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Joseph William Roos
Louisiana State University and Agricultural & Mechanical College

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Specific cell adhesion and its application to monitoring and control of mixed culture bioreactors

Roos, Joseph William, Ph.D.
The Louisiana State University and Agricultural and Mechanical Col., 1990
SPECIFIC CELL ADHESION AND ITS APPLICATION TO
MONITORING AND CONTROL OF MIXED CULTURE BIOREACTORS

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Chemical Engineering

by
Joseph William Roos
B.S. University of Notre Dame, 1981
M.S. Rice University, 1984
May 1990
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ABSTRACT

Specific cell adhesion was proposed as a means to separate and selectively remove or recycle a population in a mixed culture reactor. Theoretical analysis indicated that stable coexistence steady states of competitive mixed cultures and unstable recombinant cultures are possible with this method. Specific cell adhesion was used to separate populations of *Escherichia coli* based on their expression of maltoporin an outer membrane protein. This separation was possible due to maltoporin binding immobilized starch. Various methods for operating a packed bed of the immobilized starch support are reported. Operating conditions are identified that allow quantitative estimates of populations based on their expression of functional maltoporin. This system was used as a means of obtaining real time estimates of the population balance in mixed culture fermentations. Specific adhesion was incorporated in a reactor designed to separate and selectively recycle strains of *E. coli* and allowed direct of the mixed culture population balance.

The selective release of specifically adhering cells was addressed as a method of separating cell populations. A discrete kinetic model was developed for the binding and release of the receptor-ligand pair on an adhering cell. Various factors including receptor and immobilized ligand density, removal forces, receptor-ligand binding kinetics, receptor mobility and soluble competing ligands were incorporated into this framework. The effect of these parameters on fractionation of cell populations based on receptor expression was investigated. Several analytical solutions for the dynamic model of cell release were developed for limiting cases. One of these models was used to successfully predict the release of specifically adhering cells from a packed bed.
CHAPTER 1
INTRODUCTION

1.1

The goal of this work was to develop a method to control the population balance in a mixed culture. The method was to be employed in a continuous mixed culture bioreactor to directly alter the level of a specific population. Many proposed methods for controlling the population balance rely on the manipulation of environmental parameters such as temperature, pH or dilution rate. These parameters have a global effect on the populations. Changing a parameter value directly influences all populations in the reactor. This feature can make it difficult to control a reactor containing several populations. The method also restricts the environmental parameters to the range required for maintenance of the desired population balance. This limits the use of these variables as a means to manipulate cell metabolism or reactor productivity. For these reasons, it was desired to develop a method in which the control of the population balance was uncoupled from manipulation of the environmental parameters.

An alternative to using environmental parameters are methods in which a designated population is physically retained in the reactor or removed from the reactor and the level of the population directly controlled. This translates into a problem of cell separation. Once the populations are separated, retention or removal of a population is possible.

Specific cell adhesion is potentially one of the most powerful means available for separating cell populations and appears to be well suited for incorporation into reactor control schemes. To use this method, an adhesion surface is prepared by immobilizing ligands that bind to a component of the cell outer surface. The formation of discrete bonds between the immobilized ligand and the cell surface component, the receptor, mediates cell adhesion to the surface. Adhesion specificity arises from the selectivity of the immobilized ligand-receptor interaction and can be extremely selective as observed for antibodies raised against a particular cell surface antigen. Due to this selectivity, cell populations that are similar in all respects except for the expression of a single outer surface component can exhibit different adhesion characteristics. It is this difference in adhesion that is used to separate cell populations.
The reactor design that was developed used specific cell adhesion to separate two populations of a mixed culture reactor and then recycled the desired population back into the reactor. This was termed a selective recycle reactor. To test this reactor, two competing cultures of *Escherichia coli* were used. The populations were similar, except for the expression of a functional lamB gene product, the maltoporin, on their outer surface. One population expressed the maltoporin at high levels, while the other population did not express a functional maltoporin.

The maltoporin is an outer membrane protein of *E. coli*. It is involved in the transport of maltodextrins across the outer membrane of the cell and displays an affinity for binding linear maltose polymers and starch. When starch is immobilized on a support, the interaction between the maltoporin and the starch leads to specific adhesion of the cell. The adhesion characteristics of the cell populations can be used to separate them on the basis of maltoporin expression.

The following chapters present results of investigations on specific adhesion and its application in bioreactor control. Most of the chapters are written so that they are self contained and may be read in any order. The Materials and Methods, Chapter 2, is a compilation of experimental procedures. It contains a complete, detailed account of experimental procedures. Most of the chapters also contain the necessary information on methods pertaining to their subject.

In Chapter 3, the characteristics of specific adhesion of *E. coli* to immobilized starch are reported. Batch adhesion and cell retention in a packed bed of starch-Sepharose are used to evaluate the role of maltoporin availability on specific adhesion. The effect of fluid velocity, temperature and pH on the retention of cells in the packed bed is also reported.

The equilibrium constants and the intrinsic rate constants for maltoporin binding a variety of ligands, are presented in Chapter 4. Various experimental procedures for studying maltoporin ligand interaction are analyzed. Methods for obtaining intrinsic rate and equilibrium constants are developed and used to analyze data reported in literature. The rate and equilibrium constants calculated by these methods are tabulated and compared.

In the chapter on selective elution, Chapter 5, a kinetic framework for the selective release of specifically adhering cells is presented. The effect of various operating conditions on the release of the adhering cells is explored using the kinetic model. The criteria that govern the use of simplified forms of the general model are determined and their limitations discussed. A version of the kinetic model is used to investigate selective release as a cell separation method. Model predictions are also compared with experimental data from the release of specifically adhering cells in a packed bed.
The application of specific adhesion as a means to monitor the mixed culture population balance is the topic of Chapter 6. Quantitative separation of two E. coli populations based on their expression of maltoporin is achieved using specific adhesion. Once separated, estimates of the biomass fraction for each population are obtained. This procedure produces real time measurements of the population balance. It also proves to be less sensitive to cell aggregation, which can effect estimates obtained using the traditional method of plate counts.

Operating characteristics of reactors that employ specific adhesion for maintaining a desired population balance are dealt with in chapters 7 and 8. In chapter 7, a new method for manipulating the steady-state behavior of a mixed culture is presented. Specific adhesion is used to retain a population in the reactor. Analysis and simulations using models for competitive mixed cultures and unstable recombinant cultures are employed to demonstrate the characteristics of this control method. The results of using specific adhesion to operate a selective recycle reactor are reported in chapter 8. Adhesion differences of two strains of E. coli are used to separate the populations. Recycling the disadvantaged population back into a continuous competitive culture is shown to allow retention of the population for a longer time than observed without recycle.

In the chapter Miscellaneous Results, the preliminary investigation of several alternative systems for studying specific cell adhesion and reactor control is reported. The first section deals with a novel method of exploiting the specific interaction between an immobilized ligand and a cell surface receptor to control an unstable recombinant culture. The second section addresses the reactor dynamics of several mixed cultures. Operating conditions are reported that yield a state at which two competing populations have similar growth rates. The applicability and limitations of these culture systems as a means to investigate population selective recycle or retention is discussed.
CHAPTER 2
MATERIALS AND METHODS

2.1 Introduction

The following chapter gives a description of the experimental methods used in this work. The various strains of *E. coli* are listed with genotype and a brief statement on their use is included. All assay procedures are included along with the experimental procedures for plasmid construction, colicin E1 production and purification and support preparation. Some of the methods are repeated as needed in the manuscripts.

2.2 Organisms, Plasmids and Media

2.2.1 Organisms

The strains of *Escherichia coli*, used in this work, are listed below in Table 2-1, with genotype and origin. The genotype abbreviations and their related function are compiled in Table 2-2. The yeast *Saccharomyces cerevisiae* ATCC 18790 (American Type Culture Collection, Rockville, Maryland) was also used in some mixed culture work.

<table>
<thead>
<tr>
<th>E. coli strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>MC4100</td>
<td>F araD139, Δ(argF-lac)U169 rasI150</td>
<td>Dr. Silhavy, Department of Molecular Biology, Princeton University</td>
</tr>
<tr>
<td>MCR106</td>
<td>MC4100 with ΔlamB106</td>
<td>Dr. Silhavy</td>
</tr>
<tr>
<td>pop3132</td>
<td>MC4100 with malT&lt;sub&gt;183&lt;/sub&gt;</td>
<td>Dr. Silhavy</td>
</tr>
<tr>
<td>GW1000</td>
<td>thr-1, leu-6, thi-1, lacY1, galK2, ara-14, xyl-5, mtl-1, proA2, his-4, argE3, str-31, tsp-33, sup-37, pro&lt;sup&gt;+&lt;/sup&gt;, lac&lt;sup&gt;+&lt;/sup&gt;, SllA11(sulA) ilv(Ts) tif-1(recA441)</td>
<td>Dr. Geoffrey Zubay, Fairchild Center for Biological Sciences, Columbia University.</td>
</tr>
<tr>
<td>JM83</td>
<td>ara, rpsL, Δ(lac-proAB), 680 lacZ ΔM15</td>
<td>Dr. Eric Achberger, Department of Microbiology, Louisiana State University.</td>
</tr>
<tr>
<td>NK5012</td>
<td>supE, supF</td>
<td>Dr. Achberger</td>
</tr>
<tr>
<td>ATCC 23716</td>
<td>K12 wild type</td>
<td>American Type Culture Collection, Rockville, Maryland.</td>
</tr>
</tbody>
</table>

The strain MC4100 and the two strains derived from it, MCR106 and pop3132, were used in the the work on specific adhesion to immobilized starch. The property of interest, and the difference in the three strains, involves the expression of the *lamB* gene. The *lamB* gene is part of the mal regulon and codes for the outer
Table 2-2. Genotype of *E. coli* with Related Function.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Related Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>araD</td>
<td>arabinose</td>
</tr>
<tr>
<td>argF</td>
<td>arginine</td>
</tr>
<tr>
<td>deoC</td>
<td>deoxyriboaldolase</td>
</tr>
<tr>
<td>flb</td>
<td>flagelum biosynthesis</td>
</tr>
<tr>
<td>gal</td>
<td>galactose</td>
</tr>
<tr>
<td>his</td>
<td>histidine</td>
</tr>
<tr>
<td>ilv</td>
<td>isoleucine, valine</td>
</tr>
<tr>
<td>lac</td>
<td>lactose operon</td>
</tr>
<tr>
<td>lacZ</td>
<td>β-Galactosidase</td>
</tr>
<tr>
<td>lamB</td>
<td>lamB porin, lambda phage binding, maltose transport</td>
</tr>
<tr>
<td>malT</td>
<td>positive regulatory gene of mal regulon</td>
</tr>
<tr>
<td>mtl</td>
<td>D-mannitol utilization</td>
</tr>
<tr>
<td>proA</td>
<td>proline</td>
</tr>
<tr>
<td>pts</td>
<td>phosphotransferase</td>
</tr>
<tr>
<td>rbsR</td>
<td>ribose</td>
</tr>
<tr>
<td>recA</td>
<td>recA protein SOS response</td>
</tr>
<tr>
<td>rel</td>
<td>relaxed; regulation of RNA synthesis</td>
</tr>
<tr>
<td>rpsL</td>
<td>streptomycin resistance</td>
</tr>
<tr>
<td>str</td>
<td>streptomycin resistance</td>
</tr>
<tr>
<td>supE</td>
<td>suppressor</td>
</tr>
<tr>
<td>thi</td>
<td>thiamine</td>
</tr>
<tr>
<td>thr</td>
<td>threonine</td>
</tr>
<tr>
<td>tif</td>
<td>temperature induced</td>
</tr>
<tr>
<td>tsx</td>
<td>phage T6 or colicin K resistance/sensitivity</td>
</tr>
<tr>
<td>xyl</td>
<td>xylose</td>
</tr>
</tbody>
</table>

surface porin, the maltoporin, through which maltose and large maltooligosaccharides cross the outer membrane (Benz, 1988). The lamB porin displays a higher specificity for binding maltooligosaccharides than other carbohydrates (Luckey and Nikaido, 1980; Ferenci et al, 1986; Benz et al, 1987). The expression of the lamB porin is regulated by the expression of the *malT* gene product (Debarbouille et al, 1978).
In strain MCR106, the AlamB106 impairs the function of the maltoporin. This strain was used in separation and reactor control experiments because it did not adhere to the starch-Sepharose columns under the chosen operating conditions. This deletion in the lamB gene also results in MCR106 displaying decreased growth rates at low maltose concentrations compared to a strain with a normal maltoporin.

The strain pop3132, also derived from strain MC4100, contains a constitutive mutation in the malT gene, the positive regulator of the mal regulon. Constitutive production of the gene product yields constitutive expression of the components of the mal regulon including lamB (Debarbouille et al., 1978). Since pop3132 expresses lamB at high levels independent of maltose induction, specific adhesion of populations grown on other carbon sources was possible. Competition between the carbon source and the immobilized starch can thus be controlled.

For colicin E1 production, strain GW1000 was used. The important feature of this strain is the tif mutation in the recA gene. At elevated temperatures, this mutation results in high expression levels of the recA protease (Spangler et al., 1985). The RecA protease degrades the LexA protein, which binds to the operator of the cea gene on the ColE1 plasmid and prevents colicin production (Ebina et al., 1983). Following degradation of the lexA protein, high levels of colicin E1 are produced. This method of inducing colicin E1 formation was more attractive than the standard method of inducing the cells SOS response with mytomycin C, a strong mutagen.

2.2.2 Plasmids

Various plasmids, all based on the ColE1 wild type plasmid, were used throughout this work. The names of these plasmids and their pertinent properties are listed below. The details of construction are covered in the section on plasmid construction.

- ColE1: wild type colE1 plasmid
- PXT-5: ColE1 with tet resistance, functional ColE1 regions

Both ColE1 and PXT-5 in GW1000 were obtained from Dr. Geoffrey Zubay, Fairchild Center for Biological Sciences, Columbia University.

- pRAH12: ColE1 with a kanamycin resistance cartridge inserted at the Smal site in the cea gene (Figure 2-1). The reading frame of the kanamycin cartridge is opposite that of the cea gene.
- pRAH17: ColE1 with kanamycin resistance cartridge inserted at the Sma site in the cea gene (Figure 2-1). The reading frame of the kanamycin cartridge is same as that of the cea gene.
pRAH20 ColE1 with a Tn5 transposon carry kanamycin resistance, inserted at approximately 6066 bp of the colE1 plasmid. This is in the cea gene (Figure 2-1).

Figure 2-1. Construction of pRAH12, pRAH17 and pRAH20
2.2.3 Media

The media used for growth of the *E. coli* strains and *S. cerevisiae* are listed below with their composition (Table 2-3).

<table>
<thead>
<tr>
<th>Media</th>
<th>Composition</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>L agar</td>
<td>tryptone, 10 g/l; yeast extract, 5 g/l; sodium chloride, 5 g/l; Difco agar, 15 g/l. For top agar use Difco agar, 8 g/l.</td>
<td>Spangler <em>et al.</em> 1985</td>
</tr>
<tr>
<td>Nutrient agar</td>
<td>beef extract, 3 g/l; peptone, 5 g/l; Difco agar, 15 g/l. Used as supplied by Difco.</td>
<td></td>
</tr>
<tr>
<td>EMB agar</td>
<td>peptone, 10 g/l; lactose, 5 g/l; sucrose, 5 g/l; dipotassium phosphate, 2 g/l; eosin Y, 0.4 g/l; methylene blue, 0.065 g/l; Difco agar, 13.5 g/l. Used as supplied by Difco.</td>
<td></td>
</tr>
<tr>
<td>L broth</td>
<td>tryptone, 10 g/l; yeast extract, 5 g/l; sodium chloride, 5 g/l;</td>
<td>Spangler <em>et al.</em> 1985</td>
</tr>
<tr>
<td>M63</td>
<td>potassium phosphate monobasic, 13.6 g/l; ammonium sulfate, 2.0 g/l; ferrous sulfate sephosphate, 5.0 mg/l. Adjust the to pH 7.0 with sodium hydroxide. After autoclaving add 1 ml/l magnesium sulfate sephosphate, 1M; and carbon source. Supplement with vitamins at 40 mg/l and amino acids at 20 mg/l as required. For growth of MC4100, MCR106 and pop3132, M63 was supplemented with thiamine and argine.</td>
<td>Miller, 1972</td>
</tr>
<tr>
<td>Minimal Medium with Yeast Extract</td>
<td>ammonium sulfate, 3.0 g/l; potassium phosphate monobasic, 3.0 g/l; sodium chloride, 2.0 g/l; calcium chloride dihydrate, 25 mg/l; magnesium sulfate sephosphate, 25 mg/l; yeast extract, 1.0 g/l; glucose or maltose added as desired, pH adjusted to 6.5 with sodium hydroxide. Media was filter sterilized.</td>
<td>Davison and Stephanopoulos, 1986</td>
</tr>
<tr>
<td>Yeast Minimal Medium</td>
<td>ammonium sulfate, 12.0 g/l; magnesium chloride hexahydrate, 0.52 g/l; 85% phosphoric acid, 1.6 ml/l; potassium chloride, 0.12 g/l; calcium chloride dihydrate, 90 mg/l; sodium chloride, 60 mg/l; manganese sulfate monohydrate, 3.8 mg/l; copper sulfate pentahydrate, 0.5 mg/l; boric acid, 7.3 μg/l; sodium molybdate dihydrate, 3.3 μg/l; nickel chloride, 2.5 μg/l; zinc sulfate heptahydrate, 2.3 μg/l; cobalt sulfate heptahydrate, 2.3 μg/l; potassium iodide, 1.7 μg/l; ammonium iron sulfate hexahydrate, 35 mg/l; m-inositol, 125 mg/l; pyridoxine hydrochloride, 6.25 ml/l; calcium-D-pantothenate, 6.25 mg/l; thiamine hydrochloride, 5 mg/l; nicotinic acid, 5 mg/l; D-biotin, 0.125 mg/l. pH adjusted with sodium hydroxide.</td>
<td>Davison and Stephanopoulos, 1986</td>
</tr>
</tbody>
</table>
2.3 Assays

2.3.1 Estimation of Cell Population

Several methods of estimating cell concentration were employed. The total biomass of a cell suspension was estimated by measuring its absorbance (optical density O.D.) at 650 nm (6-550 UV/VIS Spectrophotometer, Sargent Welch) with a 1 cm path length. Another instrument used for determining total biomass was the Klett-Sommerson colorimeter equipped with a blue filter. This biomass estimate, expressed in Klett units, was very convenient since the instrument took readings from a side arm culture flask.

Viable counts were used to obtain estimates of cell densities. A sample of cells was diluted to give a final count between 50 to 150 colonies per plate. A 50 μl volume of the cell dilution was spread on the plate and the plate was incubated at 37°C. The colonies arose from single cells or cell aggregates. Since the colony origin can not be determined, all counts are considered to arise from the same source. This can be a limitation of live counts when aggregation is prevalent (Snyder, 1947; Roos and Hjortso, 1989). Live counts were also used to estimate cell densities of individual populations in a mixed culture. This was done by plating cell suspensions on the selective media, agar with neomycin or EMB agar.

The number of colonies on a plate was determined by manual counting or with an automatic colony counter (Autocount, Artek). Using the automatic counter, accurate counts for E. coli plated on L agar or nutrient agar, were obtained when colonies were approximately 1 mm diameter. The size control was set at 0.5 mm and the sensitivity was adjusted to a setting which gave minimal changes in the count number with small changes in the sensitivity.

Estimates of cell or aggregate number were also obtained using a 180+ Elzone particle counter (Particle Data). Samples were diluted with electrolyte to give final counts between 30,000 and 150,000 per 50 μl. The electrolyte used was particle free 6% sodium chloride in distilled, deionized water (DDI). It was filtered through a 0.2 μm membrane filter and stored in a covered particle free container with another 0.2 μm filter on the removal line. All utensils and glassware that contact the electrolyte were rinsed with particle free electrolyte prior to use. This was necessary to avoid particulate contamination. The lower size limit for the E. coli count window overlaps with the noise from "dirty" electrolyte. The parameter setup for counting E. coli is given in Table 2-4. The parameter setup for counting E. coli and S. cerevisiae is also shown in Table 2-4. Using this setup, S. cerevisiae counts fall in the upper window while the E. coli counts lie between the Lo and Hi trigger.
Table 2-4. Setup for Elzone 180+ Particle Counter.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Setting</th>
<th>E. coli</th>
<th>E. coli and S. cerevisiae</th>
</tr>
</thead>
<tbody>
<tr>
<td>Orifice</td>
<td></td>
<td>30.232</td>
<td>30.232</td>
</tr>
<tr>
<td>Current</td>
<td></td>
<td>6.5</td>
<td>6.0</td>
</tr>
<tr>
<td>Gain</td>
<td></td>
<td>5.5</td>
<td>5.0</td>
</tr>
<tr>
<td>Size</td>
<td>Diameter</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calibration Size</td>
<td></td>
<td>1.31</td>
<td>1.31</td>
</tr>
<tr>
<td>Calibration Trigger</td>
<td></td>
<td>251</td>
<td>251</td>
</tr>
<tr>
<td>Lo Trigger</td>
<td></td>
<td>0.573</td>
<td>0.507-0.573</td>
</tr>
<tr>
<td>Hi Trigger</td>
<td></td>
<td>1.83</td>
<td>1.69</td>
</tr>
<tr>
<td>Volume</td>
<td></td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Time</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Flow Pressure</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Metering</td>
<td>Volume</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Impedence</td>
<td></td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td>Percentiles</td>
<td>L</td>
<td></td>
<td>L</td>
</tr>
</tbody>
</table>

Each of the methods for estimating cell population density measure different quantities. A comparison of population estimates for Klett units, live counts and particle counts versus O.D_{650} are shown in Figure 2-2.
Figure 2-2. Comparison of Population Estimates.
Population of *E. coli* grown in batch culture on M63 estimated by A) particle counts; B) plate
counts; and C) Klett meter. All estimates compared to optical density at 650 nm.

2.3.2 Carbohydrate Assay

The sulfuric acid-phenol method of Dubois (Dubois et al., 1956) was used to determine total carbohydrates. For this assay, a 2 ml sample containing 10-70 ug of carbohydrate or a blank containing DDI, 2 ml, was mixed with 5 % phenol, 1 ml. Concentrated sulphuric acid, 5 ml, was added to the liquid and immediately mixed. The sample was allowed to stand at room temperature for 10 minutes. It was then vortexed and placed in a water bath at 25 to 30 °C for 10 to 20 minutes. The absorbance was measured at 490 nm. Standard curves
were prepared for each run using either glucose or α-amylase digestion of potato starch. The total carbohydrate content of the sample was determined using the standard curve. An example of a standard curve for glucose and the α-amylase digestion of potato starch is shown in Figure 2-3.

![Standard Curve for Total Carbohydrates](image)

**Figure 2-3. Sample Standard Curve for Total Carbohydrates.**

### 2.3.3 Protein Assay

The Bio-Rad Protein Assay (Bio-Rad) was used for determination of protein concentration. This is a Coomassie Blue method, and provides accurate protein determination in the range of 0.2 to 1.4 mg/ml using the standard assay procedure.

To carry out the standard assay, 1 volume of dye reagent was diluted with 4 volumes of DDI and filtered through a Whatman No. 1 paper. This dye could be used for approximately 2 weeks after preparation. Several standards of known protein concentration between 0.2 and 1.4 mg/ml were prepared. A 0.1 ml volume of the standards, properly diluted sample and buffer, to be used as a blank, were placed in test tubes. To each tube, 5.0 ml of the prepared dye solution was added. The tubes were mixed by gently vortexing. After 5 to 60 minutes, the spectrophotometer was zeroed to the blank at 595 nm, and the adsorption of the standards and samples were determined. A new standard curve was prepared for every run and the protein concentration was estimated from this standard curve. A sample of the standard curve is presented in Figure 2-4.
2.3.4 Stab Test for Colicin Production

The ability of individual colonies to produce colicin was determined by stab tests (Spangler et al., 1985). Colonies were transferred to nutrient agar with an inoculation needle and stabbed into the agar. The plates were incubated overnight at 37 °C. The resulting colonies were removed by pressing the plate on a sterile Kimwipe draped over a replicate plating block. Top agar at 45 °C, containing 20% indicator culture was layered over the stab plate. ATCC 23716, grown overnight in L-broth was used as the indicator culture. Within 6 - 8 hours, clearing of the indicator culture in the soft agar could be observed over stabs from colicin producing colonies.

2.3.5 Spot Test for Colicin Production

The spot test was used to determined the colicin titer in a liquid sample (Spangler et al., 1985). Samples to be tested for colicin activity were treated with chloroform (5% v/v), and dilutions of the sample were made with sterile DDI. A lawn of 100 μl ATCC 23716 from overnight culture in L broth was spotted with 15 μl of the diluted sample. The reciprocal of the greatest dilution that gave clearing on the lawn was taken as the colicin titer.
2.3.6 Horizontal Agarose Gel Electrophoresis

Horizontal agarose gel electrophoresis was performed on either the BioRad minigel or BioRad double wide minigel apparatus. For the mini gel, the gels were prepared from agarose, 0.4 g; 10X TBE, 5 ml; and dionized water, 45 ml., melted and tempered to approximately 65 °C before pouring. The 10x TBE buffer is made of Trizma, 108 g/l; boric acid, 55 g/l; sodium EDTA, 9.3 g/l. The gel recipe was doubled for the double wide mini gel. To every 9 µl of sample, BPB (1 % bromyl phenol blue; 26 % ficol), 3 µl; 10X TBE, 1 µl; RNase, 2 mg/ml, 0.5 µl; was added. The gels were run at 80 volts until the dye front had left the wells. The voltage was increased to 90 to 100 volts. The gels were run until the dye front had reached the end of the gel. With the 0.8% agarose gel, it was observed that 500 base pair fragments ran with the dye front.

2.3.7 Sodium Dodecyl Sulfate Polyacrylamide Slab Electrophoresis

Polyacrylamide slab electrophoresis was performed using a modified Laemmli technique (Laemmli, U. K., 1970). The solutions used for sodium dodecyl sulfate (SDS) polyacrylamide slab electrophoresis are given in Table 2-5. Solutions one through nine were degassed and used to prepare the final gel solutions.

The 7.6 % acrylamide separating gel was prepared by combining solution 2, 0.25 ml; solution 3, 5.6 ml; solution 4, 8.4 ml; solution 6, 30 µl; solution 7, 120 µl; and DDI, 9.4 ml. This mixture was poured between glass plates that had previously been sealed with solution 1. The top of the separating gel was covered with solution 8, 2 ml; and allowed to polymerize for 45 minutes. After the polymerization was complete, the n-butanol was poured off, the top of the gel was rinsed with DDI and dried. The sample well comb was inserted and the stacking gel prepared by combining solution 6, 10 µl; solution 7, 40 µl; and solution 10, 10 ml. This mixture was poured in and allowed to polymerize.

The protein samples, containing at least 10 µg of protein, were prepared by mixing one volume of sample with one volume of solution 11 and boiling for 5 minutes. This denatures the proteins. A molecular weight standard (Sigma Chemical; SDS-6H) was treated in the same manner.

The gels were run at 60 volts, until the tracking dye passed the bottom of the stacking gel, at which time the voltage was raised to 120 volts. The run was allowed to proceed until the tracking dye was within 3 mm of gel bottom. The gel was removed from between the glass plates and fixed for two hours in solution 12. After fixing, the gel was stained in solution 13 for 18 hours and destained electrophoretically in solution 14. The positions of the protein bands were determined by scanning the gel with a Joyce Loebl Ephortec Densitometer with a 530 nm filter, an apperature of 0.05 mm x 3 mm and the transmission at 2.0A.
TABLE 2-5. Solutions for SDS Polyacrylamide Electrophoresis

<table>
<thead>
<tr>
<th>Solution Number</th>
<th>Solution Name</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Sealing Agar</td>
<td>1.5 % w/v Noble agar</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.1 % w/v Sodium dodecyl sulfate (SDS)</td>
</tr>
<tr>
<td>2.</td>
<td>Sodium dodecyl sulfate</td>
<td>10 % w/v</td>
</tr>
<tr>
<td>3.</td>
<td>Standard acrylamide stock</td>
<td>30 % w/v Acrylamide</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.2 % w/v Bis acrylamide</td>
</tr>
<tr>
<td>4.</td>
<td>Separating gel buffer</td>
<td>1 M Tris-HCl pH 8.7</td>
</tr>
<tr>
<td>5.</td>
<td>Stacking gel buffer</td>
<td>1 M Tris-HCl pH 6.8</td>
</tr>
<tr>
<td>6.</td>
<td>Tetraethylmethylethylenediamine (TEMED)</td>
<td>(TEMED): Commercial solution</td>
</tr>
<tr>
<td>7.</td>
<td>Ammonium persulfate (APS)</td>
<td>10 % w/v Ammonium persulfate</td>
</tr>
<tr>
<td>8.</td>
<td>n-Butanol</td>
<td>n-Butanol saturated with DDI</td>
</tr>
<tr>
<td>9.</td>
<td>5X Running buffer</td>
<td>Tris base 15 g/l</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Glycine 72 g/l</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SDS 5 g/l</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pH 8.3</td>
</tr>
<tr>
<td>10.</td>
<td>Stacking gel stock</td>
<td>Solution 2 1.0 ml</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Solution 3 16.7 ml</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Solution 5 12.5 ml</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DDI bring to 100 ml</td>
</tr>
<tr>
<td>11.</td>
<td>Sample buffer</td>
<td>Tris base 1.21 g/l</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2-Mercaptoethanol 0.1 % v/v</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SDS 0.1 % w/v</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EDTA 0.001 M</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Glycerol 40.0 % v/v</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bromothymol blue 0.05 % w/v</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pH 8.3</td>
</tr>
<tr>
<td>12.</td>
<td>Fixative solution</td>
<td>Isopropanol 25 % v/v</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Acetic acid 10 % v/v</td>
</tr>
<tr>
<td>13.</td>
<td>Stain solution</td>
<td>Coomassie blue R-250 0.1 % w/v</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Methyl alcohol 25 % v/v</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Acetic acid 10 % v/v</td>
</tr>
<tr>
<td>14.</td>
<td>Destain solution</td>
<td>Methyl alcohol 25 % v/v</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Acetic acid 10 % v/v</td>
</tr>
</tbody>
</table>

2.4 Plasmid Miniprep

Plasmid DNA was isolated by the method of Birnboim (Birnboim, 1983). The plasmid DNA was extracted from cells grown on 2 ml of L broth overnight or to early stationary phase. The cells were pelleted in a sterile microcentrifuge tube and the supernatant was discarded. The cell pellet was suspended in the residual broth
associated with the pellet by vortexing. To this slurry was added 110 μl of freshly prepared solution I (Table 2-6). The cells and solution I were thoroughly mixed by inverting the tube several times. After incubation for 30 minutes at 0 °C, solution II, 220 μl, was added. The sample was mixed by inverting the tube until the liquid was clear and slightly viscous. The sample was stored at 0 °C for 5 minutes, and solution III, 165 μl, added. After addition of solution III, the tube was mixed by inversion until a clot of DNA formed. The clot contains chromosomal DNA, while the plasmid DNA remains in suspension.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>lysozyme, 2 mg/ml; glucose 5 mM; EDTA, 10 mM; and Tris, 25 mM pH 7.9</td>
</tr>
<tr>
<td>II</td>
<td>sodium hydroxide, 0.2 M; sodium dodecyl sulfate (SDS), 1 %</td>
</tr>
<tr>
<td>III</td>
<td>Dissolve 3 moles of sodium acetate in a minimal amount of water and titrate to pH 4.8 with glacial acetic acid. Bring volume to 1 liter with distilled water and store at room temperature.</td>
</tr>
</tbody>
</table>

After storage at 0 °C for 30 to 60 minutes, the sample was centrifuged in an Eppendorf microfuge for 5 minutes and a 400 μl aliquot of the supernatant was transferred to a sterile microfuge tube. This step was performed on no more than eight samples at a time, as the chromosomal DNA precipitate tends to go back into solution. To the supernatant, 1 ml of cold ethanol was added, mixed by inverting and stored at -60 °C for 15-20 minutes or -20 °C for 1 hour. This causes the plasmid DNA to precipitate. The samples were centrifuged for 5 minutes in the Eppendorf microfuge and the supernatant discarded. The pellet was dissolved in 100 μl of 0.1 M sodium acetate, 0.05 M Tris, pH 8.0 and mixed by thumping the tube bottom. To avoid damaging the DNA by fluid shear, a vortex mixer was not used. To the suspension, 1 ml cold ethanol was added, mixed by inversion and stored at -60 °C for 15-20 minutes or -20 °C for 1 hour. This mixture was centrifuged for 5 minutes and the supernatant discarded. The pellet of plasmid DNA was washed with cold 80 % ethanol, to remove residual salts, centrifuged, and the supernatant discarded. The pellet was dried in a vacuum for about 10 minutes.

### 2.5 Transformation of *E. coli*

A culture of *E. coli* was made competent by the method of Lederberg and Cohen (Lederberg and Cohen, 1974). An overnight culture was used to provide a 2 % v/v inoculum in 50 ml of L broth. The culture, grown at 37° C in a shaker, was harvested before reaching an O.D. of 0.6 at 600 nm. A higher O.D. results in a large
decrease in the number of competent cells. The culture was harvested, chilled and pelleted by centrifugation. The pellet was suspended in 50 ml of ice cold 0.1 M magnesium chloride, centrifuged and the supernatant discarded. The pellet was resuspended in 25 ml, ice cold 0.1 M calcium chloride and chilled in an ice bath for 20 minutes, centrifuged and the supernatant discarded. The competent cells were suspended in 2.5 ml of ice cold 0.1 M calcium chloride and stored on ice.

To transform a 0.2 ml sample of competent cells, 0.1 ml of chilled DNA sample in 0.02 M Tris pH=8.0, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.02 M sodium chloride (TEN buffer) was added and the mixture allowed to incubate on ice for 30 minutes. The competent cell-DNA mixture was heat shocked at 42 °C for 2 minutes to facilitate uptake of the DNA. The cells were chilled, diluted 10-fold with prewarmed L broth and incubated at 37 °C for 60 to 90 minutes. Samples of the transformed cells, 0.05 to 0.2 ml, were then plated out on a selective medium.

2.6 Plasmid Construction

For the investigation of colicin E1 as a means to control a mixed culture of E. coli, a plasmid was required that possessed an easily identifiable marker, conferred immunity to colicin E1 and was stably maintained in culture. Such a plasmid would also be useful as a marker in the other work with mixed cultures. This plasmid was constructed by placing an antibiotic resistance gene within the cea gene of the ColEl plasmid. Two methods were used to construct this plasmid. The first employed recombinant techniques to insert a kanamycin resistance cartridge within the cea gene. In the second case, genetic techniques were employed to insert the Tn5 transposon within the cea gene.

The E. coli strain JM83 was transformed with the ColEl plasmid isolated from the strain GW1000 ColEl. The transformed cells were plated on L agar which had previously been spread with 20 or 40 µl of filter sterilized cell lysate from GW1000 ColEl. This lysate contained colicin E1 and thus, exerted a selective pressure in favor of cells that contained the ColEl plasmid with its immunity to colicin E1. Control plates of competent JM83, that was not transformed, were also prepared. All modification to the ColEl plasmid, was carried out on plasmid DNA isolated from the transformed JM83 growing on the colicin E1 plates.

2.6.1 Ligation of Kanamycin Resistance into Wild Type ColEl.

The ColEl plasmid was purified from the transformed JM83 by the Birnboim procedure and the plasmid preparations were suspended in 40 µl of water. The presence of the plasmid was verified by electrophoresis on a horizontal agarose minigel. Two distinct plasmid bands appeared (Figure 2-5). These bands are not believed to be caused by dimerization. Both bands appeared in plasmid preparations that were digested with
**Sma**I. This digest results in a single break in the circular plasmid DNA. Dimers should have been resolved and run as the same molecular weight on the gel. Upon transformation of cells with DNA removed from one of the bands, it was found that both bands appeared in subsequent plasmid preparations. The origin of these dual bands remains a mystery.

![Image](image.png)

**Figure 2-5. ColE1 with Insertions at Smal.**

<table>
<thead>
<tr>
<th>Lane</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ColE1 wild type</td>
</tr>
<tr>
<td>2</td>
<td>kan' cartridge cut from pUC4K</td>
</tr>
<tr>
<td>3</td>
<td>ColE1 with kan' at Smal</td>
</tr>
<tr>
<td>4</td>
<td>λ phage, EcoR1, HindIII restriction</td>
</tr>
<tr>
<td>5</td>
<td>pRAH17, EcoR1, HindIII restriction</td>
</tr>
<tr>
<td>6</td>
<td>pRAH12, EcoR1, HindIII restriction</td>
</tr>
</tbody>
</table>

From each of three Birnboim preparations of ColE1, 25 μl aliquots were removed and pooled. A sample of 5 μl was removed as a control and the remaining 70 μl was subjected to digestion by the restriction enzyme **Sma**I (BRL). There is one **Sma**I site on the Col E1 plasmid (Figure 2-6), located in the cell gene which codes
for colicin production. This digestion should result in a single linear strand of DNA. The digestion was performed by combining React 4 10x buffer (BRL), 8 μl; and SmaI, 10 U/μl, 5 μl; with the Birnboim preparation, 70 μl; and allowed to proceed for 2 hours at 37 °C.

The plasmid cut with SmaI was purified by electrophoresis on a horizontal agarose minigel. The gel was stained in the dark with ethidium bromide. There appeared two distinct bands. The lower plasmid band, smaller molecular weight, was cut from the gel. The agarose slab containing this band was placed in a dialysis bag filled with TBE buffer. The plasmid DNA was removed from the gel by electro-elution in the electrophoresis chamber at 90 volts for 1 hour with TBE as the buffer. The agarose slab was removed from the bag and the sides of the bag were gently rubbed together to suspend the plasmid DNA in the buffer. The TBE-plasmid DNA solution was transferred to a plastic tube and stored at 4 °C.

To remove residual agarose from the DNA, a phenol extraction was performed. The phenol was distilled and stored under a water blanket at 4 °C to insure purity and prevent oxidation. One volume of phenol was added to one volume of the sample. The tube was shaken for 60 seconds and centrifuged at 10000 rpm for 15 minutes. The top, aqueous, layer was carefully removed so as not to disturb the phenol interface. This aqueous supernatant was made approximately 0.3 M in acetate by the addition of 0.1 volume 3 M sodium acetate. Three volumes of absolute ethanol were added. This solution was mixed by inverting the tube several times,
and the sample stored at -40 °C for 20 minutes or -20 °C for 60 minutes. The sample was centrifuged at 10000 rpm for 15 minutes and the supernatant discarded. The precipitate was suspended in 0.3 M sodium acetate and transferred to a microcentrifuge tube. Approximately 1 ml of absolute ethanol was added, the sample mixed by inverting the tube and again stored at -40 °C for 20 minutes or -20 °C for 60 minutes. The sample was centrifuged in an Eppendorf microfuge for 5 minutes and the supernatant discarded. The precipitate was washed with 1 ml of 80% ethanol to remove salts and centrifuged again. The remaining precipitate, containing the plasmid DNA, was dried at room temperature under a vacuum and stored at -20 °C.

The sample of Smal cut Col E1 was dissolved in 20 µl of water. A control was prepared by combining Smal cut ColE1, 2 µl; 5x BRL ligation Buffer, 4 µl; 40 mM ATP, 0.5 µl; T4 DNA ligase (BRL), 0.8 µl; water, 14 µl. A second reaction mixture was prepared in the same manner but also contained 4 µl of the kanamycin cartridge preparation in place of an equal volume of water. The kanamycin resistance cartridge (Figure 2-7) was cut from pUC-4K (Pharmacia LKB) with HincII to give blunt ends and was provided courtesy of T.K Ross (Department of Microbiology, LSU). Both mixtures were allowed to react overnight at 16 °C.

Strain JM83 was transformed using both the control and the kanamycin cartridge reaction mixture. The transformed cells were plated on neomycin (200 µg/ml) L agar with and without colicin E1 lysate for selection and incubated overnight at 37 °C. Colonies from the neomycin plates and the colicin E1 plates, containing cells transformed with the ColE1-Kan' ligation mixture, were picked. The presence of the plasmid with the inserted kanamycin cartridge was confirmed in the picked colonies by electrophoresis (Lane 3, Figure 2-5). The insertion of the kanamycin resistance cartridge in the ceg gene and its disruption of colicin production was tested by performing the stab assay for colicin production.

Several of the transformants displayed evidence of colicin production. In an attempt to determine the source of this behavior, plasmid DNA was isolated from several such transformants and from several transformants that did not produce colicin. These plasmids were then digested with Hind III and EcoRI. The digestion solution contained EcoRI (BRL), 0.5 µl; Hind III (BRL) 0.5 µl; 10x React 3 buffer, 0.5 µl; and 10x React 2

![Figure 2-7. Restriction Map of Kanamycin Cartridge.](image)
buffer 0.5 µl for each digestion. These were mixed and 2.0 µl of the mixture was added to the plasmid preparation, 9 µl. The digestion was conducted at 37 °C for 2 hours. The plasmid digests were run on a horizontal agarose gel (Lane 5 and 6, Figure 2-5). The size of the fragments was determined from its position on the gel relative to the position of the bands from the molecular weight standard, the λ DNA digest (Lane 4, Figure 2-5).

The ColEl plasmid does not possess a Hind III site and contains a unique Eco RI site located near the end of the cea gene (Figure 2-6). The kanamycin cartridge contains a Hind III site that is asymetrically located within the cartridge (Figure 2-7). The digest with EcoRI and HindIII, produced two fragments when the kanimycin cartridge was present and the size of the smaller fragment indicated the orientation of the cartridge in the cea gene. The transformants that were suspected of retaining colicin production were found to carry a plasmid, pRAH 17, where the direction of transcription was the same for both the kanimycin cartridge and the cea gene. The orientation of the cartridge was the opposite in the other transformants (Figure 2-5). The plasmid with this orientation was titled pRAH 12.

The purpose of the placing the kanamycin cartridge at the Sma I site in the cea gene was to disrupt colicin production. A possible explanation of the observation of residual colicin E1 production by pRAH 17, lies in the organization of the cea gene (Suit et al., 1985). The C terminal end of the cea gene must be intact if the colicin E1 is to kill the cell. The central region of the gene is necessary for the recognition and binding of the colicin E1 to the cell surface receptor. The N terminal end is reported to be necessary for colicin function and has been suggested to be involved in interactions with the outer membrane (Luria and Suit, 1987). The Sma I restriction site lies near the N terminal end. The kanamycin resistance cartridge in pRAH 17 lies with transcription in the same direction as the cea gene and the production of active colicin is suspected to be continue from this plasmid.

### 2.6.2 Tn5 mutagenesis of Col E1 in JM83

Strain JM83 was transformed with the colE1 plasmid. The Tn5 transposon was introduced into the cell by infection with a lambda phage carrying Tn5. This phage is λ b221 rex::Tn5 cI857 O888 P_mP (Ruvkun and Ausubel, 1981). The E. coli strain NK5012 has an amber supressor supE44 required for this mutant phage to produce progeny. Strains JM83 ColEl and the permissive host, NK5012, were grown in an overnight culture. L broth, 50 ml, supplemented with maltose, 0.1 mg/ml; was inoculated from the overnight cultures and incubated at 37°C. To 2 ml of these cultures, were added 2 M magnesium sulfate, 10 µl; and λ phage lysate,
200 μl (provided by Dr. Achberger, Department of Microbiology, LSU) were added. This was mixed and allowed to stand at 37 °C for 10 minutes. The cultures were incubated at 37 °C for 1 hour with shaking. This was to allow for the expression of the neomycin phosphotransferase type II carried by Tn5.

After this time, the permissive host, NK5012, showed signs of cell lysis while JM83 did not. Samples of the cultures, 50, 100, and 200 μl, were spread on neomycin (100 μg/ml) L agar and cultured overnight at 37 °C. The plates of JM83 were scraped to obtain all colonies that expressed neomycin resistance and the cells suspended in 1 ml of L broth. The cells in this suspension were pelleted by centrifugation and the plasmid purified by the Birnboim procedure. In this case, due to the large amount of cell mass, the volumes of the three solutions in the Birnboim prep (Table 2-6) were increased to solution I, 200 μl; solution II, 400 μl; and solution III, 300 μl.

The plasmid DNA was used to again transform JM83 with selection on neomycin L agar. This procedure of purifying the plasmid DNA and transforming another culture greatly increases the odds that the neomycin resistance is the result of Tn5 insertion in the plasmid DNA as opposed to insertion into chromosomal DNA.

Birnboim plasmid preps were performed on cultures derived from colonies that did not display colicin activity as determined by the stab plate method. These plasmids were screened by electrophoresis on a horizontal agarose minigel for insertion of the Tn5 into the plasmid (Lane 1 and 2, Figure 2-8). Insertion of the Tn5 transposon results in a plasmid which is 5.8 kb larger than the original plasmid.

The position of the Tn5 insertion was determined by restriction mapping. A digest was performed using Dral to determine if the insertion occurred within the cea gene. Dral, 1 μl and 10x React I, 1 μl, were added to 9 μl of the Birnboim preparations of the plasmids that contain Tn5. The restriction reaction was allowed to proceed at 37 °C for 3.5 hours. The size of restriction fragment for the plasmids with the Tn5 insert were compared to fragments from a Dral digestion of wild type ColEl plasmid (Figure 2-8). The Dral digestion of wild type colEl gives three fragments of 4.3, 1.22, 1.1 kb (Figure 2-6). The Tn5 transposon does not possess a Dral restriction site (Figure 2-9). The digestion of ColEl::Tn5 with Dral produces a fragment that contains the Tn5 transposon and is 5.7 kb larger than expected. If the Tn5 insertion occurs between the Dral sites that bracket most of the cea gene at 5117 and 6338 bp, the 1.22 kb band of the ColEl::Tn5 Dral digest increases to 6.92 kb (Lane 3 and 4, Figure 2-8). If the 4.3 kb fragment is found to increase to 10.1 kb, then the Tn5 may have inserted in the end of the cea gene that runs from 6338 bp through the EcoRI site to 60 bp.
Figure 2-8. ColE1 with Tn5 Inserions.

Lane 1  ColE1 wild type
Lane 2  ColE1 with Tn5
Lane 3  ColE1 wild type, Dral restriction
Lane 4  ColE1 ceg::Tn5, Dral restriction
Lane 5  λ phage, EcoRI, HindIII restriction
Lane 6  ColE1 ceg::Tn5, EcoRI, HindIII restriction
Lane 7  ColE1 ceg::Tn5, EcoRI, HindIII restriction
Lane 8  ColE1 ceg::Tn5, EcoRI, HindIII restriction

The EcoRI, HindIII digestion of lambda phage yields fragments of 23130, 9416, 6557, 4361, 2322, 2027, 564 and 125 base pair.

An EcoRI, HindIII digestion of colE1::Tn5 was performed using the same procedure described in the section on ligation of the kanamycin resistance cartridge. From this digestion the distance from the EcoRI site of Tn5 insertion was determined. The ColE1 plasmid has a unique EcoRI site and does not possess any HindIII sites (Figure 2-6). The Tn5 transposon does not possess an EcoRI site, but does contain two HindIII sites (Mazodier et al, 1985) (Figure 2-9). The digestion of ColE1::Tn5 will yield a 3.4 kb piece of DNA.
from between the two Hind III sites of the Tn5. There will also be two other fragments. The size of the smaller of these fragments indicates the distance from the EcoR I site to the site of Tn5 insertion (Lane 6, 7 and 8, Figure 2-8). If the Tn5 lies in the cea gene the small fragment must be less than 2.7 kb. With the distance of insertion and the information from the colEl::Tn5 Dra I digest, the positions of various ColE1::Tn5 mutations in the cea gene were mapped (Figure 2-10).

![Restriction Map of Tn5](image)

**Figure 2-9. Restriction Map of Tn5.**

2.7 Colicin Production and Purification.

2.7.1 Colicin Production

Colicin was produced using a modified procedure of Spangler et al (Spangler, et al. 1985). Cultures of GW1000 were established in L broth supplemented with 1 g/l glucose from colonies maintained on nutrient agar plates. This culture, grown overnight, was used as a 10% (v/v) inoculum in all colicin E1 production studies.

The effect of the temperature shift and the duration of the incubation period at 42 °C was investigated. Batch cultures of GW1000 were started at 30 °C. One culture was maintained at 30 °C for 8 hours. The temperature in the other cultures was shifted to 42 °C after 2 hours. At 3, 4 and 6 hours after the temperature
shift, the cells were harvested by centrifugation. The cell pellet was suspended in 0.1 M phosphate, pH=7.0, and the cells were lysed in a french press. The cell fragments were pelleted by centrifugation at 10000 rpm (10000 g for a 872 IEC rotor) for 10 minutes and the supernatant used for spot tests to determine colicin activity (Table 2-7). The temperature shift to 42 °C had a great effect on the colicin E1 activity in the culture. The duration of the shift from 3 to 6 hours, did not noticeably alter the colicin E1 activity.

<table>
<thead>
<tr>
<th>Culture Temperature Profile</th>
<th>30 °C for 8 h</th>
<th>30 °C for 2 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Shift to 42 °C for 3 h</td>
<td>Shift to 42 °C for 4 h</td>
</tr>
<tr>
<td>Colicin Activity (U/ml of Culture broth)</td>
<td>8.8x10⁶</td>
<td>2.8x10⁶</td>
</tr>
</tbody>
</table>

Colicin E1 was produced in stirred batch reactors with air sparging at 1 vol/(vol min) and temperature control. The reactors were operated at 30 °C for two hours. Colicin production was induced by increasing the temperature to 42 °C. The reactors were harvested approximately four hours after the temperature shift and the cells pelleted in a Sharples continuous centrifuge operated at 20 - 25 psig steam. The cell slurry was suspended in 0.1 M potassium phosphate buffer, pH 7.0 to give a final volume of approximately 50-200 ml for every liter of cell broth spun down.

2.7.2 Colicin Purification

Two methods for purification of colicin E1 were tested. The first method, was a modified procedure of Schwartz and Helinski (Schwartz and Helinski, 1971). Initially, sodium chloride and guanidine hydrochloride were tested for releasing membrane associated colicin E1. Cells were suspended in 0.1 M phosphate buffer, pH=7 which was 1 M in either sodium chloride or guanidine hydrochloride. The cells were agitated at 37 °C for 16 hours. Neither method resulted in the release of measurable quantities of colicin E1. As a result, cell lysis was used to release colicin E1 from the cells.
The cells were lysed in a french press and the lysate placed in an ice bath. Ammonium sulfate was slowly added with stirring to yield a 50% saturated solution. Stirring was continued for 30 minutes and the precipitate was sedimend by centrifugation at 10000 rpm (10000 g for a 872 IEC rotor) for 20 minutes. The precipitate, the 0-50 fraction, was suspended in 0.1 M phosphate buffer pH=7.0 and dialyzed against this buffer overnight.

Purification of the colicin was performed by ion exchange chromatography in a 1.6 x 40 cm jacketed chromatography column (Pharmacia LKB) maintained at 4°C and packed with DEAE Sephadex A50 cation exchange (Pharmacia LKB). The bed volume was 58 ml. A 2 ml sample of the 0-50 fraction was introduced onto the column and eluted with 0.1 M phosphate buffer, pH=7.0, containing 0.85% sodium chloride. The flow rate of the buffer through the column was 0.3 ml/min or 8.9 ml/(cm² h). The maximum colicin activity, as determined by the spot test, was found in the peak that eluted at 17.5 ml. The activity, protein content and specific activity of this colicin fraction are given in Table 2-8. The purity of the colicin fraction was evaluated using SDS-PAGE electrophoresis. The area under the peaks in the gel scan (Figure 2-11) was used to estimate the purity of colicin E1 (Table 2-8).

Table 2-8. Comparison of Colicin E1 Purification Methods.

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Colicin Activity (U/ml)</th>
<th>Specific Activity (U/mg)</th>
<th>Purity by SDS PAGE†</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-50 % Fraction</td>
<td>2.67x10⁵</td>
<td>8.59x10³</td>
<td></td>
</tr>
<tr>
<td>20-50 % Fraction</td>
<td>1 x 10⁴</td>
<td>5.1 x 10²</td>
<td></td>
</tr>
<tr>
<td>Crude Lysate</td>
<td>1.3 x 10⁶</td>
<td>1.8 x 10³</td>
<td></td>
</tr>
<tr>
<td>DEAE-Sephadex 0-50 % Fraction</td>
<td>n.d.</td>
<td>n.d.</td>
<td>56%</td>
</tr>
<tr>
<td>CM-Sepharose 20-50% Fraction</td>
<td>2.7 x 10⁴</td>
<td>6.0 x 10³</td>
<td>66%</td>
</tr>
<tr>
<td>CM-Sepharose Crude Lysate</td>
<td>1.3 x 10⁶</td>
<td>4.4 x 10³</td>
<td>79%</td>
</tr>
<tr>
<td>n.d. - not determined, †Sodium dodecyl sulfate polyacrylamide Electrophoresis (see section 2.3.7)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The second procedure tested for the purification of colicin E1 was a modified procedure of Cleveland et al (Cleveland et al, 1983). In this method, the relatively high, though disputed, value for the pI of colicin E1 (pI=10.5, Cleveland et al., 1983; pI=9.05, Schwartz and Helenski, 1971) is used to separate colicin by
adsorption onto CM-Sepharose at a pH of 9.0. Two methods of sample preparation prior to introduction to the CM-Sepharose columns were investigated. In one case the crude lysate was subject to fractionation by ammonium sulfate and the 20 - 50 fraction was used. In the second case the crude cell lysate was used. In both preparations, the sample was dissolved in a 0.1 M phosphate buffer, pH=7.

For both preparations, the purification of colicin E1 was performed in a 1.0 x 20 cm chromatography column (Pharmacia LKB), packed with CM-Sepharose 6CL (Pharmacia LKB). The column was maintained at 4 °C. The bed volume was approximately 12.5 ml. The sample was introduced on the column and washed through the column with 0.05 M Borate buffer pH=9.0 at a flow rate of between 0.3 and 0.75 ml/min, 23 to 57 ml/(cm² h). After the sample was washed through the column with 3 to 5 column volumes of buffer, elution was started with 0.05 M borate pH=9.5 with 0.3 M sodium chloride. The peak containing colicin activity began to elute from the column in the 7.5 ml fraction or approximately 0.6 bed volumes. This peak was collected and the colicin activity determined (Table 2-8). The purity of the fraction collected by both methods
was determined by SDS-PAGE (Figure 2-12, Table 2-8). The colicin E1 was purified, as calculated from the specific activities, by a factor of approximately 12 when the 20-50 fraction was passed through CM-Sepharose. A purification factor of 24 was obtained over the crude lysate.

![Scan of SDS-PAGE analysis of colcin E1 purified on a CM-Sepharose.](image)

**Figure 2-12. Colicin E1 Purified on CM-Sepharose.**
Scan of SDS-PAGE analysis of colcin E1 purified on a CM-Sepharose. Starting material was either a 20-50 % cut from ammonium sulfate precipitation or crude cell lysate.

The large peak in the densitometer scan of the SDS PAGE gel (Figure 2-11 and 2-12) represents the colicin E1. From the relative mobility of these peaks and the relative mobility of standard proteins, it is possible to
estimate the molecular weight of the colicin E1. The estimates for the colicin E1 purified by each method (Table 2-9) agree quite well with a previous estimate of 56,000 (Schwartz and Helinski, 1971) and that calculated from the genetic sequence of 57,279 (Luria and Sui, 1987).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Retention Factor</th>
<th>M.W.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.224</td>
<td>66,000</td>
</tr>
<tr>
<td></td>
<td>0.376</td>
<td>45,000</td>
</tr>
<tr>
<td></td>
<td>0.473</td>
<td>36,000</td>
</tr>
<tr>
<td></td>
<td>0.592</td>
<td>29,000</td>
</tr>
<tr>
<td></td>
<td>0.65</td>
<td>24,000</td>
</tr>
<tr>
<td></td>
<td>0.822</td>
<td>20,000</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>14,200</td>
</tr>
<tr>
<td>Colicin E1 from 0-50 Fraction on DEAE-Sephadex</td>
<td>0.288</td>
<td>54,500</td>
</tr>
<tr>
<td>Colicin E1 from 20-50 Fraction on CM-Sepharose</td>
<td>0.286</td>
<td>54,390</td>
</tr>
<tr>
<td>Colicin E1 from Crude Lysate on CM-Sepharose</td>
<td>0.289</td>
<td>54,720</td>
</tr>
</tbody>
</table>

The CM-Sepharose column was used to purify colicin E1 from the crude lysate in all the preparative work. This method was not only more simple than the other methods, requiring only one purification step after cell lyses, but CM-sepharose was easier to equilibrate than DEAE-Sephadex. The CM-Sepharose did not swell or shrink in the range of ionic strength buffers used as did the DEAE-Sephadex. The purification of colicin E1 from cell lysate by CM-Sepharose resulted in similar purities as observed in the other steps and also gave high recoveries.

2.8 Support Preparation

2.8.1 Immobilization of Colicin E1

Colicin E1 was coupled to a support activated with 1,1'-Carbonyldiimidazole (CDI) (Figure 2-13). This procedure was chosen over other activation/coupling methods because it yields a bond free of charge, coupling can be performed under mild conditions and the activated support displays good stability (Hearn et al, 1979; Bethell et al, 1981)
Figure 2-13. CDI Activation and Coupling.

Activation of Carbohydrate support is shown. Activated support can couple with amino group or hydrolize.
Initially, colicin E1 was coupled to a React-Gel 6X CDI-activated support (Pierce, Rockford, IL). This is a cross linked agarose support. The activated agarose was washed on a vacuum filter with acetone:water (7:3), acetone:water (3:7), water and suspended in 0.1 M borate buffer, pH=8.5. The washes were done rapidly so the gel was not allowed to dry. The washed gel was split into three fractions, placed in polypropylene tubes and varying concentrations of colicin E1 were added (Table 2-10). The gel and colicin E1 were allowed to react at 4 °C. After 64 hours, the gel was separated and washed five times with 3 volumes of 0.1 M phosphate buffer, pH=7.0 with 0.05 M sodium chloride. The colicin E1-agarose was suspended in this buffer and stored at 4 °C.

<table>
<thead>
<tr>
<th>Colicin E1 in Coupling Solution</th>
<th>Colicin E1 Coupled to Support</th>
</tr>
</thead>
<tbody>
<tr>
<td>(mg/ml)</td>
<td>(mg/ml Support)</td>
</tr>
<tr>
<td>React-Gel 6x</td>
<td>0.664</td>
</tr>
<tr>
<td></td>
<td>0.332</td>
</tr>
<tr>
<td></td>
<td>0.133</td>
</tr>
<tr>
<td>Sephadex G25</td>
<td>1.24</td>
</tr>
<tr>
<td></td>
<td>0.688</td>
</tr>
<tr>
<td></td>
<td>0.344</td>
</tr>
</tbody>
</table>

There was a large quantity of colicin E1 immobilized in the agarose support, as most of the pore volume was accessible. However, the colicin E1 immobilized in the pore would not be accessible to a cell. The simplicity of using preactivated support is thus outweighed by the waste of immobilizing colicin E1 where it is inaccessible to a cell. Sephadex support, activated with CDI, was used as a means of immobilizing colicin E1 only on the outer surface of the support. The support, Sephadex G25, (Pharmacia LKB) has a small pore size so colicin E1 can not penetrate into the pores.
The Sephadex G25 was allowed to swell for about 36 hours in 0.1 M phosphate buffer pH=7.0. The excess buffer was removed from the swollen Sephadex G25 by vacuum filtration on a coarse fritted glass filter. The moist Sephadex G25 was weighed and washed on the coarse fritted glass filter with 20 ml per 3 g of moist gel of the following solutions, 1-4 dioxane:water, 3:7; 1-4 dioxane:water, 7:3; and anhydrous 1-4 dioxane. The gel was suspended in anhydrous 1-4 dioxane and CDI, 0.12 g per 3 g of moist gel cake was added. This was shaken gently at room temperature for 4 hours. The gel was washed on the fritted glass filter with anhydrous acetone and stored at 4 °C in anhydrous acetone.

To couple colicin E1 to the CDI-Sephadex G25, the solvent was changed by successive washes with acetone:water, (7:3), acetone:water, (3:7), water and finally the coupling buffer, 0.1 M borate, pH=8.5. All of these washes were carried out with ice cold solvents in an ice bath. Colicin E1 was added to the activated support in polypropylene tubes and the coupling allowed to proceed at 4 °C for 48 hours. Several concentrations of colicin E1 were used in this coupling procedure (Table 2-10).

In all cases, the amount of bound colicin E1 was below that bound in the agarose support. This is not surprising, only the outer surface of the Sephadex G25 is available for colicin E1 immobilization. There is no explanation for the very low levels of colicin E1 bound in the solutions where it was added in a concentration of 0.688 and 0.344 mg/ml.

2.8.2 Immobilization of Starch

Starch was covalently coupled to Sepharose 6B (Pharmacia LKB) using the oxirane activation procedure (Figure 2-14) (Sundberg and Porath, 1974; Ferenci and Lee, 1982). Sepharose 6B was suction dried on a coarse fritted glass filter. The dried gel was removed and weighed. The gel was returned to the fritted glass filter and washed with DDI. To every 1 gram of moist Sepharose, 1,4-butanediol diglycidyl ether, 1 ml; and 0.6 M sodium hydroxide containing 2 mg/ml sodium borohydride (freshly prepared), 1ml; was added. This was vigorously mixed on a rotary shaker for 8 hours at 25 °C. The gel was transferred to a fritted glass filter and the reaction stopped by washing with large volumes of DDI. Oxirane-Sepharose is obtained by this procedure.

To effect ligand coupling, 1 volume of oxirane-Sepharose was washed on a fritted glass filter with 20 volumes of DDI followed by 20 volumes of 0.1 M sodium hydroxide. The gel was suction dried, transferred to a flask and 2.5 volumes of 0.1 M sodium hydroxide containing 20 mg/ml potato starch (Sigma) was added. This mixture was shaken in a rotary shaker at 45 °C for 20 hours. The excess starch was washed away from the gel with 0.1 M sodium hydroxide and the gel suspended in 2.5 volumes of 1 M ethanolamine. This was shaken in a rotary shaker for 4 to 6 hours at 45 °C. The gel was removed and washed on a fritted glass filter.
sequentially with 10 to 15 volumes of DDI, 0.1 M sodium acetate pH=4, 0.1 M sodium hydroxide, DDI and finally 10 mM Tris-HCl, pH=7.2 containing 0.02% sodium azide and 1 mM EDTA. The gel was stored in this buffer at 4 °C.

To estimate the starch coupled to the Sepharose, the amount of starch released from the gel by α-amylase was determined. Starch-Sepharose, 0.5 ml, was suspended in 50 mM potassium phosphate, pH=6.8. An equal volume of the phosphate buffer containing 40 μg/ml α-amylase was added. This mixture was shaken at 30 °C for 30 minutes and centrifuged to separate the gel. The supernatant was removed and the starch concentration determined by the total carbohydrate assay.
2.9 Cell Adhesion

2.9.1 Batch Adhesion

Cells used in batch adhesion were harvested from a batch culture in exponential growth, pelleted, washed in distilled, deionized water (DDI). They were pelleted again and resuspended in DDI. Maltose was added for the batch adhesions in the presence of a competing ligand. Two volumes of the suspended cells were added to one volume of starch-Sepharose in a polypropylene tube. The support and cells were immediately mixed. The tube was periodically gently inverted throughout the remainder of the experiment to keep the starch-Sepharose suspended.

At the appropriate time, a homogeneous sample of cells and support were removed from the tube. The sample was centrifuged in an IEC Clinical centrifuge at a setting of two for one minute to sediment the starch-Sepharose support. A sample from the supernatant was removed and cell concentration was estimated using an Elzone 180+ particle counter (Particle Data). Samples of cell suspension were treated in the same manner. The centrifugation did not alter the number of suspended cells. From this control, cell growth was not detected over the period of the adhesion experiment.

2.9.2 Selection of Subpopulations with Altered Adhesion Properties

To select for a subpopulation of ATCC 23716 with a high degree of specific adhesion, the method of Ferenci and Lee was employed (Ferenci and Lee, 1982). An adhering subpopulation was isolated by passing a pulse of cells through 1 ml of starch-Sepharose in an Econo column (Bio Rad). The packing was washed with approximately 10 volumes of the M63 salts buffer, pH=7.0. The adhering population was then eluted with M63 salts buffer, pH=7.0, containing 0.2 M maltose, collected and cultured in M63 with 1 g/L maltose.

The population of cells thus isolated, displayed a high degree of specific adhesion to the starch-Sepharose 6B support. This culture was maintained on L agar and used as inoculum for further adhesion studies. The adhesion property of this population was assayed before any transfer or experimental work.

2.9.3 Quantification of Specific Cell Adhesion

The characteristics of specific adhesion of cells to a starch-Sepharose 6B was quantified in a system using a 1.0 x 20 cm chromatography columns (Pharmacia LKB) fitted with a 20 μm bed support. A pulse of cells was introduced into the column. The cells were washed through the column at a constant flow rate with the wash buffer, M63 salts, pH=7.0. The specifically adhering cells were eluted using an elution buffer, M63 salts.
pH=7.0, containing a specific competing ligand. For elution of all adhering cells M63 salts, pH=7.0, with 0.2 M maltose was used. The eluant from the columns was directed through a flow through spectrophotometer (V4, Isco) and biomass concentration was continuously monitored as absorbance at 650 nm.

The signal from the spectrophotometer was analyzed by a Spectra Physics 4270 integrator set in the data slice mode. The data slice information was sent to an IBM PC XT and stored on disk. From this information the peaks of cell mass eluting from the column were integrated. The area under the peak was an estimate of the total biomass in the pulse and compares well with the total cell count in the pulse (Figure 2-15). This system allowed for rapid, reproducible comparison of the total biomass eluting from the column.

![Figure 2-15. Peak Area Versus Cell Number.](image)

The starch-Sepharose packing could be reused many times when properly treated. After each run, the packing was slowly back flushed, 0.5 ml/min, with approximately 0.25 ml of the 0.2 M maltose elution buffer. The bed was then washed with the wash buffer for several minutes at 0.75 to 1.0 ml/min. The packing was backwashed again using the wash buffer and the wash continued until the signal returned to its baseline. The purpose of this procedure was to dislodge any sterically entrapped cells. The packing was washed with 2% formaldehyde, which was left in the packing when the column was not in use.
2.9.4 Estimation of Mixed Culture Population Balance by Adhesion

The population balance in a mixed culture was determined by vortexing a sample, passing it through the chromatographic column and integrating the O.D. peaks of the exit stream. The first peak corresponds to the non-adhering strain. The second peak, obtained by eluting the column with maltose buffer, corresponds to the adhering strain. The fraction of one of the subpopulations is found as the fraction of its peak area to the sum of the two peak areas.

2.10 Population Specific Recycle

The apparatus used for specific population recycle consisted of the reactor, the packed beds of starch-Sepharose for population separation, the buffer delivery system and the detector (Figure 2-16). Separation of the two populations was performed in one of two 1.0 x 20 cm chromatography columns (Pharmacia LKB) fitted with a 20 \( \mu \)m bed support and packed with 1 ml settled volume of starch-Sepharose. The eluant from the columns was directed into a flow through spectrophotometer (Isco, V4) and biomass concentration was continuously monitored as absorbance at 650 nm. The eluant stream was directed to either a waste container or back to the reactor. The buffer delivery system allowed either column to be washed with the wash buffer, M63 salts, the elution buffer, M63 salts with 0.2M maltose, or sterilized using 1% formaldehyde.

The reactor and the buffer reservoirs with tubing and valves were steam sterilized at 15 psig for 20 minutes. The columns were packed with starch-Sepharose in M63 buffer and placed online. The columns, packing and the detector flow cell were then washed for one hour with 1% formaldehyde at a flow rate of 15 ml/h. During the wash period the flow was occasionally reversed to provide back washing. The formaldehyde was left in the system for 4-8 hours. The columns were again washed with formaldehyde for 1 hour at a flow of 15 ml/h with occasional back wash. Large volumes of sterile M63 salts were then washed through the column and detector to flush all the formaldehyde from the system. This procedure resulted in sterilization of the population recycle system.

During operation of the specific recycle, a continuous stream was taken from the reactor and circulated through the sample loop. The residence time in this loop was on the order of 2 minutes. Samples were removed from this stream and introduced into the column at a flow rate of 0.75 ml/min. After the pulse was placed on the column, the cells were washed through the column with M63 salts buffer. The first peak to come off the column was composed of the non-adhering cells and was returned to the reactor. The specifically adhering cells were eluted with M63 salts buffer containing maltose, 0.2 M, and discarded. After this peak had eluted,
the bed was washed with 10 to 15 volumes of M63 buffer to remove the maltose from the column. The separation was alternated between the two columns to allow sufficient time for column washing. This procedure allowed reactor cell samples to be separated on a column every 30 minutes.
2.11 References


Miller, J.H. (1972), Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.


CHAPTER 3
SPECIFIC ADHESION OF ESCHERICHIA COLI
TO STARCH-SEPHAROSE

3.1 Abstract

The specific adhesion of Escherichia coli to a starch-Sepharose support was investigated in a batch and a packed bed adhesion system. Available maltoporin level was manipulated through induction, the presence of soluble maltose and mutant cell strains. In the batch system, maltoporin availability, as determined by induction or soluble maltose concentration altered the initial rate of cell adhesion but did not significantly change the final fraction of bound cells. Cell retention in a packed bed was found to be a function of maltoporin induction and cells with deletions in the lamB gene were not retained. Retention of cells in the packed bed as a function of fluid flow rate, temperature, pH and maltoporin expression are also reported.

3.2 Introduction

The specific adhesion of a cell to a surface is mediated by the binding between a receptor on the cell surface and an immobilized ligand. The formation of these discrete bonds is the dominant mechanism by which cells are retained at the surface. The affinity of the receptor-ligand binding mechanism is reflected in the specificity of cell adhesion. The adhesion of cells to a surface is a function of several system properties. The receptor and immobilized ligand density can greatly alter adhesion (Rutishauser and Sachs, 1975; Weigel et al., 1979). The hydrodynamic forces on the cells (Hertz et al., 1985), the properties of the cell membrane such as receptor mobility (Rutishauser and Sachs, 1975) or membrane elasticity (Marquardt and Gordon, 1975; Van Blitterswijk et al., 1976) as well as temperature and pH also play a part in the specific adhesion of cells (Rutishauser and Sachs, 1975; Ferenci, 1983; Clune et al., 1984; Hertz et al., 1985).

Specific cell adhesion has been investigated using many different cell-ligand systems (Sharma and Mahendroo, 1980; Ferenci and Lee, 1982; Murray et al., 1987). Perhaps, the most versatile system for studying cell adhesion is that of Escherichia coli adhering to immobilized starch. The adhesion is mediated by the interaction between the immobilized starch and the lamB gene product, the maltoporin (Ferenci and Lee, 1982). The system has been used to assay maltoporin function (Charbit et al., 1988; Heine et al., 1988), isolate strains with altered maltoporin properties (Clune et al., 1984), monitor and control mixed cultures of E. coli (Roos and Hjortso, 1989a,b), and bind cells in an immobilized cell reactor (Ferenci, 1983). Despite these applications, like other specific adhesion systems, the quantitative aspects of the specific adhesion and the effect of the various system parameters, are still unclear.
In this work, the adhesion characteristics of *E. coli* to starch-Sepharose in mixed batch adhesion and in packed beds is reported. The role of receptor density on adhesion in the mixed system and in the packed bed are compared. The adhesion of cells in the packed bed as a function of the fluid velocity, pH and temperature are reported. These results are discussed in light of published data and model predictions.

### 3.3 Materials and Methods

#### 3.3.1 Organisms and Growth Conditions

Three strains of *E. coli* were used in this study. ATCC 23716, a K12 wild type, was obtained from the American Type Culture Collection. Strains MCR106 and pop3132 were a gift of T. Silhavy, Princeton University. Both strains are derived from strain MC4100 (Benson and Silhavy, 1983). MCR106 has ΔlamB106, and strain pop3132 is malTc

The cultures used for the batch adhesion experiments were grown on a defined media, pH=6.5 (Stephanopoulos et al, 1985) or a complex media; (NH₄)₂SO₄, 3.0 g/l; KH₂PO₄, 3.0 g/l; MgSO₄·7H₂O, 25 mg/l; NaCl, 2.0 g/l; Yeast Extract, 1.0 g/l; pH=6.5. The cultures used in the packed bed adhesion experiments were grown on M63, pH=7.0 (Miller, 1972). For induction of maltoporin, cells were grown with maltose, 1 g/l, as the carbon source. All other culture used 1 g/l glucose as the carbon source. All batch cultures were performed in shake flasks at 37 °C.

#### 3.3.2 Specific Cell Adhesion

Starch-Sepharose was prepared as described by Ferenci and Lee (Ferenci and Lee, 1982) using potato starch (Sigma) and Sepharose 6B (Pharmacia LKB). The immobilized starch concentration was determine to be 7.15 mg/ml of support, using the method of Ferenci and Lee (Ferenci and Lee, 1982).

Cells used in batch adhesion were harvested from a batch culture in exponential growth. The cells were pelleted and washed in distiled, deionized water (DDI), pelleted and resuspended in DDI. Maltose was then added for the study of batch adhesion in the presence of a competing ligand. Two volumes of the suspended cells were added to one volume of starch-Sepharose in a polypropylene tube. The support and cells were immediately mixed. The tube was periodically inverted throughout the period of the experiment to keep the starch-Sepharose suspended.

At the appropriate time, a homogeneous sample of cells and support were removed from the tube. The starch-Sepharose support was separated from the suspended cells by centrifugation. This procedure took approximately 1 minute. A sample from the supernatant was removed and cell concentration was estimated
using an Elzone 180+ particle counter (Particle Data). As a control, samples of cell suspension were treated in the same manner. The centrifugation did not alter the number of suspended cells and cell growth was not detected over the period of the adhesion experiment.

To select for a subpopulation of ATCC 23716 with a high degree of specific adhesion, a pulse of cells grown on M63, 1.0 g/l maltose, was passed through 1 ml of starch-Sepharose in an Econo column (BioRad). The packing was washed with approximately 10 volumes of the M63 salts buffer, pH=7.0. The adhering population was then eluted with M63 salts buffer, pH=7.0, containing 0.2 M maltose, collected and cultured in M63, 1 g/l maltose. A population, 23716A, which displayed high retention in the packed bed of starch-Sepharose was obtained after two selections. This culture was maintained on L agar and used as inoculum for further adhesion studies in packed beds. The adhesion property of this population was assayed before any transfer or experimental work and remained stable.

The characteristics of specific adhesion of cells in a packed bed of starch-Sepharose was quantified in a 1.0 x 20 cm, jacketed chromatography column (Pharmacia LKB) fitted with a 20 μm bed support. Approximately 1 ml of starch-Sepharose was packed in this column. Using 1.33 μm latex beads, the void fraction of the bed was estimated to be approximately 0.41. Cells were harvested from late exponential batch cultures or continuous cultures. A pulse of cells was introduced into the column and washed through the column at a constant flow rate with the wash buffer, M63 salts, pH=7.0. Adhering cells were eluted using M63 salts pH=7.0, containing 0.2 M maltose. In the studies of adhesion as a function of pH, the pH of the cell suspension, the wash and elution buffer were adjusted to the desired value with sodium hydroxide. The eluant from the columns was directed through a flow through spectrophotometer (V4, Isco) and biomass concentration was continuously monitored as absorbance at 650 nm.

### 3.4 Results

Batch adhesion was employed to follow the rate of specific adhesion of *E. coli* 23716 to starch-Sepharose. The adhesion of cultures grown on maltose in complex and defined media was tested. The media composition did not effect adhesion. Cells from both cultures were observed to adhere to starch-Sepharose at about the same rate and to the same extent (Figure 3-1). The cells did not adhere to unmodified Sepharose.

Maltose grown cells adhered more quickly than cells grown with glucose as the carbon source (Figure 3-2). The final fraction of bound cells was approximately the same for the two cultures. Batch adhesion was also performed in the presence of maltose using cells with maltoporin expression induced by growth on maltose. Maltose is a soluble ligand which competes with immobilized starch for the maltoporin. Adhesion in the
presence of 0.1 M maltose was initially slower than observed for the an induced culture without maltose present (Figure 3-2). The time course of adhesion was similar to that observed for the culture grown on glucose. The final fraction of adhered cells in the presence of 0.1 M maltose, was about the same as the glucose and maltose grown cultures. The addition of 0.25 M maltose was found to further decrease the initial rate of adhesion. Due to sampling problems the final level of adhesion was not determined.

The adhesion of several E. coli strains in the packed bed was estimated by washing a pulse of cells through the bed and then eluting specifically adhering cells with a buffer containing maltose. Strain 23716 displayed very low adhesion in the bed of starch-Sepharose. Therefore, adhering strain 23716A was used in the packed bed experiments. When grown on maltose, an average of 95% of the 23716A added to the column were retained in the packed bed under liquid flow rates of 0.75 ml/min. If the population was cultured on glucose, approximately 11% retention was observed. Under the same conditions, the retention of MCR106, the strain with the jamB deletion, was very low, below the level of accurate detection. The strain with constitutive jamB production, pop3132, displayed an average retention of 54%.

Figure 3-1. Adhesion of Cultures Induced with Maltose.

Batch adhesion of 23716 to starch-Sepharose; pH= 6-7, T=25 °C. Cultures grown on defined and complex media with 1.0 g/l maltose. Initial cell concentration was approximately 2.4x10⁸ cells/ml.
Figure 3-2. Adhesion for Induced and Uninduced Cultures.

Batch adhesion of cultures grown on complex media with 1.0 g/l maltose, induced, or 1.0 g/l glucose, uninduced; pH=6.5, T=25 °C. For adhesion in the presence of maltose, maltose and cells were mixed 5 minutes prior to addition to starch-Sepharose for case of 0.1 M maltose and 1 minute prior to addition to starch-Sepharose for case of 0.25 M maltose. Initial cell concentration was approximately 3.6x10^8 cells/ml.

The effect of the fluid flow rate through the column was investigated using the strain 23716A grown in batch culture. It was observed that at the low flow rates, essentially all cells were retained in the bed and could be eluted with 0.2 M maltose (Figure 3-3). As the flow rate increased, the fraction of retained cells decreased. The flow rate of 1.5 ml/min was close to the maximum fluid flow rate that could be used. At higher flow rates, cell recovery from the packed bed fluctuated, suggesting that cells were becoming entrapped in the packed bed.

At a flow rate of 0.5 ml/min, cell retention remained high over the pH range of 5 to 7 (Figure 3-4). Below pH=5, there is a significant drop in the fraction of cells retained in the packed bed. Over the pH range of 4.5 to 7, good cell recovery was obtained. At pH=4.0, the fraction of cells eluted by maltose decreased, but the total recovery of cells fluctuated (data not shown). This suggests that some non-specific retention of cells occurs at pH=4.0. Although low pH values decreased the fraction of cells that adhere to the support, lowering the pH of the wash buffer to 4.0 did not result in the elution of already adhering cells.
Retention of cells in a packed bed of starch-Sepharose as a function of flow rate; pH=7.0, T=25 °C. Approximately 4x10⁷ cells were washed into column.

Retention of cells in a packed bed of starch-Sepharose as a function of pH; T=25 °C, flow rate=0.5 ml/min. Approximately 2-3.75x10⁷ cells were washed into column.

The adhesion of 23716A grown on maltose in a chemostat at pH=7.0, 37 °C, D= 0.5 h⁻¹ was investigated at 4, 25 and 37 °C. Three flow rates 0.5, 0.75 and 1.5 ml/min were used. The temperature appeared to have
the same relative effect on adhesion at all flow rates. The fraction of cells retained in the column by specific adhesion was about the same at 37 and 25 °C, and decreased as the temperature was dropped to 4 °C (Figure 3-5).

![Graph showing cell retention as a function of temperature]

**Figure 3-5. Cell Retention as a Function of Temperature.**

Retention of cells in a packed bed of starch-Sepharose as a function of temperature; pH=7.0, flow rate = 0.5, 0.75 and 1.5 ml/min. Approximately 1.6x10^8 cells were washed into column. Population grown in a chemostat, D=0.5 h^{-1}, pH=7.0, T=37 °C.

The retention of 23716A in the column measured at 25 °C, pH=7.0 and a flow rate of 0.75 ml/min, increased slightly over the course of the continuous culture. The effect of the culture condition on the retention is apparent from a comparison of the data presented in Figures 3-3 and 3-5. At a flow rate of 0.75 ml/min, 25 °C, a slightly higher fraction of the cells from the continuous culture were retained than cells grown in the batch culture. At a flow rate of 1.5 ml/min, 25 °C, there is a much greater difference in the fraction of cells retained for cultures grown in the continuous and batch culture.

### 3.5 Discussion

The adhesion experiments indicate that the induction of maltoporin directly influences the rate of adhesion. The cells grown on maltose have maltoporin expression induced and are expected to express about one to two orders of magnitude more maltoporin than the uninduced cells (Debarbouille et al, 1978; Brass et al, 1985;
Bukau et al., 1986). In the batch adhesion experiments, the induced cells adhere more quickly than the uninduced cells but in the end approximately the same fraction of the population adheres to the support. This suggests that the expression level of maltoporin directly influences the rate of adhesion but not the equilibrium level of cell adhesion.

Adding maltose to the cells prior to the adhesion experiment has the effect of decreasing the number of available maltoporin. The maltose binds reversibly to the maltoporin and during the initial contact of the cell and the support, the maltoporin occupied by maltose are unavailable to bind immobilized starch. If the binding of maltose to the maltoporin is assumed to reach an equilibrium level prior to mixing the cells and the support, the fraction of the maltoporin available for binding the immobilized starch can be estimated. Using an equilibrium constant for maltose-maltoporin binding of 100 M^{-1} (Benz et al., 1987), with 0.1 M maltose present, about 9\% of the maltoporin is expected to be available to bind immobilized starch during the initial cell-surface contact. For the induced cells, the addition of 0.1 M maltose would drop the cell surface density of free maltoporin to approximately the same level expected for the uninduced cells. It is interesting to note that the rate of adhesion of the uninduced cells and the induced cells mixed with 0.1 M maltose are about the same. The addition of 0.25 M maltose results in approximately 4\% of the maltoporin remaining unoccupied. The rate of adhesion for cells treated in this manner is seen to decrease even further.

For cells adhering to the support, the maltose competes with the immobilized starch for the maltoporin. Eventually, some equilibrium level of maltose and starch binding to the maltoporin is reached. This equilibrium state between the two ligands does not appear to alter the final equilibrium adhesion of E. coli. At this state, a sufficient number of bonds between the cells and the support form to immobilize the cells. This is interesting, considering that all of the specifically adhering cells in a packed bed of starch-Sepharose are released in the presence of 0.1 M maltose and a flow rate of 0.5 ml/min (Roos and Hjortso, 1989c).

The adhesion characteristics of a population in the batch system is quite different from that displayed in the packed bed. The population 23716, that adhered in the batch system, did not adhere significantly in the packed bed. Using a population that did adhere in the packed bed, the dependence of cell adhesion on the level of maltoporin expression becomes quite apparent. Induction of maltoporin yields higher cell retention compared to cells that are not induced. The strain, MCR106, which carries a deletion in the lamB gene, displayed no detectable adhesion in the packed bed. An intermediate level of retention was observed for pop3132, the strain with a constitutive mutation in the regulatory gene, malT. This suggests pop3132 produce maltoporin at a level between that of induced and uninduced 23716A.
The level of the fluid forces on the cells appears to greatly increase the effects of the other system parameters on adhesion. The role of receptor density is more pronounced in the packed beds than in the batch adhesion. Comparison of the results presented here for dependence of retention on pH and temperature and those of Ferenci (Ferenci, 1983) suggests that parameter values that have only mild effects on cell retention at one flow rate have a more pronounced effect on retention at higher flow rates. These results and observation are in qualitative agreement with the prediction from the model for specific cell adhesion advanced by Hammer and Lauffenburger (Hammer and Lauffenburger, 1989).

The system of *E. coli* adhering to a support through the interaction between the maltoporin and an immobilized maltooligosaccharide, appears ideal for the study of specific cell adhesion. The role of the basic interactions on the adhesion of the cells can be readily determined. The outer membrane of *E. coli* is well studied and the mechanism by which the maltoporin binds to maltooligosaccharides is becoming better understood. There are many strains of *E. coli* available that express a mutant maltoporin with different adhesion properties and the control of their expression is readily obtained. There are also numerous cheap ligands that can be used for immobilization or specific elution that display a wide range of intrinsic rates and affinities for binding to the maltoporin. Using this system the role of a variety of system properties on specific adhesion could be determined. Such information would prove invaluable for developing a quantitative understanding of specific cell adhesion.

### 3.6 References


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CHAPTER 4
RATE CONSTANTS FOR
MALTOPORIN-LIGAND BINDING

4.1 Introduction

The specific adhesion of *Escherichia coli* to starch-Sepharose, is mediated by the interaction between the maltoporin on the cell surface and the immobilized starch. As proposed by general models for specific cell adhesion, the fraction of cells that adhere to the support and the rate of this adhesion is a function of the kinetics of bond formation between the ligand and the cell surface receptor (Bell, 1978; Bell et al., 1984; Hammer and Lauffenburger, 1989). To understand and predict specific cell adhesion using these theories, the kinetics of the bond formation must be known over a wide range of conditions. Unfortunately, only relative rate constants for the binding of soluble ligand to the maltoporin have been reported (Benz et al., 1987). The relative constants are determined using soluble ligand. These relative rate constants can not be applied to immobilized ligand interacting with a cell surface receptor, where motion of the receptor and ligand with respect to each other is restricted to the plane of the cell membrane. The information on ligand binding obtained using soluble ligand, however, can be useful if true apparent rate constants are determined. From these constants, the intrinsic rate constant for bond formation between a particular ligand-receptor can be calculated and then used to estimate the kinetics of binding between immobilized ligand and cell surface receptors.

In this chapter, a rigorous treatment for the calculation of equilibrium constants and apparent and intrinsic rate constants for porins is presented. Experimental results, reported in the literature, are analyzed to yield the intrinsic rate constants for several carbohydrate ligands and the maltoporin. The complicating factors that arise from the various methods for studying ligand binding are analyzed and their effect on determination of the rate constants is discussed.

4.2 Intrinsic Rate Constants

Before considering the experimental estimation of rate constants, the relationship between apparent rate constants and intrinsic rate constants for the binding of a soluble ligand and a porin in a membrane is reviewed. The general case of a soluble ligand binding to a porin on a cell is used to develop the relationship. This binding is believed to occur in a two step process (Bell, 1978). First, in a mass transfer step, the ligand, $L$, moves to the surface of a cell, $C$, and into the vicinity of an unoccupied porin, $R$, where an encounter complex, $L-R$, is
formed. Once in an encounter complex, the ligand is within the proper distance and is correctly oriented so that bond formation is possible. The second step is the reaction in which the ligand-porin bond, LR, is formed. This process is represented below.

\[
\begin{align*}
\text{L + C} & \xrightleftharpoons[\kappa_-]{\kappa_+} \text{L-R} \\
\text{L-R} & \xrightarrow{\kappa_-} \text{LR} \\
\end{align*}
\]

The intrinsic reaction rate constants for the formation or breakup of a ligand-porin bond are \( \kappa_+ \) and \( \kappa_- \). The mass transfer constants \( \dot{a}_+ \) and \( \dot{a}_- \) represent the movement of the ligand to the cell surface and out of the encounter complex, respectively. Porins do not cover the whole cell surface and the rest of the surface does not interact with the ligand. The rate at which ligands reach a porin to form an encounter complex is, therefore, somewhat smaller than the rate at which ligand reaches the cell surface. To determine the overall rate at which an encounter complex is formed, the effect of the nonreactive portion of the cell surface is introduced using the probability, \( P \), that a ligand at the cell surface will diffuse away from the surface, back to the bulk solution, before finding a free receptor (Berg and Purcell, 1977). This probability reflects the two-dimensional nature of ligand movement when it is near the cell surface. It is a function of the cell radius, \( \alpha \), the number of free receptors on a cell, \( R_c \), and the characteristic radius of the encounter complex, \( s \). This probability is:

\[
P = \frac{\pi \alpha}{R_c s + \pi \alpha}
\]

To determine the rate at which encounter complexes break up, the two-dimensional nature of ligand movement near the cell surface must again be considered. The ligand may leave the encounter complex, but it still is closely associated with the cell surface and may enter into another encounter complex with an unoccupied porin before escaping to the bulk solution. The rate at which ligand leaves the encounter complex and then moves into the bulk solution is, therefore, also a function of the probability, \( P \) (DeLisi, 1980; Lauffenburger and DeLisi, 1983).

If the ligand-receptor complex is at quasi-steady state, the rate of ligand-receptor bond formation can be described in terms of the cell and ligand concentration and a set of apparent rate constants (Bell, 1978; DeLisi, 1980).

\[
\frac{d(LR)}{dt} = \dot{k}_f(C)(L) - \dot{k}_r(LR)
\]

where;
The mass transfer step of encounter complex formation accounts for both the translational and the rotational alignment of the ligand in the encounter complex. The rate of rotational alignment is dependent on the shape of the ligand and steric requirements for access to the porin binding site and is expected to be unique for each ligand-porin system. If it is deemed important, the rotational effects can be included as a proportionality factor in the rate constant expression as suggested by DeLisi (DeLisi, 1980). For this analysis, the effect of rotational orientation are explicitly ignored in the determination of the rate constants for encounter complex formation. This is valid for situations where the translational movement of the ligand to the porin is rate limiting.

The diffusional rate constants for movement of the ligand into the encounter complex are dependent on the geometry of the system. For a spherical cell or particle, the diffusional rate constant, $d_s$, is (Bell, 1978);

$$d_s = 4\pi a D$$

where $D$ is the ligand diffusion coefficient.

For the ligand-porin complex, disassociation occurs when the ligand moves out of the encounter complex with the characteristic radius $s$. For the case where $s$ is small compared to the space in which diffusion occurs and the porin has a single binding site, the disassociation diffusive rate constant is given by (Lauffenburger and DeLisi, 1983);

$$d_s = \frac{3D}{s^2}$$

In many situations, one process of ligand receptor binding, either diffusion or reaction, occurs at a much slower rate than the other process and becomes the rate limiting step of ligand-porin bond formation. The relative values of $d_P$ and $k_1$ indicate whether the overall rate is diffusion or reaction limited.

In the case where binding is reaction limited, $d_P \gg k_1$;
The mass transfer equilibrium constant for encounter complex formation, $K_{apt}$, is based on the encounter complex radius.

If $d_P < k_{+1}$ the overall reaction is diffusion limited and

$$
\begin{align}
\dot{k}_f &= \dot{d}_s (1 - P) \\
\dot{k}_r &= \frac{\dot{d}_P}{K_{ran}}
\end{align}
$$

The equilibrium reaction constant, $K_{ran}$ is the ratio of the intrinsic reaction rate for bond formation and breakage, $k_{+1}/k_{-1}$.

When the product of the encounter complex radius and the number of free receptors is much smaller than $\pi$ times the cell radius, $R_s < \pi a$, the forward and reverse apparent rate constants become:

$$
\begin{align}
\dot{k}_f &= \frac{k_d e}{d_+ + k_1} R_c \\
&= k_f R_c \\
\dot{k}_r &= \frac{k \dot{d}_s}{d_+ + k_1} \\
&= k_r
\end{align}
$$

where $d_s = 4sD$. 
Here, the diffusion rate constant $d$, is based on the encounter complex radius. Notice, in the simplified expressions obtained for the case of reaction limitation (Eqn. 5) and when $Rs \ll \pi a$ (Eqn. 8), the forward apparent rate constant is proportional to the number of free receptors on the cell. This is not true in the general case or when diffusion control prevails.

4.3 Estimation of Equilibrium and Rate Constants for Porins

4.3.1 in vitro Methods

There are several methods used to estimate the affinity or kinetic characteristics of porins. Porins that act as ion channels can be studied in vitro using a lipid bilayer containing the porin of interest. The bilayer is situated so that it separates two aqueous reservoirs and the conductance across the bilayer is recorded. If a ligand binding to a porin prevents the passage of ionic compounds through the porin, the conductance across the membrane will decrease as the porins are plugged with bound ligand. Equilibrium constants are calculated from the change in membrane conductance with change in the ligand concentration in the reservoirs (Benz et al., 1987).

To determine this constant, $K$, consider a lipid membrane containing $R_p$ porins. The membrane separates two reservoirs containing the ligand at concentration of $L'$ and $L''$. The ligand is able to pass through the porin and cross the membrane as depicted in Figure 4-1. In this analysis it is assumed that the porin possess a single binding site accessible from both ends of the porin. The apparent rate constants for the binding of the ligand are, in the most general case, assumed to be dependent on the end of the porin through which ligand enters or leaves the binding site. This reflects the difference in binding site accessibility from the two ends of the porin. If only one ligand can occupy the porin at a time, the balance on filled porins, $LR$, in the bilayer is:

$$\frac{d(LR)}{dt} = k'(L') + k''(L'') - (LR)(k'_p + k''_p)$$  \hfill (9)

Where the primes indicate concentration and rate constants for a particular side of the membrane (Figure 4-1).

The apparent rate constants are of the same form as those presented for binding to a cell surface (Eqn. 3). However, for the binding of ligand to a porin incorporated in a flat membrane, the rate constants for encounter complex formation and the probability, $P$, must reflect the planar geometry of the membrane and the hydrodynamic conditions near the membrane. In lipid bilayer experiments, the reservoir on either side of the membrane is stirred to insure mixing (Benz et al., 1987). Although there is convective transport in the liquid bathing the membrane, it is assumed that the limiting mechanism for ligand transport to the membrane surface is diffusion through the stagnant layer of fluid near the membrane. The nature of the probability, $P$, would not
be altered as it is a measure of the behavior ligand that are in close proximity to the membrane (Berg and Purcell, 1977; Lauffenburger and Cozens, 1989). For the case of planar geometry, $P$ is given as (Lauffenburger and Cozens, 1989)

$$P' = \frac{r_1}{R_2 + r_1}$$

where $r_1$ is the radius of the membrane and $R$ is the total number of unoccupied porin.

In the lipid bilayer experiment, as ligand is added to one or both sides of the membrane, the conductance across the membrane quickly drops to a characteristic level as porin are filled with ligand. At this point, the number of occupied porins is assumed to have reached a steady state value. From Equation 9 this is;

$$LR = \frac{(k'_r L' + k''_{r} L'')}{(k'_r + k''_{r})}$$

The ratio of conductance at this state to the conductance across the membrane when ligand is not present is taken to be proportional to the fraction of unoccupied porin.
The encounter complex radius, \( s \), may be different for either end of the porin which would result in a unique probability, \( P \), and rate constants for encounter complex formation at each end. When both ends exhibit the same effective encounter complex radius, the rate constants for encounter complex formation and break up and the probabilities would be the same at either end of the porin. The apparent rate constants on either end of the porin would also be equal. The apparent rate constants for ligand binding to the porin in this system would also be the same if the porins are randomly oriented in the membrane.

In the experiment where soluble ligand is added in equal concentration to each side of the membrane and the apparent rate constants are equal, at equilibrium the ligand concentration is expected to be equal in both reservoirs. In this case, the equilibrium constant is defined as:

\[
K = \frac{\langle LR \rangle}{\langle L \rangle \langle R \rangle}
\]  

Equilibrium binding to porins is also investigated by placing the ligand on one side of the membrane. In this case, the conductance of the membrane quickly drops and then levels off. Repeating this experiment with different concentrations of ligand, an "equilibrium" constant is calculated. This constant is determined at a true equilibrium condition for ligands that do not pass through the porin. But for ligands that permeate a porin, the point of stable conductance probably does not occur at equilibrium. This point, more likely occurs when the binding of ligand to porin is at a quasi-steady state.

The relationship between this "equilibrium" constant and the one defined in Equation 12, is determined by assuming the conductance measurement is made at a quasi-steady state. Upon achieving the quasi-steady state, the ligand concentration on one side of the membrane is zero and the concentration on the other side is at its original value, \( L \). The number of occupied porins is determined from Equation 11 to be:

\[
\frac{R}{R_0} = \frac{1}{1 + KL}
\]  

Using the total balance on the porin, with \( R_0 \) as the total number of porin, and Equation 12, the fraction of occupied porin is calculated to be:

\[
\frac{R}{R_0} = \frac{1}{1 + KL}
\]  

Equilibrium binding to porins is also investigated by placing the ligand on one side of the membrane. In this case, the conductance of the membrane quickly drops and then levels off. Repeating this experiment with different concentrations of ligand, an "equilibrium" constant is calculated. This constant is determined at a true equilibrium condition for ligands that do not pass through the porin. But for ligands that permeate a porin, the point of stable conductance probably does not occur at equilibrium. This point, more likely occurs when the binding of ligand to porin is at a quasi-steady state.
For the case where the apparent rate constant is the same for ligand that enter either end of the porin the fraction of unoccupied porin as a function of the equilibrium constant is:

\[
\frac{R}{R_e} = \frac{1}{1 + \frac{K}{2} L}
\]  \hspace{1cm} (15)

The "equilibrium" constant determined from conductance through the membrane after adding ligand to only one reservoir is half of the true equilibrium constant. This is consistent with the results of Benz et al (Benz et al, 1987). For the addition of maltopentaose to one side of a membrane containing the maltoporin, an equilibrium constant that is one half the value of a constant calculated for the addition of ligand to both sides of the membrane was obtained.

To determine the apparent rate constants of porins, the liposome swelling assay can be used (Luckey and Nikaido, 1980; Nakae et al, 1986). In this procedure, liposomes, containing the porin, are formed in a solution of dextran or stachyose. The liposomes are then transferred to an isotonic solution of the ligand to be studied. The ligand binds to the porins and enter the liposome while the dextran or stachyose are retained. As ligands enter, the liposome swells due to the entrance of water driven by osmotic pressure. The initial rate of liposome swelling, measured as a decrease in optical density, is assumed proportional to the flux of the ligand into the liposome. From this relative flux, the relative apparent rate constants for ligand porin interaction are calculated (Benz et al, 1987).

The molecular flux of ligand into the liposomes, which occupy a fraction, \( f' \), of the total volume is:

\[
J = -V_i \frac{d((1 - f')L')}{dt} = V_i \frac{d(f'L'')}{dt}
\]  \hspace{1cm} (16)

where \( V_i \) is the total volume and \( L' \) and \( L'' \) are the concentration of ligand outside and inside the liposome respectively. It is assumed in this equality that the rate of ligand accumulation in the porins is small compared to the rate at which ligand number inside or outside the liposome change.

From the data of Luckey and Nikaido (Luckey and Nikaido, 1980), it is observed that the increase in the flux into the liposome is proportional to the porin number. This would occur under two conditions, the binding is reaction limited or \( (R_s)/C \) is much less then \( \pi a \). Here, \( a \) is the characteristic radius of a liposome, and \( C \) is the number of liposomes present. In either case, the simplification of the apparent rate constants (Eqn. 5 or 8) allows Equation 16 to be written as:

\[
\frac{d((1 - f')L')}{dt} = -k_f'(1 - f')(L') (R) + k_r'(LR)
\]

\[
= k_r'' f'(L'')(R) - k_r''(LR)
\]  \hspace{1cm} (17)
Where, $k'_r$ and $k'_r$ are given by Equation 5 or 8. If the apparent rate constants for the ligand porin interaction are the same for ligand inside or outside the membrane Equation 17 can then be solved for the number of occupied porins.

$$LR = \frac{k'_f}{2k_r} (\frac{R}{((1 - f')(L') + f'(L''))})$$

(18)

Combining this expression with the total receptor balance, yields an expression for the number of free porins,

$$R = \frac{R_0}{1 + \frac{b}{2a}((1 - f')(L') + f'(L''))}$$

(19)

Using this expression, the balance on the porins and Equations 16 and 17, the expression for ligand flux into the liposome becomes;

$$J = V'_f \frac{k'_f(1 - f')(L') - f'(L''))(R_0)}{2 + K((1 - f')(L') + f'(L''))}$$

(20)

The initial flux into the liposome is taken to be proportional to the initial rate of liposome swelling, $Y$. During this period of the experiment, the concentration of ligand in the liposome is close to zero. If the total volume of the liposomes is small compared to the total volume of the system, $f' \ll 1$, the relative apparent rate constant, $k'_f$ is calculated to be;

$$k'_f = \left(\frac{Y}{V'_f(R_0)}\right)(2 + K(L'))$$

(21a)

and the relative rate constant for a ligand vacating a porin is

$$k'_r = \frac{k'_f}{K}$$

(21b)

Thus, for the same liposome preparations, relative rate constants for different ligands or various operating conditions can be determined.

4.3.2 in vivo Methods

Using labelled ligands, that will not cross the cell membrane, equilibrium concentrations of bound versus free ligand can be measured in vivo (Ferenci et al., 1980). From such experiments, equilibrium constants and the mean number of binding sites can be determined. Once the equilibrium constant of a labeled ligand is known, the equilibrium constant for other ligands that bind to the porin can be evaluated from competition experiments.
The apparent association and disassociation rate constants for ligands that do not permeate the porins, can be measured \textit{in vivo} from ligand accumulation in the cell. The disassociation rate can also be estimated by placing cells, to whose porins are bound labeled ligand, in solutions without the ligand and monitoring ligand release. For ligands that permeate porins, if the apparent rate constants for porin interacting with ligand in the bulk solution and in the periplasmic space are taken to be equal, the initial flux of ligand into the cell can be used to estimate the apparent rate constants. During this initial period, the concentration of ligand in the periplasmic space is assumed to be zero and Equation 20 is used to estimate the apparent rate constants.

Another method used to determine flux through a porin \textit{in vivo} is based on the degradation of the ligand in the periplasmic space of the cell (Nikaido and Nakae, 1979). At the quasi-steady state the diffusion rate across the membrane is equal to the rate of ligand destruction in the periplasmic space. The concentration of the ligand analog and the degradation products in the periplasmic space and the concentration of ligand analog in the bulk fluid are constant. A compound that is produced during the enzymatic degradation of the ligand analog is released into the bulk fluid where the rate of accumulation is measured. A necessary condition for valid application of this method is that during the period that the quasi-steady state assumption holds, the concentration of degradation compounds in the bulk fluid is low and does not alter ligand movement through the porin. This has method been applied to porins where the flux was estimated using Fick's law for diffusion. Recently, the experimental method was employed to determine flux of a chromogenic analog of a ligand through the maltoporin (Freundlieb et al, 1988). Analysis of this system is quite different from the case of simple diffusion across the membrane.

Freundlieb et al. used a Michaelis-Menten type equation to correlate the rate of ligand analog crossing the membrane to the bulk fluid concentration. The results were reported in terms of a $V_{\text{max}}$ and $K_M$ for ligand transport. Here, an analysis of their experiment is presented that allows estimation of the apparent rate constant and equilibrium constant for the ligand analog-porin interaction and the apparent rate constant for the degradation products binding to the porin. The effect of competition between degradation products and the analog as well as the effect of cell uptake of the degradation products are explicitly addressed.

The interactions between the various components and the porins for a system of the type described by Freundlieb et al (Freundlieb et al, 1988) is depicted in Figure 4-2. The ligand analog and some degradation product cross the outer membrane through the porin. The degradation products can also cross the cytoplasmic membrane by a specific transport system, while the ligand analog is not capable of crossing the cytoplasmic membrane. The concentration of the ligand analog in the bulk fluid and the periplasmic space is represented by $L'$ and $L''$, respectively. The apparent rate constants for the binding or release of the ligand analog are $k_1$, $k_2$, $k_3$, $k_4$, $k_5$, and $k_6$. 
and $k_r$. When the system is at quasi-steady state, the rate at which ligand enters the periplasmic space through the porin is equal to the rate of its degradation in the periplasmic space. The rate at which the degradation product are produced, $r_d$, is related to ligand transport by:

$$\frac{f''r_d}{\xi} = -f''\hat{k}_rL''C + \hat{k}_r(LR)$$

$$= (1-f')\hat{k}_rL'C - \hat{k}_r(LR)$$

where $\xi$ is the stoichiometric coefficient for formation of the degradation product. The factor $f'$ is the total cell volume as a fraction of the total volume, $f''$ is the total volume of the periplasmic space as a fraction of the total volume and $(LR)$ is the concentration of porin that are binding the ligand analog. Due to the presence of excess degrading enzyme, a requirement for using this method, the concentration of ligand analog in the periplasmic space is assumed to be low. The concentration of porin that are filled with the ligand analog is calculated to be:

$$(LR) = \frac{\hat{k}_rC}{2\hat{k}_r}(1-f')L'$$

Some of the degradation products may compete with the ligand analog for unoccupied porin. The concentration of degradation products in the outer fluid is assumed to be small compared to the concentration of the ligand analog and the competition for sites on the outer surface of the cells is negligible. However, the concentration of the degradation products in the periplasmic space can be much higher than the ligand analog and effectively compete for the unoccupied porin. The concentration of porin filled with the degradation product, $L_dR$, is taken to be at the equilibrium value.
The chromogenic analog of a ligand, L, enters the periplasmic space through the porin. L is degraded and the chromogenic compound, $L''$, and other degradation products, $L_{dl}$, are formed. The chromogenic compound diffuses through the outer membrane into the bulk solution. The other degradation products, $L_{dl}$, can compete with L for the porin or cross the cytoplasmic membrane and enter the cell.

\[
(L_d R) = \frac{f'' k'_{d} L'' C}{2 k'}
\]
The total volume of the periplasmic space and the cells are often negligible compared to the total volume of the system. Thus, \( f'' L' \ll (1-f') L' \) and \( f' \ll 1 \). Using these assumptions and the expression for the number of porin that are filled with ligand analog (Eqn. 23), the rate at which the degradation products, \( L_d \), are formed (Eqn. 22) can be written as;

\[
\frac{f''}{\delta} r_d = \frac{k_p L'C}{2}
\]  

(25)

For degradation products that compete with the ligand analog for unoccupied porin and are transported into the cytoplasm, \( L_d \), the steady state balance in the periplasmic space is;

\[
f'' r_d = f'' k_p L_d'C + f'' r_m
\]  

(25)

The rate \( r_m \), represents the rate at which the degradation products that interfere with binding between ligand analog and porin are transported into the cytoplasm. The apparent rate constant for the binding of the degradation products to the porin is \( k_j \) and the concentration of the degradation product in the periplasmic space is \( L_d'' \).

The transport of the degradation product across the cytoplasmic membrane is represented as a specific transport pathway. The degradation product binds reversibly to the transport protein through a process described by mass action kinetics. If the rate constants that describe the release of the degradation product into the periplasmic space and the cytoplasm are the same, at quasi steady state the rate at which the degradation compound is removed from the periplasmic space and transported into the cytoplasm is;

\[
r_m = \frac{k_f''}{2} L_d'' R_mC
\]  

(27)

where \( k_f'' \) is the apparent rate constant for the binding of the degradation product to the site for transport across the cytoplasmic membrane and \( R_m \) is the concentration of these sites available.

At this point some assumption on the characteristics of the binding of either ligand to the porin must be made. The binding of the ligand analog and the degradation products to the porin are taken to be reaction limited. The apparent rate constants for the interaction of the ligand analog or degradation product with the porin are of the form expressed in Equation 5. Similar results are obtained if the assumption used in Equation 8 is valid. In either case, the apparent rate constant is proportional to the free porin concentration. Using the balance on the porin and the equilibrium values for the concentration of ligand analog and degradation product bound to porin (Eqn. 23 and 24), the concentration of free porin is found to be;
\[ R = \frac{R_0}{1 + \frac{\xi}{2} L + \frac{k_{d}'}{2} L_{d}''} \]  

(28)

where \( K_d \) is the equilibrium constant for the degradation product binding to the porin.

The expression for the production of the degradation products (Eqn. 25) can be rewritten using the apparent rate constant given by Equation 5 or 8 and substituting the expression for R (Eqn. 28).

\[ \frac{f_{d}}{\xi} = \frac{k_{d}' L_{d}'}{2 + K L + K_d L_{d}'' f''} \]  

(29)

Combining this with Equation 25, 26 and 27 gives;

\[ \frac{f_{d}}{\xi} = k_{d}' L_{d}' \left( 2 + L \left( K + \frac{\xi k_{d} K_{d}}{k_{f} + k_{f}'' \left( \frac{R_{m}}{R} \right)} \right) \right)^{-1} \]  

(30)

The rate of at which degradation products are formed is usually followed by monitoring the appearance of the chromogenic compound in the bulk fluid. If the compound is small, it can diffuse out of the periplasmic space through the general porins and not interfere with the binding at the specific porin. This is the case for the experiments described by Freundlieb et al. The rate at which this compound appears in the bulk fluid when accumulation in the membrane is negligible is;

\[ r_c = \frac{f_{d}}{\xi} \]  

(31)

\[ r_c = k_{d}' R_{d}' \left( 2 + L \left( K + \frac{\xi k_{d} K_{d}}{k_{f} + k_{f}'' \left( \frac{R_{m}}{R} \right)} \right) \right)^{-1} \]

Notice in Equation 31 the terms R and \( R_m \) are both functions of \( L_{d}'' \) or \( L' \). Solution of this expression would require information on the kinetics of transport across the cytoplasm. The expression in Equation 31, however, can be used to explore the two limiting cases for transport of the degradation products into the cytoplasm, rapid transport or no transport. If the transport into the cytoplasm is very fast, the maximum rate at which the products cross the cytoplasmic membrane is equal to the rate at which the degradation products are formed. From the balance on degradation products in the periplasmic space and bound to porin, it can be shown that \( L R \) approaches zero. The rate, \( r_c \) in Equation 31 can then be estimated as;

\[ r_c = \frac{k_{d}' R_{d} L}{2 + L K} \]  

(32)

At the other limit, the degradation products that compete for the porin are not transported into the cytoplasm, \( k_{f}'' \) is zero. In this case the rate at which the monitored compound appears in the bulk fluid is;
4.4 Intrinsic Rate Constants for Maltoporin of *Escherichia coli*

Using the analysis methods presented above, quantitative descriptions of porin binding behavior and direct comparison of results obtained from various methods is possible. This quantitative information is also necessary for estimating the intrinsic rate constants for porin ligand systems. In this section the binding properties of the maltoporin are estimated using the published results from several different experimental methods.

4.4.1 Calculation of Equilibrium Constants

The equilibrium constants for a variety of carbohydrates binding to the maltoporin have been reported by Benz et al. (Benz et al., 1987). These were determined *in vitro* using lipid bilayers containing the maltoporin and adding ligand to both sides of the membrane. Equilibrium constants have also been reported for wild type and mutated maltoporins specifically orientated in the membrane (Dargent et al., 1988). In this case, ligand was added to one side of the membrane and the constants measured are of the form given in Equation 15. Using wild type maltoporin, the constants were reported to be independent of the membrane side to which ligand was added. This indicates that equilibrium binding characteristics are the same at either end of the porin.

Estimates of the equilibrium constants determined *in vivo* were obtained from data reported by Ferenci et al. (Ferenci et al., 1980). The equilibrium constant for FITC-amylopectin was calculated using data presented in their Figure 2, to be 2.94 ml/mg. The number of binding sites for amylopectin was estimated at 0.74 mg/10⁹ cells. The equilibrium constant for several maltooligosaccharides were obtained by calculating the inverse of the concentration necessary to release half the amylopectin previously bound to the porins. These estimates are expected to be slightly low due to the competition between the tested compound and amylopectin for unoccupied porins. Taking this into account, corrected values for the equilibrium constants were calculated. As a check on the consistency of the equilibrium rate constants, the mean number of maltoporins per cell of a *malT*⁻ strain was calculated. Using the binding data of [¹⁴C]maltodecaose (Ferenci et al., 1980) maltoporin expression is calculated to be approximately 1.5x10⁵ copies per cell. This compares favorably to a value of 3x10⁴ to 10⁵ reported for maltose induced cells (Braun and Krieger-Brauer, 1977; Ferenci, 1980; Schwartz, 1983). From this set of experiments the equilibrium constant for amylose was calculated to be 0.300 ml/mg. The equilibrium constants calculated from the data of Ferenci et al., and those obtained from the reports of Ferenci et al., Benz et al. and Dargent et al. are compared in Table 4-1.
Table 4-1. Equilibrium Constants for Maltoporin

<table>
<thead>
<tr>
<th>Ligand</th>
<th>K (M⁻¹)</th>
<th>Benz¹</th>
<th>Dargent²</th>
<th>Ferenci³</th>
<th>Corrected⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maltose</td>
<td>100</td>
<td>222</td>
<td>71</td>
<td>85</td>
<td></td>
</tr>
<tr>
<td>Maltotriose</td>
<td>2500</td>
<td>2800</td>
<td>1540</td>
<td>1800</td>
<td></td>
</tr>
<tr>
<td>Maltotetraose</td>
<td>10000</td>
<td>6700</td>
<td>3330</td>
<td>4000</td>
<td></td>
</tr>
<tr>
<td>Maltopentaose</td>
<td>17000</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maltohexaose</td>
<td>15000</td>
<td></td>
<td>13300⁵</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maltoheptaose</td>
<td>15000</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maltodecaose</td>
<td>13300</td>
<td>16000</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

¹Benz et al, 1987; ²Dargent et al, 1988; ³Ferenci et al, 1980; ⁴Calculated from Ferenci et al, 1980, accounting for competition. ⁵Freundlieb et al, 1988, from their Table 3.

Several of the ligands used by Ferenci et al, (Ferenci et al, 1980) maltose, maltotriose and maltotetraose, permeate through the maltoporin. The equilibrium constants are calculated assuming that the concentration of these ligands is the same on either side of the outer membrane. This is reasonable since the cell population used lacks a malE gene product and there is no transport across the cytoplasmic membrane.

4.4.2 Apparent and Intrinsic Rate Constants

Using the equilibrium constants calculated from the in vivo data of Ferenci et al (Ferenci et al, 1980) and the information presented on the accumulation of maltose by E. coli, estimates of the apparent rate constants for maltose binding were obtained. The initial rate of maltose accumulation was calculated from reported data to be 0.028 nM/(10⁸ cells sec). This is the total flux into the cells and from Equation 20, assuming the cells express approximately 1.55 x 10⁵ maltoporin, the apparent forward rate constant was estimated to be 1.24 x 10⁶ (M sec)⁻¹. There are two more experiments reported by Ferenci et al, in which the initial accumulation of maltose in E. coli was measured in the presence of competing ligands. The cells were exposed to either amylopectin or amylose prior to addition of labeled maltose. It is assumed that equilibrium was reached before the maltose addition and due to the small concentration of maltose used, this equilibrium was maintained after maltose addition. Total cell volume is assumed to be small compared to the total system volume. The concentration of receptor filled with competing ligand, L₂R, is;
Taking the mass balance on receptors and solving for free receptor number gives;

\[ (L,R) = \frac{k_f^L}{k_c^L} \frac{(L_2)(R)}{1 + \frac{k_f^L}{2k_c^L} ((L^+)_1 + (L^+)_2) + \frac{k_f^L}{k_c^L} (L_2)} \]  

The estimate of the apparent rate constant (Eqn. 20) for the accumulation of maltose in the presence of a competing compound that does not permeate the porin becomes;

\[ k_f = \left( \frac{J}{(R)_C} \right) \left[ \frac{2 + K(L^+) + 2k_f^L(L_2)}{(L^+)} \right] \]  

In the presence of amylopectin, the apparent rate constant for binding of maltose to the maltoporin, \( k_f \), was found to be 7.625 \( \times \) \( 10^5 \) (M sec\(^{-1} \)). With amylose as the competing ligand, the rate constant was calculated to be 4.61 \( \times \) \( 10^5 \) (M sec\(^{-1} \)). The three estimates are in fairly good agreement. The value for the apparent rate constant for maltose binding to the maltoporin is taken as the average of the three estimates, 8.202 \( \times \) \( 10^5 \) (M sec\(^{-1} \)).

Apparent rate constants for other maltooligosaccharides were determined using the maltose flux into cells calculated from the data of Ferenci et al, and the values for the relative flux given by Luckey and Nikaido (Luckey and Nikaido, 1980) and Nakae et al (Nakae et al, 1986) (Table 4-2). Of all the in vivo data reported, the maltose accumulation data reported by Ferenci et al (Ferenci et al, 1980) is believed to be the most representative. The time period of data collection was short and the rate constants determined in the presence of competitors agree quite well.

The two sets of apparent rate constants determined by liposome swelling, appear to be similar. However, it should be noted that Nakae et al used stachyose in preparing the liposomes for their study. Recently, it was reported that stachyose interacts with the maltoporin (Benz et al, 1987). The effect of this interaction is not accounted for in the calculation of the apparent rate constants. Also presented in Table 4-2 are the equilibrium constants for the compounds binding to the maltoporin determined by Benz et al (Benz et al, 1987) and several estimates of apparent rate constants for ligand binding determined from in vivo ligand accumulation data. All constants were calculated assuming that there was 1.55 \( \times \) \( 10^5 \) maltoporin per cell.

To estimate the intrinsic rate constants, the binding to the maltoporin was assumed to be reaction limited. This assumption appears reasonable in light of the results of Luckey and Nikaido (Luckey and Nikaido, 1980). From the apparent rate constants obtained from the relative permeation rates reported by Luckey and Nikaido,
Table 4-2. Apparent Rate Constants for the Maltoporin.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>$K$ ($M^{-1}$)</th>
<th>$k_j$ ($Ms^{-1}$)</th>
<th>(x10^5)</th>
<th>$K_j$</th>
<th>$k_j$</th>
<th>$k_j$</th>
<th>$K_j$</th>
<th>$k_j$</th>
<th>$K_j$</th>
<th>$k_j$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(Benz)</td>
<td>(Luckey)</td>
<td>(Nakae)</td>
<td>in vivo</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maltose</td>
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<td>8.20</td>
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<td></td>
<td></td>
<td>4.08</td>
<td></td>
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<tr>
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<td>92.0</td>
<td>87.8</td>
<td>4.60</td>
<td>4.00</td>
<td>2.31</td>
<td>0.77</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maltotetraose</td>
<td>10000</td>
<td>104.4</td>
<td>197.8</td>
<td>4.00</td>
<td>2.31</td>
<td>0.77</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Maltopentaose</td>
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<td>83.9</td>
<td>107</td>
<td>2.31</td>
<td>0.77</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Maltohexaose</td>
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<td>0.77</td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Maltoheptaose</td>
<td>15000</td>
<td>6.89</td>
<td>3.44</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trehalose</td>
<td>46</td>
<td>3.99</td>
<td>4.30</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactose</td>
<td>18</td>
<td>0.33</td>
<td>1.23</td>
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</tr>
<tr>
<td>Sucrose</td>
<td>67</td>
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<td>&lt;0.38</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Gentibiose</td>
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<td>6.89</td>
<td>3.44</td>
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<td></td>
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</tr>
<tr>
<td>Melibiose</td>
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<td>8.05</td>
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<td></td>
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<tr>
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</tr>
<tr>
<td>D-glucose</td>
<td>9.5</td>
<td>9.43</td>
<td>3.64</td>
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<td></td>
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</tr>
<tr>
<td>D-galactose</td>
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<td>9.1</td>
<td>4.49</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>D-fructose</td>
<td>1.7</td>
<td>3.81</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-mannose</td>
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<td>4.92</td>
<td>3.26</td>
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<td></td>
<td></td>
<td></td>
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<td></td>
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</tr>
</tbody>
</table>

1Benz et al., 1987; 2 Luckey and Nikaido, 1980, or Nakae et al., 1986, with maltose flux as described in text as basis. 3Apparent rate constants calculated from in vivo accumulation rates using Equation 22; 4Ferenci, 1987; 5Ferenci, 1980

intrinsic rate constants and the reaction equilibrium constant for the bond formation were calculated using Equations 3, 4 and 5 (Table 4-3). The encounter complex radius was estimated to be 0.4 nm, the diameter of the pore (Benz et al., 1987).

With the analysis presented earlier, apparent rate constants can be determined in vivo using chromogenic analogs of a ligand (Freundlieb et al., 1988). In these experiments, the transport of p-nitrophenyl-α-D-maltohexaoside, PG6, a chromogenic analog of maltohexaoside, was studied. The transport properties were
Table 4-3. Intrinsic Reaction Rate Constants

<table>
<thead>
<tr>
<th>Ligand</th>
<th>$K^1$ (M⁻¹)</th>
<th>$K_{ran}$ (s⁻¹)</th>
<th>$k_{r1}$ (x10³)</th>
<th>$k_{-1}$ (x10³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maltose</td>
<td>100</td>
<td>1.95</td>
<td>1.60</td>
<td>8.2</td>
</tr>
<tr>
<td>Maltotriose</td>
<td>2500</td>
<td>48.6</td>
<td>17.9</td>
<td>3.68</td>
</tr>
<tr>
<td>Maltotetraose</td>
<td>10000</td>
<td>195.2</td>
<td>20.3</td>
<td>1.04</td>
</tr>
<tr>
<td>Maltoheptaose</td>
<td>15000</td>
<td>285.7</td>
<td>4.0</td>
<td>0.14</td>
</tr>
<tr>
<td>Trehalose</td>
<td>46</td>
<td>0.90</td>
<td>0.78</td>
<td>8.67</td>
</tr>
<tr>
<td>Lactose</td>
<td>18</td>
<td>0.35</td>
<td>0.065</td>
<td>1.83</td>
</tr>
<tr>
<td>Sucrose</td>
<td>67</td>
<td>1.29</td>
<td>0.031</td>
<td>0.24</td>
</tr>
<tr>
<td>Gentibiose</td>
<td>250</td>
<td>4.87</td>
<td>1.34</td>
<td>2.75</td>
</tr>
<tr>
<td>Melibiose</td>
<td>180</td>
<td>3.52</td>
<td>0.81</td>
<td>2.30</td>
</tr>
<tr>
<td>Celliobiose</td>
<td>6.7</td>
<td>0.13</td>
<td>0.078</td>
<td>5.97</td>
</tr>
<tr>
<td>D-glucose</td>
<td>9.5</td>
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<td>99.3</td>
</tr>
<tr>
<td>D-galactose</td>
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<td>0.47</td>
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<td>37.9</td>
</tr>
<tr>
<td>D-fructose</td>
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<td>0.03</td>
<td>0.74</td>
<td>224.1</td>
</tr>
<tr>
<td>D-mannose</td>
<td>6.3</td>
<td>0.12</td>
<td>0.96</td>
<td>78.0</td>
</tr>
</tbody>
</table>

¹Benz et al, 1987

determined by assuming the rates of transport of PG6 into the periplasmic space and hydrolysis of the PG6 were equal. Hydrolysis of PG6 was carried out by the malS gene product, a periplasmic amylase (Freundlieb and Boos, 1986). The malS region was incorporated on a multicopy plasmid to maintain excess enzyme in the periplasmic space. Still, in several experiments, it is debatable whether the requirement of excess enzyme is met. Hydrolysis of PG6 yields maltohexaose and p-nitrophenyl. Maltohexaose is the only product that interferes with the transport of PG6, the stoichiometric coefficient, $\xi$, in Equation 31 is one. The rate of hydrolysis was calculated by analyzing the release of p-nitrophenyl into the bulk fluid. If the transport and degradation of the ligand analog are at quasi-steady state, the rate at which p-nitrophenyl accumulates in the bulk fluid is equal to the rate of PG6 hydrolysis.
The experimental results reported by Freundlieb et al were analyzed using the model given in Equation 31. The rate of p-nitrophenyl release was reported for a range of PG6 concentrations and several strains of *E. coli* with different levels of lamB, *malE* and *malF* expression. The rate of hydrolysis for all strains displayed saturation behavior as a function of the PG6 concentration. The data for strains that had functional *malE* and *malF* gene products, was analyzed by the authors using Michaelis-Menten type equation to determine a $V_{\text{max}}$ and $K_M$. The maltohexaose in the periplasmic membrane was assumed to be rapidly transported into the cytoplasm in these strains, it should not compete with PG6 for the maltoporin. Taking the inverse of the expression for rapid transport of the degradation products (Eqn. 32), the expressions for the $V_{\text{max}}$ and $K_M$ can be broken down into the apparent rate constants (Table 4-4).

<table>
<thead>
<tr>
<th>Plot component</th>
<th>Michaelis-Menten</th>
<th>Eqn. 32 Active</th>
<th>Eqn. 33 Inactive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slope</td>
<td>$\frac{K_M}{V_{\text{max}}}$</td>
<td>$\frac{2}{k_f \gamma \xi}$</td>
<td>$\frac{2}{k_f \gamma \xi}$</td>
</tr>
<tr>
<td>Intercept</td>
<td>$\frac{1}{V_{\text{max}}}$</td>
<td>$\frac{K}{k_f \gamma \xi}$</td>
<td>$\frac{K + \frac{\gamma \xi}{\xi}}{k_f \gamma \xi}$</td>
</tr>
<tr>
<td>Intercept</td>
<td>$\frac{-1}{K_M}$</td>
<td>$\frac{K}{2}$</td>
<td>$\frac{-1}{2} \left( \frac{K + \frac{\gamma \xi}{k_f \xi}}{k_f \gamma \xi} \right)$</td>
</tr>
</tbody>
</table>

1Used to analyze transport and hydrolysis of p-nitrophenyl-α-D-maltohexaoside in *E. coli* (Freundlieb et al, 1988)

The two populations that expressed the functional *malE* and *malF*, differed in the level of the maltoporin. This should not effect the value of $K_M$ (Table 4-4). The $K_M$ reported for the two populations was 1.3x10^4 M, and the equilibrium constant for PG6 binding to the maltoporin is calculated to be 1.54x10^4 M^-1. This is the same as the equilibrium constant determined for maltohexaose using lipid bilayer (Benz et al, 1987). The kinetic model predicts that $V_{\text{max}}$ for these cell populations should decrease linearly with the expression of maltoporin (Table 4-4). For a population which expressed about 25% less maltoporin than a strain with
constitutive expression, the $V_{\text{max}}$ dropped to 30% that of the constitutive strain. Using estimates of $3 \times 10^4$ maltoporins per cell for the constitutive strain, the apparent rate constants for PG6 binding to the maltoporin, $k_f$ and $k_r$, were calculated to be $1.1 \times 10^4 \text{ (M s)}^{-1}$ and $0.714 \text{ s}^{-1}$ respectively.

For the populations with mutations in the malE and malF regions, the maltohexaose is not transported into the cytoplasm. The rate of PG6 hydrolysis for these populations was reported to decrease at all PG6 concentrations. From Equation 33, it is expected that this was caused by the competition between maltohexaose and PG6 for the maltoporin. The values for $V_{\text{max}}$ and $K_M$, were not reported, however they could be estimated from the graphical data presented in the paper. Using the expressions for the slope and intercept defined by Equation 33 (Table 4-4), the apparent rate constant, $k_f$, was calculated from three sets of experiments to be $1.01 \times 10^4 \text{ (M s)}^{-1}$. This is in good agreement with the value calculated for cells with functional malE and malF. The apparent rate constant for release of the degradation product, maltohexaose, from the maltoporin was calculated to be $k_r^d = 0.42 \text{ s}^{-1}$. This gives a value of $3.3 \times 10^3 \text{ (M s)}^{-1}$ for the apparent rate constant for binding, $k_f$. This is several orders of magnitude lower than rate constants for maltohexaose estimated by other methods (Table 4-2).

The estimates for the apparent rate constant of PG6 are expected to be lower than that of maltohexaose from consideration of the molar flux into the cells (Freundlieb et al, 1988). At approximately the same concentration, the flux of maltohexaose is reported to be several orders of magnitude greater than the flux of PG6. An apparent rate constant for PG6 can be obtained from the flux data using Equation 20, if the concentration of PG6 and maltohexaose in the periplasmic space are assumed to be close to zero. While this is probably not the case this estimate does give a lower limit for $k_f$ of $6.8 \times 10^3 \text{ (M s)}^{-1}$. This indicates that the estimates for the apparent rate constants of PG6 obtained using Equations 31 and 33 are consistent. This limit further supports the view that the apparent rate constants for maltohexaose binding to maltoporin calculated using Equation 33 are several orders of magnitude too small.

### 4.5 Discussion

From previously published experimental results, intrinsic rate constants for several carbohydrates binding to the maltoporin were estimated. The analysis assumed that the interaction between the carbohydrate and the maltoporin could be represented as interaction with a single site (Benz et al, 1987). This is a reasonable approximation since it appears that only one carbohydrate can occupy a pore at a time and the wild type maltoporin is symmetric with respect to its binding (Dargent et al, 1988).
The ligand-porin binding was considered a two step process. The rate of ligand movement into the proper position and orientation for binding was lumped into a diffusion rate constant in which translational motion was considered the dominant process for encounter complex formation and break up. This gave consistent results for most reported experimental results. However, for the mutant maltoporin discussed by Dargent et al (Dargent et al, 1988), this formulation could not satisfactorily account for the nonlinear dependence of the equilibrium constant on the ligand concentration. This dependence is believed to be the result of steric hindrance at the pore mouth and in this case, translational motion of the ligand does not appear to be the rate determining process for encounter complex formation. This is an example where ligand orientation or conformation must be addressed. The role of orientation and rotational motion on the apparent rate constant of bond formation has been addressed by several investigators (Schmitz and Schurr, 1972; Hill, 1975; DeLisi, 1980).

Throughout the estimation of the apparent rate constants, it was assumed that the rate constants were linear functions of the number of free porins. For the in vivo and liposome experiments, using most of the ligands reported in Table 4-3, this is valid as the binding appears to be reaction limited. This assumption may not be very good for the binding of maltotriose or maltotetraose when all the receptors on a cell surface are unoccupied. In the case of maltotetraose, the product $d_P$ has a value of approximately $1.6 \times 10^8$. This is slightly less than the estimated intrinsic reaction rate constant. The intrinsic rate constants calculated in Table 4-3 may still serve as a good first estimate. These values suggest that diffusion limitation could become important for binding of maltotriose or maltotetraose to the maltoporin. It should be noted that calculation of the intrinsic reaction rate constants and the value of $P$ are dependent on the maltoporin expression level and the stoichiometry that relates occupied porins to those available for binding.

To calculate the apparent rate constants from flux experiments or the degradation rate of PG6, accurate estimates of available maltoporin are necessary. It was assumed in this treatment that a single maltoporin protein acts independently of other maltoporin molecules to bind and transport a ligand. This may not be strictly true. The maltoporin is believed to exist on the surface of the cell as a trimer (Nakae and Ishii, 1982). Dargent et al (Dargent et al, 1988) interpret recently reported structural data (Lepault et al, 1988) to mean that the maltoporin trimer exhibits three pores on the outer surface that merge into a single outlet to the periplasmic space. The trimer is proposed to function as a single conducting unit. If the trimer can bind only one ligand at a time, it appears the role of the trimer is to increase ligand access to the binding site within the porin channel. Because of the preliminary nature of these observations, this stoichiometry between unoccupied and occupied porins was not used in this analysis.
In the treatment of ligand binding to the porin on a cell or liposome, the apparent rate constants were assumed to be the same on both sides of the membrane. The experiments of Dargent et al. (Dargent et al., 1988) with maltoporin symmetrically oriented in a planar membrane suggest that the intrinsic reaction rate constants are the same for ligand approaching the porin binding site from either end. This, however, does not necessarily mean that the apparent rate constants in the liposome or cell are the same for either end of the maltoporin. It would be expected that the rate constant describing the reversible formation of the encounter complex would be different for ligand inside or outside the membrane. The apparent geometry of the membrane is different from each point of view. Considering the ligand in the periplasmic space, its diffusion may not be adequately described by the diffusion coefficient for ligand in the bulk solution. The viscosity of the fluid in the periplasmic could differ from that of the bulk solution and steric restrictions could hinder ligand movement. These factors may, in part, explain the difference between the values for the apparent rate constants for maltohexaose leaving a porin calculated from flux experiments and the degradation rate of PG6.

The values for the rate constants obtained in this section can thus, be considered 'first' estimates. They were obtained using the ligand-receptor encounter theory as developed by Berg and Purcell (Berg and Purcell, 1977), Bell (Bell, 1977) and DeLisi (DeLisi, 1980) and assuming the rate constants are the same on either side of the membrane. It is expected that taking into account the peculiarities of ligand movement in the liposomes or periplasmic space, more representative values of the rate constants could be obtained. However, with the estimates presented here, the kinetics of the process of ligand-maltoporin binding can be directly compared to the kinetics of other processes of interest. This information is of particular importance in the investigation of maltoporin mediated specific adhesion.

4.6 Nomenclature

- \( a \) radius of cell or liposome (length)
- \( C \) cell or liposome (amount/volume)
- \( D \) Diffusion coefficient of ligand in solution (area/time)
- \( d_{+} \) rate constant for ligand entering encounter complex, valid when \( R_s/C << \pi a \) (volume/(amount time))
- \( d_{-} \) rate constant for ligand encountering surface containing liposome (volume/(amount time))
- \( d_{\pm} \) rate constant for ligand leaving encounter complex (1/time)
- \( f \) volume fraction
- \( J \) flux through porins (amount/time)
$K$-equilibrium constant for ligand-porin binding (volume/amount)

$K_{eq}$-reaction equilibrium constant

$K_{eqm}$-mass transfer equilibrium constant for encounter complex formation (volume/amount)

$k_f$-apparent rate constant for formation of the ligand-porin bond in situation of reaction limitation or $R_s/C << \pi a$, independent of $R$ (volume/(amount time))

$k'_f$-relative apparent rate constant for formation of the ligand-porin bond in situation of reaction limitation or $R_s/C << \pi a$, independent of $R$ (volume/(amount time))

$k_r$-apparent rate constant for break up of the ligand-porin bond in situation of reaction limitation or $R_s/C << \pi a$, independent of $R$ (1/time)

$k'_r$-relative apparent rate constant for break up of the ligand-porin bond in situation of reaction limitation or $R_s/C << \pi a$, independent of $R$ (1/time)

$k_f$-apparent rate constant for the formation of the ligand-porin bond (volume/(amount time))

$k_r$-apparent rate constant for the break up of the ligand-porin bond (1/time)

$k_+\text{, intrinsic reaction rate constant for formation of ligand porin bond (1/time)}$

$k_-\text{, intrinsic reaction rate constant for break up of ligand porin bond (1/time)}$

$L\text{, ligand (amount/volume)}$

$L_d\text{, degradation products that bind to porin (amount/volume)}$

$L-R\text{, ligand-porin encounter complex (amount/volume)}$

$LR\text{, ligand binding to porin (amount/volume)}$

$P\text{, probability that a ligand at a membrane surface will escape to bulk solution before encountering an unoccupied porin.}$

$R\text{, total concentration of unoccupied porin (amount/volume)}$

$R_e\text{, number of unoccupied porin on a cell or liposome (amount)}$

$R_m\text{, total concentration of sites available for ligand transport across cytoplasmic membrane}$

$r_i\text{, radius of flat membrane (length)}$

$s\text{, radius of the encounter complex (length)}$

$t\text{, time}$

$V_t\text{, total volume (volume)}$

$Y\text{, relative flux into liposome (amount/time)}$

$\xi\text{, stoichiometric coefficient for degradation products produced from ligand}$
4.7 References


CHAPTER 5
SELECTIVE RELEASE OF SPECIFICALLY ADHERING CELLS:
A METHOD OF CELL SEPARATION

5.1 Abstract

A kinetic model for the selective release of specifically adhering cells is developed. Simplified cases of the general model under limiting conditions are considered. The effect of hydrodynamic forces and competitive free ligand concentration on the release of specifically adhering cells are investigated using the kinetic model. This model is used to evaluate selective cell release as a means to fractionate cell populations. Experimental data on selective release of a population adhering in a packed bed is presented. Prediction of the selective release of cells obtained from the kinetic model agree reasonably well with experimental results.

5.2 Introduction

Specific cell adhesion is a process that could prove important in cell separation (Edelman and Rutishauser, 1974; Jovin and Arndt-Jovin, 1980; Braun and Kumel, 1986). The adhesion is mediated by cell surface receptors that bind to particular ligands. The ligands are immobilized on a surface, which leads to selective immobilization of cells with matching receptors. There are several applications of specific adhesion reported in the literature, such as separation of lymphocytes (Hertz et al., 1985; Tlaskalova-Hogenova et al., 1986; Braun and Kumel, 1986) and neural cells (Au and Sharon, 1979), determination of oral microbial lectins (Murray et al., 1987), isolation of mutant populations with altered expression of surface receptors (Ferenci and Lee, 1982) or receptor-ligand affinity (Clune et al., 1984) and monitoring of mixed culture bioreactors (Roos and Hjortso, 1989).

The conditions that alter the onset of specific adhesion and the manipulation of these parameters to bring about the separation of cell populations, has been the subject of several theoretical and experimental investigations (Edelman and Rutishauser, 1974; Bell, 1978; Bell, 1981; Bell et al., 1984; Hertz et al., 1985; Hammer and Lauffenburger, 1987). In these cases, separation is based on different adhesion characteristics of the cell populations. An alternate approach to separating cells is to employ selective elution of adhering cells.

Cells that are adhering specifically to a surface have formed a network of ligand-receptor bonds. Examples of these bonds are those between antigens and antibodies, lectins and carbohydrates or transport proteins and their substrates. These bonds retain the cell on the surface against forces that would resuspend the cell in bulk solution. It has been observed, that with respect to the time scales of interest in cell separation, such cells can
be considered to be irreversibly bound to the support (Rutishauser and Sachs, 1975a,b; Ferenci and Lee, 1982; Braun and Kumel, 1986; Roos and Hjortso, 1989). The individual bonds, however, are often reversible and under proper conditions adhering cell are released from the support.

To address the release of specifically adhering cells and its application for cell separation, a discrete kinetic model of cell elution is developed. The behavior of this model is explored over various operating conditions and special cases are discussed. The possibility of using the release characteristics of specifically adhering cells to separate two adhering populations is explored using a special case of the general release model. The behavior of cell release, experimentally determined in a packed bed, is presented and discussed in light of predictions from the release model.

5.3 The Specifically Adhering Cell

Theoretical framework has been proposed to describe the role of various contributing factors in the specific adhesion of cells (Bell, 1978; bell et al, 1984; Evans 1985a,b; Hammer and Lauffenburger, 1987). The general outline of this work depicts a cell coming into contact with a support to which it can adhere. The transport mechanisms include convective, diffusion or gravitation induced transport to the surface. There is a short period of time after the initial contact, in which a sufficient number of ligand receptor bonds must form to retain the cell against removal and repulsive forces. This time period is dependent on the fluid environment, repulsive forces and cell properties such as membrane elasticity.

The probability that the cell adheres to the support is dependent on its success in forming the required number of ligand-receptor bonds. The rate of bond formation is a function of cell properties such as receptor density and receptor mobility in the membrane. The ligand density and the rate constants for the formation of bonds between the immobilized ligand and the cell surface receptor are also important.

When a sufficient number of bonds are formed to overcome repulsive forces, the cell is adhering to the support. At this time, the area on the cell membrane and the surface in which the bond formation occurs, the contact area, is expected to be determined by the forces acting on the cell and cell deformation (Bell et al, 1984). The adhering cell is considered to be fixed at its adhesion site, the discrete bonds being the only forces holding it at the surface.

During the initial period of bond formation between immobilized ligand and receptors, the short range diffusion of receptors in the contact area is important (Rutishauser and Sachs, 1975b). After this period, the long range diffusion must be considered (McCloskey and Poo, 1986). The receptors diffuse from other areas
of the cell surface into the contact area where they can form bonds with immobilized ligand. In this manner they are trapped and the receptor number in the contact area increases with time. It would be expected that at some point a steady state level or a maximum number of receptors would be reached.

The adhesion state of a cell can be defined by the number of bonds between immobilized ligands and receptors. These bonds are in general reversible and the cells, therefore, are considered free to move through the various adhesion states as bonds break and form. The distribution of cells in the adhesion states and the rate at which a cell moves between states is expected to be a function of the same parameters that regulate bond formation during the initial stage of adhesion. The only mechanism through which cells are removed from the distribution over adhesion states is through removal from the surface. To be eligible for removal, the cell must reside in the state without immobilized ligand-receptor bonds. The sum of the cells in the various adhesion states accounts for all cells adhering to the support.

Taking these factors into account yields a description of the total number of adhering cells, the movement of cells through the adhesion states and changes in the number of adhering cells due to release or readhesion. This model, however, can be greatly simplified by considering the physical conditions that encourage the adhesion or release of the cells. Under conditions that encourage cell adhesion it has been observed that once the cells adhere, very few are released unless steps are taken to promote release (Edelman and Rutishauser, 1974; Weigel et al., 1979; Ferenci, 1983). It has also been reported that under conditions that severely limit cell adhesion, already adhering cells are not necessarily released (Rutishauser and Sachs, 1975a). It is therefore reasonable to assume that conditions which result in cell release do not permit significant, concurrent adhesion of cells and in the model development readhesion of cells will not be considered.

**5.4 Cell Adhesion States**

The distribution of adhered cells is readily represented using two integers. These integers represent the number of bonds between receptors and ligands that were in solution and the number of bonds between receptors and immobilized ligands. Only cells in states with no receptor-immobilized ligand bonds are eligible for removal. The specific rate of removal is expected to depend on the dominant mechanism of cell transport, convection or Brownian motion. It is reasonable to assume that this rate could be increased by altering the attributes of the fluid flow. However, the effectiveness of this approach will be limited if the distribution of adhesion states is shifted away from the state without immobilized ligand-receptor bonds. Thus, to achieve release of specifically adhered cells in a reasonable time frame, a shift of cells toward the adhesion state without any immobilized ligand-receptor bonds is required. This can be accomplished either through increasing
the rate at which immobilized ligand-receptor bonds break, or decreasing the rate at which they form. There are several approaches that can yield either result, such as stressing the ligand-receptor bonds by increasing the fluid drag on the cells or adding a competitive free ligand or receptor. To estimate the effect of these steps on cell elution a quantitative description of the adhesion state is formulated.

**Figure 5-1. Adhesion States of Specifically Adhering Cells.**

Adhesion states for cells adhering to a support in the presence of soluble ligand. The state is defined by the number of immobilized ligand-receptor and soluble ligand-receptor bonds.
Consider the general case where soluble ligand is present to compete for the cell surface receptor. The state of a cell is defined by the number of receptors in the contact area that are occupied by immobilized and soluble ligand (Figure 5-1). The cells move through the various states by breaking or forming ligands bonds. For example, the specific rate at which cells leave the state with \( i \) immobilized ligand-receptor bonds and \( j \) soluble ligand-receptor bonds by forming an immobilize ligand-receptor bond is \( r_{i+1,j}^- \). These specific rates are dependent on the state of the cell.

A balance on the total number of cells, \( N_{ij} \), in the state with \( i \) immobilized ligand bonds and \( j \) soluble ligand bonds in the contact area, \( A \), can be written as:

\[
\frac{dN_{ij}}{dt} = r_{i,j}^+ N_{i-1,j} + r_{i+1,j}^- N_{i+1,j} + r_{i,j+1}^- N_{i,j+1} + r_{i,j}^- N_{i-1,j} - (r_{i,j}^+ + r_{i+1,j}^- + r_{i,j+1}^- + r_{i,j}^-) N_{ij}
\]

where, \( r_{i,j}^+ \) and \( r_{i,j}^- \) represent, respectively, the specific rate at which a cell moves out of an adhesion state due to formation or breakup of a bond between a receptor and a soluble ligand. The specific rates at which a cell leaves a state because of the formation or breakup of immobilized ligand-receptor bonds are \( r_{i,j}^- \) and \( r_{i,j}^- \). All cells are assumed to have the same number of receptors in the contact area, \( n \).

5.4.1 Specific Rates for Cells Moving between Adhesion States

In the development of the model for adhesion states, several simplifying assumptions are made. When determining the rates of reversible ligand binding during cell elution, it is sufficient to consider only the receptors in the contact area. The process of receptor diffusion into and out of this area and the trapping by bond formation are assumed to be at steady state, the total number of receptors in the contact area is thus a constant. For a cell in the state \( N_{ij} \) there are \((n-i-j)\) unoccupied receptors. All of the receptors in the contact area are considered to be equivalent, and each of the unoccupied receptors has the same probability of forming the next ligand-receptor bond. The soluble ligand is assumed to be free to enter the region between the cell and the adhesion surface. Thus, the concentration is constant and equal to that of the bulk fluid. The immobilized ligand density, \( C_L \), in this region is constant since these ligands are fixed. With a fixed contact area, the number of immobilized ligands that are free to bind to receptors is only dependent on the cell state and is given as \( C_L - i/A \), for a cell in the state \( N_{ij} \).
To develop an expression for the formation of immobilized ligand-receptor bonds, the orientation of the cell and the surface to which it is adhering must be considered (Figure 5-2). Due to the elasticity of the cell membrane, the cell deforms to some extent to match the contours of the adhesion surface. For this model, the cell membrane and the surface are viewed as lying parallel to each other. The immobilized ligands extend away from the adhesion surface and lie in the same plane as the cell surface receptors.

![Diagram of Cell and Adhesion Surface](image)

**Figure 5-2. Orientation of Membrane and Adhesion Surface.**
Orientation of the contact area between the adhering cell and the surface. The view is from the edge of the plane in which receptor-immobilized ligand interaction occurs.

In the model of Equation (1) the specific rates describe four basic processes. These processes will now be considered individually. The specific rates will be expressed in terms of fundamental rate constants, cell state and system parameters such as fluid forces.

### 5.4.2 Formation of Immobilized Ligand-Receptor Bonds

Binding of a ligand to a cell surface receptor can be viewed as a two step process (Bell, 1978). First, the ligand and receptor must move close enough to each other to allow reaction to occur. This is the formation of an encounter complex. The second step is the reaction step, where a ligand receptor pair, in the encounter complex, form a bond. Since ligand receptor binding is reversible, both steps have an associated release process. For a ligand, L, and a receptor, R, the formation of the encounter complex, L-R, and the ligand-receptor bond, LR, can be represented as:
where $d_+^-$ and $d_-\!^-$ are the rate constants for encounter complex formation and breakup and $k_+^-$ and $k_-^-$ are the intrinsic reaction rate constants for reversible bond formation.

It is frequently assumed that the concentration of the encounter complex quickly reaches a quasi-steady state (Bell, 1978; Delisi, 1980). The apparent rate constants for the reaction network then become:

$$k_-^\pm = \frac{d_+^\pm}{k_-^\pm + d_-\!^\pm} \quad (2a)$$

$$k_-^\pm = \frac{d_+^\pm}{k_-^\pm + d_-\!^\pm} \quad (2b)$$

The rate constants for formation and breakup of the encounter complex, $d_+^-$ and $d_-\!^-$, are expressible in terms of more fundamental parameters. These rate constants describe the diffusion of receptors in and out of the encounter complex. To calculate the forward rate constant, $d_+$, the mean encounter times for the ligand-receptor system are determined (Berg and Purcell, 1977).

$$t_e = \frac{s_c^4}{2D_m(s_c^2-s_0^2)^2} \left( \ln \left( \frac{s_c}{s_0} \right) - \frac{1}{4} \left( 3 - \left( \frac{s_c}{s_0} \right)^2 \right) \left( 1 - \left( \frac{s_c}{s_0} \right)^2 \right) \right) \quad (3)$$

This time is a function of the receptor membrane diffusion coefficient, $D_m$, the mean separation radius between the unbound immobilized ligands, $s_0$, and the radius of the encounter complex, $s_c$. Using the mean encounter time, the net flux of ligand into the encounter complex is found to be,

$$J^* = \frac{\pi s_c^3 [R]}{t_e} \quad (4)$$

where $[R]$ is the surface density of receptors in the contact area.

The rate constant for the formation of the encounter complex is determined from this flux as:

$$d_+^\pm = \frac{J^*}{[R]} \quad (5)$$

$$= \frac{2\pi D_m \left( 1 - \left( \frac{s_c}{s_0} \right)^2 \right) \left( \ln \left( \frac{s_c}{s_0} \right) - \frac{1}{4} \left( 3 - \left( \frac{s_c}{s_0} \right)^2 \right) \left( 1 - \left( \frac{s_c}{s_0} \right)^2 \right) \right)}{\left( \ln \left( \frac{s_c}{s_0} \right) - \frac{1}{4} \left( 3 - \left( \frac{s_c}{s_0} \right)^2 \right) \left( 1 - \left( \frac{s_c}{s_0} \right)^2 \right) \right)}$$
This expression is valid as long as the characteristic separation radius between ligands is greater than the radius of the encounter complex, \( s > s_c \). The separation radius, and thus the rate constant \( d_+ \), is a function of the cell adhesion state. As more ligands are bound, the mean separation radius between available ligands will increase and the rate constant for encounter complex formation decreases.

In the reverse process, an encounter complex is said to have broken when the receptor moves beyond the characteristic radius of the encounter complex. This is a more restrictive definition than previously proposed (DeLisi, 1980). Using an approach similar to the one used in deriving equation (3), a characteristic time for a receptor within the encounter complex to cross beyond the complex boundary is found to be,

\[
t = \frac{s^2}{8D_m}
\]  

(6)

It is assumed that only one ligand and receptor can inhabit an encounter complex so the concentration of receptor within an encounter complex is unity. The flux out of an encounter complex thus is;

\[
J^- = \frac{1}{t_+}
\]  

(7)

The rate constant for ligand leaving the encounter complex becomes;

\[
d_+ = J^-
\]  

(8)

The intrinsic reaction rate constant for bond formation, \( k_+ \), used in Equation 2a and 2b, is dependent on the particular ligand-receptor system. It will not change with the state of a cell.

The rate at which one immobilized ligand-receptor bond forms is expected to be first order with respect to immobilized ligand concentration. The total rate of formation of the next immobilized ligand-receptor bonds is proportional to the total number of available receptors in the contact area. The specific rate at which cells leave the state \( N_{ij} \) through the formation of an immobilized ligand-receptor bond becomes;

\[
r_{ij}^* = \left( \frac{k_+ d_+}{d_+ + k_+} \right) \left( C_L - \frac{(i - 1)}{A} \right) (n - i - j + 1)
\]  

(9)

5.4.3 Breakage of Immobilized Ligand-Receptor Bonds

The specific rate at which a cell in state \( N_{ij} \) leaves due to break up of an immobilized ligand-receptor bond is proportional to the number of these bonds.

\[
r_{ij} = k^* \cdot i
\]  

(10)
The rate constant, \( k^* \), is given in equation (2b). However, the intrinsic rate constant for breaking the immobilized ligand-receptor bond, \( k_r \), changes when forces such as fluid drag on an immobilized cell stress the bonds. The result of the stress on the bond is to decrease the lifetime of the bond (Bell, 1978). This is reflected by an increase in the intrinsic reaction rate constant for breaking of the bonds. If the stress is evenly distributed over the immobilized ligand bonds, the stress on an individual bond will decrease as more bonds are formed.

Forces on an adhering cell that can induce stress on the bonds can be non-specific interactions or the influence of the hydrodynamic environment on the cell. While, under some circumstances, the non-specific repulsive forces could prove significant, they cannot easily be manipulated and will not be dealt with explicitly. The forces arising from the hydrodynamic environment are more readily controlled and thus, more useful in engineering applications.

The velocity gradient of a fluid flowing past an adhering cell subjects the cell to a torque and the cell has a tendency to roll over the surface. For the cell to remain fixed, a sufficient number of bonds must form between the cell and surface to resist this force. The bonds on the upstream side of the contact area will experience a greater stress than those on the downstream side. Several estimates of the stress placed on bonds between the immobilized ligand and the cell surface receptor have been proposed (Evans, 1985; Hammer and Lauffenburger, 1987). The phenomena of rolling over the surface will not be considered here. A simple stress distribution will be used: the total removal forces on the cell are assumed equally distributed over all immobilized ligand-receptor bonds. The ligand-receptor bonds, regardless of their position in the contact area relative to the fluid flow, will possess the same mean life. The intrinsic rate constant for breaking bonds, therefore, is not a function of the bond position in the contact area.

Due to the small size of an adhered cell, the flow over the cell is clearly Stokes flow. Thus the drag force on the cell must be proportional to viscosity, fluid velocity and cell radius. For lack of a better estimate, the proportionality constant will be assumed to be \( 6\pi \), the same as in Stokes’ law. The effect of the hydrodynamic environment is idealized by restricting the cell to two types of behavior. It either remains fixed at its point of adhesion or is released from the support.

The total force, \( F_t \), is estimated for a cell of radius \( a \), experiencing Stokes drag from a fluid of viscosity \( \eta \) with a characteristic velocity, \( v \). The force per bond, \( F_b \), is the Stokes drag divided by the number of immobilized ligand-receptor bonds.

\[
F_b = \frac{F_t}{i} = \frac{6\pi \eta a v}{i}
\]  

(11)
The intrinsic rate constant for breaking a stressed bond is a function of the force exerted on the bond, \( F_b \), and the rate constant for the bond if no hydrodynamic forces were present, \( k^o \). The expression proposed by Bell (Bell, 1978) is used here for the intrinsic rate constant of a stressed bond;

\[
k_\text{-} = k^o \exp \left( \frac{6\gamma \pi a v}{k_b T i} \right)
\]  

(12)

where \( \gamma \) is the characteristic bond length, \( k_b \) is the Boltzman constant and \( T \) is the absolute temperature.

Thus, the specific rate at which a cell leaves the state \( N_i \) and enters \( N_{i+1} \) by breaking a ligand-receptor bond is;

\[
r_{ij} = \left( \frac{d k^o}{d_+ + k_\text{-}} \right) \exp \left( \frac{6\gamma \pi a v}{k_b T i} \right)
\]  

(13)

5.4.4 Formation of Soluble Ligand-Receptor Bonds

The formation of the soluble ligand-receptor bond is taken to occur after the ligand-receptor encounter complex has formed. In the region between the cell and the adhesion surface, diffusion is considered the primary transport mechanism for soluble ligand. The rate constant for encounter complex formation between a soluble ligand and a cell surface receptor describes the process of ligand diffusion to the cell surface and then the movement into the encounter complex. This rate constant is calculated from the flux of soluble ligand to receptors in the contact area.

To estimate the flux, a cell with uniform receptor density equal to that found in the contact area of the adhering cell is considered. The soluble ligand flux to the surface of this cell is calculated from the mean collision time between the cell and ligand in three dimension or from diffusion in a continuum (Delisi, 1980). This, however, is not the flux to the receptors since receptors do not cover the entire surface of a cell. The flux to an unoccupied receptor is calculated from the probability that a ligand, having diffused to the cell surface, will escape from the surface before reaching an unoccupied receptor. This probability is related to the number of receptors, \( N \), available for binding on the cell. The probability accounts for the action of the ligand, which, having diffused to a cell surface, will remain within the vicinity of the surface, colliding several times, before diffusing away. This probability, \( P \), is of the form (Berg and Purcell, 1977; DeLisi, 1980);

\[
P = \frac{\pi a}{N s_+ + \pi a}
\]  

(14)

The flux to receptors in the contact area, \( J_{\text{ca+}} \), is assumed to be equal to the product of the total flux to receptors over the whole cell, \( J^* \), and the surface fraction, \( \beta \), of the adhering cell that lies in the contact area. The flux to receptors in the contact area becomes;
\[ J'_{c,a} = J'_{i,a} \beta \]
\[ = 4\pi a D \left( 1 - \frac{\pi a}{N_{S_c} + \pi a} \right) C \beta \]

where \( C \) is the concentration of soluble ligand and \( D \) is the diffusion coefficient of the ligand in solution.

The rate constant for the formation of the encounter complex between the receptor and the soluble ligand becomes:

\[ \hat{d}_{i,j} = \frac{J_{c,a}}{C} \]
\[ = 4\pi a D \left( 1 - \frac{\pi a}{N_{S_c} + \pi a} \right) \beta \]
\[ = 4\pi a D \left( \frac{S_c}{N_{S_c} + \pi a} \right) (n - i - j + 1) \]

where \( N = (n - i - j + 1)/\beta \). Note that this rate constant is a function of the adhesion state of the cell.

For the cell, the flux of a soluble ligand out of an encounter complex, \( J_{w,c} \), is proportional to the rate of diffusion of the ligand out of the complex and the probability that the ligand will escape to the bulk solution, before encountering an unoccupied receptor. The process of the soluble ligand leaving the encounter complex is taken to be similar to a soluble ligand diffusing away from a receptor in free solution (Lauffenburger and Delisi, 1983). The flux out of an encounter complex is multiplied by the total number of ligand in an encounter complex, \( J_{e,c}/\beta \), to give total flux away from the cell.

\[ J'_{w,c} = \frac{3D J_{e,c}}{S_c^2} \left( \frac{\pi a}{N_{S_c} + \pi a} \right) \beta \]

The quantity \( J_{e,c} \), is the number of soluble ligand-receptor encounter complexes in the contact area. The flux out of the contact area, \( J_{c,a} \), is the product of the area fraction of the contact area and the flux for the whole cell. The rate constant for breakup of the encounter complex, is:

\[ \hat{d}_{i,j} = \frac{J_{c,a}}{J_{i,e}} \]
\[ = \frac{3D}{S_c^2} \left( \frac{\pi a}{N_{S_c} + \pi a} \right) \]

and the rate constant is function of the adhesion state.
As in the case of the immobilized ligand, the encounter complex composed of soluble ligand and receptor is considered to be at quasi-steady state. The apparent rate constant for the process of ligand diffusion to the cell surface in the contact area, into an encounter complex and then forming a bond is of the form given in Equation 2a. It is based on the cell and describes the process of formation of the next soluble ligand-receptor bond in the contact area. The intrinsic reaction rate constant \( k_* \) is a property of the specific soluble ligand receptor pair, it is not a function of the adhesion state or the hydrodynamic environment. The rate constants for encounter complex formation and break up are given by Equation 16 and 18.

The specific rate at which a cell enters the state \( N_{ij} \) due to the formation of the \( j^{th} \) soluble ligand-receptor bond is proportional to the apparent rate constant for the formation of this bond in the contact area and the concentration of soluble ligand:

\[
\hat{r}^*_{ij} = \frac{k_* \hat{d}^*_{ij}}{\hat{d}^*_{ij} + k_*} C
\]

5.4.5 Breakage of a Soluble Ligand-Receptor Bond

A cell can leave the state \( N_{ij} \) by breaking a soluble ligand-receptor bond in the contact area. The specific rate at which this process occurs, \( \hat{r}^*_{ij} \), is determined by the rate at which the individual bonds break and the number of bonds. Using the quasi-steady state assumption for the encounter complex (Eqn. 2b), the specific rate becomes:

\[
\hat{r}^*_{ij} = \frac{k_* \hat{d}^*_{ij}}{\hat{d}^*_{ij} + k_*} j
\]

The intrinsic reaction rate constant for bond breakage \( k_* \), is dependent on the particular ligand and receptor. It is not a function of the cell state. The rate constant for encounter complex breakup \( \hat{d}^*_{ij} \) is given in Equation 18.

5.4.6 Removal Rate of Unbound Cells

For removal of a cell from a surface, all ligand-receptor bonds must be broken and the cell must travel away from the adhesion surface. If all of the immobilized ligand bonds of a cell are broken and the cell is not removed from its adhesion site, an immobilized ligand-receptor bond can form. In this case, the process of binding an immobilized ligand is dependent only on receptor diffusion to form the encounter complex. A cell that is removed from the surface must go through a more involved process to form an encounter complex requiring movement in the bulk fluid. A cell is considered removed from the surface when it is displaced a sufficient distance that receptor diffusion is not the dominant process involved in encounter complex formation.
between the immobilized ligand and receptor. At this point, the mechanism of encounter complex formation is the same as described for the onset of adhesion. When these conditions are met, the cell is considered indistinguishable from cells in the bulk fluid. Cells in the bulk fluid are viewed as removed from the system of the adhering cells and the surface and do not interfere with the release of adhering cells. As discussed earlier, once a cell is released, it is assumed that there is no reassociation and adhesion to the surface.

The rate with which cells in the states \( N_{0,i} \) are removed from the surface is proportional to the number of cells in the state. The proportionality constant is the specific rate of cell release, \( k_r \). This rate is estimated as the characteristic velocity of the fluid near the surface divided by the characteristic cell radius.

### 5.4.7 Cell Balance over Adhesion States in the Presence of Soluble Competing Ligand

Complete balances for cells in all adhesion states and balances on cells that have been released can now be formulated:

**for \( i = j = 0 \)**

\[
\frac{dN_{0,0}}{dt} = r_{1,0}N_{1,0} + \rho_{0,1}N_{0,1} - (r_{1,0}^* + r_{0,1}^* + k_r)N_{0,0} \tag{21a}
\]

**for \( i = 0, \ 0 < j < n \)**

\[
\frac{dN_{0,j}}{dt} = r_{0,j}^*N_{0,j-1} + \rho_{0,j+1}N_{0,j+1} + r_{i,j}N_{1,j} - (r_{0,j}^* + r_{0,j+1}^* + r_{i,j}^* + k_r)N_{0,j} \tag{21b}
\]

**for \( j = 0, \ 0 < i < n \)**

\[
\frac{dN_{i,0}}{dt} = r_{i,0}^*N_{i-1,0} + r_{i+1,0}N_{i+1,0} + r_{i,1}N_{i,1} - (r_{i,0}^* + r_{i+1,0}^* + r_{i,1}^*)N_{i,0} \tag{21c}
\]

**for \( i + j < n, \ i \neq 0, \ j \neq 0 \)**

\[
\frac{dN_{i,j}}{dt} = r_{i,j}^*N_{i-1,j} + r_{i+1,j}N_{i+1,j} + \rho_{i,j+1}N_{i,j+1} + r_{i,j}N_{i,j} - (r_{i,j}^* + r_{i+1,j}^* + \rho_{i,j+1}^* + r_{i,j}^*)N_{i,j} \tag{21d}
\]

**for \( i = n, \ j = 0 \)**

\[
\frac{dN_{n,0}}{dt} = r_{n,0}^*N_{n-1,0} - r_{n,0}N_{n,0} \tag{21e}
\]

**for \( j = n, \ i = 0 \)**

\[
\frac{dN_{0,n}}{dt} = \rho_{0,n}^*N_{0,n-1} - (\rho_{0,n}^* + k_r)N_{0,n} \tag{21f}
\]
For $i+j = n, \ i \neq 0, \ j \neq 0$

\[
\frac{dN_{i,j}}{dt} = r_{i,j}^+ N_{i-1,j} + r_{i,j}^- N_{i+1,j} - (r_{i,j}^+ + r_{i,j}^-) N_{i,j}
\]  

(21g)

\[
\frac{dN_f}{dt} = k \left( \sum_{j=0}^{\infty} N_{0,j} \right)
\]  

(21h)

where $N_i$ is the number of cells released from the support.

For a cell population with $n$ receptors in the contact area, this would consist of $(1/2)(n+1)(n+2)+1$ ordinary differential equations.

5.4.8 Special Cases

Simplification of the specific rates at which cells change states is often reasonable under certain limiting conditions. If the diffusive rate constant for removal of ligand from the encounter complex is much larger than the intrinsic reaction rate constant of bond formation, the process is reaction limited. For the case of immobilized ligand-receptor pair, the following substitution can be made for the specific rate of a cell entering or leaving a state due to formation and breaking of immobilized ligand-receptor bonds

\[
r_{i,j}^+ = k_K a \exp \left( \frac{C_L - (i - 1)}{A} \right)
\]  

(22a)

\[
r_{i,j}^- = i k^0 \exp \left( \frac{6 \eta \pi n v}{k_T T_i} \right)
\]  

(22b)

The constant, $K_{eq} = d_v d_s$, is the equilibrium diffusion constant. This constant is not a function of the membrane diffusion coefficient, but is dependent on the adhesion state, through the dependence of $d_v$ on the mean spacing between unbound immobilized ligands (Eqn. 5).

If the immobilized ligand-receptor interaction is diffusion limited, $k_r \gg d_s$, the specific rates for cells moving between states by reversible bond formation with immobilized ligands become;

\[
r_{i,j}^+ = d_v (n - i - j + 1) \left( \frac{C_L - (i - 1)}{A} \right)
\]  

(23a)

\[
r_{i,j}^- = i \frac{d_v}{K_{eq}} \exp \left( \frac{6 \eta \pi n v}{k_T T_i} \right)
\]  

(23b)

where $K_{eq}$ is the intrinsic reaction equilibrium constant.

If the number of immobilized ligands in the contact area is much greater than the number of receptors, the surface concentration of immobilized ligands remains essentially constant over adhesion states and Equation 9 reduces to;
Similar simplifications can be applied to the specific rates for cells moving between adhesion states due to the binding of receptors and soluble ligands. If this process is reaction limited, the specific rates become:

\[ r_{i,j}^* = \frac{k_d d_i}{d_i + k_s} (n - i - j + 1)C_z \]  

(24)

Further simplification of the specific rates is possible if the factor \( N_{sc} \) of Equation 14 is much smaller than the factor \( n_a \). In this extreme, the probability, \( P \), approaches unity. The rate constant for encounter complex formation reduces to;

\[ r_{i,j}^* = \tilde{k}_e K_{quin} \]  

(25a)

\[ = \tilde{k}_e K_{quin}^{'} (n - i - j + 1)C \]  

(25b)

\[ r_{i,j}^* = \tilde{k}_j \]  

where \( K_{quin}^{'} = 4s_j^2/3 \).

When diffusion limitation prevails, the following substitutions for the specific rates are valid;

\[ r_{i,j}^* = \tilde{d}_{i,j} C \]  

(26a)

\[ r_{i,j}^* = \frac{\tilde{d}_{i,j}}{K_{run}} j \]  

(26b)

The equilibrium constant for soluble ligand-receptor bond formation is \( \tilde{K}_{run} \).

Further simplification of the specific rates is possible if the factor \( N_{sc} \) of Equation 14 is much smaller than the factor \( n_a \). In this extreme, the probability, \( P \), approaches unity. The rate constant for encounter complex formation reduces to;

\[ \tilde{d}_{i,j} = 4s_j D (n - i - j + 1) \]  

(27)

and remains a function of the adhesion state. The diffusion rate constant for encounter complex breakup becomes independent of the adhesion and is estimated to be;

\[ \tilde{d}^- = \frac{3D}{s_i^2} \]  

(28)

A simplified expression for the dynamic model (Eqn. 21) can be obtained if it is valid to apply the quasi-steady state assumption to the adhesion state distribution. The steady state values for the number of cells in the adhesion states without immobilized ligand-receptor bonds are then used in Equation 21h. With this assumption, an analytical solution for dynamics of cell release is possible.

The quasi-steady state assumption implies that the cell distribution over the adhesion states reaches steady state quickly and it is maintained throughout the process of cell release. The validity of this assumption depends on the relative magnitude of the specific rates at which cells leave the state without immobilized ligand bonds.
The processes of interest are the removal of cells from the support and the formation of the first immobilized ligand bond, and the specific rates for these processes are \( k_r \) and \( r_{ij} \) respectively. To illustrate this criteria, a sum is taken over all states without immobilized ligand bonds,

\[
\sum_{j=0}^{n-1} \frac{dN_{0,j}}{dt} = \sum_{j=0}^{n-1} r_{ij}N_{1,j} - \sum_{j=0}^{n-1} (r_{ij} + k_r)N_{0,j}
\]

(29)

If \( k_r \ll r_{ij} \) over all \( j \), the specific rate of removal has little effect on the dynamics of the steady state distribution.

The steady state distribution is easily determined. For any given number of immobilized ligand-receptor bonds, \( i \), there is a distribution over the possible states of receptors filled with soluble ligand;

\[
\text{for } j \leq n-i
\]

\[
N_{i,j} = \hat{K}_{i,j} \cdots \hat{K}_{i,0}N_{i,0}
\]

(30)

The constant, \( \hat{K}_{i,j} \), is the ratio of the specific rate for a cell entering state \( N_{i,j} \) through the formation of a soluble ligand-receptor bond (Eqn. 19) and rate at which the \( j \)th bond is broken (Eqn. 20),

\[
\hat{K}_{i,j} = \frac{r_{ij}^+}{r_{ij}^-} = \frac{r_{ij}^+}{r_{ij}^-} \frac{K_{rpm}^+}{}\left(\frac{n-i-j+1}{j}\right)C
\]

(31)

The total number of cells in the adhesion states with \( i \) immobilized ligand bonds, \( N_i^T \), is found by summing over all such states.

\[
N_i^T = \sum_{j=0}^{n-i} N_{i,j}
\]

(32)

\[
= N_{i,0}(1 + \hat{K}_{rpm}^+ C)^{n-i}
\]

The number of cells in a state \( N_{i,0} \) is;

\[
N_{i,0} = K_{i,0} \cdots K_{i,0} N_{0,0}
\]

(33)

The constant \( K_{i,0} \) is the ratio of the specific rate at which a cell enters the state with \( i \) immobilized ligand bonds by the addition of an immobilized ligand bond to the specific rate a cell leaves the state due to bond breakup. Substitution of these rates into Equation 33 gives,

\[
N_{i,0} = N_{0,0} \left( \frac{K_{rpm}^+}{\tau_{i,0}} \right)^{i} \prod_{m=1}^{N} d_e \left( \exp \left( -\frac{\gamma F_t}{k_B T m} \right) \left( C_e - \frac{(m-1)}{\Lambda_e} \right) \right)
\]

(34)
The only cells that are eligible for release are those in states without bonds to immobilized ligands. The number of cells without an immobilized ligand bond is found by setting $i=0$ in Equation $32$;

$$N^T_0 = N_{0,0} (1 + \tilde{K}_{run} \tilde{K}_{ap} C)^n$$  \hspace{1cm} (35)

The quantity $N_{0,0}$ is unknown, however, the fraction, $f_0$, of the total adhering cells in the state $N^T_0$ is readily calculated.

$$f_0 = \frac{\sum_{j=0}^{n_0} N_{0,j}}{\sum_{i=0}^{n_0} \sum_{j=0}^{n-i} N_{i,j}}$$  \hspace{1cm} (36)

$$= \left(1 + \sum_{i=1}^{n} \left(\frac{K_{ran}}{d_i}\right)^{n_i} \prod_{m=1}^{n_i} \left(\frac{\gamma F_i}{k_B T m} \left(C_m - \frac{(m-1)}{A_c}\right)\right)^{-1}\right)^{-1}$$

With this estimate of the cell population without immobilized ligand bonds, an analytical solution for Equation 21h is obtained. The number of cells released from the support as a function of time is,

$$N_f = N_T (1 - \exp(-k_f t))$$  \hspace{1cm} (37)

where $N_T$ is the total number of cells initially adhering to the support.

For the special case where the immobilized ligand is in excess and the hydrodynamic stress on the immobilized ligand-receptor bond can be ignored, further simplification is possible. The concentration of immobilized ligand available for binding would remain constant with adhesion states, as would $d_i$. The intrinsic reaction rate constant for the breakup of the immobilized ligand bonds also becomes independent of the adhesion state. With these assumptions, the estimate of the fraction of cells in the adhesion state without bonds to immobilized ligands simply becomes,

$$f_0 = \left(\frac{1 + \tilde{K}_{ran} \tilde{K}_{ap} C}{1 + \tilde{K}_{ran} \tilde{K}_{ap} C + K_{ran} K_{ap} C_L}\right)^n$$  \hspace{1cm} (38)

and the release of cells from a support as a function of time is,

$$N_f = N_T \left(1 - \exp\left(-k_f \left(\frac{1 + \tilde{K}_{ran} \tilde{K}_{ap} C}{1 + \tilde{K}_{ran} \tilde{K}_{ap} C + K_{ran} K_{ap} C_L}\right)^n\right)\right)$$  \hspace{1cm} (39)

When the receptors are in excess, an expression similar to that of Equation 39 can be derived if the rate constant for encounter complex formation is considered to be independent of adhesion state. Excess receptor implies that the immobilized ligand number in the contact area is low. The mean separation radius between the immobilized ligand is therefore, expected to be much larger than the radius of the encounter complex. The expression for the diffusion rate constant (Eqn. 5) reduces to;
From this expression, it is seen, that the values of the rate constant would vary over a limited range, from the value for only one immobilized ligand available to the value when all immobilized ligand in the contact area are available. If this variation is small, the rate constant is considered unchanging with the cell state and cell release is;

\[ N_f = N_f \left( 1 - \exp \left( -k_r t \left( \frac{1 + K_{rn} K_{qm} C}{1 + K_{rn} K_{qm} C + K_{rn} K_{qm} C_s} \right)^l \right) \right) \]  

where \( C_s \) is the surface concentration of the receptors in the contact area and \( l \) is the number of immobilized ligands in the contact area.

### 5.5 Model Predictions

In the models for the release of specifically adhering cells, the release mechanism was broken down into the underlying processes. It is necessary to determine the extent to which individual processes contribute to or retard cell release and how altering these processes would alter the release of cells. For various systems the rates of these individual processes can change greatly. This is illustrated by the range of values reported for parameters such as the intrinsic rate constants, membrane diffusion coefficients and immobilized ligand concentrations (Table 5-1). To illustrate the behavior of cell release and its dependence on the different processes, a range of parameters values were used (Table 5-1).
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Reported Range</th>
<th>Values Used</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solution Diffusion Coefficient $D$ (cm$^2$/sec)</td>
<td>Dependent on M.W.</td>
<td>$10^{-5}$ to $10^{-7}$</td>
<td>Reid et al, 1977</td>
</tr>
<tr>
<td>Membrane Diffusion Coefficient $D_m$ (cm$^2$/sec)</td>
<td>$10^{-8}$ to $10^{-12}$</td>
<td>$5 \times 10^{6}$</td>
<td>Kotyk et al, 1988</td>
</tr>
<tr>
<td>Intrinsic Reaction Rate Constant for Bond Formation $k_+$ or $k_-$ (1/sec)</td>
<td>$10^5$ to $10^9$</td>
<td>$27.4$ to $1.6 \times 10^4$</td>
<td>Lauffenburger and DeLisi, 1983; Bell, 1978; Roos, 1989</td>
</tr>
<tr>
<td>Intrinsic Reaction Rate Constant for Bond Breakage $k_-$ (1/sec)</td>
<td>$10^{-5}$ to $10^6$</td>
<td></td>
<td>Bell, 1978; Roos, 1989</td>
</tr>
<tr>
<td>Cell Radius $a$ (cm)</td>
<td>$5 \times 10^{-5}$ to $5 \times 10^{-4}$</td>
<td>$5 \times 10^{-5}$</td>
<td>Bell, 1978; Benz et al, 1986</td>
</tr>
<tr>
<td>Encouter Complex Radius $s_e$ (cm)</td>
<td>$4 \times 10^{-8}$ to $2 \times 10^{-7}$</td>
<td>$4 \times 10^{-8}$</td>
<td>Bell, 1978; Benz et al, 1986</td>
</tr>
<tr>
<td>Immobilized Ligand Concentration $C_L$ (mole/cm$^2$)</td>
<td>$10^{-14}$ to $10^{-11}$</td>
<td>$10^{-15}$</td>
<td>Rutishauser and Sachs, 1975a; This work</td>
</tr>
<tr>
<td>Bond Length $\gamma$ (cm)</td>
<td>$5 \times 10^{-8}$ to $5 \times 10^{-8}$</td>
<td>$5 \times 10^{-8}$</td>
<td>Bell, 1978</td>
</tr>
<tr>
<td>Fraction of Cell Surface in Contact Area $\beta$</td>
<td>$\frac{1}{4\pi}$ to $10^{-4}$</td>
<td>$0.1$</td>
<td>Hammer and Lauffenburger, 1989</td>
</tr>
<tr>
<td>Fluid Velocity $v$ (cm/sec)</td>
<td>0 to 0.15</td>
<td>0 to 0.2</td>
<td>Hertz et al, 1985; This work</td>
</tr>
</tbody>
</table>
5.5.1 Dynamic Model

To study cell release using the kinetic models, cells are assumed to have been associated with the support for a sufficient period that the distribution of cells over the adhesion states has reached a steady state. During this period, the cells do not experience hydrodynamic stresses and there is no soluble ligand present. This initial condition is estimated using the steady state form of the cell balance (Eqn. 21). The distribution is a function of the number of receptors in the contact area and the immobilized ligand concentration. With other variables held constant, an increase in the total receptor number in the contact area causes the mean number of immobilized ligand-receptor bonds to increase (Figure 5-3) until the immobilized ligand becomes limiting. The distribution of cells through the states is not symmetric. The asymmetry arises as receptor number becomes limiting.

![Figure 5-3. Initial Distribution Versus Receptor Number.](image)

Steady state distribution of cells through adhesion states as a function of receptor number in contact area. There is no soluble ligand present. The parameters are:

\[ D_a = 10^{-10} \text{ cm}^3/\text{sec}, \quad k_1 = 1.28 \times 10^7/\text{sec}, \quad k_2 = 2.74 \times 10^3 \text{ sec}^{-1}, \quad D = 5 \times 10^{-6} \text{ cm}^2/\text{sec}, \quad \dot{k}_1 = 5.06 \times 10^4 \text{ sec}^{-1} \]

\[ \dot{k}_2 = 1.64 \times 10^4 \text{ sec}^{-1}, \quad k_r = 0, \quad a = 10^{-4} \text{ cm}, \quad s_c = 4 \times 10^{-6} \text{ cm}, \quad C_L = 10^{-13} \text{ mole/cm}^2 \]

\[ C = 0, \quad \gamma = 5 \times 10^{-8} \text{ cm}, \quad \nu = 0 \text{ cm/sec}, \quad T = 310 \text{ K} \]
For a given receptor number in the contact area, the distribution of cells over the adhesion states also varies with the surface concentration of immobilized ligand (Figure 5-4). At high ligand concentration, the distribution is skewed as receptors become limiting. An immobilized ligand concentration of $10^{12}$ mole/cm$^2$ results in cells residing in the states with almost all receptors bound to ligands. As the surface concentration of ligand decreases, the cells shift to states that have fewer receptors bound to immobilized ligand. At a surface concentration of $10^{15}$ mole/cm$^2$, immobilized ligand is limiting and almost all the cells reside in a state with from zero to two receptors bound.

![Figure 5-4. Initial Distribution Versus Immobilized Ligand.](image)

Steady state distribution of cells through adhesion states as a function of immobilized ligand concentration. There is no soluble ligand. The parameters are:

- $n = 20$, $C_L = 10^{-15}$, $10^{-14}$, $10^{-13}$ and $10^{-12}$ mole/cm$^2$
- Other parameters same as in Figure 5-3.

Cells would be more quickly removed from a surface as the distribution is skewed toward adhesion states with fewer immobilized ligand bonds. The two parameters, receptor number and ligand density, that greatly alter the steady state distribution in the adhesion states, also are important in determining the initial cell adhesion (Edelman and Rutishauser, 1974; Weigel et al., 1979). It is questionable whether low receptor number
or immobilized ligand densities would permit cell adhesion. With immobilized ligand densities that favor adhesion, the cells are probably distributed such that few cells lie in states with low number of immobilized bonds. In order to elute such cells, it is necessary to alter the conditions so that a shift towards the distribution to states with few immobilized ligand bonds occurs.

As stated earlier, one method of shifting the distribution toward states with fewer immobilized ligand bonds is to increase the rate at which these bonds are broken. A widely used approach is to increase the hydrodynamic forces on the cells. As the hydrodynamic forces on the cell become larger, the intrinsic reaction rate constant for breaking immobilized ligand-receptor bonds for any adhesion state will increase (Eqn. 12). The steady state cell distribution, in turn, shifts toward states with fewer immobilized ligand-receptor bonds (Figure 5-5). At the lower fluid velocities, the forces on the cells are not sufficient to drastically change the steady state distribution of cells from that observed for unstressed cells. This indicates that the magnitude of the hydrodynamic forces imposed on cells alters their release.

![Figure 5-5. Initial Distribution Versus Fluid Velocity.](image)

Steady state distribution of cells through adhesion states as a function of fluid velocity past adhering cells. There is no soluble ligand. The parameters are:

\( n = 10, \ v = 0.01, 0.12, 0.16 \) and \( 0.2 \text{ cm/sec} \)

Other parameters same as in Figure 5-3.
The second approach to shift the cell distribution over adhesion states toward fewer immobilized ligand bonds is to decrease the rate of bond formation. A widely applied method, is to reduce the number of receptors available for binding immobilized ligand. This is achieved by introducing a soluble ligand that competitively binds to the receptor, effectively decreasing the number of unoccupied receptors. As the number of receptors binding soluble ligand increases, the rate of immobilized ligand-receptor bond formation decreases (Eqn. 2a), shifting the cell distribution toward states with fewer immobilized ligand bonds (Figure 5-6). For example, with soluble ligand concentration of 0.2 M, the cell distribution moves such that a substantial number of the adhering cells occupy a state without any immobilized ligand-receptor bonds (Figure 5-6).

An alternate approach entails the use of a soluble receptor that binds to the immobilized ligand. This interaction alters the specific rate for immobilized ligand-receptor bond formation. The term for unbound immobilized ligand must be altered to account for binding of the soluble receptor. The specific rates for cells
moving through adhesion states due to the formation of immobilized ligand bonds are all reduced in the presence of the soluble compound. As the soluble receptor concentration increases, the cell distribution is expected to shift to adhesion states with fewer immobilized ligand bonds. In this work only the case where soluble ligand binds to the cell surface receptor is addressed. Consideration of soluble receptors would not add significantly to the description of cell release and is not addressed further.

The rate at which cells are removed from the surface is modeled as a first order process with respect to cells in the state with no immobilized ligand bonds. The specific rate of removal, $k_r$, is the ratio of a characteristic velocity and length for the system, considered here to be the fluid velocity near the surface and the cell radius. Cell release from a support is greatly influenced by the specific removal rates (Figure 5-7). In this work, removal rates of 10 to 100 s$^{-1}$ are used. These are obtained assuming a characteristic length of 1.0 $\mu$m, an approximate radius for a bacterial cell, and a characteristic velocity of 0.001 cm/s.

![Figure 5-7. Cell release Versus Specific Removal Rate.](image)

Release of adhering cells versus specific removal rate. Estimates obtained using the dynamic model (Eqn. 21). The parameters used are:

$n = 10$, $C = 0.002 \, M$, $v = 0.01 \, cm/sec$, $k_r = 10, 10^2, 10^3$ and $10^4 \, 1/sec$

Other parameters same as in Figure 5-3.
As expected from the behavior of the adhesion state distribution, the release of cells increases as the cells are subject to greater hydrodynamic forces (Figure 5-8). In these simulations, a constant specific release rate was used to uncouple this effect from that of stress on the immobilized ligand bonds arising from hydrodynamic forces (Eqn. 11). At the lower flow rates, cell release is not much different from that observed when bonds are unstressed. While these results suggest that larger hydrodynamic forces will increase cell release, there is a practical limit to the forces that can be placed on the adhering cells due to physical constraints such as pressure drop across a packed bed. In many cases, high flow rates are not practical since high fluid shears may damage the adhering cells.

![Graph](image)

**Figure 5-8. Cell Release Versus Fluid Velocity.**
Release of adhering cells versus fluid velocity imposed on adhering cells. Hydrodynamic force on cell is approximated by that in Stokes' drag (Eqn. 11). The parameters used are:

- $n = 10$, $C = 0.002 \, M$, $k_r = 100 \, 1/\text{sec}$, $v = 0, 0.01, 0.12, 0.16$ and $0.2 \, \text{cm/sec}$

Other parameters same as in Figure 5-3.

The addition of soluble ligand was also found to increase the release of cells (Figure 5-9). In a system with very low hydrodynamic forces on the cells, the release of adhering cells increases with the soluble ligand
concentration. This is the result of the soluble ligand binding to the receptor and hence, decreasing the rate at which immobilized ligand-receptor bonds form (Eqn. 9). The cells shift toward the states without immobilized ligand bonds, and become eligible for release.

![Graph showing cell release versus soluble ligand concentration.](image)

**Figure 5-9. Cell Release Versus Soluble Ligand.**
Release of adhering cells versus time as a function of soluble ligand concentration. The parameters used are:

- \( n = 10 \)
- \( v = 0.01 \text{ cm/sec} \)
- \( k_r = 100 \text{ l/sec} \)
- \( C = 2 \times 10^{-4}, 2 \times 10^{-3}, 2 \times 10^{-2} \) and \( 2 \times 10^{-1} \text{ M} \)

Other parameters same as in Figure 5-3.

At high surface concentration, immobilized ligand serves to decrease the total cell release (Figure 5-10). The cells are expected to be residing in adhesion states with relatively large number of immobilized ligand bonds. At low surface concentrations, a region is reached were the immobilized ligand is limiting and many of the adhering cells are in states with low number of immobilized ligand-receptor bonds.

The release of cells from the support is also a function of the total number of receptors in the contact area. Under the situation where receptor number is limiting, the greater the number of receptors available, the more immobilized ligand bonds will be formed (Figure 5-3). For any adhesion state, \( i,j \), the greater the total receptor number, the higher the specific rate \( r_{ij}^* \). As a result, an increase in receptor number in the contact area yields a decrease in the rate of cell release (Figure 5-11).
Figure 5-10. Cell Release Versus Immobilized Ligand.

Release of adhering cells versus immobilized ligand concentration. The parameters used are:

\[ n = 10, \quad v = 0.01 \text{ cm/sec}, \quad k_r = 100 \text{ 1/sec} \quad \text{and} \quad C = 0.02 \text{ M} \]

\[ C_L = 2 \times 10^{-4}, \quad 2 \times 10^{-3}, \quad 2 \times 10^{-2} \quad \text{and} \quad 2 \times 10^{-1} \text{ mole/cm}^2 \]

Other parameters same as in Figure 5-3.

Figure 5-11. Cell Release Versus Receptor Number.

Release of adhering cells versus the number of receptors in the contact area. Parameters used are:

\[ C = 0.02 \text{ M}, \quad v = 0.01 \text{ cm/sec}, \quad k_r = 100 \text{ 1/sec} \quad \text{and} \quad n = 3, 5, 7, 10 \quad \text{and} \quad 15 \]

Other parameters same as Figure 5-3.
The dependence of cell release on receptor number is quite dramatic. Cells with only slightly different number of receptors in the contact area can display very different levels of release at a given time. This behavior is illustrated in Figure 5-12. Cell removal is shown as a function of receptor number at a given time. There is a steep front separating the cells that are almost completely released and those cells, with only a few more receptors, that show very little release. This front moves with time but moves more slowly as time goes by.

\[ \text{Figure 5-12. Release of Cells with } n \text{ Receptors Versus Time.} \]

Release of adhering cells versus number of receptors in the contact area as a function of time. The parameters used are:

- \( n = 3 \) through 18,
- \( C = 0.02 \) \( M \),
- \( v = 0.01 \) \( cm/sec \) and \( k_r = 10^5 \) \( 1/sec \)
- Other parameters same as Figure 5-3.

5.5.2 Quasi-Steady State Model

The valid application of the quasi-steady state assumption is based on the relative magnitude of the specific cell release rate and the rate at which formation of the first immobilized ligand-receptor bond occurs. Estimates of cell release using the dynamic model (Eqn. 21) and the quasi-steady state model (Eqn. 39), for different
values of the specific rate of cell removal, $k_r$, are shown in Figure 5-13. The predictions using the two models agree fairly well at the lowest value for the specific release rate, but become quite different as the specific release rate becomes larger.

The differences in predicted cell release can be explained by considering the relative magnitude of the specific rates for cell removal and formation of the first immobilized ligand bonds. The specific bond formation rate for a cell with the lowest number of free receptors is the most convenient rate to use for this comparison, since it has the smallest value of all the $r^{*}_{ij}$. For the case where soluble ligand is present, this is the rate corresponding to the formation of the bond between the immobilized ligand and a cell that has all the receptors but one filled with soluble ligand, $r^{*}_{i,n-1}$. Using Equation 9, and the parameter values for the simulation in Figure 5-13, $r^{*}_{i,n-1}$ is calculated to be 110 1/sec. The quasi-steady state assumption is not valid for conditions that yield a specific removal rate that is not much less than this value.

![Dynamic and Quasi-Steady State Model](image)

**Figure 5-13. Quasi-Steady State and Dynamic Model.**

Release of adhering cells versus specific removal rate using quasi-steady state and dynamic model. The parameters used are:

- $n = 10$, $C = 0.002M$, $v = 0.01cm/sec$, $\hat{K}_{rn} = 3.08 \times 10^2$, $K'_{pm} = 5.14 \times 10^{-2}$ M
- $K_{rn} = 4.67 \times 10^4$, $K_{pm} = 3.02 \times 10^8cm^2/mole \land k_r = 10$, $10^3$, $10^4$ 1/sec
- Other parameters same as in Figure 5-3.
When \( k_r \gg r_{i,a} \), the removal of cells from the support becomes independent of the specific removal rate and it is limited by the rate at which cells shift through the adhesion states to states without immobilized ligand bonds. Once cells reach this state, they are removed before they can reform a bond with an immobilized ligand. The quasi-steady state assumption does not account for this limiting process. It assumes that immediately after a cell is removed from the support, the remaining cells instantaneously obtain the new steady state distribution. When the specific removal rate is significantly less than the rates at which cells leaves the states \( N_{0,i} \) through formation of the first bond with immobilized ligand, the overall cell release rate is controlled by the specific removal rate and the cell release predicted by the quasi steady state models and the complete model.

### 5.5.3 Cell Separation

The elution curves (Figure 5-12) indicate that cells with only a slight difference in receptor expression can exhibit quite different release properties. This suggests that selective release of specifically adhering cells may be used in cell separation. Selective fractionation of cell populations should be possible based on the expression of receptors in the contact area. To illustrate the use of selective release as a cell separation tool, conditions are chosen such that Equation 39 gives a valid description of cell release. The immobilized ligand are in excess, fluid forces have a negligible effect on the lifetime of the immobilized ligand-receptor bond and the quasi-steady state assumption is valid.

The time required to release a given fraction of a cell population with \( n \) receptors in the contact area is obtained from Equation 39:

\[
\xi^n \frac{k_r}{K_r} \ln \left( 1 - \frac{N_f}{N_r} \right)
\]

where:

\[
\xi = 1 + \frac{K_{qn}K_{qn}C_L}{1 + \hat{K}_{qn}K_{qn}C}
\]

The time required to release a given fraction of the adhering cell is proportional to the quantity \( \xi \) raised to the \( n \)th power, \( n \) being the number of receptors in the contact area. For a constant immobilized ligand concentration, the maximum value of \( \xi \), \( 1 + K_{qn}K_{qn}C_L \), occurs when there is no soluble ligand present. The value of \( \xi \) will decrease as \( \hat{K}_{qn}K_{qn}C \) increases, until a minimum value of unity is reached. At this condition, all cell populations follow the same release behavior, independent of the receptor number in the contact area.

Consider two populations of cells adhering to the same surface with respectively \( n_1 \) and \( n_2 \) receptors in the contact area. From Equation 42a, the ratio of the time to release half of the two populations is found to be:
This simple expression illustrates two points. First, for any given condition, the ratio of the release time for two populations is dependent on the difference in the number of receptors in the contact area. Second, the ratio of release times for two populations increases as the quantity $\xi$ increases. Better separation of two populations would be expected from increasing the immobilized ligand concentration or using a lower concentration of soluble competing ligand to release the cells.

The time to release a desired fraction of a population will decrease as the quantity $K_{\text{ran}} \cdot K_{\text{com}} \cdot C$ increases.

The effect of the soluble ligand concentration on the separation of cell types can therefore best be compared at the same relative time. Here, comparison is made using the time for the release of half of a population with ten receptors. In Figure 5-14, the fraction of a cell population released at this time by different concentrations of the soluble ligand are compared. Notice the shape of the release curves. As the soluble ligand concentration decreases the curves become sharper, yielding improved separation of cell populations with different receptor numbers. The half times for release, however, become longer. Thus, a balance must be obtained between purity and the time to obtain a desired yield in a process using cell release.

Similar behavior is observed if the number of receptors is in excess. Using Equation 41, the release time for a desired fraction of adhering cells is:

$$\frac{t^{1/2}}{t^{1/2}} = \xi^{n_2-n_1}$$
Figure 5-14. Cell Release for Excess Immobilized Ligand.

Release of adhering cell populations versus number of receptors in the contact area as a function of the soluble ligand concentration. The immobilized ligand is in excess. Profiles determined at the time which half of the adhering population with 10 receptors is released. The parameters used are:

\( n = 3 \) through \( 20 \), \( K_{rn} = 4.67 \times 10^5 \), \( K_{spr} = 3.02 \times 10^8 \text{cm}^2\text{mole} \) and \( k_r = 10 \ \text{sec}^{-1} \), \( v = 0.01 \ \text{cm/sec} \)
\( \dot{K}_{rn} = 3.14 \times 10^5 \), \( K_{spr} = 5.14 \times 10^{-21} \text{mole/cm}^2 \), \( C_L = 5 \times 10^{-13} \text{mole/cm}^2 \) and \( C = 0.01, 0.05, 0.1, 0.5, \) and \( 1.0 \) M

Other parameters same as in Figure 5-3.

\[
t = \frac{\alpha}{k_r} \ln \left( 1 - \frac{N_t}{N_f} \right) \quad (44a)
\]

where

\[
\alpha = 1 + \frac{K_{rn}K_{spr}C_s}{1 + \dot{K}_{rn}K_{spr}C} \quad (44b)
\]

The release time is dependent on receptor density, raised to the power, 1, the number of ligands in the contact area. The maximum time to release a desired fraction of adhering cells occurs when there is no soluble ligand present and the term \( \alpha \) has a value of \( 1 + K_{rn}K_{spr}C_n \). The minimum time is obtained as \( \alpha \) approaches one, at high values of \( \dot{K}_{rn}K_{spr}C \). At this extreme, the release time is independent of receptor or immobilized ligand density.
The ratio of the half release times for two populations with different numbers of receptors in the contact area is:

\[
\frac{t_{1/2}^{n_2}}{t_{1/2}^{n_1}} = \left( \frac{1 + \kappa_{\text{ran}} K_{\text{apn}} C + K_{\text{ran}} K_{\text{apn}} C_{n_2}}{1 + \kappa_{\text{ran}} K_{\text{apn}} C + K_{\text{ran}} K_{\text{apn}} C_{n_1}} \right)^i
\]

(45)

As the quantities, \(K_{\text{ran}} K_{\text{apn}} C_{n_1}\) and \(K_{\text{ran}} K_{\text{apn}} C_{n_2}\) become much greater than \(K_{\text{ran}} K_{\text{apn}} C\) and unity, the ratio of the half times for release approaches:

\[
\frac{t_{1/2}^{n_2}}{t_{1/2}^{n_1}} = \left( \frac{C_{n_2}}{C_{n_1}} \right)^i
\]

(46)

and the time for the release of the populations (Eqn. 44a) nears the maximum. The lower limit on the ratio of the half release times occurs when the quantity \(\kappa_{\text{ran}} K_{\text{apn}} C\) is large compared to \(K_{\text{ran}} K_{\text{apn}} C_{n_1}\) and \(K_{\text{ran}} K_{\text{apn}} C_{n_2}\). In this case the ratio of the half times for release approaches unity.

At constant relative time, the half time for release of a population with 1000 receptors in the contact area, a decrease in the soluble ligand concentration significantly sharpens the curve for cell release (Figure 5-15). Decreasing the soluble ligand concentration, results in much longer times to obtain a desired release of a population.

The purpose of cell separation through the release of specifically adhering cells is to obtain a cell mixture that is enriched with respect to a particular population. Among cells that carry the same receptor, this would be possible if the populations express the receptor at slightly different levels. Within a population, there will be cells with varying levels of receptor expression distributed about the mean level of expression (Titus giai, 1984). Subpopulations of cells that express a certain range of receptors can be enriched with respect to the rest of the population or one population separated from another that has a different mean expression of receptors.

The separation may also be based on the binding characteristics of the receptor. For either ligand or receptor limitation, release times are dependent on the equilibrium constants for receptor binding to immobilized or soluble ligand (Eqn. 39 and 41). Cell populations that express the receptor at the same level but possesses different affinity for the ligands can display differential released from the support.

To illustrate the behavior of cell separation via the release of specifically adhering cells, two populations of cells are considered. The two populations express the same receptors, but, at different levels. The distribution of receptors in the contact area as modeled by a log-normal distribution is shown in Figure 5-16. Initially there are equal amount of both populations adhering to the surface.
Figure 5-15. Population Release for Excess Receptors.
Release of adhering cells versus number of receptors in the contact area as a function of the soluble ligand concentration. Profiles determined at the same relative time, the time for release of half of the adhering population with 1000 receptors in the contact area. Estimates obtained using the quasi-steady state model, receptors in excess. The parameters used are:

\[ n = 520 \text{ through } 2000, \quad C_L = 10^{14}\text{cm}^2\text{mole} \]

Other parameters same as in Figure 5-3.

The goal of the separation is to obtain a cell mixture that is either enriched or depleted of a target population. Enrichment of population 1 (Figure 5-16) will occur through preferential release of the population. To obtain a mixture of cells that is enriched with respect to population 2, population 1, with a lower number of receptors, is preferentially eluted. When the adhering cells contain the desired fraction of population 2, they are eluted and collected.

To carry out the cell separation different release strategies can be employed. All rely on manipulating the soluble ligand concentration to obtain the desired yields and purities. In this case, release using a constant concentration of soluble ligand is considered. Other approaches such as step changes in soluble ligand concentration or gradient elution may also be used, however, they add little to the illustration of cell separation and are not discussed further.
Figure 5-16. Distribution of Receptors among Populations.

Distribution of receptors in contact area for two populations to be separated. Both distributions are estimated to be log-normal distributions. The fraction of the population with \( k \) receptors in the contact area is:

\[
N_k = \frac{1}{(k - \theta)\sigma\sqrt{2\pi}} \exp\left(-\frac{1}{2}\left(\frac{\ln(k - \theta) - \mu}{\sigma}\right)^2\right)
\]

The distributions are defined by:

- Population 1: \( \theta = 5.0 \), \( \sigma = 0.3 \) and \( \mu = 3.5 \)
- Population 2: \( \theta = 15.0 \), \( \sigma = 0.26 \) and \( \mu = 3.9 \)

The mean number of receptors in population 1 is 40 and the mean for population 2 is 66.

Conditions for this illustration were chosen so that the use of quasi-steady state model with excess immobilized ligand is valid (Eqn. 39). The elution of each population with time is a function of the soluble ligand concentration (Figure 5-17 and 5-18). Population 1 is more readily removed from the support at all levels of soluble ligand used. At short times, the fraction of population 1 in the released mixture is quite high (Figure 5-17). The use of low concentrations of soluble ligand results in a released cell population that is highly pure with respect to population 1 but gives low yields. The purity of the released cell mixture decreases over time when higher soluble ligand concentrations are used but a greater fraction of population 1 is released.
Figure 5-17. Selective Release of Population 1.

The selective release of population 1 using different soluble ligand concentrations. A) Fraction of population 1 released versus time as a function of soluble ligand concentration. B) Purity of released cells, the fraction of released cells that are population 1. Initial distribution of adhering populations is shown in Figure 5-16. The parameters used are:

\[ C = 0.10, \ 0.15, \ 0.20, \ 0.25, \text{ and } 0.30 \ M \]

Other parameters same as in Figure 5-13.

The effect of the different soluble ligand concentration on the purity of the remaining cells is depicted in Figure 5-18. Using low soluble ligand concentration has little effect on the population balance of the adhering cells. Higher concentrations release more cells of both populations, however, due to the preferential release of population 1, the purity of the adhering cells increases rapidly with time (Figure 5-18).
Figure 5-18. Preferential Retention of Population 2.

The preferential retention of population 2 at different soluble ligand concentrations due to higher receptor number. A) Fraction of population 2 released from the support versus time as a function of soluble ligand concentration. B) Purity of adhering cells, the fraction of adhering cells that are population 2. Initial distribution of adhering populations is shown in Figure 5-16. The parameters used are; 

\[ C = 0.10, \ 0.15, \ 0.20, \ 0.25, \text{ and } 0.30 \ M \]

Other parameters same as in Figure 5-13.

Figures 5-19 and 5-20, illustrate the distribution of released cells among the two populations at specific times. The distribution of each population is normalized with respect to the total amount the population initially adhering to the support. The distributions are displayed against the initial distribution of the adhering populations. For comparison, two soluble ligand concentrations are presented.
The cells released after ten seconds, (Figure 5-19 and 5-20) are almost entirely those belonging to population 1, the population with the lowest expression of receptors in the contact area. As time increases, more of the cells with a greater number of receptors in population 1 are released. Also, cells from the lower end of the population 2 start to be released. This corresponds to the drop in the fraction of population 1 in the released cell mixture. For the case where soluble ligand at a concentration of 0.2 M is used (Figure 5-19), between the time 500 to 1000 seconds, most of population 1 has been released. The number released does not significantly change with the additional time. During this period, there has occurred a noticeable release of population 2 from the support. The majority of the cells of population 2 released, are from the lower part of the distribution, the end that overlaps the distribution of population 1.
Similar behavior is observed at a soluble ligand concentration of 0.3 M (Figure 5-20). At ten seconds, a larger fraction of population 1 is eluted. At a hundred seconds, most of population 1 has been released from the support. However, a large fraction of population 2 has also been released. As time reaches 1000 seconds, it is noticed, that only a small fraction of population 1 remains adhering to the support (Figure 5-20). All of the cells of population 2 that lay in the segment of the distribution that overlapped population 1 have also been released. At this point, the cells that are adhering to the support are almost totally from population 2 and consist of cells that lie in the region of the distribution with a large number of receptors in the contact area.

### 5.6 Experimental Results

*Escherichia coli*, adhering to starch-Sepharose in a packed bed, was used to investigate the release of specifically adhering cells. The cell adhesion is mediated by the interaction between the maltoporin, the *lamB*
gene product, on the cell surface and the immobilized starch (Ferenci and Lee, 1982). A range of different maltooligosaccharides, with varying equilibrium constants, are available for use as a soluble ligand to release the cells from the support (Ferenci et al., 1986; Benz et al., 1987). Experimental procedures used are similar to those described by Roos and Hjortso (Roos and Hjortso, 1989).

Release of the adhering population of *E. coli* was carried out using two separate ligands, maltose and maltotriose. Both ligands bind to the maltoporin as a step in their transport across the outer membrane. The equilibrium constant for maltose and maltotriose is $100 \text{ M}^{-1}$ and $2500 \text{ M}^{-1}$, respectively (Benz et al., 1987). As expected, because of its higher equilibrium constant, less maltotriose than maltose is required to elute the same fraction of adhering cells under the same conditions (Figure 5-21).

![Figure 5-21. Elution of Specifically Adhering Cells.](image)

**Figure 5-21. Elution of Specifically Adhering Cells.**

Elution of specifically adhering *E. coli* from starch-Sepharose support versus soluble ligand concentration. Maltose used as soluble ligand at volumetric flow rates of 0.5 and 0.75 ml/min. Maltotriose used as soluble ligand at a flow rate of 0.75 ml/min. Included are predictions of release obtained using Equation 39.

As predicted by cell release models, increasing soluble ligand concentration released a greater fraction of adhering cells in a given time. For each soluble ligand, there is a critical concentration above which all cells,
within limits of measurement, are eluted from the column during a finite period. Below this concentration, only a fraction of the cells are released during the release period. As the soluble ligand concentration decreases, released cells decrease until none are detectable.

For release using maltose, two flow rates through the column were studied. At the lower flow rate, 0.5 ml/min, a higher concentration of maltose was generally required to release the same fraction of adhering cells than was the case for a flow rate of 0.75 ml/min. This indicates that the flow rate through the column gives a detectable change in the release of a cell population.

For this system, the immobilized ligand, appears to be limiting. E. coli, induced with maltose, has been reported to possess on the order of 3x10^4 to 10^6 copies of the maltoporin (Braun and Krieger-Brauer, 1977; Ferenci, 1980). If the characteristic radius of E. coli is 0.5 μm, the surface density of maltoporin is approximately 1 - 3.2x10^12 porins/cm^2. To determine the surface concentration of starch, the starch was assumed to be equally distributed throughout the accessible portion of the support. The mean molecular weight of the starch was estimated to be about 5x10^5, by size exclusion chromatography. For a branched starch molecule with a length of 1-10 η, a surface concentration of between 7x10^9 to 7x10^10 molecules/cm^2 was estimated to be available for binding to the maltoporin.

The excess of receptors available suggests that cell release from the starch-Sepharose beads could be described using Equation 41, if the effects of stress on bond breakage are ignored. The applicability of the model was tested, assuming 6.5x10^4 maltoporin are expressed by the adhering cells. The equilibrium constant, K_\text{p,m}K_\text{m,m} for immobilized starch binding to the maltoporin was estimated to be 1.67x10^12 cm^2/mole from binding data for soluble amylopectin (Ferenci et al, 1980) and the expressions for rates of encounter complex formation (Eqns. 5, 8, 16 and 18). The radius of the encounter complex was assumed to equal the radius of the maltoporin pore, 0.4 ηm (Benz et al, 1986).

The release rate constant k, was estimated using the cell radius, 5x10^{-5} cm and the interstitial fluid velocity through the packed bed. Taking t as the time over which the release of cells from the column was monitored, 7 minutes, the product k,t was estimated to be 3.3x10^5 1/sec at a flow rate of 0.75 ml/min. The contact area was assumed to be 1% of the total surface area of the E. coli. The mean of the estimated surface concentration of immobilized ligand was used to yield an estimate of 12 ligands in the contact area and Equation 41 was used to determine the fraction of cells released as a function of the soluble ligand concentration (Figure 5-21).
The fluid velocity has a great effect on the amount of maltose required to release cells from the column. The model does not quantitatively predict the experimental behavior. This may be because fluid velocity is reflected only in the parameter, $k_t$. In general, the model predictions agree surprisingly well with the experimental results considering the simplifications and the uncertainty in parameter estimation.

It should be noted that the model is applicable only to perfectly mixed conditions. These conditions apply only to small slices of the column behind the soluble ligand front. The requirement for a mixed environment is particularly important when considering the assumption that released cells do not reaggregate. After cell release in a bed packed with a porous support, the cell, excluded from the intraparticle volume, moves ahead of the soluble ligand front. The soluble ligand concentration around the cell is low and reversibly bound soluble ligand would start to release. Also, for this experimental system, maltose and maltotriose are transported into the E. coli. As a result, maltoporins become unoccupied and the cell has a greater chance to reaggregate as they move through the bed. Adhesion of the cells to a support is a function of both available receptors and fluid velocity past the support (Hertz et al., 1985; Hammer and Lauffenburger, 1987; Roos and Hjortso, 1989b).

The cell peaks eluted from the bed by various concentrations of maltotriose and the final elution with 0.2 M maltose are shown in Figure 5-22. The area under the peak for cell elution using 0.2 M maltose becomes smaller as the concentration of the preceding maltotriose wash is increased. The shape and width of the peaks, however, changes very little. The shapes of the cell peaks eluted with the different maltotriose concentration change dramatically as the maltotriose concentration decreases. The peak maxima are eluted at longer times, although the beginning of the peak is detected at the same time.
Figure 5-22. Shape of Eluted Cell Peaks.

Release of *E. coli* from a packed bed of starch-Sepharose with fixed concentration of maltotriose followed by release with 0.2 M Maltose. Maltotriose concentration used in the first elution step are A) 0.00075 M; B) 0.001 M; C) 0.0015 M; D) 0.004 M. Absorbance of bed eluant was monitored continuously at 650 nm as an estimate of biomass released.

5.7 Conclusions

A general kinetic model that describes the release of specifically adhering cell was developed. This model accounts for the properties of individual cell populations such as receptor density, their mobility in the membrane and affinity for various ligands. The density of immobilized ligands and the concentration of soluble ligand that bind to the receptor are included in this formulation. The role of the hydrodynamic environment is addressed using a simplified view of the effect of fluid drag on the average lifetime of the immobilized ligand-receptor bond.

Various limiting cases of the general model were discussed. Cases in which the distribution of cells over the adhesion states was assumed at quasi-steady state greatly reduced the complexity of the release model. Under this assumption, an analytical solution was obtained. This solution, while limited by the restrictions behind its formulation, yields a simplified expression with which the applications of selective cell release was readily explored.
The release of specifically adhering cells in response to changes in the hydrodynamic environment and the presence of soluble ligand which competitively binds to the receptor were studied using these models. It was found that modest hydrodynamic forces alone have a limited effect on the release of specifically adhering cells. The presence of soluble ligand can greatly increase the rate at which specifically adhering cells are released. These observations agree with the experimental results for several systems (Hertz et al., 1985; Clune et al., 1984).

Selective release of adhering cells as a means of separating populations was investigated. Soluble ligand was used to release cells from the support based on the receptor density in the contact area. Cells which displayed a difference of only a few receptors could display dramatically different release properties. It was shown that populations that exhibit only slightly different levels of receptor expression could be fractionated.

The general behavior of the release of cells adhering in a specific manner to support in a packed column was predicted by the kinetic release models. There was a lower limit of soluble ligand required to release a fraction of the population in a finite time. This concentration was dependent on the flow rate through the bed and the equilibrium constants for soluble ligand-receptor binding. The fraction of adhering population that was released from the column was found to lie within the range predicted by the kinetic model under conditions of immobilized ligand limitation and the quasi-steady state assumption for the adhesion state distribution.

The cell release models predict several characteristics of release behavior that were tested experimentally. The models predict that at any given time the number of cells released from a support increases with the concentration of soluble competing ligand. The soluble ligand concentration required to release a given fraction of cells is a function of the ligand binding affinity. The fluid velocity is also expected to alter the release characteristics of the cells. As the time after introduction of the soluble ligand becomes large, the release rate of cells drops off dramatically and at long time periods the number of cells released becomes very small. All of these characteristics were observed in the packed bed experiments using E. coli.

There are several other methods of releasing adhering cells that were not dealt with. Any change in operating condition that either increases the specific rate at which cells move through adhesion state by breaking immobilized ligand bonds or decreases the specific rate for changing states through formation of these bonds can be used to obtain selective release of cells. A soluble compound that competitively binds to the immobilized ligand would have a similar effect on release as obtained using a soluble ligand. The intrinsic rate constant for reversible bond formation could be altered through changes in temperature or pH. Any one of these approaches alone or combined could prove sufficient to obtain selective release of cell populations.
The separation of specifically adhering cells based on selective release could prove to be a valuable method for the enrichment of a desired population. The separation of the populations would be based on receptor expression or ligand-receptor affinity. The proposed model suggests that a large difference in release time can be obtained for cells that differ only slightly in the expression of the surface receptor. Manipulation of soluble ligand and immobilized ligand concentrations and other parameters allows precise discrimination over release of the cell populations.
### 5.8 Nomenclature

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>contact area (area)</td>
</tr>
<tr>
<td>a</td>
<td>cell radius (length)</td>
</tr>
<tr>
<td>C</td>
<td>soluble ligand concentration (amount/volume)</td>
</tr>
<tr>
<td>C_I</td>
<td>immobilized ligand density (amount/area)</td>
</tr>
<tr>
<td>C_s</td>
<td>surface concentration of receptors in the contact area (amount/area)</td>
</tr>
<tr>
<td>d_e</td>
<td>rate constant for formation of immobilized ligand-receptor encounter complex (area/(amount time))</td>
</tr>
<tr>
<td>d_b</td>
<td>rate constant for breakup of immobilized ligand-receptor encounter complex (1/time)</td>
</tr>
<tr>
<td>d_i,j</td>
<td>rate constant for formation of j\textsuperscript{th} soluble ligand-receptor encounter complex on cell in state N_{i,j} (volume/(amount time))</td>
</tr>
<tr>
<td>d_i,j</td>
<td>rate constant for breakup of j\textsuperscript{th} soluble ligand-receptor encounter complex on cell in state N_{i,j} (1/time)</td>
</tr>
<tr>
<td>D</td>
<td>soluble ligand diffusion coefficient (area/time)</td>
</tr>
<tr>
<td>D_m</td>
<td>receptor membrane diffusion coefficient (area/time)</td>
</tr>
<tr>
<td>f_0</td>
<td>fraction of adhering cells without any immobilized ligand-receptor bonds</td>
</tr>
<tr>
<td>F_b</td>
<td>force per immobilized ligand bond</td>
</tr>
<tr>
<td>F_t</td>
<td>total hydrodynamic force exerted on cell</td>
</tr>
<tr>
<td>j_e.c.</td>
<td>number of soluble ligand-receptor encounter complexes in the contact area</td>
</tr>
<tr>
<td>j^+</td>
<td>net flux of receptors into encounter complex with immobilized ligand (amount/time)</td>
</tr>
<tr>
<td>j^+e.c.</td>
<td>net flux of soluble ligand into encounter complex with receptors in contact area (amount/time)</td>
</tr>
<tr>
<td>j^+w.c.</td>
<td>net flux of soluble ligand into encounter complex with receptors on whole cell (amount/time)</td>
</tr>
<tr>
<td>j^-</td>
<td>net flux of receptor out of encounter complex with immobilized ligand (1/time)</td>
</tr>
<tr>
<td>j^-e.c.</td>
<td>net flux of soluble ligand out of encounter complex with receptor in contact area (1/time)</td>
</tr>
<tr>
<td>j^-w.c.</td>
<td>net flux of soluble ligand out of encounter complex with receptor on whole cell (1/time)</td>
</tr>
<tr>
<td>k_b</td>
<td>Boltzmann constant</td>
</tr>
<tr>
<td>k_r</td>
<td>specific rate of cell release (1/sec)</td>
</tr>
<tr>
<td>k^+</td>
<td>apparent rate constant for immobilized ligand-receptor bond formation (area/time)</td>
</tr>
<tr>
<td>k^-</td>
<td>apparent rate constant for immobilized ligand-receptor bond breakup (1/time)</td>
</tr>
</tbody>
</table>
$k_1$ intrinsic reaction rate constant for formation of immobilized ligand-receptor bond (1/time)

$k_2$ intrinsic reaction rate constant for breakup of immobilized ligand-receptor bond (1/time)

$k^e$ intrinsic reaction rate constant for breakup of unstressed immobilized ligand-receptor bond (1/time)

$k_i$ intrinsic reaction rate constant for formation of soluble ligand-receptor bond (1/time)

$k_i$ intrinsic reaction rate constant for breakup of soluble ligand-receptor bond (1/time)

$K_{ran}$ equilibrium constant for immobilized ligand-receptor bond formation

$K_{sprot}$ equilibrium constant for immobilized ligand-receptor encounter complex formation (area/amount)

$\hat{K}_{ran}$ equilibrium constant for soluble ligand-receptor bond formation

$\hat{K}_{sprot}$ equilibrium constant for soluble ligand-receptor encounter complex formation for the contact area (volume/amount)

$\hat{K}_{i,j}$ ratio of the specific rate at which a cell enters state $N_{i,j}$ through formation of a soluble ligand-receptor bond and the rate it leaves the state through breaking a soluble-ligand receptor bond

$K'_{sprot}$ equilibrium constant for soluble ligand-receptor encounter complex formation for a receptor (volume/amount)

1 number of immobilize ligands in the contact area

L ligand

L-R ligand-receptor encounter complex

LR ligand binding to receptor

n total number of receptors in contact area

N number of unoccupied receptors on a cell if surface density of the cell is the same as in the contact area

$N_T$ total number of cells initially adhering to support

$N_f$ number of free cells

$N_{i,j}$ number of cells in an adhesion state with $i$ immobilized ligands and $j$ soluble ligands bound to receptors in the contact area

$N_T^n$ total number of cells in an adhesion state with $i$ immobilized ligand-receptor bonds

P probability that a ligand at the cell surface will diffuse away from the cell before diffusing into an encounter complex

$r^+_{i,j}$ specific rate at which a cell enters state $N_{i,j}$ by the formation of an immobilized ligand-receptor bond (1/time)

$r^-_{i,j}$ specific rate at which a cell leaves state $N_{i,j}$ by breaking an immobilized ligand-receptor bond (1/time)

$\hat{r}^+_{i,j}$ specific rate at which a cell enters state $N_{i,j}$ by the formation of a soluble ligand-receptor bond (1/time)

$\hat{r}^-_{i,j}$ specific rate at which a cell leaves state $N_{i,j}$ by breaking a soluble ligand-receptor bond (1/time)
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
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<tbody>
<tr>
<td>R</td>
<td>receptor</td>
</tr>
<tr>
<td>$s_e$</td>
<td>encounter complex radius (length)</td>
</tr>
<tr>
<td>$s_l$</td>
<td>mean separation radius between unbound immobilized ligand (length)</td>
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<tr>
<td>$t$</td>
<td>time</td>
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<tr>
<td>$t_e$</td>
<td>mean encounter time between immobilized ligand and receptor (time)</td>
</tr>
<tr>
<td>$t_r$</td>
<td>mean time for receptor to leave encounter complex (time)</td>
</tr>
<tr>
<td>$T$</td>
<td>Temperature</td>
</tr>
<tr>
<td>$v$</td>
<td>characteristic fluid velocity near surface (length/time)</td>
</tr>
<tr>
<td>$\alpha$</td>
<td>separation factor for case of excess receptors in the contact area</td>
</tr>
<tr>
<td>$\beta$</td>
<td>fraction of surface of adhering cell that lies in contact area</td>
</tr>
<tr>
<td>$\gamma$</td>
<td>characteristic bond length (length)</td>
</tr>
<tr>
<td>$\eta$</td>
<td>fluid viscosity</td>
</tr>
<tr>
<td>$\xi$</td>
<td>separation factor for case of excess immobilized ligand in the contact area</td>
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5.9 References


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CHAPTER 6

DETERMINATION OF POPULATION BALANCES
IN A MIXED CULTURE BY SPECIFIC CELL ADHESION

by
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6.1 Summary

Specific cell adhesion can be used to monitor the population balance in a mixed culture. A mixture of two Escherichia coli strains were separated and monitored based on their different expression of the LamB protein. The results are compared to those obtained by differential plate counts.

6.2 Introduction

Mixed cultures are used deliberately in processes such as waste treatment and energy or food production, or may arise as an undesirable consequence of plasmid loss in recombinant cultures. In either case, a quick, accurate method of elucidating the population balances is required before any real time control scheme of the culture can be implemented.

The method of plate counts can yield fairly accurate estimate of the fractions of different viable populations present in a culture if suitable selective conditions can be identified. Direct microscopic counting (Beaty et al.), electronic particle counting, with size discrimination (Davison et al.) or centrifugal separation (Beaty et al.) can be employed if the various populations display gross morphological differences. Flow cytometry has been used to determine the ratio of plasmid-bearing to plasmid-free cells in an unstable recombinant culture (Srienc et al.). Biochemical methods (Beaty et al.) and labeled, population specific probes, such as antibodies (Tlaskalova-Hogenova et al.), have also been applied to the problem of quantifying the cell populations of a mixed culture.

Cell affinity chromatography has recently received much attention as a tool for separating cell mixtures, and could possibly also be used to quantify subpopulations in a cell mixture. The method uses specific cell adhesion to separate populations with different adhesion properties. Differential adhesion may arise from gross differences in outer surface characteristic, such as hydrophobicity (van Loosdrecht et al.) or from the different levels of expression of outer surface components, such as carbohydrates (Hertz et al.), pili (van der Mei et al.) or receptors (Ferenci and Lee).
In this paper, it is shown, that specific cell adhesion can quantitatively separate two microbial populations and determine their absolute amounts in a mixed culture. A mixture of two *Escherichia coli* strains, one that expresses the *lamB* protein and one that does not, is separated on a starch-Sepharose support. The *lamB* protein, a transport protein for maltose and higher maltodextrins, binds to starch (Ferenci and Lee), thus immobilizing only the population that is *lamB*+. This population can then be quantified by eluting it with a solution of maltose, a competitive ligand to the *lamB* receptor.

6.3 Materials and Methods

6.3.1 Organisms, Media and Cultivation

The organisms used in these experiments were *Escherichia coli* MCR106, donated by T.J. Silhavy, Princeton University and *E. coli* K12 ATCC 23716. MCR106 is strain MC4100 (Benson and Silhavy) with the modification Δ*lamB*106. The strain 23716 was transformed with a stable plasmid coding for neomycin resistance, to allow for easy identification by plate counts. All organisms were maintained on L agar; tryptone, 10 g/l; yeast extract, 5 g/l; NaCl, 5 g/l; Bacto agar, 15 g/l. For the selective growth of the plasmid bearing strains, 50 mg/l of neomycin was added to the L agar before it solidified. Liquid cultures were grown in M63 media (Miller, 1972) which consisted of M63 salts, KH2PO4, 13.6 g/l; (NH4)2SO4, 2 g/l; FeSO4·7H2O, 0.5 mg/l; supplemented with MgSO4·7H2O, 1 mM; arginine, 20 mg/l; thiamine, 20 mg/l; and maltose, 1 g/l. The pH was adjusted to 7.0 with NaOH. All fermentations were performed at 37°C.

6.3.2 Cell Separation

The support used for specific cell adhesion was starch-Sepharose 6B, prepared by a modified method of Ferenci and Lee. A 1.0 x 20 cm chromatography column (Pharmacia LKB) fitted with a 20 um bed support, was packed with 1 ml settled volume of starch-Sepharose in M63 salts buffer, pH 7.0. The column was sterilized with 1% formaldehyde for 30 minutes. The optical density of the effluent from the column was monitored continuously at 650 nm. A 15 second pulse of cells was pumped into the column and washed through the packing with M63 salts buffer. The first peak to come off the column was composed of the non-adhering cells. After this peak had eluted, cells adhering to the packing were eluted with M63 salts buffer containing maltose, 0.2 M, and the bed washed with 10 to 15 volumes of M63 buffer at a flow rate of 1 ml/min. This procedure took approximately 25 to 30 minutes per sample.

The strain ATCC 23716, as received, did not exhibit a sufficiently high degree of binding to the starch-Sepharose support. An adhering subpopulation was isolated by first passing a pulse of cells through the column. The adhering cells were eluted with the M63 salts buffer, with 0.2 M maltose, collected and cultured in M63
containing maltose, 1 g/l. The process was repeated four times. The population of cells thus isolated, displayed a high degree of specific adhesion to the starch-Sepharose support. This culture was maintained on L agar and used as inoculum for further adhesion studies. The adhesion property of this population was assayed before any transfer or experimental work. The strain MCR106 did not adhere to the support.

The population balance in a mixed culture was determined by vortexing a sample, passing it through the chromatographic column and integrating the O.D. peaks of the exit stream. The first peak corresponds to the non-adhering strain, MCR106. The second peak, obtained by eluting the column with maltose buffer, corresponds to the adhering strain, 23716. The fraction of one of the subpopulations is found as the fraction of its peak area to the sum of the two peak areas. Population balances were also determined from replicate plating, making use of the plasmid coded neomycin resistance in strain 23716.

6.4 Results

Using pure cultures, a calibration curve relating area under an eluted peak with the optical density of the original sample was prepared. A linear response was observed throughout the range of biomass concentration encountered. The ability of strain 23716 to adhere to the starch support was determined at several points on the growth curve. After growth had started, the fraction of cells that adhered to the starch-Sepharose packing, using a flow rate of 0.5 ml/min, was found to be greater than 97% throughout the growth curve.

The ability of the column to quantitatively separate cell mixtures, was tested using defined mixtures of strains 23716 and MCR106. The two strains were grown separately in shake flasks and samples were taken in late exponential phase. The O.D. at 650 nm of each sample was determined and a mixture was prepared containing a known fraction of each strain. These mixtures were vortexed vigorously before a pulse was placed on the column. The flow rate through the column was set at either 0.5 or 0.75 ml/min.

The fraction of strain 23716, as determined by specific cell adhesion, versus the fraction determined from mixing of pure cultures is plotted in Figure 1. Excellent agreement is observed at a flow rate of 0.75 ml/min. At a flow rate of 0.5 ml/min, the data suggests a systematic deviation from a line passing through the origin.

We believe the deviation at the lower flow rate is caused by cell-cell interactions. Examination of the two cultures, by light microscopy, revealed the presence of cell aggregates in the MCR106 culture. The strain 23716 existed as cell singlets or doublets. In the column, interactions between the adhering and the non-adhering populations could lead to non-specific cell adhesion and poor separation. However, at a flow rate of 0.75 ml/min, fluid shear forces are high enough to prevent significant cell-cell interactions. A flow rate of 0.75 ml/min was used in all subsequent work.
Mixed culture fermentations were prepared by inoculating a flask of M63 medium with equal volumes of overnight culture of the two organisms. Once growth had started, samples were removed and vortexed until most cell aggregates were broken up as determined by microscopic examination and O.D. measured. The fraction of strain 23716 during the growth curve was determined by cell adhesion and plate counts (Figure 2). The growth curve for the mixed culture was determined by plate counts and by the sum of the area of the two peaks from the cell adhesion assay. The growth curve for strain 23716 was calculated from the plate counts on selective media and the peak area for the adhering cells. These growth curves are shown in Figure 3. For comparison purposes, the curves have been normalized such that the maximum values of the mixed culture curves is unity.
The two methods give similar results during the early part of the growth curve but the results differ as the coculture reaches late exponential phase (Figure 2 and 3). As noted earlier, the strain MCR106 tended to form cell aggregates and the number of aggregates were observed to increase during the later stages of growth. The vortexing procedure, although it appeared effective under microscopic examination, apparently did not break up cell aggregates enough for accurate plate count determinations. The presence of aggregates would skew the results obtained by plate counts toward higher fractions of strain 23716 and explain the inconsistencies of the measured fraction (Figure 2). Occasional inclusion of a 23716 cell in an aggregate, would further skew the results in the direction observed.

Through vortexing the sample and eluting the cells from the column at a higher flow rate, the effect of the cell-cell interactions is believed to be minimized in the adhesion assay. The relatively consistent behavior of the mixed culture as determined by the adhesion assay (Figure 2 and 3), suggests that this assay procedure is not as sensitive to the populations' aggregation properties as the method of plate counts.

Figure 6-2. Fraction 23716 in Mixed Batch Culture.
Fraction of 23716 in mixed batch culture as determined by adhesion and plate counts. Culture inoculated at t=0 h.
6.5 Conclusions

Specific cell adhesion has been employed to monitor the population balance in a coculture of two strains of \textit{E. coli}. Separation was achieved based on the expression of an outer membrane protein, \textit{lamB}. In a coculture in which one population displayed strong aggregation tendencies, the adhesion method yielded more logical results than those obtained by plate counts. The distinct advantages of the cell adhesion method are, that estimates of the population balance are obtained within 30 minutes, and that the two populations are not required to display gross physical differences. In particular it may prove valuable in monitoring fermentations of unstable recombinant organisms. Since adhesion relies on the expression of a single cell surface component, placing the expression of this component under control of the plasmid would link a cell's adhesion properties to the presence of the plasmid.
6.6 Acknowledgments

The authors would like to thank Dr. Eric Achberger for his valuable assistance, and Dr. Silhavy for donating strain MCR106.

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6.7 References


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CHAPTER 7

CONTROL OF MIXED MICROBIAL CULTURES VIA SPECIFIC CELL ADHESION

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7.1 Abstract

A new method for manipulating the steady state behavior of a mixed culture is introduced. The method makes use of differences in adherence properties between competing populations to maintain a desired population ratio. The very specific nature of some ligand to cell interactions allows precise manipulation of even closely related populations. The control method is illustrated by analysis and simulations of models of a competitive mixed culture and a culture of an unstable recombinant organism. In both cases, retention of the disadvantaged population via cell adhesion results in creation of a stable coexistence steady state over a range of operating conditions.
Mixed culture fermentations are used in a wide variety of production systems. They can generally use more complex substrates and produce a wider range of products than pure cultures. Through the various interactions between the populations of the mixed cultures, conversions can be performed that are generally energetically unfavorable in pure culture (1). Usually, the consortia of microorganisms of mixed culture fermentations are derived from stable natural populations. The prospects, however, of designing a microbial consortia, whether naturally stable or unstable, and maintaining strict control, have led to many recent advances in the operation and control of mixed culture fermentations.

The possible interactions between two microbial populations is quite varied (2). However, the competition between two organisms for a common limiting substrate is the case of greatest interest. In a continuous system, four steady states can possibly be obtained. Two of the steady states correspond to the existence of either population alone, one steady state is the coexistence steady state and the other is the sterile steady state. Investigations into controlling the population balance of competitive mixed cultures in a continuous system have been aimed at maintaining coexistence by manipulating which population will dominate. The control methods depend frequently on an ability to manipulate the specific growth rates of the populations through changes in the growth conditions.

In competitive mixed cultures where the specific growth rates of the two populations are equal at some environmental condition, a metastable coexistence steady state occurs at the conditions of equal growth rates (3,4). Altering the dilution rate, pH or substrate concentration can be used as a method to maintain this steady state or to bring one of the populations into dominance (5,6,7). When specific growth rate curves do not intersect, a periodic change in the dilution rate or the substrate concentration can, under certain conditions, allow control of a mixed culture. (8). This method requires that the two populations differ in the rate at which they adapt to environmental changes.

The mixed population that arises from the loss of an unstable plasmid by a recombinant organism is an increasingly important mixed culture system. These cultures are unstable in a continuous reactor system if some form of growth advantage is not provided for the plasmid-bearing population (9). Several methods have been used to confer a growth advantage upon the plasmid-bearing cells including plasmid coded relief of substrate inhibition (10), resistance to inhibitory compounds (11), and the production and immunity to bacteriocins (12). Reactors that have entrapped the plasmid-bearing cells as a means of maintaining the population have been reported (13).
These control methods for mixed and unstable plasmid cultures all rely on manipulation of the specific growth rates or killing of specific populations. However, it is clearly desirable to be able to use growth rate as a tool for optimizing the reactor productivity. The addition of toxic compounds to control the cell populations may complicate downstream processing. An alternative strategy that does not suffer from these drawbacks has been proposed by Ollis for recombinant cultures (14). The method entails retention of the growth disadvantaged population in the reactor through the use of selective cell recycle. Davison et al. (15) applied this strategy successfully to a mixed culture of *Saccharomyces cerevisiae* and *Escherichia coli*. Based on its relatively large size, *S. cerevisiae* was selectively returned to the reactor and a stable coexistence steady state was obtained.

In the case of *S. cerevisiae* and *E. coli*, the gross difference in cell size provides a convenient method for separating the populations, but competing organisms may be phenotypically very similar and hence, more difficult to separate. However, cell affinity chromatography has recently emerged as a powerful method for separating mixtures of cell populations (16,17,18,19). This method separates populations based on cell surface characteristics and could be used as a tool for recycling the disadvantaged population in a mixed culture fermentation. When used for this purpose there is no need to provide a separate recycle stream or separator section. The surface for cell adhesion can be incorporated within the reactor, thus reducing the complexity of the overall system. As will be shown, the retention of the disadvantaged cells provides the "advantage" necessary to allow stable coexistence of two competing populations over a wide range of operating parameters. By changing the amount of surface available for adhesion, the steady state ratio of the populations in a mixed culture can be controlled.

### 7.3 Cell Adhesion

The success of affinity binding as a tool for stabilizing mixed cultures depends on the ability to utilize differences in adhesion properties of the two cell populations. Specific cell adhesion, perhaps the most selective mechanism of adhesion, is defined as involving stereochemical restrictions on a molecular level of the interactions between active groups on the cell and the adhesion surface. The binding capabilities of microbial surface lectins (18,20,21,22), interactions between lectins and cell surface structures (17,23,24), binding of cell surface receptors to their respective ligands (16), and the interactions between antigen and antibodies (25,26) are representative of specific interactions that can be exploited to mediate adhesion.
Nonspecific cell adhesion may also be used to separate populations if the cells differ greatly in adhesion properties. Long range interactions between the cell and a surface, primarily van der Waals and ionic forces, or short range interactions, generally of an ionic or hydrophobic nature mediate nonspecific cell adhesion (27,28). Both the hydrophobic and electrokinetic potential of the cell surface interact to influence cell adhesion (29,30,31). Factors that effect cell adhesion through cell surface hydrophobicity include pili expression (20,32,33) and growth rate (31). Cell adhesion is also influenced by the carbon source available (20,34,35) and cell starvation (36).

Whether specific or nonspecific interaction mediate cell adhesion, it is postulated to occur in two distinct steps, followed by a phase of surface colonization (37). In the first step, the cells contact the solid surface and adhere in a reversible fashion. The second step is the irreversible adherence of the cells.

Application of cell adhesion for control of a reactor requires the solid surface, to which cells adhere, to be retained within the reactor. This surface is subject to hydrodynamic forces from the bulk fluid motion. Thus, cells adhering to the surface experience detachment forces due to the shear stresses. At some characteristic shear stress cells would not remain bound to the surface long enough to allow irreversible adhesion (38). Thus the dominating adhesion mechanism in the reactor is likely to be the initial, reversible phase of cell adhesion. The irreversible adhesion of the cells to a surface has been reported not to occur until after some critical residence time on the surface (39).

There are various solids available that display low non-specific adhesion and may be readily derivatized to exploit cell adhesion (24,40). Surfaces have been modified to take advantage of hydrophobic (20,41,42) and ionic (43,44) interaction. The surfaces can also be readily derivatized to exploit specific cell adhesion, as is the case in cell affinity chromatography (16,17,18,19).

Cell adhesion is a function of many interactions. Theoretical models, that describe cell adhesion in terms of the fundamental underlying processes, have been advanced for adhesion between cells (45,46,47,48) and for cell adhesion to a solid support (17,19,49). These models attempt to describe cell adhesion in terms of the fundamental underlying processes. Adhesion occurs when reversible bonds form between specific compounds on the surfaces of two cells or between a cell and a solid support. The formation of these bonds must compete against nonspecific repulsion forces (46) and hydrodynamic forces (17,19,45,49,50,51). The receptor and ligand density within the contact area along with the specific rate of bond formation are important in determining whether adhesion will occur (17,19,45,46,49). Many of the components of the cell surface are free to diffuse
within the plane of the membrane. It has been found, that following the initial contact, the cell surface com-
ponents responsible for adhesion will accumulate in the area of contact (52). The effect of diffusion of receptors
over the cell membrane has recently been incorporated into a model for cell adhesion (49).

There are no simple comprehensive models that account for the role of all these factors in cell adhesion
and release rates. However, quite a few stochastic (53,54,55) and mass action (56,57,58,59,60,61) models
have been introduced that appear to describe the rates of adhesion and release quite well under limited con-
ditions.

In this work, Langmuir type equilibrium between free and adhered cells will be assumed, giving rise to the
following expression;

\[ b = \frac{XL_0}{K + X} \]  

(1)

In this equation, \( L_0 \) represents the finite number of identical adhesion sites on the surface. \( K \) represents the
ratio of the rate constants for release of cells to the rate constant for adherence. The concentration of free cells
is \( X \), and the concentration of bound cells is \( b \). Clearly, as \( X \) becomes large, the adhesion sites will become
saturated and \( b \) will reach its maximum value, \( L_0 \). Irreversible adhesion occurs as a special case when the rate
constant for release of cells become zero. As this happens, \( K \) goes to zero. This equilibrium relationship has
been employed by several investigators to quantitate the equilibrium of cell adhesion (56,57,58,60). A physical
interpretation of equation (1), is that \( L_0 \) represent a finite number of identical adhesion sites that are initially
available.

The use of a steady state expression implies that the characteristic time scale for cell adhesion is short
compared to the characteristic time for the cells metabolic processes or for the reactor. This is a common
simplification employed during simulation of cell adhesion (27,46).

7.4 Control of a Competitive Mixed Culture.

We will first analyze the case of two populations in competition for a common limiting substrate. The
specific growth rates of both populations will be assumed to follow Monod kinetics with parameters such that
the specific growth rate of population 1 is always less then that of population 2. It will also be assumed that
only the slower growing population binds to the adhesion surface in the reactor. The two populations compete
for the same limiting substrate but, otherwise do not interact.
The cultures are maintained in a continuous stirred tank reactor (CSTR) with an inlet flow rate $F$, of substrate concentration $S_0$. The volume of the reactor is $V$. The cell number concentration, specific growth rate and yield of the populations are $X_i, \mu_i$, and $Y_i$, respectively, where $i=1,2$. A solid support is retained in the reactor and provides an area, $A$, to which cell population 1 adheres. There are no interactions between the adhered cells and cells of either population in suspension. The concentration of adhered cells of population 1 on the surface is $b$ described by equation 1. The cells adhering to surface are taken to have the same growth kinetics as the cells in suspension. The balance on the total amount of population 1 can be written:

$$\frac{d}{dt}(X_1 V + bA) = \mu_1 (X_1 V + bA) - FX_1$$

(2)

In the reactor, population two, the faster growing population, does not adhere to the surface. The balance on this population thus yields:

$$V \frac{dX_2}{dt} = V \mu_2 X_2 - FX_2$$

(3)

A balance on the limiting substrate will then give:

$$V \frac{dS}{dt} = F(S_0 - S) - \frac{\mu_1}{Y_1} (VX_1 + bA) - \frac{\mu_2}{Y_2} VX_2$$

(4)

7.4.1 Working Model

Equation 1, is used to eliminate the concentration of bound cells from the balance on population 1 (eqn. 2). Monod growth kinetics are applied to both populations. After differentiation, equation 2, as well as the other balance equations are rendered dimensionless.

$$\frac{dx}{d\tau} = \left( \frac{Uz(K_2 + 1)}{K_1 + z} x \left( \frac{1}{1 + \frac{z}{\beta + x}} \right) - \theta x \right) \left( 1 + \frac{\xi \beta}{(\beta + x)^2} \right)$$

(5)

$$\frac{dy}{d\tau} = \left( \frac{z(K_2 + 1)}{K_2 + z} - \theta \right) y$$

(6)

$$\frac{dz}{d\tau} = \theta(1-z) - \frac{Uz(K_2 + 1)}{K_1 + z} x \left( \frac{1}{1 + \frac{z}{\beta + x}} \right) \frac{z(K_2 + 1)}{K_2 + z} y$$

(7)
where the dimensionless variable are defined as:

\[ x = \frac{X_1}{S_0 Y_1} \quad y = \frac{X_2}{S_0 Y_2} \quad z = \frac{S}{S_0} \quad (8) \]

\[ \theta = \frac{D(K_2 + 1)}{\mu_2^{\max}} \quad \tau = \frac{\mu_1^{\max}}{K_2 + 1} \quad \beta = \frac{K}{S_0 Y_1} \]

\[ \xi = \frac{A L_0}{V Y_1 S_0} \quad U = \frac{\mu_1^{\max}}{\mu_2^{\max}} \quad K_i = \frac{k_i}{S_0} \quad \text{for } i = 1, 2 \]

The parameter, \( \xi \), is the ratio of the total number of binding sites available to the theoretical maximum size of population one. Its value can be set for a given system simply by adjusting the available surface area in the reactor. By changing this area it will be possible to fix the ratio of the two population concentrations at a desired value, leaving the dilution rate and the inlet substrate concentration free to be used to optimize the productivity of the fermentation.

The \( k_i \) are the Monod constants and \( \mu_i^{\max} \) are the maximum growth rates for the \( i \)th population.

### 7.4.2 Region of Coexistence

Now, the steady state operating properties of this reactor will be investigated. The steady state equations for the total biomass concentration of populations 1 and population 2 and the steady state balance for substrate concentration are obtained by setting the right hand side of the balance equations equal to zero.

Of all possible solutions to these equations, only those that give rise to non-negative values of \( x, y \) and \( z \) are meaningful from a biological standpoint. Four different types of steady states can be envisioned: a coexistence steady state, a sterile steady state, and two single organism steady states. The appearance of a coexistence steady state in this model is of course not guaranteed a priori, and if it does exist it must be stable in order to be of any practical value. As we will proceed to show, such a stable steady state can exist for a range of operating conditions. To achieve this, the value of the parameter \( \xi \) must lie in an interval the size of which depend on the growth parameters and the operating conditions.

For any steady state in which population 2 exists, i.e. a steady state for which \( y \) is non-zero, equation (6) can be solved for \( z \);

\[ z = \frac{K_2 \theta}{K_2 + 1 - \theta} \quad (9) \]

For a steady state that also contains population 1, equation (9) is substituted into equation (5) and the steady state value of \( x \) determined;
Finally, the steady state value of \( y \) can be found by substitution of equations (9) and (10) into the dimensionless substrate balance (eqn. 7) and solving for \( y \),

\[
y = 1 - \frac{\theta K_2}{K_2 + 1 - \theta} \frac{\xi}{K_r(K_r + 1) - 1} - \frac{\xi}{K_r(K_r + 1) - 1} + \beta
\]

(11)

As mentioned above, \( x \) must be larger than zero for the steady state to be biologically meaningful. Applying this demand to the result in equation 10 and rearranging yields the following lower limit on the value of \( \xi \);

\[
\xi > \beta \left( \frac{K_r(K_r + 1 - \theta) + K_r \theta}{U K_2(K_2 + 1)} \right) - 1
\]

(12)

The same argument can be applied to \( y \) and the result in equation 11 to yield an upper bound on \( \xi \);

\[
\xi < \left( 1 - \frac{\theta K_2}{K_2 + 1 - \theta} + \beta \right) \left( \frac{K_r(K_r + 1 - \theta) + K_r \theta}{U K_2(K_2 + 1)} \right) - 1
\]

(13)

Both inequality (12) and (13) must be satisfied for a coexisting steady state to exist. We see that the upper and lower bounds on \( \xi \) are in fact different, implying that coexistence is found at a range of operating conditions, and not just on a separatrix between two single organism domains.

The lower bound on \( \xi \) represent the value at which \( x \) becomes zero. Thus, a minimum surface area for adhesion is required before coexistence can be achieved. The upper limit on \( \xi \) implies that if this area becomes too large, the faster growing but non-adhering species will be unable to compete successfully with the slower growing but adhering species and will be washed out of the reactor. The range of \( \xi \)-values that give rise to a coexistence steady state are shown as functions of the dimensionless dilution rate \( \theta \) in region I of Figure 7-1.

Also shown in this diagram are the curves that separate the single organism steady states from the washout steady state. The analytical expression for these curves are easily found by an analysis similar to the one above.

A pure culture of species 1 will not washout if;

\[
\xi > \beta \left( \frac{\theta(K_1 + 1)}{U(K_2 + 1)} - 1 \right)
\]

(14)

and a pure culture of species 2 will not wash out if;

\[
\theta < 1
\]

(15)

is satisfied.
The conditions imposed by inequalities (12) through (15) divide the operating diagram in Figure 7-1 into several domains. Domain I is the coexistence region. The straight line that separates domains III and IV from the rest of the diagram, is the washout curve for population 1 (eqn. 14). Above this line, the slower growing population can exist in pure culture. The equivalent line for population 2 is the vertical line at $\theta$ equal to 1. To the left of this line population 2 can exist as a pure culture. Thus, for instance, in domain II, either population can exist in pure culture, but they are unable to coexist. All the domains meet at a common point given by:

$$\xi = \beta \left( \frac{K_1 + 1}{U(K_2 + 1)} - 1 \right)$$  \hspace{1cm} (16)$$

and

$$\theta = 1$$  \hspace{1cm} (17)$$
Close to this point, very small changes in the system parameters will result in significant changes in the steady state values or in the type of steady state.

From the results above, it also follows that coexistence can only occur if $\theta$ is less than 1. This inequality turns out to be very useful when investigating the stability of the steady states.

The nature of the steady state solution in the coexistence region can be elucidated by plotting isoratio curves through this region. Along these curves, the concentration of organism 1 divided by the concentration of organism 2 remains constant. The value of $\xi$ that is required to obtain a given ratio of $x$ and $y$ is easily found to be;

$$\xi = \left( \frac{\frac{x}{y} \left( 1 - \frac{\theta K_2}{K_2 + 1 - \theta} \right)}{1 + \frac{x}{y}} + \beta \right) \left( \frac{K_1(K_2 + 1 - \theta) + K_2\theta}{UK_2(K_2 + 1)} - 1 \right)$$ (18)

Several of these isoratio curves are presented in Figure 7-2. The lower bound on the coexistence region is approached as the ratio of the two populations approaches zero. As the ratio of population 1 to population 2 becomes very large, the upper bound of the coexistence region is approached.
7.4.3 Stability of the Coexistence Steady State

It is important to determine the stability characteristics of the coexistence steady state. The local stability of any steady state is determined from the sign of the eigenvalues of the system Jacobian matrix evaluated at the steady state in question. Thus, the equation for the eigenvalues is;

$$\det \begin{pmatrix} a_{11} - \lambda & a_{12} & a_{13} \\ a_{21} & a_{22} - \lambda & a_{23} \\ a_{31} & a_{32} & a_{33} - \lambda \end{pmatrix} = 0 \quad (19)$$

Figure 7-2. Isoratio Curves in Coexistence Domain.
Isoratio curves in the coexistence domain. The parameters are the same as those used in Figure 7-1.
where;

\[
\begin{align*}
 a_{11} &= \left( \frac{U_z(K_2 + 1)}{K_1 + z} - \frac{\theta}{1 + \frac{\xi}{(\beta + x)^2}} \right) + \left( \frac{U_z(K_{sub2} + 1)}{K_1 + z} \right) \left( \frac{\xi}{\bar{\beta} + x} - \theta \right) \left( \frac{2\xi \beta}{(\beta + x)^2} \right) \left( \frac{1 + \frac{\xi \beta}{(\beta + x)^2}}{1 + \frac{\xi}{(\beta + x)^2}} \right)^2 \\
 a_{12} &= 0 \\
 a_{13} &= \frac{U K_z (K_2 + 1) x}{(K_1 + z)^2} \left( \frac{1 + \frac{\xi}{\bar{\beta} + x}}{1 + \frac{\xi \beta}{(\beta + x)^2}} \right)^2
\end{align*}
\]

\[
\begin{align*}
 a_{21} &= 0 \\
 a_{22} &= \frac{z(K_2 + 1)}{K_2 + z} - \theta \\
 a_{23} &= \frac{K_2 + 1}{(K_2 + z)^2} y \\
 a_{31} &= -\frac{U z(K_1 + 1)}{K_2 + z} \left( \frac{1 + \frac{\beta z}{(\beta + x)^2}}{1 + \frac{\xi}{(\beta + x)^2}} \right) \\
 a_{32} &= -\frac{z(K_2 + 1)}{K_2 + z} \\
 a_{33} &= -\theta - \frac{U K_1 (K_2 + 1) x}{(K_1 + z)^2} \left( \frac{1 + \frac{\xi}{\bar{\beta} + z}}{1 + \frac{\xi \beta}{(\beta + x)^2}} \right) \frac{K_2 (K_2 + 1) y}{(K_2 + z)^2}
\end{align*}
\]

and \( x, y \) and \( z \) are the steady state values of these variables at the steady state being investigated.

The determinant in the characteristic equation above can be evaluated, and the equation written as a polynomial in \( \lambda \);

\[
\lambda^3 + \gamma_1 \lambda^2 + \gamma_2 \lambda + \gamma_3 = 0
\]  

(21)

where;

\[
\begin{align*}
 \gamma_1 &= -a_{11} - a_{33} \\
 \gamma_2 &= a_{11} a_{33} - a_{13} a_{23} - a_{23} a_{32} \\
 \gamma_3 &= a_{23} a_{32} a_{11}
\end{align*}
\]

Through application of the Routh-Hurwitz criteria and some algebra, it can be shown that the real parts of the eigenvalues are always negative when the inequalities (12) and (13) are satisfied (Appendix). Therefore, whenever a coexistence steady state occurs, it will be locally stable.
It is considerably more difficult to determine if the coexistence steady state is globally stable or to determine what initial conditions will cause the system to reach the coexistence steady state. However, an eigenvalue analysis of the two pure culture steady states and the sterile steady state reveals the following: If the inequalities (12) and (13) are satisfied, i.e. if the reactor is operated under conditions such that a coexistence steady state does exist, then both of the pure culture steady states are unstable with respect to a contamination of the other organism, and the sterile steady state is unstable with respect to a contamination of either organism (Appendix). This result strongly implies that when the coexistence steady state exists, it can be reached from any initial condition that contains both organisms. In other words, no start-up strategy is required to reach the desired steady state.

It is, of course, possible that the system can exhibit bizarre dynamic behavior that will make it more difficult to attain the coexistence steady state. For instance, the eigenvalue analysis does not eliminate the possibility that the coexistence steady state is surrounded by an unstable limit cycle, nested within a stable limit cycle. However, we have carried out extensive numerical simulations of the model equations, using a range of operating parameters and initial conditions, and have always found that the dynamic behavior is quite tame. Never was a limit cycle observed. The dynamic behavior is illustrated in a phase plane diagram (Figure 7-3) that shows the trajectories in the x-y plane for different initial conditions.
Figure 7-3. Phase Plane Trajectories in Mixed Culture.
Phase plane trajectories in a competitive mixed culture when the conditions for coexistence are satisfied. The coexistence steady state is shown by the square. The parameters are the same as those used in Figure 7-1.

7.5 Control of a Unstable Recombinant Culture

The second example chosen to demonstrate the utility of this control scheme, is the case of a mixed culture that arises from plasmid instability. In this case, we consider a plasmid that codes for the adhesion of the cell. The mixed culture that occur upon loss of this plasmid is composed of two phenotypes. One is the adhering phenotype. This population contains plasmid-bearing cells and cells that have recently lost their plasmids, but due to phenotypic lag, retain the plasmid coded properties. The other population is of the non-adhering phenotype and is composed solely of plasmid-free cells. The phenotypic lag is the result of the finite time required for turnover of cell components after plasmid loss. At this stage it is assumed that the turnover rate is independent of the growth rate and is similar for all components. Unless some growth advantage is given to the adhering phenotype, the culture is unstable in a continuous system and the non-adhering phenotype will eventually dominate (9).
In this example, it will be assumed that the only interaction between the two populations is competition for a limiting substrate. The yields for the two populations are taken to be the same. The growth characteristics of the adhering phenotype are considered to be the same as a plasmid-bearing cell. This characteristic is again attributable to phenotypic lag. The implications of this simplifying assumption will be discussed later.

7.5.1 Working Model

Unstructured models of recombinant cultures have been presented by Ollis (14). In these models the specific growth rate of recombinant cells is decreased by an amount $P$ - the ratio of the rate of plasmid loss to the specific growth rate. Simultaneously, the specific rate of formation of plasmid free cells is increased by the same amount. A similar approach is used in the formulation of the models to represent two phenotypically defined populations. The parameter $P$, now represents the ratio of the specific rate of loss of adhesion capabilities by the adhering population to the specific growth rate. The specific rate of loss of adhesion capability is dependent on the specific rate of plasmid loss and the rate at which the phenotype will change upon plasmid loss. In using this formulation, it is required that at any initial condition or perturbation in which the adhering phenotype is present, there must be some plasmid-bearing cells in the adhering population. This restriction is easily satisfied under normal reactor startup procedure. Following directly from equations (5), (6) and (7) and introducing $P$, the total biomass balance on each population can be developed. For the adhering population, $x$, the dimensionless balance becomes;

$$\frac{dx}{d\tau} = \left( \frac{Uz(K_2+1)}{K_1+z} x \left( 1 + \frac{\xi}{\beta+x} \right) (1-P) - \theta x \left( 1 + \frac{\xi\beta}{(\beta+x)^2} \right) \right)$$

The balance on the non-adhering population, $y$, contains a term for production from the adhering population;

$$\frac{dy}{d\tau} = \left( \frac{z(K_2+1)}{K_2+z} - \theta \right) y + \frac{Uz(K_2+1)}{K_1+z} x \left( 1 + \frac{\xi}{\beta+x} \right) P$$

The balance for the limiting substrate is;

$$\frac{dz}{d\tau} = \theta(1-z) - \frac{Uz(K_2+1)}{K_1+z} x \left( 1 + \frac{\xi}{\beta+x} \right) - \frac{z(K_2+1)}{K_2+z} y$$

where the dimensionless parameters are defined as before (eqn. 8).

As in the example of a mixed competitive culture, Monod growth kinetics have been assumed. The differences between the balance equations of the competitive mixed culture (eqns. 5 - 7) and the system model for the
unstable plasmid (eqns. 23 - 25), are the term that accounts for the decrease in adhering cells, \((1 - P)\), in equation 23 and the term used to describe the production of non-adhering cells through phenotypic change in equation (24).

7.5.2 Region of Coexistence

In a culture that contains an unstable plasmid, plasmid-free cells are generated whenever plasmid-bearing cells are present. Hence, it is not possible to obtain a pure culture of plasmid containing cells, no matter how much surface area is available to which plasmid-bearing cells may adhere. There is, therefore, no upper limit on \(\xi\) and the operating diagram for this system will have fewer domains than the diagram for a competitive culture. There will be two lower limits on \(\xi\). One that will prevent total washout of the reactor, and one that will prevent washout of adhering cells while retaining the non-adhering cells. Determination of these bounds proceeds in a manner similar to that of a competitive mixed culture. As a first step, combination of the steady state form of equations (23) and (24) gives;

\[
\frac{x\theta P}{1 - P} = \left( \frac{\theta - \frac{z(K_2 + 1)}{K_2 + z}}{K_2 + z} \right)^y
\]  

(26)

Since all three dependent variables must be positive, the expression inside the brackets must be positive. This gives rise to the following inequality for \(z\);

\[
z < \frac{K_1 \theta}{K_2 + 1 - \theta}
\]  

(27)

When \(z\) becomes equal to this upper bound, \(x\) becomes equal to zero without \(y\) becoming zero simultaneously.

Thus, inequality (27) represents the boundary between the coexistence region and the region of pure plasmid-free cells. Inequality (27) can be rewritten in terms of \(\xi\) by using the steady state version of equation (23). Keeping in mind that at this limit \(x\) equals zero, one obtains;

\[
\xi > \beta \left( \frac{K_1(K_2 + 1 - \theta) + K_2\theta}{K_1U(K_2 + 1)(1 - P) - 1} \right)
\]  

(28)

Notice the similarity between inequalities (12) and (28).

The second boundary on the coexistence domain is found when the concentration of non-adhering and adhering cells approach zero simultaneously. When this occurs the dimensionless substrate concentration will approach 1 from below, leading to the obvious restriction that \(z < 1\). Using this inequality as a constraint for \(z\) in equation (23) gives;

\[
\xi > \beta \left( \frac{\theta(K_1 + 1)}{U(K_2 + 1)(1 - P) - 1} \right)
\]  

(29)
Both of these inequalities must be satisfied to obtain coexistence. At values of $\theta$ less than 1, inequality (27) is the most demanding, while at $\theta$ values greater than 1 it is inequality (29) that is the most demanding. A $\theta$ value of 1 equals the washout dilution rate in a pure plasmid-free culture.

An example of an operating diagram is shown in Figure 7-4. In domains I and IV coexistence is possible, but a pure culture of plasmid-free cells can only exist in domain I, not in domain IV. A pure culture of plasmid-free cells is also possible in domain II, while a sterile steady state is the only possibility in domain III. All four domains meet in a common point given by:

$$\xi = \beta \left( \frac{K_1 + 1}{U(K_2 + 1)(1 - P)} - 1 \right)$$  \hspace{1cm} (30)

and

$$\theta = 1$$  \hspace{1cm} (31)

Figure 7-4. Recombinant Culture Operating Diagram.
Operating diagram for an unstable recombinant culture. Region of coexistence is shaded. See text for explanation of the different regions. The parameters used are: $P = .2$ others are the same as used in Figure 7-1.
A solution for the population ratio is not easily obtained analytically, but can be determined numerically. A form of the steady state equations that is convenient for numerical solution is;

$$\frac{x}{y} = \frac{1-P}{P\theta} \left( \frac{z(K_2+1)}{K_2+z} + \right)$$

where

$$z = 1-x-y$$

The result of a numerical solution is shown in Figure 7-5. The plot shows the population ratio as a function of the dimensionless dilution rate. A family of curves exists, one for each value of $\xi$. The fraction of adhering cells keeps increasing with dilution rate until inequality (29) is violated. When this happens, both populations wash out as shown by the vertical lines. The washout occurs at increasingly higher dilution rates as the surface area available for binding is increased. The theoretical maximum ratio is obtained as $z$ becomes zero;

$$\left( \frac{x}{y} \right)_{\text{max}} = \frac{(1-P)}{P}$$

This equation is dependent only on the parameter $P$.

The stability of the steady state containing the adhering population was analyzed in the same fashion as the competitive mixed culture. The steady state containing the adhering and non-adhering populations is locally stable if the inequalities (28) and (29) are satisfied. Global stability of this steady state was investigated through analysis of the other possible steady states. It is found, that when the conditions for coexistence are satisfied, both the sterile steady state and the pure plasmid-free cell culture are unstable with respect to contamination that contains at least one plasmid-bearing (Appendix). Numerical simulations of the time dependent model equations displayed only a very tame behavior. This suggests that the coexistence steady state is globally stable, and that no particular start-up strategy, other than an initial presence of plasmid-bearing cells, is required to reach this steady state.
7.6 Discussion

This study was carried out to determine if specific cell adhesion can be used to alter the steady state behavior of a mixed culture. Analysis of mathematical models of a competitive mixed culture and an unstable plasmid culture show that the method can stabilize these otherwise unstable cultures. Using a simple model to represent the growth and adhesion kinetics of the two populations, conditions for coexistence or dominance of the populations were determined.

The success of this control method relies on effective utilization of differences in adhesion properties between competing populations. The mathematical models were developed assuming that cell adhesion was reversible. Frequently, this assumption may not be valid, but the effect of irreversible adhesion is easily deduced. The ratio of the release to the adhesion rate constants is incorporated in the parameter $\beta$. As the adhesion becomes irreversible, this parameter becomes zero. The effect on the limiting inequalities for the
mixed culture (eqns. 12 and 13) and the unstable plasmid culture (eqns. 28 and 29) is readily apparent. For either culture, the minimum $\xi$ that allows coexistence is proportional to $\beta$. As the adhesion tends towards irreversible adhesion, the lower limit on \( \xi \) approaches zero. In response to an increasingly irreversible adhesion the upper bound of coexistence for the mixed culture will decrease to a limiting value determined by the growth characteristics of the two populations and the reactors dilution rate (eqn 13).

It was also assumed that adhesion is completely specific, i.e. only one population can adhere to the support. In practice, both populations may contain cell surface components that lead to adhesion, such as surface lipopolysaccharides, pili or protein. In either specific or non-specific adhesion, it is the bonding between these components and structures on a surface that lead to adhesion. However, according to the current theory of specific cell adhesion, a sufficient number of bonds must form between the cell and a surface to resist repulsive and removal forces before cell adhesion will occur (45,46,49).

As predicted by one model of specific cell adhesion, if a population were to experience a 25% decrease in the number of receptors on the cell surface, the population could lose its adhesion capability (49). It is the removal forces exerted by the fluid shear on the cells that allow this differentiation between adhesion capabilities. The fluid shear is an easily controlled parameter in a reactor. It would be assumed that in an analogous manner, the ability of a cell population to adhere to a surface through non-specific adhesion could be controlled.

In this work, the manipulation of an unstable recombinant culture relies on a plasmid coded adherence property. Following loss of plasmid, a cell will pass through a phenotypic lag before it converts to the non-adhering phenotype. The length of this phenotypic lag will depend on the turnover rate of surface receptors and m-RNA coding for these receptor. A conservative estimate would be that the total number of surface receptors remains constant, and that cells cease to adhere when cell divisions have brought the number of receptors per cell down to some critical value. As mentioned above, a decrease of as little as 25% in the number of receptors per cell, can result in loss of adhesion capability. Thus, the phenotypic lag would typically be less than the duration of the cell cycle.

Reactors designed to retain the plasmid-bearing cells through adhesion, could result in the natural selection of cells with high levels of plasmid expression. If expression of adherence and product formation are controlled by the same promoter, cells with strong adhesion characteristics would likely also express the product at a high level. Hence, conditions that exert large removal forces on the cells, such as high shear stress, would preferentially retain cells with high rates of product formation.
An alternative approach for control of an unstable plasmid culture would have the plasmid control the repression of the adhesion property. The plasmid-bearing cells would not display adherence, but upon loss of the plasmid, the cells would express the adherence properties. In this system, plasmid-bearing cells may be retained by specific recycle as suggested by Ollis (14). The time period for the cell to change phenotype after plasmid loss, would essentially be the time needed to initiate expression of the gene that codes for adhesion.

The choice of a Langmuir model to describe adhesion equilibrium means several implicit assumptions were made. First, that it is reasonable to assume that there are a fixed number of adhesion sites; second, all of these adhesion sites are identical and third, the interactions between adhered cells are not significant. These assumptions imply that the cells adhere in monolayers. This is reasonable if the rate of cell aggregation is low and most cells exist as singlets.

Proper preparation of the surface for adhesion can minimize differences between adhesion sites. However, in several cases adhesion kinetics have suggested that several types of adhesion sites may exist (57,58). It is believed that even for properly prepared surfaces, site differences can exist due to non-uniform adsorption of macromolecules to the surface.

The possible interactions between adhered cells are either attraction or repulsion. Attraction between neighboring cell could result in cell-cell binding on the surface which could stabilize adherence. Cell-cell repulsion would result in less stable adherence of the cells. In a similar manner adhered cells can affect the properties of adjacent sites. The cell-cell interactions between the two populations can also invalidate the assumption of selective adhesion (62). When these interactions are significant, deviations from the Langmuir kinetics could be observed. Such interactions could also result in apparent surface saturation as adhesion rates are modified by the cell-cell interactions (63).

The utilization of cell adhesion to control mixed cultures is not limited to systems with competing populations. It may be readily extended to populations that exhibit any type of interactions. Incorporation of adhesion of several populations is also straightforward, but beyond the scope of this work. While many proposed methods for control of mixed cultures rely on manipulations of the growth rates via changes in the environment, this method does not. Because of this, control of mixed cultures by affinity adhesion allows the environmental parameters to be used to optimize the fermentation. Thus, it provides a powerful and flexible approach to control of a wide range of mixed cultures.
7.7 Appendix.

7.7.1 Eigenvalue Evaluation for Mixed Culture

The general problem is that of determining the sign of the eigenvalues in the Jacobian matrix when it is evaluated at one of the steady states, and the conditions for a coexistence steady state are satisfied. This means determining the signs of the roots in the equation (21) when the system parameters are such that equations (12) and (13) are satisfied.

The eigenvalues at the coexistence steady state are evaluated first. At this steady state the element $a_{22}$ is zero, and the characteristic equation is:

$$\lambda^3 + \gamma_1 \lambda^2 + \gamma_2 \lambda + \gamma_3 = 0$$

where

$$\gamma_1 = -a_{11} - a_{33}$$

$$\gamma_2 = a_{11}a_{33} - a_{13}a_{31} - a_{22}a_{32}$$

$$\gamma_3 = a_{32}a_{33}$$

Utilizing the steady state form of equations (5) to (7), the Jacobian elements are written as;

$$a_{11} = \frac{U(K_2 + 1)}{(K_1 + z)} x z \left(1 + \frac{\xi \beta}{(\beta + x)^2}\right)^{-1}$$

$$a_{13} = \frac{\theta x K_1}{z(K_1 + z)} \left(1 + \frac{\xi \beta}{(\beta + x)^2}\right)^{-1}$$

$$a_{23} = \frac{\theta y}{z(K_2 + z)}$$

$$a_{31} = -\frac{U z(K_2 + 1)}{K_1 + z} \left(1 + \frac{\xi \beta}{(\beta + x)^2}\right)$$

$$a_{32} = -\theta$$

$$a_{33} = -\theta \left(1 + \frac{x K_1}{z(K_1 + z)} + \frac{y K_2}{z(K_2 + z)}\right)$$

All elements not listed are identically equal to zero.

The Routh-Hurwitz criteria can be applied to equation (21) to determine the sign of the roots. The real part of the roots are negative if $\gamma_1$, $\gamma_2$, $\gamma_3$ and $\gamma_1\gamma_2 - \gamma_3$ are all greater than zero.
It is obvious that because all model parameters are positive, a physical necessity, \(a_{13}\), and \(a_{23}\) are positive and all other elements are negative. Therefore, the \(\gamma_i\) s in equation (22) are positive. To prove that \(\gamma_1 - \gamma_3\) is also positive, expand this expression to;

\[
\gamma_1 - \gamma_3 = (-a_{11} - a_{33}) (a_{11} a_{33} - a_{31} a_{13} - a_{23} a_{32}) - a_{32} a_{23} a_{11}
\]

As above, only \(a_{13}\), and \(a_{23}\) are positive, and thus the right hand side of equation (A.2) can be seen to be a sum of positive terms. Hence, the eigenvalues of the Jacobian matrix all have negative real parts, and the steady state is locally stable.

The sign of the eigenvalues at the other steady states can be found in a similar manner. At the steady state where population 2 exists alone, i.e. where \(x = 0\), the Jacobian matrix reduces to;

\[
J = \begin{pmatrix}
a_{11} & 0 & 0 \\
0 & 0 & a_{23} \\
a_{31} & a_{32} & a_{33}
\end{pmatrix}
\]

The first row in the Jacobian matrix represents the equation for population 1, the absent population. Clearly, a contamination of this organism will grow up if the eigenvalue of this equation,

\[
a_{11} = \left( \frac{U K_2(K_2 + 1)}{K_1(K_2 + 1 - \theta) + K_2 \theta \left( 1 + \frac{\xi}{\beta} \right)} - 1 \right) \left( 1 + \frac{\xi}{\beta} \right)^{-1}
\]

is greater than zero.

Equation (12), one of the conditions for coexistence, can be rearranged to show that the first factor in the above expression is positive. The two other factor are clearly also positive, so \(a_{11}\) must be positive.

The remaining two eigenvalues govern the behavior of a pure culture of population 2. It can be shown using the Routh-Hurwitz criteria as above, that these eigenvalues have negative real parts at this steady state. In other words, a pure culture of population 2, operating at the conditions of coexistence, is unstable to small contaminations of population 1. To other perturbations, the system behaves as the classical Monod chemostat.

The other pure culture steady state occurs when only population 1 is present, \(x>0, y=0\). The terms \(a_{12}\), \(a_{21}\) and \(a_{23}\) of the Jacobian matrix are zero, and the Jacobian thus becomes;

\[
J = \begin{pmatrix}
a_{11} & 0 & a_{13} \\
0 & a_{22} & 0 \\
a_{31} & a_{32} & a_{33}
\end{pmatrix}
\]
The eigenvalue associated with population 2 is \( \alpha_{22} \). If this eigenvalue is positive, the system will be unstable with respect to a contamination of population 2. Although the expression for \( \alpha_{22} \) is quite simple, it is not immediately obvious whether it is positive or negative. It can be shown to be positive by the following argument.

Eliminate \( x \) from the steady state version of equation (5) and (7), and solve for \( \xi \):

\[
\xi = (1 - z + \beta) \left( \frac{\alpha(K_1 + z)}{Uz(K_2 + 1)} - 1 \right) \tag{A.6}
\]

Using this result in equation (13), the equation for the upper bound on \( \xi \), yields, upon rearrangement, the following inequality;

\[
(1 - z + \beta) \left( \frac{\alpha(K_1 + z)}{Uz(K_2 + 1)} - 1 \right) < (1 - \alpha + \beta) \left( \frac{\alpha(K_1 + \alpha)}{U\alpha(K_2 + 1)} - 1 \right) \tag{A.7}
\]

where,

\[
\alpha = \frac{\theta K_2}{K_2 + 1 - \theta}. \tag{A.8}
\]

It is easy to confirm that this inequality is satisfied if and only if, \( z > \alpha \), which upon rearrangement yields,

\[
\alpha_{22} = \frac{z(K_2 + 1)}{K_2 + z} - \theta > 0. \tag{A.9}
\]

The remaining eigenvalues are found to have negative real parts by a straight forward application of the Routh-Hurwitz criteria as discussed previously.

For the sterile steady state, \( x=0 \), \( y=0 \), and \( z=1 \), the Jacobian matrix becomes;

\[
J = \begin{pmatrix}
a_{11} & 0 & 0 \\
0 & a_{22} & 0 \\
a_{31} & a_{32} & a_{33}
\end{pmatrix} \tag{A.10}
\]

The eigenvalue associated with population 1 equals \( a_{11} \)

\[
a_{11} = \frac{\beta}{\beta + \xi} \left( \frac{U(K_2 + 1)}{K_1 + 1} \left( \frac{\xi}{\beta} + 1 \right) - \theta \right) \tag{A.11}
\]

The restriction from equation (12) can be rearranged to give;

\[
1 + \frac{\xi}{\beta} > \frac{K_1(K_2 + 1 - \theta) + K_2 \theta}{U(K_2 + 1)K_2} \tag{A.12}
\]

This inequality can now be used in conjunction with equation (A.11) to obtain a lower bound on \( a_{11} \):
The expression in the parenthesis is easily shown to be positive when $\theta < 1$, which is always satisfied in the coexistence region. Therefore, $a_{11}$ must also be positive.

The eigenvalue associated with population 2 is:

\[ a_{22} = 1 - \theta \]

which is clearly always positive.

The last eigenvalue is given by

\[ \lambda_3 = a_{33} = -\theta \]

and is obviously negative.

The sterile steady state is thus unstable with respect to contamination of either organism.

7.7.2 Eigenvalue Evaluation for Unstable Recombinant Culture

For the model of unstable recombinant cultures the elements in the Jacobian matrix becomes:

\[
\begin{align*}
    a_{11} &= \frac{\beta}{\beta + \xi} \left( \frac{K'(K_2 + 1) + \theta}{K'(K_1 + 1)} - \theta \right) \\
    a_{12} &= 0 \\
    a_{13} &= \frac{U K'(K_2 + 1) x(1 - P)}{(K_1 + z)^2} \left( 1 + \frac{\xi}{\beta + x} \right) \left( 1 + \frac{\xi \beta}{(\beta + x)^2} \right)^{-1} \\
    a_{21} &= \frac{U z(K_2 + 1)Y}{K_1 + z} \left( 1 + \frac{\xi \beta}{(\beta + x)^2} \right) \\
    a_{22} &= \frac{z(K_2 + 1)}{K_2 + z} - \theta \\
    a_{23} &= \frac{K'(K_2 + 1)}{(K_2 + z)^2} x + \frac{U K'(K_2 + 1)}{(K_1 + z)^2} \left( 1 + \frac{\xi}{\beta + x} \right) x P \\
\end{align*}
\]
\[ a_{31} = -\frac{Uz(K_2+1)}{K_2+z} \left( 1 + \frac{\beta \xi}{(\beta+x)^2} \right) \]

\[ a_{32} = -\frac{z(K_2+1)}{K_2+z} \]

\[ a_{33} = -\theta - \frac{UK_1(K_2+1)x}{(K_1+z)^2} \left( 1 + \frac{\xi \beta}{\beta+z} \right) - \frac{K_2(K_2+1)y}{(K_2+z)^2} \]

where \( x, y \) and \( z \) should be set equal to their values at the steady state being investigated.

Because all the model parameters and variables are positive, it is immediately obvious that \( a_{13}, a_{21}, \) and \( a_{23} \) are positive, and that \( a_{31}, a_{32}, \) and \( a_{33} \) are negative. The sign of \( a_{11} \) and \( a_{22} \) must be evaluated for each steady state.

At the coexistence steady state \( a_{11} \) can be rewritten as:

\[ a_{11} = \frac{Uz(K_2+1)}{(K_1+z)} (1-P) \left( 1 + \frac{\xi \beta}{(\beta+x)^2} \right) \left( 1 + \frac{\xi \beta}{(\beta+x)^2} \right)^{-1} \] (A.17)

Substitution of \( \theta \), as determined from the steady state balance for population 1 into equation (A.17) and rearrangement yields:

\[ a_{11} = -\frac{Uz(K_2+1)}{(K_1+z)} (1-P) \left( \frac{x \beta}{(\beta+x)^2} \left( 1 + \frac{\xi \beta}{(\beta+x)^2} \right)^{-1} \right) \] (A.18)

which is negative.

To determine the value for \( a_{22} \), the steady state balance for population 2 is rewritten to give:

\[ \frac{z(K_2+1)}{(K_2+z)} - \theta = -\frac{Uz(K_2+1)}{(K_1+z)} x \left( 1 + \frac{\xi \beta}{(\beta+x)^2} \right)^{-1} \] (A.19)

The left hand side of equation (A.20) is \( a_{22} \) as defined in equation (A.16), so \( a_{22} \) is negative.

The characteristic equation is of the same form as that of the mixed culture (eqn 21). The coefficients are different, however and are:

\[ \gamma_1 = -a_{11} - a_{22} - a_{33} \]

\[ \gamma_2 = a_{11}(a_{22} + a_{33}) + a_{22}a_{33} - a_{22}a_{32} - a_{33}a_{13} \] (A.20)

\[ \gamma_3 = a_{11}(a_{22}a_{33} - a_{23}a_{32}) - a_{13}(a_{21}a_{32} - a_{22}a_{31}) \]
According to the Routh-Hurwitz criteria, the eigenvalues will be negative if \( \gamma_1, \gamma_2, \gamma_3 \) and \( \gamma_2 \gamma_3 - \gamma_1 \) are all positive quantities. From the knowledge of the sign of the \( a_i \)'s it is clear that this in fact the case. Hence, the coexistence steady state is locally stable.

The steady state containing only the plasmid-free population, i.e. when \( x = 0 \) and \( y > 0 \), can occur when \( 0 < \theta < 1 \). Utilizing the steady state solutions to simplify the Jacobian yields:

\[
J = \begin{pmatrix}
a_{11} & 0 & 0 \\
a_{21} & 0 & a_{23} \\
a_{31} & a_{32} & a_{33}
\end{pmatrix}
\]  

(A.21)

There is only 1 non-zero eigenvalue associated with the plasmid-bearing cells, and it clearly equals \( a_{11} \).

Evaluation of \( a_{11} \) using the steady state value for \( z \), from equation (24), indicates that the associated eigenvalue will be positive if

\[
1 < \frac{U K_2 (K_2 + 1)(1 - \theta)}{K_1 (K_2 + 1 - \theta) + K_3 \theta} \left( \frac{\xi}{\beta} \right)
\]

(A.22)

Rearrangement of inequality (28), which defines a bound on the coexistence region, gives us the value of the right hand side of inequality (A.23) to be greater than 1. Thus, the eigenvalue in question must be positive, and the steady state unstable with respect to a contamination of plasmid-bearing cells. The two remaining eigenvalues can be shown to be negative using the Routh-Hurwitz criteria. The pure culture steady state is therefore stable to perturbations in the other two dependent variables.

The Jacobian for the sterile steady state may be written

\[
J = \begin{pmatrix}
a_{11} & 0 & 0 \\
a_{21} & a_{22} & 0 \\
a_{31} & a_{32} & a_{33}
\end{pmatrix}
\]  

(A.23)

Since all elements above the diagonal are zero, the eigenvalues are simply the three diagonal elements.

The first eigenvalue is given by,

\[
\lambda_1 = a_{11}
\]

(A.25)

\[
= \left( \frac{U(K_2 + 1)}{K_1 + 1} \left( 1 + \frac{\xi}{\beta} \right) (1 - \theta) \right) \left( 1 + \frac{\xi}{\beta} \right)^{-1}
\]

(A.24)

In the part of the coexistence region where \( \theta > 1 \), rearrangement of inequality (29) shows that the eigenvalue will always be positive.
For the part of the coexistence region where $0 < 1$, it can be shown, in a manner directly analogous to that used in the sterile steady state of the mixed culture (eqns. A.11 - A.13), that the eigenvalue defined by equation (A.25) is positive. Hence, a contamination of plasmid-bearing cells will not wash out.

The second eigenvalue is found to be:

$$\lambda_2 = a_{22}$$

$$= 1 - \theta$$

It is obvious that this eigenvalue is negative if $\theta$ is greater than 1, otherwise it is positive. Clearly, a contaminant of plasmid free cells will only proliferate if $\theta$ is less than 1.

The third eigenvalue is obviously always negative.

$$\lambda_3 = a_{33}$$

$$= -\theta$$

7.8 Acknowledgments.

This work was supported by NSF grant EET-8710512

7.9 Nomenclature.

- $a_{ij}$ Element in Jacobian matrix.
- $A$ Area available for binding.
- $b$ Surface concentration of bound cells.
- $F$ Flow rate through reactor.
- $J$ Jacobian matrix.
- $k_i$ Monod constant of species i.
- $K$ Ratio of release to adhesion rate constants.
- $K_i$ Dimensionless Monod constant of species i.
- $L_0$ Binding site concentration.
- $P$ Growth rate decrease due to plasmid loss.
- $S$ Substrate concentration.
- $S_0$ Inlet substrate concentration.
- $t$ Time.
$U$ Ratio of maximum specific growth rates.
$V$ Reactor volume.
$x$ Dimensionless concentration of adhering species.
$X_i$ Concentration in suspension of species $i$.
$y$ Dimensionless concentration of non-adhering species.
$z$ Dimensionless substrate concentration.
$Y_i$ Yield factor of species $i$.
$\beta$ Dimensionless adhesion parameter.
$\theta$ Dimensionless dilution rate.
$\lambda$ Eigenvalue of Jacobian matrix.
$\mu_i$ Specific growth rate of species $i$.
$\xi$ Dimensionless number of binding sites.
$\tau$ Dimensionless time.

7.10 References


CHAPTER 8
SPECIFIC CELL ADHESION USED TO OPERATE
A MIXED CULTURE REACTOR WITH POPULATION SPECIFIC RECYCLE

8.1 Abstract
A chemostat, with population specific recycle, was employed to alter the dynamics of a competitive mixed culture of Escherichia coli. Based on differential expression of a functional maltoporin, the two populations were separated by specific adhesion on starch-Sepharose. The slower growing population was then recycled to the reactor. The specific recycle was successful in maintaining the slower growing population at a higher level than in comparable reactors without recycle.

8.2 Introduction
Analysis of the dynamics and control of mixed culture reactors is generating substantial interest. Much of this work is directed at two populations competing for a common limiting substrate (Aris and Humphrey, 1977; Davison et al., 1985; Stephens and Lyberatos, 1987; Goochee et al., 1989) and the special case of competition that arises in an unstable recombinant culture (Ollis, 1982; Stephanopoulos and Lapidus, 1988; Stephens and Lyberatos, 1988). In continuous culture, the coexistence state of both of these systems is generally unstable. One of the populations will grow faster than the other and become the dominant population and with time take over the reactor. The slower growing population will be washed out. The only stable steady state consists of the faster growing organism in monoculture. In the unstable recombinant culture, unless selective pressure is applied, the plasmid-free population dominates the reactor and the plasmid-bearing population is eventually washed out. Control strategies are applied to these reactors with the goal of either changing the dominant population or maintaining the disadvantaged population. Obtaining a stable coexistence state is a primary goal of these control strategies. However, it is also desirable to be able to obtain a predetermined ratio of the two populations at a stable coexistence steady state.

The methods that have been proposed for control of competing populations in a chemostat fall into two general classes. One strategy is to alter environmental parameters of the reactor such as pH, dilution rate or temperature and thus, manipulate the specific growth rates of the two populations. This method has been used when the specific growth rates of the two populations are equal at some reactor condition. At this point there is an unstable coexistence steady state (Aris and Humphrey, 1977). Through periodic changes of environmental parameters, the reactor can be switched to operate on either side of this point and the dominant population changed or coexistence maintained (Davison and Stephanopoulos, 1986; Goochee et al., 1987a). Periodic
Forcing has also been proposed as a means of maintaining two populations whose specific growth rates never equal (Stephens and Lyberatos, 1987). A similar approach has been applied to unstable recombinant cultures (Stephens and Lyberatos, 1988; Weber and San, 1989).

The second category of control methods entails altering the residence times of the populations in the continuous reactor. The proper distribution of the residence times over the populations can alter the dominant population or yield a state where both populations are maintained (Ollis, 1982; Sheintuch, 1987; Davis and Parnham, 1989; Roos and Hjortso, 1989a).

Immobilizing a population is one method to ensure the population is retained in a reactor. An example is the retention of an unstable recombinant population in a continuous reactor by immobilization of the plasmid bearing population (Nasri et al, 1988). The formation of biofilm in the reactor is a similar situation. One or several populations may form a biofilm in the reactor, thus, preventing their washout. The population balance in this case is determined by reactor conditions and the kinetics of biofilm formation (Bryers, 1986).

If populations can be separated, a specific recycle of the desired population can be used to maintain this population in a reactor or alter the population balance (Ollis, 1982; Bungay, 1984). This approach has been used to design reactors that separate and recycle populations based on their sedimentation rate. These reactors have been successfully used with mixed populations that differ significantly in size (Davison et al, 1985) or flocculation properties (Sheintuch, 1987; Davis and Parnham, 1989).

Cell adhesion is a powerful method of separating cell populations based on small differences in cell surface composition. One mechanism of adhesion is the result of non-specific ionic or hydrophobic interactions between cells or cells and a surface. The cell-cell interaction leads to aggregate formation while the interaction with a surface can cause cell adhesion. When non-specific interactions are the dominant adhesion mechanism, some differences in adhesion between populations can be observed (Goochee et al, 1987b; Van Loosdrecht et al, 1987a,b). Populations of microorganisms, however, are often quite similar with respect to gross surface properties such as charge or hydrophobicity and separation based on non-specific adhesion can be difficult.

If the non-specific attractive forces are negligible, specific adhesion to a surface can provide a very selective means of separating cell populations. In this case, adhesion is mediated by interaction between an immobilized ligand and a receptor on the outer surface of a cell. The ligand-receptor bonds are the dominant interaction responsible for adhesion and their formation is often quite specific. Therefore, only populations that express a complimentary receptor for the immobilized ligand adhere to the surface. Antigen-antibody (Evans et al,
1969; Tlaskalova-Hogenova et al, 1986), lectin-carbohydrate (Edelman and Rutishauser, 1974; Hertz et al, 1985; Murray et al, 1987) and substrate-transport protein interactions (Ferenci and Lee, 1982; Roos and Hjortso, 1989b) are examples of ligand-receptor systems used to promote specific cell adhesion to a surface.

Using specific cell adhesion, cells that are otherwise similar can be separated if they differ in the expression or functioning of a single outer surface component. In this work, specific cell adhesion is employed to separate a disadvantaged, slower growing population in a mixed culture and recycle it back to the reactor. Two populations of Escherichia coli that differ in the expression of an outer surface transport protein, the maltoporin, are used. This protein, the lamB gene product, is part of the maltose regulon, and plays a role in the transport of maltooligosaccharides into the cell. It displays a binding specificity for maltooligosaccharides and starch (Ferenci et al, 1980, Ferenci et al, 1986; Benz et al, 1987). This interaction is used to specifically remove an E. coli population that expresses the maltoporin from the reactor by adhesion to a starch-Sepharose support.

8.3 Material and Methods

8.3.1 Organisms, Culturing and Support Preparation

The two strains of E. coli used in this study were 23716A and MCR106. The strain, 23716A is a population isolated from ATCC 23716 of the American Type Culture Collection as previously described (Roos and Hjortso, 1989b). It binds to the to the starch-Sepharose support under the operating conditions used in this work. It was transformed with the plasmid pRAH12, a wild type ColE1 plasmid with a neomycin resistance cartridge inserted in the sma1 site on the cec gene. This construct did not display colicin production in 23716A and was observed to be stably maintained. No plasmid loss was detectable over more than 50 residence times in continuous culture. Neomycin resistance was used as a marker for strain identification. Strain MCR106 was obtained from Dr. Silhavy (Princeton University). It is strain MC4100 (Benson and Silhavy, 1983) with the modification AlamB106. Due to this deletion, the strain did not produce a functional lamB gene product, the maltoporin, and did not adhere to the starch-Sepharose support under the conditions used in the experiment.

Cultures were maintained on L agar; tryptone, 10 g/l; yeast extract, 5 g/l; NaCl, 5 g/l; Bacto agar, 15 g/l. For the selective growth of 23716A, 50 mg/l of neomycin was added to the L agar. Liquid cultures were grown in M63 media (Miller, 1972) which consisted of M63 salts, KH₂PO₄, 13.6 g/l; (NH₄)₂SO₄, 2 g/l; FeSO₄·7H₂O, 0.5 mg/l; supplemented with MgSO₄·7H₂O, 1 mM; arginine, 20 mg/l; thiamine, 20 mg/l; and maltose, 1 g/l. The pH was adjusted to 7.0 with NaOH. All fermentations were performed at 37°C.
The competition experiments in continuous culture were started with inoculum from the shake flask cultures. This inoculum was always taken at the same point in the exponential phase of the growth curve as determined by optical density. This was done to minimize differences in culture dynamics due to differing physiological states of the inoculum.

One reactor used for continuous culture was a modified Bioflow (New Brunswick Scientific) with a liquid volume of 350 ml. A custom reactor, with a liquid volume of 146 ml, was also used. The volume of both reactors was maintained by media overflow. The reactors were operated at 37 °C with an air flow of 0.6 L/min, agitation at 400 rpm and a pH of 7.0. The feed stream was M63 media with 0.5 g/l maltose. The make-up stream for the reactors without recycle was composed of M63 salts, pH=7.0.

The support used for specific cell adhesion was starch-Sepharose, prepared by a modified method of Ferenci and Lee. The starch bound to the support was assayed using the method of Ferenci and Lee. There was 7.15 mg of starch coupled to 1 ml, settled volume, of Sepharose 6B (Pharmacia LKB).

8.3.2 Standard Adhesion Assay

A 1.0 x 20 cm chromatography column (Pharmacia LKB) with 20 μm nylon mesh bed support and flow controllers was packed with 1 ml of starch-Sepharose. The column and packing were washed with M63 salts, pH=7.0, at a flow rate of 0.5 or 0.75 ml/min. A pulse of cells was introduced into this stream and washed through the column. After non-adhering cells were washed from the column, specifically adhered cells were released from the support by elution with 0.2 M maltose in M63 salts. The same flow rate was used for specific elution as in the other steps. The column eluant was continuously monitored at 650 nm by a flow through spectrophotometer (Isco, V4). The area under the peaks for the eluting cells was determined and used to estimate the fraction of specifically adhering cells (Roos and Hjortso, 1989b).

8.3.3 Population Specific Recycle

The setup used for specific population recycle consisted of the reactor, the packed beds of starch-Sepharose for population separation, the buffer delivery system and the detector (Figure 8-1). Separation of the two populations was performed in one of two 1.0 x 20 cm chromatography columns (Pharmacia LKB) fitted with a 20 μm bed support and packed with 1 ml settled volume of starch-Sepharose. The eluant from the columns was directed into a flow through spectrophotometer (Isco, V4) and biomass concentration was continuously monitored as absorbance at 650 nm. The eluant stream was directed to either a waste container or back to the reactor. The buffer delivery system allowed either column to be washed with the wash buffer, M63 salts, the elution buffer, M63 salts with 0.2 M maltose, or sterilized using 1% formaldehyde.
Figure 8-1. Schematic of Selective Recycle Reactor.
The reactor and the buffer reservoirs with tubing and valves were steam sterilized at 15 psig for 20 minutes. The columns were packed with starch-Sepharose in M63 buffer and placed online. The columns, packing and the detector flow cell were then washed for one hour with 1% formaldehyde at a flow rate of 15 ml/h. During the wash period the flow was occasionally reversed to back flush the packing. The formaldehyde was left in the system for 4-8 hours. The columns were again washed with formaldehyde for 1 hour at a flow of 15 ml/h with occasional back wash. A large volume of sterile M63 salts was then washed through the column, packing and detector to flush all the formaldehyde from the system. This procedure was required to sterilize the population recycle system.

During operation of the specific recycle, a continuous stream was taken from the reactor and circulated through the sample loop. The residence time in this loop was on the order of 2 minutes. Samples were removed from this stream and introduced into the column at a flow rate of 0.75 ml/min. After the pulse was placed on the column, the cells were washed through the column with M63 salts buffer. The first peak to come off the column was composed of the non-adhering cells and was returned to the reactor. The specifically adhering cells were eluted with M63 salts buffer containing maltose, 0.2 M, and discarded. After this peak had eluted, the bed was washed with 10 to 15 volumes of M63 buffer to remove the maltose from the column. The separation was alternated between the two columns to allow sufficient time for column washing. This procedure allowed reactor samples to be separated on a column every 30 minutes.

8.3.4 Estimation of Population Balance

Samples were removed from the reactors periodically to determine the culture O.D. at 650nm and the population balance. Population balances were estimated from differential plates counts, making use of the plasmid coded neomycin resistance in strain 23716A. The population balance was determined online by integrating the O.D. peaks of the exit stream from the separation columns (Roos and Hjortso, 1989b).

8.4 Results

8.4.1 Column Operating Characteristics

For use in reactor control, the recycle stream from the columns should be highly enriched with respect to strain MCR106, the non-adhering and slower growing population. To enrich this stream, the populations must be separated in the adhesion column. The strains displayed the desired adhesion characteristics when placed on the column alone. Better than 90% of population 23716A was retained in the column under conditions of the standard adhesion assay. The adhering cells could be eluted from the column using M63 salts with 0.2 M maltose. The fraction of the population retained in the packing by specific adhesion was found to remain
steady or increase slightly when grown in a continuous culture with maltose as the carbon source. MCR106 eluted in the void volume of the column, displaying no retention to the packing. The effect of fluid velocity and cell loading on the separation of two strains was investigated.

The flow rate through the column, over a range of 0.5 to 0.75 ml/min, was found to influence the separation of the two populations (Roos and Hjortso, 1989b). At a flow rate of 0.5 ml/min, MCR106 was partially retained in the column with 23716A. This retention was minimal at a flow rate of 0.75 ml/min. The flow rate that yields the best separation is important in determining the amount of cells that can be recycled in a given period. Higher flow rates allow a larger volume of cell mixture to be separated in a given time. Therefore, cell separation was investigated over a wider range of flow rates.

A mixture of the cell populations was prepared that contained 40% 23716A, the population that is capable of specific adhesion to the support. A 0.75 ml sample of the mixture containing approximately 8x10^8 cells/ml was placed on the column. The cell addition and subsequent wash and elution were carried out at the desired flow rate. At 0.5 ml/min, a larger fraction of cells than expected are found to adhere and elute with maltose buffer (Figure 8-2). As the flow rate is increased, to 0.75 ml/min, the fraction of adhering cell decreases to the level that is expected. At higher flow rates, a smaller fraction of cells adhere than is expected.

A likely explanation of this behavior is that at a flow rate of 0.5 ml/min, the removal forces exerted by the fluid are insufficient to prevent non-specific cell-cell adhesion between MCR106 and 23716A. As the fluid velocity increases, fluid removal forces become sufficient to prevent non-specific adhesion. At still higher flow rates, the fluid forces become strong enough to alter the specific adhesion of 23716A. The flow rate of 0.75 ml/min, was found to give the best separation. At this flow rate the specific adhesion is essentially unaffected, while the non-specific adhesion appears to be minimal.

In order for the recycle strategy to work, the columns must be used repeatedly at high loadings without loss of separation capacity or plugging. The ability of the starch-Sepharose packed bed to separate the strain 23716A from MCR106 over a range of cell loadings was, therefore, investigated. At a constant flow rate of 0.75 ml/min, pulses of cell mixture of increasing duration were pumped onto the column. The pulse times ranged from 0.167 to 5.0 minutes or 0.125 to 3.75 ml of cell mixture. The total number of cells placed on the column ranged from 10^8 to 3x10^9 and contained a biomass fraction of approximately 0.31 strain 23716A. The loading did not significantly alter the separation of the two populations (Figure 8-3). Using the data from separations at all loadings the mean biomass fraction of adhering cells was 0.302 with a standard deviation of 0.072 compared to the expected value of 0.31. Good separation was obtained at high loadings for 15-20 repetitions.
Figure 8-2. Effect of Flow Rate on Separation.
Separation of MCR106 and 23716A as a function of volumetric flow rate through column. Expected fraction of population adhering was 0.40.

Figure 8-3. Effect of Cell Loading.
Separation of MCR106 and 23716A as a function of the duration of pulse addition to column. Flow rate was 0.75 ml/min. Expected fraction of population adhering was 0.31.
8.4.2 Estimation of Cell Population Balance

Two methods were used to estimate the population balance within the mixed culture reactors, differential plate counts and adhesion. It was reported that for competing populations in batch culture the two methods can yield different estimates of the population balance (Roos and Hjortso, 1989b). To determine if this happens during competition in a chemostat, estimates obtained by plate counts and adhesion were compared. For a reactor without specific recycle, a sample was removed and plate counts were performed. Sample pulses of 0.167 and 1.0 minutes were placed on the column and separation was carried out following the procedures of the standard adhesion assay at a flow rate of 0.75 ml/min. The population fractions of MCR106 and 23716A competing in continuous culture, determined by differential plate counts and adhesion, are shown in Figure 8-4. The population balance, as determined by the two methods, is in general agreement.

Figure 8-4. Continuous Mixed Culture Population Balance.
Mixed culture started by inoculation with 23716A at t=0 h. Dilution rate was 0.232 h\(^{-1}\). See Table 8-1 for specific reactor conditions.
8.4.3 Growth Rates of MCR106 and 23716A

The specific growth rates of the two E. coli strains 23716A and MCR106 were modeled by Monod growth kinetics;

\[ \mu_i = \frac{\mu_{\text{max}} S}{K_i + S} \]

where \( \mu_{\text{max}} \) is the maximum specific growth rate and \( K_i \) is the substrate saturation constant. These parameters were estimated from growth rates in batch culture using a Haynes plot (Davis and Parnham, 1989). A maximum growth rate of 0.525 h\(^{-1}\) and 0.75 h\(^{-1}\) and a saturation constant of 22.7 \( \mu M \) and 187 \( \mu M \) were determined for strain 23716A and MCR106 respectively. The specific growth rate curves cross at a maltose concentration of approximately 360 \( \mu M \). Conditions at which the specific growth curves cross and the cultures coexist could not be obtained experimentally. This is not surprising, as the calculated dilution rate for coexistence is close to the washout dilution rate for 23716A. In all experiments, MCR106 was the slower growing population and consequently, 23716A would dominate in a chemostat.

8.4.4 Population Specific Recycle

The operation of population specific recycle required periodic removal and addition of cells suspension from the mixed culture reactor. The operation is best described by considering the idealized flow diagram of the reactor (Figure 8-5, Table 8-1). The reactor was inoculated with MCR106 and allowed to reach steady state. The flow rates of the media inlet stream F1 and the make up stream, F4, determine the steady state. The make up stream flow rate was adjusted to yield the same average dilution rate for the reactors with and without recycle.
Figure 8-5. Flow Diagram for Selective Recycle Reactor.

Flow rates for the various streams and period of operation given in Table 8-1. Concentration of population MCR106 and 23716A are represented by $X_1$ and $X_2$, respectively. The concentration of limiting substrate in the inlet stream and the reactor are $S_0$ and $S$, respectively. Concentration of MCR106 and the limiting substrate in recycle stream back to the reactor is $B_{X_1}$ and $B_{S}$. $B_{X_2}$ is the concentration of 23716A in the waste stream.

After steady state was achieved, the population 23716A was inoculated into the reactor. In the experiments without specific recycle, the flow rates of the two inlet streams were not changed. For reactors with specific recycle, the make up stream $F_4$ was turned off after inoculation with population 23716A and the recycle was started. Samples not exceeding 2-3% of the reactor volume were removed and the population balance assayed by plate counts. For one of the reactors without recycle, population balance of this sample was also determined using the adhesion assay offline.

In an attempt to maintain MCR106, the slower growing population, non-adhering cells were recycled back into the reactor. During operation of the specific recycle, one cycle consisted of removing a volume of cell suspension from the reactor and introducing it as a pulse into the packed bed. Population 23716A adhered to the support and the non-adhering cells, population MCR106, were washed through the bed and back into the reactor. The cells that were adhering to the support were eluted and discarded.
Table 8-1. Reactor Operating Conditions

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Constant Volume</th>
<th>Variable Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>with recycle</td>
<td>without recycle</td>
</tr>
<tr>
<td>Average Dilution Rate&lt;sup&gt;1&lt;/sup&gt; $D_{avg} (h^{-1})$</td>
<td>0.192</td>
<td>0.191</td>
</tr>
<tr>
<td>Volume (ml)</td>
<td>350</td>
<td>350</td>
</tr>
<tr>
<td>F1 (ml/h)</td>
<td>56.8</td>
<td>56.8</td>
</tr>
<tr>
<td>F2 (ml/h)</td>
<td>45</td>
<td>0</td>
</tr>
<tr>
<td>F3 (ml/h)</td>
<td>45</td>
<td>0</td>
</tr>
<tr>
<td>F4 (ml/h)</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>T1-T0 (time of use, h)</td>
<td>0.05 (0-3.0)</td>
<td>0.083 (0-10.5)</td>
</tr>
<tr>
<td></td>
<td>0.067 (3.0-13.0)</td>
<td>0.1 (10.5-15.5)</td>
</tr>
<tr>
<td></td>
<td>0.083 (13.0-18.0)</td>
<td></td>
</tr>
<tr>
<td>T3-T2 (time of use, h)</td>
<td>0.167 (0-5.5)</td>
<td>0.162 (0-10.5)</td>
</tr>
<tr>
<td></td>
<td>0.184 (5.5-13.0)</td>
<td>0.167 (10.5-15.5)</td>
</tr>
<tr>
<td></td>
<td>0.2 (13.0-18.0)</td>
<td></td>
</tr>
<tr>
<td>T4 (h)</td>
<td>0.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>

<sup>1</sup>Average dilution rate calculated during recycle period based on average volumetric flow rate out of reactor. Calculated as:

$$D_{avg} = \left( F1 + \frac{(T3-T2)F3-(T1-T0)F2}{T4} \right) / Vol$$

The recycle period was started by removing cell suspension from the sample loop in stream F2 at time T0 and directing the stream through the column packed with starch-Sepharose (Figure 8-5, Table 8-1). At time T1, flow stream F2 was turned off and the cells were washed through the packing with M63 salts, flow stream F3. At T2, the leading edge of the pulse of non-adhering cells had reached V2, where non-adhering cells were directed back into the reactor. This addition was continued for the period (T3-T2). Column elute was then directed to a waste container and the adhering cells were eluted using a 0.2 M maltose buffer. The clock was reset at time T4 to T0 and a new cycle started using an alternate column. The eluants from both columns were
monitored and the biomass peaks recorded to obtain estimates of the population balance. Operation of the recycle in this manner resulted in a periodic forcing of the reactor through the dilution rate, the addition of the non-adhering population and the addition of substrate washed through the column.

Recycle operation results in the two types of reactor modes, constant volume (C.V.) and variable volume (V.V). In the constant volume reactor, the flow rate of stream F2 was less than the flow rate of stream F1. The reactor, operated with an overflow, remains at constant volume during the period of cell removal (T1-T0). The flow rate of stream F2 was greater than that of F1 in the variable volume reactor. The overflow becomes zero and the working volume decreases over the period (T1-T0).

Population dynamics for the constant volume recycle reactor were compared to those in a reactor without recycle and the same media inlet flow rate and time averaged dilution rate (Table 8-1). The population of MCR106, as determined by plate counts, was quickly washed out of the reactor without recycle (Figure 8-6). In the reactor with a specific recycle of the non-adhering cells, the population of MCR106 was maintained at a relatively constant level for approximately 12 hours, more than two residence times. After this period, the population of MCR106 decreased slowly. The recycle reactor retained MCR106 throughout the 18 hours of recycle operation. After the recycle was discontinued, the population level of MCR106 dropped quickly.

Strain 23716A is the dominant population and in the reactor without recycle the concentration of 23716A quickly increases (Figure 8-7). With the recycle operating, the population level of 23716A increases more slowly. The population level of 23716A continued to increase slowly after the recycle was stopped.

The total biomass in the reactors, measured as optical density at 650 nm (O.D.) changed over the course of the reactor runs (Figure 8-8). It decreased throughout most of the run in the reactor without recycle as MCR106 was washed out of the reactor. The O.D. increased slightly toward the end of the experiment as the population of 23716A took over the reactor. In the reactor with specific recycle, the O.D. increased initially, and then decreased through the rest of the experiment.
Figure 8-6. MCR106 Concentration in C.V. Reactor.
Concentration of MCR106 determined by plate counts. Results are from a constant volume reactor with recycle and a reactor with similar dilution rate without recycle. Mixed culture started by inoculation of population 23716A at t=0 h. Recycle started at t=0 h and stopped at t=18 h. Recycle operation described in Table 8-1. Error bars show standard deviation.

Figure 8-7. 23716A Concentration in C.V. Reactor.
Concentration of 23716A determined by plate counts. Results are from a constant volume reactor with recycle and a reactor with similar dilution rate without recycle. Mixed culture started by inoculation of population 23716A at t=0 h. Recycle started at t=0 h and stopped at t=18 h. Recycle operation described in Table 8-1. Error bars, which in some cases are hidden by data symbols, show standard deviation.
Biomass, as determined by O.D., for constant and variable volume reactors with recycle and reactors with similar dilution rate without recycle. Mixed culture started by inoculation of population 23716A and recycle started at t=0 h. For constant volume reactor, recycle stopped at t=18 h. In the variable volume reactor, recycle stopped at t=15.5 h. Recycle operation described in Table 8-1.

The drop in the O.D. was due to the washout of MCR106 and the slow increase in the population of 23716A. Another perspective of the effect of the recycle on the population balance is obtained by considering the fraction of MCR106 or 23716A in the total population (Figure 8-9). The strain MCR106 is maintained as a higher fraction of the total population in the reactor with recycle than is observed for the case without recycle. Upon discontinuing the recycle stream, the fraction of MCR106 quickly drops and 23716A increases to a much higher percentage of the population.
Figure 8-9. Population in C.V. Reactor by Plate Counts.

Population balance is determined as a fraction of the total population using plate counts. Results are for a constant volume reactor with recycle and a reactor of similar dilution rate. Mixed culture started by inoculation of population 23716A and recycle started at t=0 h. Recycle stopped at t=18 h. Recycle operation described in Table 8-1.

Similar behavior was observed in the recycle reactor with variable volume. The culture O.D. increased slightly after the recycle was started and then decreased slowly (Figure 8-8). A reactor without recycle, but with a similar dilution rate (Table 8-1) displayed a decrease in the O.D. which leveled off toward the end of the experiment.

The fraction of the two populations in the reactors was determined by plate counts and the adhesion assay (Figure 8-10 and 8-11). Both estimation methods indicate MCR106 is maintained as a higher fraction of the total population in the reactor with specific recycle compared to the case without recycle. The dominant strain, 23716A, was observed to take over the reactor more quickly for the case without recycle.
Population balance is determined as a fraction of the total population using plate counts. Results are for a variable volume reactor with recycle and a reactor of similar dilution rate. Mixed culture started by inoculation of population 23716A and recycle started at t=0 h. Recycle stopped at t=15.5 h. Recycle operation described in Table 8-1.

The total area of the eluted cell peaks for both recycle reactors was compared to the culture O.D. The total peak area is an estimate of the biomass passing through the column. This comparison can be used to monitor the recovery of cells from the bed over the time the recycle is operating. For the recycle reactor with constant volume, the total peak area closely followed the trend of the culture O.D. In the variable volume reactor with recycle there is evidence that at about 10 to 11 hours into the run, a fraction of the biomass placed on the columns was not being eluted. The total peak area dropped off more quickly than culture O.D. Despite previous results that column fouling should not be a problem under these operating conditions, it appears that cells were becoming entrapped in the packing. This entrapment became worse as the run progressed and the recycle was discontinued at 15.5 hours.
Figure 8-11. Population of V.V. Reactor by Adhesion.

Population balance is determined as a fraction of the total population using adhesion data. Results are for a variable volume reactor with recycle and a reactor of similar dilution rate. Mixed culture started by inoculation of population 23716A and recycle started at t=0 h. Recycle stopped at t=15.5 h. Recycle operation described in Table 8-1.

8.5 Discussion

Separating populations with specific adhesion allowed a particular population to be recycled into the reactor. In this manner, the population balance was directly influenced. To compare the population dynamics between reactors with and without recycle, the recycle, though operated in a discontinuous manner, was treated as continuous. For both recycle reactors reported in this work, the period for the discontinuous recycle was a half hour. The constant volume reactor experienced the perturbation in the dilution rate and addition of the recycled population at a frequency of approximately 10 per residence time. The frequency in the variable volume reactor was slightly lower, 8 per residence time. In both cases, the average residence time was used. The high frequency of the recycle was used to justify comparison of the reactors with recycle to those without recycle, based on the average dilution rate. For both recycle reactors studied, the disadvantaged population was maintained as a larger fraction of the total population than in a comparable reactor without recycle.
Reactor models using the assumptions of continuous recycle and Monod growth kinetics gave fairly good prediction of the effect of recycle in the constant volume reactor. They did not describe the behavior of the variable volume reactor very well. The models were modified to account for the discontinuous nature of the recycle and the finite time required for a cell to respond to shifts in culture conditions (Stephens and Lyberatos, 1987). These modified models resulted in better qualitative fit of the reactor behavior. However, the parameters that describe culture adaption were not determined independently but by fitting the reactor data. Thus, the capability of the model to describe the reactor behavior may be due simply to the increase in the number of adjustable parameters.

The key point in operating the recycle reactors was the ability to enrich the fraction of a desired population in a recycle stream. In this case specific adhesion based on the expression of the maltoporin was used to separate the cell populations. Only toward the end of the run in the variable volume reactor did separation suffer. This is not a problem with the control algorithm, but with the design of the separation column. In this case, separation in the column declined as the column became fouled. The effect of the recycle decreases as a fraction of the non-adhering cells are not returned to the reactor. A decrease in the recycle of non-adhering cells would cause a drop in the fraction of MCR106 in the variable volume reactor as observed using the plate counts (Figure 8-11). Both the decrease of population MCR106, as determined by plate counts, and the simultaneous fouling of the packed beds could be caused by increase aggregation of MCR106.

The fraction of MCR106 in the recycle reactor population, estimated from the adhesion data, is consistently higher than the fraction determined using plate counts. With the exception of the samples from the first several hours, the same is true for the reactor without recycle operating at a dilution rate of 0.232. The results of the standard adhesion assay at different loading rates indicate a slight over-estimation of the fraction of MCR106 in a population. This could account for the difference between the two estimates in a reactor. The difference could also, in part, be due to the effect of cell aggregation on the plate counts (Roos and Hjortso, 1989b). Both methods of estimating population, however, show that specific recycle of cells has the same qualitative effect on reactor dynamics.

In general, specific cell adhesion could prove to be a versatile method for separating populations and allowing preferential removal or retention of a desired population. In our investigation with mixed populations, specific adhesion proved an effective means of rapidly separating large quantities of the two populations.
Specific adhesion for cell separation is potentially applicable to any group of populations that display a
difference in their cell outer surface structure. These surface differences may arise from different species or
strains of organisms. The expression of the surface component that interacts with the immobilized ligand may
be under plasmid coded control. Plasmid-bearing and plasmid-free populations would express different levels
of the cell surface component. Population separation by specific adhesion could then be used to control unstable
recombinant cultures (Roos and Hjortso, 1989a). To separate populations using specific adhesion it is not
necessary that one population express a surface component for adhesion while the other does not. One theory
of specific cell adhesion in a hydrodynamic flow indicates that a large difference in adhesion is expected for
a population that expresses half of the surface component of the adhering type (Hammer and Lauffenburger,
1987). Analysis of the selective elution of adhering cells also suggests this method may be applied to separate
populations with similar expression levels of the surface component (Roos and Hjortso in preparation).

A variety of reactor designs that employ specific cell adhesion in the control of the population balance are
possible. In this work, a packed bed was used to separate the populations. With this design either population
could be recycled. Separation via specific adhesion is not limited to packed beds. Good separation with
minimal physical entrapment may be possible if fluidized beds or tubular systems such as hollow fibers were
used. In any of these systems, specific adhesion would allow large numbers of cells to be rapidly separated.
Incorporation of a support for adhesion of the disadvantaged population in a reactor allows for a simple reactor
design that is still capable of altering the mixed culture population balance (Roos and Hjortso, 1989a). The
complete range of reactor designs that use specific cell adhesion is readily applied to the control of any type
mixed culture. With this means of directly manipulating a particular population, it is possible to alter the
population balance in almost any mixed culture. This control tool could prove valuable for the investigation
of population interactions in a mixed culture. It could also allow the maintenance of novel mixed cultures and
provide a means of controlling mixed culture yields and conversions.

8.6 References

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CHAPTER 9
ALTERNATIVE SYSTEMS FOR USE OF
SELECTIVE RECYCLE VIA SPECIFIC ADHESION

9.1 Abstract

In mixed culture reactors, specific cell adhesion provides a means to operate the reactor with population
selective recycle or removal. This concept was demonstrated in previous work. Presented in this chapter are
preliminary results of applying specific adhesion for reactor control of other mixed microbial cultures. One
method is aimed at the control of unstable recombinant cultures. In this system, the interaction between the
immobilized ligand and the cell surface receptor, that can lead to adhesion, also proves fatal to cells that do
not posses a particular plasmid. Also included in this chapter are reports on the characteristics of various other
mixed culture that could be controlled using the interaction between a population and a starch-sepharose
support. In this work, the suitability of the mixed cultures for use in reactors with specific cell adhesion was
investigated. The advantages and limitations of the various mixed culture systems are discussed.

9.2 Immobilized Colicin E1 for use in
Control of Unstable Recombinant Cultures

9.2.1 Introduction

Today, genetically engineered organisms can be made that will produce a wide range of commercially
important compounds. This potential is widely recognized and substantial work has been directed toward
understanding and manipulating growth kinetics of recombinant organisms so that their use may become
practical on an industrial scale (Adams et al, 1979; Ollis, 1982; Skogman et al, 1983; Hjortso and Bailey,
1984; Ryder and DiBiasio, 1984; Lauffenburger, 1985; Pierce and Gutteridge, 1985; Siegel and Ryu, 1985;
An important aspect of recombinant cell behavior, that is of particular importance in this context, is plasmid
instability. Plasmids carrying the recombinant genes can be lost through segregative (Pierce and Gutteridge,
1985) or structural instability (Kadam et al, 1987). These revertant cells are formed continuously and can
proliferate to the extent that they, and not the desired recombinant cells, are the dominant cell population.

An obvious approach to minimize this deactivation problem, is to subject the populations to selective
pressure that inhibits or kills the revertant cells. This could be done by using a host cell that exhibits better
growth with the assistance of the product from a plasmid borne gene. For instance, the plasmid could carry a
gene that confers antibiotic resistance or allows the synthesis of an essential growth factor not present in the media. Antibiotic selection has been applied successfully (Pierce and Gutteridge, 1985). This method, however, is expensive and removal of antibiotic from the final product is a potential problem. Employing a media that lacks an essential growth factor works well under laboratory conditions (Skogman et al, 1983), but defined media are required for this technique and costs could prove prohibitively high for large scale cultivation.

Other strategies for maintaining productivity in recombinant cultures have been suggested. Siegel and Ryu (Siegel and Ryu, 1985) used a two stage system in which recombinant cells were grown in the first stage under conditions that gave minimal plasmid loss. Conditions in the second stage were set for maximum product formation. Ryder and DiBiasio (Ryder and DiBiasio, 1984) modeled a system in which the plasmid relieved substrate inhibition in the host cell. It was determined that stable coexistence between the plasmid-bearing and plasmid-free populations could be achieved using feedback control. The incorporation of bacteriocin production and immunity on a plasmid was used as a method to maintain high levels of plasmid-bearing cells in a chemostat (Adams et al, 1979). The bacteriocin, produced by the plasmid bearing cells, selectively inhibits growth of the plasmid-free cells. The plasmid bearing cells are minimally affected due to the immunity coded on the plasmid. The system where an unstable plasmid is responsible for bacteriocin production and immunity has been analyzed (Lauffenburger, 1985; Kubota et al, 1987) and the necessary conditions for stability of the recombinant culture determined. Differences in the adaption times of plasmid-bearing and plasmid-free populations have been explored as a means to maintain plasmid-bearing populations in continuous reactors (Stephens and Lyberatos, 1988; Impoolsup et al, 1989; Weber and San, 1989). This approach entails the use of periodically forced reactors to exploit the different adaption rates.

Ollis (Ollis, 1982) suggested that selective recycle of plasmid-bearing cells could provide a means of maintaining this population in a reactor if a method could be developed for separating cells with and without plasmids. This separation is clearly not easy to achieve. The difference in phenotype between cells with and without plasmids are expected, in most cases, to be quite subtle, rendering classical cell separation methods too inefficient to be of practical value. However, cell affinity chromatography has emerged as a novel method for separating cell populations (Hertz et al, 1984). In this method cells are separated by adhesion based on the expression of a cell surface receptor. The crucial aspect of the separation method is its specificity.

Cells adhesion is the result of bond formation between an immobilized ligand and a cell surface receptor. The bonds thus formed, can be highly specific and adhesion is based on such subtle attributes as the macromolecules, receptors, expressed on the cell outer surface. The receptor expression could be placed under the control of the plasmid in the recombinant culture. Separation of plasmid-bearing and plasmid-free populations
would be possible based on the plasmid coded adhesion characteristics. For control of the population balance, reactors with selective recycle or retention would be employed. These reactor configurations were discussed in previous chapters. An alternative control strategy for unstable recombinant populations, based on the formation of the discrete bonds between the receptors and the surface, is introduced here.

In this scheme, both the plasmid-free and the plasmid-bearing cells adhere to a support through specific ligand-receptor interactions. However, only the plasmid-bearing population remains viable after adhesion. The ligand-receptor interaction that leads to adhesion selectively kills the plasmid-free population. Such a system could be realized employing colicin E1 as the immobilized ligand and *Escherichia coli* as the host organism. Colicin E1 is coded for by the ColEl plasmid, and in free solution it will bind to the vitamin B12 receptor of *E. coli*, the *btuB* gene product (Braun and Hantke, 1981), and eventually cause cell death in sensitive cells. The action of the colicin E1 is to depolarize the cell membrane (Luria and Suit, 1987). Colicin E1 has previously been immobilized on Sephadex beads (Lau and Richards, 1976), and was found to retain its specificity and its ability to induce death in sensitive cells. Immunity to the action of colicin E1 is conferred by the product of the *imm* gene on the ColEl plasmid. Because both the gene that codes for colicin formation and immunity are located on the same plasmid, they are easily accessible for manipulation.

This strategy for eliminating plasmid-free cells is different from the commonly used method of antibiotic selection. Here the bacteriocin, colicin E1, is not in free solution, but immobilized under conditions such that leakage is insignificant (Lau and Richards, 1976). Therefore, the colicin could be used repeatedly. The expense incurred when applying antibiotic to each fermentation batch, as well as the concern over the presence of antibiotic in the final product is minimized.

In this investigation, several aspects of the use of immobilized colicin were addressed. The methods for production and purification of colicin E1 were tested (see methods). The immobilization of colicin E1 to two different supports and the adhesion of *E. coli* to the supports was investigated. Also, a plasmid was constructed for use in investigations of reactor dynamics.

### 9.2.2 Material and Methods

The *E. coli* strains used in this study were GW1000, a gift from Dr. G. Zubay, Columbia University, and ATCC 23716, a K12 wild type obtained from American Type Culture Collection. GW1000 contained a wild type ColEl plasmid and was used for production of colicin E1. The strain ATCC 23716, transformed with the plasmid, pRAH12, was used in the studies of cell adhesion to immobilized colicin E1.
The plasmid pRAH12 was a ColE1 wild type plasmid containing a kanamycin resistance marker. The kanamycin resistance GenBlock (Pharmacia LKB) was inserted in the *smal* site of the *cea* gene using standard techniques. The *cea* gene codes for colicin E1 formation. The direction of transcription of the kanamycin cartridge was opposite that of the *cea* gene. When 23716 was transformed with this plasmid, colicin E1 was not detected and 23716 pRAH12 displayed colicin E1 immunity. The cells were cultured in M63 supplemented with 1 g/l maltose, pH=7.0, at 37 °C. Cultures grown overnight were used for the adhesion experiments.

Colicin E1 was produced as described by Spangler (Spangler et al, 1985) and purified from a cell lysate using a modified method of Cleveland (Cleveland et al, 1983). This procedure yields a solution that contained about 78% colicin E1 as determined by SDS-PAGE. The colicin was immobilized in one case to React-Gel 6x CDI activated support (Pierce). In the other case, CDI activated Sephadex was used (Hearn, 1987). The colicin E1 and the activated support were suspended in a 0.1 M borate buffer, pH=8.5 and the coupling performed in polypropylene tubes at 4°C for 48 hours. From the difference in protein concentration of the buffer before and after coupling, it was determined that there was 2.5 mg of colicin E1 immobilized per ml of Sepharose and 0.4 mg immobilized per ml of Sephadex.

Cell adhesion studies were performed as previously described (Roos and Hjortso, 1989) in a packed bed of the immobilized colicin support. The wash buffer was M63 salts, pH=7.0. Vitamin B12 in the wash buffer was used as a soluble ligand for specific inhibition of adhesion or for specific elution of adhering cells.

### 9.2.3 Results and Discussion

The adhesion characteristic of *E. coli* 23716 to the supports with immobilized colicin E1 was tested in packed beds. The cells were introduced into a column packed with either colicin E1-Sepharose or colicin E1-Sephadex, and washed through the column at constant fluid velocity. This experiment was repeated at several fluid velocities. For a range of superficial fluid velocities, 0.637-0.318 cm/sec, there was no measurable cell retention in the bed for either support. All the cells applied to the column were eluted in the wash buffer.

The shape of the cell peaks washed out of the colicin E1-Sephadex packed beds, suggest that there is interaction between the cells and the support. The peak for cells added to a column packed with Sephadex that had been activated and then allowed to hydrolyze, is shown in Figure 9-1. Also displayed are peaks for cells washed off a column packed with colicin E1-Sephadex. These peaks are more spread out and in one case there appears a bimodel shape. When cells are added to the column containing colicin E1-Sephadex and a wash buffer is used that contains vitamin B12, the peak shapes revert back to the shape recorded for packing without colicin E1. A similar broadening of the wash peaks was observed when the column was packed with colicin E1-Sepharose.
Adhesion to colicin E1-Sephadex was also tested in a stop-flow system. The cells were pumped into the packing and the fluid flow was turned off. After 1 or 5 minutes in the packing the flow through the column was resumed. For both cases no cell retention was observed. After four cycles of this stop-flow operation where the cells were allowed to sit in the packing for one minute, the column was washed with M63 buffer containing 2 μg/ml vitamin B12. No cells were eluted from the packing.

The peak shape of cells washed off Sephadex with and without immobilized colicin E1 (Figure 9-1) suggests that there is interaction between the cells and the colicin E1-Sephadex packing. Vitamin B12 binds to the same receptor on the cell surface as colicin E1 and is reported to block the receptor making it inaccessible for colicin E1 binding (DiMasi et al, 1975). The restoration of the peak shape of cells applied to colicin E1-Sephadex in the presence of vitamin B12 supports the hypothesis that the broadening of the cell peaks is due to specific

Figure 9-1. Cell Peaks Washed off Colicin E1-Sephadex.

Two cell pulses were placed on each column. The wash buffer for colicin E1-Sephadex with B12 contained 2 μg/ml vitamin B12. The superficial velocity of the buffer washing the cells through the packing was 0.42 cm/min.

Adhesion to colicin E1-Sephadex was also tested in a stop-flow system. The cells were pumped into the packing and the fluid flow was turned off. After 1 or 5 minutes in the packing the flow through the column was resumed. For both cases no cell retention was observed. After four cycles of this stop-flow operation where the cells were allowed to sit in the packing for one minute, the column was washed with M63 buffer containing 2 μg/ml vitamin B12. No cells were eluted from the packing.

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interaction between the cells and the immobilized colicin E1. This interaction does not allow cells to be retained on the support under continuous flow conditions. It is also does not lead to measurable adhesion of cells under the conditions of the stop-flow experiments.

It has been reported that *E. coli* adheres to colicin E1 immobilized on Sephadex (Lau and Richards, 1976). In this report, there was 0.8-1.5 mg of colicin E1 immobilized on 1 ml of Sephadex. This is a higher surface density than used in this work and could result in the observed difference in adhesion. It has been reported that the surface density of immobilized ligand can dramatically affect specific adhesion of cells (Rutishauser and Sachs, 1975; Weigel et al., 1979). In the report of Lau and Richards, adhesion was carried out by placing the support in a cell suspension and mixing for one hour. Binding was reported to be substantial. This procedure could also be a factor contributing to the difference in cell adhesion. It has been observed that a population that readily adheres when placed in suspension with a support, displays low retention in a packed bed (Roos and Hjortso, 1989).

A possible explanation for this behavior can be advanced considering the results of other specific cell adhesion experiments. The number of cell surface receptors (Roos and Hjortso, 1989) and the fluid forces on the cell (Hertz et al., 1985; Roos and Hjortso, 1989) have been reported to alter the ability of a cell to adhere in a specific manner to a support. In this study, the fluid forces on the cells appear to large to allow specific cell adhesion, an insufficient number of colicin E1-receptor bonds form to retain the cells on the surface. The number of bonds is determined by the number of cell surface receptors and the number of immobilized ligand that are in the contact area between the cell and the support. The total number of vitamin B12 binding sites on *E. coli*, the colicin E1 receptor, has been reported to be on the order of 200 (White et al., 1973). This is several orders of magnitude lower than the expression of maltoporin, a receptor that mediates specific adhesion of *E. coli* under similar fluid velocities (Roos and Hjortso, 1989). This suggests that higher surface densities of immobilized colicin E1 would be required to yield adhesion of *E. coli* to the immobilized colicin E1 under fluid flow conditions observed in the packed bed.

9.2.4 References


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9.3 Mixed Culture Reactor Dynamics

9.3.1 *Escherichia coli* 23716 and *Saccharomyces cerevisiae*

The competitive mixed culture of *E. coli* and *S. cerevisiae* posses several attributes that make it attractive as a model system to study the interactions and control of mixed cultures. The yeast, *S. cerevisiae*, is significantly larger than the bacteria, *E. coli*. This allows determination of the mixed culture population balance using a particle counter with size discrimination (Davison and Stephanopoulos, 1986). The specific growth rate of *E. coli* is reported to decrease with pH over the range from 6 to 4, while the specific growth rate of *S. cerevisiae* is relatively unaffected (Davison and Stephanopoulos, 1986). Manipulating the culture pH is a convenient means to alter the growth rate difference between the two populations. At the higher pH range *E. coli* will dominate the mixed culture. As the pH is lowered a value is reached where the specific growth rate of *E. coli* becomes less than *S. cerevisiae* and *S. cerevisiae* becomes the dominant population. The mixed culture of *E. coli* and *S. cerevisiae* is also particularly well suited for the purpose of reactor control using specific cell adhesion. There are unique ligand-receptor systems that can be employed to yield specific adhesion of either population (Ferenci and Lee, 1982; Horisberger, 1976)
9.3.2 Results and Discussion

Continuous cultures were performed with *E. coli* and *S. cerevisiae* in mixed culture using the defined yeast media described in Material and Methods, Chapter 2. The mixed culture experiments were performed by allowing the *S. cerevisiae* to reach a steady state then inoculating the culture with *E. coli*. The population level was determined using a particle counter with the size cut off set to discriminate between the two populations. An operating condition for the mixed continuous culture was identified that was close to conditions of the unstable coexistence steady state (Figure 9-2). Identification of this state was important because operating at the same conditions but with a lower pH should yield a mixed culture in which *S. cerevisiae* dominates. Using a higher pH should cause *E. coli* to dominate the continuous mixed culture.

![Graph showing concentration over time](image)

**Figure 9-2. Mixed Culture of *S. cerevisiae* and *E. coli*.**
Continuous culture of *S. cerevisiae* and *E. coli* in competition. *E. coli* inoculated at t=0 h. Reactor conditions; pH=5.6; T=26°C; D=0.16 h⁻¹. Defined yeast media used with 1 g/l glucose as carbon source.

In an attempt to separate the two populations, the specific adhesion of *E. coli* to a starch-Sepharose support was employed. The characteristics of this separation were studied using the batch adhesion technique reported in Chapter 2. For this experiment the organisms were grown in shake flasks on the defined yeast media supplemented with 1 g/l maltose. *S. cerevisiae* did not adhere to the starch-Sepharose after a 10 minute contact.
*E. coli* did adhere to the support (see Chapter 3). When the two populations were mixed, and then added to the starch-Sepharose support, adhesion of *E. coli* was detected as determined by the decrease in the number of cells in suspension (Figure 9-3). There was also a dramatic decrease in the number of *S. cerevisiae* in solution.

**Figure 9-3. Batch Adhesion of *S. cerevisiae* and *E. coli*.**

Batch adhesion on starch-Sepharose performed at pH=6.1. Population balance determined by particle counts with size discrimination.

This coadhesion was believed to be caused by interactions between the two populations. It has been reported that *E. coli* adhere to yeasts through the interaction of pili on *E. coli* and the structural carbohydrates in the cell wall of yeasts (Ofek et al, 1977; Jann et al, 1981; Nowicki et al, 1985; Goochee et al, 1987). The expression of pili by 23716 was not verified by other means, but their existence would explain the experimental results. If pili expression leads to cell-cell adhesion, the *E. coli* would adhere to the *S. cerevisiae* during the initial mixing of the two populations. The *S. cerevisiae*, coated with *E. coli*, then competes with the *E. coli* in suspension for the adhesion sites on the support. If this is the dominant mechanism that leads to the adhesion of both cells to the support, the populations are not suitable for use in a reactor controlled by specific adhesion. However, employing a strain or population of *E. coli* that does not express pili, should alleviate the problem of coadhesion (Goochee et al, 1987).
9.3.3 Mixed Cultures of E. coli

The dynamics of two strains of E. coli in a mixed culture were investigated. The purpose of the work was to identify culture conditions at which the relative difference between the specific growth rates of the populations becomes small. Control of the mixed culture reactor using selective retention or recycle, requires separation of a smaller fraction of the total population as the relative difference in specific growth rate decreases. This operational simplification would facilitate investigations of this control method.

Two mixed cultures were studied. The mixed culture of MCR106 and pop3132 was grown on glucose as the carbon source. This is possible since maltoporin expression in pop3132 is not dependent on induction with maltose. Because high levels of maltoporin expression in strain 23716 pRAH12 must be induced, the mixed culture containing this strain were performed using maltose as the carbon source. For both systems, the properties that allow separation of populations by specific adhesion to starch-Sepharose are unchanged.

9.3.4 E. coli Strains MCR106 and pop3132

The two strains of E. coli, MCR106 and pop3132, are derived from the same parent strain MC4100 (personal communication, Dr. Silhavy, Princeton University). Strain MCR106 carries a deletion in the lamB gene, while pop3132 constitutively expresses the malT gene product. This results in expression of the maltoporin, the lamB gene product, at high levels independent of the presence of an inducer. The difference in expression and function of the lamB gene product between the two populations effects there relative specific growth rates on maltose. At low maltose concentrations, pop3132 would dominate a mixed culture. Because of its high expression of a functioning maltoporin, the specific growth rate of pop3132 under these conditions is expected to be much higher than MCR106 and dominate the reactor after a few residents times. This would necessitate the removal or retention of large fractions of the desired population from the reactor. As a system to explore the reactor control, this mixed culture is expected to behave much the same as 23716 and MCR106 (see Chapter 8). It is desirable, therefore, to identify operating conditions that yield specific growth rates for the two populations that are more nearly the same.

The approach taken to decrease the relative difference in specific growth rates between the two populations was to minimize the growth advantage constitutive expression of the maltoporin confers to pop3132. This was done by changing the carbon source to glucose. The maltoporin is still expressed in pop3132 due to the constitutively mutation in malT. This expression does confers a growth advantage to pop3132, however, the relative difference in specific growth rates is expected to be decreased. The reasoning was that cells grow very well on low concentration of glucose without induction of the maltoporin, therefore, its expression would have minimal effect on growth. To determine if this reasoning leads to the desired mixed culture behavior, a con-
Continuous mixed culture was performed using M63 supplemented with 0.5 g/l glucose. A high dilution rate, 0.74 h\(^{-1}\), was used. The steady state at a high dilution rate is expected to have a higher concentration of glucose present than observed at a lower dilution rate. A kanamycin marker in Tn5 was introduced into the strain pop3132 using the method described in Materials and Methods (Chapter 2) for Tn5 mutation of the CoIE1 plasmid. This marker was used to identify the two populations. The reactor contained a well made of nylon mesh. In later studies using this reactor, a biofilm was observed to form on this well. This biofilm formation was not detected in this run.

The competitive mixed culture was initiated by allowing MCR106 to reach steady state in monoculture. The inoculum of pop3132 was obtained from a batch culture in exponential growth. The state of this culture as determined by optical density was the same as that used in competition experiments between MCR106 and 23716. After inoculation of pop3132, the concentration of each population in the reactor remains relatively constant for about 30 hours, approximately 22 residence times (Figure 9-4). After this period, pop3132 begins to dominate the reactor and by 49 hours after inoculation the concentration of pop3132 has increased almost ten fold.

![Figure 9-4. Mixed Culture of MCR106 and pop3132.](image)

Continuous mixed culture of MCR106 and pop3132 grown on M63 with 0.5 g/l maltose. Reactor conditions: \(D=0.74\) h\(^{-1}\); pH=7.0; \(T=37\) °C. Reactor containing MCR106 at steady state was inoculated with pop3132 at \(t=0\) h. 95% confidence levels displayed for all points.
Under the reactor conditions used in this experiment, specific growth rates of MCR106 and pop3132 appear to be similar, the population balance remained almost constant for about 20 residence times. These conditions could prove ideal to explore the application of specific retention or recycle of a population as a reactor control method. The growth advantage of the maltoporin may be further minimized, if not eliminated by using glycerol as the carbon source.

9.3.5 MCR106 and 23716

During mixed culture cultivation at low maltose concentration, 23716 pRAH12 expresses a growth advantage over MCR106 due to the induced levels of maltoporin. The specific growth rates of these organisms can be represented by the Monod model. For growth on M63 with maltose at pH=7 and T=37°C, the maximum growth rate was calculated to be 0.525 and 0.75 \( \text{h}^{-1} \) and the Monod saturation constant was calculated to be 22.7 \( \mu \text{m} \) and 187 \( \mu \text{m} \) for 23716 pRAH12 and MCR106 respectively. The Monod model predicts that at sufficiently high maltose concentration, MCR106 becomes the dominant organism. At this point, the growth advantage of the maltoporin expression for 23716 pRAH12 would be overcome by the higher maximum growth rate of MCR106. Using the Monod model for specific growth rate and the derived parameters, the reactor operating conditions at which this shift occurs are predicted to be at a dilution rate of 0.49 \( \text{h}^{-1} \). To investigate whether MCR106 dominates the reactor at dilution rates greater than 0.49 \( \text{h}^{-1} \), competitive mixed cultures were performed at high dilution rates. The procedure for performing these experiments was the same as used for other competitive mixed cultures of MCR106 and 23716 as reported in Chapter 8.

Reactor conditions were not found that resulted in MCR106 dominating the reactor. In a continuous culture with a dilution rate of 0.55 \( \text{h}^{-1} \), it appeared that a coexistence state was obtained (Figure 9-5). The concentration of 23716 pRAH12 and MCR106 remain relatively constant for over 12 hours of reactor operation, more than 6 residence times. This is interesting since the dilution rate of the reactor is above the predicted washout dilution rate for 23716 pRAH12. Either the limited accuracy of the calculated model parameters should be questioned or the reactor was not monitored for a sufficient period for washout to occur.
Figure 9-5. MCR106 and 23716 at High Dilution Rate.
Continuous mixed culture of MCR106 and 23716 pRAH12 grown on M63 with 0.5 g/l maltose. Reactor conditions; \( D=0.55 \text{ h}^{-1} \); \( pH=7.0 \); \( T=37 \text{ °C} \). Reactor containing MCR106 at steady state was inoculated with 23716 pRAH12 at \( t=0 \text{ h} \). 95% confidence levels displayed for all points.

9.3.6 Summary

The results of the continuous reactors containing mixed cultures of *E. coli* indicate, that for both mixed cultures investigated, conditions can be set to minimize the growth advantage conferred by expression of the maltoporin. In the experiment with strains MCR106 and pop3132, a coexistence state was maintained for more than 20 residence times before pop3132 began to dominate and MCR106 was washed out of the reactor. Over the limited period that the mixed culture of MCR106 and 23716 pRAH12 was observed, conditions allowed maintenance of a coexistent state. However, the ultimate fate of the culture is unknown.

One important conclusion can be drawn from these results. Reactor conditions can be manipulated to decrease the relative difference in the specific growth rates. This allows mixed culture population balances to be altered by exerting less "pressure" in reactors with selective retention or removal of a population. Under these conditions the control of the reactor could be investigated without the added complication of separating a large fraction of the reactor cell population.

9.3.7 References


VITA

On June 7, 1959 in Belleville, Illinois Joseph W. Roos was born, son of Liz and C. William Roos. He attended grade school and high school in Belleville before enrolling at the University of Notre Dame. Upon graduation, after living through several beautiful South Bend winters, he pledged to leave the north behind and enrolled in the Chemical Engineering Department at Rice University< Houston Texas. At Rice he met Wendy L. Firmin, the department secretary. He graduated from Rice with his M.S. and enrolled in the Ph.D. program at Louisiana State University. Within a year he married Wendy. This proved a wise decision as he probably would have never obtained his degree without her support.
DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Joseph William Roos

Major Field: Chemical Engineering

Title of Dissertation: Specific Cell Adhesion and Its Application to Monitoring and Control of Mixed Culture Bioreactors

Approved:

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Major Professor and Chairman
Dean of the Graduate School

EXAMINING COMMITTEE:

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Date of Examination:

December 4, 1989