion may not prevent the development of concentration gradients around cells (2).

As a result of mass transfer-limited transport to the cell, the fluid mechanical environments in which microorganisms exist (quiescence, advection, and fluid shear) can affect overall observed uptake kinetics. Adveotive flow past cells has been shown to increase bacterial uptake of low-molecular-weight compounds. $[\text{H}]$-leucine uptake rates of Zoogloea ramigera fixed on filter membranes in an advective flow field of 1 mm s$^{-1}$ were found to be 55 to 65% higher than leucine uptake rates by suspended cells (11). Using a marine bacterium, Logan and Kirchman (15) found that $[\text{H}]$-leucine uptake rates by bacteria fixed on filters in an advective flow field of 0.23 to 0.81 mm s$^{-1}$ were eight times higher than uptake rates of cells at velocities of 0.035 mm s$^{-1}$. In contrast, laminar fluid shear rates of <50 s$^{-1}$ were found to have no discernible effect on bacterial uptake of compounds with low molecular weights, and, therefore, high diffusion coefficients, such as leucine and glucose (11, 15). However, it has been predicted that fluid shear could increase bacterial uptake of low-diffusivity macromolecular substrates at similar shear rates (12, 13).

This purpose of this investigation was to demonstrate that fluid shear can increase macromolecule uptake by suspended bacteria. Using pure bacterial cultures, we examined uptake of two types of compounds as models of macromolecules, a protein (bovine serum albumin [BSA]) and a polysaccharide (dextran). Uptake was monitored in stirred and nonstirred reactors, using both radiolabeled compounds and oxygen consumption. The effects of fluid shear on macromolecule uptake were contrasted with those obtained in identical studies with representative macromolecule monomers of proteins (leucine) and polysaccharides (glucose).

**MATERIALS AND METHODS**

**Culture conditions and cell preparation.** Pure cultures of *Z. ramigera* (ATCC 19623), a gram-negative rod, were used in all protein and amino acid experiments. Long-term cultures were maintained on nutrient agar (Difco Laboratories) and transferred to sterile liquid medium for each experiment. Liquid medium consisted of 1.0 g of BSA (65,000 amu; U.S. Biochemical Corp.) per liter in a mineral salt buffer (MSB) containing the following, per liter of ultrapure water (Milli-Q system; Millipore Corp.): 0.57 g of $\text{NH}_4\text{NO}_3$, 0.5 g of $\text{KH}_2\text{PO}_4$, 0.2 g of $\text{MgSO}_4$, 0.04 mg of $\text{FeCl}_3$, and 0.02 mg of vitamin $\text{B}_12$. Cells used in experiments were harvested either from a 1.0-liter bench top fermentor (VirTis Co.) operated as
a continuously stirred tank reactor maintained at an average detention time of 16 h or during late exponential growth from 500-ml Erlenmeyer flasks shaken at 120 rpm. Prior to all experiments, cells were centrifuged (5,200 × g for 25 min), rinsed twice, resuspended in fresh MSB, and then filtered through a 5.0-μm cellulose acetate filter (Millipore), pre-rinsed with ultrapure water, to remove floccs. Pure cultures of Escherichia coli (ATCC 15957) were used in all dextran and glucose experiments since we were unable to grow Z. ramigera with dextran as a sole carbon source. Cell growth, storage, and preparation procedures were identical to those used for protein and amino acid experiments except that dextran (70,800 amu; Sigma Chemical Co.) or glucose (American Drug and Chemical) was used as the substrate, and only Erlenmeyer flasks were used to grow cultures. Cell densities in all experiments were approximately 10^{14} liter^{-1}.

**Bacterial uptake using macromolecules.** The effect of fluid shear on uptake was determined by comparing uptake of substrate by sheared (stirred) and undisturbed (still) bacterial suspensions. Since we are interested in examining whether fluid shear increases the rate of macromolecule transport to the cell, we consider "uptake" in this study to be the successful hydrolysis of a macromolecule into smaller molecules that are subsequently either discarded or used by the cell for energy production or growth. All uptake experiments with radiolabeled compounds were performed in 60-ml biochemical oxygen demand (BOD) bottles (Wheaton) containing Teflon magnetic stir bars (2.5 cm long). Sheared environments were obtained by vigorous mixing with magnetic stir plates (VWR Scientific) covered with an insulating pad to prevent sample heating. Undisturbed bottles contained stir bars, but were not mixed.

We determined net ^3H-BSA uptake by Z. ramigera by resuspending washed cells in MSB, adding 1.5 mg of ^3H-BSA per liter, 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 10-place manifold (Hofer Scientific) at 250 mm Hg (ca. 33 kPa), and rinsing the filters with two 5-ml aliquots of MSB. The filters were radioassayed in a Beckman 3801 liquid scintillation counter with H-number correction for sample quenching. Cells were not treated with a chemical such as formaldehyde since this chemical cross-links proteins and prevents unassimilated ^3H-BSA from being washed through the filter.

Dextran uptake by E. coli in stirred and still BOD bottles was determined by using the same procedures used in BSA uptake experiments, with dextran concentrations prepared with ^3Hdextran (0.3 mg liter^{-1}) and nonradiolabeled dextran (10 mg liter^{-1}) prepared as described below.

To ensure that changes in filterable radioactivity were not due to biosorption, we compared our observed uptake of ^3H-BSA with uptake in other reactors containing either sodium cyanide (0.1 M) or sodium m-arsenite (0.1 M), since both of these compounds inhibit oxidative metabolism. Cyanide binds to cytochrome oxidase, preventing the transfer of electrons to oxygen (9). Arsenite reacts with diethylthiocarbamate to form a chelate, thus inactivating oxidative enzymes requiring lipoic acid and causing the accumulation of pyruvate and other α-keto acids (16).

**Bacterial uptake of low-molecular-weight substrates.** To compare the effects of fluid shear on bacterial uptake of macromolecules with uptake of compounds with lower molecular weights, we also measured uptake of leucine and glucose. ^3H]leucine uptake by Z. ramigera was determined as described above when ^3H-BSA was used as a substrate, except formaldehyde (final concentration, 3%) was used to kill cells and stop uptake prior to filtration. ^3H]leucine (60 Ci mmol^{-1}; ICN Biomedicals, Inc.) was used at a concentration 0.27 µg liter^{-1}. Previous studies under different mixing conditions have shown that leucine uptake by Z. ramigera is unaffected by fluid motion (11).

**Molecular size fractionation.** During macromolecule degradation, bacteria can release ^3H_{2}O, hydrolyzed ^3H-BSA fragments, and low-molecular-weight metabolites into solution. The accumulation of low-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. We monitored the accumulation of radiolabel in a lower-molecular-weight size fraction (<10,000 amu), using low-protein-binding ultrafiltration units (Millipore Ultrafree-MC 10,000 NMWL). Samples (2 ml) were centrifuged at 2,040 × g for 15 min, and the filtrate was radioassayed.

**Oxygen uptake.** We measured dissolved oxygen (DO) concentrations to use oxygen uptake as a surrogate parameter for substrate uptake. This requires that substrate uptake be coupled to oxygen utilization. In BSA and glucose experiments, oxygen uptake by two stirred suspensions was compared with uptake by a still sample. The stirred suspensions were either 60-ml BOD bottles analyzed by using a DO meter and mixing electrode (Yellow Springs Instruments model 57) or a DO chamber (Yellow Springs Instruments model 5300 Biological Oxygen Monitor) that permitted continuous monitoring of oxygen in a small (<5-ml) mixed chamber. For stirred conditions, replicate BOD bottles were periodically sacrificed and oxygen was measured by using the mixing (Yellow Springs Instruments model 57) electrode. In dextran uptake experiments, the mixing oxygen probe used for BOD bottle measurements was replaced with a nonconsumptive DO probe (Wheaton 60 Second BOD System electrode) that allowed accurate oxygen measurement with or without mixing.

**Preparation of radiolabeled macromolecules.** ^3H-BSA was prepared from nonradiolabeled BSA as the ^3H-formal derivatization in a reaction that used ^3H]formaldehyde (33 mCi ml^{-1}; DuPont), sodium cyanoborohydride (Sigma), and BSA, using a modified procedure of Jentoft and Dearborn (8). We added to a centrifugal ultrafiltration unit (Millipore Ultrafree-MC 10,000 NMWL; 10,000-amu cutoff) 150 µl of BSA (4.2 mg ml^{-1} in 100 mM HEPES [N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid] buffer, pH 7.5), 50 µg of formaldehyde (4 mM), and 50 µg of cyanoborohydride (100 mM), which was reacted at room temperature for 2 h and centrifuged at 2,040 × g for 15 min; the retentate was resuspended in 400 µl of MSB three times, and the mixture was refrigerated at 4°C until use.

^3H]dextran (138 mCi g^{-1}; average molecular weight, 70,800 amu; Amersham) was dissolved in 0.5 ml of ultrapure water (Millipore Milli-Q system). The low-molecular-weight fraction (<10,000) was removed by a washing procedure, using ultrafiltration membranes. We placed 0.5 ml of the ^3H]dextran solution in centrifugal ultrafiltration units (Millipore Ultrafree-MC 10,000 NMWL), which were centrifuged at 2,040 × g for 15 min, and diluted the retentate with 0.5 ml of ultrapure water (three times). The final retentate (0.2-µm polycarbonate membrane filter units; Poretics Corp.) was sterile filtered and stored at 4°C until use. The final activity of the dextran was 0.046 µCi ml^{-1}.

Nonradiolabeled dextran was also prepared by using a washing procedure to remove material of <10,000 amu, using dextran in ultrapure water in a centrifugal cell
(Amicon 8200 series) containing a 10,000-amu-cutoff membrane (Amicon YM-10). The retentate was ultrafiltered to ~20 ml and resuspended in ultrapure water three consecutive times, and the solution was sterile filtered (0.2-μm Gelman Acrodisc syringe filters) prior to immediate use. The final concentration of the nonradioactive dextran stock solution was determined to be 1.5 g liter⁻¹ by the anthrone method (20).

**Mass transfer model.** The mass transfer model has been described in detail elsewhere (10–13). The effect of fluid shear on substrate uptake by dispersed cells under unsaturated conditions can be determined by using a mass transfer analysis. The rate of mass transfer, Q, to a cell is:

\[ Q = 4\pi aDSh_pC \]

where \( a \) is the cell radius, \( D \) is the substrate molecular diffusivity, \( Sh \) is the Sherwood number (defined as unity for a cell in a stagnant fluid), \( E_p \) is the collector efficiency, and \( C \) is the bulk solution substrate concentration. 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 at which substrate disappears from solution is:

\[ r_s = Q N \]

where \( N \) is cell concentration (liter⁻¹). Likewise, the rate of oxygen disappearance is:

\[ r_o = Y Q N \]

where \( Y \) is a yield coefficient (grams of O₂ gram of substrate⁻¹)

The Sherwood number correlations selected to calculate the effect of shear on mass transfer of substrate to spheres in fluid shear environments (11) were:

\[ Sh_G = 1 + 0.26 \text{Pe}_G^{0.5} \quad (\text{Pe} \ll 1) \]

\[ Sh_G = 1.54 \text{Pe}_G^{0.153} \quad (0.1 \leq \text{Pe} \leq 10) \]

where the Peclet number, \( \text{Pe}_G \), is defined as \( \alpha^2G/D \), and \( G \) is the fluid shear rate.

The ratio of mass transfer to a cell in a shear field, \( Q_G \), to that of a cell in a undisturbed fluid, \( Q \), is consequently:

\[ \frac{Q_G}{Q} = Sh_G \]

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 10² to 10⁷ amu, Polson (19) provided the empirical correlation:

\[ D = 2.74 \times 10^{-5} \text{ M}^{-\frac{1}{3}} \]

where \( D \) is in square centimeters per second, and \( M \) is the molecular weight. An empirical correlation for the diffusion coefficients of dextrans in water was obtained by Frigon et al. (4) as:

\[ D = 7.04 \times 10^{-5} \text{ M}^{-0.47} \]

The diffusivity of leucine in water was calculated by using the Wilke-Chang (23) correlation, which is valid for compounds in water with molecular weights of \(<1,000 \text{ amu} \), using:

\[ D = 5.1 \times 10^{-6} \frac{T}{\mu V_p} \]

where \( T \) is the temperature (Kelvin), \( \mu \) is the dynamic viscosity of water (centipoise), and \( V_p \) is the compound molal volume at normal boiling point, which is estimated from standard calculations as \( V_p = 171 \text{ cm}^3 \text{ g-mol}^{-1} \) for leucine (23). The diffusivity of glucose at 20°C is \( 0.6 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1} \) (22).

**Kinetics.** To test the mass transfer model, it was necessary to ensure that all substrate concentrations were in the range expected for unsaturated enzyme kinetics (15). We determined the Michaelis-Menten half-saturation constant, \( K_s \), for uptake of all substrates. We measured uptake rates in shaken flasks and calculated \( K_s \) by using double-reciprocal (Lineweaver-Burke) plots. Rates were normalized with cell concentrations. Bacterial numbers were determined by using acridine orange epifluorescence direct counts (7). For BSA, substrate concentration was measured with a Coomassie protein assay kit (Pierce Chemical Co.). For dextran and glucose, we used the anthrone method (20). Leucine uptake in Z. ramigera is unsaturated for values below 100 mg liter⁻¹ (11), 4 to 5 orders of magnitude higher than the leucine concentrations used in these experiments.

**RESULTS**

**BSA uptake.** The rate of BSA uptake by a suspended culture of Z. ramigera was monitored over a 52-h period for a cell suspension split into two samples, A and B, in 60-ml BOD bottles. During the first 8 h, BSA uptake was 12.6 times higher in reactor A, which was stirred, than in reactor B, which was still (Fig. 1). The uptake rates in the stirred and still reactors during this time were \( 4.3 \pm 0.2 \times 10^{-19} \) and \( 3.4 \pm 0.9 \times 10^{-20} \text{ g of BSA min}^{-1} \text{ cell}^{-1} \), respectively (Table 1). The measured amount of radiolabel from still samples was nearly constant, suggesting that the label on the BSA molecules may not have been incorporated into cell proteins (as discussed below). After 25 h, 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 h at a rate of \( 3.6 \times 10^{-19} \text{ g of BSA min}^{-1} \text{ cell}^{-1} \), 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 that there was a substantial release...
Table 1. Comparison of uptake rates of stirred and still reactors

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Type</th>
<th>Bacteria</th>
<th>Measured variable</th>
<th>Uptake rate (g min⁻¹ cell⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg liter⁻¹</td>
<td></td>
<td>Per liter (10¹⁵)</td>
<td>Stirred</td>
</tr>
<tr>
<td>BSA</td>
<td>1.5</td>
<td>Z. ramigera</td>
<td>4</td>
<td>4.3 × 10⁻¹⁹</td>
</tr>
<tr>
<td>BSA</td>
<td>40</td>
<td>Z. ramigera</td>
<td>0.1</td>
<td>1.5 × 10⁻¹⁵</td>
</tr>
<tr>
<td>Dextran</td>
<td>10.3</td>
<td>E. coli</td>
<td>2</td>
<td>4.7 × 10⁻¹⁵</td>
</tr>
<tr>
<td>Dextran</td>
<td>10.3</td>
<td>E. coli</td>
<td>2</td>
<td>1.2 × 10⁻¹⁶</td>
</tr>
<tr>
<td>Glucose</td>
<td>50</td>
<td>E. coli</td>
<td>1</td>
<td>1.6 × 10⁻¹⁵</td>
</tr>
<tr>
<td>Leucine</td>
<td>0.00027</td>
<td></td>
<td>2</td>
<td>7.2 × 10⁻²³</td>
</tr>
</tbody>
</table>

of radiolabel back into solution, is likely a result of microbial utilization of BSA for energy production.

To verify that radiolabel was being released into solution, we periodically monitored the amount of radiolabel in a lower-molecular size fraction (<10,000 amu). Since the molecular weight of BSA is approximately 68,000 amu, this smaller-size fraction is composed of hydrolyzed protein fragments, exported cellular metabolites, or the unassimilated formal compound containing the radiolabel released during hydrolysis. By measuring the small-size fraction, we obtained an additional measure of substrate uptake by the cell. The concentration of radiolabel in the stirred sample filtrate increased 7.1 times faster than that in the nonmixed (still) samples, indicating more active metabolic processes in the stirred sample (Fig. 2a). 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. 2b).

In separate experiments, we verified that the accumulation of radiolabel in cells was not due to biosorption. We added sodium cyanide and sodium m-arsenite to shear reactors to inhibit prevented cellular accumulation of radiolabel (Fig. 3).

In the noninhibited control culture, ⁴H accumulation is comparable to that in Fig. 1. This indicated that the observed radiolabel increases are due to increased metabolic activity and not to increased biosorption due to stirring. Similarly, abiotic adsorption of radiolabeled compounds on the reactor vessel walls was insignificant in these experiments. The total amount of radiolabel in each reactor was tracked during the course of each experiment by monitoring suspension radioactivity (solution plus cells, no filtration). Suspension radioactivity varied less than 5% during the course of any experiment.

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 to 7.4 mg liter⁻¹ in a separate, undisturbed reactor containing similar concentrations of BSA (2.0 mg liter⁻¹). Since K₅₅ values for O₂ are <1 mg liter⁻¹ (6), oxygen uptake by bacteria is saturated at concentrations used in these experiments.

Dextran uptake. Using ⁴H]dextran, we also observed that fluid mixing increased uptake of dextran by E. coli. Cultures of E. coli were acclimated to dextran, washed, resuspended in MSB, and split into two samples, stirred and still, each containing 10 mg of dextran liter⁻¹. The uptake rate in the mixed reactor, (4.7 ± 1.3) × 10⁻¹⁵ g of dextran min⁻¹ cell⁻¹, was 6.2 times the uptake of (7.6 ± 5.6) × 10⁻¹⁶ g of dextran min⁻¹ cell⁻¹ determined in the still reactor over a 90-min period (Fig. 4 and Table 1). The mixed reactor initially had a slightly higher activity than the still reactor. This difference in initial activity between shear and still conditions probably is a result of a more rapid binding of substrate to cell receptor sites in the stirred sample immediately upon mixing than occurred in the still sample.

Oxygen uptake. Oxygen utilization was monitored during macromolecule degradation experiments to determine the net activity of stirred samples in comparison to still samples. We added 40 mg of BSA liter⁻¹ to a washed suspension of Z. ramigera and separated the suspension into several BOD bottles, half of which were mixed on stir plates. We also placed 4 ml of this suspension into a DO chamber. Bottles

FIG. 2. (a) Amount of ⁴H label (converted to grams of BSA) appearing in filtrate (<10,000 amu) collected during the experiment reported in the legend to Fig. 1) during utilization of ⁴H-BSA by Z. ramigera in stirred (A) and still (B) reactors. (b) Similar, replicate experiment, but after reactor A was switched at 7 h from stirred to still and reactor B was switched from still to stirred.
were periodically sacrificed and analyzed for DO over a period of 240 min. The rates of oxygen utilization in the stirred BOD bottles and the DO chamber were 2.7 and 2.3 times faster, respectively, than the rates in the still bottles (Fig. 5) during the 80- to 240-min interval. The steady-state oxygen uptake rates in the stirred reactors were $(1.7 \pm 0.2) \times 10^{-15}$ g of O$_2$ min$^{-1}$ cell$^{-1}$ in the BOD bottle and $(1.5 \pm 0.2) \times 10^{-15}$ g of O$_2$ min$^{-1}$ cell$^{-1}$ in the DO chamber versus $(6.4 \pm 0.8) \times 10^{-16}$ g of O$_2$ min$^{-1}$ cell$^{-1}$ in the still reactor (Table 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 is 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.

We conducted a similar oxygen utilization experiment with E. coli grown on dextran, except we continuously monitored the still bottle with the nonconsuming DO probe, and we used only the DO chamber for the stirred sample. The rate of oxygen utilization of the stirred sample was 2.9 times faster than that of the still sample (Fig. 6). Oxygen concentrations decreased in the stirred bottle to 38% saturation $[(1.23 \pm 0.02) \times 10^{-16}$ g of O$_2$ min$^{-1}$ cell$^{-1}$] versus 75% saturation in the still sample after 200 min $[(4.2 \pm 0.2) \times 10^{-17}$ g of 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.

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 (11, 15) did not affect the uptake of low-molecular-weight substrates such as glucose and leucine. However, we were 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, we examined oxygen utilization by *E. coli*, using glucose as the sole carbon and energy source. We 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 $1.6 \pm 0.1 \times 10^{-15}$ g of O$_2$ min$^{-1}$ cell$^{-1}$ (Fig. 7). This indicates that the effects we measured because of stirring were due to macromolecular substrates used and that increased cell respiration rates were not an artifact of the experimental setup.

We also examined $[^3]$H-leucine uptake by cultures of *Z. ramiugera* grown on BSA and resuspended in MSB with no substrate. We found that $[^3]$H-leucine uptake in the stirred reactor was $(7.2 \pm 0.2) \times 10^{-23}$ g cell$^{-1}$ min$^{-1}$, 1.15 times the still reactor rate of $(6.3 \pm 0.2) \times 10^{-23}$ g cell$^{-1}$ min$^{-1}$ (Fig. 8). This result indicates that shear slightly increased uptake of the leucine. The magnitude of this effect is compared with calculated values below.

**Mass transfer models.** The effect of fluid shear on mass transfer to microorganisms can be calculated by using equations 5 to 8. These equations require cell size (radius), substrate diffusion coefficients, and reactor shear rates. The effective radius, determined during cell counting, of either *Z. ramigera* or *E. coli* was approximately 0.7 μm. The calculated diffusion coefficients, which range from $37 \times 10^{-8}$ to $874 \times 10^{-8}$ cm$^2$ s$^{-1}$ for each substrate, are shown in Table 2. The shear rate of the reactors used could not be directly measured. We 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 (3). In Fig. 9, we show the predicted effect of fluid shear rate on the rate of mass transfer to microorganisms over a shear rate range of 10 to 1,000 s$^{-1}$ for the various substrates used in this study. For BSA, we calculate that uptake would increase by factors of 1.07, 1.63, and 2.08 at shear rates of 10,200, and 1,000 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.

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 transfer model. For BSA, mass transfer calculations indicate that uptake should be 1.6 times greater in the stirred bottle than in the still bottle. However, the measured rate of stirred samples was 12.6 times the still reactor rate when radiolabeled substrates were used. The oxygen utilization rates indicated an overall increase of 2.9 times, which is much closer to the model value. For dextran, we calculated an increase by a factor of 1.8 for stirred samples, versus observed increases by factors of 6.2 with radiolabeled compounds and 2.9 with oxygen.

For the low-molecular-weight substrates, we calculated increases of 1.12 and 1.16 for leucine and glucose as a result

![FIG. 7. Oxygen consumption by *E. coli* growing on glucose in stirred (+, DO chamber; ×, BOD bottle) and still (□, BOD bottle) samples.](image)

![FIG. 8. Uptake of $[^3]$H-leucine by *Z. ramiugera* cultures in stirred (+) and still (□) bottles.](image)

![FIG. 9. Predicted effect of fluid shear of bacterial uptake of different compounds when uptake is limited by mass transfer to the cell surface. With the model, the ratio of bacterial uptake in a sheared fluid to uptake in an undisturbed fluid is equal to the dimensionless Sherwood number (equation 6).](image)

### Table 2. Comparison of predicted and observed ratios of uptake by stirred samples and still samples

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_r$ (mg liter$^{-1}$)</th>
<th>Diffusivity (cm$^2$ s$^{-1}$)</th>
<th>Ratio of stirred/still uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>[^3]$H</em></td>
</tr>
<tr>
<td>BSA</td>
<td>50</td>
<td>68</td>
<td>1.6</td>
</tr>
<tr>
<td>Dextran</td>
<td>60</td>
<td>37</td>
<td>1.8</td>
</tr>
<tr>
<td>Glucose</td>
<td>40</td>
<td>600</td>
<td>1.16</td>
</tr>
<tr>
<td>Leucine</td>
<td>&lt;100</td>
<td>874</td>
<td>1.12</td>
</tr>
</tbody>
</table>

* — not determined.
of stirring. This result for leucine compares favorably with the factor of 1.15 observed with radiolabeled substrates. We were not able to measure any increases in glucose uptake as a result of fluid shear when oxygen measurements were used.

**DISCUSSION**

The uptake of macromolecules, measured using radiolabeled chemicals and oxygen consumption, are higher in stirred than in still environments. When oxygen consumption was measured by using BOD bottles, BSA and dextran uptake rates of stirred samples were 2.3 and 2.9 times higher, respectively, than those of 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.

The increased rates of macromolecule uptake measured for stirred bottles were larger than expected from mass transfer calculations. We calculated increases in uptake by factors of 1.6 for BSA and 1.8 for dextran as a result of stirring. The differences between measured and predicted rates are likely due to both the assumptions made in deriving the mass transfer model and the series of correlations used to make the calculations. Microbial utilization of macromolecules involves several steps, including transport to the cell and binding to hydrolytic enzymes; macromolecule hydrolysis into sizes of compounds able to be transported across the cell membrane; and metabolism of components for either energy (catabolism) or cell growth (anabolism). The mass transfer calculations assume that the first transport step is limiting and describe transport to the cell, using a Sherwood number, as well as the rate of binding of macromolecules to enzymes, through a collector efficiency. However, the mass transport equations do not incorporate the effect of time required for hydrolysis and metabolism.

The total rate of macromolecule transport to cells was probably not estimated accurately with radiolabeled chemicals since the amount of radiolabel in the cell reflects uptake and anabolism, but not catabolism. In our experiments with stirred reactors, macromolecule utilization for anabolism was much larger than that for catabolism. In experiments with BSA as a sole carbon source, stirred cultures accumulated label in cells at a rate 12.6 times that of still cultures, but the total amount of label in the stirred samples was only 8 times larger than in still samples after 25 h (Fig. 1). When we stopped stirring the cultures, the amount of radiolabel in the cell decreased, while the amount of lower-molecular-weight (<10,000 amu) materials in solution increased, indicating a net loss of labeled macromolecules in the cells because of catabolism. If anabolism had dominated, the amount of radiolabel in the solution would not have substantially changed when the sample was removed from the stirring plate.

Oxygen consumption appears to predict more accurately the effect of shear, and our experimental results more closely agree with predictions of the mass transfer model for macromolecular substrates when oxygen consumption is coupled with growth. In experiments with dextran and BSA as sole carbon sources, oxygen uptake in still and sheared reactors were similar for the first 40 and 100 min (Fig. 5 and 6). Thereafter, oxygen utilization rates in stirred samples were higher than in still samples. The measured increases caused by stirring are approximately 50% higher than predicted. This difference can be a result of inaccuracies in mass transfer correlations or estimated effective diffusivities being lower than calculated values. Although we filtered out macromolecules smaller than 10,000 amu in preparing substrates, both BSA and dextran could have contained size fractions of higher molecular weight than average and could have undergone intermolecular bonding, resulting in average molecular sizes much larger than 68,000 or 70,000 amu.

**Implications for research.** We have demonstrated that fluid mixing can substantially increase the uptake rate of macromolecular components of culture media. Laboratory kinetic studies conducted with complex or defined medium containing macromolecules will be altered by mixing conditions. This would result in kinetic constants, determined by using conventional double-reciprocal plots, becoming a function of mixing conditions, such as flasks on shaker tables, magnetic stir plates, and bench top reactors. Since fluid shear is a function of energy input per unit volume of medium, the mixing rate and volume of medium used could also be important variables. It is suggested that mixing conditions should be reported in future studies, recognizing that kinetic data obtained in one reactor configuration may not be repeatable in another.

**Conclusions for wastewater treatment studies.** The biochemical oxygen (BOD) test is the standardized test used to evaluate the oxygen requirements of all wastewaters and the efficiency of wastewater treatment bioreactors such as trickling filters and activated sludge (6). 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 hydrodynamic conditions of a BOD test, i.e., an undisturbed reactor, wastewater reactors such as the activated sludge reactor are characterized by high-shear environments of 90 to 220 s⁻¹ (18). 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 (14, 17). This suggests that we may need to alter the conditions of a BOD test to reflect more accurately the biological hydrodynamic conditions of wastewater treatment bioreactors.

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