

**EFFECTS OF IMMOBILIZATION
ON THE METABOLISM OF YEAST**

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Abstract

The composition and kinetic properties of *Saccharomyces cerevisiae* were found to be substantially different when the cells were immobilized on gelatin. Batch fermentation experiments conducted in a gradientless reaction system allowed comparison of immobilized cell and suspended cell performance.

In complete nutrient medium, the specific rate of ethanol production by the immobilized cells was 40-50% greater than for the suspended yeast. The immobilized cell consumed glucose twice as fast as the suspended cells, but their specific growth rate was reduced by 45%. Yields of biomass from the immobilized cell population were lower at one-third the value for the suspended cells.

Cellular composition was also affected by immobilization. Measurements of intracellular polysaccharide levels showed that the immobilized yeast stored larger quantities of reserve carbohydrates and contained more structural polysaccharide than suspended cells. Flow cytometry was used to obtain DNA, RNA and protein frequency functions for suspended and immobilized cell populations. These data showed that the immobilized cells have higher ploidy than cells in suspension. The level of stable, double-stranded RNA in immobilized cells was only one-quarter that measured for suspended cells. The observed changes in immobilized cell metabolism and composition may have arisen from disturbance to the yeast cell cycle by cell attachment, causing alterations in the normal patterns of yeast bud development, DNA replication and synthesis of cell wall components.

Recovery from hydroxyurea-induced DNA synthesis inhibition was indicated by measurements of growth rate, DNA content and light scatter properties. The immobilized cells quickly developed an effective means for overcoming hydroxyurea-induced inhibition of replication, and proceeded to synthesize large

amounts of DNA while still in the presence of the inhibitor. Stable RNA levels for immobilized cells remained low at 25% of the measured quantity for hydroxyurea-treated suspended cells. Synthesis of protein and RNA was not adversely affected in either cell type. Suspended cell protein pools increased by a factor of 1.8 following inhibition, while the immobilized cells contained 2.6 times the level of protein before hydroxyurea treatment.

Fermentation properties of immobilized and suspended cells were changed by hydroxyurea. The specific rate of ethanol production by immobilized cells increased by an average of 24%, while, for the suspended cells, specific productivity was up to three times higher. Glucose consumption rates for both cell types also increased under the influence of hydroxyurea. Suspended cell polysaccharide content was reduced by 65%, while the immobilized cells, in contrast, contained 30% more polysaccharide after hydroxyurea treatment.

Biotin starvation of immobilized yeast was effective in reducing synthesis of DNA. Biotin-deficient immobilized *S. cerevisiae* contained approximately the same quantity of DNA as starved suspended cells, while RNA and protein levels were reduced. Glycerol was synthesized at the expense of ethanol during fermentation by biotin-deficient immobilized and suspended cells.

The character and occurrence of glycolytic oscillations were affected by immobilization. Endogenous metabolism by immobilized cells gave rise to relaxation oscillations in the absence of external substrate. Addition of glucose to starved immobilized yeast also generated relaxation-type behaviour, while suspended cells produced only sinusoidal waveforms. Interpretation of these immobilized cell dynamics is discussed in terms of the kinetics and regulatory properties of energy metabolism in yeast.

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CHAPTER 1

INTRODUCTION

The use of microorganisms to catalyse biochemical reactions is an old technology. Although fermentation systems now provide a large number of pharmaceutical and food products, process development in these industries proceeds along mainly empirical lines. Typically, the conversions are conducted in batch, and product formation is often coupled with cell growth.

Immobilization of microorganisms represents a new approach to established fermentation processes. By fixing cells onto or within solid particles, many of the principles of heterogeneous catalysis can be applied to biological processing. The affinity of many microorganisms for attachment and growth on solid surfaces is historically well established. Microbial films growing on soil particles and in water may be treated as naturally occurring immobilized cell systems. In addition, cells adhering to stones and pebbles have long been used in trickling filter reactors for waste treatment, while vinegar production by bacteria attached to wood chips has been a commercial proposition since 1880.

Application of immobilized cells offers several advantages over conventional suspended cell culture. In terms of process variables, high cell densities can be achieved in immobilized cell reactors without affecting the viscosity or rheological character of the solution. Since the cells are spatially fixed, culture washout cannot occur, and continuous operation at high dilution rates is possible. Separation of product from biomass is also easier when the cells are immobilized.

Together with these factors which influence reactor design and operation, there are other advantages associated with reuse of the cells for batch or continuous processing. When the desired product is formed by the organism in stationary

phase, the preparatory lag and growth phases can be eliminated by recycling the immobilized cell population. In addition, once the biocatalyst is produced, maintenance of the culture in a non-growing but productive state is a potential means for improving yields. In its most sophisticated form, protocol for use of immobilized cells involves manipulation of cell functioning so that undesirable enzymes are deactivated and by-product formation minimized. Periodic regeneration of catalytic activity under more favourable conditions allows the immobilized cell preparation to provide long-term utility.

In their immobilized state, cells may be partially or completely ruptured, they may be intact yet nonviable, or they may be whole living organisms capable of growth. Transformations requiring only single enzyme function can be effected using immobilized cell supports containing only cell debris. Of more academic interest is the application of immobilized viable cells for sequential reaction of multiple enzymes with or without cofactor regeneration.

When a cell is immobilized, it is impossible to tell *a priori* whether its metabolism will follow the same pattern as in the freely suspended organism. It is conceivable that contact with a solid will interfere with some cell functions, especially those involving the cell wall. These modifications are in addition to the more familiar and easily represented diffusional effects, since transport of substrates and products may also be hindered by the immobilization support.

As yet, there is no general formula relating properties of the support and immobilization procedures to subsequent alterations in cellular metabolism. Interactions between surfaces and cells are poorly understood. The most direct way of evaluating the effects of immobilization on cell behaviour is by comparison with suspended cells. When the properties of immobilized cells are examined, the results have most meaning when they are related back to what is known about the

simpler suspended cell system. Unless this comparison is followed through, the influence of cell-solid interactions on immobilized cell functioning will always be unclear.

Although research into immobilized cells has continued for more than ten years, most of the work is concentrated on evaluating overall productivities and operational stability for immobilized cell reactors. The outcome is a haphazard collection of results which, oftentimes, has not included important information about the biochemical, morphological and genetic states of the cells investigated. In terms of progress towards a better understanding of immobilized cells, a more comprehensive and systematic characterization of their properties is required.

CHAPTER 2

OBJECTIVES

This study was undertaken with the following aims:

- (i) to compare immobilized cell and suspended cell metabolism; and
- (ii) to examine the causes for any differences between immobilized and suspended cell behaviour.

The investigation was designed so that the kinetic and compositional properties of immobilized cells could be properly characterized in the absence of diffusional or other artifacts. Comparison of immobilized and suspended cell growth and activity was proposed as a means for identifying the changes to metabolism wrought by immobilization. Evaluation of the factors responsible for altered behaviour with reference to the modified environment of the immobilized cell was pursued as an additional problem.

Within the framework of this general purpose, two further subjects were investigated. Additional work with immobilized and suspended cells was aimed at:

- (i) determining the effects of growth inhibition on cell activity; and
- (ii) examining the suitability of NADH fluorescence measurement as a non-invasive monitor of metabolism.

CHAPTER 3

BACKGROUND

This investigation was prompted by results from previous studies which suggested that microbial metabolism is altered by immobilization. In addition, several of the methods used in execution of this work were selected after consideration of many techniques developed by others.

The purpose of this literature survey is to outline some of the factors which gave direction to the present study and helped shape its aims. The following topics are reviewed:

- (i) methods for cell immobilization;
- (ii) the differences already observed between immobilized and suspended cells ;
- (iii) results of previous work with metabolically inhibited cells;
- (iv) the properties of the yeast, *Saccharomyces cerevisiae*, which are pertinent to this study;
- (v) the use of flow cytometry to characterize cell populations; and
- (vi) glycolytic oscillations, the conditions under which they may be observed, and the mathematical treatment of the phenomena.

3.1 IMMOBILIZATION TECHNIQUES

Many immobilization procedures have been tested. In principle, microorganisms may be immobilized by either:

- (i) entrapment; or

(ii) surface attachment.

Entrapment is the most common method of cell immobilization, probably because of its broad applicability. Nearly all cells can be entrapped; however, surface attachment techniques require a more specific cell-support interaction.

3.1.1 Entrapment Methods

In general, entrapped cells are confined within a solid lattice through which substrates and products must diffuse. In many cases where polymeric gels are used, the entrapped cells lyse (Chibata, et al., 1974a; Mason, et al., 1978; Koshcheenko, et al., 1978; D'Souza and Nadkarni, 1980). Loss of activity may then occur due to leakage of the enzymes from the support lattice (Mosbach and Mosbach, 1966).

A problem with entrapped cells which have remained viable is their ability to divide and eventually break open the support (Krouwel, et al., 1982; Cheetham, et al., 1979). Gas evolution may also disrupt the entrapment matrix (Krouwel and Kossen, 1980).

3.1.1.1 Synthetic Polymers

Polyacrylamide is the most commonly used synthetic polymer for cell entrapment. The immobilization procedure is described by Mattiasson, et al. (1977). Polymerization may also be initiated by UV-radiation (Mosbach and Larsson, 1970). Advantages associated with use of prepolymerized polyacrylamide and controlled chemical crosslinking are discussed by Pines and Freeman (1982).

The acrylamide monomer as well as the polymerization conditions are toxic to cells. Siess and Divies (1981) report that polymerization destroys 40-80% of cells entrapped in the gel.

Difficulties with cell counting and separation of cells from polyacrylamide are reported by Mason, et al. (1978).

Other synthetic materials available for cell entrapment are polyurethane (Tanaka, et al., 1979) and methacrylate polymers (Fukui, et al., 1978; Kumakura, et al., 1978; Tanaka, et al., 1982). Several synthetic ionic network polymers have been tested by Klein, et al. (1979). Methods for the entrapment of organisms in porous epoxy beads were also developed (Klein and Eng, 1979).

3.1.1.2 Natural Polymers

Alginate was first used by Hackel, et al. (1975) for the biodegradation of phenol by immobilized cells. The method is mild and simple, and for these reasons has been adopted in many immobilized cell studies (Cheetham, et al., 1979; Hahn-Hägerdal and Mattiasson, 1982). One difficulty in the use of calcium alginate is solubilization of the gel by moderate concentrations of calcium chelating agents, such as phosphate, EDTA, Mg^{2+} and K^+ . This property may be used to advantage for release of cells for analysis (Kierstan and Bucke, 1977). Several techniques for gel stabilization have been suggested (Veliky and Williams, 1981; Birnbaum, et al., 1981). Another problem is leakage of cells from the relatively open pores of the support lattice (Nilsson and Olson, 1982).

κ -carrageenan, a seaweed polysaccharide, can be used to entrap microbial cells under mild conditions (Takata, et al., 1977; Wada, et al., 1979). Hardening agents such as glutaraldehyde or hexamethylenediamine improve the stability of the immobilized cell preparation (Chibata, 1979).

Agar and agarose gels have also been tested (Toda and Shoda, 1975). Agars with low gelation temperatures are now available. However, the mechanical strength of agar gels is relatively poor.

Collagen membranes (Vieth, et al., 1973; Constantinides, et al., 1981) and gelatin (Parascandola and Scardi, 1981; Marek, et al., 1981) are protein materials which have been used for cell entrapment. Many of the procedures involve glutaraldehyde as crosslinking or tanning agent. Under these conditions, it is unlikely that immobilization is due solely, as described by Venkatasubramanian, et al. (1974), to non-covalent interactions. In the presence of glutaraldehyde, protein residues, especially ϵ -amino lysine, form stable covalent bridges with cell wall protein.

Other biopolymers used for entrapment include cellulose and its derivatives (Linko, et al., 1977) and crosslinked albumin (Petre, et al., 1978; Tanaka, et al., 1978).

3.1.1.3 Hydrous Metal Oxides

Entrapment of cells using these materials relies on the reaction between carbonyl and amino groups on the cell surface and metal hydroxide in the gel (Kennedy, et al., 1976). Several transition metal salts have been tested (Kennedy, 1978).

3.1.1.4 Silica Hydrogel

A procedure for cell entrapment in silica gel films was tested by Rouxhet, et al. (1981). Multiplication of cells inside the gel caused the film surfaces to crack. Cell counting was possible, however, after mixing the films with water and applying ultrasound to the suspension.

3.1.1.5 Porous Glass and Ceramics

Cells may be entrapped in porous glass if the pores are sufficiently large (Navarro and Durand, 1977). The procedure can be modified for use with glass fibre pads (Arcuri, et al., 1980; Arcuri, 1982). Cells immobilized in the pads are

either attached to or caught between the glass fibres.

A similar system is the controlled-pore ceramic support developed by Messing (1982). The relationship between cell dimensions and accumulation of organisms in structures of specified pore size has been investigated (Messing, et al., 1979).

3.1.1.6 Hollow-Fibre Membranes

Cells can be immobilized by entrapment within the macroporous matrix of ultrafiltration hollow-fibre membranes (Inloes, 1982; Hopkinson, 1983). The fibres are arranged either singly or as multifibre modules in dialysis units. Growth within the packed macropores may result in disruption of the membrane and loss of cells (Inloes, 1982).

3.1.1.7 Flocculation, Pelletization and Cell Crosslinking

These means for aggregating cells may be viewed as entrapment techniques which do not require a support matrix. Aggregated cells constitute an immobilized cell system which can be readily separated from the reaction solution and reused.

Flocculated cell reactors using naturally flocculent strains are described by Smith and Greenshields (1973), Arcuri, et al. (1980) and Hsiao, et al. (1983). Cell flocs are usually very weak mechanically, and gas release can readily cause floc break-up and cell flotation.

Growth of moulds in pellet form has been examined by Metz and Kossen (1977). Bacteria may be induced to flocculate under the influence of strong electrolytes (Lee and Long, 1974), and the resultant precipitate granulated into catalyst particles (Boersma, et al., 1979).

Entrapment by crosslinking is accomplished by initiating covalent bonding between cells. Tetrazotized benzidine (Lartigue and Weetall, 1978), 2,4-toluene diisocyanate (Chibata, et al., 1974a) and glutaraldehyde (Chibata, et al., 1974a;

Navarro and Monsan, 1976; Lantero, 1978) have been used as coupling agents. The toxicity of many polyfunctional reagents limits the general applicability of cell crosslinking techniques.

Immobilization using any of these aggregation methods creates high cell densities. However, internal diffusion resistances within the cell aggregates can be great. Larger pellets may become hollow due to autolysis of cells following nutrient limitation (Trinci and Righelato, 1970; Metz and Kossen, 1977).

3.1.1.8 Microencapsulation

Microencapsulated cells are retained inside a semi-permeable membrane (Chang, 1971; Mohan and Li, 1974) which, in general, allows substrate and product to diffuse more freely than in the preparations already described. The average size of the microcapsules is also much smaller (20-300 μm) than the solid particles usually prepared for cell entrapment. Despite these differences, however, diffusion often remains the limiting factor (Mohan and Li, 1975).

Procedures for encapsulation of cells in ethylcellulose and cellulose acetate butyrate are given by Ado, et al. (1979). The droplets containing cells produced by these methods are very fragile. In addition, sedimentation of cells within the microcapsules may occur in the absence of adequate agitation.

Aqueous two-phase immobilized cell systems have been tested in several studies. The most common two-phase components are poly(ethylene glycol) and dextran (Mattiasson and Hahn-Hägerdal, 1983).

3.1.2 Surface Attachment Methods

The ability of cells to attach to surfaces is an important property in soil and aquatic microbiology (Zobell, 1943; Lahav and Keynan, 1962; Stotzky and Rem,

1966, 1967). Despite this, the mechanisms of many support-cell interactions are poorly understood (Weiss and Harlos, 1972; Marshall, 1976).

Loading of biomass using surface immobilization methods is limited by the surface area of the support particle. In addition, since the cells are fully exposed to the medium, they may be easily sheared from the carrier surface by rapid fluid flow or by turbulence created by release of gas bubbles (Marcipar, et al., 1979). Changes in medium pH or ionic strength during reaction can also reduce the strength of cell attachment (Hattori and Furusaka, 1960).

3.1.2.1 Adsorption

Electrostatic adsorption of cells to ion-exchange materials is a convenient immobilization method. At normal pH, most organisms are negatively charged and are attracted to anionic resins. Extents of adsorption for various combinations of organisms and ion-exchange supports are discussed by Zvyagintsev (1962), Daniels and Kempe (1966), Gainer, et al. (1980) and Daugulis, et al. (1981).

From the data provided, the affinity of a specific species for a given ion-exchange resin appears somewhat unpredictable. Configuration of charged sites and accessibility of the functional groups may play subtle roles in mediating ion-exchange interactions. For example, affinity of cells for certain resins may alter with cell age due to increased accessibility of charged groups on the cell surface (Rotman, 1960).

Any substance which interacts with surface charge can influence the success of ion-based adsorption methods. Cells are routinely eluted from ion-exchange resins by addition of acid or salt solutions. These procedures allow recovery and recycle of the support.

Attraction of cells to glass (Rouxhet, et al., 1981), sand (Panikov, et al., 1981)

and ceramic surfaces (Marcipar, et al., 1979) occurs as a result of the charge usually present on these materials (Nordin, et al., 1967). Surface-bound water or organic molecules may also facilitate cell adsorption (Gerson and Zajic, 1979). Adhesion to glass can be improved by treatment of the surface with metal ions or charged colloidal particles (Rouxhet, et al., 1984). Affinity of cells for ceramic supports increases with cell age (Marcipar, et al., 1979).

Adsorption of microorganisms has been demonstrated on several plastic surfaces (Kolpakchi, et al., 1976; Fletcher and Loeb, 1979), including polystyrene (Wolfe and Colby, 1981), cellophane (Reed and McKercher, 1948) and polyethylene (Gerson and Zajic, 1979). Metal surfaces may also be colonized (Gerson and Zajic, 1979).

Derivatized cellulose, and to a lesser extent unmodified cellulose is able to adsorb cells (Kaliuzhny, 1964; Helmstetter, 1967; Leung, et al., 1983). Adhesion to sawdust has recently been studied using zeta potential measurements (Michaux, et al., 1982; Thonart, et al., 1982). Wood chips were observed by Moo-Young, et al. (1980) and Gencer and Mutharasan (1981) to have a high capacity for adsorption of microorganisms. Strength of adhesion is improved in the presence of flocculating agents (Moo-Young, et al., 1980).

In general, adsorption is a mild surface immobilization method. The adsorption process can usually be reversed by chemical effectors or physical means. Stability is limited by desorption of cells from the support.

3.1.2.2 Covalent Bonding

Covalent linkage of cells to activated surfaces is achieved via reactive components on the cell wall. Amino, carboxyl, sulphydryl, hydroxyl or phenol groups of cell wall proteins can be coupled with agents such as carbodiimide (Jack and Zajic, 1977) and cyanuric chloride (Gainer, et al., 1980).

Binding of cells to many supports can be effected using glutaraldehyde as linking agent. Gelatin (Griffith and Compere, 1975; Sitton and Gaddy, 1980), chitosan (Nilsson and Mosbach, 1980) and silica (Navarro and Durand, 1977) have been used as reactive bases for glutaraldehyde-cell coupling. Glutaraldehyde may also provide the last link in longer chemical spacers joining cell and solid support (Turková, 1979; Gulaya, et al., 1979). Wold (1967) describes procedures for the use of multifunctional reagents in protein chemistry, many of which may be applied for the immobilization of cells.

The reaction of glutaraldehyde with cells is not clearly understood. Glutaraldehyde reacts predominantly with the lysinyl residues of proteins, and has generally been assumed to form a Schiff base. There is evidence, however, that precludes the existence of simply α,ω -bis-Schiff bases (Richards and Knowles, 1968). Foremost is the fact that protein modification by glutaraldehyde is irreversible, surviving treatment with urea, semicarbazide and wide ranges of pH, temperature and ionic strength. The complexity of the reaction has been attributed to the presence of impurities in commercial preparations, and to the tendency of glutaraldehyde to form polymeric chains in aqueous solution (Richards and Knowles, 1968; Korn, et al., 1972).

Several cleavable bifunctional reagents for protein crosslinking may be substituted for glutaraldehyde. These are described by Coggins, et al. (1976), Smith, et al. (1978), Carlsson, et al. (1978), Abdella, et al. (1979), Baskin and Yang (1980) and Zarling, et al. (1980).

The use of metal salts to activate surfaces for covalent bonding of cells is discussed by Vijayalakshmi, et al. (1979) and Cabral, et al. (1983). This method is a variation of that described by Kennedy (1979) for cell entrapment.

Covalent bonding of cells is an irreversible process. Application of this tech-

nique has been limited due to the toxicity and denaturation properties of many crosslinking agents.

3.1.2.3 Biospecific Interaction.

Certain macromolecules are able to bind specific sites on the surface of microorganisms. When these molecules are attached to solid supports, they provide a selective base for cell immobilization. This principle has been adopted in the development of affinity chromatography.

Lectins are naturally occurring proteins which are able to agglutinate certain types of cells. There are many examples of the use of immobilized lectins for adsorption of animal cells (Zabriskie, et al., 1973; Rutishauser and Edelman, 1976; Kinzel, et al., 1977; Mattiasson and Borrebaeck, 1978). Schmer, et al. (1977) have used lectin-bound resealed blood cells in a semi-artificial organ for blood treatment. Application of lectins for surface immobilization of microorganisms is much less widespread (Horisberger, 1976).

Concanavalin A appears to be the most widely used lectin. Its physicochemical properties are described by Sharon and Lis (1972), and Wang, et al. (1975). Concanavalin A combines with a variety of polysaccharides (Goldstein, et al., 1965). It is strongly bound to α -linked D-mannopyranosyl and D-glucopyranosyl units. Mechanisms for cell agglutination by concanavalin A are discussed by Rutishauser and Sachs (1974) and Walther (1976).

In addition to sugars, concanavalin A binds Mn^{2+} and Ca^{2+} ions. Transition metal ions are required for binding of Ca^{2+} , and both are necessary for the reaction with saccharides (Lis and Sharon, 1973).

Lens culinaris lectin binds directly with α -glucans and α -mannans as does concanavalin A, but the interaction involves different parts of the polysaccharide

molecules. The binding constant for agglutination with *Lens culinaris* lectin is approximately 50 times lower than that for concanavalin A (Kinzel, et al., 1976a).

Cells bound to either concanavalin A or *Lens culinaris* lectin can be detached. This is accomplished by eluting with solutions containing carbohydrates which bind more strongly to the lectin than do the cell wall components. Addition of α -methyl-D-glucopyranoside allows almost complete recovery of cells (Inbar and Sachs, 1969). Mild mechanical agitation aids the chemical release process (Edelman, et al., 1971; Kinzel, et al., 1976a).

D-glucose is also a competitor for the lectin binding sites (Goldstein, 1976). D-galactose has a much lower binding constant, although small numbers of cells may be non-specifically removed in its presence (Kinzel, et al., 1976b).

In addition to reactive sites for lectin binding, cells may have antigenic properties which allow coupling to activated surfaces on an immunological basis. The procedure has been demonstrated with animal cells (Evans, et al., 1969; Truffa-Bachi and Wofsy, 1970). Many microorganisms also carry specific surface antigens (Björk, et al., 1972; Dazzo and Hubbell, 1975); however, the potential of this means for surface immobilization of microorganisms has not been fully realized.

Coupling of enzymes to yeast and bacteria is described by Emery, et al. (1972). This technique has not yet been used for cell immobilization *per se*. It has been applied to suspended cultures in wine clarification and fermentation (Hartmeier, 1981).

In some cases, the bridge between cell and support is already present on the cell surface in the form of microscopic polysaccharide or protein exopolymers (Costerton, et al, 1978). These structures are found primarily on bacteria in their natural environment and allow adhesion to surfaces and to other cells (Harris and Mitchell, 1973; Geesey, 1982).

Biospecific surface attachment is a relatively sophisticated method for immobilizing cells. It has not been applied routinely to microorganisms. The binding is usually specific and strong, and occurs under physiological conditions. However, the ligands used have biological function. Interaction with them may impart information to the cell that subsequently modifies some aspect of behaviour or metabolism (Kaplan, 1981; Ryazanova, et al., 1982). In many cases, reversal of attachment is possible.

3.1.3 Conclusions

The choice of immobilization method will depend on the particular application. In an investigation of immobilized cell metabolism and kinetics, it is necessary that the immobilization procedures:

- (i) be mild and, as far as possible, non-damaging to the organism;
- (ii) allow release of cells for counting and other analyses; and
- (iii) minimize the barriers between nutrient medium and cells to avoid problems with intraparticle diffusion.

Many of the entrapment techniques do not fulfil any of these requirements, although diffusion is clearly dependent on particle size.

Covalent bonding reactions employing toxic linking agents are also unsuitable, especially for multienzyme conversions. Covalent attachment presents additional problems with recovery of cells, since the reactions are generally irreversible.

Several of the adsorption and milder crosslinking methods, and in particular, the bioaffinity techniques, merit further consideration.

3.2 COMPARISON OF IMMOBILIZED AND SUSPENDED CELL PROPERTIES

The properties of microorganisms change in response to their environment. For immobilized cells, close proximity of a solid surface affects conditions at the cell envelope. These alterations may be complex in the case where the cell is bonded to the solid. Modifications to cell behaviour can be expected to occur as a result of cell-solid interaction.

Immobilized and suspended cells may be characterized by:

- (i) conditions for growth and activity;
- (ii) morphology;
- (iii) composition; and
- (iv) metabolic kinetics and product yields.

These properties are discussed in the literature. Explanations for observed differences between immobilized and suspended cells are also given.

3.2.1 Conditions for Growth and Activity

Temperature and pH optima, thermal stability and the effects of substrate concentration on activity have been determined for many immobilized cell preparations in terms of bulk fluid properties.

For entrapped cell preparations, the effects of pH, temperature and other parameters on the observed reaction rate may be disguised by diffusion. Some generalizations about the influence of immobilization on apparent activity and its parametric sensitivity have been offered by Ollis (1972) and Karanth and Bailey (1978) in reference to immobilized enzyme catalysts. Many of these principles apply in the analysis of entrapped cell kinetics.

3.2.1.1 Temperature

Temperature affects the rate of enzyme reaction. It also determines to some extent which metabolic processes may occur, since all proteins are heat labile.

Optimal activity with immobilized cells has been observed at temperatures both higher (Yamamoto, et al., 1974) and lower (Wheatley and Phillips, 1984; Williams and Munnecke, 1981) than those for highest activity in free cells. In some cases, the temperature profiles of immobilized and suspended cell activities are virtually coincident (Chibata, et al., 1974a).

Activity at a given temperature depends on the thermal stability of enzymes as well as their kinetics. Again, both increased (Yamamoto, et al., 1974) and decreased (Wheatley and Phillips, 1984) heat stabilities have been reported for immobilized cells.

3.2.1.2 pH

Like temperature, pH affects enzyme kinetics. pH shifts for optimal activity of immobilized cells were first observed in soil bacteria (McLaren and Skujins, 1963). Most often, pH optima for immobilized cells are lower than for suspended cells (Yakovleva, 1978). However, Shimizu, et al. (1975) report maximum rates at higher pH values with entrapped cells. Clearly, if diffusion limits transport to and from the cell, the direction of the pH shift will depend on whether H^+ ions are produced by the cell or taken up from the medium.

Reduced parametric sensitivity may be expected in transport limited immobilized cell preparations (Ollis, 1972; Karanth and Bailey, 1978). Accordingly, the broad pH-activity profiles obtained in several studies with immobilized cells (D'Souza and Nadkarni, 1978; Nabe, et al., 1979; Williams and Munnecke, 1981) provide further evidence that diffusional effects in entrapped systems often interfere

with kinetic measurements.

Another factor affecting pH profiles in immobilized cell systems is the electrostatic properties of support material. support. A cationic layer on the surface of anion-exchange resin was postulated by Hattori and Furusaka (1960) to explain the pH shift observed for optimal adsorbed cell activity.

Immobilized cell, suspended cell and free enzyme pH-activity profiles were compared by Wheatley and Phillips (1984) and Chibata, et al. (1974b). Although displaced to pH values lower than for the suspended cells, the immobilized cell profile was very similar to that for the enzyme in solution. Lysis of cells inside immobilized cell preparations may be responsible for many of the reported differences between immobilized cell and suspended cell properties.

3.2.1.3 Substrate Concentration

Observed values of the Michaelis constant, K_m , for immobilized cells are usually higher than for suspended cell reaction. This is the expected result, since diffusional limitation of enzyme kinetics classically leads to an increase in the apparent K_m (Thomas, et al., 1974). In some cases, however, reduced K_m values for immobilized cells have been found (Petre, et al., 1978; D'Souza and Nadkarni, 1980; van Keulen, et al., 1981). These results suggest that changes in binding affinities or other enzyme properties occur in immobilized cells.

Immobilized and suspended cell activities at varying substrate concentrations were determined by Hattori and Furusaka (1959a). The electrostatic properties of the carrier influenced the results. For cells adsorbed to ion-exchange resin, the apparent kinetic dependence on substrate concentration varied with the charge carried by the substrate molecule.

In other work, the activity of immobilized cells was found to decline with

moderate increases in substrate concentration, even though suspended cells under the same conditions retained their maximum reaction rates (Nabe, et al., 1979; Takamatsu, et al., 1980; Fusee, et al., 1981).

3.2.2 Morphology

Immobilized cells are usually in contact with a solid surface or matrix. Such interaction could conceivably affect cellular morphology and, especially, interfere with reproduction of the organism.

No detailed examination of immobilized cell morphology has been carried out. However, the following observations were made in the course of other immobilized cell studies.

Production of filamentous chains of bacteria in the pores of glass microbeads is described by Ou and Alexander (1974). While suspended cells are able to double their length before division unimpeded, pore size may restrict septum formation or cell separation in dividing immobilized bacteria.

Bacteria were observed by Koshcheyenko, et al., 1978 to produce extracellular processes after entrapment. These structures disappeared in subsequent generations.

Chain-like filaments of yeast on the surface of an immobilization support were observed by Jirků, et al. (1980). Budding was unusually polarized. The immobilized cells grew as rods rather than displaying the normal ellipsoidal shape of suspended yeast. Elongation of yeast immobilized on controlled-pore glass is described also by Bandyopadhyay and Ghose (1982). In contrast, reduced length-to-width ratios for immobilized yeast were observed by Kazantsev, et al. (1979).

Detachment of cells from glass (Rouxhet, et al., 1981) and ion-exchange resin

(Hattori, et al., 1972b) at the time of bud emergence or cell elongation suggests that these processes strain the cell-solid interaction in immobilized cell preparations.

3.2.3 Cell Composition

The composition of cells changes with nutrient availability, environmental conditions and cell age. Analysis of immobilized cell composition has not often been carried out. However, Hattori, et al. (1972a) report that cells desorbed from ion-exchange resin contained 25% less RNA than freely suspended cells. DNA levels were approximately 10% higher in the desorbed cells.

3.2.4 Metabolic Kinetics and Product Yields

The effect of immobilization on the intrinsic kinetics of intracellular enzymes is unpredictable. However, where there are diffusional limitations, measured activities will most likely be reduced. Even for surface-bound cells, some parts of the cell wall may be rendered unavailable for nutrient uptake so that access to substrate and product removal is impaired. In most studies of immobilized cell kinetics, reduced rates of growth and product formation are observed.

Product yields reflect the extent to which different metabolic pathways are functioning in the cell. Altered yields may imply a rearrangement of cellular metabolism to satisfy changing demands within the cell for energy or intermediate compounds. Again, transport limitations in immobilized cell systems can mask these effects. When diffusion of material away from the cells is hindered, inhibition of enzymes whose regulation is sensitive to product concentrations can occur.

Most often, activities and yields for immobilized cells are found to be reduced

compared with suspended cells. In some systems, however, rate enhancement occurs, and the results from these studies are summarized below.

3.2.4.1 Product Formation

The literature contains many claims that productivity is enhanced in immobilized cell systems. However, interpretation of the published data is often difficult. Calculations of productivity are usually inadequately described.

Volumetric productivities, which are not useful for comparing intrinsic rates of cellular activity, are most frequently reported (Suzuki and Karube, 1979; Wada, et al., 1980; Sitton, et al., 1980; Daugulis, et al., 1981). In some cases, it is not clear which productivity is reported (Holcberg and Margalith, 1981; Hahn-Hägerdal, et al., 1982; Mattiasson and Hahn-Hägerdal, 1982). Even when volumetric rates are specified, the basis for the measurements, whether total reactor volume, void volume or bed volume, is usually not given.

Other workers who report specific productivities do not describe the methods used for determining the numbers of immobilized cells present during the measurement (Somerville, et al., 1977; Venkatasubramanian and Toda, 1980). Most entrapment methods are irreversible, and microscopic counting of cells on surfaces is difficult, so it is not obvious how changes in cell loading are indicated in many experiments. When cell numbers are not carefully monitored, measurements of enhanced activity may easily be due to proliferation within or on the support.

For adsorbed immobilized cells, concentrations of cells in buffer before and after adsorption are sometimes used to calculate support loadings (Hattori and Furusaka, 1959b; Vijayalakshmi, et al., 1979). This procedure does not allow for loss of viability or residual growth which can occur in the absence of nutrients. In addition, citrate or acetate buffers are often used. Both these solutions contain

compounds which provide many microorganisms with a source of carbon for metabolism.

Nevertheless, relatively careful and systematic experiments by Navarro and Durand (1977) and Brodelius, et al. (1979) have indicated higher specific rates of productivity for immobilized cells compared with suspended cells.

Variations in product yields between immobilized and suspended cells are described by Kalyuzhny (1957), Navarro and Durand (1977), Brodelius, et al. (1979) and Tyagi and Ghose (1982).

There is some evidence that immobilized cells may be able to tolerate higher concentrations of ethanol than suspended cells (Holcberg and Margalith, 1981; Pines and Freeman, 1982). Immobilized cell activity has been found to be unaffected at concentrations up to 22% v/v ethanol. The reported results are subject to the effects of transport limitations, since entrapped cell preparations were used in these studies.

3.2.4.2 Growth

Kalyuzhny (1957), Hattori (1972) and Navarro and Durand (1980) have reported faster growth rates for immobilized cells compared with suspended cells. Doubling times were, respectively, 0.40, 0.45 and 0.37 the generation times measured with suspended cells.

In other work, cell yields from glucose with immobilized cells were approximately one-eighth (Brodelius, et al., 1979) and one-fifth (Tyagi and Ghose, 1982) the values obtained with suspended cells.

Induction of synchrony in surface immobilized bacteria and yeast cells is reported by Navarro and Durand (1981). Synchrony lasted 4-7 generations for adsorbed cells and 10-12 generations for covalently bound cells. Similar synchrony

induction was observed by Helmstetter (1967) and Bandyopadhyay and Ghose (1982).

3.2.4.3 Substrate Consumption

Oxygen uptake has been measured in several immobilized cell systems. Higher rates of oxygen consumption by adsorbed organisms in soil and marine environments are reported in the older literature (Zobell, 1943; Zvyagintsev, 1959). It is often difficult to determine whether the immobilized and suspended cell rates were measured using the same numbers of cells.

Studies in which measurements of immobilized cell respiration were better controlled include those by Marcipar, et al. (1979) and Vijayalakshmi, et al. (1979). Specific oxygen consumption rates for adsorbed cells were, respectively, 6.8 and 5.1 times those for suspended cells.

Hattori and Furusaka (1961) observed shorter lag times for oxygen uptake by adsorbed bacteria. The enzymes required for metabolizing the substance may have been more readily induced in immobilized cells. This effect was observed also with cells which had been desorbed, indicating that the effects of immobilization were not immediately reversed once the support was removed.

Glucose consumption has been examined by Navarro and Durand (1977). Specific substrate consumption by immobilized cells was calculated from measurements of uptake rates in solutions containing both free cells and adsorbed cells. The results indicated that glucose consumption by immobilized cells was approximately five times faster than for suspended cells.

Uptake of amino acid by bacteria isolated from aquatic slime has been measured. The value of V_{\max} for arginine consumption was ten times higher in the attached cells than in suspended cells (Geesey, 1982).

The ability of immobilized cells to metabolize very low concentrations of glucose is reported (Heukelekian and Heller, 1940; Ou and Alexander, 1974). At concentrations where suspended cell activity had ceased due to nutrient depletion, immobilized cells continued to grow and consume substrate.

3.2.4.4 *Explanations of Observed Kinetic Effects*

There are several explanations commonly offered when enhanced activity is found for immobilized cells.

(a) *Cell Membrane Permeability:* Modification of membrane permeability by immobilization has been suggested as an explanation for higher activities in immobilized cells (Marcipar, et al., 1979; Vijayalakshmi, et al., 1979). It is implied that transport into the immobilized cell is the rate-limiting step in metabolism. For some substrates, this may be true. However, there has been no direct experimental evidence of membrane effects in intact immobilized cells.

(b) *Microenvironment:* For surface attached cells, it is often postulated that the concentration of nutrients in the microenvironment of the cell is increased (Kalyuzhny, 1957; Marcipar, et al., 1979; Ghose and Bandyopadhyay, 1980; Bandyopadhyay and Ghose, 1982). Macromolecules are known to be preferentially adsorbed at surfaces (Marshall, 1976), although the details of this phenomena are not well known. For charged surfaces, attraction of ions and locally charged substrate molecules is obvious.

A number of difficulties arise in association with this theory. In terms of direct utilization of substrates, enzyme kinetics are dependent on substrate concentrations only for $S < \sim 2K_m$. This applies also to the cell membrane substrate carriers. In immobilized cell systems where the bulk $S \gg K_m$, such as in continuous cultivations at fast dilution rates or initially in batch systems, exposure of the

cells to higher concentrations of substrate will not improve kinetics to any large extent. Substrate inhibition may, instead, be possible.

In addition, it is unclear to what extent nutrients which have been attracted to the surface by electrostatic or other forces are available to the cell. These molecules may be bound strongly to the solid and, as such, can no longer be considered solubilized.

Other changes may occur due to elevated concentrations near the cell of molecules and ions not directly metabolized. It seems unlikely, though, that alterations in pH, ion concentration, water activity, surface tension or osmotic pressure occurring at the interface can enhance immobilized cell activity to the extent sometimes observed. Relative to suspended cells growing at near-optimal rates, a doubling of growth rate for immobilized cells (Hattori, 1972), for example, would appear to require significant environmental modifications.

Altered behaviour is known to persist even after cells are detached from the support (Hattori and Furusaka, 1961). Other more complicated consequences of immobilization are given by Larsson and Mosbach (1979). Daughter cells produced in an entrapped cell preparation were more active than the parent cells, even though both were immobilized in the same way.

These results suggest the immobilization can have rather far-reaching consequences with regard to microbial metabolism. In explaining enhanced activity in immobilized cells, factors beyond those related to the sorptive properties of surfaces should be considered.

In addition, higher concentrations of nutrients in the vicinity of immobilized cells have never been verified experimentally.

(c) *Protection from Environment:* Entrapped cells are often considered to be protected by the support matrix (Chibata, 1979). This may be taken as a statement of diffusional limitation in the system. Unless the bulk solution contains inhibitory substances which are partitioned with lower concentration in the cell microenvironment, it is unclear how protection by the support solid would benefit the cells.

(d) *Excretion of Product:* Some cellular products are usually stored in cell vacuoles. This refers generally to secondary metabolites. Brodelius, et al. (1979) has suggested that a greater tendency to release alkaloids may be responsible for the higher rates observed for product accumulation in solution.

(e) *Extracellular Polymers:* Geesey (1982) describes the role of adhesive bacterial extracellular polymers in sequestering nutrients for the cell. Again, this function will be advantageous to the immobilized cell only when nutrients are present at low concentrations in the medium.

3.2.5 Conclusions

The properties of immobilized cells have already been the subject of much research. However, examination of previous work reveals three major shortcomings:

- (i) diffusional intrusions frequently mask kinetics of reaction and the dependence of cell function on environmental parameters;
- (ii) the number of cells present is usually not measured, so that specific activities are unknown; and
- (iii) the reasons most often cited to explain variations in immobilized cell and suspended cell behaviour remain unsubstantiated by experiment.

As a consequence of these problems, immobilized cells are not well characterized.

3.3 APPLICATIONS OF METABOLICALLY INHIBITED IMMOBILIZED AND SUSPENDED CELLS

One of the advantages in the use of immobilized cells is that they cannot wash out at high dilution rates, even when cell proliferation has ceased. This property allows long-term continuous production using non-growing active cells, a mode of operation impossible with suspended cell chemostat cultures.

In addition, productivity may benefit if cells are maintained in a no-growth state. The implications for improving production of secondary metabolites are obvious. However, formation of what are usually considered "growth-associated" products may also be enhanced as substrate is diverted away from synthesis of biomass and is routed into more desirable metabolic reactions.

Reduction of growth and by-product formation by cells can be effected using:

- (i) nutritional starvation;
- (ii) chemical agents;
- (iii) non-permissive operating conditions; and
- (iv) mutant strains.

Little attention has been given to the production potential of non-growing cells, although starvation and chemical inhibitors have been widely used in biochemical studies of cell functioning.

3.3.1 Starvation

Cells grow only if certain nutrients and growth factors are provided in assimilable form. Some organisms sporulate as a response to nutritional deprivation (Hanson, et al., 1970). The metabolic compromises involved in growth of cells in nutrient-limited environments are discussed by Trinci and Thurston (1976) and Tempest and Neijssel (1981).

Although specific growth requirements will vary depending on the organism used, some generalizations about cell nutrition are possible.

3.3.1.1 Carbon Source

Cells derive energy from catabolism of carbon compounds. Carbon source starvation induces breakdown of intracellular reserves of polysaccharide (Panek, 1963). When these stores are depleted, the cells rapidly lose viability and lyse (Wilson, et al., 1977). Protein and nucleic acid turnover in carbon-starved cells has been studied by Halvorson (1958) and Mandelstam (1963).

The effects of restricting supplies of substrate to the maintenance level in chemostat culture was studied by Righelato, et al. (1968) and Bainbridge, et al. (1971). A number of reports based on chemostat experiments suggest that steady growth rates cannot be maintained below a finite minimum substrate concentration (Pirt, 1972; Tempest, et al., 1967). Population heterogeneity at very low dilution rates was observed by Koch and Coffman (1970). Substantial reorganization of metabolism was evident in all these studies.

3.3.1.2 Nitrogen Source

Nitrogen is required for amino acid and nucleotide synthesis. As well as inorganic nitrogen, some organisms must be supplemented with specific amino acids or

purine and pyrimidine bases which cannot be made intracellularly from simpler nitrogen compounds. Some consequences to the cell of nitrogen starvation are described by Halvorson (1958), Mandelstam (1963), Imahori and Kudo (1963), López and Gancedo (1979) and Lagunas, et al. (1982).

Nitrogen starved cultures may increase in biomass for some time after the onset of starvation. Growth must be considered unbalanced under these conditions. In general, viability is lost less rapidly than with carbon source starvation.

Fermentation by nitrogen-deficient suspended yeast cells was studied by Harrison and Graham (1970). More glycerol and organic acid were formed in the absence of amino acids as nitrogen source. Accumulation of ethanol from 10% glucose medium continued for more than three days in batch culture. Similar results were found by Haukeli and Lie (1973). In other work, a loss of activity was observed within eight hours of nitrogen starvation during aerobic fermentation of 2% sugar solutions (Lagunas, et al., 1982).

Nitrogen starvation of immobilized cells has been examined in several studies. For cells entrapped in κ -carrageenan beads (Wada, et al., 1980), death and autolysis commenced immediately following transfer to nitrogen-deficient medium. Production of ethanol dropped rapidly as a consequence of this viability loss. In contrast, cells entrapped in calcium alginate gel (Kierstan and Bucke, 1977) continued to be active for 13 days in minimal glucose media without nitrogen. Yeast in hollow-fibre membranes also remained active; in this case, for more than 35 days under conditions of periodic starvation and regeneration (Inloes, 1982). Incomplete growth inhibition was observed in the nitrogen-deficient medium, probably as a result of cryptic growth. Nitrogen deficiency reduced both ethanol productivity and yield. Higher amounts of acetaldehyde and glycerol were formed, and there was also evidence of enhanced synthesis and storage of lipids.

3.3.1.3 Other Requirements for Growth

Magnesium and phosphorus are essential for microbial growth. Magnesium is the most common enzyme activator, and is of particular importance in mediating phosphate-transfer reactions. Protein and nucleic acid turnover during magnesium starvation is described by Kennell (1967).

Phosphate compounds are used by cells for energy transfer and are important in lipid synthesis. Starvation in phosphorus causes an increase in the rate of protein breakdown, but the effects are less severe than with nitrogen starvation. Depending on the capacity of the organism for polyphosphate storage, deprivation of phosphate may not occur for some time after it is omitted from the medium (López and Gancedo, 1979).

3.3.2 Chemical Inhibitors

There are a large number of inhibitors and enzyme modifiers routinely used in biochemical research. Many of the more widely used inhibitors of macromolecular synthesis are discussed by Schindler and Davies (1975).

Several compounds may be used to block energy metabolism. Iodoacetate and fluoride compounds inactivate glyceraldehyde 3-phosphate dehydrogenase and enolase, respectively, in the glycolytic sequence (Chance, et al., 1965). Modifiers of other glycolytic enzymes are described by Horecker, et al. (1967). Sodium bisulphite blocks acetaldehyde utilization, while hydroxylamine combines with the acyl compounds of coenzyme A to prevent their further reaction. Metabolism of α -oxo-acids is affected by semicarbazide and arsenite compounds (Dagley and Chapman, 1971). Azide, cyanide and sulphide have been used to block respiration (Betz and Chance, 1965a).

Transport processes and other cell envelope-associated functions are affected by 2,4-dinitrophenol (Chipley, 1974; Kotyk, 1967), N,N'-dicyclohexylcarbodiimide (Chipley, 1974), 1-ethyl-3-(3'-dimethylaminopropyl) carbodiimide (Jack and Zajic, 1977) and uranyl ions (van Steveninck and Rothstein, 1965; Kotyk, 1967).

3.3.2.1 *Hydroxyurea*

Hydroxyurea is a preferential inhibitor of DNA synthesis (Slater, 1973). It has been widely used in the past as an anti-cancer drug (Timson, 1975), as an inducer of cell synchrony (Miyata, et al., 1979; Yamada and Ito, 1979) and as a tool in investigations of cell cycle event interdependency (Hartwell, 1976; Kassir and Simchen, 1978; Sloat, et al., 1981).

In *Escherichia coli*, bacteriophage T4, several animal systems and presumably also in yeast, hydroxyurea inhibits DNA synthesis by deactivating the enzyme, ribonucleotide reductase, which catalyses reduction of ribonucleotides to deoxyribonucleotides, the precursors of DNA. (Elford, 1968; Larsen, et al., 1982). This inhibition occurs as a result of a specific interaction between hydroxyurea and a subunit of the active enzyme complex (Krakoff, et al., 1968) and has been correlated with depletion of cellular pools of deoxyribonucleoside triphosphates (Neuhard, 1967). When DNA synthesis is prevented by hydroxyurea, DNA replication, and consequently, nuclear division cannot occur. Hydroxyurea has no direct effect on template function or on the DNA polymerization reaction (Schindler and Davies, 1975).

There is some evidence that exposure to hydroxyurea damages DNA by causing breaks or nicks in the molecule (Jacobs and Rosenkranz, 1970). Degradation of the polynucleotide structure has been related to the lethal action of hydroxyurea on bacteria and its potential for devitalizing cellular activity. However, this type of effect is time and concentration dependent. For short incubations up to 12 hours,

and with low inhibitor concentrations of 0.1M or less, hydroxyurea treatment of yeast has minimal effect on RNA and protein synthesis. Total cell mass may increase normally for several hours after addition of the drug (Silva-Lopez, et al., 1975). Hydroxyurea does not affect the structure of ribosomes or the properties of RNA (Rosenkranz, et al., 1966).

3.3.2.2 Application of Inhibitors in Immobilized and Suspended Cell Culture

The use of chemical inhibitors to reduce cell growth and by-product formation has been the subject of several recent studies. The effects of dinitrophenol, azide and arsenate on fermentation were examined by Amin, et al. (1984). In the presence of these inhibitors, conversion of substrate was improved. There was evidence that intracellular stores of glycogen were released for fermentation, and that by-product formation was reduced. The inhibitors were also tested with cells immobilized in carrageenan. In this case, it was necessary to alternate feeding with and without inhibitor, presumably because of accumulation of the substances inside the cells.

Hahn-Hägerdal and Mattiasson (1982) report a shift towards ethanol production in aerobically cultured immobilized cells after addition of sodium azide. Yields were temporarily raised to twice the theoretical value, probably due to the effect of azide on mobilization of intracellular carbohydrate reserves (Trevelyan, et al., 1952).

Glycerol production by immobilized cells may be enhanced by addition of sodium sulphite to the medium (Bisping and Rehm, 1982).

Substances which interfere with transport of polar molecules, such as succinate, were tested by Duro and Serrano (1981) with suspended cells. Succinate by-product formation during fermentation was reduced by transport inhibition.

3.3.3 Operating Conditions Unfavourable for Growth

The optimum conditions for growth of an organism may be different from those for formation of other products. For example, ethanol fermentation by yeast is most rapid at 40–42°C, whereas growth is maximized at 30°C (Stokes, 1971). Maintenance energy requirements may be responsible for reducing biomass yields from substrate during growth at superoptimal temperatures (van Uden and Madeira-Lopes, 1976).

As yet, operating conditions unfavourable for growth have not been applied to reduce cell formation and improve productivity in immobilized cell systems.

3.3.4 Mutants

Many temperature sensitive mutants have been isolated. Generally, these mutants have a maximum growth temperature of 23°C-25°C. When exposed to 36°C, they exhibit a preferential loss of ability to carry out synthesis of protein, RNA or DNA, or to complete cell division and cell wall formation (Hartwell, et al., 1973).

Other cell mutants are auxotrophic for selected growth factors. Commonly used mutants in this category include those which require provision of specific amino acids or nucleotides in the medium. Strains may also be engineered so that metabolism of certain carbon source compounds cannot occur.

This property has not been exploited for preventing cell proliferation in immobilized systems.

3.3.5 Conclusions

Techniques for the application of intact, non-growing immobilized cells to improve yields in continuous reaction are not widely practised.

Manipulation of cellular metabolism to increase productivity involves a balance between successfully blocking undesired pathways and damaging the cell to such an extent that activity is lost. Cell lysis often occurs as a result of unintentional interference with those metabolic processes necessary for survival of the organism. In general, agents which block macromolecule synthesis are preferable to those which directly affect enzyme activity. This allows the cell to degrade its reserves of many compounds to ensure short-term survival.

In order to take best advantage of the no-growth state, a high level of specificity is required of the methods used to prevent cell reproduction. Starvation will affect many functions in the cell, whereas chemical inhibitors may modify only selected reactions. Greatest specificity, however, is offered in the use of non-proliferating mutant strains. Many mutations of this type are lethal, but conditional mutants such as those whose disfunction is temperature or nutrient sensitive provide the opportunity for periodic regeneration of the culture.

3.4 PROPERTIES OF *SACCHAROMYCES CEREVISIAE*

In an investigation aimed at examining the effects of immobilization on cell metabolism, it is advantageous to use an organism which is well characterized in suspended form. When the properties of the suspended cell are known, unusual or abnormal behaviour in the immobilized state is more easily recognized. In addition, an understanding of the biochemistry of the organism may be useful in explaining the mechanism for any differences observed when the cell is immobil-

ized.

The yeast, *Saccharomyces cerevisiae*, has a long history of utility in the brewing and baking industries. Consequently, its culture requirements have been thoroughly examined. *S. cerevisiae* has proven particularly suitable for studies of enzyme regulation, since it can grow under both aerobic and anaerobic conditions. The vegetative cell cycle of yeast has been given much attention, and many of its features have been distinguished from experiments with *S. cerevisiae*. As a eukaryotic organism, and because of the large amount of information available concerning its growth behaviour, *S. cerevisiae* is also used extensively as a model system for higher organisms in genetic engineering research. Many genetically modified mutants are available for study.

Some of the properties of *Saccharomyces cerevisiae* have particular bearing in the present study. They include:

- (i) cell composition;
- (ii) vitamin and trace oxygen requirements;
- (iii) fermentation characteristics; and
- (iv) cell cycle operation.

3.4.1 Composition

The macromolecular composition of *S. cerevisiae* reflects the biosynthetic priorities of the organism under the conditions imposed during its growth. Production of DNA, RNA, protein, and storage and structural polysaccharides account for most of the biosynthetic activity of the cell. Levels of the various constituents may fluctuate with position in the cell cycle or phase of culture growth; however, a general description of cell composition is given below.

3.4.1.1 DNA

The amount of chromosomal DNA in *S. cerevisiae* cells depends proportionately on the organism's ploidy. The *S. cerevisiae* haploid genome consists of approximately 14,000 kbp (Fangman and Zakian, 1981). During each cell cycle, the chromosomal DNA content exactly doubles, so that the precise quantity of DNA in the cell depends on its position in the cycle.

In addition to chromosomal DNA, yeasts contain mitochondrial DNA which carries the genetic information for a few essential mitochondrial components. The relative proportions of mitochondrial and nuclear DNA under various growth conditions were determined by Fukuhara (1969). No major differences were found between aerobically- and anaerobically-grown cells. On average, mitochondrial DNA accounts for about 15% of total cell DNA.

Inhibitors of DNA synthesis may have differential effects on nuclear and mitochondrial DNA. Fluorodeoxyuridine and cytosine arabinoside (Pica Mattoccia and Roberti, 1974) and hydroxyurea (Pica Mattoccia and Roberti, 1974; Dujardin, et al., 1978) have a limited action on mitochondrial DNA while effectively inhibiting nuclear DNA synthesis. In contrast, ethidium bromide inhibits only mitochondrial DNA replication (Pica Mattoccia and Roberti, 1974).

Most *S. cerevisiae* strains also contain multiple copies of a $2\mu\text{m}$ circle of double-stranded DNA. The molecule contains approximately 6 kbp (Fangman and Zakian, 1981). Most evidence indicates that $2\mu\text{m}$ DNA is located in the nucleus, and is reproduced during the chromosomal DNA synthesis phase of the cell cycle.

3.4.1.2 RNA

Only about 4% of the total cell nucleic acid is DNA, the remainder being RNA. Cellular RNA may be resolved into three distinct fractions: ribosomal RNA (rRNA),

messenger RNA (mRNA) and transfer RNA (tRNA). These may be differentiated by analysis of nucleotide base composition, stability, sedimentation and saline solubility characteristics.

Ribosomal RNA accounts for 85% of the RNA in *S. cerevisiae* (Warner, 1971). In a study of *S. cerevisiae* by Gatti and Fredericq (1974), 60-70% of this rRNA was found to be double-stranded.

Messenger RNA accounts for about 2% of cellular RNA. The instability and high turnover of mRNA is related to its function as an intermediate carrier of the genetic information required for protein synthesis. In rapidly dividing yeast, a large fraction of extracted ribosomes is found in clusters called polysomes. These are of various sizes, and are bound together by a strand of mRNA to serve as templates for protein synthesis.

There are transfer RNA molecules specific for each amino acid. tRNA transfers activated amino acids to the ribosome-mRNA complex.

3.4.1.3 Protein

The amount of protein in *Saccharomyces cerevisiae* varies considerably with growth rate and nitrogen source. The nature of the protein molecules is very diverse, reflecting the multitude of structural, enzymatic and other functional roles played within the cell.

Yeasts contain a pool of free amino acids, made up mainly of glutamic and aspartic acids and α -alanine (Harrison, 1963). Approximate proportions of the amino acids present in *S. cerevisiae* are given by Eddy (1958). Estimates of the amounts of several enzymes in crude extracts of *Saccharomyces* yeast are listed by Fraenkel (1982). The glycolytic enzymes account for an appreciable proportion of soluble protein in *S. cerevisiae*. Hess, et al. (1969) reports this fraction to be

65%.

3.4.1.4 *Storage Polysaccharides*

Yeast energy reserves consist largely of carbohydrate, and make up approximately 25% of the dry matter in the cell (Sols, et al., 1971). Glycogen accounts for 60-70% of the carbohydrate content of *S. cerevisiae* cells, while the proportion of trehalose is usually 5-8% (Manners, 1971).

The storage of reserve carbohydrate is markedly dependent on the metabolic state of the cell. It has been found that the carbohydrate level of slower growing cells is higher than that of faster growing ones (Küenzi and Fiechter, 1969). Restricted growth, whether due to carbon, nitrogen, or phosphorous limitation (Lillie and Pringle, 1980; Küenzi and Fiechter, 1972) is a signal for carbohydrate accumulation.

During starvation of yeast, glycogen is broken down to provide energy for the cell. Trehalose is not usually utilized during endogenous metabolism. However, it is rapidly consumed during lag phase in the presence of nutrients (Panek, 1963).

3.4.1.5 *Structural Polysaccharides*

The structural carbohydrates, glucan and mannan, form about 10% of the dry weight of *S. cerevisiae* (Manners, 1971). Three components, glucan, mannan, and chitin, make up over 90% of the yeast cell wall, whereas only small and variable amounts of lipid have been reported (Phaff, 1971). Synthesis of structural polysaccharides is known to be little affected by growth rate (McMurrough and Rose, 1967). Glucan and mannan are not degraded during endogenous metabolism (Berke and Rothstein, 1957; Panek, 1963).

In *S. cerevisiae*, glucan polysaccharide is composed predominately of β -linked D-glucopyranose units. α -linked mannose units account for much of the

mannoprotein structure (Ballou, 1982).

3.4.1.6 Lipids

Yeast accumulate lipids as reserves of energy when there is an excess of oxygen and an abundant sugar supply. Even so, *S. cerevisiae* normally are composed of only about 5% lipid (Sols, et al., 1971).

3.4.2 Vitamin Requirements

Vitamins function as activators in many enzyme reactions. While some may be synthesized, others must be assimilated directly from the medium.

Saccharomyces cerevisiae must be provided with biotin, pantothenic acid and nicotinic acid. Other vitamins promote growth but may not be essential depending on the other growth factors in the medium. In particular, yeasts are able to synthesize riboflavin, para-aminobenzoic acid and folic acid (Suomalainen and Oura, 1971).

3.4.2.1 Biotin

S. cerevisiae cannot synthesize biotin from simple precursors (Keränen, 1969). However, aspartic acid is able to partly replace, and together with oleic acid, completely replace biotin as a growth factor (Suomalainen and Keränen, 1963).

Biotin has several roles in biosynthesis. Growth of biotin-requiring yeast under conditions of biotin deficiency has been shown to bring about certain changes in the chemical composition of the cells. Biotin-deficient *Saccharomyces cerevisiae* contain less DNA, RNA, and total protein (Ahmad, et al., 1961) compared with exponentially growing yeast. Activity of several enzymes, particularly those concerned with the metabolic transfer of carbon dioxide, are dimin-

ished (Cazzulo, et al., 1968; Mishina, et al., 1980). Fatty acid synthesis (Suomalainen and Keränen, 1963) and formation of purine and pyrimidine bases (Rose, 1960) are also impaired.

Biotin deficiency may lead to damage of the plasma membrane (Dixon and Rose, 1964). Increases in membrane permeability have been measured (Rose, 1963). Biotin-starved yeast were found to secrete nicotinic acid (Rose and Nickerson, 1956).

Microscopic examination of budding *S. cerevisiae* cells grown in biotin-deficient medium has shown that normal separation of buds from the parent cells does not occur. Large aggregates of cells may instead be formed (Dunwell, et al., 1961). The ratio of glucan to mannan was found to be elevated in these cells.

Inside the cell, biotin is concentrated 1100-fold over extracellular levels (Cooper, 1982). Uptake is stimulated by glucose, and is very rapid. Inhibition of biotin transport following long-term growth of cells in the presence of the vitamin has been attributed to repression (Rogers and Lichstein, 1969). Active transport of biotin is seen only when the cells are grown in the presence of concentrations of biotin less than about 0.25 ng/ml.

3.4.3 Oxygen Requirements

Under completely anaerobic conditions, the need for trace amounts of oxygen in biosynthesis must be met by supplying ergosterol (Andreasen and Stier, 1953) and oleic acid (Andreasen and Stier, 1954) in the medium. These requirements are also supplied by yeast extract in undefined medium (Rogers and Stewart, 1973).

3.4.4 Fermentation

Saccharomyces cerevisiae is a heterotrophic organism which can use sugars and a variety of other compounds as nutrient sources. From these compounds, carbon skeletons are derived for use in biosynthesis of cellular constituents, and energy is produced to allow the biosynthetic reactions to proceed.

During oxidation of sugars by fermentation, organic compounds serve as the terminal electron acceptors. In *S. cerevisiae*, the major end-products of fermentation are ethanol and carbon dioxide, with ATP being the physiological end-product useful to the cell.

3.4.4.1 Sugar Uptake

As the first step in the utilization of sugars by *S. cerevisiae*, transport across the cell membrane must occur.

The mechanism of glucose uptake is not completely understood (Bisson and Fraenkel, 1983). Glucose appears to enter *S. cerevisiae* by way of a constitutive facilitated diffusion system. The phosphorylated sugar may be found inside the cell before the free sugar (Meredith and Romano, 1977), suggesting a close relationship between transport and phosphorylation. Affinity for uptake changes depending on whether the cell is fermenting or respiring. The rate of glucose utilization is greater during fermentation (Azam and Kotyk, 1967).

The internal concentration of glucose is very low in *S. cerevisiae* cells (Kleinzeller and Kotyk, 1967; Becker and Betz, 1972). It appears that glucose uptake may limit the rate of fermentation, since the glycolytic enzymes are able to remove the sugars as fast as they enter (van Steveninck and Rothstein, 1965; van Uden, 1967).

Other sugars besides glucose may be fermented by *S. cerevisiae*. Fructose and mannose enter the cell in a similar fashion and at the same rate as glucose (Heredia, et al., 1968). The affinity of the *S. cerevisiae* membrane carriers for several other hexose sugars are given by Kotyk and Janacek (1975).

Galactose transport occurs via an inducible facilitated diffusion system (Cirillo, 1968). Uptake of galactose is prevented when glucose is present, possibly as a result of destruction or alteration of the galactose carrier (Matern and Holzer, 1977). *S. cerevisiae* is also able to take up trehalose, a polysaccharide normally thought of as a storage material (Kotyk and Michaljanicová, 1979). The transport system for trehalose has not been identified.

3.4.4.2 Metabolic Pathway

Fermentation of glucose to ethanol in *Saccharomyces cerevisiae* requires functioning of the Embden-Meyerhof-Parnas pathway. This multistep reaction sequence converts glucose to pyruvate, with a nett production of two ATP molecules per molecule of glucose. Intermediates of the EMP pathway provide the carbon required for biosynthesis via the pentose phosphate shunt.

The distinguishing feature between fermentation and respiration is the fate of pyruvate formed in the EMP pathway. In respiring yeast, pyruvate is oxidized to carbon dioxide and water. When coupled to the respiratory chain, the tricarboxylic acid cycle acts as the main energy producer of the cell. Intermediates of the tri-carboxylic acid cycle also serve as precursors for biosynthesis.

During fermentation, pyruvate is instead decarboxylated and reduced in two steps to form the fermentation products, ethanol and carbon dioxide, which are excreted from the cell. The route from glucose to ethanol is outlined in Figure 3-1. The overall reaction is:

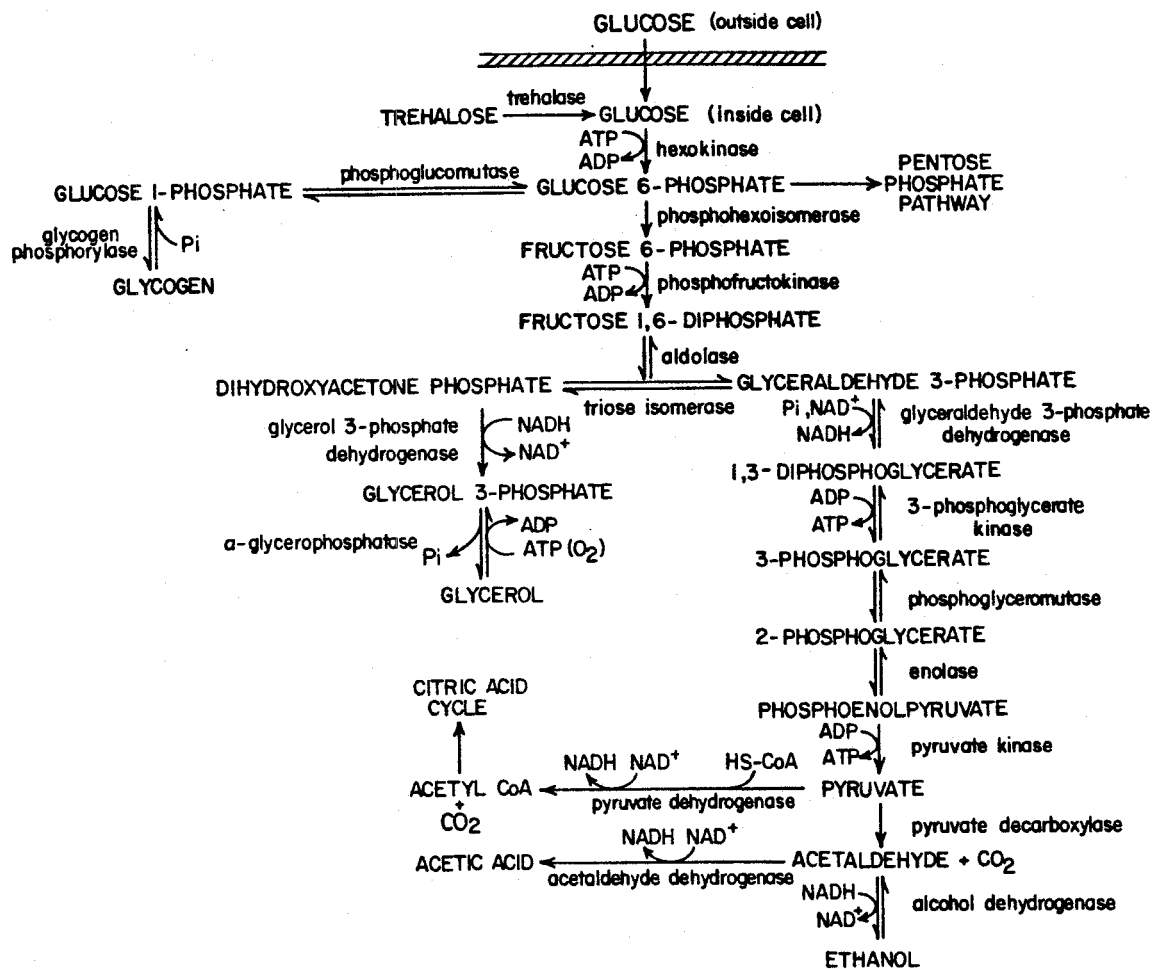
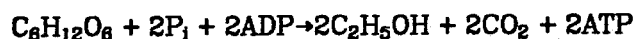


Figure 3-1

Reactions of the Embden-Meyerhof-Parnas and related pathways



Based on this stoichiometric relationship between ethanol produced and glucose consumed, the theoretical product yield is 51.1% ethanol and 48.9% carbon dioxide by weight.

Also shown in Figure 3-1 are two alternative fermentation pathways. When the enzyme alcohol dehydrogenase is not operative, regeneration of NAD^+ occurs by reduction of dihydroxyacetone phosphate (Gancedo, et al., 1968). The main fermentation product in this case is glycerol, which diffuses out of the cell. Unlike ethanol fermentation, this route does not provide the cell with either energy or biosynthetic precursors.

Under alkaline conditions, acetaldehyde may be converted into acetic acid instead of ethanol. Since the enzyme which accomplishes this conversion requires NAD^+ (Black, 1951), the glycerol route must also operate to reoxidize the NADH produced. It is likely that the physiological role of this process is to lower the pH of the medium to within a range more favourable to the yeast cell (Sols, et al., 1971).

3.4.4.3 Fermentation of Galactose and Storage Carbohydrates

Utilization of galactose in the EMP pathway requires induction of an enzyme system which converts galactose to glucose 1-phosphate. Galactokinase converts galactose to galactose 1-phosphate, and uridyl transferase converts galactose 1-phosphate and UDP-glucose to glucose 1-phosphate and UDP-galactose. UDP-galactose is reconverted to UDP-glucose by an epimerase enzyme. These galactose pathway enzymes are susceptible to glucose repression.

Two other enzymes belong to the galactose pathway, but have broader roles in the cell. Phosphoglucomutase interconverts glucose 1-phosphate to glucose 6-phosphate, and UDP-glucose pyrophosphorylase forms UDP-glucose from glucose 1-

phosphate and UTP (Fraenkel, 1982).

Glycogen degradation occurs via glycogen phosphorylase, which forms glucose 1-phosphate. Again, phosphoglucomutase is involved in the transformation to glucose 6-phosphate. Trehalose is hydrolysed to glucose by the enzyme, trehalase. Trehalase appears to be an inducible enzyme sensitive to the level of ATP in the cell (Sols, et al., 1971).

3.4.4.4 Oxygen Effects

In the transition from anaerobic to aerobic conditions, facultative organisms increase their production of biomass. This effect stems from the contribution of mitochondrial oxidative phosphorylation to the overall energy economy of the cell. Growth yield is related to ATP production (Stouthamer and Bettenhausen, 1973), and complete oxidation of glucose allows production of up to 18 times more ATP per mole of catabolized sugar than that obtained from the fermentative pathways. Since energy production is more efficient, substrate utilization rates are usually lower during respiration. Inhibition of catabolism by oxygen is known as the Pasteur effect.

Although enhancement of growth in the presence of oxygen is observable with *Saccharomyces cerevisiae*, it has been shown that the nett benefit from aerobic metabolism of glucose is actually much less than predicted from theoretical considerations (Lagunas, 1979). Under normal conditions, glucose utilization during aerobiosis is not greatly altered from that in the absence of oxygen, so that *S. cerevisiae* is considered not to show a noticeable Pasteur effect. High glucose concentrations are generally responsible for this insensitivity to oxygen (Oura, 1974). Inhibition of glucose fermentation by aerobiosis is possible at very low sugar concentrations (Rogers and Stewart, 1973) and during nitrogen starvation (Lagunas, 1982).

Respiration is more important during growth on galactose (Fraenkel, 1982). Under aerobic conditions with galactose as substrate, about 88% of the cell's ATP production occurs as a result of respiration. With glucose, this fraction is only 34% (Lagunas, 1979).

3.4.4.5 Substrate Effects

Respiratory capacity and the levels of many respiratory components and enzymes in *Saccharomyces cerevisiae* depend on the carbon source used for growth. Glucose concentrations higher than 0.2 mM can be expected to induce catabolic repression of many respiratory enzymes (Akbar, et al. 1974). Repression is most effective with glucose, but galactose causes a similar response (Polakis and Bartley, 1965). This suggests that catabolism rather than glucose itself signals control of the repression.

A distinction should be made between catabolic repression of enzyme synthesis and inhibition of enzyme activity. Although the data of Lagunas (1979) and others show that high glucose concentrations have little effect on respiratory activity, catabolic inactivation of specific gluconeogenic enzymes has been demonstrated. This is discussed further by Fraenkel (1982).

There have been many attempts to identify the controlling sequences leading to catabolic repression and inactivation of respiratory enzymes. However, the mechanism is still not known.

3.4.4.6 Product Inhibition

During alcohol fermentation by *Saccharomyces cerevisiae*, increasing concentrations of ethanol adversely affect activity of the cells. Specific growth rate (Bazua and Wilke, 1977), viability (Thomas, et al., 1978), specific fermentation rate (Aiba, et al., 1968) and transport rate for sugars and amino acids (Thomas and

Rose, 1979) decline as ethanol accumulates in batch reaction. According to Holzberg, et al. (1967), these effects become evident as ethanol concentrations rise above about 25 g/l. Cessation of growth occurs at about 70 g/l ethanol. The effects of ethanol inhibition become more severe with increasing temperature (Leão and van Uden, 1982).

Carbon dioxide also has an inhibitory effect on *S. cerevisiae* growth. Chen and Gutmanis (1976) report that inhibition is negligible at concentrations less than 20% carbon dioxide in the aeration mixture. Under normal fermentation conditions, dissolved carbon dioxide in the fermentation broth is usually less than 20% of this inhibitory level.

3.4.5 Cell Cycle Operation

The cell cycle is a process of asexual cellular reproduction. Under normal conditions, one cell gives rise to two genetically identical cells. Coordination of the various biochemical and morphological events has been investigated using cell division cycle, or *cdc* mutants, of *Saccharomyces cerevisiae*. By definition, a *cdc* mutation leads to a defect in a particular stage-specific function of the cell cycle.

In principle, a *cdc* mutation could either block completely or produce only abnormalities in the affected function. In practice, however, most *cdc* mutations studied have produced complete blockage of events essential for cell cycle progress (Pringle and Hartwell, 1981). Since mutations of this type are lethal, conditional mutants such as those which exhibit temperature sensitivity have been used in most studies.

3.4.5.1 Landmark Events

The temporal order of events is shown in Figure 3-2 (Pringle and Hartwell,

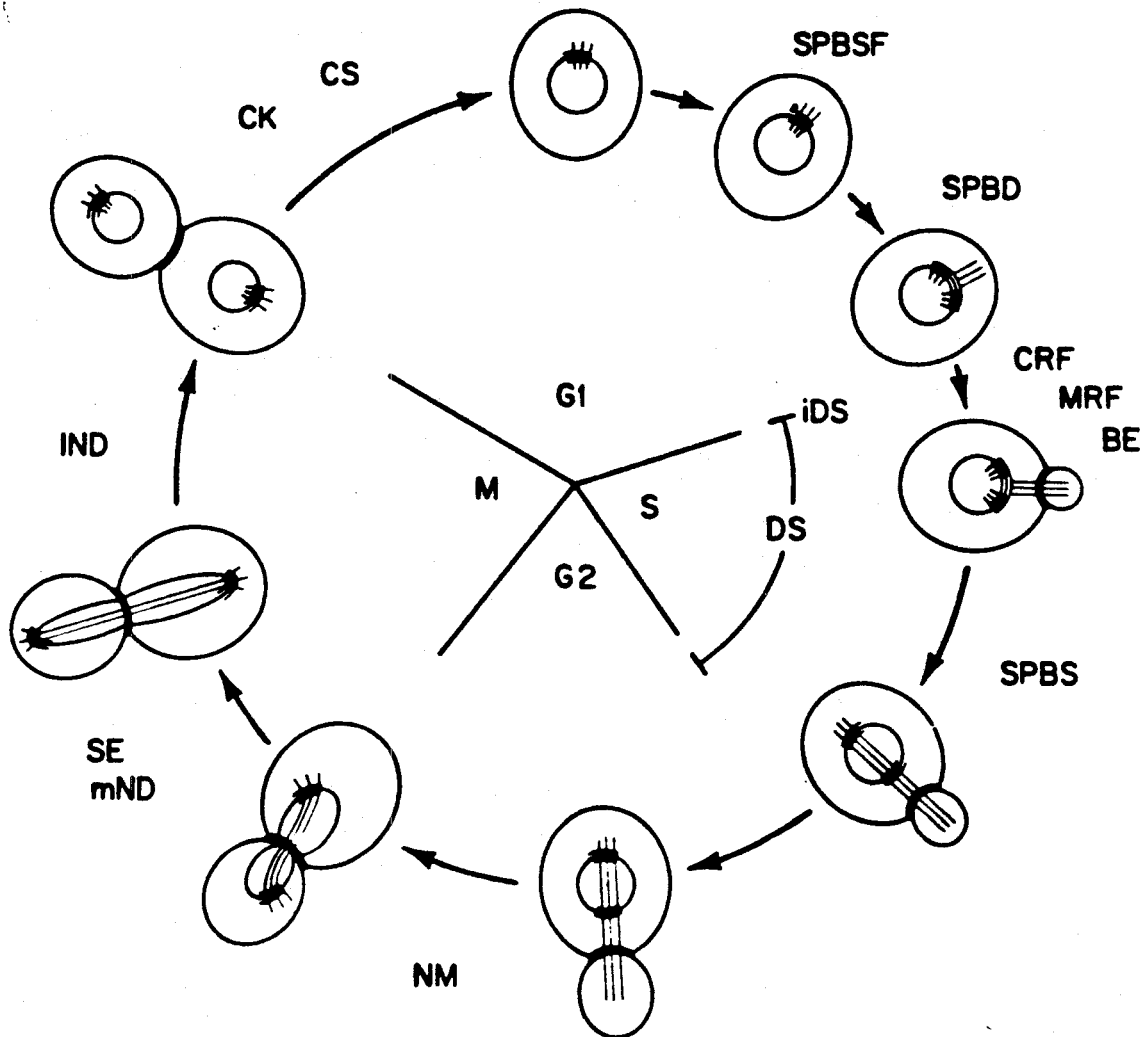


Figure 3-2

Major events of the *Saccharomyces cerevisiae* cell cycle (Pringle and Hartwell, 1981)

Abbreviations

BE -	bud emergence	mND -	medial nuclear division
CK -	cytokinesis	MRF -	microfilament ring formation
CRF -	chitin ring formation	NM -	nuclear migration
CS -	cell separation	S -	DNA synthesis phase
DS -	chromosomal DNA synthesis	SE -	spindle elongation
G1 -	first growth phase	SPBD -	spindle-pole-body duplication
G2 -	second growth phase	SPBS -	spindle-pole-body separation
iDS -	initiation of chromosomal DNA synthesis	SPBSF -	spindle-pole-body satellite formation
IND -	late nuclear division		
M -	mitosis phase		

1981) and a detailed description of the individual events is given by Hartwell (1974).

Like cell cycles of other eukaryotes, the yeast cell cycle may be divided into four stages. The G₁-phase denotes the unbudded stage of the cycle preceeding initiation of chromosomal DNA replication. Chromosomal and 2 μ m-circular DNA synthesis occurs during the S-phase. The bud grows in size throughout the subsequent G₂-phase. The end of the G₂-phase is marked by migration of the nucleus into the neck of the cell-bud unit. Nuclear division, cytokinesis, or cell membrane separation, and cell wall separation occur in the M- or mitosis-phase.

Two unbudded cells are formed; however, the daughter is usually smaller than the parent cell by an amount which depends on growth rate (Slater, et al., 1977). For slow-growing cells, most of the increase in generation time results from increases in the duration of the G₁-phase (Johnston, et al., 1980). Growth is usually required prior to initiation of budding. In this way, yeast cell growth and cell division are coordinated (Johnston, et al., 1977). Development to a critical size is often taken as the criteria for cell cycle "start". However, the variability of cell volume at bud emergence under different conditions suggests that some parameter only loosely correlated with cell size is the controlling factor (Pringle and Hartwell, 1981). The fraction of cells in each phase of the cell cycle, together with an age distribution expression for the population, allows calculation of the timing for cell development in the cycle (Slater, et al., 1977; Bailey, et al., 1981).

3.4.5.2 Functional Sequence

Events of the cell cycle may be organized as shown in Figure 3-3 (Pringle and Hartwell, 1981). Only the middle pathway of events involved primarily with DNA replication and nuclear division must be completed before commencement of a second cycle. Bud emergence and nuclear migration occur independently of the

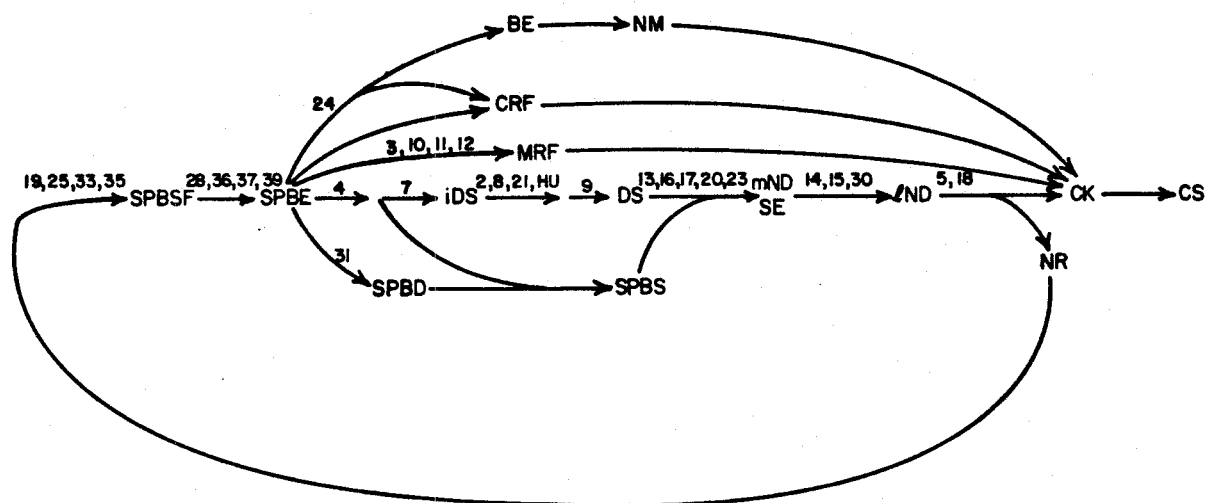


Figure 3-3

Functional dependence of events in the *Saccharomyces cerevisiae* cell cycle (Pringle and Hartwell, 1981). CDC genes associated with particular steps are indicated by their numbers.

Abbreviations

BE -	bud emergence	NM -	nuclear migration
CK -	cytokinesis	NR -	nuclear reorganization
CRF -	chitin ring formation	SE -	spindle elongation
CS -	cell separation	SPBD -	spindle-pole-body duplication
DS -	chromosomal DNA synthesis	SPBE -	spindle-pole-body enlargement
HU -	hydroxyurea	SPBS -	spindle-pole-body separation
iDS -	initiation of chromosomal DNA synthesis	SPBSF -	spindle-pole-body satellite formation
IND -	late nuclear division		
mND -	medial nuclear division		
MRF -	microfilament ring formation		

events in the inner cycle. The pathways diverge at spindle-pole-body elongation and converge for cytokinesis (Hartwell, et al., 1974).

As indicated in Figure 3-3, DNA synthesis and nuclear division can occur in the absence of bud emergence. Conversely, bud emergence and nuclear migration may proceed without synthesis of DNA. Cytokinesis and cell separation are dependent upon completion of nuclear division as well as bud development.

3.4.5.3 Bud Emergence

Bud formation can occur at any place on the yeast cell; however, there is preferential occurrence at the poles of ellipsoidal diploid *S. cerevisiae* cells (Streiblová, 1970; Bartnicki-Garcia and McMurrough, 1971; Sloat, et al., 1981). Synthesis of new cell wall material is directed into the bud tip, as demonstrated by autoradiographic analysis (Johnson and Gibson, 1966), fluorescent labelling of mannan (Tkacz and Lampen, 1972) and by staining of acid phosphatase (Field and Schekman, 1980).

There is limited information about the structural changes which must occur at the cell wall for budding to take place. The mechanism of localized wall expansion, whether through the weakening action of hydrolytic enzymes acting on the cell wall polysaccharides, or through physical stretching of the glucan fibres, has not been resolved.

Moor (1967) postulated that disulphide reductase was the agent used to soften the cell wall. Evidence from electron microscope studies suggested that this enzyme was carried by vesicles which appeared just prior to budding.

The behaviour of intranuclear spindles and spindle plaques in relation to bud emergence has been studied by Byers and Goetsch (1975). It was hypothesised that microtubules extending from the duplicated plaque into the bud neck were

influential in the choice of budding site and in the transport of vesicles needed for bud development. The two events, spindle-pole-body duplication and bud initiation are now known to be independent, following the isolation of *cdc 31* mutants which can bud and initiate DNA synthesis without plaque duplication (Pringle and Hartwell, 1981).

An investigation of *cdc 24* mutant strains by Sloat, et al. (1981) provided evidence that the *CDC 24* gene product plays a key role in selection of budding site, although the mechanism remains unclear. Many abnormalities in budding morphology were observed with these mutants, so it is difficult to pinpoint the precise sequence which determines the location and appearance of the nascent bud.

3.4.5.4 Growth during the Cell Cycle

RNA and protein accumulate continuously during the cell cycle. Their synthesis is independent of DNA replication, as shown by experiments with hydroxyurea (Slater, 1973) and various *cdc* mutants (Hartwell, 1971).

Mitochondrial DNA synthesis is also continuous through the cell cycle and does not cease when nuclear DNA replication is inhibited (Cottrell, et al., 1973; Cryer, et al., 1973).

Cyclic changes in reserve carbohydrate levels during the *S. cerevisiae* cell cycle are described by Küenzi and Fiechter (1969). Cells accumulate glycogen and trehalose during the cycle between successive buddings. Part of these reserves are rapidly degraded immediately prior to swelling of the new bud. Relatively more reserve material is mobilized for budding in slower growing populations than in faster growing cells.

Synthesis of the major cell wall polysaccharides appears to be continuous throughout the cell cycle, although there may be a decrease in rate near the time

of cell division (Küenzi and Fiechter, 1969; Hayashibe, et al., 1977). In contrast, chitin synthesis is a discontinuous process, occurring in one or two bursts (Hayashibe and Katohda, 1973; Cabib and Bowers, 1975).

3.4.6 Conclusions

Saccharomyces cerevisiae has been used extensively as an experimental organism in many biochemical, morphological and genetic studies. Many of the features of its growth and fermentation behaviour have been elucidated. The extent of this information base renders *S. cerevisiae* particularly useful in work which aims to identify changes in metabolism resulting from cell immobilization.

3.5 FLOW CYTOMETRY

Flow cytometry is a technique which allows rapid measurement of the properties of single cells. About 1000-5000 cells/second may be analysed, enabling measurements of high statistical significance to be made in a short time.

3.5.1 Instrument and Applications

As shown in Figure 3-4, the flow cytometer comprises a laminar flow system which transports cells, one at a time, past a laser excitation source. If a specific component of the cells, such as DNA, RNA or protein, has been fluorescently labelled prior to measurement, the stained component will emit fluorescence as the cell passes the laser beam. Since each cell is exposed to the laser for only a few microseconds, problems with fluorescent decay are minimized. Two or more fluorochromes may be used if the emissions are of different wavelengths and may be resolved optically.

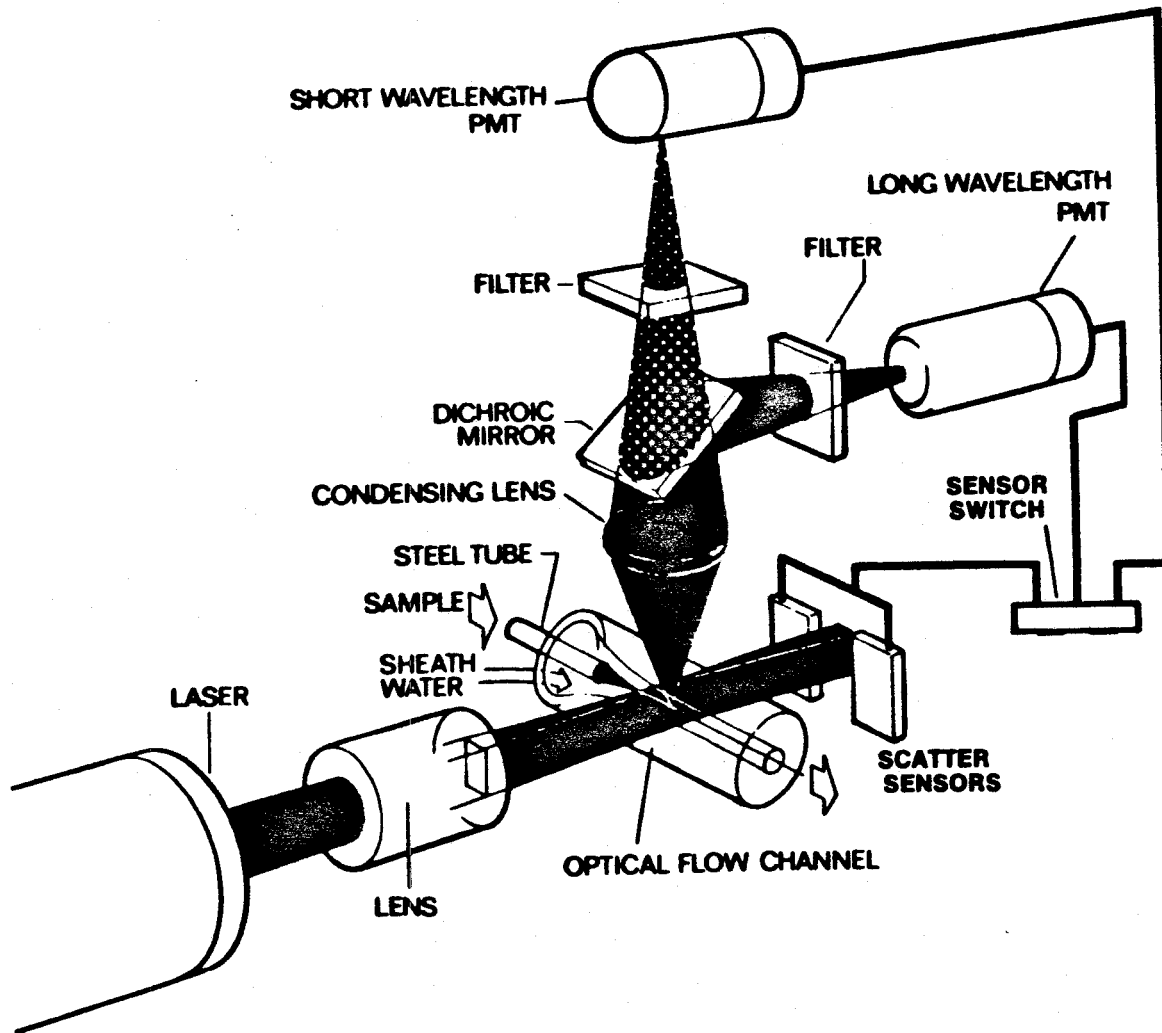


Figure 3-4
Flow cytometer components (Super, 1979)

The fluorescence emitted by the cell is detected and analysed electronically to give a pulse with amplitude proportional to the amount of fluorescence. These pulses are sorted and stored to give a frequency function of single-cell fluorescence level for a population of cells.

Light scatter capability is also included in the system of Figure 3-4. As the cells pass through the laser, the extent to which they scatter the light depends on cell size.

As a cytological technique, flow microfluorometry is relatively new. Information about the method and its applications with mammalian cells is given by Crissman, et al. (1975). The use of flow cytometry in the study of microorganisms is an even more recent development. The smaller size of the cells and the lower levels of the various components require a more intense excitation source and sensitive emission detectors.

Measurement of RNA and protein content for various strains of *Saccharomyces cerevisiae* during vegetative growth was first performed by Hutter (1974). Methodology for other applications, such as viability measurement (Hutter and Eipel, 1978) and ploidy evaluation (Hutter and Eipel, 1979) have since been developed. Gilbert, et al. (1978) and Hutter (1978) have studied the distributions of DNA, RNA and protein at various stages of the batch growth of *S. cerevisiae*. In addition, the cell cycle behaviour of yeast has been investigated using flow cytometry to determine relative numbers of cells in the G₁-, S- and G₂- + M- phases (Slater, et al., 1977).

3.5.2 Fluorescent Staining

For laser excitation of stained cellular components, the dye of choice must be excitable at one of the given laser wavelengths. In addition, it should bind very

strongly with its specific component molecules and should not leach out into the suspending fluid.

3.5.2.1 DNA

The Feulgen reaction is probably the most widely used protocol for quantitative staining of cellular DNA. Acid hydrolysis is used to remove purines from the DNA. The aldehydes of deoxyribose sugars exposed by hydrolysis then react with an aldehyde-specific agent. RNA is not involved in the staining, since it is removed during acid hydrolysis.

Feulgen staining of cells using acriflavine is described by Crissman, et al. (1975). A laser wavelength of 488nm is used.

Mithramycin has also been used to fluorescently stain DNA. This substance is highly selective for DNA and does not interfere significantly with RNA. Magnesium is required for mithramycin-DNA binding. Procedures taking only 20 minutes to perform and using unfixed cells are described by Crissman, et al. (1975). Alternative staining methods are given by Boye, et al. (1983).

Mithramycin has a maximum excitation peak at about 395nm and an emission peak at 530nm. Since the lowest efficient wavelength available from an argon-ion laser is 457nm, the technique may suffer from suboptimal excitation.

3.5.2.2 RNA

Intercalating dyes, such as ethidium bromide and its analogue, propidium iodide, may be used to stain stable double-stranded RNA (Gatti and Fredericq, 1974). These substances selectively and quantitatively intercalate into the double-stranded regions of nucleic acids, and may also be used for DNA staining following pretreatment of the cells with RNAase. Since DNA represents only a small fraction of total cell nucleic acid, its fluorescence during RNA analysis does not affect the

measurement significantly.

3.5.2.3 Protein

Among the most commonly used fluorochromes for protein labelling is fluorescein isothiocyanate (FITC). Maximum excitation and emission peaks lie at about 490nm and 530nm respectively (Crissman, et al., 1975).

3.5.3 Conclusions

Flow cytometry has proven itself as a rapid means for quantifying the physical and biochemical characteristics of cells. It is a valuable analytical tool in immobilized cell research when recovery of cells from the immobilization support is possible.

3.6 GLYCOLYTIC OSCILLATIONS

Under certain conditions, perturbances of concentration or temperature in the glycolytic pathway evoke an oscillatory response which involves all intermediates and cofactors in the sequence. These oscillations afford an opportunity to monitor the properties of enzymes in the cell which are responsible for energy production during fermentation.

3.6.1 Detection of Glycolytic Oscillations

Fluorescence measurement of intracellular NADH levels is a convenient way of monitoring glycolytic oscillations. Techniques have been developed which enable detection of fluorescence from as few as 10^5 NADH molecules. Each yeast cell contains, at most, 10^8 molecules of NADH (Chance, et al., 1967). About 2×10^{-8} moles of NADH per gram of yeast are involved in the oscillations (Chance, et al., 1964a).

Spectrophotometric studies have indicated that the main component recorded by fluorometry or spectroscopy is protein-bound NADH (Hess, et al., 1973). In yeast, maximum absorbancy occurs at 335-350 nm. Fluorescence emission is at 450 nm (Chance, et al., 1964a).

Glycolysis is thought to occur entirely in the cytoplasm (Pye, 1972; Fraenkel, 1982). Two essentially independent pools of NADH occur in yeast, cytoplasmic and mitochondrial, but considerable evidence from inhibitor studies indicates that it is the glycolysis-associated cytoplasmic pool which oscillates (Chance, et al., 1964a; Pye, 1972).

Oscillations have been measured in both intact cells and in cell-free extracts. Generally, extract oscillations are less damped than when glycolysis operates in conjunction with other cell functions, particularly the sugar transport (Pye, 1969; Hess and Boiteux, 1971) and carbohydrate storage (Betz and Hinrichs, 1968) systems. Glycolytic flux and frequency of oscillations may be greater by an order of magnitude in intact cells than in cell-free extracts (Betz and Moore, 1967).

3.6.2 Methods for Inducing Glycolytic Oscillations

Damped oscillations are usually induced in yeast by adding glucose to aerobically-grown starved cells. After a period of rapid respiration which exhausts the supply of oxygen present, transition to anaerobiosis occurs, triggering the oscillatory response (Hommes, 1964).

Anaerobiosis is required for the oscillations to appear. Mitochondrial control of intracellular ATP/ADP levels may prevent the glycolytic system from oscillating under aerobic conditions (Chance, et al., 1964a; Berridge and Rapp, 1979). Cyanide and sulphide are inhibitors of respiration in common use to induce the transition to higher levels of reduced pyridine nucleotides. Enhancement of oscillations by

cyanide, and the inhibitory action of other substances is discussed by Betz and Chance (1965a).

In cell-free extracts of yeast, substrate addition may not be necessary for oscillations to occur. A temperature change from 0°C to room temperature was found by Chance, et al. (1964b) to induce periodic NADH level oscillations.

3.6.3 Oscillation Characteristics

The frequency and amplitude of oscillation depends on prior treatment of the cells as well as the conditions used to perturb the system. Factors which affect the character of glycolytic oscillations include:

- (i) phase of cell growth;
- (ii) starvation time;
- (iii) type of substrate added;
- (iv) substrate concentration;
- (v) temperature; and
- (vi) addition of intermediate compounds and substances which modify the energy charge of the cell.

3.6.3.1 Phase of Growth

The occurrence of oscillations and their duration depends to a large extent on the growth phase of the culture used in the fluorescence measurements.

For the diauxic growth behaviour exhibited by yeast under aerobic conditions, cells harvested during the first logarithmic phase do not usually show NADH oscillations. Cells harvested late in the second logarithmic phase during growth on ethanol are likely to display short trains of continuously damped oscillations.

However, for cells at the end of their first logarithmic growth phase just before the intermediary lag phase, anaerobiosis may initiate long trains of oscillations (Pye, 1966, 1969; Kreuzberg and Betz, 1979).

The oscillatory response is associated with the state of the glycolytic pathway as the supply of sugar in solution is diminished. Kreuzberg and Betz (1979) have suggested that these effects are related to increased glyconeogenesis through fructose 1,6-diphosphatase activity.

Synthesis of enzymes which were previously not required commences as ethanol is exchanged for glucose as carbon source. Oscillations in enzyme synthesis are discussed by Goldbeter and Nicolis (1976). However, interaction between these events and glycolytic oscillations have not been examined. Presumably, the time scale for the glycolytic oscillations is smaller than that involved in enzyme synthesis.

3.6.3.2 Starvation

For cells harvested and starved prior to fluorescence measurements, the period and amplitude of the oscillations (Kreuzberg and Betz, 1979) and their duration (Chance, et al., 1964a) vary with the length of the starvation time. In general, the amplitude of oscillation increases and the period decreases with longer starvation times.

Although cells may be starved before measurement of their NADH levels, a considerable amount of intracellular endogenous substrate is still available. Apparently, this supply of substrate for the glycolytic pathway does not affect the oscillatory reductions (Hommes, 1964).

After addition of glucose to starved cells, approximately 50% of the substrate is channelled into production of storage reserves. Periodic fluctuations in this

pathway and interaction with oscillatory controlled glycolysis in yeast is discussed by Betz and Hinrichs (1968).

3.6.3.3 Nature of Substrate

Different sugars produce different transient response characteristics. All other conditions kept constant, the frequency of oscillation with glucose > mannose > fructose.

Concentrations of fructose greater than about 50mM suppress the oscillatory response in yeast (Kreuzberg and Betz, 1979). This effect is not evident with glucose as substrate. Betz, et al. (1975) have examined inhibition of phosphofructokinase by fructose. D-fructose has an allosteric effect on phosphofructokinase, and in addition, competes with fructose 6-phosphate for the phosphofructokinase binding site. Fructose 1-phosphate may also accumulate in cells grown on fructose. This substance is a potential substrate for phosphofructokinase, but has different kinetic properties than fructose 6-phosphate (Kreuzberg, et al., 1977). Allosteric activation and competitive inhibition of yeast phosphofructokinase by fructose and the consequences to production of glycolytic oscillations are discussed by Betz, et al. (1975).

With single additions of glucose or fructose, only damped oscillations have been produced. However, additions of trehalose to cell-free extract were found to cause sustained oscillations lasting about four hours (Pye and Chance, 1966; Hess, et al., 1966). Trehalase has a lower activity than hexokinase, and the decreased flux into the pathway may be responsible for production of self-oscillations (Sel'kov, 1968a; Pye, 1969).

3.6.3.4 Sugar Concentration

Sugar concentration determines the rate of substrate entry into the glycolysis

pathway. The critical role played by this parameter in determining the occurrence and character of the oscillations is illustrated in the work of Hess and Boiteux (1968, 1973). In a cell-free extract, there exists a range of substrate injection rates which induces NADH oscillations. Outside this range, glycolysis settles without periodicity into a steady state regime. The waveform, as well as the period and amplitude, is controlled by the rate of substrate input. Double periodicity was observed at low injection rates of about 20 mM/hour (Hess and Boiteux, 1968). With intact cells and high concentrations of substrate, saturation of the membrane sugar carriers may remove dependence of the oscillations on the actual concentration added.

Periodic addition of substrate to yeast cell-free extract showed that glycolysis could synchronize with the period of substrate supply. Synchronization to a subharmonic of the driving frequency within a certain range was recorded (Boiteux, et al., 1975).

3.6.3.5 Temperature

Frequency of oscillation is highly temperature dependent (Chance, et al., 1964a; Betz and Chance, 1965a). Variation of temperature from 15°C to 38°C reduces the period of oscillation by a factor of ten (Kreuzberg, et al., 1977).

3.6.3.6 Addition of Intermediates and Other Substances

Several compounds may be used to modify the character of glycolytic oscillations in cell-free extract. Additions of ADP, pyruvate and acetaldehyde cause phase-dependent phase shifts (Chance, et al., 1964c; Betz and Becker, 1975).

The effects of acetaldehyde and pyruvate were cited as a possible mechanism for the apparent synchrony which exists between oscillating yeast cells (Chance, et

al., 1973). At first, the damping of glycolytic oscillations after single additions of glucose was thought to occur due to loss of synchrony between cells in suspension, resulting in an average NADH fluctuation of zero for the population. However, microfluorometer techniques developed by Chance and Legallais (1959) helped identify the damped oscillations as a result of single cells themselves undergoing periodic fluctuations of decreasing intensity.

When two populations of yeast 180° out of phase are mixed, synchronized oscillations develop within about 20 seconds (Pye, 1969). The magnitude of the disturbance created upon mixing of such out-of-phase cultures depends on the relative phase angles (Chance, et al., 1973). Since pyruvate and acetaldehyde are readily excreted by yeast and may be present in the medium at approximately the same concentration as in the cell, these substances may provide a physical basis of information exchange between single cells in suspension (Betz and Becker, 1975).

The effects of apyrase on oscillation characteristics were examined by Frenkel (1966) and Pye (1969). Apyrase, which functions as an ATPase in cell-free extracts, interferes with the energy charge of the cell by lowering the ATP/ADP ratio. The waveform as well as the frequency of oscillation is affected by apyrase additions.

3.6.4 Control Sites in Glycolysis

All the intermediary compounds along the glycolytic pathway (Hess and Boiteux, 1968), together with glucose utilization rate (Hommes, 1965; Maitra, 1966), pH (Hess and Boiteux, 1968; Hess, et al., 1969; Hess and Boiteux, 1973; Hess, et al., 1973) and pCO_2 (Hess and Boiteux, 1968; Hess, et al., 1969; Hess and Boiteux, 1973) fluctuate during glycolytic oscillations. Most reports describe ethanol and glycerol concentrations rising at a constant rate, despite fluctuating levels of

pyruvate and NADH (Betz and Chance, 1965b; Betz, 1966; Pye, 1973). However, very slight oscillations of ethanol concentration have been detected in phase with NADH by Giersch, et al. (1975). This result probably reflects improved instrumentation.

Phase relationships between the glycolytic intermediates are described by Hess, et al. (1969). Each metabolite concentration changes with the same frequency as the rest, but they may have different phase angles relative to each other. Two groups are apparent, differing by a phase angle, α . The magnitude of α is dependent on experimental conditions, particularly the rate of entry of substrate into glycolysis (Hess and Boiteux, 1973).

Location of the control points of the oscillatory mechanism can be demonstrated (Ghosh and Chance, 1964) using the crossover theorem developed by Chance and Williams (1956). The enzymes, phosphofructokinase, pyruvate kinase and glyceraldehyde 3-phosphate dehydrogenase, are control sites, the last reaction corresponding to the variable phase angle, α . The extent of the phase shifts for these enzymes shows that phosphofructokinase and pyruvate kinase are the strongest control points, while the glyceraldehyde 3-phosphate dehydrogenase - 3-phosphoglycerate kinase system exerts a weaker influence.

All three control sites identified by the crossover diagram involve the adenosine phosphate system. The ATP/ADP balance is a crucial factor in the propagation of oscillations down the pathway. In particular, ATP, ADP and AMP exert strong direct control on the phase of the oscillatory components of glycolysis (Hommes and Schuurmans Stekhoven, 1964; Chance, et al., 1965; Pye, 1971). The adenylate kinase reaction links the three adenosine phosphates and, as such, must also be considered an important regulatory step for glycolysis.

3.6.5 Modelling of Glycolytic Oscillations

When oscillations in anaerobic yeast cell preparations were first discovered, it was expected that rare and relatively unique biochemical mechanisms would be associated with the phenomena. However, recognition of feedback and the role of allosteric enzymes in metabolism led to the prediction, on theoretical grounds, that limit cycle oscillations and other non-linear behaviour should be commonplace in biochemical kinetics.

For the glycolytic pathway, there are many mechanisms corresponding to distinct control situations which could explain the observed oscillatory dynamics. Once a basic understanding of allosterism and non-linearity in enzyme kinetics was provided, the problem became one of determining the details which could account for the variation in response under different conditions. Some of the models proposed for glycolytic oscillations are outlined below. Interactions between phosphofructokinase, the adenosine nucleotides, enzymes of the lower glycolytic pathway, glucose transport and ATP sink reactions are discussed.

3.6.5.1 Higgins (1964,1967)

Reaction Mechanism

The first model proposed for the oscillating phosphofructokinase reaction was by Higgins (1964). The model was based on one-step feedback activation of phosphofructokinase by fructose diphosphate, and removal of the product by a Michaelis-Menton type reaction. The main purpose of the model was to show that phosphofructokinase, by itself and under ordinary conditions, could produce limit cycle behaviour. No attempt to involve the lower glycolytic pathway was made.

The results showed that sustained oscillations occurred only for a certain range of rate constants and glucose concentrations. Subsequent models (Higgins,

1967) extended these results by addition of enzymatic input, reaction kinetics, multiple feedback and variable inhibition and activation stoichiometries.

3.6.5.2 Sel'kov (1968a, 1968b, 1972); Sel'kov and Betz (1973)

Back Activation and Multiple Singular Points

Sel'kov (1968a) used a one-substrate enzyme model for phosphofructokinase, and considered the cooperative effect of ADP with substrate inhibition and product activation. The model proposed that the substrate, ATP, is supplied at a constant rate, and that the product, ADP, is removed by an irreversible first order sink reaction. Phosphofructokinase becomes active in the form $PFK.ADP^\gamma$, where γ represents the order of activation of phosphofructokinase by glycolytic intermediates, ADP and AMP. A quasi-stationary state hypothesis for the enzymatic forms allowed description of this system by a set of two kinetic equations for ATP and ADP (Sel'kov, 1968b).

Again, it was found that the substrate supply rate must be within a certain range. Sel'kov's model also predicted the experimentally observed effect of apyrase on the frequency of glycolytic oscillations (Frenkel, 1966).

The model was later altered (Sel'kov and Betz, 1973) to include phosphorylation of substrates. Coenzyme inhibition of enzyme reaction was also considered (Sel'kov, 1972). Although the earlier models by Sel'kov exhibit only one limit cycle, later models have multiple solutions, both oscillatory and non-oscillatory. As such, they provide some of the earliest evidence for kinetic solutions in biological systems more complex than a simple limit cycle.

3.6.5.3 Dynnik and Sel'kov (1973)

Lower Glycolytic Pathway

The possibility of self-oscillations in the lower part of the glycolytic system was

investigated by Dynnik and Sel'kov (1973). This treatment made it clear that inhibition of pyruvate kinase by fructose diphosphate alone can induce oscillations, even under conditions of non-fluctuating fructose diphosphate supply.

3.6.5.4 Hahn, Ortoleva and Ross (1973)

Variable Boundary Permeability

The ability of changing membrane permeability to give rise to oscillations and multiple steady states was demonstrated theoretically by Hahn, et al. (1973). When transport across a membrane is a function of the concentration of substance transferred or any other metabolite, there is a coupling of reaction and permeation which provides feedback. Even for simple subsequent reaction mechanisms, this situation allows multiple steady states, hysteresis effects, oscillatory approaches to stable steady states and limit cycles.

3.6.5.5 Dynnik, Sel'kov and Semashko (1973)

Coexistence of Stable Limit Cycle and Stable Singular Point

The effects of ATP on the activity of phosphofructokinase were considered in this model. The character of the multiple singular points of the model as a function of energy charge in the cell is discussed.

3.6.5.6 Higgins, Frenkel, Hulme, Lucas and Rangazas (1973)

Double Periodicity

Double periodicity can be generated when a periodic function interacts with itself after it has been phase shifted. This situation is possible in glycolysis if oscillations in fructose diphosphate are projected down the glycolytic sequence and are phase shifted in the 3-phosphoglycerate and phosphoenolpyruvate reactions. These oscillations can then interact with the unshifted ATP/ADP oscillations at 3-phosphoglycerate kinase and pyruvate kinase to induce beat frequencies.

Higgins, et al. (1973) showed that secondary minima in traces of ATP, ADP and AMP induced under certain experimental conditions can be modelled by interactions of these type.

3.6.5.7 Goldbeter and Nicolis (1976)

Concerted Transition Theory Model for Phosphofructokinase

The concerted transition theory was formulated by Monod, et al. (1965) to describe cooperative interactions in multisubunit enzymes. Using this approach to model the allosteric nature of phosphofructokinase, the cooperative interactions of the enzyme with the substrate, ATP, and product, ADP, are described on a molecular level.

Phosphofructokinase is considered to be a monosubstrate allosteric enzyme consisting of two protomers. The protomers are able to exist in either of two configurations, R and T, which differ in catalytic activity and in affinity for the substrate. The transition between these conformations is reversible and fully concerted.

The enzyme complex obtained by binding the substrate to the R and T forms decomposes irreversibly to yield the product, which is a positive effector of the enzyme and binds exclusively to the R conformation. The R conformation also has a higher affinity towards the substrate (K-effect), and a larger turnover number (V-effect) than the T state.

In this model for glycolysis, phosphofructokinase is considered to be a dimeric molecule, and the second substrate, fructose 6-phosphate, is ignored. Examination of the concentrations of the various enzyme species and their variable affinities for substrates and products yields thirteen differential equations. This set may be reduced to two after quasi-stationary state assumptions. These two equations are used to define the changes in ATP and ADP as a function of the source rate, sink

rate and saturation level for the phosphofructokinase reaction.

The substrate input rate was considered to be constant for solution of this model. This situation occurs, for example, when glucose is injected into cell-free extracts at a fixed rate. The kinetics of the sink reaction were taken to be those of a non-saturated Michaelian-type enzyme.

A detailed study of this model is presented by Goldbeter and Nicolis (1976). The analysis shows that it is important to represent the non-linearity of the oscillator by an autocatalytic-allosteric type mechanism in order to achieve a proper representation of the dynamic properties of oscillating glycolysis. The dominant source of non-linearity in the model is the allosteric behaviour of phosphofructokinase. However, incorporation of the allosteric constant for phosphofructokinase without the feedback terms yields only a unique stable state, with no limit cycle. Activation of the enzyme by its product is essential for destabilization of the steady state and oscillations to occur (Hess, et al., 1978; Hess, 1979).

The model admits a single steady state solution that becomes unstable for critical values of the parameters. For a certain range of substrate injection rates, the system undergoes sustained oscillations. The period and amplitude variation with substrate supply rate closely matches the experimentally observed results for yeast (Hess, et al., 1969), as does the phase shift induced in the model by titration with ADP (Pye, 1969).

3.6.5.8 Goldbeter and Nicolis (1976)

Stability Analysis in the Presence of Diffusion

In formulation of the concerted transition theory model, diffusion *in vivo* or in unstirred cell extracts was entirely neglected. Periodicities in both time and space were examined by adding substrate and product diffusion terms. It was assumed that the enzymes were uniformly distributed and underwent negligible diffusion.

Both periodic and Dirichlet boundary conditions were considered.

This theoretical study showed that sharp wavefronts of chemical activity could propagate over macroscopic dimensions in cell-free extracts. This pattern can lead to development of a spatio-temporal organization of reactants and products. The theoretical analysis was supported by experiments where periodic structure formation synchronized with the glycolytic oscillations (Boiteux and Hess, 1978).

The occurrence of intracellular spatial inhomogeneities has not been verified. Since the molarity of glycolytic enzymes is high in yeast, the mean molecular distance between enzymes is only 40-50Å, and transit time for diffusion of metabolites is computed to be in the range of 1μsec. These characteristics make spatial propagation of low concentration intermediates experimentally difficult to observe. However, if some enzymes are partially immobilized within the cell compartment and diffusion is controlled, concentration waves may be produced in the cytoplasm (Hess, et al., 1978).

3.6.5.9 Hess, Goldbeter and Lefever (1978)

Synchronization of Yeast Populations

The reaction and diffusion equations developed by Goldbeter and Nicolis (1976) were used to describe interactions between cells of two yeast populations oscillating out of phase. The relationship between the synchronization phase and the initial phases of oscillation for the population was derived in the analysis. The model behaves as a quasirelaxation oscillator, and exhibits a region of triple discontinuity.

3.6.5.10 Boiteux, Goldbeter and Hess (1975); Goldbeter and Nicolis (1976)

Substrate Entry Effects

The model proposed by Goldbeter and Nicolis (1976) was extended to examine

the response to external perturbations (Boiteux, et al., 1975). In the original formulation, a constant source rate was applied. However, fluctuating substrate inflow is more likely to represent the situation *in vivo*. For a stochastic input of substrate, the enzyme retains periodic behaviour and, furthermore, behaves as a narrow band-pass filter centred at the mean frequency. For a periodic source, the enzyme may entrain to the driving frequency or show subharmonic entrainment depending on the period of the source fluctuations.

3.6.5.11 Hess and Plessner (1978)

Two-Enzyme Concerted Transition Model

Stability analysis of the allosteric model described by Goldbeter and Nicolis (1976) demonstrates that the control properties of the glycolytic system can be reduced to the properties of phosphofructokinase as master oscillator. However, full understanding of the overall dynamics of the glycolytic pathway under all experimental conditions requires consideration of the other regulatory enzymes of the glycolytic sequence.

An extension of the model to include the *n*-protomer enzymes, phosphofructokinase and pyruvate kinase of glycolysis and adenylate kinase, is given by Hess and Plessner (1978). The phosphofructokinase and pyruvate kinase reactions are both described with two-substrate rate laws. The system is open for substrates fructose 6-phosphate and phosphoenolpyruvate, while the products, fructose diphosphate and pyruvate, accumulate. The limiting situation where the enzyme species are in equilibrium is considered. The waveforms and periods of oscillation obtained using this model closely resemble the experimental findings.

3.6.5.12 Termonia and Ross (1981a, 1981b, 1982)

Comprehensive Model

A comprehensive model of glycolysis, including fully concerted enzyme kinetics for phosphofructokinase and pyruvate kinase, and with an expression for the lumped reactions connecting these two enzymes has been formulated by Termonia and Ross (1981a). Problems with the choice of allosteric constant were avoided by incorporation of activation-inhibition reactions for phosphofructokinase and pyruvate kinase. Resonance and entrainment effects in glycolysis are discussed in relation to this model (Termonia and Ross, 1981b, 1982; Ross, 1981).

3.6.5.13 Other Theoretical Considerations

Dissipation of free energy during glycolytic oscillations is discussed by Richter and Ross (1980, 1981). The efficiency of free energy conversion from glucose and ADP to the products of anaerobic fermentation and ATP is estimated to be 5-10% higher during the oscillatory transient.

Oscillations in a compartmentalized phosphofructokinase system are described by Hervagault and Thomas (1983). Metabolite diffusion coupled with non-linear enzyme reaction allows long-term oscillations which were verified experimentally (Hervagault, et al., 1983).

Evidence for oscillations of the relaxation type in glycolyzing yeast extracts is discussed by Sel'kov and Betz (1973), Heinrich, et al. (1977) and Das, et al. (1979).

3.6.6 Conclusions

There are several points in the above discussion which may have implications in an examination of immobilized yeast cell glycolytic oscillations.

- (i) The nature and occurrence of the oscillations is dependent to a large extent on the kinetic properties of the glycolytic enzymes. These may be altered in immobilized cells.
- (ii) The efficiency of substrate uptake can be indirectly monitored by observing the oscillatory response to carbohydrate additions. Since immobilization involves the cell wall, modifications in the kinetics of transport may be expected to occur in immobilized cells.
- (iii) Oscillation characteristics reflect, through the ATP/ADP balance, functioning of endogenous metabolism in the cell and the priority given to carbohydrate storage. If immobilized cell growth rates are different compared with suspended cells, the pattern of accumulation and breakdown of cellular energy reserves will most likely also be altered.
- (iv) The observation of glycolytic oscillations depends on synchronization of the cell population. The interactions of individual immobilized cells with their environment may be less than optimal due to the presence of the immobilization support matrix. In this case, a greater degree of heterogeneity may be expected for an immobilized cell population. On the other hand, forced physical contact between immobilized cells may enhance the mechanism of synchrony induction.

The nature of NADH oscillations in immobilized cell populations may be useful as an index for the state of glycolysis in the cells and for the interaction of the pathway with other cell functions.

CHAPTER 4

MATERIALS AND METHODS

Experimental equipment and procedures are described in this chapter. The basic aims of the experimental work were:

- (i) to immobilize cells;
- (ii) to set up an apparatus for immobilized cell fermentation;
- (iii) to measure cell growth and productivity during fermentation with immobilized and suspended cells; and
- (iv) to analyse the composition and morphology of immobilized and suspended cells.

The procedures varied depending on the immobilization support and nutrient medium used in each fermentation experiment. Two immobilization supports were tested. They were:

- (i) glutaraldehyde-crosslinked gelatin-coated glass beads; and
- (ii) Sepharose beads activated with concanavalin A.

Four types of nutrient medium were used in the fermentations. They were:

- (i) undefined yeast extract medium with glucose as limiting substrate;
- (ii) undefined medium as in (i), containing hydroxyurea, an inhibitor of DNA synthesis;
- (iii) defined glucose medium deficient in biotin; and
- (iv) galactose medium containing yeast nitrogen base medium without amino acids.

In each case, the sugar substrate was fermented to ethanol and carbon dioxide

under anaerobic conditions.

In addition to the fermentation work, experiments were carried out to measure NADH oscillations in immobilized and suspended cells grown on yeast extract medium. These procedures are described separately.

4.1 ORGANISM

Saccharomyces cerevisiae ATCC 18790, a standard diploid strain, was obtained from the American Type Culture Collection. The stock culture was maintained on Sabouraud dextrose agar (Difco) plates at 4°C. This organism was used in all experiments.

4.2 PREPARATION OF CELL SUPPORTS

The following considerations directed the development of procedures for cell immobilization:

- (i) cells should be immobilized by surface attachment to avoid problems with intraparticle diffusion;
- (ii) the surface of the support should be covered by, at most, a monolayer of cells also to avoid diffusional restrictions;
- (iii) the immobilization procedure should be reversible to allow detachment of cells for analytical purposes; and
- (iv) the immobilization method should cause minimal damage to the cells.

4.2.1 Gelatin Beads

Procedures for preparation of the gelatin support were adapted from Sitton (1979).

Glass beads, 4mm in diameter, were dip-coated in a hot 25-30% solution of gelatin. The coated beads were then dropped into a chilled, rapidly stirred 3% glutaraldehyde solution, drained, and dried for at least 24 hours. For successful cell loading, it was crucial that the quantity of air bubbles in the dipping solution be minimal, and that an adequate thickness of gelatin be formed on each bead. Washing with water and drying for a second time improved the mechanical strength of the gelatin coating.

4.2.2 Concanavalin A-Sepharose Beads

Concanavalin A lectin was coupled to CNBr-activated Sepharose. The resultant conjugate provided a reactive support in the form of microspheres, 200-300 μ m in diameter, for the immobilization of yeast cells.

The coupling procedure was carried out under sterile conditions. All buffer and protein solutions were filter sterilized (0.45 μ m, Nalgene filter unit, type S).

About 1 g CNBr-activated Sepharose 6MB (Pharmacia) was swollen in 200 ml 1mM HCl for 15 minutes. The solution was filtered off under mild vacuum using a 12 μ m polycarbonate filter (Nuclepore), and the beads were washed with a further 100 ml 1mM HCl. The Sepharose was sterilized by steeping overnight in 70% ethanol at room temperature. The ethanol was removed by filtration.

The protein solution was prepared by dissolving 35 mg concanavalin A (Sigma) in approximately 30 ml borate buffer (0.05M, pH 8.3, containing 0.5M NaCl). After addition of the Sepharose gel to the protein solution, the suspension was mixed in

an end-over-end mixer for 2 hours at room temperature. Washing on a $12\mu\text{m}$ filter removed excess and non-specifically adsorbed protein. Acetate buffer (0.05M, pH 4.4, containing 0.5M NaCl) and borate buffer were used alternately for four or five washing cycles.

The lectin-Sepharose beads could be stored overnight at 4°C in Tris-HCl buffer (0.05M, pH 7.4, containing 1mM CaCl_2 , 1mM MnCl_2 , 1mM MgCl_2).

4.3 CULTURE MEDIA

Fermentation by gelatin-immobilized yeast was studied using three different glucose-based nutrient solutions. Since glucose reverses *S. cerevisiae*-concanavalin A attachment, nutrient medium containing galactose as carbon source was used for tests with the Sepharose microcarriers.

The pH of the solutions was adjusted to 4.5 by addition of concentrated H_2SO_4 . All solutions were filter sterilized (Gelman Sciences liquid filter capsule, $0.2\mu\text{m}$).

4.3.1 Glucose Media for Gelatin-Immobilized Cells

Undefined yeast extract medium containing all the essential growth factors for *Saccharomyces cerevisiae* was used for immobilized and suspended cell fermentation under conditions favourable for cell growth. In addition, behaviour of cells whose growth was inhibited by hydroxyurea or by biotin deficiency was examined in experiments using other glucose-based media.

4.3.1.1 Yeast Extract Medium

The composition of this solution was taken from Aiba, et al. (1968). The components are given in Table 4-1.

TABLE 4-1

Composition of Yeast Extract Medium

<i>Component</i>	<i>Per 1000 ml</i>
D-glucose (Mallinckrodt, A. R.)	20 g
KH ₂ PO ₄ (Mallinckrodt, A. R.)	5 g
(NH ₄) ₂ SO ₄ (Mallinckrodt, A. R.)	2 g
MgSO ₄ ·7H ₂ O (Mallinckrodt, A. R.)	0.4 g
CaCl ₂ ·2H ₂ O (Mallinckrodt, A. R.)	0.1 g
Yeast Extract (Difco)	2 g

4.3.1.2 Hydroxyurea Medium

For the experiments with hydroxyurea-inhibited cells, 5.71 g/l (0.075M) hydroxyurea (Sigma) was added to the yeast extract medium described in Table 4-1.

4.3.1.3 Biotin-Free Medium

Fermentation by *S. cerevisiae* was studied under conditions of biotin deficiency. The composition of the defined biotin-free glucose medium is given in Table 4-2.

TABLE 4-2

Composition of Biotin-Free Medium

<i>Component</i>	<i>Per 1000 ml</i>
D-glucose (Mallinckrodt, A.R.)	20 g
KH ₂ PO ₄ (Mallinckrodt, A. R.)	5 g
(NH ₄) ₂ SO ₄ (Mallinckrodt, A. R.)	2 g
MgSO ₄ .7H ₂ O (Mallinckrodt, A. R.)	0.4 g
CaCl ₂ .2H ₂ O (Mallinckrodt, A. R.)	0.1 g
trace mineral solution	1 ml
vitamin solution	10 ml

Trace Mineral Solution

<i>Component</i>	<i>Per 1000 ml</i>
ZnSO ₄ .7H ₂ O (Matheson, Coleman & Bell)	0.5 g
CuSO ₄ .5H ₂ O (Mallinckrodt, A. R.)	0.2 g
MnSO ₄ .H ₂ O (Matheson, Coleman & Bell)	0.15 g
FeSO ₄ .7H ₂ O (Mallinckrodt, A. R.)	0.1 g

Vitamin Solution

<i>Component</i>	<i>Per 1000 ml</i>
m-inositol (Sigma)	12.5 g
pyroxidine-HCl (Sigma)	0.625 g
Ca-D-pantothenate (Sigma)	0.625 g
thiamine-HCl (Sigma)	0.5 g
nicotinic acid (Sigma)	0.5 g

4.3.2 Galactose Medium for Concanavalin A-Immobilized Cells

Galactose medium was used in experiments with concanavalin A-bound *S. cerevisiae*. It was found that solutions which did not contain amino acids interfered least with the protein-Sepharose and lectin-cell interactions. The composition of the galactose medium is given in Table 4-3. The calcium and manganese salts added to this medium provided the metal ions required for optimal reactivity of the concanavalin A binding site.

TABLE 4-3

Composition of Undefined Galactose Medium

<i>Component</i>	<i>Per 1000 ml</i>
D-galactose (Sigma, <0.01% glucose)	20 g
KH ₂ PO ₄ (Mallinckrodt, A. R.)	5 g
(NH ₄) ₂ SO ₄ (Mallinckrodt, A. R.)	2 g
MgSO ₄ ·7H ₂ O (Mallinckrodt, A. R.)	0.4 g
CaCl ₂ ·2H ₂ O (Mallinckrodt, A. R.)	0.25 g
MgCl ₂ ·6H ₂ O (Mallinckrodt, A. R.)	0.2 g
MnCl ₂ ·4H ₂ O (Fisher Scientific)	0.2 g
Yeast Nitrogen Base Without Amino Acids (Difco)	6.7 g

The composition of Difco yeast nitrogen base without amino acids is given in Appendix I.

4.4 EXPERIMENTAL APPARATUS

Batch fermentation experiments were performed with both immobilized and suspended cells. Stirred fermenters with temperature and pH control were used for the suspended cell tests. The reactor configuration for the immobilized cell experiments depended on which immobilization support was tested.

4.4.1 Suspended Cell Reactors

Productivity experiments with suspended cells were carried out in two stages, each involving a separate fermenter.

A 300 ml beaker served as the first-stage fermenter. The beaker was immersed in a water bath equipped with a Haake (Saddle Brook, New Jersey) temperature controller. The contents were stirred with a magnetic stirrer. The second fermentation stage took place in a New Brunswick (Edison, New Jersey) Bioflo fermenter.

In both vessels, pH was controlled by means of an Ingold (Andover, Massachusetts) sterilizable combination pH electrode, a Horizon (Chicago, Illinois) pH controller and using 2N solutions of NaOH and H_3PO_4 . Nitrogen gas was bubbled continuously into both reactors at a rate of about 10 cc/second.

4.4.2 Immobilized Cell Reactors

Since the two immobilization supports developed in this work were very different in size, two immobilized cell reactors were designed for their use.

4.4.2.1 Column Reactor for Gelatin Beads

A glass column, 5 cm internal diameter and with an effective length of 15 cm, was constructed for experiments with gelatin-immobilized cells. The gelatin-coated glass beads were randomly packed into the column reactor.

The column operated in a recirculation loop with either of the fermenters described above for suspended cells. The immobilized cell experiments were conducted in two stages, with the immobilized cell column connected first to the beaker fermenter and then to the Bioflo. The schematic diagram of Figure 4-1 outlines the operation of these recirculation loops. A photograph of the second-stage apparatus with the immobilized cell column and Bioflo fermenter is shown in Figure 4-2. The equipment in the photograph is identified in Figure 4-3.

4.4.2.2 Stirred Reactor for Sepharose Microbeads

A 250 ml vessel for suspension culture of cells attached to microcarrier beads was obtained from Techne Incorporated (Princeton, New Jersey). It is shown in Figure 4-4.

A bulbous rod with built-in magnet suspended from a screw cap at the top of the vessel provided gentle agitation of the microcarriers. The bottom of the vessel was shaped so that the stirring action created secondary flow patterns in the vertical direction. Stagnation below the axis of rotation was avoided by this design.

The vessel has two side-arms. These were adapted to provide entry for nitrogen gas and to allow escape of nitrogen and carbon dioxide.

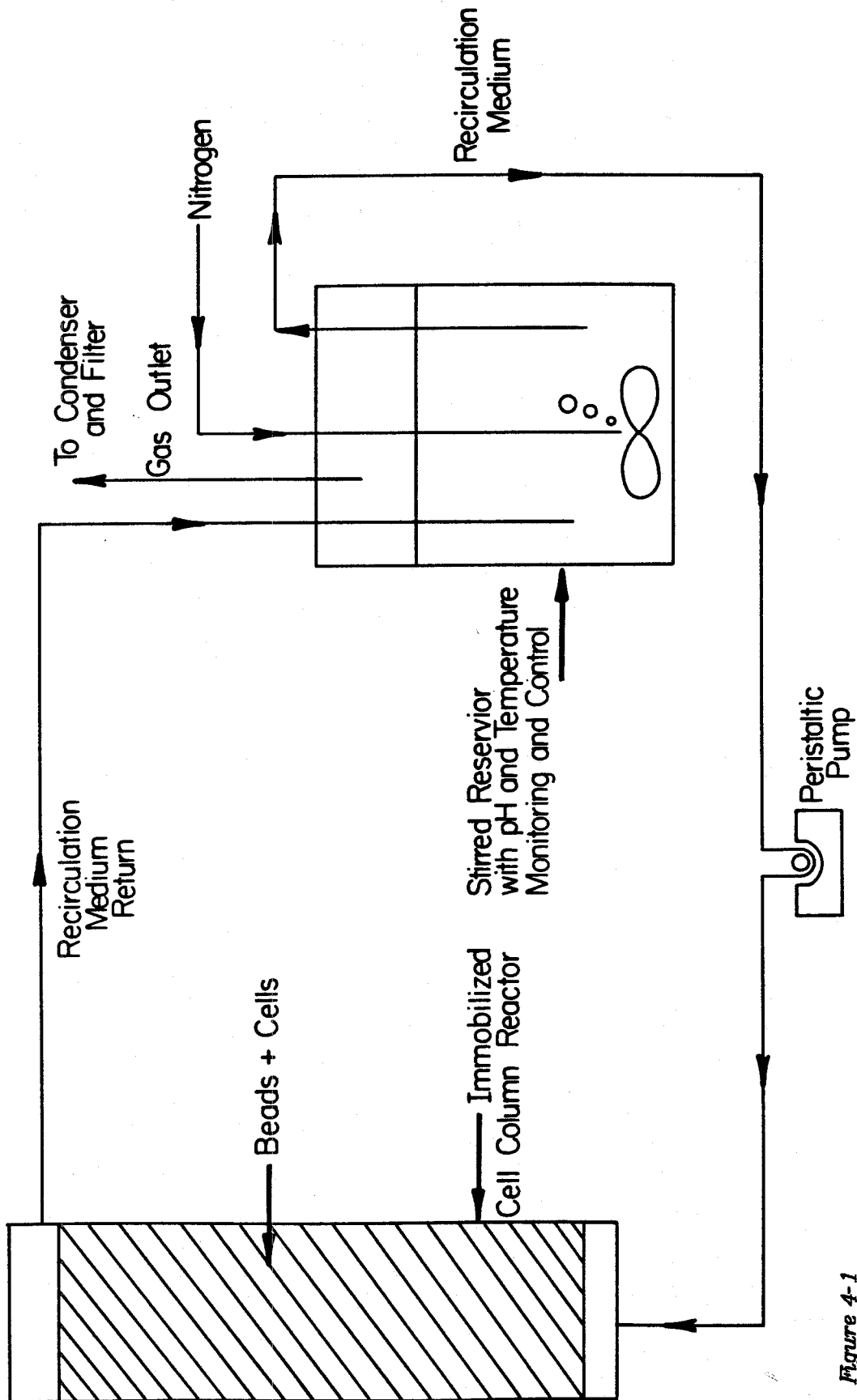
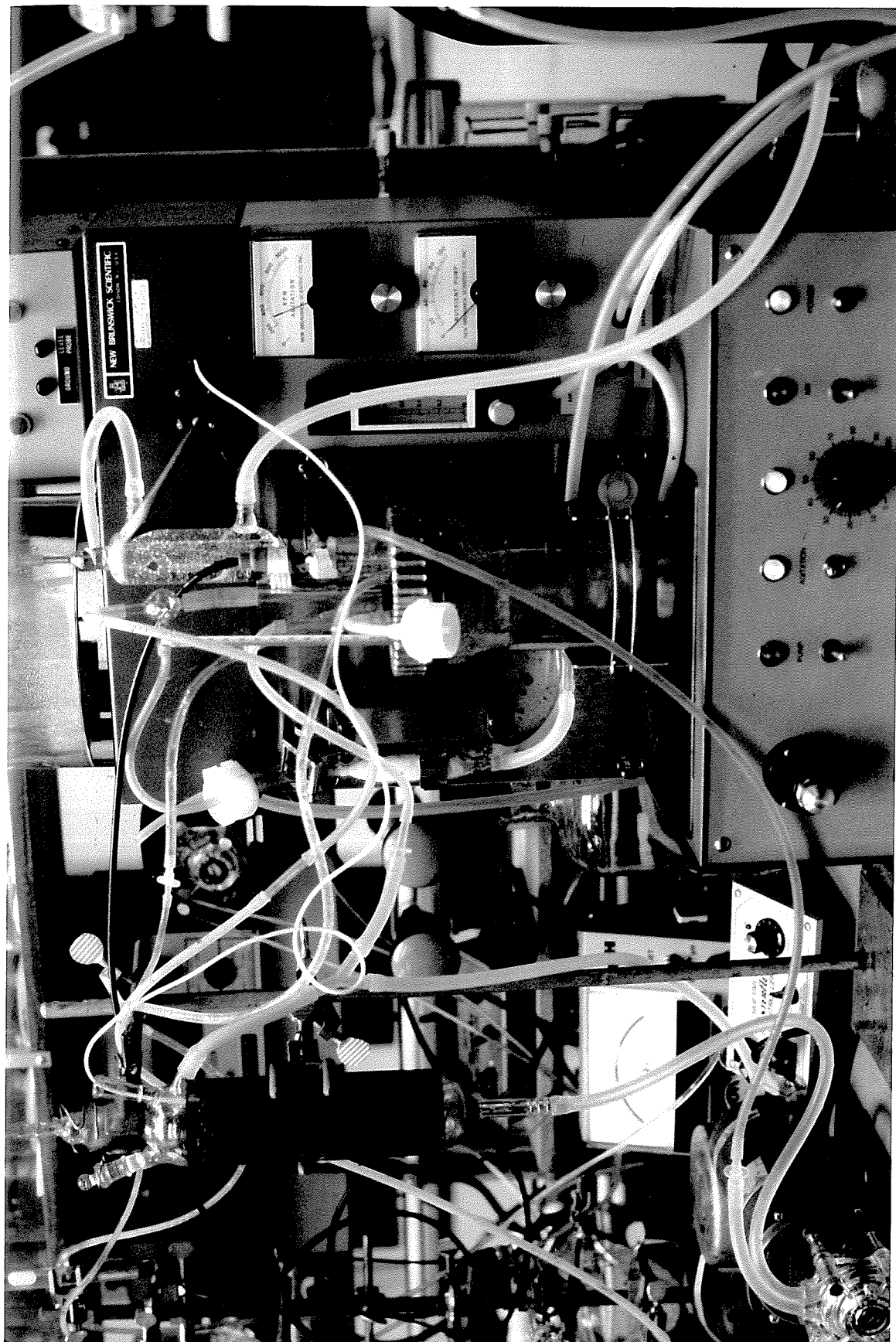


Figure 4-1
Immobilized cell recirculation reactor

Figure 4-2

Second-stage immobilized cell fermentation apparatus



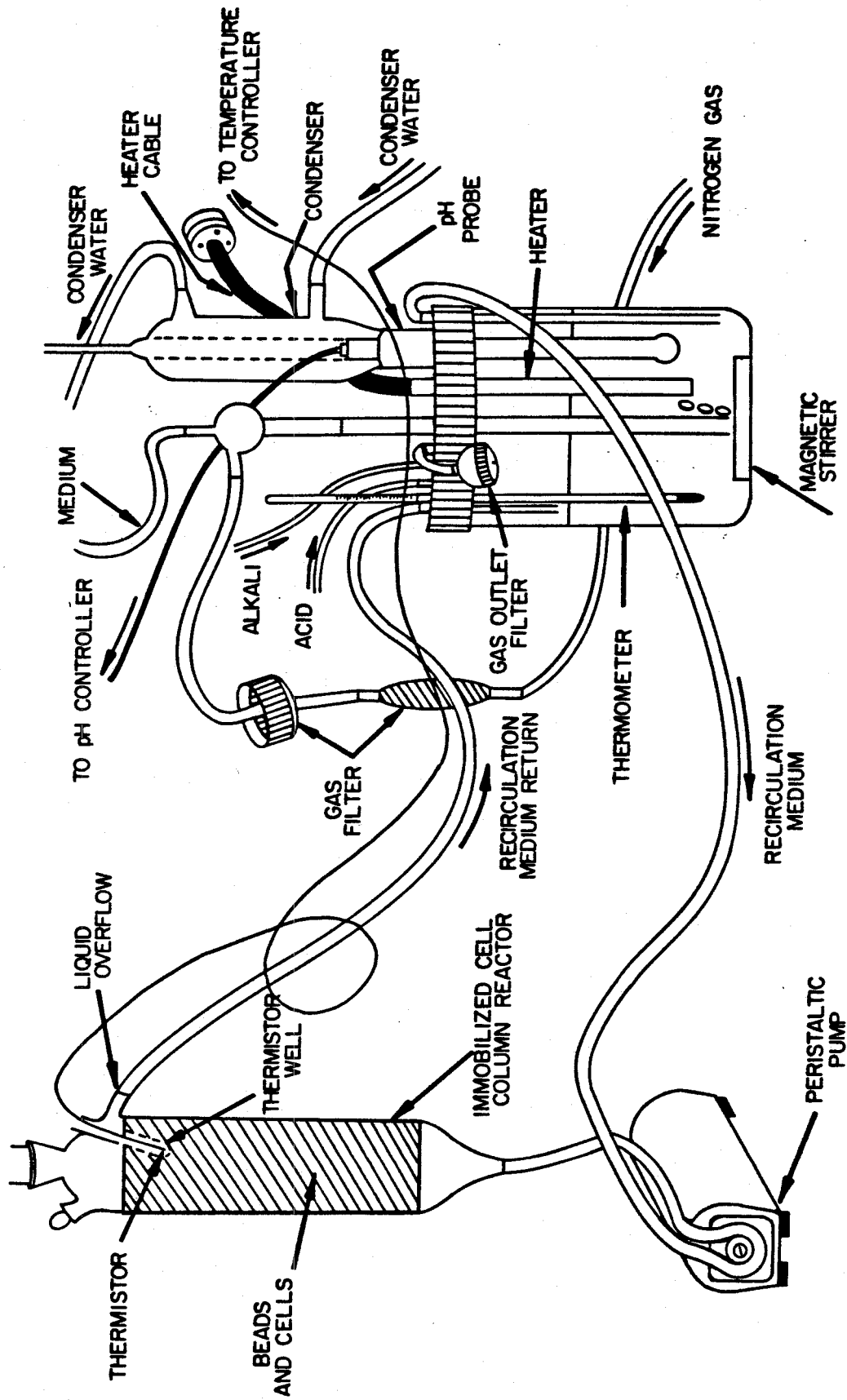


Figure 4-3
Equipment comprising immobilized cell fermenter system of Figure 4-2

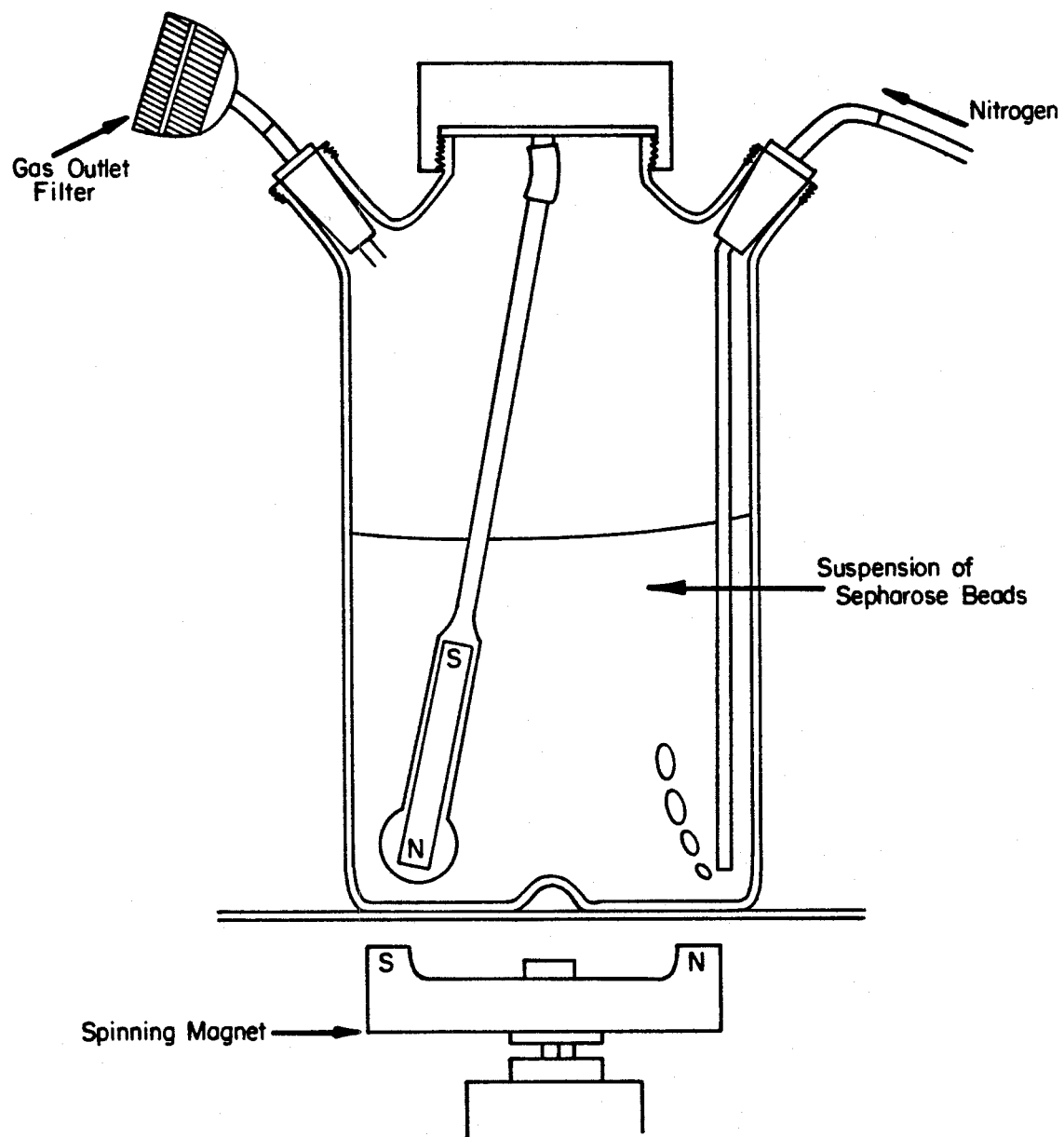


Figure 4-4
Vessel for culture of immobilized cells bound to Sepharose microbeads

4.5 FERMENTER OPERATING CONDITIONS

Each fermentation was carried out under the following conditions:

- (i) temperature: $30 \pm 0.3^\circ\text{C}$
- (ii) pH: 4.5 ± 0.1
- (iii) 0% dissolved oxygen saturation

The immobilized cell column reactor was operated so that concentration and temperature gradients were minimized in the system. Since the cell catalyst was immobilized at the surface of the carrier beads, internal diffusion was not a problem. Gradients down the length of the reactor and in the immediate vicinity of the bead surface were minimized by using high recirculation flow rates relative to the reaction rate of the cells. These high fluid velocities together with a low catalyst concentration also discouraged the build-up of gas bubbles and channelling in the column. With a recirculation rate of about 550 ml/min in an effective reactor volume of 400 ml and with an immobilized cell catalyst concentration of about 2×10^9 cells/litre, the system operated with less than 1% conversion per pass. The rate of reaction did not change when flow velocities greater than this (700 ml/min) were employed.

The insignificance of mass-diffusional intrusions in the immobilized cell reactor was also checked theoretically by calculating the Damköhler number for the system in terms of the observable reaction rate (Carberry, 1976). For both substrate consumption and product formation, including carbon dioxide, the value of Da was very small, of the order of 10^{-6} . This is an indication that mass transfer to and from the cells was very rapid relative to reaction and can be ignored.

The residence time distribution for flow through the column-Bioflo recirculation system was examined by injecting a tracer substance into the feed stream and

noting the variation of output concentration with time. Details of this procedure are given in Appendix II. The results showed that ideal CSTR macromixing occurred within the system.

4.6 FERMENTATION PROCEDURES

Methods used for the fermentation experiments are described separately for each of the culture conditions tested. All fermentations were conducted in batch.

For each type of glucose medium, two sets of experiments were carried out: one with gelatin-immobilized cells and one with suspended cells. As far as possible, the same procedures were used with the suspended cells as with the immobilized cells. This was necessary to allow a valid comparison of their behaviour.

In each experiment, exponential-phase cells were produced in a preliminary fermentation stage. These cells were then transferred to fresh medium, and their initial ethanol productivity measured under batch reaction conditions.

4.6.1 Fermentation of Glucose-Yeast Extract Medium

Fermentation of complete nutrient medium by suspended and gelatin-immobilized yeast was examined using the following methods.

4.6.1.1 *Preparation of Inoculum*

S. cerevisiae was precultured for both the immobilized and suspended cell fermentations at room temperature in yeast extract medium containing 100 g/l glucose. This procedure was followed in order to provide a solution of high cell density for loading of the immobilization support. The enriched medium was prepared

by increasing the concentrations of all components in Table 4-1 by a factor of five. The cell concentration at the end of this preculture stage was approximately 3×10^{11} cells/litre.

4.6.1.2 Immobilized Cell Fermentation

The gelatin-coated beads used in these experiments were sterilized by autoclaving. The column reactor was packed with beads and filled with PBS solution (phosphate-buffered-saline solution: NaCl, 8.0 g/l; KCl, 0.2 g/l; KH_2PO_4 , 0.2 g/l; Na_2HPO_4 , 0.15 g/l; pH 6.6). After autoclaving at 121°C and 15 psig, the solution was drained from the column and the beads thoroughly washed with sterile deionized water before loading with cells.

Yeast cells grown in preculture medium for 12 hours at room temperature were used to inoculate the gelatin-coated carrier beads. The cells were passed slowly through the packing at a rate of approximately 20 ml/min for 6-8 hours until a sufficient surface loading was obtained as observed under the microscope.

Once loaded with cells, the packing was washed with nutrient medium to remove unattached cells. The column was then connected to the beaker fermenter in the manner indicated in Figure 4-1 for the preliminary stage of the experiment.

Samples were taken every 30 minutes and analysed for ethanol concentration. Usually after 3 or $3\frac{1}{2}$ hours, the immobilized cells had entered exponential phase with respect to ethanol production. At this stage, the packing was again washed very thoroughly with nutrient medium to remove free cells. The column was then connected to the Bioflo fermenter (Figure 4-3).

Liquid samples were taken from the reactor every 10 minutes for the first hour of this second cultivation stage, and then every 20 minutes for another 2 or $2\frac{1}{2}$ hours. Samples of beads, approximately 6-8 g wet weight, were carefully

removed from the column every 20 minutes for the duration of the second-stage experiment.

Samples for analysis of substrate and product concentration were filtered immediately after removal from the reactor on 0.22 μ m membrane filters (Millipore). The filtrate was stored below 0°C. Samples taken to allow counting of suspended cells in the recirculation medium were stored on ice for a maximum of 15 minutes before analysis. Beads removed from the reactor were kept in 3% glutaraldehyde at 4°C.

Batch immobilized cell fermentation of glucose-yeast extract medium was carried out twice using these procedures.

4.6.1.3 Suspended Cell Fermentation

For the suspended cell experiments, yeast were grown at room temperature for 18-20 hours in preculture medium. This culture was used directly to inoculate the beaker fermenter for the first stage of the experiment.

As described above for the immobilized cells, the fermentation was monitored by measuring ethanol production until the cells progressed out of lag phase. About 50 ml of this culture was spun down and the cells then used to inoculate the Bioflo. Samples were taken for 3-3½ hours and were treated prior to analysis as described above for the immobilized cell experiments.

Three suspended cell fermentations of glucose-yeast extract medium were performed in order to check the repeatability of the results.

4.6.1.4 Control Experiments

Two control tests were performed in association with the immobilized cell fermentations in yeast extract medium. The purpose of these additional experiments was:

- (i) to determine whether gelatin dissolved in the medium affects yeast fermentation kinetics; and
- (ii) to verify that glutaraldehyde and other substances recirculated in the medium do not cause changes in the composition of yeast.

The procedures for each of these controls are described separately below.

(a) *Gelatin Test:* This experiment was carried out with suspended cells as described in Section 4.6.1.3, except that the nutrient medium in both fermentation stages contained 0.5 g/l gelatin. Fermentation was monitored and samples were taken as already described.

(b) *Glutaraldehyde Test:* In preparation for immobilized cell fermentation, the column reactor containing glutaraldehyde-crosslinked gelatin beads is autoclaved, drained and washed with water. An experiment was performed with suspended cells in medium containing some of this wash liquid to check that the DNA content of yeast is not affected by exposure to traces of glutaraldehyde.

The test was carried out following the procedures for suspended cells given in Section 4.6.1.3. A 25 ml volume of sterile wash liquid drained from freshly autoclaved beads was added to about 75 ml of inoculum culture 8 hours before the beginning of the first fermentation stage. In this way, exposure of yeast during the immobilized cell experiments to chemicals contained in the wash was simulated.

The first fermenter was inoculated by pipetting cells directly from the preculture medium, and 25 ml of this culture was later transferred to the second fermenter. Cell samples were removed one hour after the start of the second stage fermentation and fixed in 70% ethanol.

4.6.2 Fermentation of Glucose-Yeast Extract-Hydroxyurea Medium

Some preliminary experiments were undertaken with hydroxyurea to confirm its inhibitory effect on the yeast strain used in this study. Some modifications were made to the general procedures described in Section 4.6.1 on the basis of the results from these inhibition tests.

4.6.2.1 Hydroxyurea Inhibition Tests

Hydroxyurea was added to an asynchronous, exponentially-growing *S. cerevisiae* culture at 30°C to give a final concentration of 0.075M. Culture density, cell count and viability were monitored for approximately five hours. Formation of ethanol was also checked during this transition period.

4.6.2.2 Preparation of Inoculum

Cells were precultured for the hydroxyurea experiments as described in Section 4.6.1.1.

4.6.2.3 Immobilized Cell Fermentation

Immediately before loading of the gelatin-coated carrier beads, hydroxyurea dissolved in preculture medium was added to the inoculum culture to give a final concentration of 0.075M. Loading of the beads then proceeded for 4 hours.

The methods used in these experiments were otherwise the same as those described in Section 4.6.1.2, except that 0.075M hydroxyurea was present in all media exposed to the immobilized yeast.

In total, four experiments were performed with hydroxyurea-inhibited immobilized cells. The procedures used for each were essentially identical. The number of cells present at the beginning of each experiment varied between 10^9 and 10^{10} cells/l liquid.

4.6.2.4 Suspended Cell Fermentation

After 12 hours of growth on preculture medium, hydroxyurea was added to the suspended yeast to give a final concentration of 0.075M. Inhibition was allowed to proceed for 4 hours. These cells were used to inoculate the beaker fermenter for the first stage of the experiment. Subsequent procedures were the same as described above (Section 4.6.1.3).

Fermentation with hydroxyurea-treated suspended cells was repeated four times. As with the immobilized cells, each experiment was performed using a different inoculum size.

4.6.2.5 Control Experiment

In a control experiment, urea was substituted for the hydroxyurea otherwise added to the medium for suspended cell fermentation. The purpose of this test was to distinguish whether the extra nitrogen represented by hydroxyurea in the medium may be responsible for any changes in cell growth and productivity characteristics. The procedures followed were the same as those outlined for the suspended cell experiments (Section 4.6.2.4) except that 0.075M urea was used in all steps requiring addition of hydroxyurea.

4.6.3 Fermentation of Biotin-Deficient Medium

Cell growth in biotin-free medium was first examined in preliminary experiments. Many of the procedures used were the same as those already outlined.

4.6.3.1 Growth Inhibition due to Biotin Starvation

The medium of Table 4-2 was made up using a vitamin solution containing, in addition to the components already listed, 0.0125 g biotin/1000 ml. An

exponential-phase culture of suspended *S. cerevisiae* was produced by growth at 30°C on this complete defined medium. Cells from this culture were centrifuged down, washed twice with the biotin-deficient medium described in Table 4-2, and resuspended in biotin-free medium.

Culture density in this suspension was monitored for approximately 50 hours. After 13.5 hours, a control culture was started using the complete medium containing biotin for comparison of growth rates.

4.6.3.2 Preparation of Inoculum

For fermentation under conditions of biotin deficiency, cells were grown up on the defined medium of Table 4-2 to which was added 0.125 mg/l biotin.

After 12 hours of growth at 30°C, these cells were spun down and washed twice with biotin-free nutrient medium. This preculture medium contained all the components of Table 4-2, but the concentration of each was five times that listed. Starvation proceeded in this solution at room temperature.

4.6.3.3 Immobilized Cell Fermentation

After 20 hours of biotin deficiency, cells prepared in the preculture stage were used to inoculate the gelatin beads. Attachment of the biotin-starved yeast appeared to be a slower process than with unstarved cells. After about 8 hours, loading of the immobilization support was usually sufficient for the fermentation experiment to proceed.

Subsequent steps in the experiment are described in Section 4.6.1.2. The medium solutions used for washing were biotin-free and of the same composition as that given in Table 4-2.

4.6.3.4 Suspended Cell Fermentation

The suspended cells were precultured for 28.5 hours in biotin-free medium. These cells were centrifuged out of suspension and added to the first-stage fermenter. The remaining steps in the fermentation procedure are given in Section 4.6.1.3.

4.6.4 Fermentation of Galactose Medium

An attempt was made to measure the fermentation characteristics of concanavalin A-bound immobilized yeast cells. The immobilization procedures and fermentation conditions are described below.

4.6.4.1 Preparation of Inoculum

S. cerevisiae cells were grown for 12 hours at 30°C on the galactose medium of Table 4-3.

4.6.4.2 Immobilized Cell Fermentation

Precultured cells were centrifuged and resuspended in 10-15 ml sterile Tris-HCl buffer (0.5M, pH 7.4, containing 1mM CaCl₂, 1mM MnCl₂ and 1mM MgCl₂). This suspension was added to the Sepharose gel. Immobilization was effected by mixing in an end-over-end mixer for 2.5 hours at room temperature.

Excess cells were gently removed from the bead suspension by filtering on a 12 μ m filter. Once the cells were attached, it was essential that the Sepharose beads were kept covered by liquid. Failure to ensure this led to detachment of the yeast.

The gel was washed during filtration with Tris-HCl buffer, followed by galactose medium. When the solution surrounding the microbeads on top of the filter was

essentially of the same composition as the galactose medium, the suspension was poured into the Techne fermentation vessel (Figure 4-4) containing deaerated galactose medium at the operating pH and temperature.

Samples were removed by pipetting out aliquots of culture solution. The beads were examined under the microscope.

4.7 ANALYTICAL METHODS

The concentrations of ethanol, glycerol and glucose in the samples taken from the fermenters were measured. Immobilized and suspended cell count and viability were also routinely determined. Once these analyses were completed, progress of the batch experiments could be followed by examining the changes in these concentrations with time.

In addition to the analyses required for the concentration-time trajectories, intracellular contents of polysaccharide, DNA, RNA and protein were determined for immobilized and suspended cells. For each assay, exponential-phase cell samples were taken one hour after the start of the second stage of fermentation and stored in 70% ethanol at 4°C. Immobilized cells harvested at this time for electron microscopy were fixed in 3% glutaraldehyde.

4.7.1 Ethanol Concentration

Ethanol was measured by gas chromatography using an Antek (Houston, Texas) gas chromatograph, model 461. A 6 ft., $\frac{1}{8}$ in. stainless steel column packed with 80/100 mesh Porapak Q (Waters Associates, Milford, Massachusetts) was operated with a thermal conductivity detector. The carrier gas was helium, used at a flowrate of 30 cc/min. The column oven was operated isothermally at 175°C. The

liquid sample volume was 5 μ l.

A calibration curve generated each time ethanol was measured related ethanol concentration and chromatogram peak area. The standard solutions contained ethanol at concentrations between 0.15 g/l and 2.5 g/l. Peak area was found to be a linear function of concentration within this range.

4.7.2 Glucose Concentration

A Waters Associates (Milford, Massachusetts) high pressure liquid chromatograph with refractive index detector and Waters Associates column for carbohydrate analysis measured glucose concentrations. The solvent used was 65:35 acetonitrile:water at a flowrate of 1.7 ml/min. A liquid sample volume of 25 μ l was injected.

The instrument was calibrated each time glucose analyses were performed. A linear relationship between glucose concentration and chromatogram peak area was determined for concentrations between 2.5 g/l and 20 g/l.

4.7.3 Glycerol Concentration

Glycerol concentration was determined using an analytical kit for triglycerides (Sigma). By omitting the saponification steps, and with a sample volume of 30 μ l, glycerol could be analysed directly by procedures which coupled the enzymatic phosphorylation of glycerol with oxidization of NADH to NAD (Sigma Technical Bulletin No. 320-UV). The amount of NADH oxidized was followed spectrophotometrically (Bausch and Lomb Spectronic 21, Rochester, New York) at 340 nm.

Calibration curves for absorbance versus glycerol concentration were measured before each analysis. Standard solutions containing between 0.01 g/l and 0.5

g/l glycerol covered the range of concentrations found in the fermentation samples. The spectrophotometer calibration curves showed a linear relationship between absorbance and glycerol level.

4.7.4 Hydroxyurea Concentration

Hydroxyurea was analysed by high pressure liquid chromatography at the same time as glucose. Under the conditions described in Section 4.7.2, the hydroxyurea peak preceeded the glucose peak by approximately 40 seconds.

The chromatograph was calibrated for hydroxyurea each time the analysis was performed. Standard solutions with concentrations ranging from 0.95 g/l to 5.8 g/l were used to establish a linear relationship between hydroxyurea concentration and chromatogram peak area.

4.7.5 Cell Concentration

A Levy-Hausser (Blue Bell, Pennsylvania) counting chamber was used for measuring suspended cell concentration. Cell doublets resulting from treatment with hydroxyurea were counted as two cells.

Determination of the number of immobilized cells present in each experiment required that the cells attached to the surface of the support be first removed for counting.

4.7.5.1 Reversal of Gelatin Immobilization

Samples of beads taken during the experiments were stored in 3% glutaraldehyde for cell fixation. The glutaraldehyde was later washed from the beads with PBS (pH 6.6) on a 12 μ m filter.

The gelatin coating was dissolved by the action of proteolytic enzymes. Treatment of the bead samples with approximately 30 mg collagenase (Sigma, Type 1A) in 20ml 0.05M Tris-HCl buffer (pH 8.1, containing 0.0115M CaCl_2 to inhibit self-digestion) at 37°C for 3-4 hours resulted in the complete removal of yeast from the support. Gentle agitation or aspiration with a pipette helped dislodge the cells from the carrier. Digests containing the previously immobilized cells could then be analysed using the Levy-Hausser counting chamber in the same way as were the samples of suspended cells.

4.7.5.2 Reversal of Concanavalin A Attachment

Glucopyranosides which bind very strongly to concanavalin A may be used to displace yeast α -mannan from the lectin binding pocket. However, it was found that complete removal of yeast was not easily effected using this method. Instead, the concanavalin A protein was digested away using a similiar procedure as that for reversal of gelatin immobilization. Trypsin (Sigma) was used rather than collagenase; otherwise the method is the same as described in Section 4.7.4.1.

4.7.6 Culture Density

Cell density in suspended cell cultures was monitored by measuring absorbance using a Klett-Summerson colourimeter (New York, New York) with red filter.

4.7.7 Viability

Viability was measured in two ways. Routine analysis of cell viability was performed during the fermentations using a staining procedure. A 0.1 ml volume of 4 mg/ml trypan blue (Sigma) solution was added to 1 ml of cell suspension, and the number of stained, or nonviable, cells determined during cell counting. Percentage

viability was measured for the immobilized cells after the removal of unfixed cells from the support surface.

In addition to the staining procedures, viability of immobilized and suspended cells was determined by plating out quantities of the cell suspensions on Sabouraud dextrose agar.

4.7.8 Polysaccharide Content

Measurements of intracellular polysaccharide content were made using samples of immobilized and suspended cells taken from the reactors one hour after the start of the second fermentation stage.

Extractions of trehalose, mannan, glycogen and glucan and analysis of these fractions with anthrone were performed as described by Stewart (1975), with modifications by Jermyn (1975). A Bausch and Lomb (Rochester, New York) Spectronic 21 spectrophotometer was used for the measurements. About 4×10^6 cells were involved in each analysis.

All samples were analysed together. Polysaccharide content was measured as grams of glucose equivalent per cell. Solutions containing between 5×10^{-4} and 10^{-2} g/l glucose were used to generate a calibration curve for the instrument. A linear relationship between solution absorbance and glucose concentration was established.

4.7.9 Flow Cytometry

DNA, RNA and protein measurements were made using a flow cytometer (Cytofluorograf Model 50, Ortho Instruments, Westwood, Massachusetts) equipped with a 2W argon laser operated at the 488 nm line. The three cell components were

selectively stained with fluorescent dyes, and the fluorescence signals were detected in conjunction with small-angle light scatter. The scatter signals are used as an indication of cell size. The collected data were digitized, stored and analysed using a Cytomic 12 one- and two-parameter data processor (Kratel Instrumente GmbH, Stuttgart, FRG).

The staining procedures were adapted from Crissman, et al. (1975) and are described below. In each step, cells were centrifuged at 4°C and 6000 rpm for 10 minutes.

4.7.9.1 DNA

Cellular DNA was fluorescently labelled with acriflavine (Sigma). After fixation, the cells were washed with deionized water, hydrolysed in 4N HCl for 20 minutes at room temperature and washed again.

The staining solution consisted of 20 mg acriflavine and 500 mg potassium metabisulfite in 100 ml deionized water, to which was added 10 ml 0.5N HCl. This solution was filtered on a 0.22 μ m Millipore filter. After staining for 20 minutes at room temperature, the samples were washed three times with a solution of 1 ml concentrated HCl in 99 ml 70% ethanol.

The cells were resuspended in deionized water just before analysis. Laser output power was 0.75W and the sample fluorescence was detected at 515 nm.

4.7.9.2 RNA

Propidium iodide (Calbiochem) was used to stain stable double-stranded nucleic acid in the cell, most of which is RNA. The cell samples were washed with 0.05M phosphate buffer (pH 7.5) and stained for 30 minutes at 4°C in a solution of 0.05 mg/ml propidium iodide in 1.13% (w/v) sodium citrate. After being washed

twice with phosphate buffer, the cells were resuspended in deionized water for analysis. At a laser power of 0.5W, RNA fluorescence was detected at 575 nm.

4.7.9.3 Protein

The procedure used for staining cell protein was the same as that for RNA, except that the stain solution consisted of 0.03 mg/ml fluorescein isothiocyanate (Sigma) in 0.5M NaHCO₃ made fresh daily. The laser was operated at 0.75W for this analysis. A 510-520 nm bandwidth filter was used to isolate the protein fluorescent signal.

4.7.10 Microscopy

Yeast cells were viewed using both light and scanning electron microscopy.

4.7.10.1 Electron Microscopy

S. cerevisiae cells immobilized on both gelatin-coated glass beads and Sepharose-concanavalin A microspheres were prepared for scanning electron microscopy. Samples of gelatin beads were removed from the immobilized cell reactors one hour after the beginning of the second stage of fermentation. Sepharose beads were collected for this analysis immediately after the attachment of yeast cells. The cell samples were fixed in 3% glutaraldehyde and stored at 4°C.

The cells were dehydrated in a series of progressively more concentrated alcohol solutions - 10 minutes each in 20%, 40%, 60% and 70% ethanol - and suspended finally in 95% ethanol. After CO₂ critical point drying, the beads were mounted and then shadowed with a 100Å layer of 80:20 gold:palladium by vacuum deposition.

The immobilized cells were examined with an ETEC (Hayward, California)

Autoscan scanning electron microscope using an accelerating voltage of 20kV.

4.7.10.2 Light Microscopy

Suspended cells from a fermentation experiment with hydroxyurea were viewed under an Olympus BHB light microscope. The cells were removed from the fermenter one hour after the beginning of the second experimental stage and fixed in 70% ethanol. Microscope slides covered with a thin film of 2% agarose were streaked with these cells. Photographs were taken at a magnification of 440x in bright field using a Nikon SLR 35mm camera with 55mm f3.5 lens and Kodak 1600 ASA film.

4.8 NADH OSCILLATION MEASUREMENTS

Immobilized and suspended cell NADH level oscillations were measured as an indirect monitor of enzyme regulation properties. The immobilization support used was crosslinked gelatin. Procedures for the oscillation experiments involved several stages:

- (i) preculture of cells;
- (ii) preparation of immobilization support and immobilized cell loading;
- (iii) cell growth and harvesting;
- (iv) cell starvation; and
- (v) fluorescence measurement.

The experimental details for each step are described below. As far as possible, the methods used for preparation of the cells were the same irrespective of whether immobilized or suspended cells were tested.

4.8.1 Preculture

Saccharomyces cerevisiae cells were grown up at 30°C on a 100 g/l glucose medium containing the components of Table 4-1 at concentrations five times those listed.

After 12 hours, this culture was either used for loading of the immobilization support, or was allowed to sit at room temperature for 3 hours before the suspended cell experiments.

4.8.2 Immobilized Cell Preparation

Gelatin-coated glass microscope slides were prepared using similar techniques as those described in Section 4.2.1. Instead of dip-coating the glass, hot gelatin solution was pipetted onto the surface of the slides. After 5-10 minutes, the coating was sufficiently hard so that the slides could be immersed in cold 3% glutaraldehyde solution for crosslinking. After drying overnight, the gelatin was washed with water and dried again. The slides were autoclaved in PBS (phosphate-buffered-saline solution: NaCl, 8.0 g/l; KCl, 0.2 g/l; KH_2PO_4 , 0.2 g/l; Na_2HPO_4 , 0.15 g/l; pH 6.6).

Immobilization of *S. cerevisiae* onto the gelatin surface was achieved by placing the slides, gelatin side up, at the bottom of a jar containing the preculture broth. Loading of the support was allowed to proceed for 3 hours at room temperature with occasional stirring.

4.8.3 Batch Growth and Cell Harvesting

Yeast cells for the oscillation experiments were cultured in flasks at room

temperature with continuous aeration. The glucose-yeast extract medium of Table 4-1 was used for this batch growth.

For the suspended cell tests, cells spun down from the preculture medium were used as the inoculum. For the immobilized cell experiments, the glass slides loaded with yeast were carefully transferred to the fresh yeast extract medium after unattached cells were washed from the gelatin surface.

Growth was monitored by measurement of cell density using a Klett-Summerson (New York, New York) colourimeter with red filter. When the cells had reached late exponential-phase, they were harvested, placed in air-saturated phosphate buffer (0.05M, pH 7.6) and kept on ice.

4.8.4 Starvation

Cells were starved for varying periods of time in continuously aerated phosphate buffer at 25°C.

4.8.5 Fluorescence Measurement

NADH fluorescence was measured using a Perkin-Elmer (Oak Brook, Illinois) LS-5 fluorescence spectrophotometer. The excitation and emission wavelengths were 340 nm and 460 nm respectively. The temperature of the cuvette holder was thermostatically controlled at 25°C.

4.8.5.1 Suspended Cell Fluorescence

A 2 ml aliquot of starved cell suspension was transferred to a square quartz cuvette and placed in the fluorometer. After a steady signal was obtained, 0.5 ml of glucose solution was injected into the cuvette. The concentration of glucose in

the solution varied from 10mM to 500mM. Five minutes after addition of the sugar, 0.3 ml of 25mM potassium cyanide was injected into the cuvette in order to block respiration. Fluorescence response to this transition was followed on a chart recorder connected to the spectrofluorometer.

4.8.5.2 Immobilized Cell Fluorescence

Strips of gelatin were cut and carefully lifted off the microscope slides. Each strip was placed against one side of a quartz cuvette containing 2 ml of air-saturated phosphate buffer. Injection of glucose and KCN proceeded as described above for the suspended cell experiments (Section 4.8.5.1).

4.9 SUMMARY OF EXPERIMENTAL WORK

Saccharomyces cerevisiae cells were immobilized by surface attachment. Glutaraldehyde-crosslinked gelatin-coated beads and concanavalin A-activated Sepharose 6MB were used as immobilization supports. Techniques were developed to allow reversal of attachment.

Batch experiments conducted with immobilized and suspended cells provided samples for determination of initial fermentation rates. The activities of suspended and gelatin-immobilized yeast were monitored under conditions both favourable and unfavourable for cell growth with three different glucose-based media. Galactose fermentation was tested with concanavalin A-bound cells.

Samples of exponential-phase cells taken from the fermenters allowed comparison of immobilized and suspended cell macromolecular contents. The morphology of immobilized yeast cells was also examined by electron microscopy.

The characteristics and occurrence of NADH fluorescence oscillations were examined with immobilized and suspended *S. cerevisiae*. These measurements

gave an indication of the glycolytic activity in the cells. Crosslinked gelatin was used as the immobilization support in these experiments.

CHAPTER 5

MATHEMATICAL MODELLING

Two mathematical models were developed in the course of this study. The first describes growth and product formation by immobilized and suspended cells in batch fermentation. A second model was devised to represent the dynamics of the glycolytic pathway in yeast.

In this chapter, a discussion of the theoretical work is presented.

5.1 KINETIC MODELS FOR FERMENTATION

Models were developed to facilitate batch fermentation data fitting and parameter estimation. A mathematical treatment of immobilized and suspended cell reaction is outlined below. The general equations are given first; however, simplifications are possible depending on whether cell growth is associated with fermentation under the experimental conditions.

5.1.1 General Equations for Fermentation

Monod kinetics are assumed in the derivation of general models for cell growth and activity. Immobilized cell and suspended cell fermentations are considered separately below.

5.1.1.1 Generalized Suspended Cell Model

For suspended cells in batch culture, the equations describing growth, substrate consumption and product formation are:

$$\frac{dX_S}{dt} = \mu_S X_S - \kappa_S X_S \quad (5.1-1)$$

$$\frac{dS}{dt} = -\left(\frac{\mu_S}{Y_S} + m_S\right)X_S \quad (5.1-2)$$

$$\frac{dP}{dt} = (\mu_S Z_S + \rho_S)X_S \quad (5.1-3)$$

where

m is the maintenance coefficient, or specific rate of substrate utilization for maintenance activities (g substrate/hr cell)

P is product concentration (g/l)

S is substrate concentration (g/l)

t is time (hrs)

X is viable cell concentration (cells/l liquid)

Y is cell yield from substrate (cells/g substrate)

Z is product selectivity (g product/cell produced)

κ is specific death rate (hr^{-1})

μ is specific growth rate (hr^{-1})

ρ is the specific rate of non-growth-associated product formation (g product/hr cell)

and subscript

I denotes immobilized cells

S denotes suspended cells

5.1.1.2 Generalized Immobilized Cell Model

Consider now batch fermentation by immobilized yeast cells. Many details included in this model are based on observations made during experiments with gelatin-immobilized *Saccharomyces cerevisiae*.

In the immobilized cell reactor, live cells are attached to the surface of carrier beads. These yeast cells are able to bud and produce daughter cells. Some fraction of the daughter cells are released into the medium, while others remain attached to the surface adjacent to the immobilized mother cell. For *S. cerevisiae*, the actual proportion of daughter cells released will depend on the location of the budding site on the mother cell, as well as on the extent to which the cell is surrounded by other immobilized yeast.

Proliferation of the immobilized cells and the subsequent shedding of daughter cells creates a coexistent suspended cell population in the medium contacting the beads. These suspended cells are also able to reproduce. It is assumed that the cells in suspension may attach themselves at any time to those support particles having available free surface.

Two further assumptions are based on experimental findings:

- (i) immobilization is an irreversible process, so that any cell, once immobilized, remains on the support surface; and
- (ii) the immobilized cells are able to form, at most, a monolayer covering the particle surface.

The validity of the first assumption is attested by the fact that yeast cells attached to crosslinked gelatin are very difficult to remove. The cell-gelatin bond survives treatment with heat, 6M urea, acid and alkali as well as harsh mechanical handling. Under the conditions of the fermentation experiments, then, spontane-

ous release of cells from surface to suspension is unlikely.

The second assumption has been verified by electron microscopy. With the fast flowrates operating in the immobilized cell reactor, cellular material does not build up on the beads past a monolayer thickness. This situation implies that there exists a condition of full loading which occurs when the beads cannot support further cell attachment. Measurements of cell density on the gelatin surface have, in fact, shown that loading of the support ceases at some point which may be taken as the fully-loaded state. This is discussed further in Appendix III.

The general equations describing cell growth for such an immobilized cell system are:

$$\frac{dX_I}{dt} = [1 - \alpha(\frac{X_I}{X_F})]\mu_I X_I + \beta(1 - \frac{X_I}{X_F})X_S - \kappa_I X_I \quad (5.1-4)$$

$$\frac{dX_S}{dt} = \mu_S X_S + \alpha(\frac{X_I}{X_F})\mu_I X_I - \beta(1 - \frac{X_I}{X_F})X_S - \kappa_S X_S \quad (5.1-5)$$

where α is a factor related to the probability of an immobilized cell daughter being released into suspension

β is the rate constant for attachment of suspended cells to the support surface (hr^{-1})

X_F is the viable immobilized cell concentration at full surface loading (cells/l liquid)

The factor $\alpha(\frac{X_I}{X_F})$ represents the fraction of immobilized cell daughters which are released into suspension, and increases as the surface loading increases. The term $\beta(1 - \frac{X_I}{X_F})$ represents the specific rate of attachment of free cells to the support surface. This rate decreases as full loading is approached, since less surface is avail-

able for immobilization. The value of β can be expected to depend on, among other factors, the operating recirculation flowrate.

Substrate consumption and product formation in the generalized immobilized cell system are described as follows:

$$\frac{dS}{dt} = -\left(\frac{\mu_I}{Y_I} + m_I\right)X_I - \left(\frac{\mu_S}{Y_S} + m_S\right)X_S \quad (5.1-6)$$

$$\frac{dP}{dt} = (\mu_I Z_I + \rho_I)X_I + (\mu_S Z_S + \rho_S)X_S \quad (5.1-7)$$

5.1.2 Fermentation with Cell Growth

Simplification of the kinetic equations for immobilized and suspended cell fermentation under conditions supporting microbial growth involves the following assumptions:

- (i) cell death is negligible;
- (ii) product formation is growth-associated;
- (iii) substrate consumption for maintenance purposes is negligible; and
- (iv) the concentration of limiting substrate remains much higher than the value of K_S , the substrate constant in the Monod expression.

Trypan blue staining was used to indicate cell viability during the suspended cell and immobilized cell fermentations. Essentially 100% viability was maintained in each experiment. This justifies use of assumption (i) in the present work.

Assumptions (ii) and (iii) follow from the relative insignificance of maintenance metabolism when all nutrients are provided and cell growth occurs. Assumption (iv) is valid in batch experiments employing relatively high substrate

levels and where initial rate data are taken.

5.1.2.1 Suspended Cell Model

When cell growth is significant, the suspended cell equations (5.1-1)-(5.1-3) become:

$$\frac{dX_S}{dt} = \mu_S X_S \quad (5.1-8)$$

$$\frac{dS}{dt} = \frac{-(\mu_S X_S)}{Y_S} \quad (5.1-9)$$

$$\frac{dP}{dt} = \mu_S X_S Z_S \quad (5.1-10)$$

From assumption (iv), when $S \gg K_S$ in the Monod expression for microbial kinetics, μ may be considered constant. In this case, integration of equations (5.1-8)-(5.1-10) with initial values indicated by subscript 0 gives:

$$X_S = X_{S0} e^{\mu_S t} \quad (5.1-11)$$

$$S = [S_0 + \frac{X_{S0}}{Y_S}] - \frac{X_{S0}}{Y_S} e^{\mu_S t} \quad (5.1-12)$$

$$P = [P_0 - X_{S0} Z_S] + X_{S0} Z_S e^{\mu_S t} \quad (5.1-13)$$

5.1.2.2 Immobilized Cell Model

Equations (5.1-4)-(5.1-7) for batch immobilized cell fermentation may also be simplified by neglecting the cell death and maintenance terms:

$$\frac{dX_I}{dt} = [1 - \alpha(\frac{X_I}{X_F})] \mu_I X_I + \beta(1 - \frac{X_I}{X_F}) X_S \quad (5.1-14)$$

$$\frac{dX_S}{dt} = \mu_S X_S + \alpha(\frac{X_I}{X_F}) \mu_I X_I - \beta(1 - \frac{X_I}{X_F}) X_S \quad (5.1-15)$$

$$\frac{dS}{dt} = -\left(\frac{\mu_I}{Y_I}\right)X_I - \left(\frac{\mu_S}{Y_S}\right)X_S \quad (5.1-16)$$

$$\frac{dP}{dt} = \mu_I Z_I X_I + \mu_S Z_S X_S \quad (5.1-17)$$

Equations (5.1-14) and (5.1-15) may be simplified further for application to growing cells. The following rationale applies when fermentation conditions allow immobilized and suspended cell growth to proceed much faster than the rate of suspended cell attachment to gelatin.

Experience with yeast loading onto gelatin-coated beads has indicated that attachment of the cells is rather slow unless there are very high concentrations of suspended cells present, and unless they are passed very slowly through the immobilized cell column. A separate test was performed with non-growing cells so that the value of the parameter, β , could be measured under the fast recirculation flowrate conditions used during all immobilized cell fermentation experiments. A detailed account of the procedures followed and the data obtained is given in Appendix III.

The results show that the value of β is approximately 0.006 hr^{-1} , or about two orders of magnitude less than the specific growth rate for yeasts in complete medium. This allows the second term in equation (5.1-14) to be dropped with little effect. Equations (5.1-14) and (5.1-15) may then be rewritten as:

$$\frac{dX_I}{dt} = \left[1 - \alpha\left(\frac{X_I}{X_F}\right)\right]\mu_I X_I \quad (5.1-18)$$

$$\frac{dX_S}{dt} = \mu_S X_S + \alpha\left(\frac{X_I}{X_F}\right)\mu_I X_I \quad (5.1-19)$$

Integration gives the expressions for X_I and X_S :

$$X_I = \frac{\mu_1 e^{\mu_1 t}}{\left[\left(\frac{\alpha \mu_1}{X_F} \right) (e^{\mu_1 t} - 1) + \frac{\mu_1}{X_{I0}} \right]} \quad (5.1-20)$$

and

$$X_S = X_{S0} e^{\mu_S t} + \frac{\alpha \mu_1}{X_F} e^{\mu_S t} \int_0^t \frac{\mu_1^2 e^{(2\mu_1 - \mu_S)\tau}}{\left[\left(\frac{\alpha \mu_1}{X_F} \right) (e^{\mu_1 \tau} - 1) + \frac{\mu_1}{X_{I0}} \right]^2} d\tau \quad (5.1-21)$$

After substitution of X_I and X_S in equations (5.1-16) and (5.1-17), integration gives:

$$S = \left[S_0 + \frac{X_{S0}}{Y_S} \right] - \frac{X_{S0}}{Y_S} e^{\mu_S t} - \left(\frac{\mu_1}{Y_I} \right) \left(\frac{X_F}{\alpha} t + \frac{X_F}{\alpha \mu_1} \left[\log \left(\frac{\alpha X_{I0}}{X_F} + \left(1 - \frac{\alpha X_{I0}}{X_F} \right) e^{-\mu_1 t} \right) \right] \right) \\ - \frac{\mu_S \alpha \mu_1}{X_F Y_S} \int_0^t e^{\mu_S \tau} \int_0^\tau \frac{\mu_1^2 e^{(2\mu_1 - \mu_S)w}}{\left[\left(\frac{\alpha \mu_1}{X_F} \right) (e^{\mu_1 w} - 1) + \frac{\mu_1}{X_{I0}} \right]^2} dw d\tau \quad (5.1-22)$$

and

$$P = [P_0 - Z_S X_{S0}] + Z_S X_{S0} e^{\mu_S t} + \mu_1 Z_I \left(\frac{X_F}{\alpha} t + \frac{X_F}{\alpha \mu_1} \left[\log \left(\frac{\alpha X_{I0}}{X_F} + \left(1 - \frac{\alpha X_{I0}}{X_F} \right) e^{-\mu_1 t} \right) \right] \right) \\ + \frac{\mu_S Z_S \alpha \mu_1}{X_F} \int_0^t e^{\mu_S \tau} \int_0^\tau \frac{\mu_1^2 e^{(2\mu_1 - \mu_S)w}}{\left[\left(\frac{\alpha \mu_1}{X_F} \right) (e^{\mu_1 w} - 1) + \frac{\mu_1}{X_{I0}} \right]^2} dw d\tau \quad (5.1-23)$$

5.1.3 Fermentation with Cell Growth Inhibition

The general description of batch fermentation was modified for application to chemically-inhibited cells. Models for analysis of cell activity in the presence of hydroxyurea are outlined in this section. Again, several experimental observations are involved in the development.

When yeast growth is inhibited by the action of hydroxyurea, substrate continues to be consumed and products formed. Although not strictly maintenance metabolism, many activities in the cell proceed in the absence of growth. Total biomass increases due to continued protein and RNA synthesis, and, in addition, some residual cell division may occur. Under these conditions, growth is not balanced since some synthetic pathways are artificially blocked.

Application of Monod-based models, such as those of Section 5.1.1, cannot be recommended in this type of situation. However, due to the lack of quantitative information about alterations to metabolism arising from hydroxyurea treatment, the macroscopic descriptions provided by equations (5.1-1)-(5.1-7) will be used as the basis for a model of chemically-inhibited growth. It is assumed that substances such as hydroxyurea affect the model only by reducing the maximum specific growth rate.

Application of the general equations to experiments with hydroxyurea inhibition involves the following assumptions:

- (i) cell death is negligible; and
- (ii) the concentration of limiting substrate remains much higher than the value of K_S in the Monod expression for microbial kinetics.

The first assumption was checked by monitoring cell viability with trypan blue dye. Immobilized and suspended cells retain 98-100% viability during fermentation tests with hydroxyurea. As discussed in Section 5.1.2, assumption (ii) is valid during the initial stages of batch culture.

5.1.3.1 Suspended Cell Model

For incomplete growth inhibition of suspended cells, the general equations describing batch growth, substrate consumption and product formation become:

$$\frac{dX_S}{dt} = \mu_S X_S \quad (5.1-24)$$

$$\frac{dS}{dt} = -\left(\frac{\mu_S}{Y_S} + m_S\right)X_S \quad (5.1-25)$$

$$\frac{dP}{dt} = (\mu_S Z_S + \rho_S)X_S \quad (5.1-26)$$

For $S \gg K_S$, μ is constant, and equations (5.1-24)-(5.1-26) may be integrated directly. With initial values denoted by subscript 0:

$$X_S = X_{S0} e^{\mu_S t} \quad (5.1-27)$$

$$S = \left[S_0 + \frac{\left(\frac{\mu_S}{Y_S} + m_S\right)X_{S0}}{\mu_S} \right] - \frac{\left(\frac{\mu_S}{Y_S} + m_S\right)X_{S0}}{\mu_S} e^{\mu_S t} \quad (5.1-28)$$

$$P = \left[P_0 - \frac{(\mu_S Z_S + \rho_S)X_{S0}}{\mu_S} \right] + \frac{(\mu_S Z_S + \rho_S)X_{S0}}{\mu_S} e^{\mu_S t} \quad (5.1-29)$$

5.1.3.2 Immobilized Cell Model

If cell death is neglected, the general equations for growth in the immobilized cell reactor become:

$$\frac{dX_I}{dt} = \left[1 - \alpha \left(\frac{X_I}{X_F} \right) \right] \mu_I X_I + \beta \left(1 - \frac{X_I}{X_F} \right) X_S \quad (5.1-30)$$

$$\frac{dX_S}{dt} = \mu_S X_S + \alpha \left(\frac{X_I}{X_F} \right) \mu_I X_I - \beta \left(1 - \frac{X_I}{X_F} \right) X_S \quad (5.1-31)$$

Simplification of this model for cell growth inhibition relies on the observation that X_I changes little during hydroxyurea-inhibited batch fermentation. The following additional assumptions may then be made:

(i) $\beta \left(1 - \frac{X_I}{X_F} \right) = \beta' = \text{constant}$

$$(ii) \quad \alpha \left(\frac{X_I}{X_F} \right) = \alpha' = \text{constant; and}$$

$$(iii) \quad \alpha \left(\frac{X_I}{X_F} \right) \mu_I X_I = \alpha' \mu_I X_{Ia}$$

where X_{Ia} is an average value for the immobilized cell concentration over the duration of the experiment. For the immobilized cell tests with hydroxyurea completed in this study, X_{Ia} differs by a maximum of 10% from the initial and final X_I values, usually less.

Using these approximations, equations (5.1-30) and (5.1-31) become:

$$\frac{dX_I}{dt} = [1 - \alpha'] \mu_I X_I + \beta' X_S \quad (5.1-32)$$

$$\frac{dX_S}{dt} = (\mu_S - \beta') X_S + \alpha' \mu_I X_{Ia} \quad (5.1-33)$$

Integration with initial values denoted by subscript 0 gives:

$$\begin{aligned} X_I = & \frac{\beta' \alpha' X_{Ia}}{(\mu_S - \beta')(1 - \alpha')} + \frac{\beta' [X_{S0} + \frac{\alpha' \mu_I X_{Ia}}{(\mu_S - \beta')}]}{(\mu_S - \beta' - (1 - \alpha') \mu_I)} e^{(\mu_S - \beta')t} \\ & + [X_{I0} - \frac{\beta' [X_{S0} + \frac{\alpha' \mu_I X_{Ia}}{(\mu_S - \beta')}] }{(\mu_S - \beta' - (1 - \alpha') \mu_I)} - \frac{\beta' \alpha' X_{Ia}}{(\mu_S - \beta')(1 - \alpha')}] e^{(1 - \alpha') \mu_I t} \end{aligned} \quad (5.1-34)$$

and

$$X_S = [X_{S0} + \frac{\alpha' \mu_I X_{Ia}}{(\mu_S - \beta')}] e^{(\mu_S - \beta')t} - \frac{\alpha' \mu_I X_{Ia}}{(\mu_S - \beta')} \quad (5.1-35)$$

The form of these equations is seen more easily after combining constants:

$$X_I = \varphi + \eta e^{(\mu_S - \beta')t} + \xi e^{(1 - \alpha') \mu_I t} \quad (5.1-36)$$

and

$$X_S = \psi e^{(\mu_S - \beta')t} - \nu \quad (5.1-37)$$

where

$$\varphi = \frac{\beta' \alpha' X_{Ia}}{(\mu_S - \beta')(1 - \alpha')}$$

$$\eta = \frac{\beta' [X_{S0} + \frac{\alpha' \mu_I X_{Ia}}{(\mu_S - \beta')}] }{(\mu_S - \beta' - (1 - \alpha') \mu_I)}$$

$$\xi = [X_{I0} - \frac{\beta' [X_{S0} + \frac{\alpha' \mu_I X_{Ia}}{(\mu_S - \beta')}] }{(\mu_S - \beta' - (1 - \alpha') \mu_I)} - \frac{\beta' \alpha' X_{Ia}}{(\mu_S - \beta')(1 - \alpha')}]$$

$$\psi = [X_{S0} + \frac{\alpha' \mu_I X_{Ia}}{(\mu_S - \beta')}]$$

$$\nu = \frac{\alpha' \mu_I X_{Ia}}{(\mu_S - \beta')}$$

Substrate consumption and product formation for this system are described as follows after substituting the results for X_I and X_S into equations (5.1-6) and (5.1-7) and integrating:

$$\begin{aligned} S = & [S_0 + (\frac{\mu_I}{Y_I} + m_I) (\frac{\eta}{(\mu_S - \beta')} + \frac{\xi}{(1 - \alpha') \mu_I}) + (\frac{\mu_S}{Y_S} + m_S) \frac{\psi}{(\mu_S - \beta')}] \\ & - [(\frac{\mu_I}{Y_I} + m_I) \varphi - (\frac{\mu_S}{Y_S} + m_S) \nu] t - [(\frac{\mu_I}{Y_I} + m_I) \frac{\xi}{(1 - \alpha') \mu_I}] e^{(1 - \alpha') \mu_I t} \\ & - [(\frac{\mu_I}{Y_I} + m_I) \frac{\eta}{(\mu_S - \beta')} + (\frac{\mu_S}{Y_S} + m_S) \frac{\psi}{(\mu_S - \beta')}] e^{(\mu_S - \beta') t} \end{aligned} \quad (5.1-38)$$

and

$$\begin{aligned}
 P = & [P_0 - (\mu_1 Z_1 + \rho_1) \left(\frac{\eta}{(\mu_s - \beta')} + \frac{\xi}{(1 - \alpha') \mu_1} \right) - (\mu_s Z_s + \rho_s) \frac{\psi}{(\mu_s - \beta')}] \\
 & + [(\mu_1 Z_1 + \rho_1) \varphi - (\mu_s Z_s + \rho_s) \nu] t + [(\mu_1 Z_1 + \rho_1) \frac{\xi}{(1 - \alpha') \mu_1}] e^{(1 - \alpha') \mu_1 t} \\
 & + [(\mu_1 Z_1 + \rho_1) \frac{\eta}{(\mu_s - \beta')} + (\mu_s Z_s + \rho_s) \frac{\psi}{(\mu_s - \beta')}] e^{(\mu_s - \beta') t} \quad (5.1-39)
 \end{aligned}$$

5.1.4 Summary of Fermentation Modelling

General immobilized and suspended cell models were developed to describe growth, substrate consumption and product formation during batch fermentation. The situation in the case of immobilized cells is more complicated than for suspended cell fermentation due to coexistence of suspended daughter cells with the immobilized cell population. Two sets of simplified equations apply under conditions with and without cell growth inhibition.

The models were used to analyse batch concentration data. By fitting the data to the appropriate equations, values for the fermentation parameters incorporated into the models could be estimated.

The results from application of the fermentation models are discussed in Chapter 6.

5.2 MODELLING OF GLYCOLYTIC OSCILLATIONS

The reactions of the glycolytic pathway were represented in a kinetic model. The purpose of the model was to simulate the mechanism of NADH oscillations in immobilized and suspended yeast.

Mathematical treatment of the reactions converting glucose to ethanol was

based on experimental findings. The model includes a description of allosterism for the enzymes phosphofructokinase and pyruvate kinase, as well as many of the feedback properties of glycolysis. A lumped enzyme reaction involving production of NADH connects the principal oscillators, phosphofructokinase and pyruvate kinase.

5.2.1 Outline of the Reaction Scheme

Figure 5-1 shows the reaction sequence of the model. The scheme involves explicitly the reactions of hexokinase, phosphofructokinase and pyruvate kinase, the three most irreversible steps of glycolysis (Lehninger, 1975). Entry of substrate into the cell is incorporated into the first step of the sequence, which is denoted as the hexokinase reaction.

Glyceraldehyde 3-phosphate dehydrogenase is included to connect the phosphofructokinase and pyruvate kinase steps, since there is some evidence that this enzyme exerts some degree of control in glycolysis (Hess, et al., 1969). In addition, turnover by glyceraldehyde 3-phosphate dehydrogenase is relatively slow (Boiteux and Hess, 1981), so that this enzyme step should be considered along with the other rate-limiting reactions.

Also included in the model are the ATPase and adenylate kinase reactions. The ATPase step represents a sink reaction for consumption of ATP, the product of glycolysis. The adenylate kinase reaction provides control of the energy charge in the cell.

In deriving the scheme of Figure 5-1, two simplifying assumptions were made:

- (i) The kinetics of glucose phosphate isomerase, aldolase, triose phosphate isomerase, phosphoglycerate kinase, phosphoglycerate mutase, enolase,

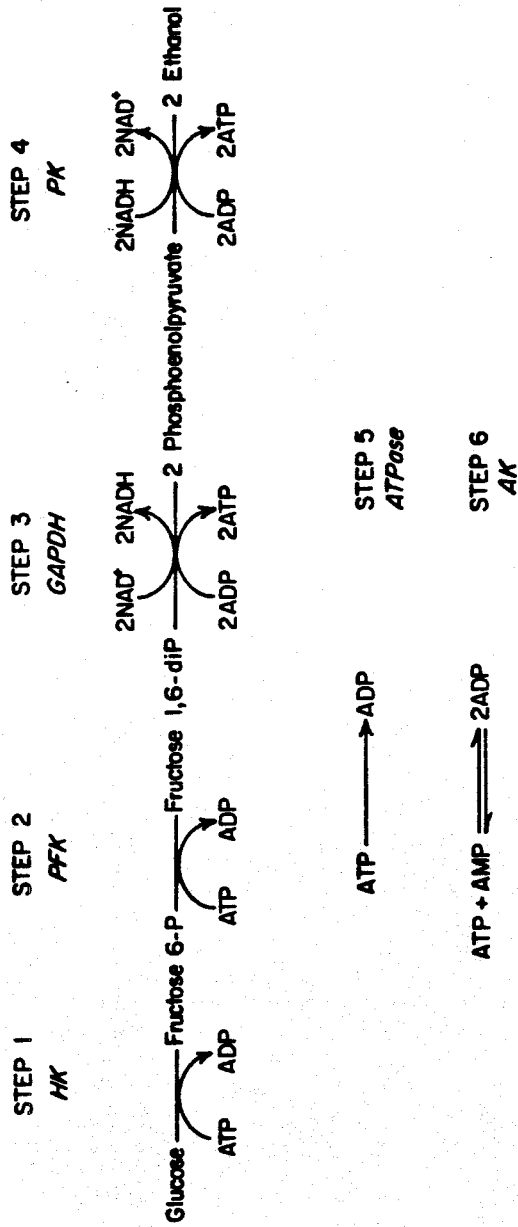


Figure 5-1

Simplified glycolytic sequence and related reactions

Abbreviations

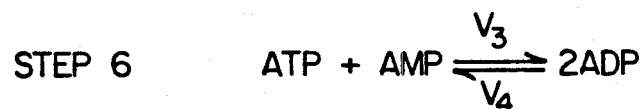
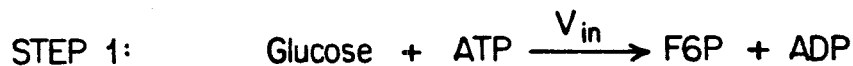
ADP -	adenosine diphosphate	HK -	hexokinase
AK -	adenylate kinase	NAD ⁺ -	nicotinamide adenine dinucleotide
AMP -	adenosine monophosphate	NADH -	nicotinamide adenine dinucleotide, reduced
ATP -	adenosine triphosphate	PFK -	phosphofructokinase
GAPDH -	glyceraldehyde 3-phosphate dehydrogenase	PK -	pyruvate kinase

pyruvate decarboxylase and alcohol dehydrogenase need not be considered explicitly. These enzymes are assumed to catalyse very fast reactions so that the appropriate equilibria are maintained, even under dynamic conditions (Hess, et al., 1969; Rapoport and Heinrich, 1975).

- (ii) The reaction of glyceraldehyde 3-phosphate dehydrogenase is considered to be irreversible. In the cell, 1,3-diphosphoglycerate, the product of the glyceraldehyde 3-phosphate dehydrogenase reaction, is consumed by phosphoglycerate kinase. This enzyme reaction is very fast and highly exergonic, and serves to pull the glyceraldehyde 3-phosphate dehydrogenase reaction to completion (Lehninger, 1975).

5.2.2 Rate and Conservation Equations

The individual reactions of the model are:



The abbreviations used are:

EtOH -	ethanol	PEP -	phosphoenolpyruvate
F6P -	fructose 6-phosphate	PFK -	phosphofructokinase
FdP -	fructose diphosphate	PK -	pyruvate kinase

It is assumed that the rates of synthesis of the pyridine and adenine nucleotides are relatively slow, so that the total concentrations of these compounds remain constant (Heinrich, et al., 1977). The conservation equations are:

$$[\text{NAD}^+] + [\text{NADH}] = \text{AN} = \text{constant} \quad (5.2-1)$$

$$[\text{AMP}] + [\text{ADP}] + [\text{ATP}] = \text{AP} = \text{constant} \quad (5.2-2)$$

The system of six ordinary differential equations describing the concentration changes for the glycolytic intermediates is as follows:

$$\frac{d[\text{F6P}]}{dt} = v_{\text{in}} - v_{\text{PFK}} \quad (5.2-3)$$

$$\frac{d[\text{FdP}]}{dt} = v_{\text{PFK}} - v_1 \quad (5.2-4)$$

$$\frac{d[\text{PEP}]}{dt} = 2v_1 - v_{\text{PK}} \quad (5.2-5)$$

$$\frac{d[\text{ATP}]}{dt} = -v_{\text{in}} - v_{\text{PFK}} + 2v_1 + v_{\text{PK}} - v_3 - v_4 + v_5 \quad (5.2-6)$$

$$\frac{d[\text{ADP}]}{dt} = v_{\text{in}} + v_{\text{PFK}} - 2v_1 - v_{\text{PK}} + v_3 + 2v_4 - 2v_5 \quad (5.2-7)$$

$$\frac{d[\text{NADH}]}{dt} = 2v_1 - v_{\text{PK}} \quad (5.2-8)$$

Kinetic expressions for each of the six steps in the glycolytic reaction sequence are discussed below.

5.2.2.1 Step 1

As a first approximation, the hexokinase reaction is assumed to operate at a constant rate. The rate of entry of substrate into the phosphofructokinase reaction is denoted by V_{in} .

5.2.2.2 Step 2

The fully concerted model of Monod, Wyman and Changeux (1965) has been used extensively for the study of the phosphofructokinase reaction. The kinetics of allosteric enzymes may be described on a molecular level using the concerted transition approach. The basic concepts involved in formulation of this model (Goldbeter and Nicolis, 1976; Termonia and Ross, 1981a) are:

- (i) The enzyme is made up of n equivalent and independent subunits, each bearing a single site for each of the stereospecific ligands;
- (ii) These protomers are able to exist in either of two configurations, R and T, which differ in catalytic activity and affinity for the ligands;
- (iii) The transition between the R and T states is supposed to be fully concerted so that the enzyme may exist in only two forms: an active state (R) containing n active protomers, and an inactive state (T) where all n protomers are inactive; and
- (iv) The enzyme saturation velocity remains constant.

The concerted transition model for allosteric enzyme activity described by Hess and Plesser (1978) is applied here to describe the kinetics of phosphofructokinase. Both fructose 6-phosphate and ATP are considered substrates of

phosphofructokinase, and modulation of the enzyme by the adenosine phosphates is included.

In the limiting situation when the enzyme species are in equilibrium at any time, V_{PFK} may be expressed as:

$$V_{\text{PFK}} = V_{\text{R,max}} v_{\text{PFK}}(\lambda_1, \lambda_2, \gamma) \quad (5.2-9)$$

where

$V_{\text{R,max}}$ is the maximum catalytic activity of the R state of phosphofructokinase

$\lambda_1 = \frac{[\text{F6P}]}{K_{\text{R,F6P}}}$, a dimensionless substrate concentration

$\lambda_2 = \frac{[\text{ATP}]}{K_{\text{R,ATP}}}$, a dimensionless substrate concentration

$\gamma = \frac{[\text{AMP}]}{K_{\text{R,AMP}}}$, a dimensionless enzyme effector concentration

and

$K_{\text{R,j}}$ is the Michaelis constant for compound j in the R state of phosphofructokinase.

The expression for v_{PFK} is taken from Hess and Plesser (1978):

$$v_{\text{PFK}} = \frac{g_{\text{R}} \lambda_1 \lambda_2 R^{n-1} + q L g_{\text{T}} c_1 \lambda_1 c_2 \lambda_2 T^{n-1}}{R^n + L T^n} \quad (5.2-10)$$

where

$g_{\text{R}}, g_{\text{T}}$ are substrate affinity coefficients for the R and T states of phosphofructokinase

$$c_j = \frac{K_{\text{R,j}}}{K_{\text{T,j}}}$$

$$q = \frac{V_{T,max}}{V_{R,max}}$$

$$L = L_o \left(\frac{1 + c_T \gamma}{1 + \gamma} \right)^n$$

$$R = 1 + \lambda_1 + \lambda_2 + g_R \lambda_1 \lambda_2$$

$$T = 1 + c_1 \lambda_1 + c_2 \lambda_2 + g_T c_1 \lambda_1 c_2 \lambda_2$$

and

n is the number of protomers comprising the enzyme

$K_{T,j}$ is the Michaelis constant for compound j in the T state of phosphofructokinase

$V_{T,max}$ is the maximum catalytic rate of the T state of phosphofructokinase

L_o is the allosteric or the equilibrium constant for the R and T states of phosphofructokinase in ligand-free solution.

5.2.2.3 Step 3

The kinetics of glyceraldehyde 3-phosphate dehydrogenase are represented by an expression adapted from Velick and Furfine (1963) with modifications based on the data of Yang and Deal (1969). Two substrates, glyceraldehyde 3-phosphate and NAD^+ , are involved in the rate equation. Product inhibition by NADH is also included, as are the inhibitory effects of AMP, ADP and ATP. NADH competes with both glyceraldehyde 3-phosphate and NAD^+ for their binding sites (Velick and Furfine, 1963), while the adenosine phosphates are competitive with NAD^+ only (Yang and Deal, 1969).

The following assumptions are involved in formulation of the glyceraldehyde 3-phosphate dehydrogenase rate expression:

- (i) the third substrate in the reaction, phosphate, is assumed to be available in excess and does not influence the kinetics of the reaction;
- (ii) product inhibition by 1,3-diphosphoglycerate is negligible (Velick and Furfine, 1963); and
- (iii) adenine-containing compounds other than AMP, ADP and ATP are either absent, or exert only a relatively weak inhibitory effect on glyceraldehyd 3-phosphate dehydrogenase so that they may be ignored (Yang and Deal, 1969).

Assumption (ii) of Section 5.2.1 allows the concentrations of glyceraldehyde 3-phosphate and fructose diphosphate to be related simply following equilibrium of the connecting reactions. From available data which characterize both the steady and oscillatory states of glycolysis (Hess, et al., 1973):

$$[\text{glyceraldehyde 3-phosphate}] \approx 0.01 [\text{fructose diphosphate}]$$

Rate laws for enzymes with crossed product inhibition and with competitive inhibition from other effectors may be derived using rapid equilibrium assumptions (Dixon and Webb, 1979). The rate of the glyceraldehyde 3-phosphate dehydrogenase reaction, V_1 , may be expressed in the following way:

$$V_1 = V_{\max,1} \left[1 + \frac{K_{GSP}}{0.01[FdP]} + \frac{K_{NAD^+}}{[NAD^+]} \left(1 + \sum_{i=1}^3 \frac{[\xi_i]}{K_{L\xi_i}} \right) + \frac{K_{GSP}K_{NAD^+}}{0.01[FdP][NAD^+]} \left(1 + \frac{[NADH]}{K_{I,NADH}} \right) \left(1 + \sum_{i=1}^3 \frac{[\xi_i]}{K_{L\xi_i}} \right) \right]^{-1} \quad (5.2-11)$$

where

$V_{\max,1}$ is the maximum velocity of the glyceraldehyde 3-phosphate

dehydrogenase reaction

K_j is the Michaelis constant for compound j

$K_{I,j}$ is the inhibition constant for compound j

ζ_1 is AMP

ζ_2 is ADP

ζ_3 is ATP

and subscript G3P represents glyceraldehyde 3-phosphate.

5.2.2.4 Step 4

In the model, the two substrate kinetic expression of equation (5.2-9) for phosphofructokinase is applied also to pyruvate kinase (Hess and Plesser, 1978). In this case, the substrates are phosphoenolpyruvate and ADP. Fructose diphosphate acts as an activator of pyruvate kinase, and appears in the rate equation for V_{PK} :

$$V_{PK} = V_{R,max} v_{PK}(\lambda_1, \lambda_2, \gamma) \quad (5.2-12)$$

where

$V_{R,max}$ is the maximum catalytic activity of the R state of pyruvate kinase

$$\lambda_1 = \frac{[PEP]}{K_{R,PEP}}$$

$$\lambda_2 = \frac{[ADP]}{K_{R,ADP}}$$

$$\gamma = \frac{[FdP]}{K_{R,FdP}}$$

and

$K_{R,j}$ is the Michaelis constant for compound j in the R state of pyruvate kinase.

The expression for v_{PK} is (Hess and Plessner, 1978):

$$v_{PK} = \frac{g_R \lambda_1 \lambda_2 R^{n-1} + q L g_T c_1 \lambda_1 c_2 \lambda_2 T^{n-1}}{R^n + L T^n} \quad (5.2-13)$$

where

g_R, g_T are substrate affinity coefficients for the R and T states of pyruvate kinase,

$$c_j = \frac{K_{R,j}}{K_{T,j}}$$

$$q = \frac{V_{T,max}}{V_{R,max}}$$

$$L = L_0 \left(\frac{1 + c_j \gamma}{1 + \gamma} \right)^n$$

$$R = 1 + \lambda_1 + \lambda_2 + g_R \lambda_1 \lambda_2$$

$$T = 1 + c_1 \lambda_1 + c_2 \lambda_2 + g_T c_1 \lambda_1 c_2 \lambda_2$$

and

n is the number of protomers comprising the enzyme

$K_{T,j}$ is the Michaelis constant for compound j in the T state of pyruvate kinase

$V_{T,max}$ is the maximum catalytic rate of the T state of pyruvate kinase

L_0 is the allosteric or the equilibrium constant for the R and T states of pyruvate kinase in ligand-free solution.

5.2.2.5 Step 5

Consumption of ATP by ATPase and other enzymes is considered to be slow (Hess, et al., 1969) and practically irreversible. First order reaction kinetics are assumed for V_2 :

$$V_2 = k_2[\text{ATP}] \quad (5.2-14)$$

where k_2 is the first order rate constant.

5.2.2.6 Step 6

The adenylate kinase reaction is assumed to be fast (Hess, et al., 1969; Sel'kov and Betz, 1973), so that the adenosine phosphates are in equilibrium at all times.

$$V_3 = k_3 [\text{ATP}] [\text{AMP}] \quad (5.2-15)$$

$$V_4 = k_4 [\text{ADP}]^2 \quad (5.2-16)$$

At equilibrium, $V_3 = V_4$, and the equilibrium constant, K , may be defined as:

$$K = \frac{k_3}{k_4} = \frac{[\text{ADP}]^2}{[\text{ATP}][\text{AMP}]} \quad (5.2-17)$$

5.2.3 Parameter Values

Values for the constants in the kinetic expressions of Section 5.2.2 were obtained from the literature. Assignment of parameter values for simulation of immobilized cell glycolysis involves some assumptions or approximations, since essentially no information is available about the kinetic constants for immobilized cell enzymes. Although values similar to those of the suspended cells were generally assumed, the range of parameter values tested for some reactions was extended to account for possible differences in immobilized cell enzyme characteristics.

The parameters associated with each step of the model are discussed below.

5.2.3.1 Step 1

Both experimental and theoretical studies have shown that the rate of substrate entry into glycolysis strongly influences the occurrence and nature of glycolytic oscillations. Consequently, V_{in} is considered as an adjustable parameter in this work.

Realistic values for V_{in} lie within the range reported by Boiteux, et al. (1975):

$$5.5 \times 10^{-3} < V_{in} < 4.4 \times 10^{-2} \text{ mM s}^{-1}$$

5.2.3.2 Step 2

Parameters in the concerted transition model for phosphofructokinase kinetics were taken directly from Hess and Plesser (1978), and are listed in Table 5-1.

5.2.3.3 Step 3

Values for the constants of equation (5.2-11) are given in Table 5-2.

$V_{max,1}$ was obtained after multiplying the maximum specific enzyme activity of 20.8 s^{-1} reported by Velick and Furfine (1963) by the *in vivo* concentration of glyceraldehyde 3-phosphate dehydrogenase in yeast, $4 \times 10^{-2} \text{ mM}$, given by Yang and Deal (1969), so that:

$$V_{max,1} = 0.83 \text{ mM s}^{-1}$$

5.2.3.4 Step 4

The fixed parameter values for pyruvate kinase are shown in Table 5-3 (Hess and Plesser, 1978).

TABLE 5-1
Kinetic Parameters for Phosphofructokinase

<i>Parameter</i>	<i>Value</i>
$V_{R,max}$	5.0 mM s^{-1}
$K_{R,F6P}$	1.0 mM
C_{F6P}	5×10^{-4}
$K_{R,ATP}$	$6 \times 10^{-2} \text{ mM}$
C_{ATP}	1.0
g_R	10.0
g_T	1.0
$K_{R,AMP}$	$2.5 \times 10^{-2} \text{ mM}$
C_{AMP}	1.9×10^{-2}
L_O	4×10^6
q	0.0
n	2

$V_{R,max}$ is considered as an adjustable parameter. Its value lies within the range used by Hess and Plesser (1978):

$$10^{-2} < V_{R,max} < 10^3 \text{ mM s}^{-1}$$

5.2.3.5 Step 5

The value of k_2 , the kinetic constant for the ATPase reaction, is considered adjustable in this representation of yeast glycolysis. According to Martiny (1972), $k_2 < 0.83 \text{ s}^{-1}$ for yeast. The range $0.4 < k_2 < 0.8 \text{ s}^{-1}$ was used during application of the model.

TABLE 5-2

Kinetic Parameters for Glyceraldehyde 3-Phosphate Dehydrogenase

<i>Parameter</i>	<i>Value</i>	<i>Source</i>
K_{GSP}	2.5×10^{-3} mM	Velick and Furfine (1963)
K_{NAD^+}	0.18 mM	Yang and Deal (1969)
$K_{I,NADH}$	0.3×10^{-3} mM	Velick and Furfine (1963)
$K_{I,AMP}$	1.1 mM	Yang and Deal (1969)
$K_{I,ADP}$	1.5 mM	Yang and Deal (1969)
$K_{I,ATP}$	2.5 mM	Yang and Deal (1969)

5.2.3.6 Step 6

The equilibrium constant for adenylate kinase was estimated as the average of values for yeast given by Martiny (1972), Sel'kov and Betz (1973) and Hess and Plesser (1978). The result was $K = 0.6$.

5.2.3.7 Other Constants

For the most part, the total concentration of adenosine phosphate compounds in the cell, AP, was taken to be 5 mM (Hess and Plesser, 1978). However, there is some variation of this value in the literature (0.48 mM, Martiny, 1972; ≥ 1 mM, Sel'kov and Betz, 1973; 1.5 mM, Betz and Moore, 1967; 3 mM, Hess, et al., 1973), so that some exploration at lower adenosine phosphate levels was carried out.

The total concentration of pyridine nucleotides in yeast is indicated by Hess, et al. (1973) to vary between about 1 mM and 3 mM. Within this range, however, $[NADH] \ll [NAD^+]$ (Hess, et al., 1973; Heinrich, et al., 1977), so that usually,

TABLE 5-3
Kinetic Parameters for Pyruvate Kinase

<i>Parameter</i>	<i>Value</i>
$K_{R,PEP}$	0.2 mM
C_{PEP}	4×10^{-8}
$K_{R,ADP}$	50.0 mM
C_{ADP}	1.0
g_R	1.0
g_T	1.0
$K_{R,FdP}$	0.2 mM
C_{FdP}	1.0×10^{-2}
L_O	3.9×10^5
q	1.0
n	2

$[NADH] \approx 0.01 [NAD^+]$.

5.2.4 Summary of Glycolysis Modelling

The glycolytic pathway and related reactions in yeast were simulated in a kinetic model. Simplifications of the reaction scheme were based on experimental findings reported in the literature. The model contains the important control features of the allosteric enzymes, phosphofructokinase and pyruvate kinase, as well as a kinetic representation of glyceraldehyde 3-phosphate dehydrogenase.

The model was devised to allow analysis of the mechanism for NADH oscilla-

tions in immobilized and suspended *Saccharomyces cerevisiae* cells. The results from both steady state and transient solutions of the model are discussed in Chapter 6.

CHAPTER 6

RESULTS AND DISCUSSION

In this chapter, results from the fermentation and glycolytic oscillation experiments are presented. The data collected in this work provide information about immobilized yeast morphology, composition and kinetics of metabolism.

Analysis of the results is aimed at establishing a comparison of immobilized cell and suspended cell properties and fermentation characteristics. The experimental findings are discussed with reference to the possible effects of cell-surface interactions on yeast metabolism and cell cycle operation.

Immobilized *Saccharomyces cerevisiae* cells were characterized under several different conditions. Results from experiments with gelatin-immobilized cells describe:

- (i) fermentation and growth on complete nutrient medium;
- (ii) fermentation while cell growth is inhibited by hydroxyurea; and
- (iii) fermentation under conditions of biotin starvation.

Some properties of concanavalin A-immobilized *S. cerevisiae* are also given.

The stability and dynamics of immobilized and suspended cell energy metabolism were studied from experimental and theoretical viewpoints. Changes in intracellular NADH levels following starvation of cells and nutritional shift-up were compared for the two cell populations. Interpretation of these data is discussed in terms of the mathematical model of glycolysis derived in Chapter 5.

6.1 FERMENTATION AND GROWTH WITH COMPLETE NUTRIENT MEDIUM

Immobilized and suspended cell fermentation was studied using yeast extract medium containing all essential nutrients for growth. The immobilization support in these experiments was crosslinked gelatin.

6.1.1 Morphology

Four scanning electron micrographs of immobilized *Saccharomyces cerevisiae* cells are reproduced in Figure 6-1.

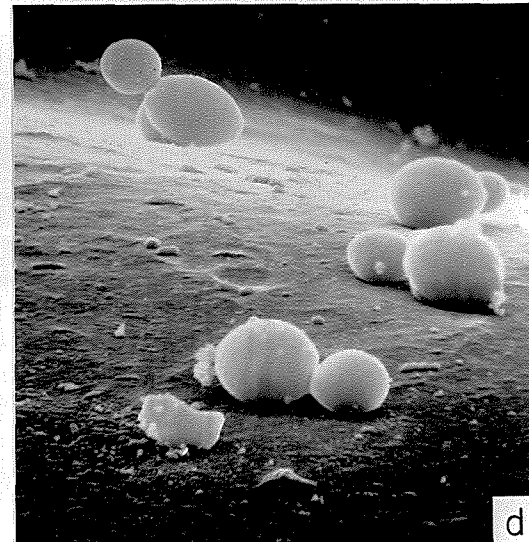
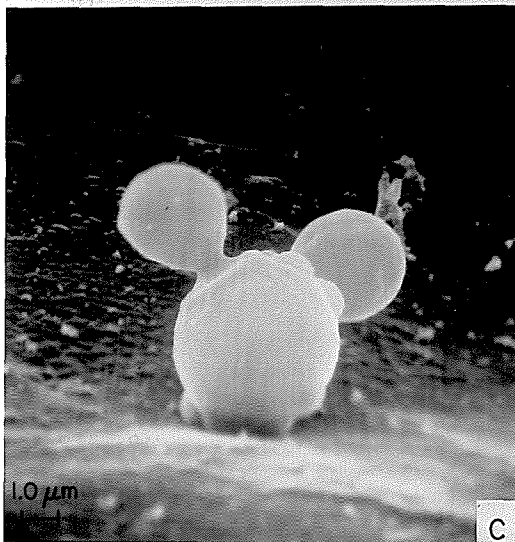
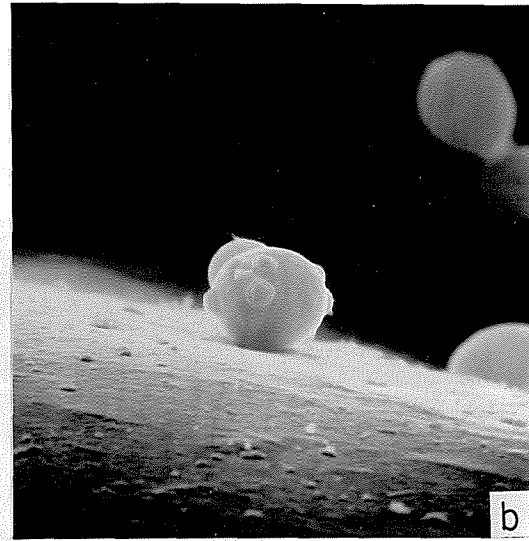
Figure 6-1a (x400) shows the extent to which the immobilization surface is covered with cells. The yeast form less than a monolayer. This result is pertinent to the development of the models for immobilized cell fermentation outlined in Section 5.1. Problems in the analysis due to diffusion were avoided after assuming that the immobilized yeast do not form thick cellular films under the fermenter operating conditions.

Figure 6-1b (x3000) shows bud scar location on an immobilized cell. It is clear from this picture that yeast cells immobilized on gelatin do not change shape or become disformed due to attachment. The proportion of cell surface which interacts with the gelatin surface is also indicated in this view of a single cell.

A budded immobilized cell is shown in Figure 6-1c (x5000) just prior to separation. Since the bud here is directed away from the surface, it will most likely be released into suspension. It is possible, though, that immobilized cells produce daughters which become attached to the gelatin during bud development, as shown in the foreground of Figure 6-1d (x3000).

Figure 6-1

**Scanning electron micrographs of immobilized yeast cells
grown on complete nutrient medium
(a) x400; (b) x3000; (c) x5000; (d) x5000**



6.1.2 Fermentation Characteristics

Typical sets of results from the immobilized and suspended cell batch fermentations are plotted in Figures 6-2, 6-3, 6-4 and 6-5.

Figure 6-2 shows ethanol, glycerol and glucose concentration data from an experiment with suspended cells. Cell concentrations are given in Figure 6-3. The initial cell concentration was 1.48×10^9 cells/l.

Figure 6-4 shows the product and substrate concentration changes during an immobilized cell fermentation, in which the initial total cell concentration was 6.19×10^9 cell/l. Immobilized and suspended cell concentrations in the immobilized cell reactor are plotted in Figure 6-5.

The ethanol, glycerol, glucose, immobilized cell and suspended cell concentration data collected during the experiments were fitted to the fermentation equations developed in Chapter 5 (Section 5.1.2) using a Marquardt routine. For the suspended cell experiments, equations (5.1-11) - (5.1-13) were used. The immobilized cell data were fitted to equations (5.1-20) - (5.1-23) after application of Simpson's rule for evaluation of the integrals. Values of the parameters in these equations allowing the best fit of the data were obtained from the Marquardt analysis.

The calculated concentration trajectories are included in Figures 6-2 - 6-5. The data are well fitted by both suspended and immobilized cell models. Initial productivities and yields for the fermentations were obtained from the derivatives at time zero of each of the concentration trajectory equations.

The suspended cell experiments, which were repeated three times, gave essentially identical results. The data from all three suspended cell fermentations are given in Tables IV-1 - IV-3 in Appendix IV. Results from duplicate immobilized cell

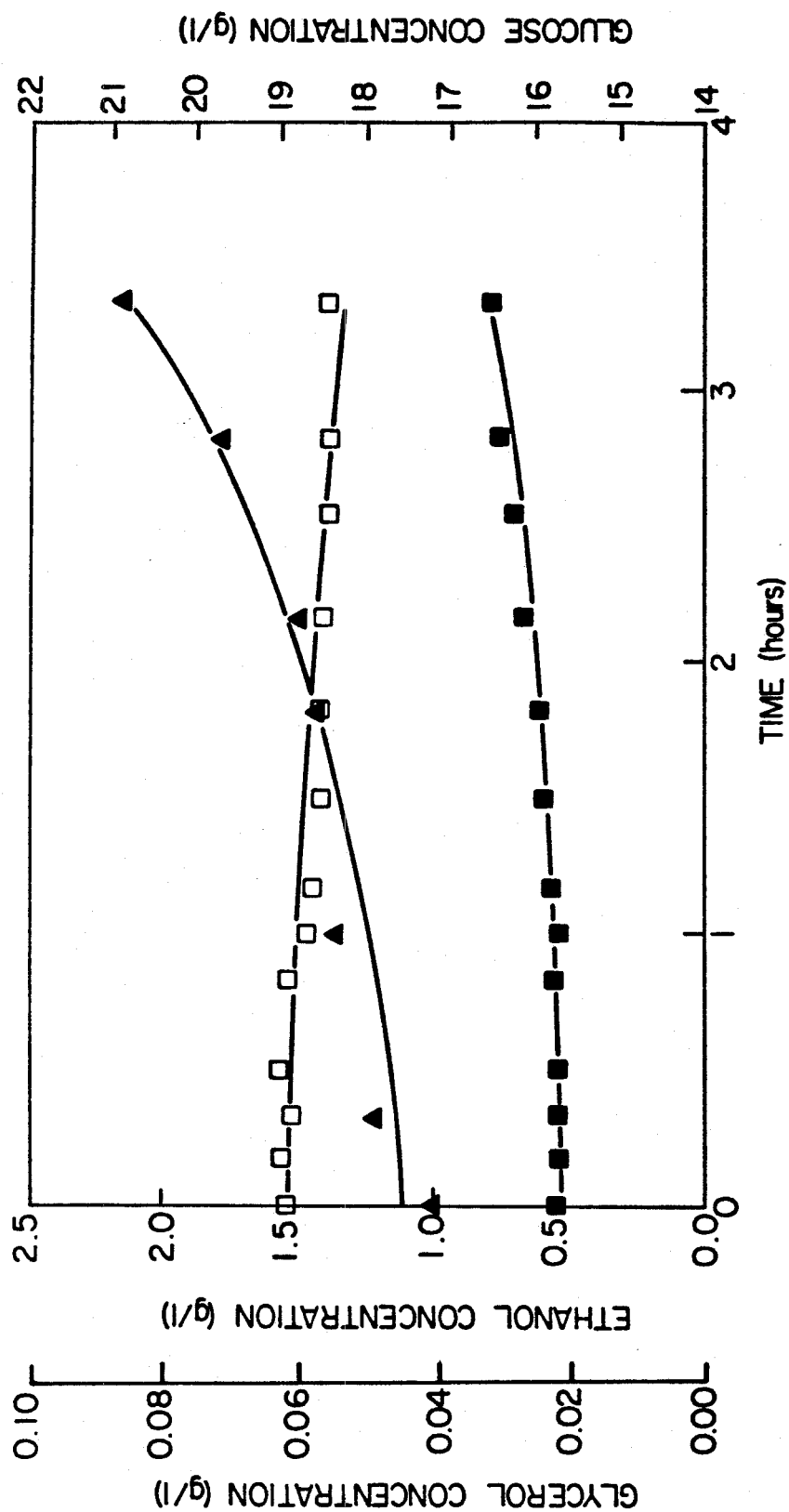


Figure 6-2
Concentration data from typical suspended cell fermentation with complete nutrient medium showing accumulation of ethanol (■) and glycerol (▲) and disappearance of glucose (□). The initial suspended cell concentration was 1.48×10^9 cells/l. The initial specific ethanol productivity for this experiment was 2.2×10^{-11} g/hr cell.

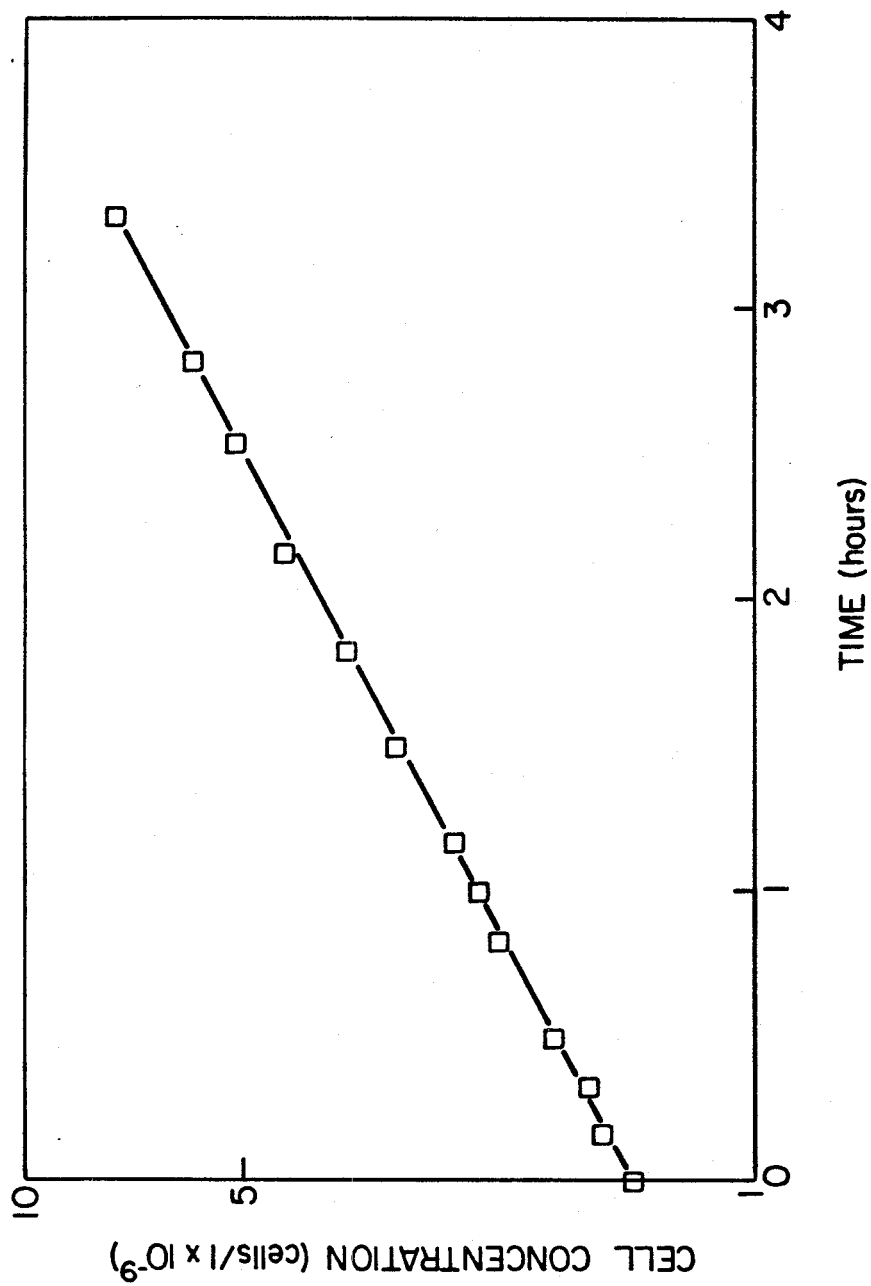


Figure 6-3
Cell concentration data from typical suspended cell fermentation with complete nutrient medium. The suspended cell specific growth rate in this experiment was 0.49 hr⁻¹.

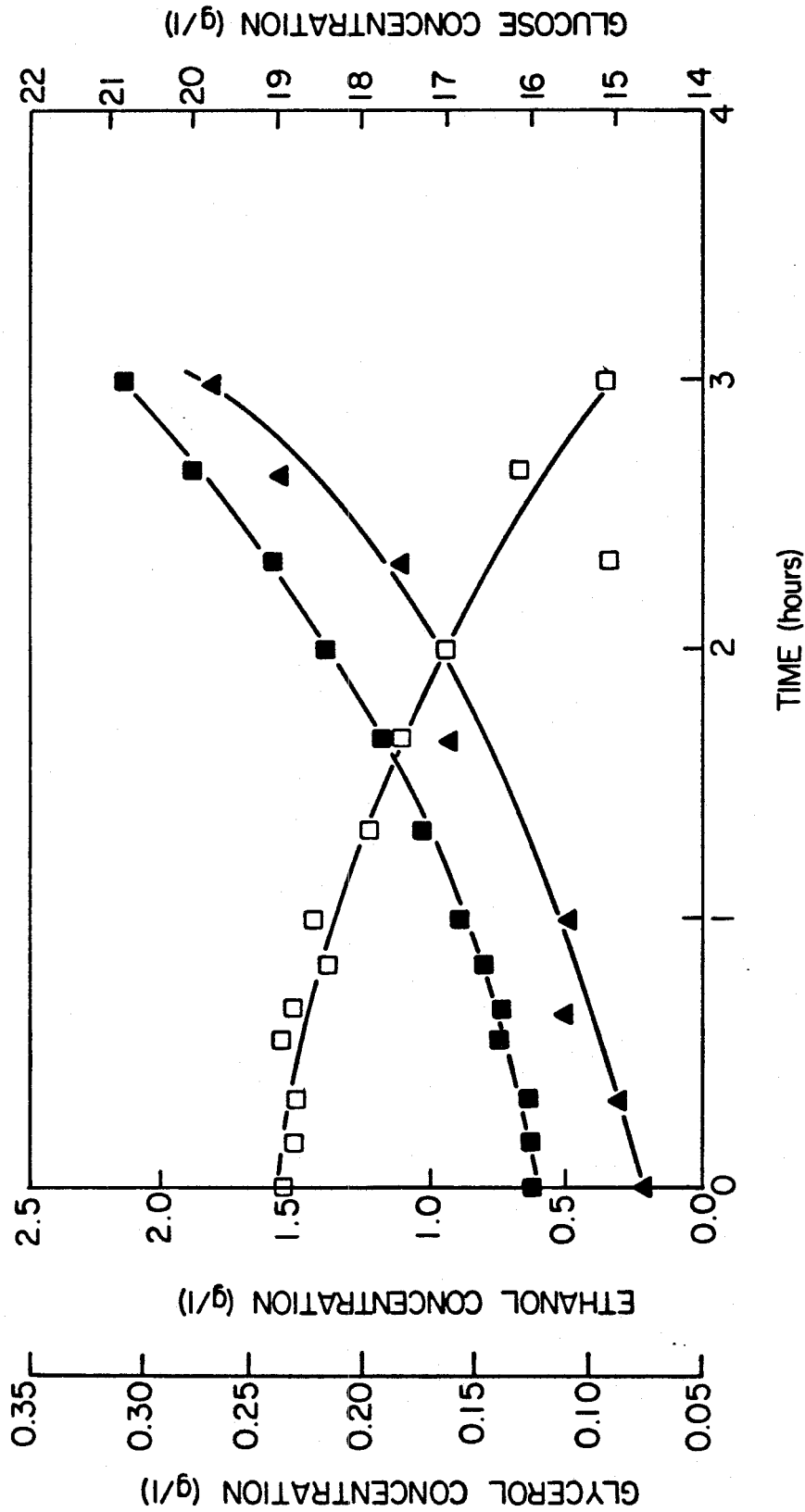


Figure 6-4
Concentration data from typical immobilized cell fermentation with complete nutrient medium showing accumulation of ethanol (■) and glycerol (▲) and disappearance of glucose (□). The initial total cell concentration was 6.19×10^9 cells/l. The initial specific ethanol productivity for this experiment was 3.7×10^{-11} g/hr cell.

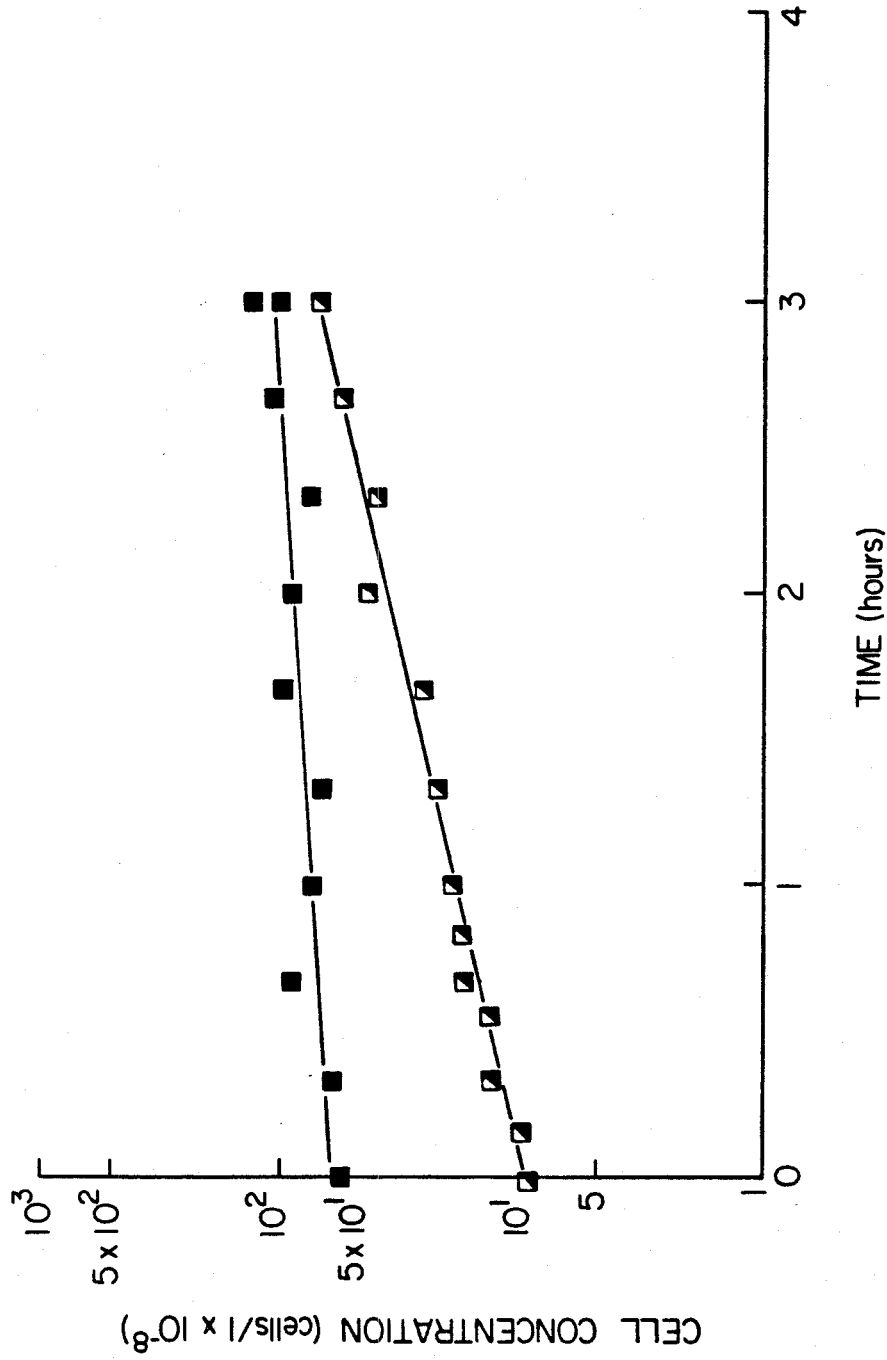


Figure 6-5
Cell concentration data from typical immobilized cell fermentation with complete nutrient medium showing accumulation of both immobilized cells on the beads (■) and suspended cells in the recirculating medium (□). Release of immobilized cell daughters into the medium contributes to the increase in suspended cell number.

experiments showed more variation, especially with respect to glucose uptake and glycerol formation rates. Tables V-1 and V-2 in Appendix V list the raw immobilized cell data.

A summary of the kinetic results together with the analysed product yields from glucose is given in Table 6-1.

TABLE 6-1
Batch Fermentation Results
for Cells Grown on Complete Nutrient Medium

	Suspended Cells	Immobilized Cells
initial specific ethanol productivity (g/hr cell $\times 10^{11}$)	2.3 \pm 4%	3.3 \pm 10%
initial specific glycerol productivity (g/hr cell $\times 10^{12}$)	2.7 \pm 2%	3.5 \pm 26%
initial specific glucose consumption (g/hr cell $\times 10^{11}$)	5.0 \pm 8%	10.5 \pm 24%
ethanol yield from glucose (mol/mol)	1.8 \pm 5%	1.4 \pm 17%
glycerol yield from glucose (mol/mol)	0.11 \pm 3%	0.06 \pm 2%
cell yield from glucose (cells/mol $\times 10^{-11}$)	18.1 \pm 3%	5.3 \pm 13%
μ_s (hr $^{-1}$)	0.51 \pm 4%	0.50 \pm 2%
μ_i (hr $^{-1}$)	—	0.28 \pm 11%

6.1.2.1 Fermentation Rates and Yields

The specific rate of ethanol production by the immobilized cells was about 45% greater than for the suspended cells. Glycerol production was also faster. Glucose was taken up by the immobilized cells at about twice the suspended cell rate, an unexpected result since the cell surface area available to the immobilized cells for transport was probably lower.

Concomitant with the increased rates of glucose consumption and ethanol and glycerol production in the immobilized cells, there was a decrease in their specific growth rate and cell yield. Apparently, the formation of daughter cells by the immobilized yeast could not proceed as readily as with the freely suspended cells. Product yields from glucose for the immobilized cells were also lower than for suspended cells.

Although consuming glucose rapidly, the immobilized *S. cerevisiae* channelled a smaller fraction of substrate into production of ethanol, glycerol and cells.

6.1.2.2 Gelatin Control Experiment

The rate of suspended cell fermentation in medium containing 0.5 g/l gelatin was measured during a control experiment. The initial batch ethanol productivity was within 10% of the value obtained without gelatin.

6.1.2.3 Viability Analysis

Immobilized and suspended cell viability was routinely measured using trypan blue staining (Section 4.7.7). In complete growth medium, essentially 100% viability was maintained by all types of cells for the duration of the experiments.

The ability of immobilized and suspended cells to produce colonies was tested by plating on agar. The immobilized cells were detached from the gelatin for this analysis; however, the enzyme treatment did not affect colony formation, as

indicated by a suspended cell control test.

Results from the plate counts showed that the proportion of immobilized cells able to initiate colony growth was reduced to only 42%.

6.1.3 Polysaccharide Content

The results shown in Table 6-2 suggest that, in immobilized cells, low cell and ethanol yields are consequences of the high priority apparently given by these cells to polysaccharide synthesis. Compared with suspended cells, the immobilized cells contained almost four times more polysaccharide, with substantially higher levels of all compounds except trehalose.

6.1.4 Flow Cytometry Results

The DNA, RNA and protein frequency functions for populations of suspended and immobilized cells are shown in Figures 6-6, 6-7 and 6-8 respectively. Fluorescence intensity is proportional to the amount of stained compound contained in the cells. The frequency, or number of cells counted, is plotted for each level of fluorescence.

Figure 6-9 gives the small-angle scatter histograms for the two populations. Small-angle scatter increases with increasing cell size.

The mean fluorescence and light scatter values for each cell type have been calculated from the frequency functions shown, and are listed in Table 6-3.

6.1.4.1 DNA

It is clear from the DNA results (Figure 6-6) that something very unusual has occurred in the immobilized cells.

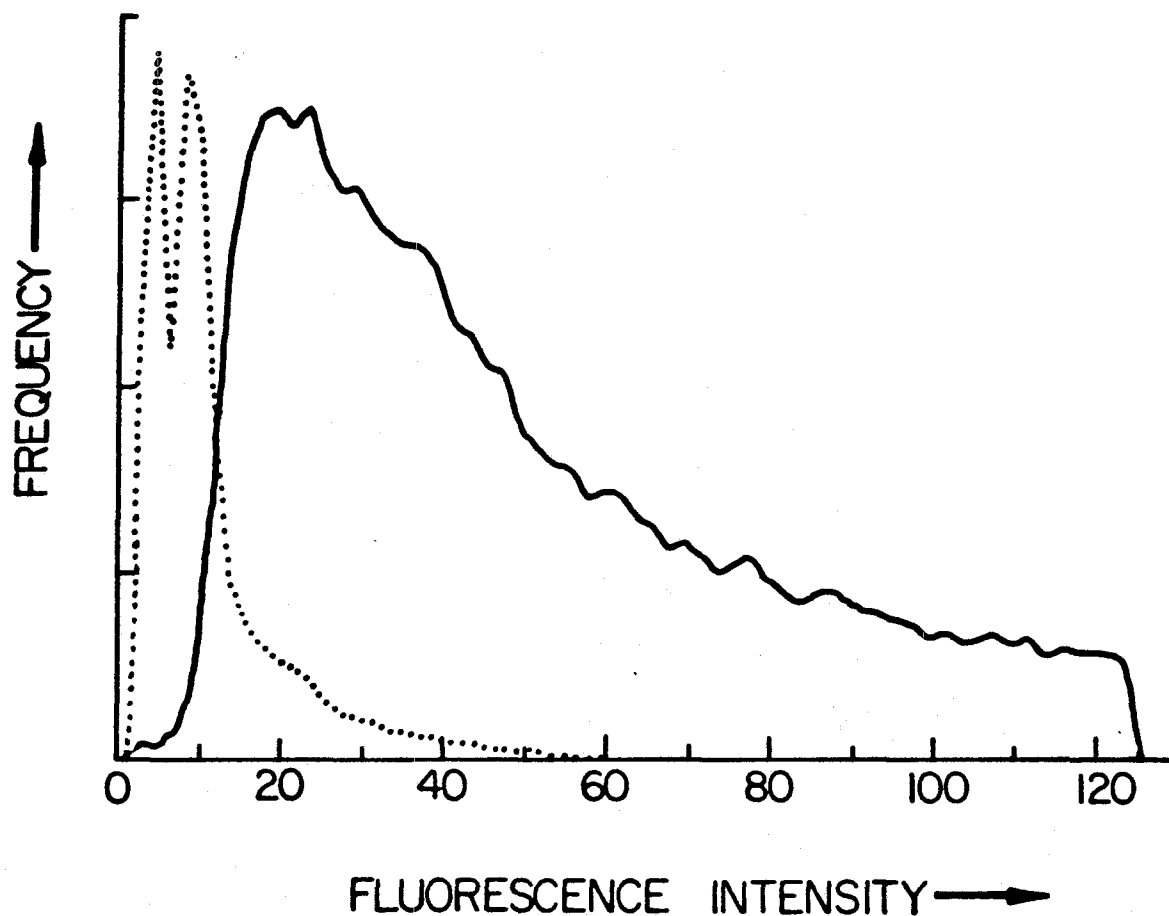


Figure 6-6

Single-cell DNA frequency functions for immobilized (—) and suspended cells (.....) grown on complete nutrient medium. The frequency, or relative number of cells counted, is plotted as a function of DNA fluorescence. The DNA fluorescence corresponding to 1 and 2 genome copies are indicated by the two sharp modes in the suspended cell histogram. The immobilized cells contain, on average, 3.8 times more DNA than do the suspended cells.

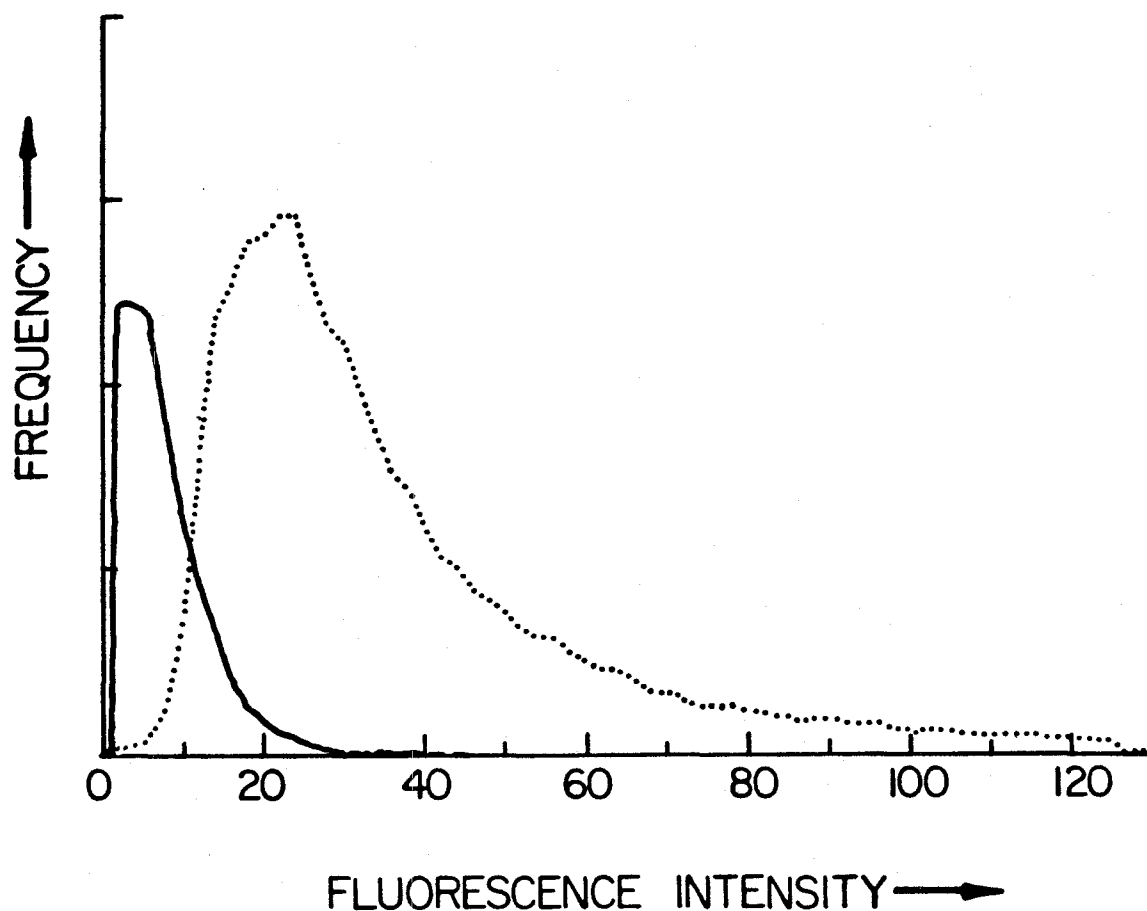


Figure 6-7

Single-cell stable double-stranded RNA frequency functions for immobilized (—) and suspended cells (.....) grown on complete nutrient medium. The frequency, or relative number of cells counted, is plotted as a function of RNA fluorescence. The immobilized cell RNA content is severely reduced compared with the suspended cells.

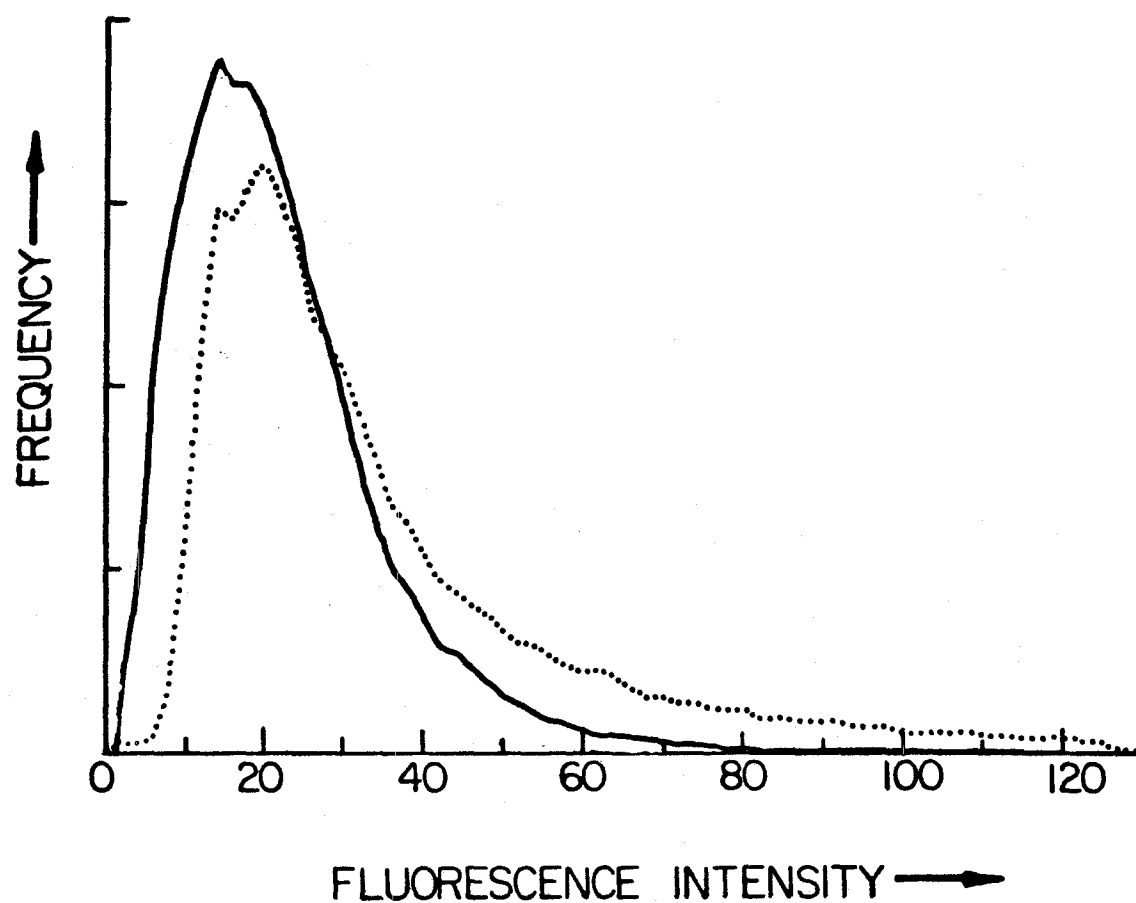


Figure 6-8

Single-cell protein frequency functions for immobilized (—) and suspended cells (.....) grown on complete nutrient medium. The frequency, or relative number of cells counted, is plotted versus single-cell stained protein fluorescence intensity.

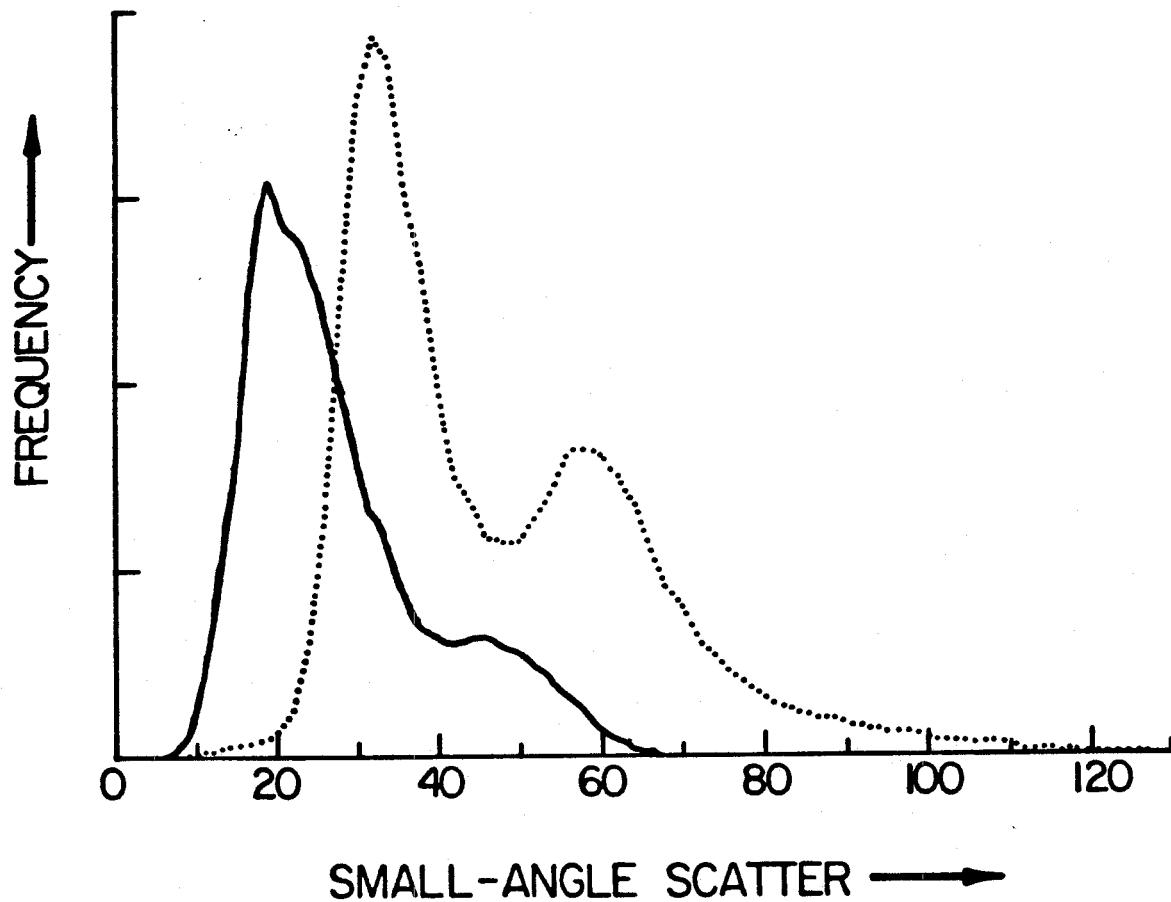


Figure 6-9

Single-cell small-angle light scatter frequency functions for immobilized (—) and suspended cells (.....) grown on complete nutrient medium. The frequency, or relative number of cells counted, is plotted for each level of single-cell light scatter detected. Light scatter increases with increasing cell size. Immobilized cells are smaller, and there are fewer budded cells than in the suspended cell population.

TABLE 6-2
Cell Polysaccharide Content
for Cells Grown on Complete Nutrient Medium

	Polysaccharide Content (g glucose equiv./cell x10 ¹⁰)	
	Suspended Cells	Immobilized Cells
trehalose (cold TCA fraction)	1.8	2.0
alkali soluble glycogen + mannan	4.6	29
acid soluble glycogen	4.1	26
glucan	6.7	11
TOTAL	17	67

An exponentially-growing yeast population normally has a bimodal DNA distribution as shown in the figure for the suspended cells. The first peak represents cells in the G1-phase of the cell cycle before initiation of chromosomal replication, while the second peak represents cells in either the G2- or M-phases.

In comparison, the frequency function for the immobilized cell population shows that these cells contained widely varying and greater amounts of DNA. Almost no immobilized cells contained the quantities of DNA corresponding to only one or two diploid yeast genomes. There were a large number with about five times the DNA content of G1-phase suspended cells, and significant populations of

TABLE 6-3

**Mean Values of Fluorescence and Light Scatter Frequency Functions
for Cells Grown on Complete Nutrient Medium**

	Frequency Function Mean Values (from Figures 6-6 - 6-9)	
	Suspended Cells	Immobilized Cells
DNA fluorescence	13.0	49.4
RNA fluorescence	35.8	8.2
protein fluorescence	33.6	21.8
small-angle scatter	47.0	33.4

immobilized cells with what appear to be six, seven and eight genomes.

From Table 6-3, the average DNA content of the immobilized cells was 3.8 times that of the suspended cells.

These results for immobilized cells are not due to clumping or aggregation of the cells as they pass through the flow cytometer. This is evident after examination of the immobilized and suspended cell two-parameter frequency functions shown in Figure 6-10. In this representation, the frequency or relative number of cells exhibiting a particular combination of single-cell DNA fluorescence and light scatter levels is indicated by the vertical coordinate. The maximum light scattering levels for both cell populations are quite similar, indicating that the

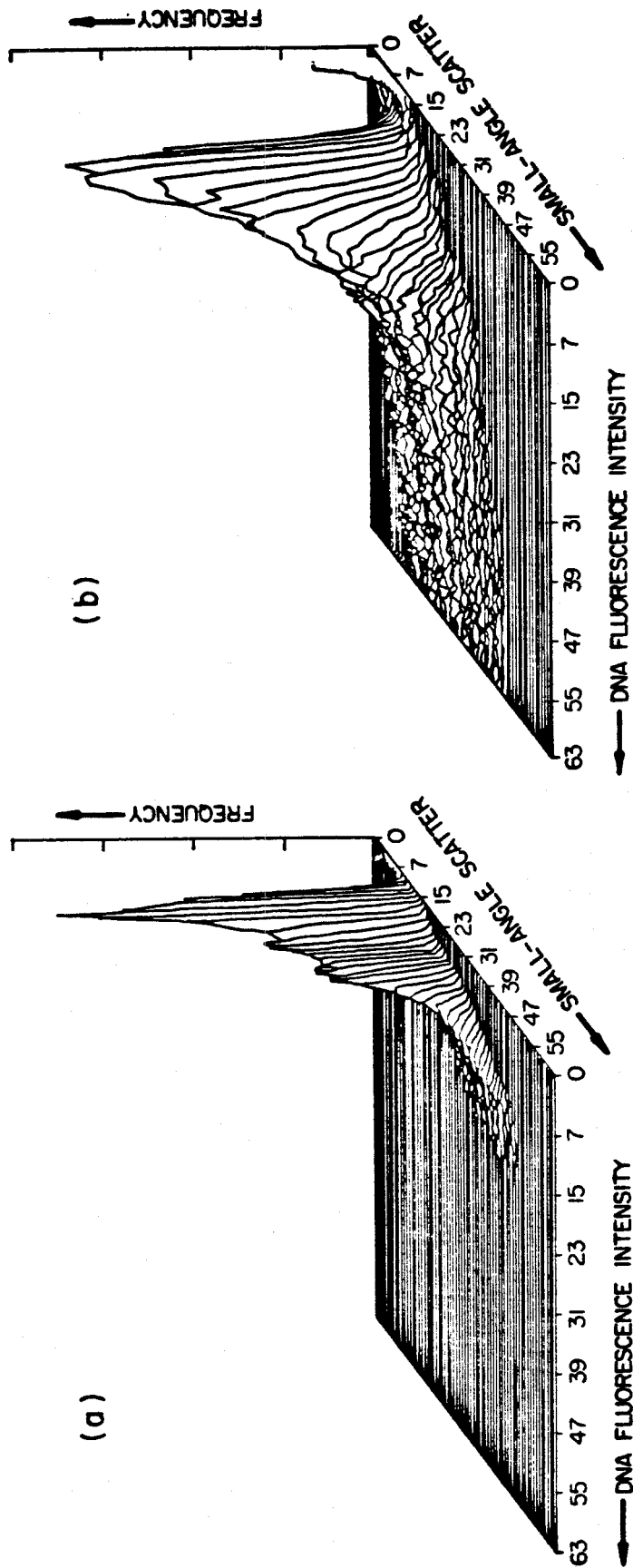


Figure 6-10
Single-cell DNA fluorescence-light scatter frequency functions for (a) suspended cells and (b) immobilized cells grown on complete nutrient medium. The frequency or relative number of cells which display a particular combination of DNA fluorescence level and light scatter level is plotted vertically for each cell type. Since the maximum scatter levels for both populations are approximately the same, the higher DNA content measured for the immobilized cells cannot be due to clumping or aggregation of the cells in the flow cytometer sample stream.

immobilized cell sizes detected by the flow cytometer during DNA measurement were no greater than those for the suspended cells.

Verification of the relative amounts of DNA in the immobilized and suspended cells was obtained by fluorescence microscopy. Compared with the suspended cell fluorescence which could barely be seen, the immobilized cell DNA was very bright, and some cells appeared to be filled with fluorescent compound.

The results from a control experiment showed that the differences in DNA levels for immobilized and suspended yeast were not due to the effects of glutaraldehyde or dissolved gelatin in the immobilized cell reactor medium. A quantity of wash liquid from the immobilized cell column approximating the liquid hold-up in the packing was added to a suspended cell fermentation. The DNA frequency function for these cells was unchanged compared with the histogram for an untreated suspended cell population.

6.1.4.2 RNA

Results from the RNA fluorescence measurements (Figure 6-7 and Table 6-3) indicate that the immobilized yeast cells contained only approximately one-fourth the amount of stable double-stranded RNA found in suspended cells.

6.1.4.3 Protein

The protein frequency functions (Figure 6-8) show that the immobilized and suspended cell populations contained the same two subgroups with respect to protein content. This may be taken as an indirect indication that there was no interference in the flow cytometry procedures due to gelatin debris. The relative number of cells in each subgroup varies with cell type, so that the average protein content for the immobilized cells is two-thirds that for the suspended cells (Table 6-3). From Figure 6-9, the mean size of the immobilized *S. cerevisiae* is also

approximately two-thirds that of the suspended cells, so that the quantity of protein per unit size for the two cell types is roughly equal.

In all, there are more similarities between immobilized and suspended cells with regard to protein distribution than are evident in the results from each of the nucleic acid measurements.

6.1.4.4 Light Scatter

Small-angle scattering results are shown in Figure 6-9. Immobilized cells have a smaller average size than the suspended cells (Table 6-3). Assuming that the second peak in the light scatter histograms represents only those yeast cells with buds, budded cells account for approximately 46% of the total suspended cell population, while this fraction in the immobilized cell population is reduced to 22%.

6.1.5 Discussion

In the immobilized cell fermentations, both ethanol and glycerol were produced faster than by suspended cells under identical fermentation conditions, even though the yields from glucose for these end-products were reduced. It appears that, in the immobilized cells, the rate of one or more enzyme reaction steps in the Embden-Meyerhof-Parnas pathway is enhanced. Since the route to glycerol in yeast is the same as that for ethanol synthesis up to dihydroxyacetone phosphate, it may be postulated further that stimulation of the pathway kinetics probably occurs in the first half of the sequence.

There is some evidence that glucose uptake limits the rate of fermentation in *S. cerevisiae* (van Steveninck and Rothstein, 1965; van Uden, 1967), although the mechanism of glucose transport in yeast is not yet completely understood (Bisson and Fraenkel, 1983). If substrate uptake does serve as the rate-limiting step for

functioning of the EMP pathway, then an increase in the kinetics of ethanol production without a parallel improvement in yield would imply that glucose was consumed faster. This, in fact, was found to be the case, with the immobilized cells taking up glucose at about twice the suspended cell rate.

Enhanced glucose utilization by immobilized cells occurred despite the possible interference to substrate transport arising from attachment of part of the cell surface to the support beads. Damage of the immobilized cell wall may have facilitated entry of glucose into the cell as suggested before in many other reports (Suzuki and Karube, 1979; D'Souza and Nadkarni, 1980), or, otherwise, an increased demand for glucose from within the cell caused greater transport activity through the immobilized yeast cell wall.

Enhanced productivity and substrate uptake by immobilized cells has often been explained in the past by assuming that the cells benefit from improved nutrient availability at the solid-liquid interface (Marcipar, et al., 1979; Ghose and Bandyopadhyay, 1980). This rationale makes good sense when rates of cellular processes are limited by the local nutrient supply. This situation occurs in immobilized cell systems if slow diffusion leads to significant substrate gradients near the cell, or if the concentration of substrate in the medium is very low.

Neither of these conditions apply in the present study. The experiments here were conducted with medium in which glucose was the limiting nutrient, and using a reactor system in which replenishment of nutrients at the cell surface occurred at a much faster rate than did utilization of substrate by the cells. This is described more fully in Chapter 4 (Section 4.5). Moreover, even if a microenvironment were established near the immobilized cells where nutrient concentrations were greater than in the bulk liquid, since the concentration of glucose in the medium (20 g/l) is already much greater than K_s , the half-saturation constant for

glucose transport (0.9 g/l, Kotyk and Kleinzeller, 1967), the effect on substrate consumption should be minimal.

With this in mind, enhanced nutrient availability at the cell surface seems an unlikely explanation for the kinetic effects discussed above, especially also in view of the more complex modifications to cell metabolism suggested by the results from the other analyses.

In studies by Hahn-Hägerdal, et al. (1982), improved ethanol productivity was obtained with *S. cerevisiae* by adding dextran and polyethylene glycol to the fermentation medium. It was hypothesized that the resultant decrease in water activity directed metabolism towards ethanol production (Mattiasson and Hahn-Hägerdal, 1982). Sodium alginate in solution was found to enhance ethanol production by *Saccharomyces cerevisiae* in other work (Holcberg and Margalith, 1981). However, the suspended cell control experiment with gelatin conducted in this work showed that the different activities found with immobilized cells are not related simply to the presence of this impurity in the medium.

Immobilized cell growth rate and cell yield were both reduced when compared with the suspended cells. In addition, the fraction of budded cells in the immobilized population was less, as shown by the results in Figure 6-9. Observations such as these suggest that, although budding and daughter cell development occurred as shown in the scanning electron micrographs of Figure 6-1, bud emergence may have been inhibited in immobilized yeast due to attachment of the cells to the gelatin support.

Bud initiation is a highly organized process (Moor, 1967; Byers and Goetsch, 1975) which is not totally understood (Sloat, et al., 1981). Bud formation can occur at almost any place on a *S. cerevisiae* cell, but initiation of any one bud does not occur randomly (Bartnicki-Garcia and McMurrough, 1971). It is possible that

attachment of yeast cells to gelatin-coated beads interferes with the cellular activities which occur normally at the cell wall in preparation for bud emergence, such as accumulation of membrane-bound vesicles (Moor, 1967; Byers and Goetsch, 1975) and microtubules (Byers and Goetsch, 1975). Effects such as these may be at least partially responsible for the lower growth rates and cell yields measured in the present study.

It is not uncommon for cells whose growth has been suppressed, as is the case with these immobilized yeast, to accumulate energy stores in the form glycogen and trehalose (Küenzi and Fiechter, 1969). The elevated polysaccharide content for immobilized cells bears this out, although the reasons for preferential storage of glycogen are unclear.

Synthesis of the structural components of the cell wall, glucan and mannan, is known to be little affected by growth rate (McMurrough and Rose, 1967). Accordingly, the elevated glucan content of the immobilized cells is unusual. During the cell cycle for *Saccharomyces cerevisiae*, part of the cell's storage reserves are degraded immediately before bud extrusion, and there ensues a period of structural polysaccharide synthesis (Küenzi and Fiechter, 1969). If normal orientation of budding sites and early bud formation does not occur in immobilized cells, then control over this synthetic activity may be affected. Abnormal patterns of cell wall synthesis have been observed in yeast mutants when bud formation is prevented. *S. cerevisiae* cells defective in the *CDC24* gene cannot bud, but continue to synthesize chitin, mannan and presumably, also glucan, with production being delocalized over the cell surface (Sloat, et al., 1981).

Generation of polyploid immobilized cells is the most unexpected result from this work and possibly the most telling with regard to immobilized cell cycle events. Polyploid yeast can be produced by mating; however, in a heterozygous diploid

population, the probability of spontaneous formation of tetraploids is extremely low, about 10^{-5} per cell (Takano, et al., 1977). Although it is possible that immobilization acted to improve this frequency, the fact that the immobilized yeast population consisted almost entirely of polyploids suggests that additional means for generation of such cells were available.

In budded yeast, it is possible for DNA synthesis to recommence before separation of the mother and bud already formed. The small peak further downfield of the main suspended cell population in Figure 6-6 represents cells in such a state. It is also known that DNA synthesis and nuclear division can be decoupled from bud formation in the *S. cerevisiae* cell cycle (Hartwell, 1971; Sloat, et al., 1981). For example, yeast spheroplasts do not possess the ability to divide, but have been found to continue synthesis of DNA, RNA and protein until the contents of these macromolecules increased more than twofold and up to eightfold (Hutchison and Hartwell, 1967). In addition, yeast mutants defective in bud emergence frequently undergo additional DNA synthesis and nuclear division cycles to become tetranucleate at the restrictive temperature (Hartwell, et al., 1974).

The low immobilized cell growth rate and cell yield and the results from the polysaccharide and DNA analyses may be explained by postulating that, depending on bud site selection during a particular cell cycle, surface attachment of the yeast acts to prevent bud emergence. The fact that synthesis of both cell wall components and chromosomal DNA can continue in the absence of budding suggests that accumulation of these compounds in immobilized *S. cerevisiae* cells follows from disfunction of the normal budding processes.

Intracellular crowding seems inevitable for the immobilized cells which, on average, are smaller than suspended yeast, as the quantities of stored macromolecular compounds increases. That the immobilized cells are stressed by their

excess DNA levels is also suggested by the fact that most of them cannot form colonies on agar (Section 6.1.2.4). Although gelatin-immobilized cells are active in ethanol production and function to prevent uptake of trypan blue viability dye, about 60% of them are apparently unable to successfully undergo repeated reproductive cycles. This figure may be even greater, since it is possible that some of the colonies counted after plating the immobilized cells were due to growth of attached buds rather than of the cells which were actually bound to the gelatin.

DNA frequency functions for cells in colonies produced by immobilized yeast were essentially the same as those of exponentially-growing suspended *S. cerevisiae*. Apparently, the effects of immobilization on DNA levels shown in Figures 6-6 and 6-10 are diluted out in successive generations.

A connection remains to be found between a blocked yeast cell cycle which allows continued DNA synthesis but only occasional bud development, and enhancement of glucose consumption and ethanol production rates. Although research in the area of yeast polyploidy has not yet unveiled the mechanism, polyploid strains have often been reported to possess superior fermentative capabilities (Kosikov, et al., 1975; Kosikov, 1977; Takagi, et al., 1983). The effect in immobilized cells can be supposed to occur along the same lines.

The postulated mechanism for cell cycle disturbance does not explain the markedly reduced contents of stable double-stranded RNA in immobilized cells. RNA is able to accumulate in yeast whose cell cycle progress has been blocked by agents such as hydroxyurea (Slater, 1973), or by various *cdc* mutations (Hartwell, 1971), so that, like DNA, its production seems decoupled from budding and cell division. Synthesis of both RNA and DNA draws from the same pool of nucleotide precursors, so it is possible that vigorous DNA replication by immobilized cells preempts formation of RNA. In any case, maintenance of reasonable levels of pro-

tein by the immobilized cells after severe reduction in their RNA content is also difficult to explain.

The picture of immobilized cell metabolism drawn in this discussion shows that the response of cells to immobilization may be complex, and that cell functioning may be affected at many different levels.

6.2 FERMENTATION WITH HYDROXYUREA-INDUCED GROWTH INHIBITION

The effects of hydroxyurea on the cell cycles of immobilized and suspended yeast were examined during growth on glucose. Batch fermentation parameters for the two cell populations were also obtained. Gelatin-coated beads provided the support for immobilization of cells in these experiments.

The results of Section 6.1 show that immobilized cells accumulate abnormally large quantities of DNA while attached to gelatin. Treatment with hydroxyurea, a preferential inhibitor of DNA synthesis, provided the opportunity to study the characteristics of immobilized cells under conditions which discouraged this response to immobilization.

6.2.1 Hydroxyurea Inhibition Induction

The initial effects of hydroxyurea on *S. cerevisiae* cell growth and ethanol production are shown in Figures 6-11 and 6-12.

Increase in cell number (Figure 6-11a) was halted 2-2½ hours after addition of hydroxyurea to an exponential culture. During this time, viability remained high at about 93%. Cell density continued to increase after hydroxyurea addition, as shown in part (b) of Figure 6-11. Ethanol production (Figure 6-12) proceeded during the induction period and continued after cell reproduction had ceased.

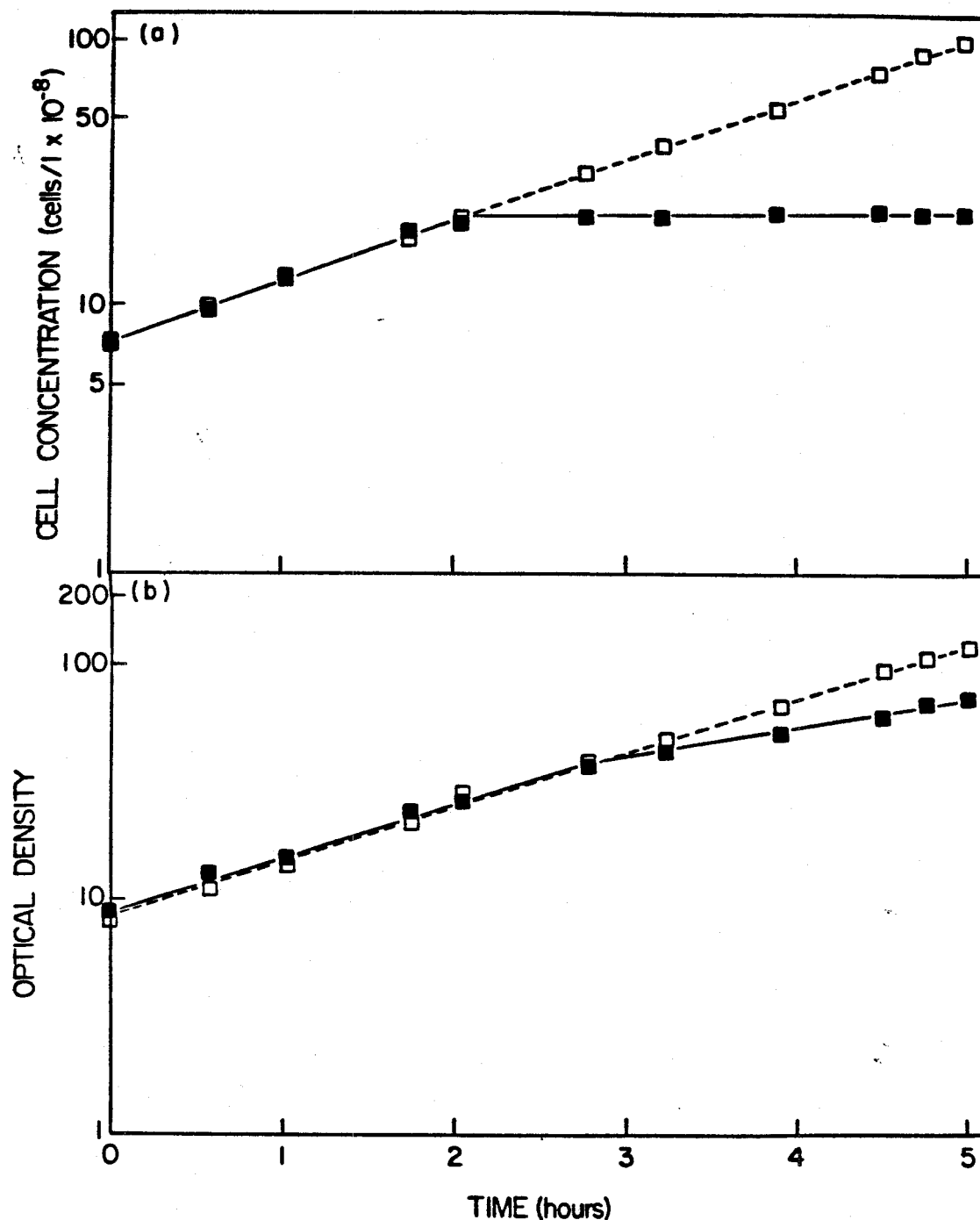


Figure 6-11

Cell concentrations and culture density during induction of hydroxyurea inhibition.

(a) Viable cell concentration data showing initial response of *S. cerevisiae* to hydroxyurea. At time=0, an asynchronous suspended culture of yeast growing exponentially at 30°C was separated into 2 flasks, and hydroxyurea was added to one to give a concentration of 0.075M. The control culture (□) continued to grow with a specific growth rate of 0.52hr⁻¹, while increase in cell number was arrested after 2-2½ hours in the inhibited culture (■).

(b) Cell density data for the cultures shown in (a). Cell density continued to increase in the inhibited culture (■), although at a slower rate than in the control culture (□) without hydroxyurea.

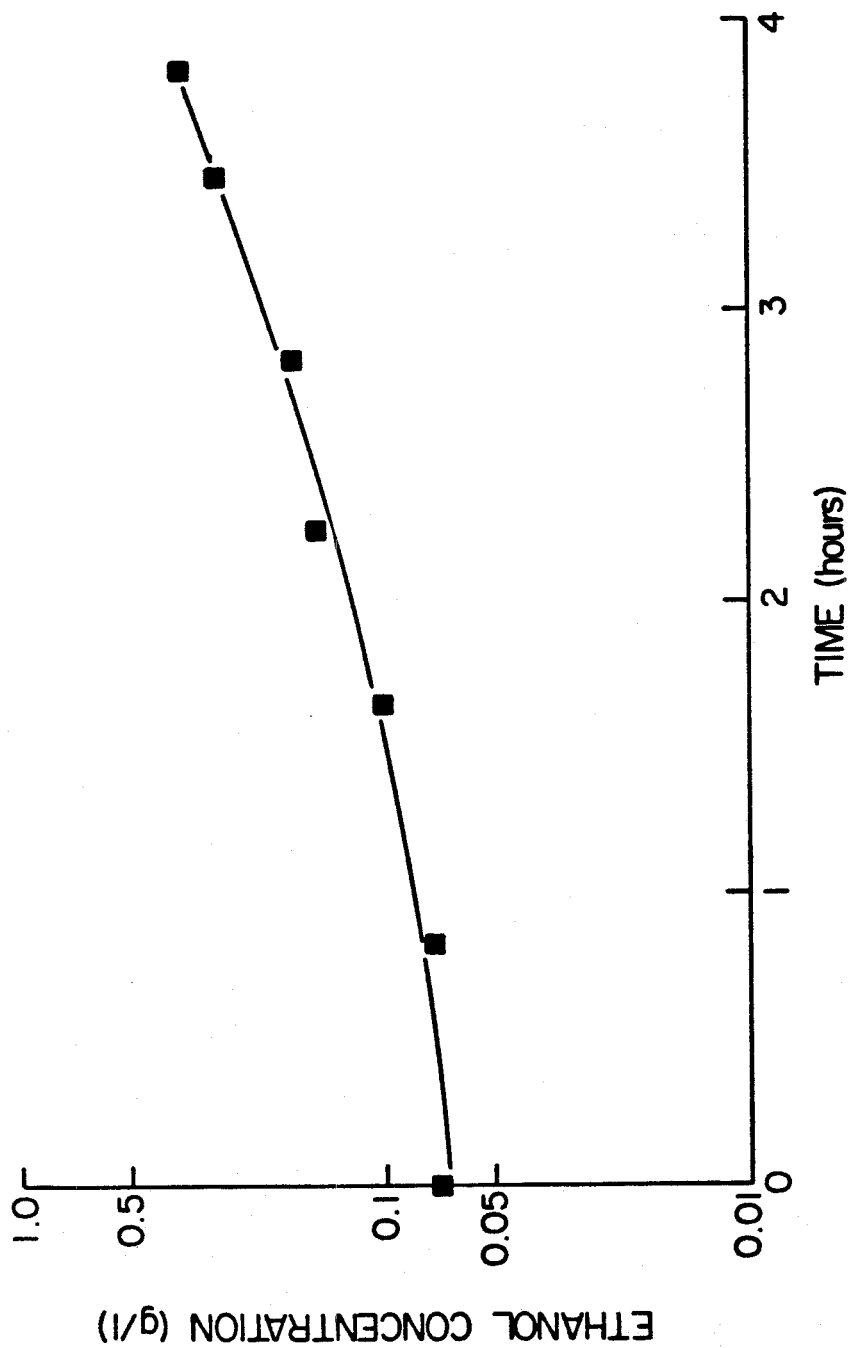


Figure 6-12
Accumulation of ethanol during induction of hydroxyurea inhibition in medium
containing suspended cells and 0.075M hydroxyurea. Hydroxyurea was added at
time=0.

In these inhibition induction tests, it was found that the concentration of hydroxyurea in solution dropped by about 10% during the first 1½ hours, but remained constant throughout the remainder of the experiment.

6.2.2 Morphology after Hydroxyurea Treatment

The effect of hydroxyurea on the morphology of suspended yeast cells is illustrated in Figure 6-13. After 8 hours of inhibition, the population was dominated by cells arrested before cell separation with large single buds (Figure 6-13a). Both bud and mother cell of the yeast doublets were often large or abnormally elongated (Figures 6-13b and 6-13c). Together with the increasing culture density data shown in Figure 6-11b, this morphology suggests that although cell separation was prevented, the cells continued to grow without dividing. The possibility of unbalanced growth in hydroxyurea-treated yeast is clearly indicated by these results.

Four scanning electron micrographs of immobilized yeast cells are reproduced in Figure 6-14. A control population of cells which were not exposed to hydroxyurea is shown in Figure 6-14a (x2000). The effects of hydroxyurea inhibition are evident in Figure 6-14b (x1700), where many attached cells are in the form of doublets, and there are few unbudded cells. The buds are large and elongated, as was found after suspended cell inhibition, and are shown more closely in Figures 6-14c (x8000) and 6-14d (x8000). The buds can generally be identified from their lack of bud scars. The neck connecting mother cell and bud has been extended, and often, the buds are attached to the gelatin surface next to the parent cell.

Figure 6-13

Morphology of suspended cells treated with hydroxyurea

- (a) light microscope photograph, x440;
- (b) scanning electron micrograph, x6000;
- (c) scanning electron micrograph, x7000.

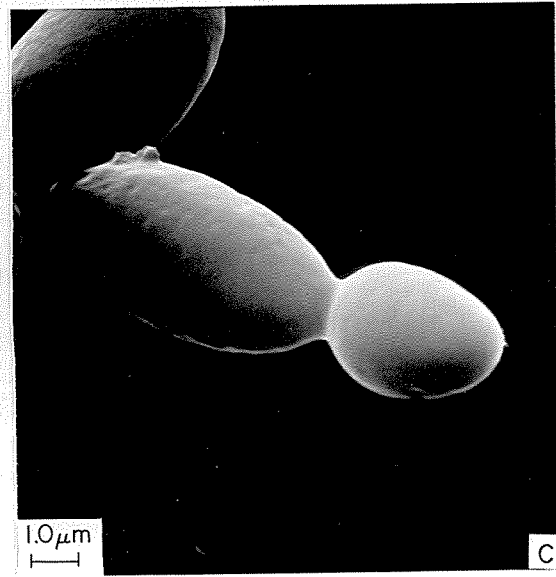
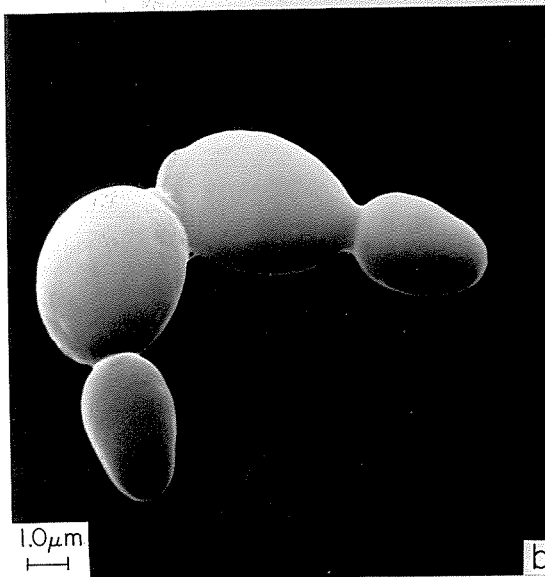
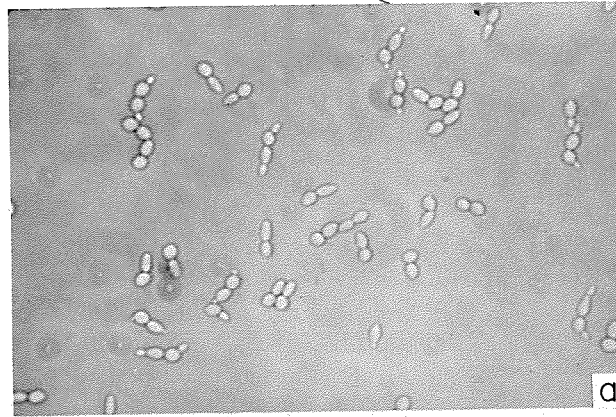
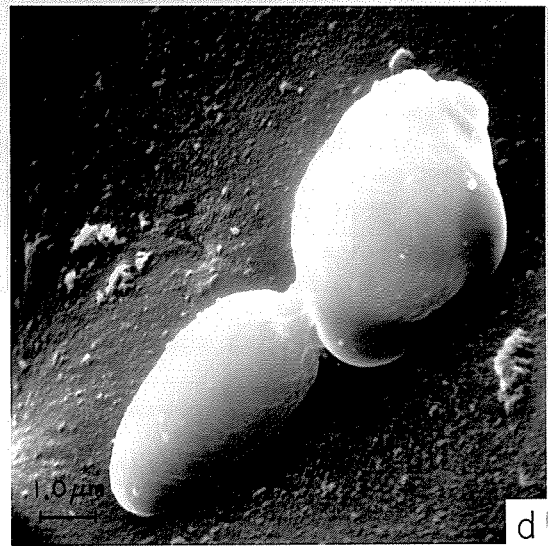
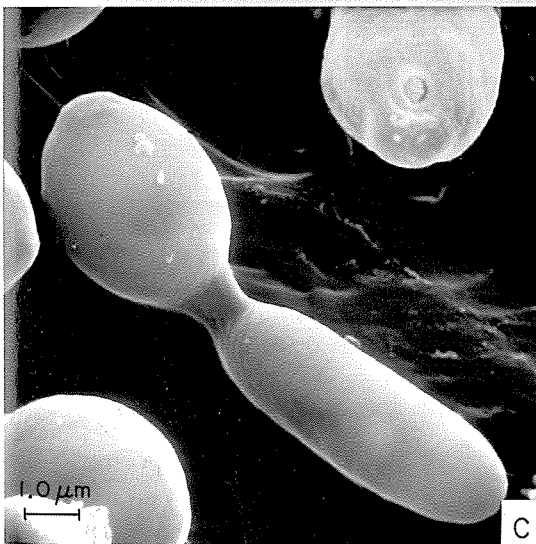
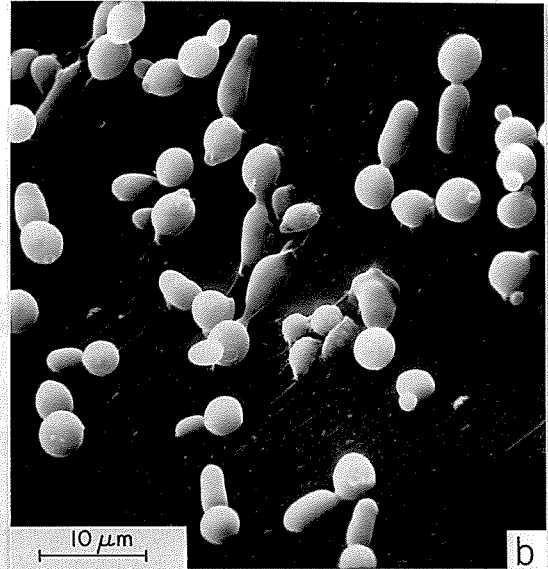
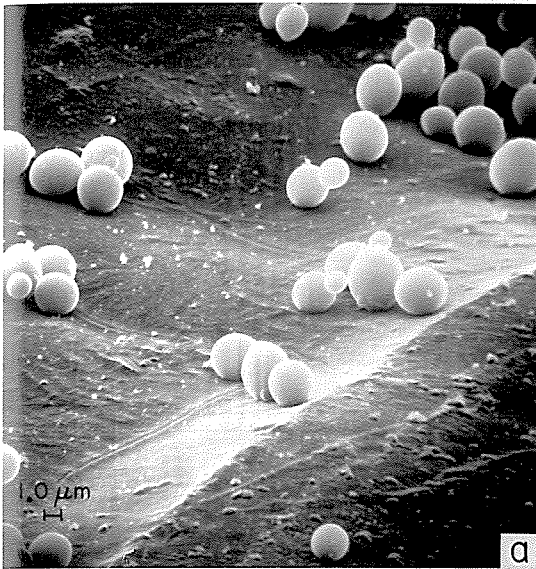


Figure 6-14

**Scanning electron micrographs of immobilized yeast cells
treated with hydroxyurea**

- (a) control population of yeast not treated with hydroxyurea, x2000;
(b) x1700; (c) x8000; and (d) x8000, cells after 8 hours of exposure
to hydroxyurea



6.2.3 Fermentation Characteristics

Sets of results for the initial trajectories of ethanol, glycerol, glucose, hydroxyurea and cell concentrations from a typical suspended cell fermentation and a typical immobilized cell fermentation, both with hydroxyurea, are reproduced in Figures 6-15, 6-16, 6-17 and 6-18.

In total, four suspended cell and four immobilized cell batch fermentations were carried out with hydroxyurea. The suspended cells data obtained from these experiments are listed in Tables IV-4 - IV-7 in Appendix IV, while the immobilized cell results may be found in Tables V-3 - V-6 in Appendix V.

Figure 6-15 shows ethanol and glycerol production and glucose consumption by hydroxyurea-treated suspended cells during a 3.67-hour batch experiment. Hydroxyurea concentration is also plotted to show that, for the duration of the fermentation, there was no apparent consumption of inhibitor by the cells. Suspended cell growth is represented by the data of Figure 6-16. The initial cell concentration was 2.4×10^9 cells/l.

The changes in ethanol, glycerol, hydroxyurea and glucose concentrations during a 3-hour immobilized cell batch fermentation are shown in Figure 6-17. Again, the concentration of hydroxyurea was essentially constant during the immobilized cell experiments. This result shows that neither consumption of inhibitor nor absorption into the gelatin coating was significant. Immobilized and suspended cell concentrations in the immobilized cell reactor are plotted in Figure 6-18. The total initial cell concentration for this experiment was 1.3×10^9 cells/l.

Product, substrate and cell concentration data from immobilized and suspended cell experiments with hydroxyurea were fitted to the equations developed in Chapter 5 (Section 5.1.3). The suspended cell model represented by

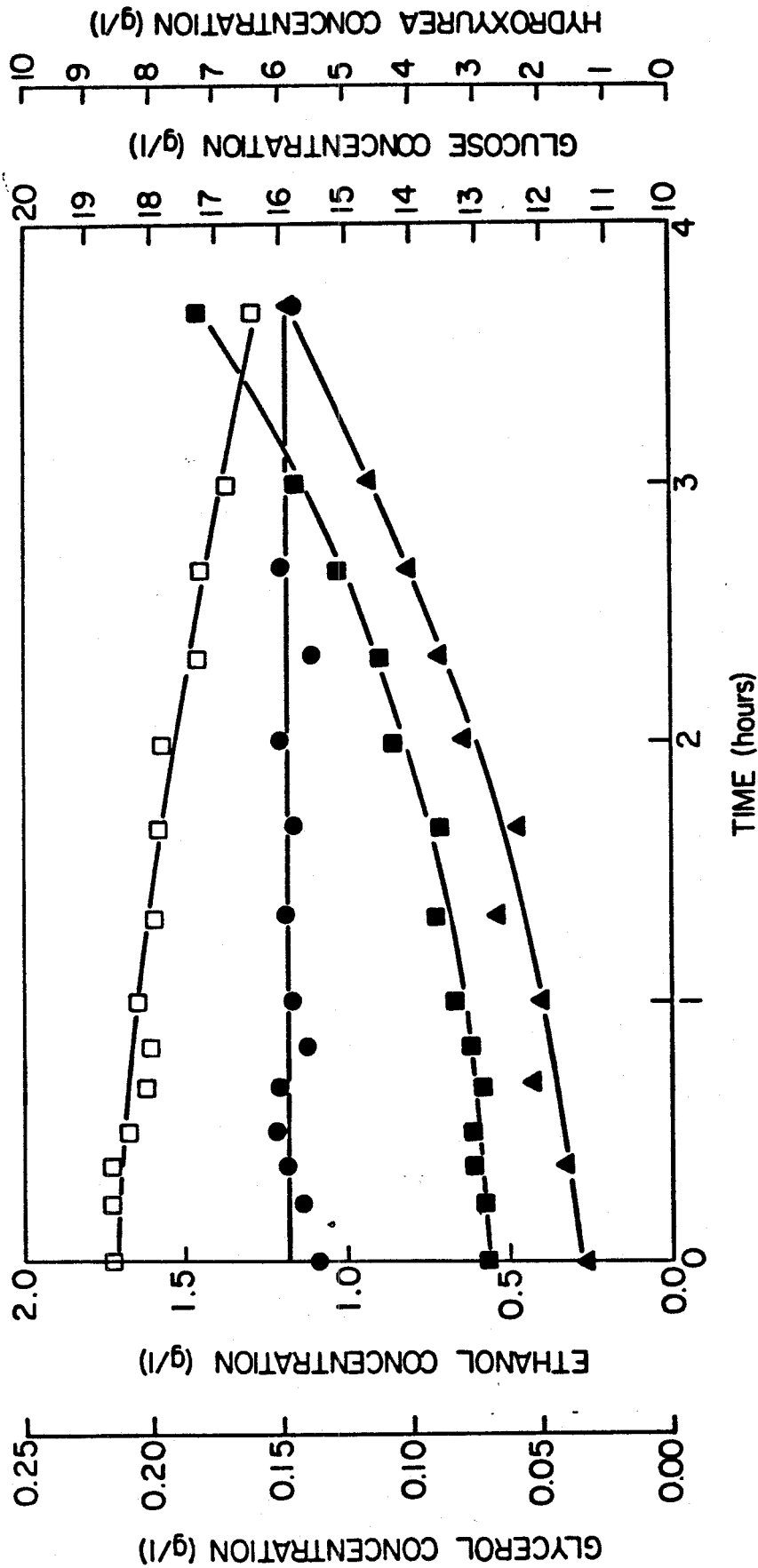


Figure 6-15
Concentration data from typical suspended cell batch fermentation with hydroxyurea showing accumulation of ethanol (■) and glycerol (▲) and disappearance of glucose (□). Hydroxyurea (●) was not consumed by the suspended cells. The initial suspended cell concentration in this experiment was 2.36×10^8 cells/l, and the initial specific ethanol productivity was 3.1×10^{-11} g/hr cell.

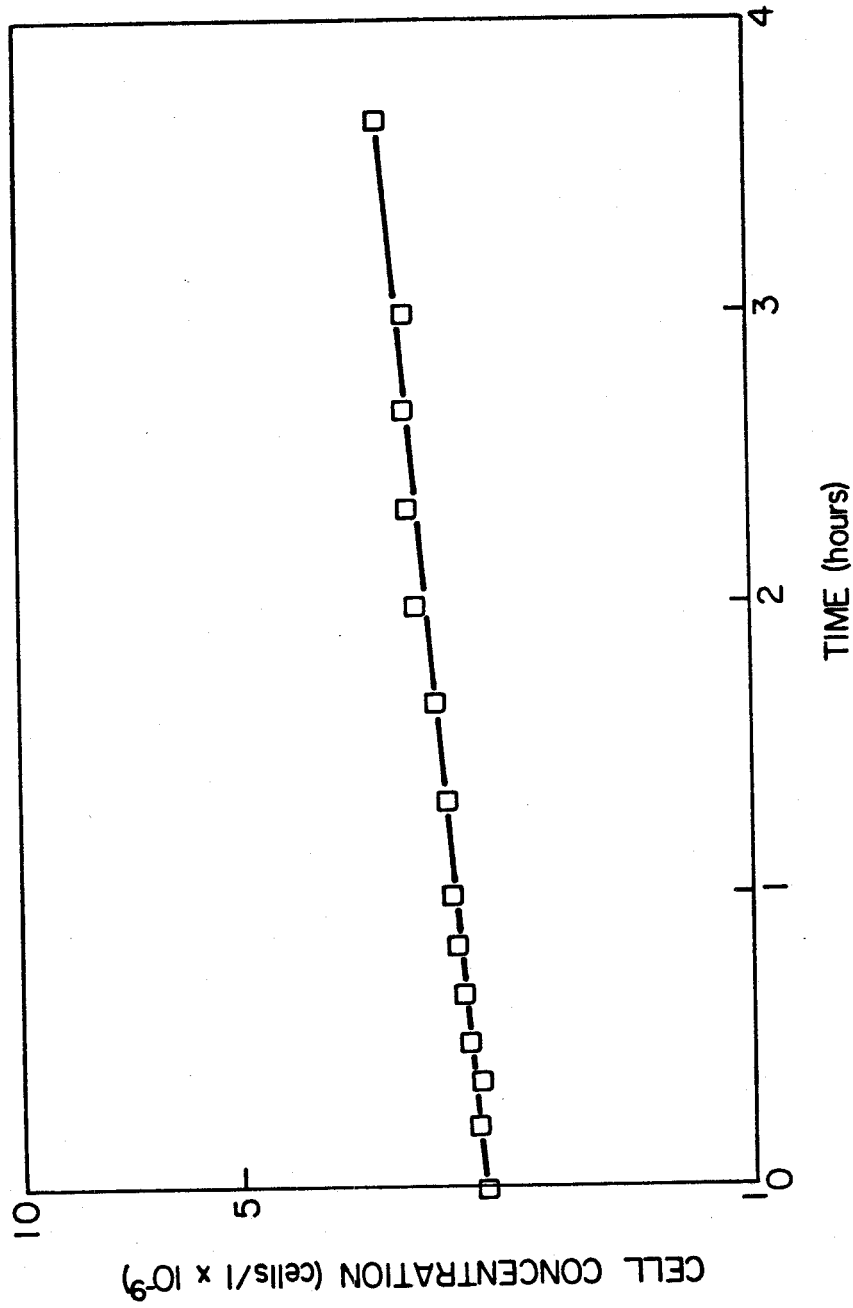


Figure 6-16
Cell concentration data from typical suspended cell batch fermentation with hydroxyurea. The specific growth rate was 0.08 hr^{-1} .

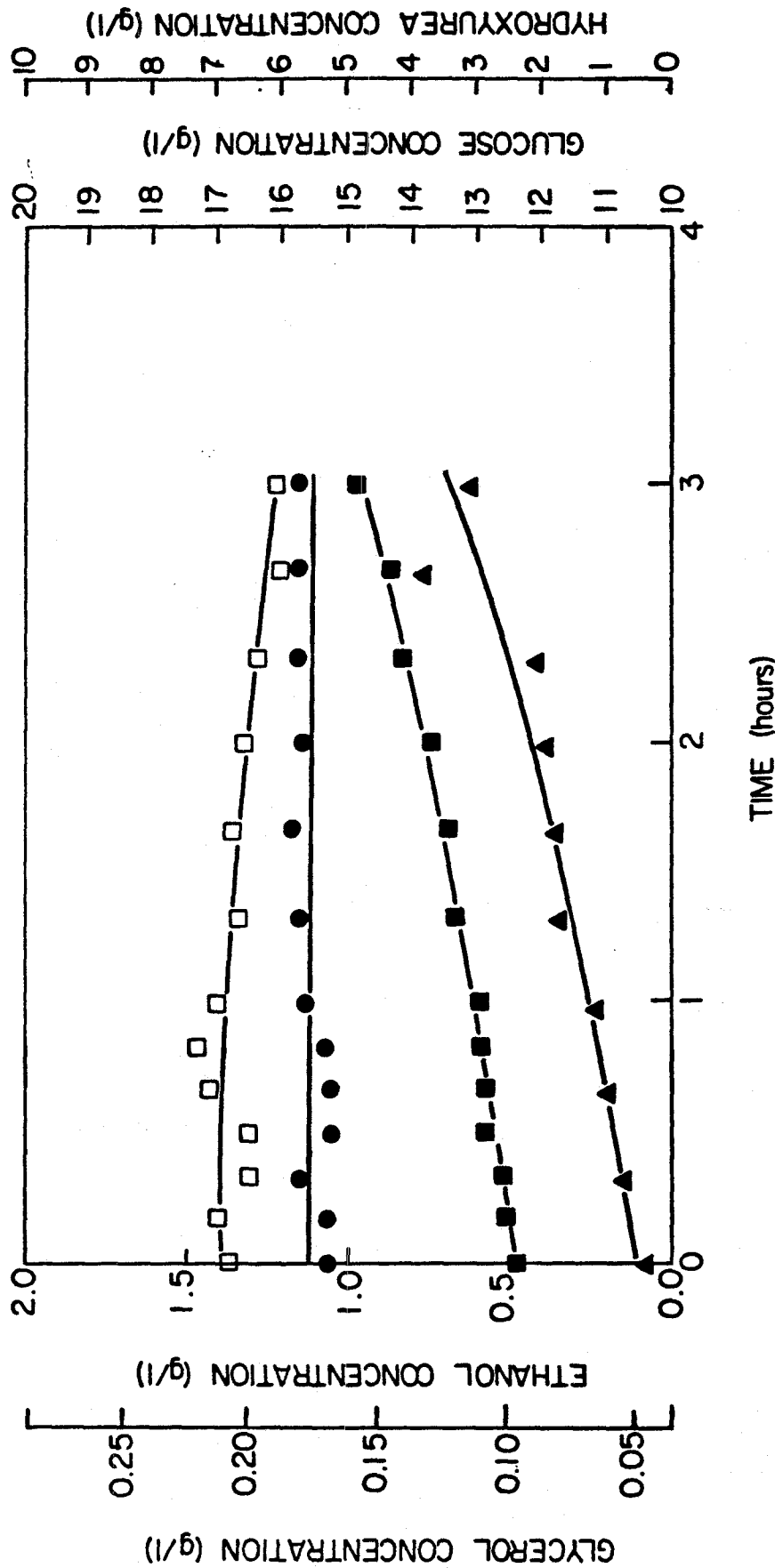


Figure 6-17
Concentration data from typical immobilized cell batch fermentation with hydroxyurea showing accumulation of ethanol (■) and glycerol (▲) and disappearance of glucose (□) during a 3-hour batch experiment. Hydroxyurea (●) was not consumed by the cells, nor was it absorbed by the gelatin. The total initial cell concentration for this experiment was 1.34×10^9 cells/l, and the initial specific ethanol productivity was 6.5×10^{-11} g/hr cell.

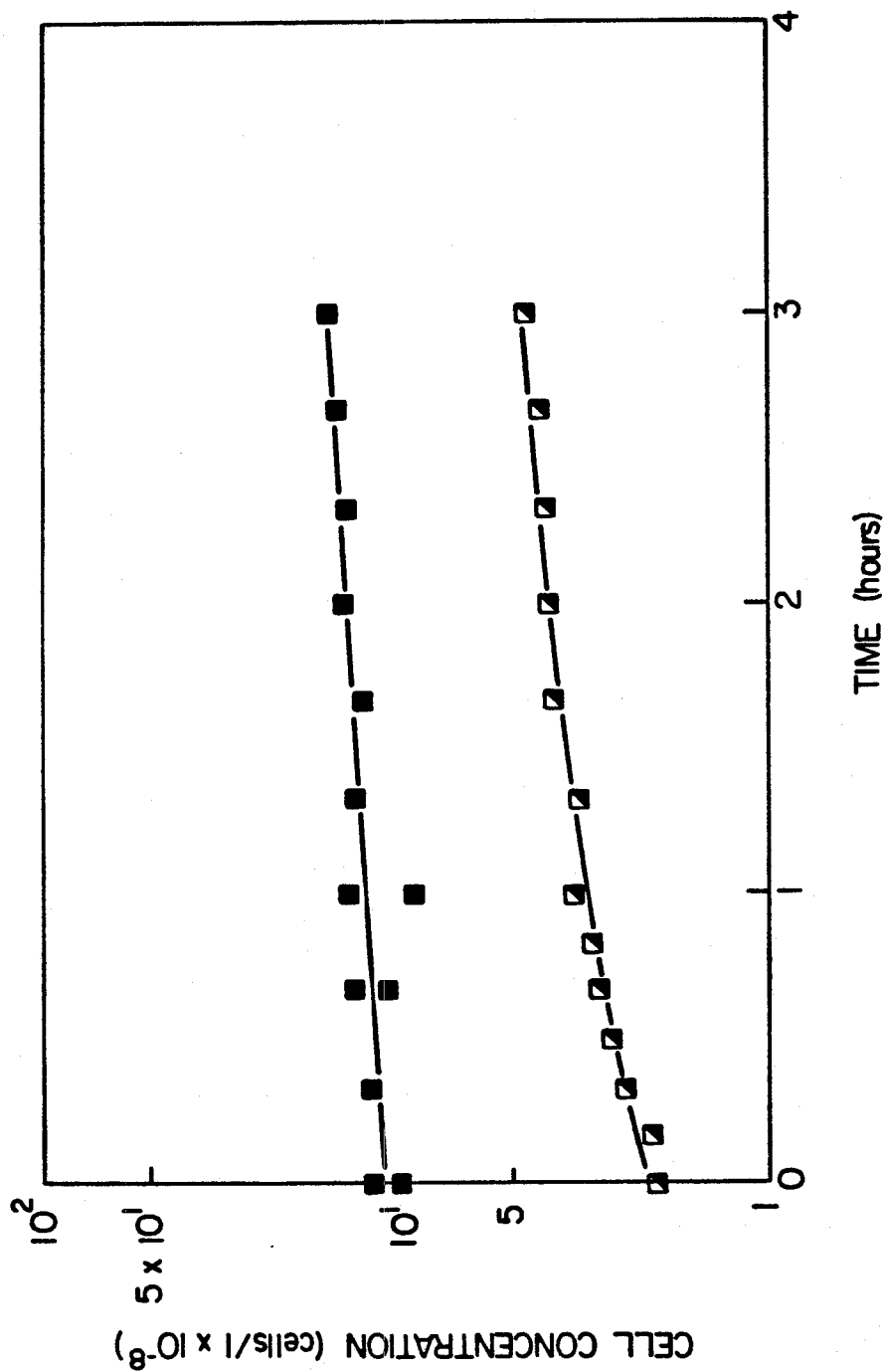


Figure 6-18
Cell concentration data from typical immobilized cell batch fermentation with hydroxyurea showing accumulation of both immobilized cells on the beads (■) and suspended cells in the recirculation medium (□). Release of immobilized cell daughters into the medium contributed to the increase in suspended cell concentration.

equations (5.1-27)-(5.1-29), and, for the immobilized cell fermentations, the set of equations, (5.1-35), (5.1-36), (5.1-38) and (5.1-39) were used. A Marquardt routine estimated the parameter values in these models to give the best fit of the equations to the data. Initial productivities and yields were calculated from the derivatives of each of the concentration trajectory equations at time zero.

Although, as discussed in Section 5.1.3, the models used for analysis of the immobilized and suspended cell fermentations are inadequate for proper treatment of unbalanced growth, the curves shown in Figures 6-15 - 6-18 show that the data fit resulting from application of these models is quite reasonable. Since the model equations are used principally for calculation of the initial slopes of these curves, the results should agree well with those obtained using any geometric method.

In the following discussion, the fermentation results are compared with some of those reported in Section 6.1.2 for cells without inhibitor.

6.2.3.1 *Suspended Cells*

The fermentation results for suspended cells are listed in Table 6-4. The four experiments with hydroxyurea were performed under essentially identical conditions.

Under the influence of hydroxyurea, the specific ethanol productivity of *Saccharomyces cerevisiae* was between 1.2 and 3.0 times higher than in the absence of inhibitor. Specific rates of glucose consumption 2.5 to 5.1 times faster than without hydroxyurea were observed.

In a suspended cell control experiment with 0.075M urea, an initial specific ethanol productivity of 2.1×10^{-11} g/hr cell was measured. This value is identical within experimental error to that obtained without inhibitor. Enhancement of

TABLE 6-4

**Batch Fermentation Results
for Suspended Cells with Hydroxyurea**

	Suspended Cells	Suspended Cells with Hydroxyurea			
initial specific ethanol productivity (g/hr cell x10 ¹¹)	2.3	2.7	3.1	7.0	4.8
initial specific glucose consumption (g/hr cell x10 ¹¹)	5.0	12.3	16.9	25.6	23.1
ethanol yield from glucose (mol/mol)	1.8	0.9	0.7	1.1	0.8
glycerol yield from glucose (mol/mol)	0.11	0.09	0.07	0.01	0.09
cell yield from glucose (cells/mol x10 ⁻¹¹)	18.1	1.9	0.9	0.7	0.9
μ_s (hr ⁻¹)	0.51	0.13	0.08	0.10	0.12

ethanol production with inhibited cells cannot, therefore, be due to the effects of the extra nitrogen in the medium represented by hydroxyurea, nor can it be due to the effects of any ionic, electrostatic or other properties shared by this analogue.

Although the rates of fermentation with hydroxyurea varied somewhat from experiment to experiment, there is a correlation between the changes in specific ethanol productivity and the corresponding values for substrate consumption rate. Improved ethanol production apparently follows from enhancement of glucose

uptake.

Difficulties were experienced in reproducing the initial rate results for suspended cell fermentation with hydroxyurea. Evidently, some factors which were uncontrolled in the experiments played a part in determining their outcome.

Ethanol yields were an average of 50% lower following hydroxyurea inhibition. Glycerol yields were also significantly reduced. As expected, growth of hydroxyurea-treated suspended cells was slower, at about one-fifth the rate without inhibitor. This level of residual cell division is higher than that expected after considering the initial effects of hydroxyurea on cell number shown in Figure 6-10a. Compared with the uninhibited culture, hydroxyurea-treated cell yields were lower, with less than one-tenth the amount of biomass produced per mole of glucose.

6.2.3.2 Immobilized Cells

The fermentation results from four repeated immobilized cell experiments with hydroxyurea are compared in Table 6-5 with the properties of immobilized cells tested without inhibitor.

There was a wide variation in specific ethanol productivity in the presence of hydroxyurea, with values ranging from 0.6 to 2.0 times the ethanol production rate for untreated immobilized cells. However, as in the suspended cell case, the changes in ethanol productivity which occurred from experiment to experiment were followed by the values for specific rate of glucose consumption.

In general, the ethanol yield for hydroxyurea-treated immobilized cells was not greatly different from that for immobilized cells without hydroxyurea, with an average reduction of only 16%. Cell yields and specific growth rates were lower in the immobilized cell experiments with inhibitor. In most cases, the suspended cells

TABLE 6-5
Batch Fermentation Results
for Immobilized Cells with Hydroxyurea

	Immobilized Cells	Immobilized Cells with Hydroxyurea			
initial specific ethanol productivity (g/hr cell $\times 10^{11}$)	3.3	2.1	4.5	3.3	6.5
initial specific glucose consumption (g/hr cell $\times 10^{11}$)	10.5	7.1	14.5	14.3	18.5
ethanol yield from glucose (mol/mol)	1.4	1.2	1.2	0.9	1.4
glycerol yield from glucose (mol/mol)	0.06	0.10	0.09	0.08	0.12
cell yield from glucose (cells/mol $\times 10^{-11}$)	5.3	1.7	0.7	1.2	1.5
μ_s (hr $^{-1}$)	0.50	0.12	0.003	0.004	0.004
μ_l (hr $^{-1}$)	0.28	0.06	0.07	0.10	0.18

in the immobilized cell reactor recirculation medium grew much slower and were presumably more affected by the inhibitor than the cells attached to the beads.

Without hydroxyurea, immobilized cells are slower growing and produce less biomass than suspended cells (Section 6.1.2.1). However, after exposure to hydroxyurea, the behaviors of both cell types with regard to frequency of budding and cell yield become very similar.

6.2.4 Polysaccharide Content

Results from measurements of the intracellular polysaccharide content of treated and untreated cells are shown in Table 6-6.

TABLE 6-6
Polysaccharide Contents
for Immobilized and Suspended Cells with Hydroxyurea

	Polysaccharide Content (g glucose equiv./cell $\times 10^{10}$)			
	Suspended Cells	Suspended Cells with Hydroxyurea	Immobilized Cells	Immobilized Cells with Hydroxyurea
trehalose (cold TCA fraction)	1.8	0.3	2.0	22
alkali soluble glycogen + mannan	4.6	2.0	29	37
acid soluble glycogen	4.1	1.8	26	20
glucan	6.7	1.8	11	11
TOTAL	17	5.9	67	89

During analysis of cell number, hydroxyurea-inhibited yeast doublets were counted as two cells, so that the results here reflect the polysaccharide content per cell subunit.

Suspended cells incubated with hydroxyurea contained only one-third the amount of polysaccharide found in untreated cells. Both storage reserves and lev-

els of cell wall material were depleted after exposure to hydroxyurea. In contrast, the immobilized cells treated with hydroxyurea contained more storage carbohydrate than those without hydroxyurea, although there was approximately the same level of structural polysaccharide in their cell walls.

Hydroxyurea-treated immobilized cells contained about 15 times more storage and structural compounds than did the inhibited suspended cells. The differences in polysaccharide content between untreated immobilized and suspended cells shown in Table 6-2 were increased further following hydroxyurea inhibition.

6.2.5 Flow Cytometry Results

The results from measurements of cell size and intracellular levels of DNA, RNA and protein for hydroxyurea-treated suspended and immobilized cells are considered below. Many of the data already reported in Section 6.1.4 for uninhibited yeast are used for comparison in this analysis. The complete set of results for mean fluorescence and light scatter is given in Table 6-7.

6.2.5.1 DNA and Small-Angle Scatter

DNA and light scatter frequency functions for suspended cells cultured with and without hydroxyurea are shown in Figures 6-19, 6-20 and 6-21. The results from these measurements for immobilized cells are shown in Figures 6-22, 6-23 and 6-24.

(a) *Suspended Cells*: The DNA results for suspended cells (Figure 6-19) illustrate the principal effect of hydroxyurea on the cell cycle of *Saccharomyces cerevisiae*. Compared with the bimodal distribution for untreated cells, the frequency function for the hydroxyurea-inhibited population shows that hydroxyurea causes accumulation of cells in the S-phase between the two peaks with quantities of DNA between

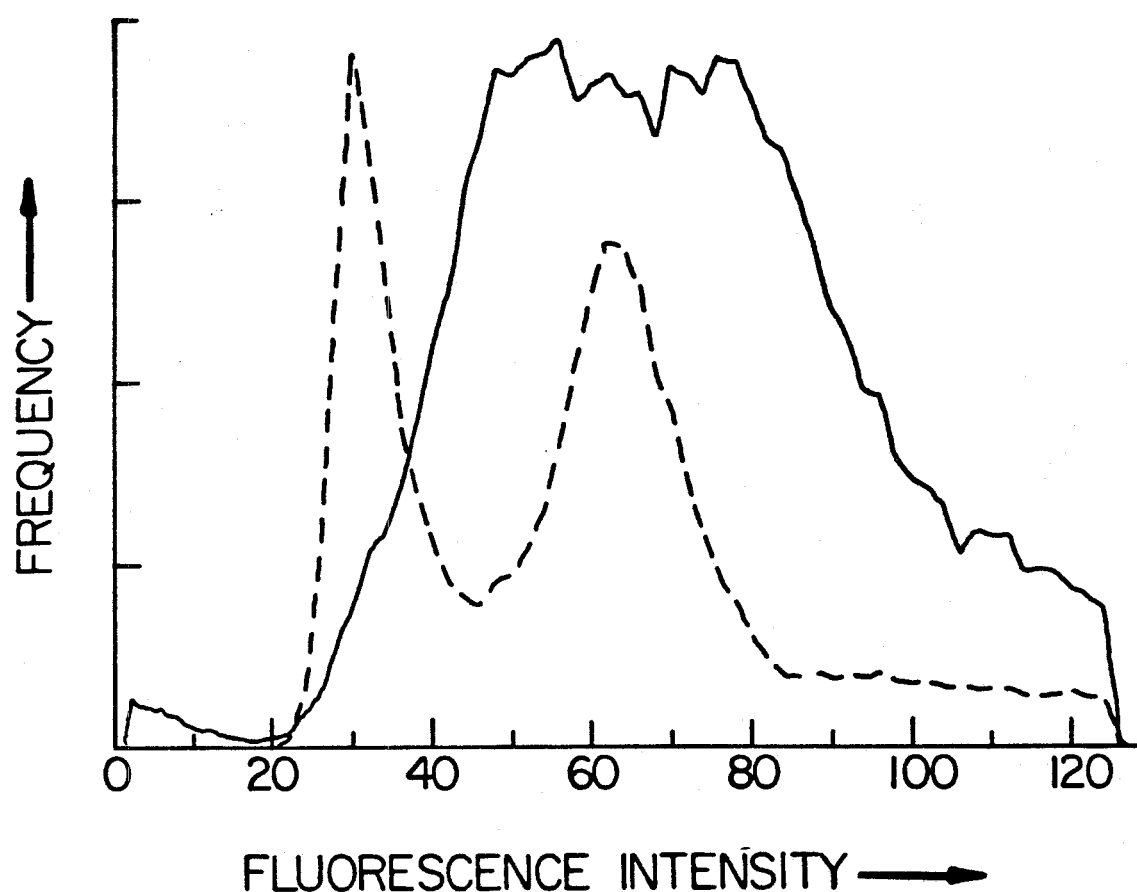


Figure 6-19

DNA frequency functions for suspended cells with (—) and without (---) hydroxyurea. The frequency, or relative number of cells counted, is plotted as a function of DNA fluorescence. The quantities of DNA corresponding to one and two diploid yeast genomes are indicated by the two modes of the suspended cell histogram. The population of hydroxyurea-treated *S. cerevisiae* contains cells arrested in DNA synthesis with between one and two sets of chromosomes. Many of the treated cells have proceeded with DNA synthesis without prior cell division and contain more than two genomes.

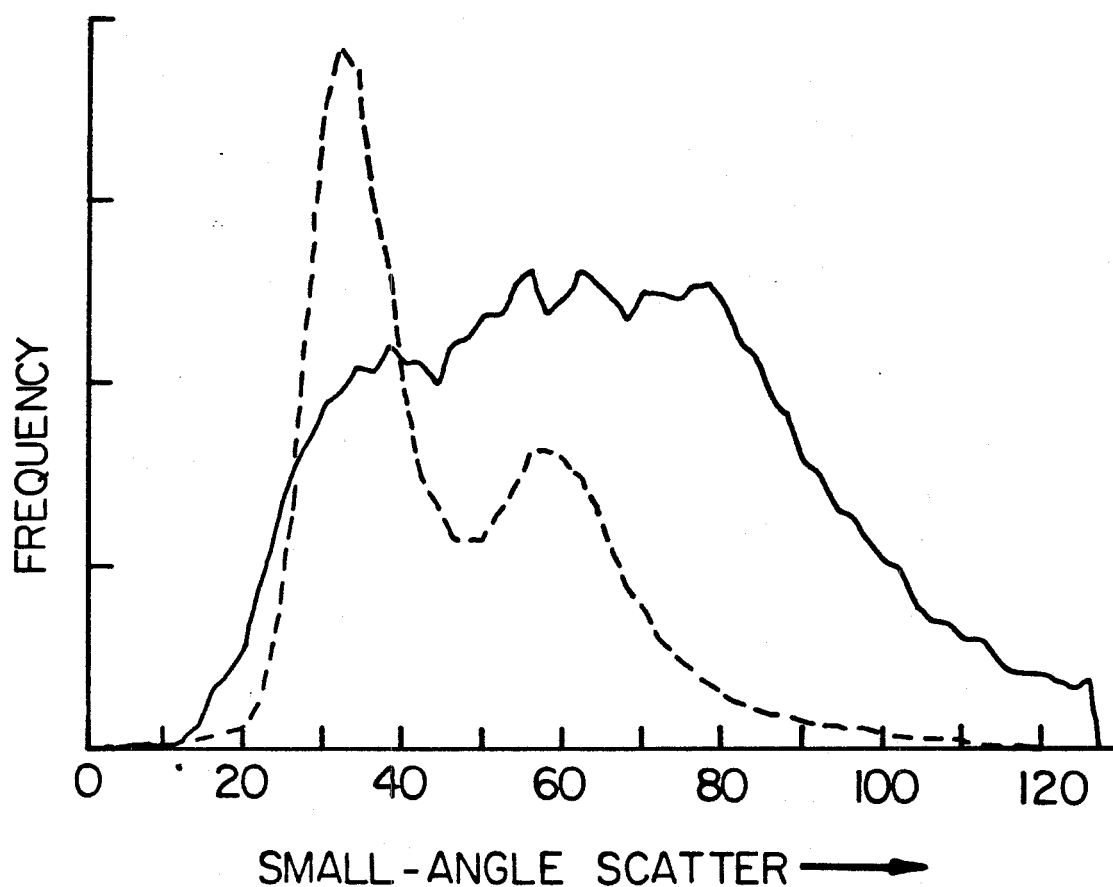


Figure 6-20

Small-angle light scatter frequency functions for suspended cells with (—) and without (---) hydroxyurea. The frequency, or relative number of cells counted, is plotted as a function of the level of small-angle light scatter detected. Light scatter increases with increasing cell size. Hydroxyurea-treated suspended cells scatter light to approximately the same extent as do budded cells in the control population.

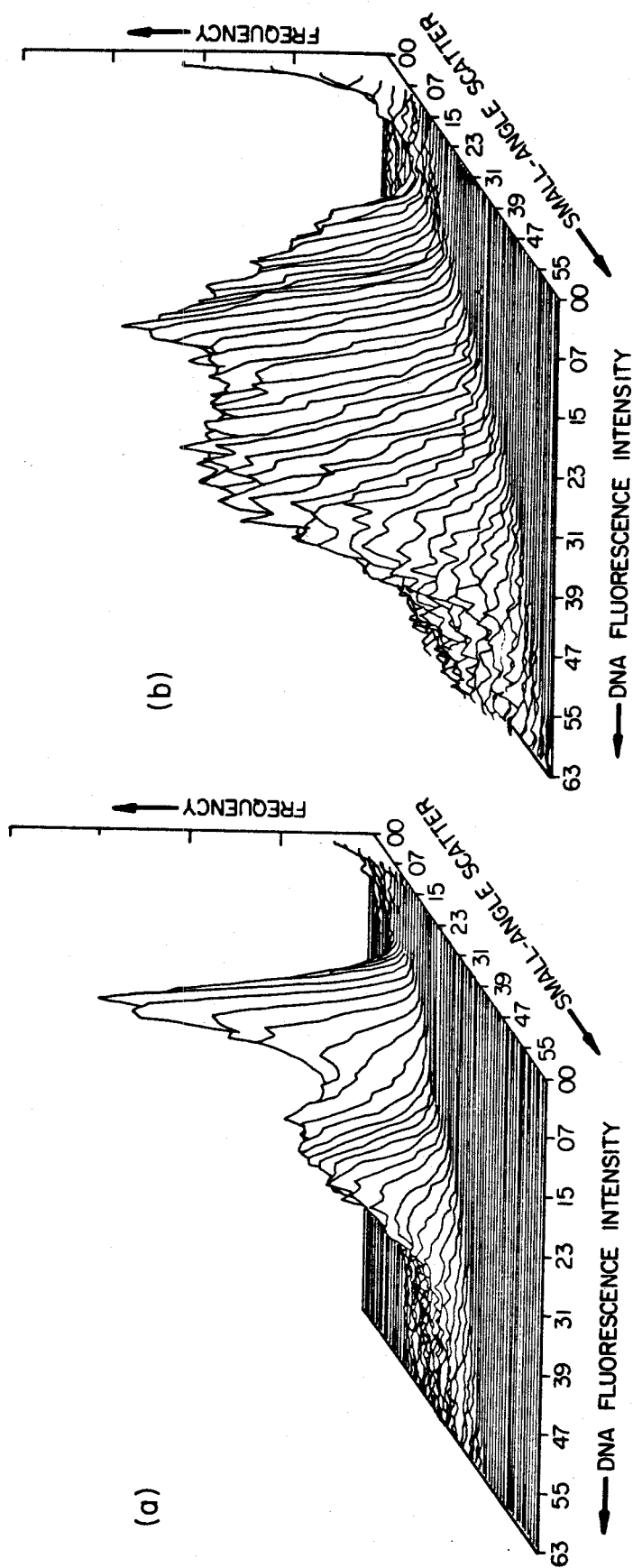


Figure 6-21
DNA fluorescence-light scatter frequency functions for suspended cells (a) without hydroxyurea; and (b) with hydroxyurea. The frequency or relative number of cells which display a particular combination of DNA fluorescence and light scatter levels is plotted vertically for each population. The larger cells of the hydroxyurea-treated population contain the higher levels of DNA.

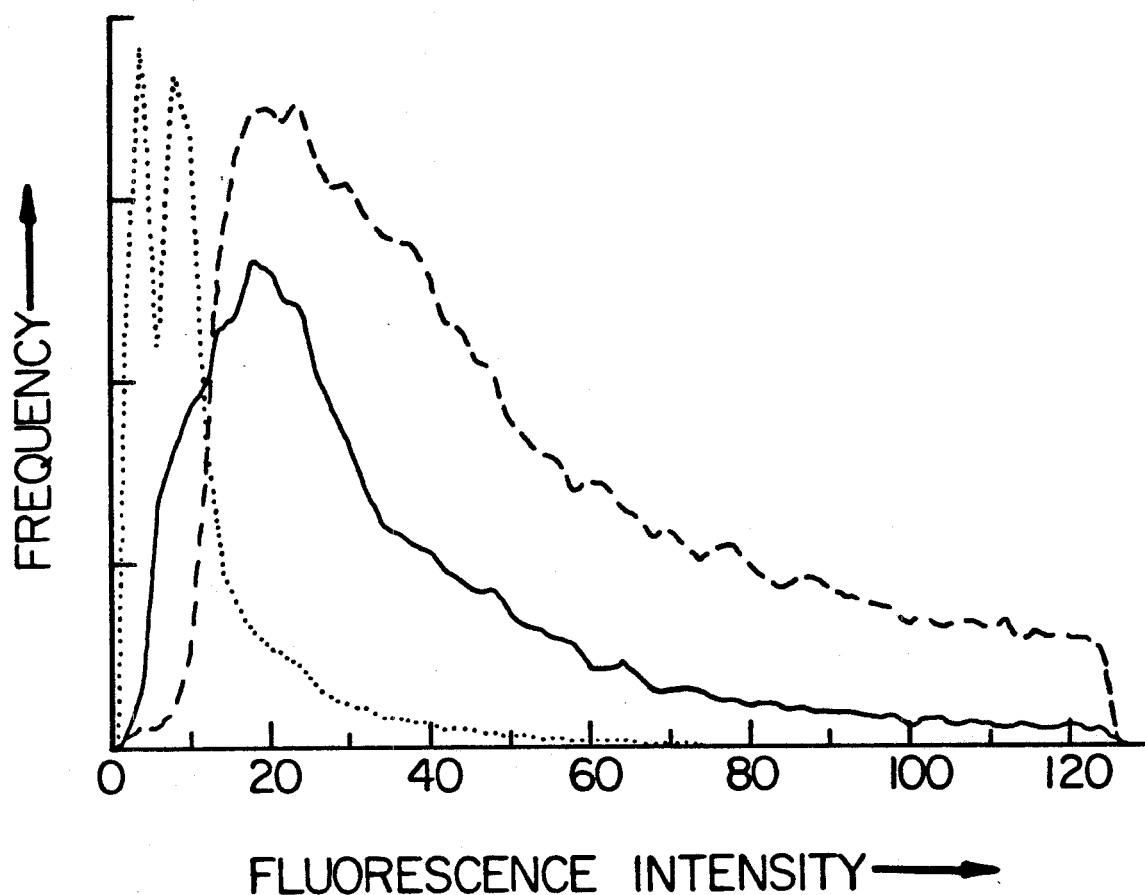


Figure 6-22

DNA frequency functions for immobilized cells with (—) and without (---) hydroxyurea. The frequency, or relative number of cells counted, is plotted for each level of DNA fluorescence. The DNA frequency function for untreated suspended cells (.....) gives an indication of the extent to which the fluorescence scale has been compressed in order to fit the immobilized cell curves into the diagram. Both treated and untreated immobilized cells contain significantly more DNA than suspended cells.

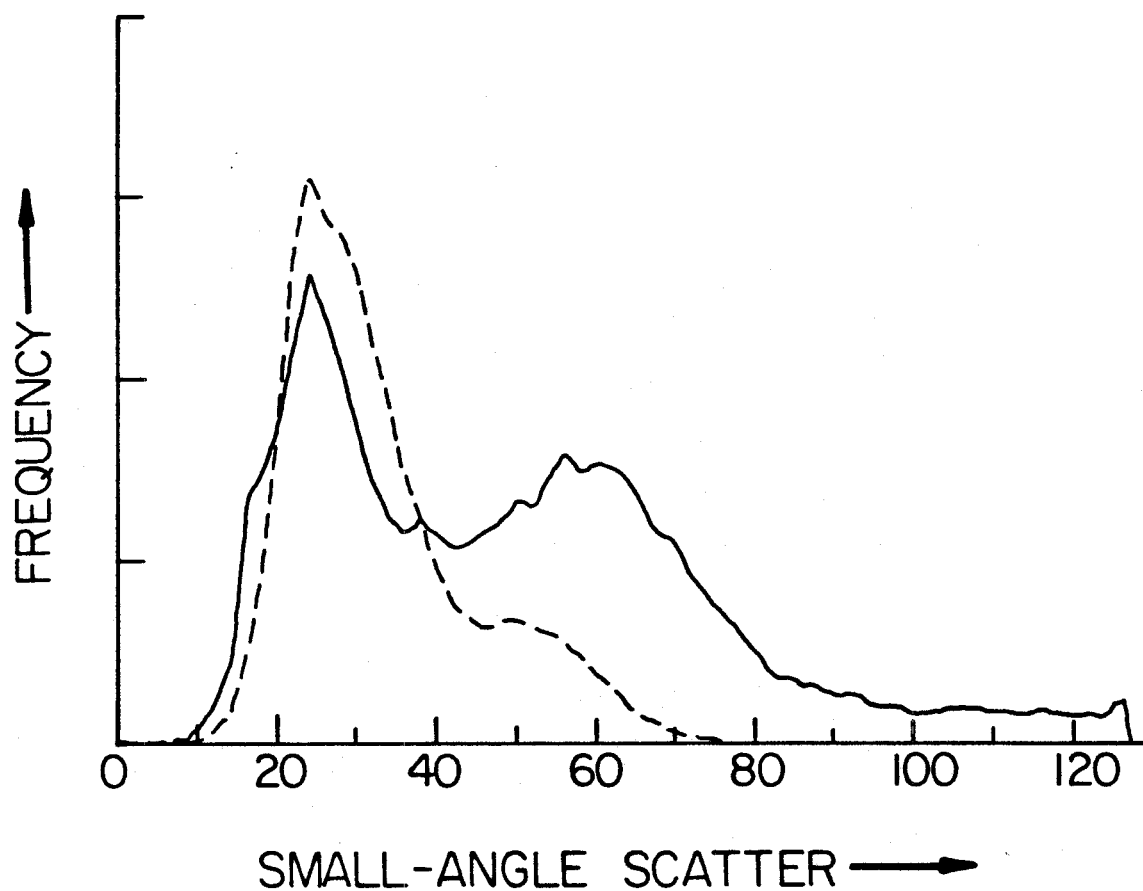


Figure 6-23

Small-angle light scatter frequency functions for immobilized cells with (—) and without (---) hydroxyurea. The frequency, or relative number of cells counted, is plotted as a function of the level of light scatter detected. Light scatter increases with increasing cell size. The hydroxyurea-treated population of immobilized cells contains a higher proportion of budded yeast than does the untreated population.

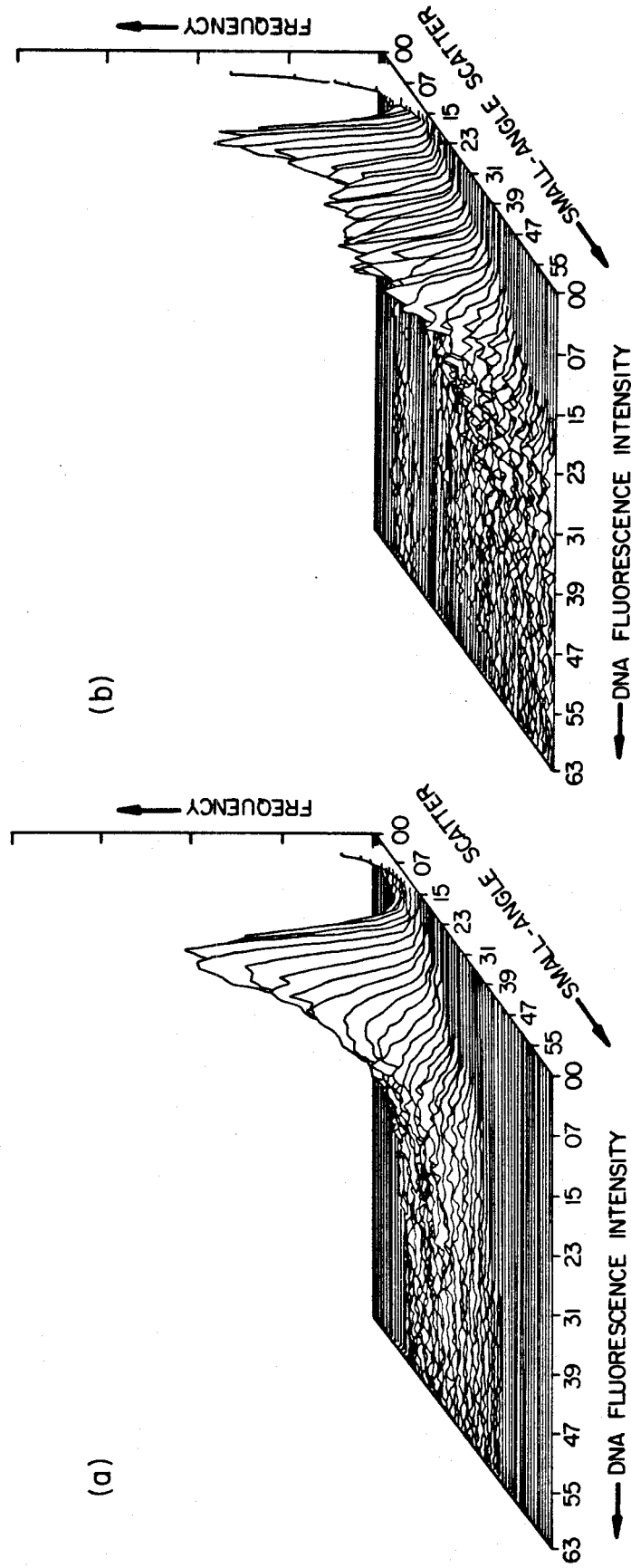


Figure 6-24
DNA fluorescence-light scatter frequency functions for immobilized cells (a)
without hydroxyurea; and (b) with hydroxyurea. The frequency or relative number
of cells which display a particular combination of DNA fluorescence and light
scatter levels is plotted vertically for each population.

TABLE 6-7

**Mean Values of Fluorescence and Light Scatter Frequency Functions
for Immobilized and Suspended Cells with Hydroxyurea**

	Frequency Function Mean Values			
	Suspended Cells	Suspended Cells with Hydroxyurea	Immobilized Cells	Immobilized Cells with Hydroxyurea
DNA fluorescence	13.0	15.2	49.4	33.9
RNA fluorescence	35.8	52.3	8.2	13.6
protein fluorescence	33.6	58.9	21.8	55.6
small-angle scatter	47.0	64.0	33.4	48.9

one and two genomes. The measurements also indicate that many cells have proceeded with a further round of DNA synthesis and contain more DNA than that found in G2- and M- phase yeast with fully replicated chromosomes.

The mean scatter level for the inhibited cells (Figure 6-20 and Table 6-7) is higher than that for the control population, and in addition, is slightly greater than the scatter levels characteristic of untreated budded cells before cell separation. These results are consistent with the terminal morphology of inhibited cells shown in Figure 6-13, since many treated cells possess unusually large buds at least the size of the mother cell. The high levels of light scatter suggest also that chains of three or more cells may be found in the hydroxyurea-inhibited population. Interpretation of the light scatter measurements for treated and untreated

cells is subject to the caveat that the shapes of these cells are quite different, and this may influence comparison between the two populations.

The relationship between DNA fluorescence and light scatter level for treated and untreated suspended cells is illustrated in Figure 6-21. In general, DNA content increases with cell size in roughly a linear fashion. Cells with between two and three genomes contributing to the right-hand tail of Figure 6-19 are also likely to be those possessing more than one or a very large bud. This suggests that, if cells treated with hydroxyurea are able to complete replication, DNA synthesis may proceed without prior or immediate separation of bud from parent cell.

(b) *Immobilized Cells*: The DNA frequency functions for immobilized *S. cerevisiae* cells with and without hydroxyurea (Figure 6-22) show that the response of immobilized cells to the inhibitor is quite different from that of cells in suspension. The immobilized cell histograms are given along with the frequency function for suspended cells without inhibitor as a control. Fluorescence intensity is scaled differently in Figures 6-19 and 6-22. This change was necessary in order to fit both the suspended cell and immobilized cell DNA functions into the picture.

As discussed in Section 6.1.4.1, untreated immobilized cells contain many times the amount of DNA corresponding to one diploid yeast genome, which is represented by the first peak of the suspended cell histogram. In the experiments with hydroxyurea-treated immobilized cells, loading of yeast onto the support beads commenced at the same time as hydroxyurea addition (Section 4.6.2.3). This means that after 2-3 hours, DNA replication should have been halted, both in the newly immobilized cells and in the inoculum culture. However, the DNA histogram for hydroxyurea-treated immobilized cells shows that many of these cells, 8 hours after exposure to the inhibitor, contain much more than 2 or even 3 times the normal diploid quantity of genetic material. In fact, the DNA distribution for

immobilized cells with hydroxyurea is not very different from that for immobilized cells without inhibitor. Hydroxyurea affects the immobilized cells to give a lower proportion of cells with very large DNA fluorescence levels, and to give more cells in the 2-4 genome range. These effects, though, are minor compared with the general result that immobilized cells are very effective at escaping the hydroxyurea block on DNA synthesis.

The light scatter frequency functions for immobilized cells with and without inhibitor are given in Figure 6-23. Analysis of these histograms, assuming that the second peak represents only those cells with buds, shows that in the hydroxyurea-treated immobilized cell population, 57% of cells are budded, whereas this proportion is much smaller at about 22% for untreated immobilized yeast.

This result confirms earlier conclusions drawn from the scanning electron micrographs of Figure 6-14 that hydroxyurea treatment leads to the accumulation of budded cells on the gelatin surface. It also implies that, in terms of preventing cytokinesis and cell separation, hydroxyurea inhibition does influence the immobilized yeast cell cycle, although DNA synthesis seems relatively unaffected. This apparent contradiction with regard to the response of immobilized cells to hydroxyurea is highlighted by the fact that, in the hydroxyurea-treated immobilized cell population, the cells whose DNA levels are lower than those found in uninhibited cells and whose DNA synthesis seems to be most effectively blocked by hydroxyurea are the small, single cells without buds. This is clear from examination of the DNA frequency function plot of Figure 6-24.

6.2.5.2 RNA and Protein

Results from suspended cell RNA and protein measurements are plotted in Figures 6-25 and 6-26. The relationships between cellular RNA and protein contents and light scatter levels for populations of inhibited and uninhibited

suspended cells are given in Figures 6-27 and 6-28.

RNA and protein frequency functions for immobilized cells appear in Figures 6-29 and 6-30. The macromolecular contents of cells of varying sizes are indicated in Figures 6-31 and 6-32.

(a) *Suspended Cells*: The frequency functions for stable double-stranded RNA and protein (Figures 6-25 and 6-26) and the mean values of stable RNA and protein fluorescence (Table 6-7) verify that hydroxyurea-treated cells continue to synthesize these macromolecules even though DNA replication is inhibited.

Since the cell cycle is blocked before cell separation, continued production of these substances can be expected to produce hydroxyurea-inhibited yeast with higher specific levels of RNA and protein compared with untreated cells. Figures 6-27 and 6-28 show, in fact, that treated cells do contain higher levels of these macromolecules than untreated cells of the same size.

(b) *Immobilized Cells*: In the absence of inhibitor, immobilized cells contain less than one-fourth the amount of RNA found in suspended yeast (Section 6.1.4.2). Although the results of Figure 6-29 and Table 6-7 indicate that hydroxyurea-treatment causes an increase in the average stable RNA level, from Figure 6-31, the larger cells produced after hydroxyurea treatment do not contain substantially greater amounts of the substance.

This is in contrast with the results for protein shown in Figure 6-32, from which it is obvious that hydroxyurea-treated immobilized cells produce proteins in amounts which even exceed those expected from their increased size. In terms of protein synthesis, immobilized cell behavior in the presence of hydroxyurea is similar to that of suspended cells.

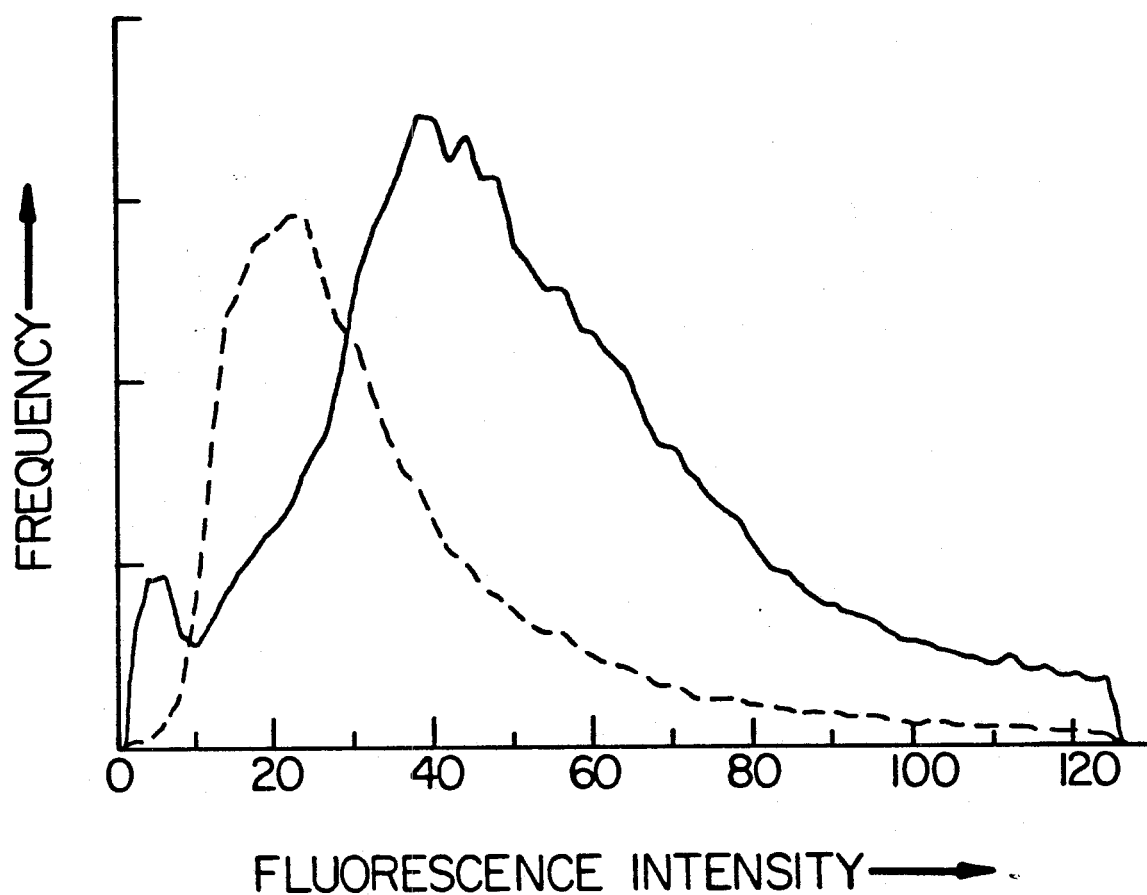


Figure 6-25

Stable double-stranded RNA frequency functions for suspended cells with (—) and without (---) hydroxyurea. The frequency, or relative number of cells counted, is plotted as a function of stable RNA fluorescence. RNA synthesis continues in hydroxyurea-treated cells in the absence of both DNA replication and cell division.

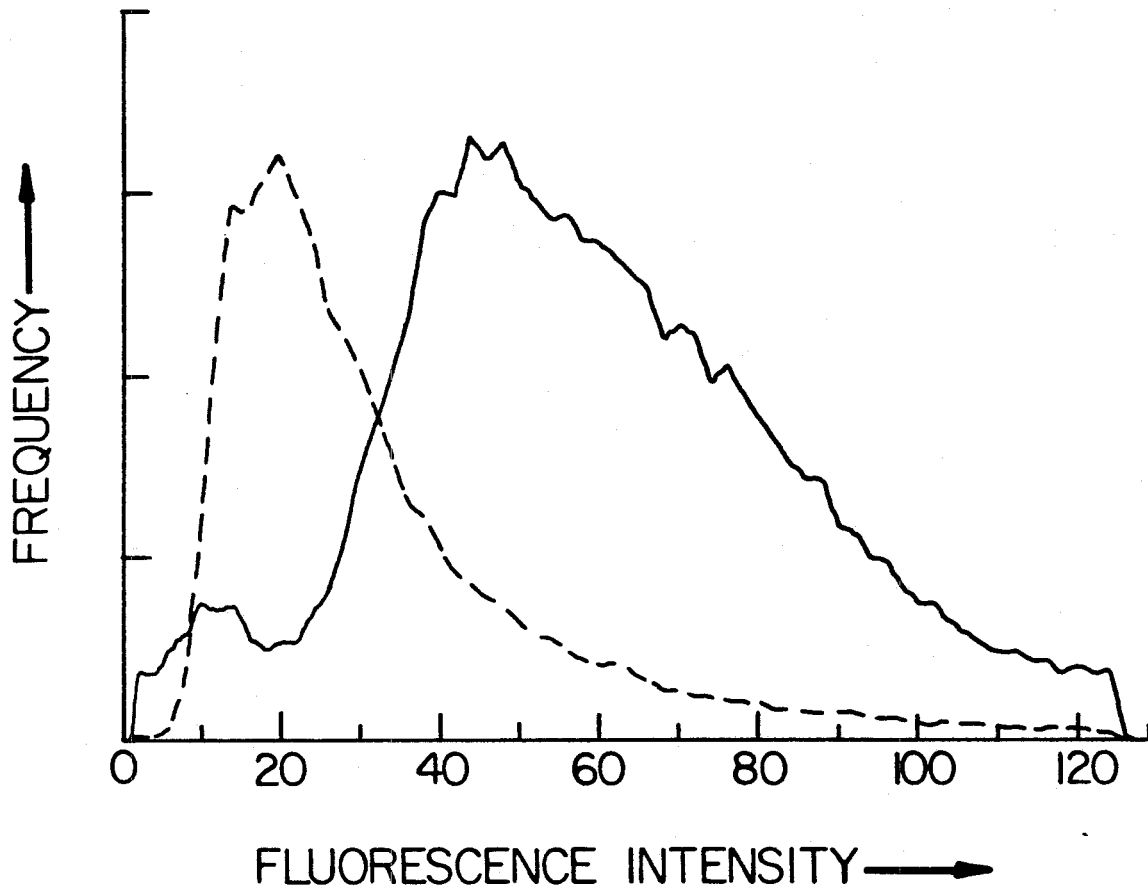


Figure 6-26

Protein frequency functions for suspended cells with (—) and without (---) hydroxyurea. The frequency, or relative number of cells counted, is plotted versus stained protein fluorescence intensity. Protein synthesis continues in hydroxyurea-treated cells in the absence of DNA replication and cell division.

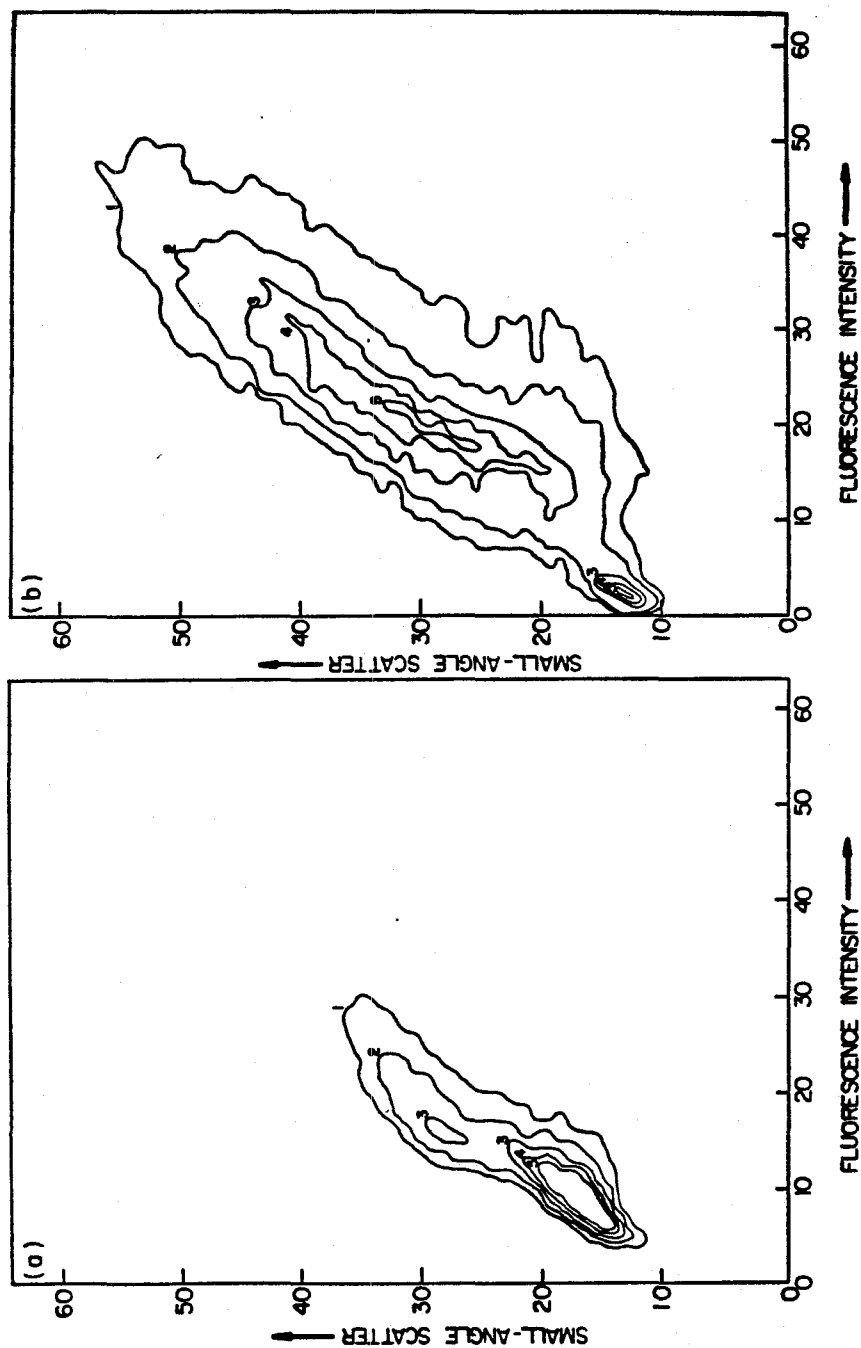


Figure 6-27
Relationship between stable double-stranded RNA fluorescence and light scatter level for suspended cells (a) without hydroxyurea; and (b) with hydroxyurea. The numbers 1-5 indicate the relative frequency or cell count measured for the particular combinations of light scatter and stable RNA fluorescence enclosed by the contour lines.

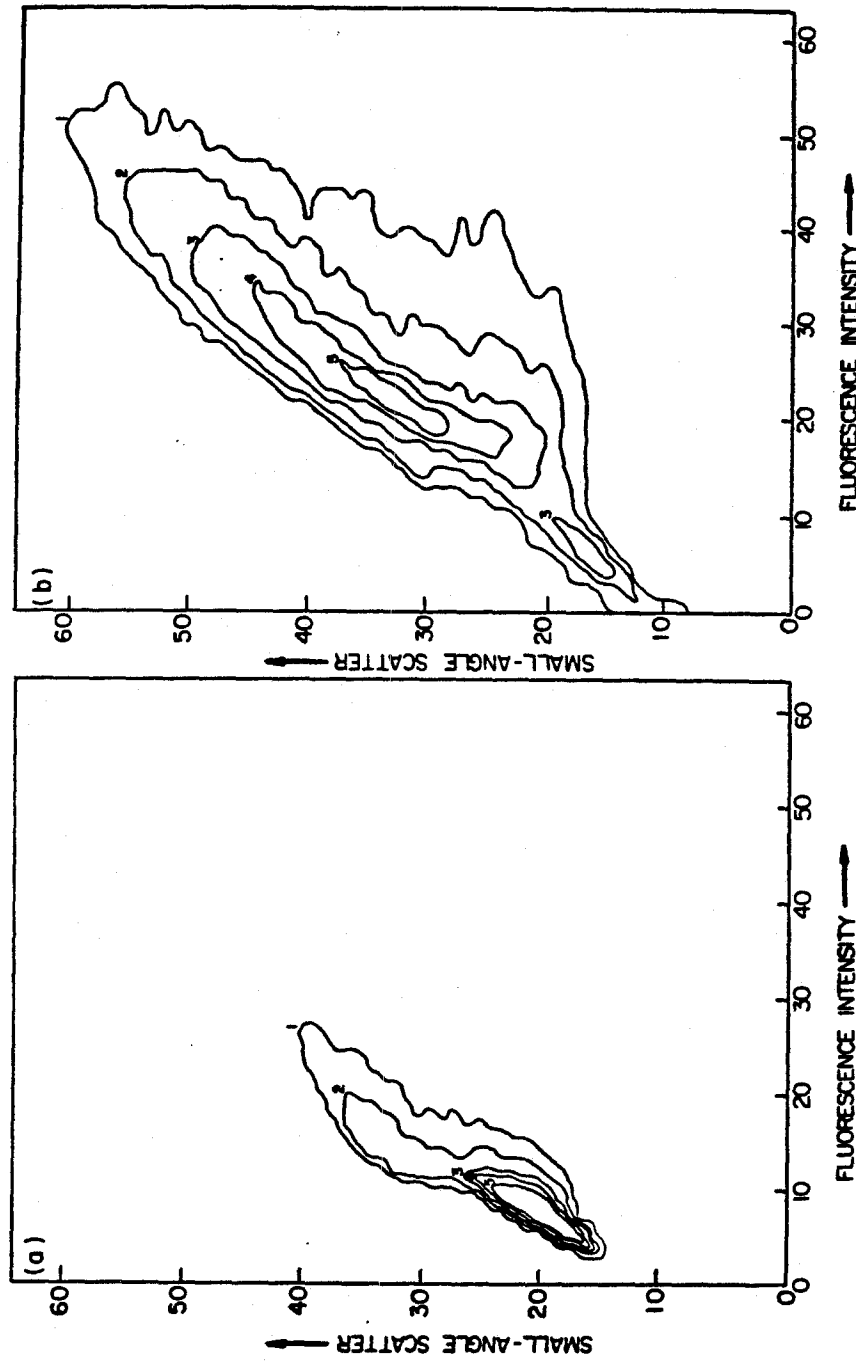


Figure 6-28
Relationship between protein fluorescence and light scatter level for suspended cells (a) without hydroxyurea; and (b) with hydroxyurea. The numbers 1-5 indicate the relative frequency or cell count measured for the particular combinations of light scatter and protein fluorescence enclosed by the contour lines. The hydroxyurea-treated cells contain even more protein than that expected from their increased size.

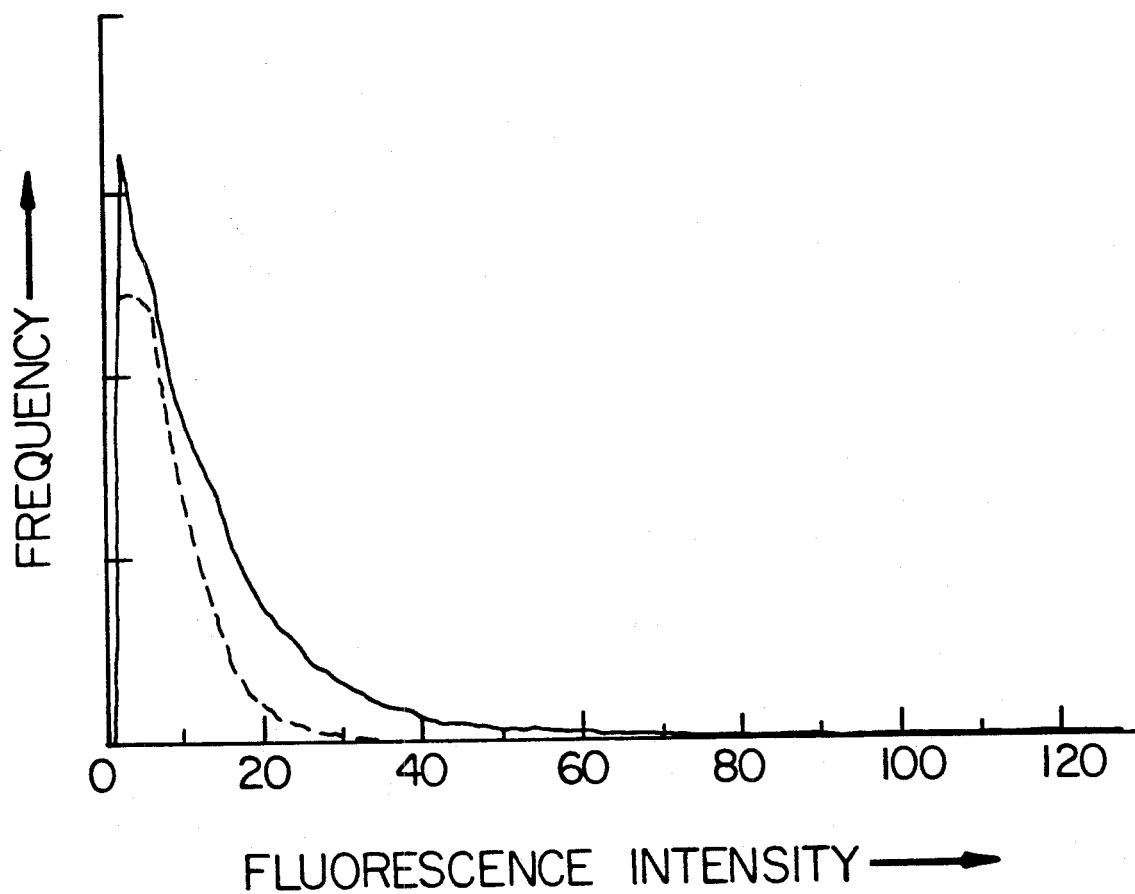


Figure 6-29

Stable double-stranded RNA frequency functions for immobilized cells with (—) and without (---) hydroxyurea. The frequency, or relative number of cells counted, is plotted as a function of stable RNA fluorescence.

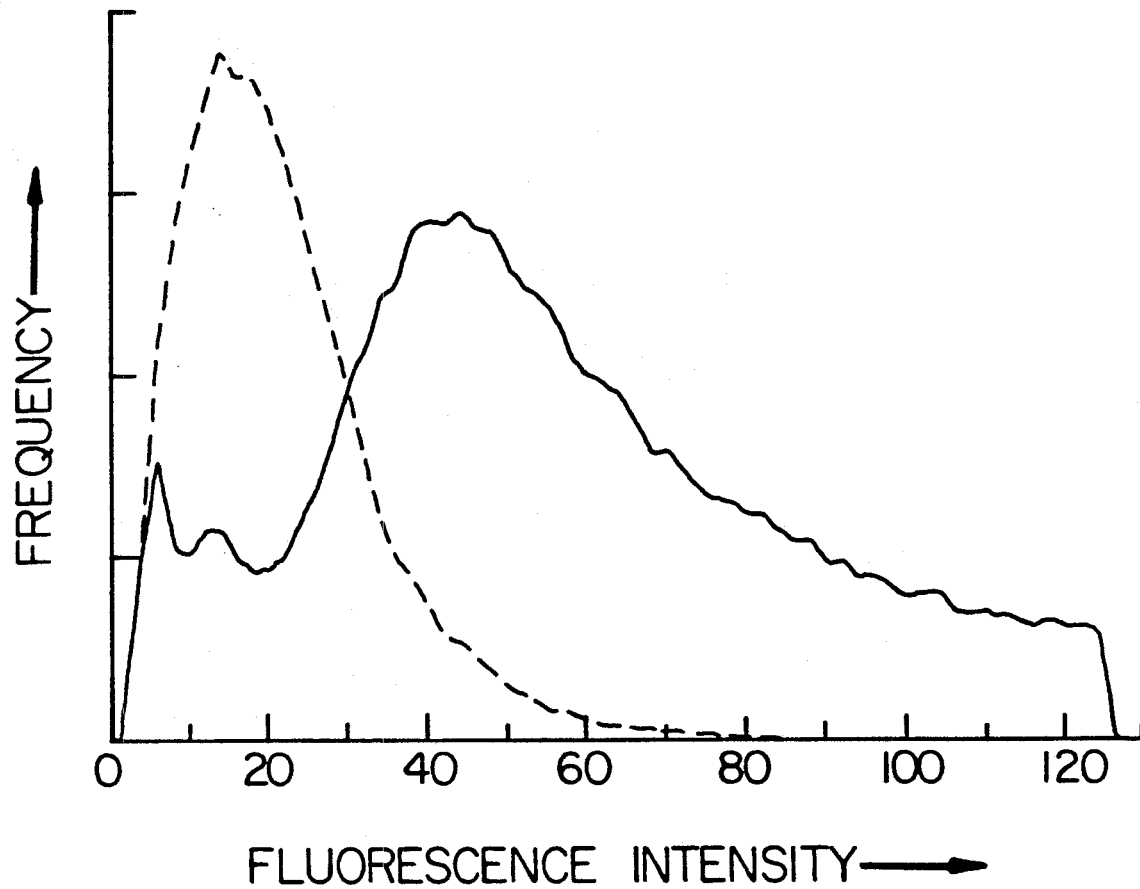


Figure 6-30

Protein frequency functions for immobilized cells with (—) and without (---) hydroxyurea. The frequency, or relative number of cells counted, is plotted versus stained protein fluorescence intensity.

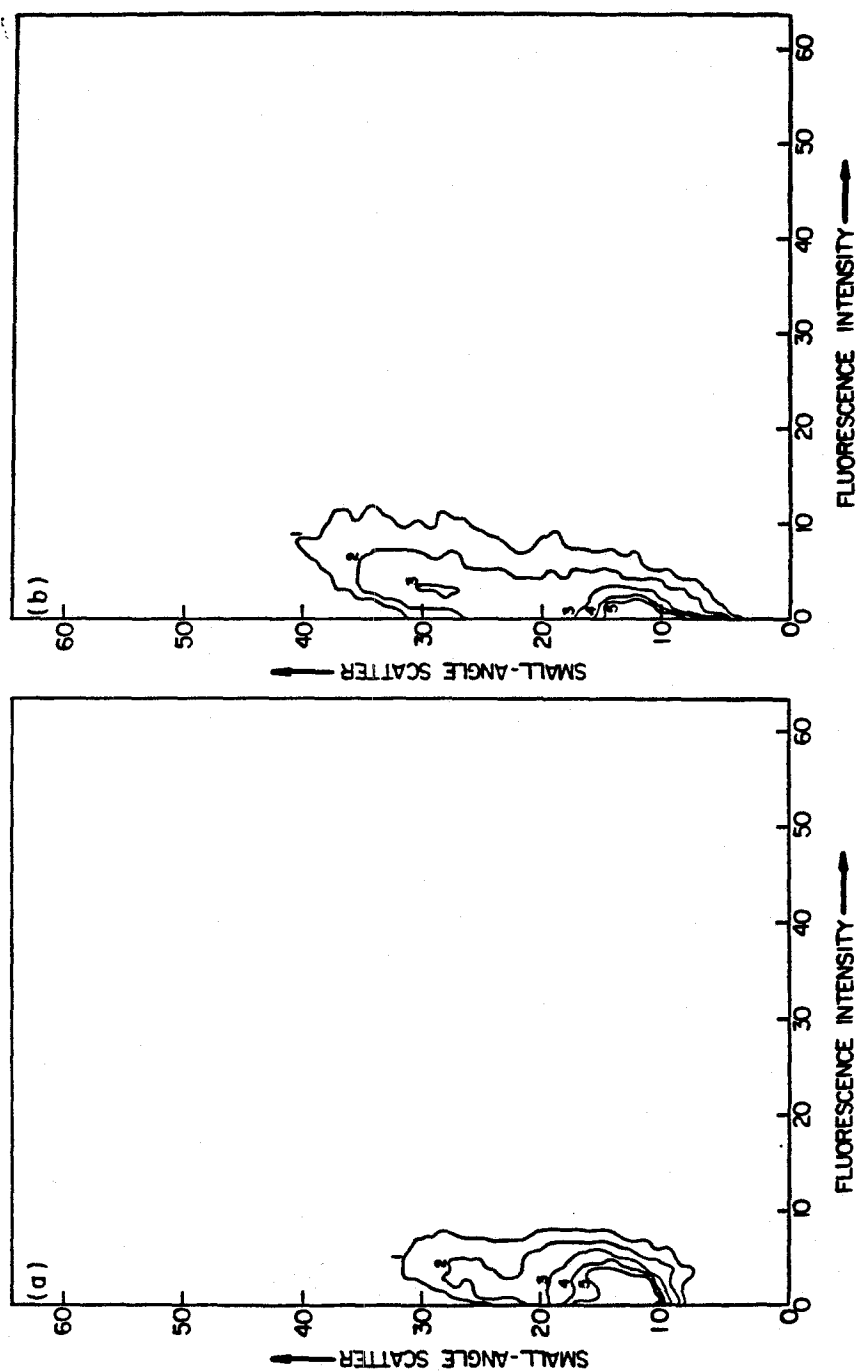


Figure 6-31
Relationship between stable double-stranded RNA fluorescence and light scatter level for immobilized cells (a) without hydroxyurea; and (b) with hydroxyurea.
 The numbers 1-5 indicate the relative frequency or cell count measured for the particular combinations of light scatter and stable RNA fluorescence enclosed by the contour lines. Hydroxyurea-treated immobilized cells do not accumulate RNA, even though the average cell size is larger.

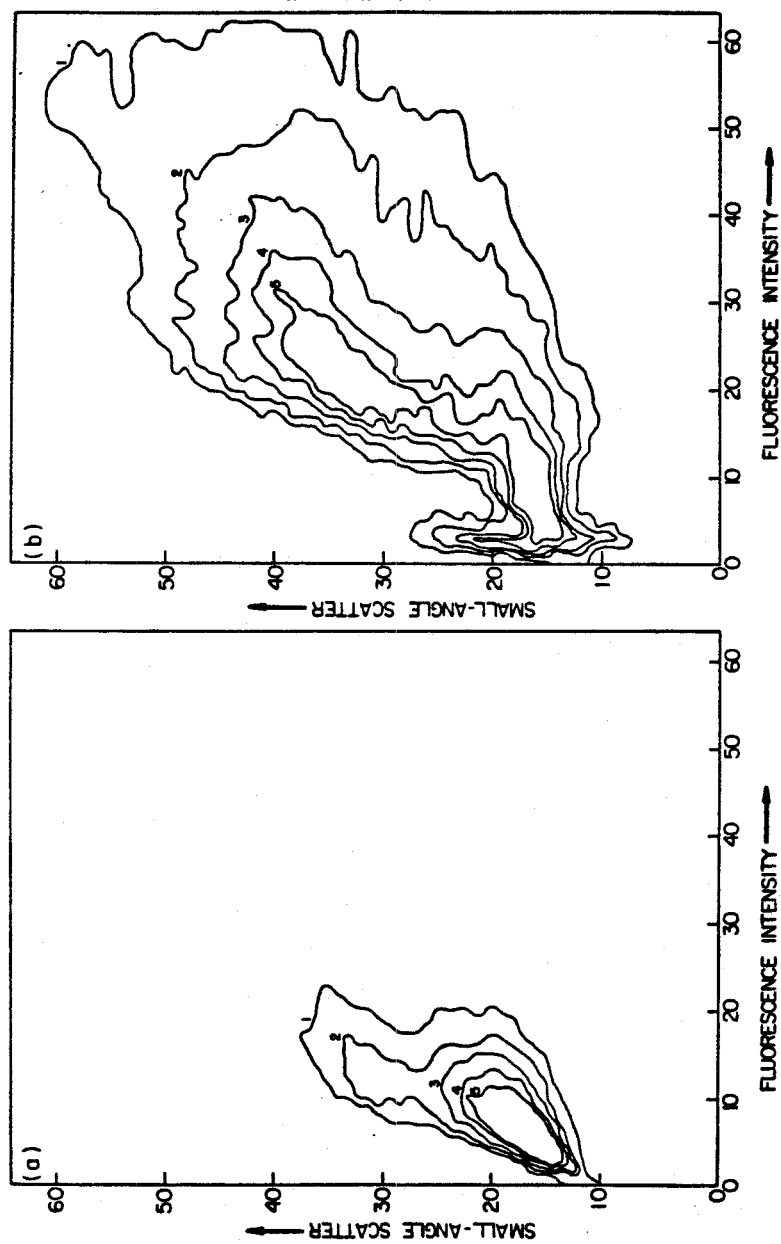


Figure 6-32
Relationship between protein fluorescence and light scatter level for immobilized cells (a) without hydroxyurea; and (b) with hydroxyurea. The numbers 1-5 indicate the relative frequency or cell count measured for the particular combinations of light scatter and protein fluorescence enclosed by the contour lines. These results imply a concerted effort on the part of the immobilized hydroxyurea-treated cells to enlarge their protein pools to an extent not seen in the suspended cells. It is possible that this activity is directed at replacing the enzymes deactivated by the inhibitor.

6.2.6 Discussion

Although hydroxyurea inhibited DNA synthesis in both immobilized and suspended cells, there is evidence that the inhibitory action was not complete. The DNA frequency functions for suspended yeast show that synthesis of DNA proceeded in some cells. A significant proportion of the population were large cells containing more DNA than that represented by two genome copies.

Continued mitochondrial DNA synthesis may have contributed to the higher DNA fluorescence levels found in some inhibited cells (Hartwell, et al., 1973; Pica Mattoccia and Roberti, 1974; Dujardin, et al., 1978). However, some residual cell division also occurred, as evidenced by the average specific growth rate for suspended cells of 0.11hr^{-1} , so that it is probable that some synthesis of chromosomal DNA persisted.

That division of cells does not halt completely under the influence of hydroxyurea has been documented by Slater (1973), while Hartwell (1976) showed that *S. cerevisiae* can recover its ability to divide even when the inhibitor is still present. These accounts suggest that yeast cells are able to overcome hydroxyurea-induced inhibition of DNA synthesis, although this process is slow, and is not appreciable until 6-8 hours after exposure to the substance (Hartwell, 1976). The mechanism for this recovery is not known.

Hydroxyurea had a favorable effect on ethanol productivity in both immobilized and suspended cells of *Saccharomyces cerevisiae*. Parallel improvements were observed for glucose uptake. The cells may have experienced an increased demand for substrate as a response to hydroxyurea, or otherwise, the substance permeabilized the yeast cell wall. Alterations to wall structure and subsequent changes in permeability in *S. cerevisiae* spores after hydroxyurea treatment have been reported (Schild and Byers, 1978).

In the present study, it was found that the polysaccharide composition of the suspended cell walls was changed following exposure to hydroxyurea, and this may have affected substrate uptake capabilities. Elongation of the buds may also be due to altered wall structure in the treated cells, but it is more likely an indication of new growth being directed into the bud tip (Tkacz and Lampen, 1972).

The variation in productivity results obtained from repeated experiments with suspended and immobilized yeast probably reflects the state of metabolic imbalance existing in the cells. Although each test was performed in the same way, the ability of the cells to synthesize DNA and complete the division cycle was changing. Recovery from hydroxyurea inhibition had most likely occurred to varying extents in each experiment, so that the state of the cells examined was not well defined.

Hydroxyurea inhibition led to a reduction in the yields of biomass for immobilized and suspended cells, but ethanol yields did not improve. In the suspended cell case, the reduction in levels of storage polysaccharides without a corresponding increase in ethanol yield suggests that these energy reserves, along with the extra glucose admitted into the cell, were channelled directly into the biosynthetic pathways. After DNA synthesis is inhibited by hydroxyurea, the cells may attempt to reduce the effect by building up their pools of ribonucleotide precursors, although this type of response occurs only to a minimal extent in *E. coli* (Neuhard, 1967). Alternatively, since the cells recover to some extent their ability to synthesize DNA, priority may be given specifically to rapid replenishment or reactivation of the enzyme affected by the inhibitor. Continued protein synthesis by hydroxyurea-treated cells is clear in the flow cytometry results. It is possible that much of the anabolic activity in hydroxyurea-treated cells is directed towards overcoming the DNA synthesis block.

Although the suspended *S. cerevisiae* cells recovered partially from the

hydroxyurea block on DNA synthesis, the immobilized cells appeared to accomplish this process with much more vigour. The fact that 2.3 times more cells were budded in the treated population than in the immobilized cell control indicates that hydroxyurea acted to prevent nuclear division and cell separation. This is also clear from the scanning electron micrographs of Figure 6-14. However, the immobilized cell DNA frequency functions show that those treated with hydroxyurea were able to overcome the inhibitory effects on DNA very quickly and proceeded to build up large quantities of DNA. This may have been possible, using the same argument as applied to the suspended cells, following a burst of protein synthesis which replaced the ribonucleotide reductase enzymes affected by hydroxyurea. After exposure to hydroxyurea, the suspended cells increased the size of their protein pool by a factor of 1.8, whereas, after the same period of time, the immobilized cell protein levels were an average of 2.6 times those found in untreated cells. This large increase in protein content in immobilized cells occurred even though only relatively small quantities of stable RNA were available for protein synthesis.

The similarity between the DNA frequency functions for treated and untreated immobilized cells may also be interpreted to mean that hydroxyurea was not taken up effectively by cells attached to the gelatin beads. Many of the results in this study, however, indicate that this was not the case. First, the scanning electron micrographs show immobilized cells arrested with abnormally large and elongated buds, a morphology characteristic of DNA synthesis and nuclear division defects. Compared with the histogram for untreated immobilized cells, the light scatter frequency function for inhibited cells also gives evidence of this block. In addition, immobilized cell growth rate was reduced by an average of 63% in the presence of hydroxyurea, compared with 78% for the suspended cells (Tables 6-4 and 6-5). Protein and RNA synthesis by immobilized cells (Table 6-7) also followed the same

trend as that of suspended cells treated with inhibitor.

In every sense except with regard to DNA synthesis, immobilized cell behavior was consistent with that expected from hydroxyurea-inhibited yeast. The conflicting implications of DNA synthesis in hydroxyurea-treated immobilized cells and their budded morphology can readily be reconciled after consideration of the known properties of cell cycle regulation. All events related to replication of DNA and nuclear division are independent of cytokinesis and cell separation, although these two events cannot occur without prior nuclear division (Hartwell, 1974). It is possible, therefore, that cell division be delayed after reactivation of DNA synthesis, as has been demonstrated by Slater (1973) in experiments with *S. cerevisiae*. After removal of hydroxyurea from the culture, cells commenced DNA synthesis immediately, but cell division and increases in cell number did not occur until after a period of two generation times.

The results of this study show that hydroxyurea blocks DNA synthesis and cell separation in immobilized yeast, but that this inhibition is readily overcome. There are many parallels between the behavior of the immobilized cells studied here and that of the yeast mutant, *cdc24* (Hartwell, et al., 1974), defective in bud emergence but able to continue DNA replication. In view of this, it is interesting to note that, in studies with hydroxyurea, this mutant was able to rapidly escape the hydroxyurea block at the restrictive temperature. In an experiment carried out by Sloat, et al. (1981), an asynchronous, exponentially-growing mutant yeast culture was shifted to 36°C 1½ hours after hydroxyurea addition, and the percentage of budded cells started to decline as soon as 3-6 hours later (Table IV, Sloat, et al., 1981).

The immobilized cells were affected by both hydroxyurea-treatment and attachment to the support. A comparison between hydroxyurea-treated immobil-

ized and suspended cells shows that the changes to cell behaviour due to immobilization, such as those discussed in Section 6.1, are still evident after hydroxyurea inhibition. For example, the hydroxyurea-treated immobilized cells contained more storage materials and cell wall components than treated suspended cells, even though hydroxyurea tends to reduce polysaccharide levels in cells (Table 6-6). The smaller average size of hydroxyurea-inhibited immobilized cells is also in line with earlier observations with untreated immobilized cells, as are the persistent low levels of RNA in immobilized yeast. These results reflect more the influence of immobilization on cell properties than the effects of hydroxyurea.

6.3 FERMENTATION WITH BIOTIN DEPRIVATION

The fermentative capabilities of biotin-starved immobilized and suspended yeast were determined in batch experiments using glucose medium. The morphology and composition of the two cell populations were also examined. Cells were immobilized by attachment to gelatin.

Biotin is involved in a broad spectrum of biosynthetic activities. DNA, RNA, protein and cell wall synthesis may be expected to decrease as a result of starvation for this vitamin. As shown in Section 6.1, yeast cells grown in complete nutrient medium proceed to build up intracellular levels of DNA and cell wall polysaccharides following immobilization on gelatin. Tests with biotin starvation were aimed at differentiating the response to immobilization under conditions unfavourable for synthesis of these compounds.

6.3.1 Induction of Biotin Starvation

A preliminary experiment showed that biotin deprivation is effective in reduc-

ing cell growth only after approximately 25 hours of biotin-free culture. This is shown in Figure 6-33. Even after this starvation time, absorbance of the suspended cell culture continued to increase slowly, reflecting unbalanced growth of the cells.

6.3.2 Morphology After Biotin Starvation

The scanning electron micrographs of Figure 6-34 show the morphology of yeast cells immobilized on gelatin after 32.5 hours of biotin starvation.

There are few budded cells in this population, as indicated in Figure 6-34a (x1500). In addition, there is some evidence that the strength and integrity of the cell walls of these yeast is affected by biotin deficiency. In Figure 6-34b (x10000), one of the cells is of abnormal morphology. At the point of contact with the gelatin surface, there is a protuberance which alters the basic spherical shape of the cell. Bulges in the cell surface were common in the population of immobilized yeast examined. Figures 6-34c (x10000) and 6-34d (x11000) show similar localized cell wall swelling. These protuberances may be associated with cell wall softening prior to bud emergence in the starved cells. However, their appearance suggests that the cell walls of starved yeast are relatively weak, so that a more general loss of wall rigidity occurs.

Attachment of the cells shown in Figures 6-34b and 6-34d involve a greater proportion of cell surface area compared with the unstarved yeast of Figure 6-1. This characteristic may also be due to a reduction of cell wall strength which causes the cells to spread over the gelatin surface.

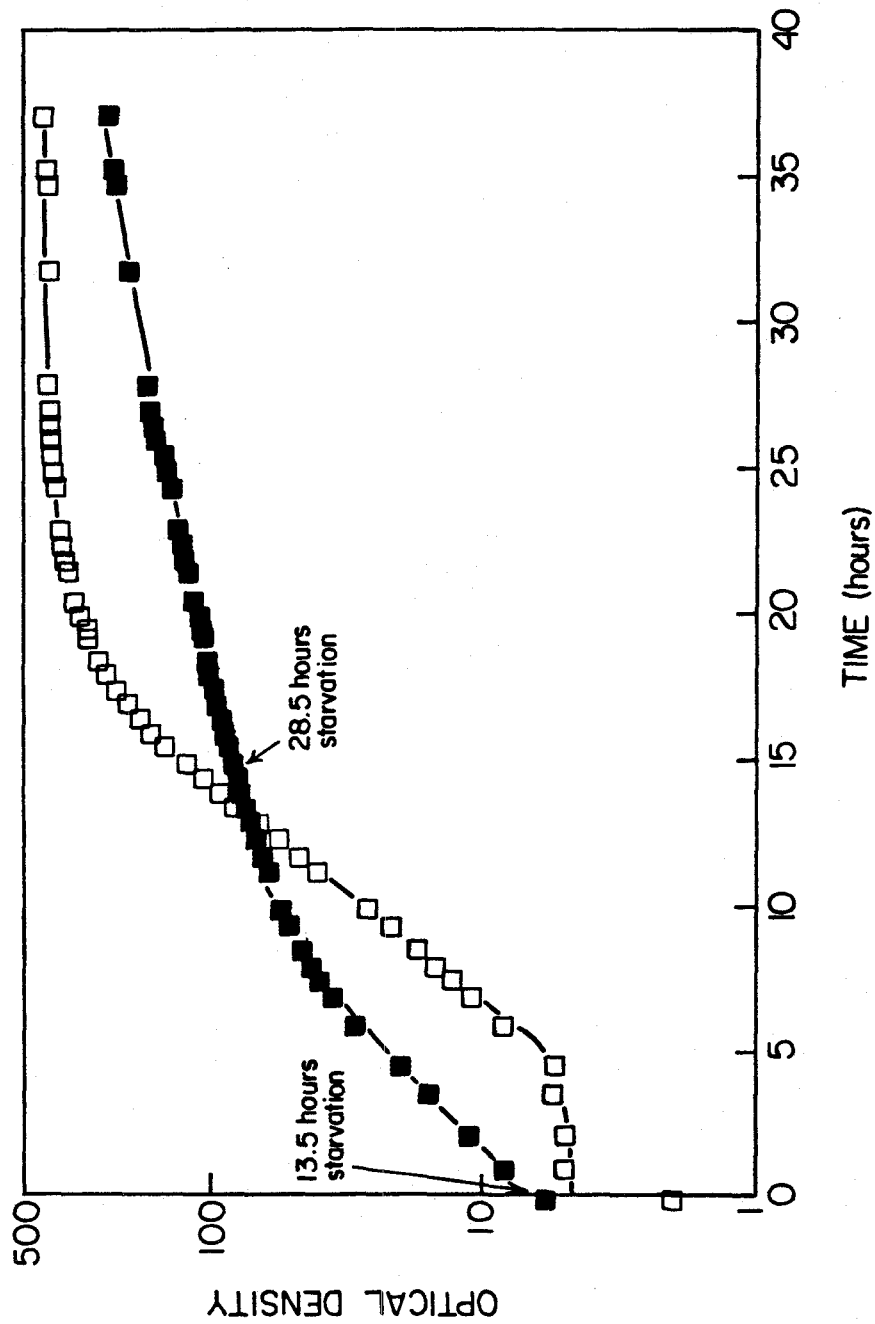
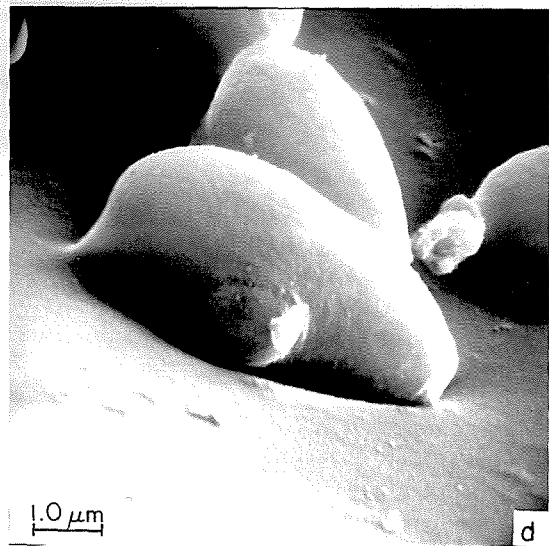
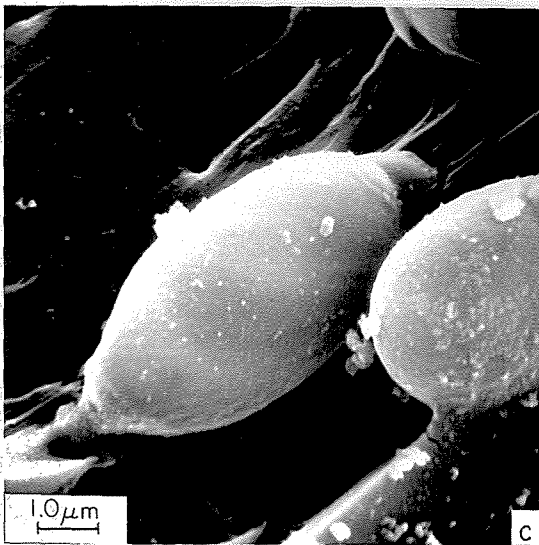
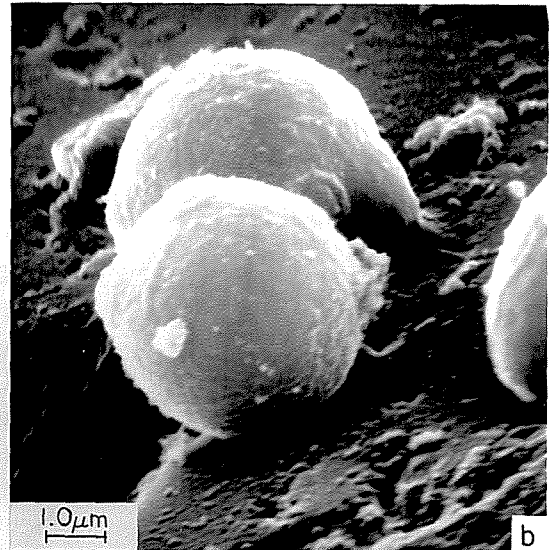
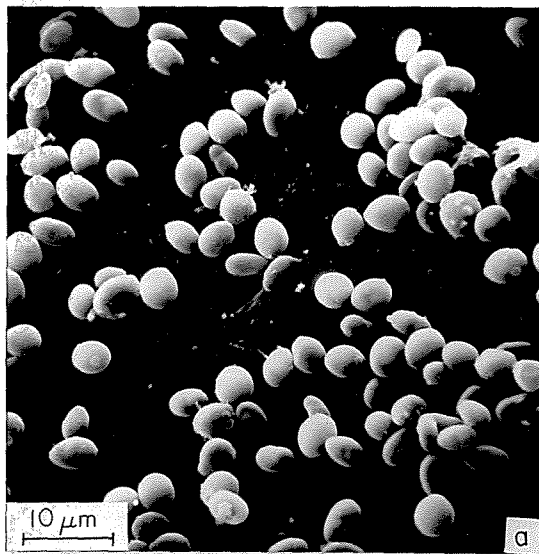


Figure 6-33
Culture density during induction of biotin starvation. Culture density for cells deprived of biotin (■) increased at a slower rate than the control culture (□) after approximately 25 hours of starvation. The control was started in medium containing an optimal concentration of biotin 13.5 hours after the beginning of starvation in the test culture. The suspended and immobilized cell batch fermentations were started using cells starved for 28.5 hours.

Figure 6-34

**Scanning electron micrographs of biotin-starved
immobilized yeast cells**
(a) x1500; *(b)* x10000; *(c)* x10000; *(d)* x11000



6.3.3 Fermentation Characteristics

Concentrations of ethanol, glycerol and glucose during suspended cell batch fermentation of biotin-free glucose medium are plotted in Figure 6-35. The changes in cell concentration during this experiment are shown in Figure 6-36. The initial suspended cell concentration was 1.13×10^9 cells/l, and the specific growth rate, assuming Monod kinetics, was 0.10 hr^{-1} . The suspended cell raw data are listed in Table IV-8 in Appendix IV.

Results from an immobilized cell experiment without biotin are given in Figures 6-37 and 6-38. The product, substrate and cell concentrations plotted in these Figures are listed in Table V-7 of Appendix V. The total initial cell concentration for the immobilized cell fermentation was 1.23×10^{10} cells/l.

Calculation of initial fermentation rates in these experiments is difficult due to the unsteady nature of activity. Overall, the immobilized and suspended cells produced negligible amounts of ethanol when starved of biotin, although the dynamic state of the metabolism of both cell populations is evident from the oscillatory nature of ethanol synthesis (Figures 6-35 and 6-37).

The amplitude of the changes in ethanol concentration during the course of the experiments is well within the resolution of the analytical procedures used. The concentration changes were greater with the suspended cells. This is despite the fact that, initially, the number of suspended cells present was less than 10% that in the immobilized cell experiment.

Ethanol concentration cycles in the immobilized and suspended cell fermenters occurred with essentially equal frequencies. After initial fluctuations, the period of oscillation was of the order of one hour.

The suspended cells did not consume an appreciable amount of glucose. How-

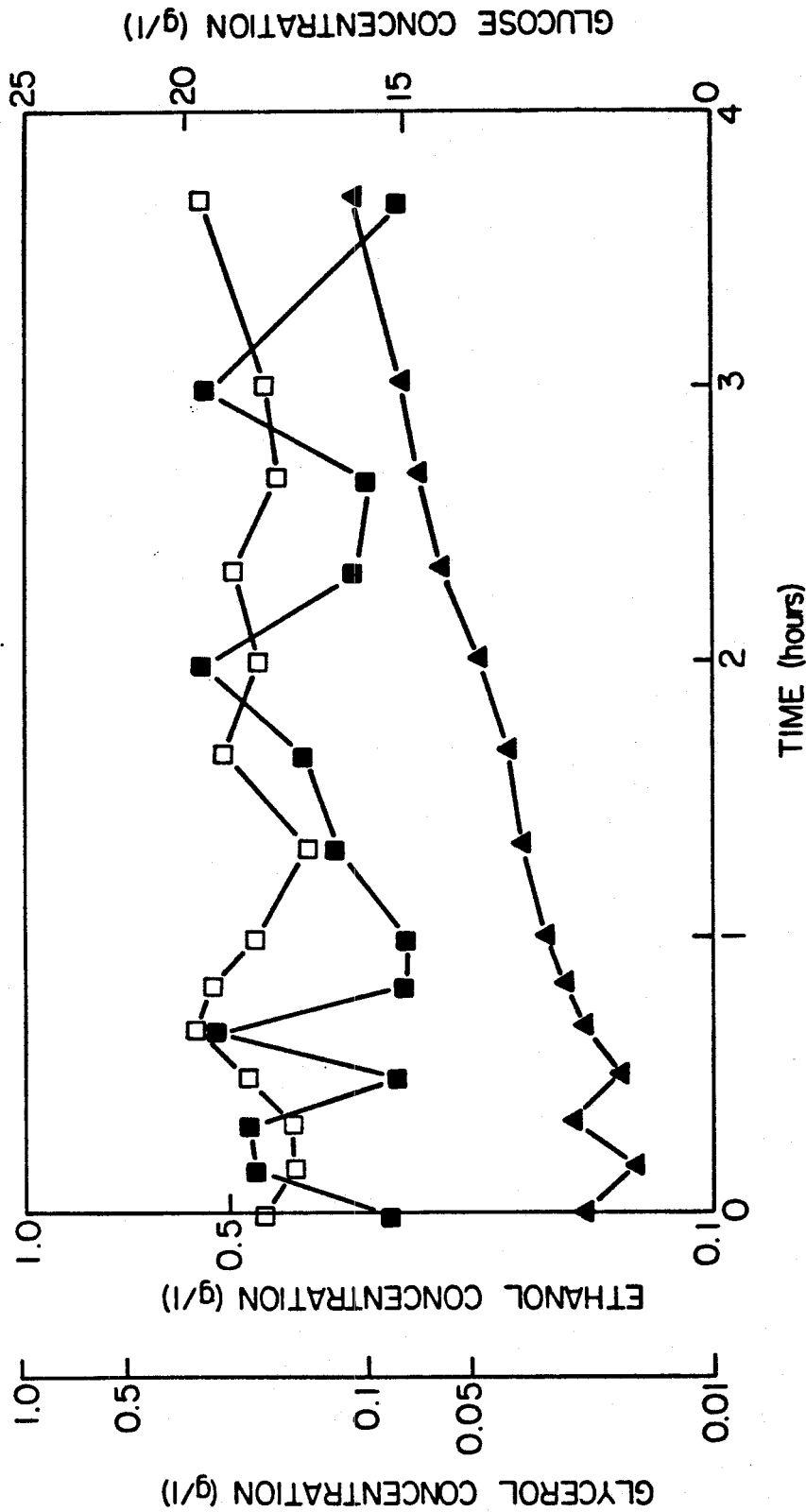


Figure 6-35
Concentration data from suspended cell fermentation in biotin-free medium
 showing ethanol (■), glycerol (▲) and glucose (□) concentrations. The initial
 suspended cell concentration was 1.13×10^9 cells/l. The dynamic state of
 suspended cell metabolism is evident in these data. Glycerol was produced by the
 suspended cells at the expense of ethanol.

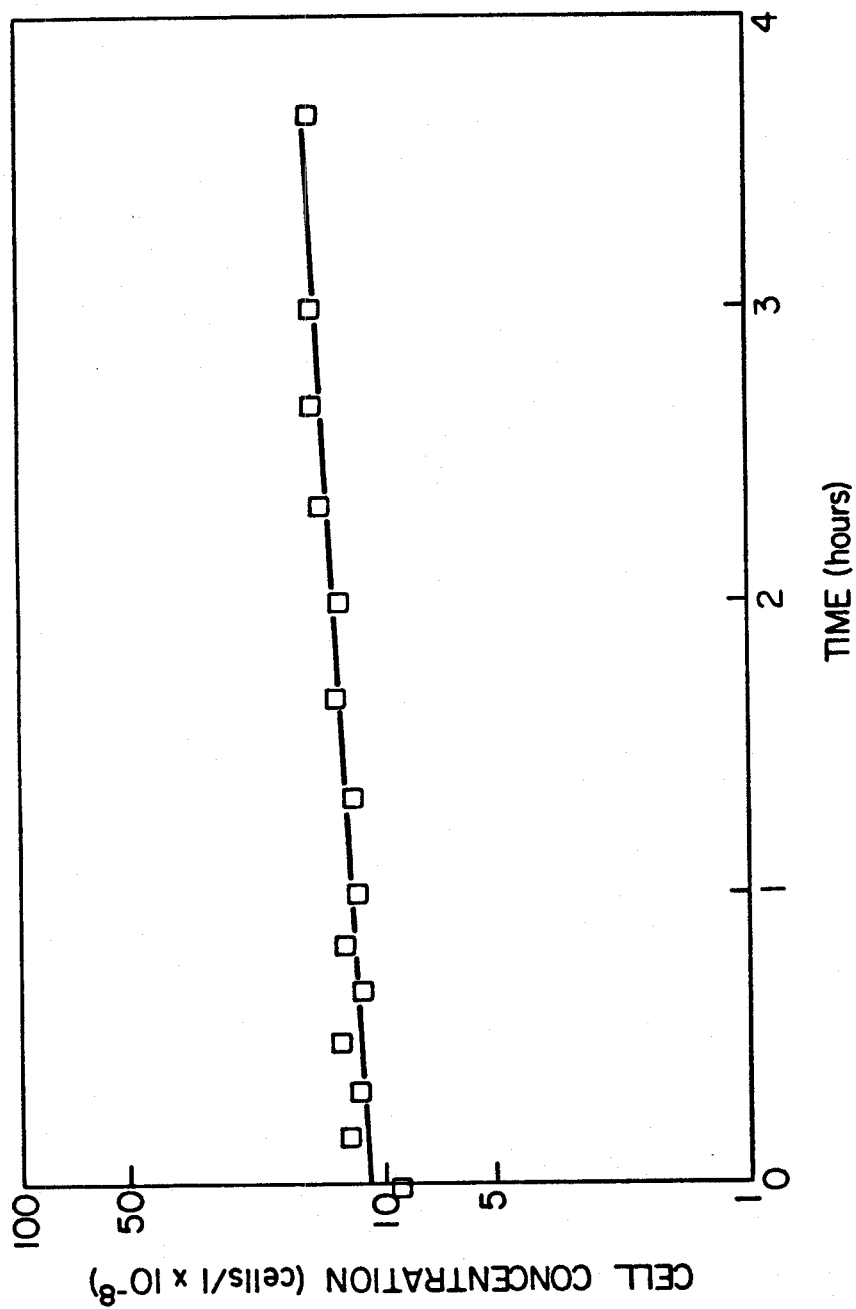


Figure 8-36
Cell concentration data from suspended cell fermentation in biotin-free medium.
The specific growth rate in this experiment was 0.10 hr^{-1} .

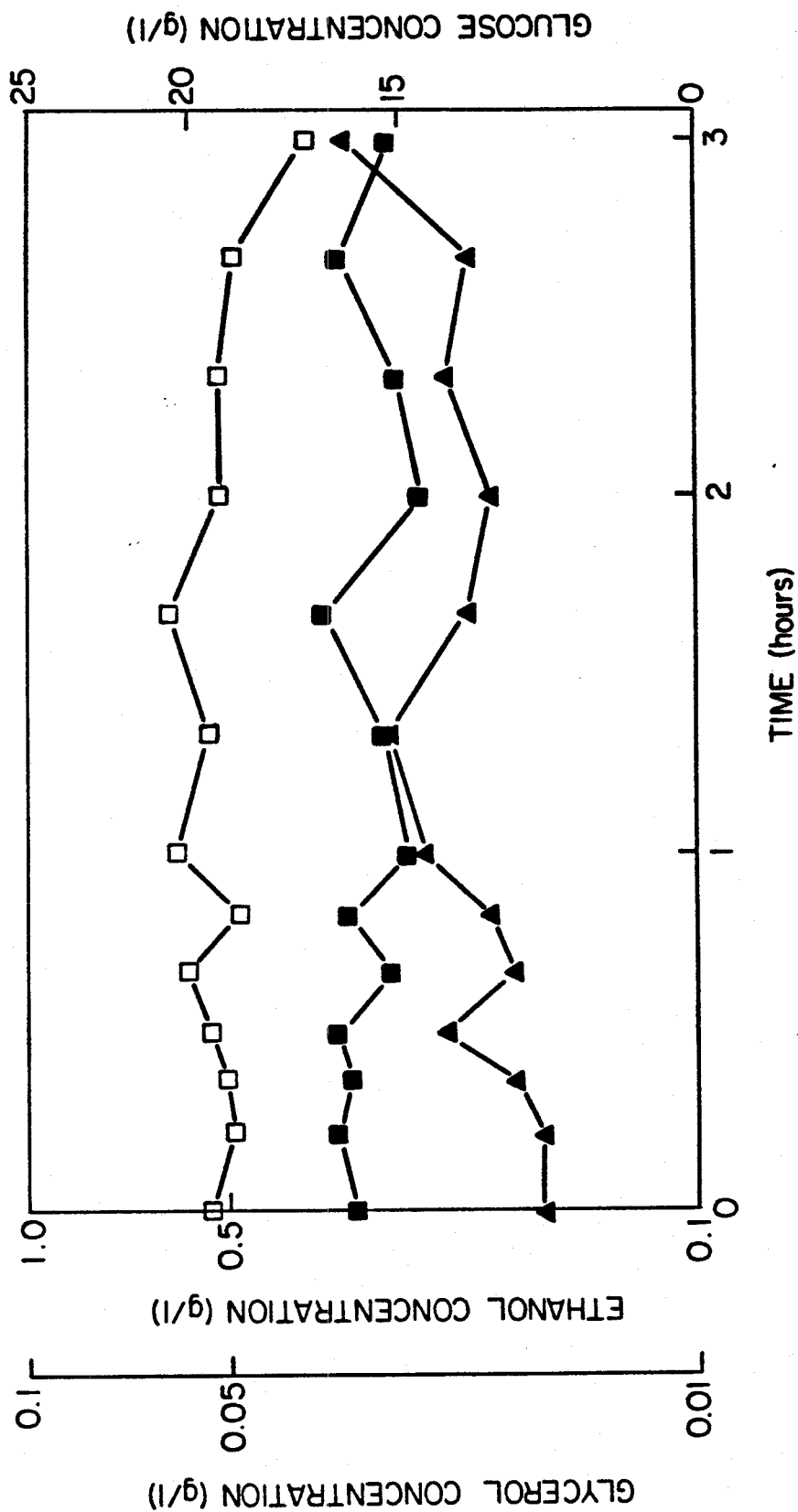


Figure 6-37
Concentration data from immobilized cell fermentation in biotin-free medium
 showing ethanol (■), glycerol (▲) and glucose (□) concentrations. The initial
 total cell concentration was 1.23×10^{10} cells/l.

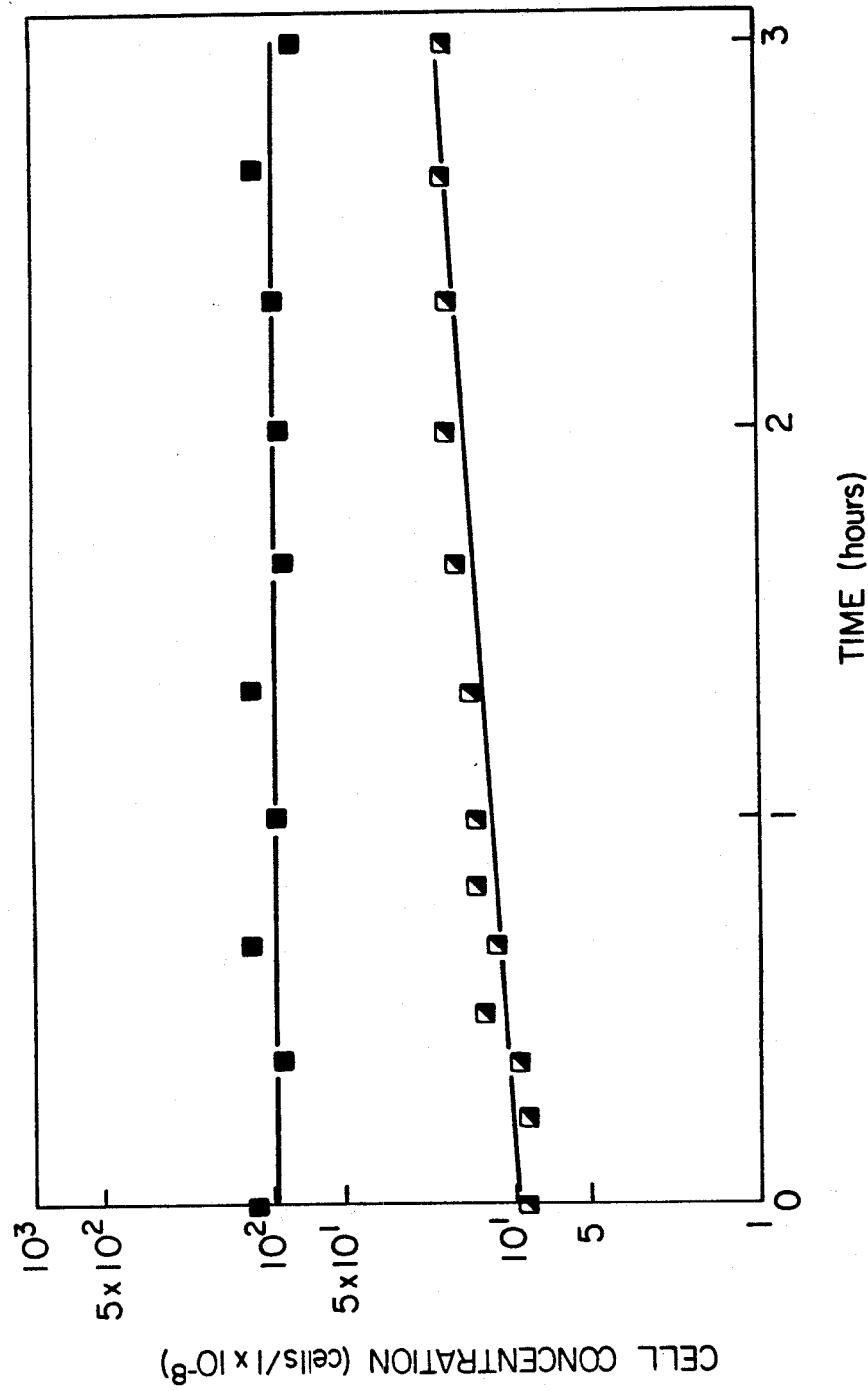


Figure 6-38
Cell concentration data from immobilized cell fermentation in biotin-free medium. There was negligible increase in immobilized cell number on the beads (■). However, the number of suspended cells in the recirculation medium (◐) approximately doubled.

ever, the number of cells in the suspended cell experiment increased by about 50% in 3.67 hours.

Accumulation of immobilized cells on the gelatin beads did not occur, although the number of suspended cells in the immobilized cell reactor doubled during the 3 hour experiment. Glucose uptake in the immobilized cell reactor occurred primarily during the last hour. The total amount consumed was about 2.4 g/l, so that the overall cell yield was 7.5×10^{10} cells/mol glucose.

There was a notable difference between immobilized and suspended cells with regard to glycerol production. After an initial lag of about 20 minutes, the suspended cells produced approximately five times more glycerol than the immobilized cells, even though the number of immobilized cells present was an order of magnitude greater. For the immobilized cells, the overall glycerol yield from glucose was 0.014 mol/mol.

6.3.4 Flow Cytometry Results

DNA, RNA and protein frequency functions were measured together with cell light scatter levels for biotin-deficient immobilized and suspended cells.

Comparisons of immobilized and suspended cell DNA, RNA and protein levels are shown in Figures 6-39, 6-40 and 6-41. Cell size was indicated by small-angle scatter, and the scatter histograms for biotin-starved immobilized and suspended cells are given in Figure 6-42. Table 6-8 lists the mean fluorescence and light scatter values obtained after analysis of Figures 6-39 - 6-42.

6.3.4.1 DNA

After biotin starvation, the DNA contents of immobilized and suspended yeast cells are roughly equal (Figure 6-39 and Table 6-8). The starved cells contain less

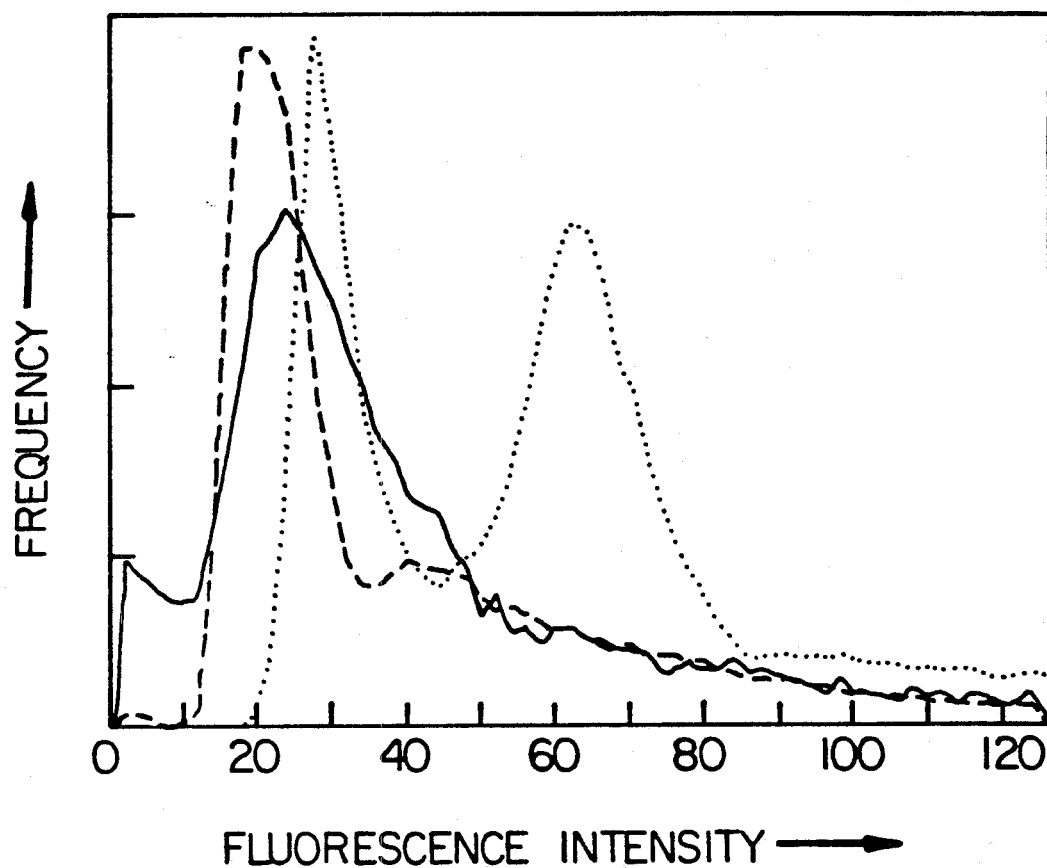


Figure 6-39

Single-cell DNA frequency functions for biotin-starved immobilized (—) and suspended cells (—). The DNA fluorescence histogram from an unstarved suspended cell control population (.....) is shown for comparison. DNA levels in the starved cells are less than in cells provided with biotin, and are apparently unaffected by immobilization.

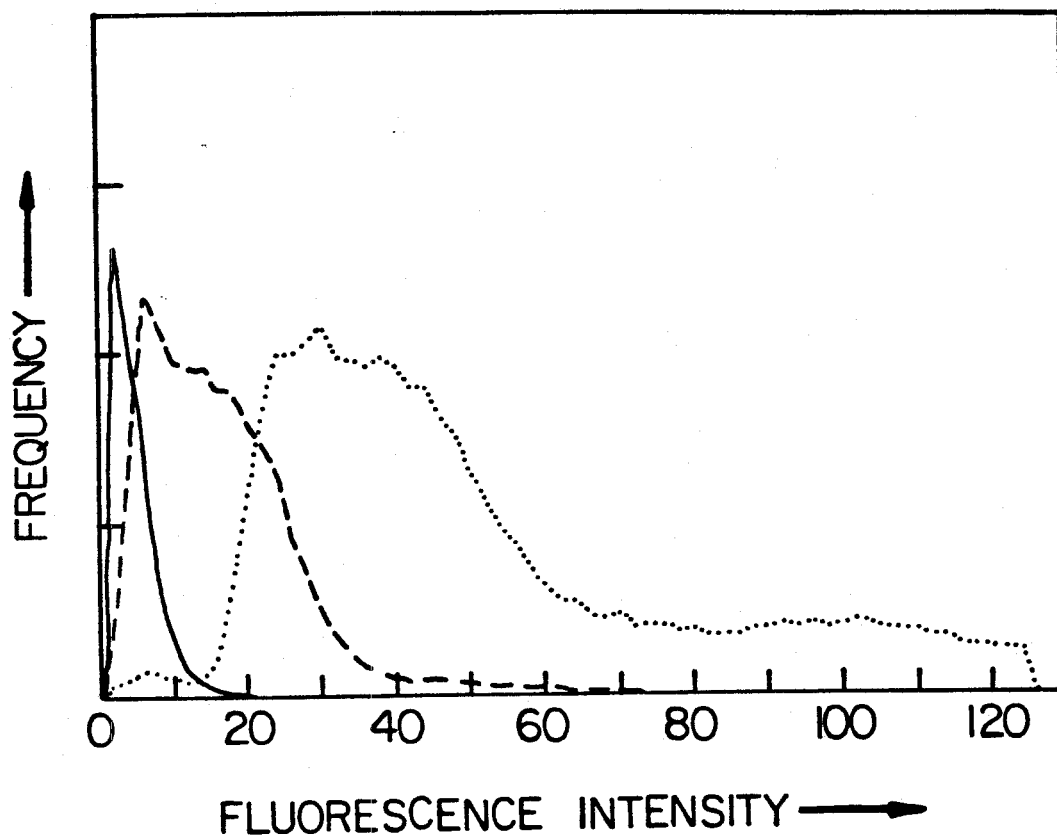


Figure 6-40

Single-cell stable double-stranded RNA frequency functions for biotin-starved immobilized (—) and suspended cells (---). Both populations of starved cells have a reduced stable RNA content compared with the suspended cell control (.....). Immobilized cells cultured without biotin have less stable RNA than starved suspended cells.

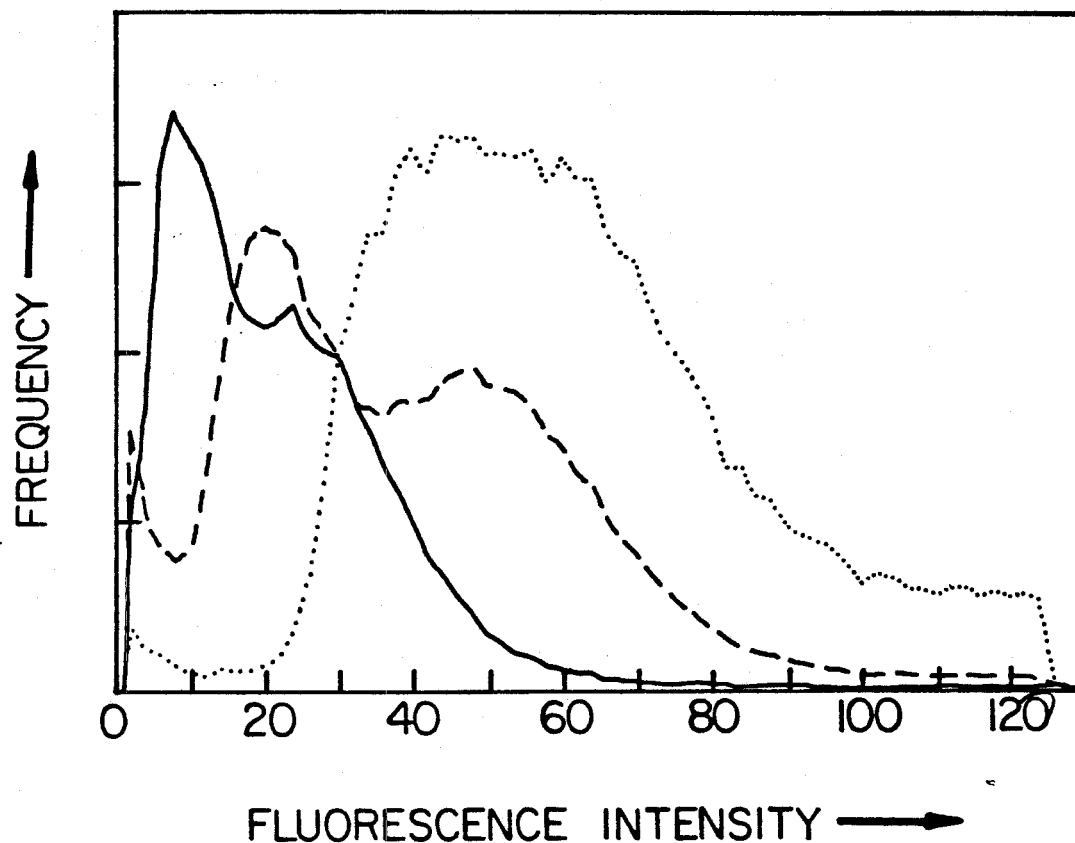


Figure 6-41

Single-cell protein frequency functions for biotin-starved immobilized (—) and suspended cells (---). Both starved populations contain, on average, less protein than the suspended cell control (.....) cultured in biotin-optimal medium. The size of the protein pools in starved immobilized cells is less than that in the starved suspended cells.

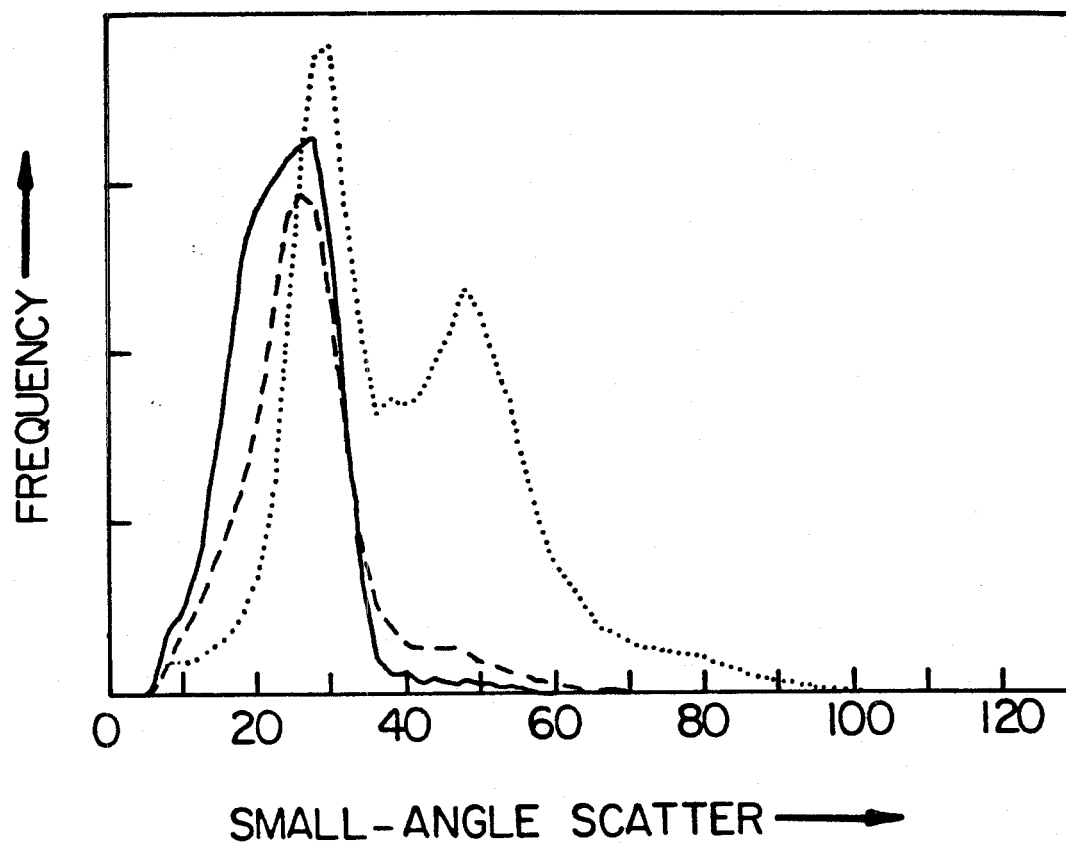


Figure 6-42

Single-cell small-angle light scatter frequency functions for biotin-starved immobilized (—) and suspended cells (—). Small-angle light scatter is an indicator of cell size. Starved immobilized and suspended cells are approximately the same size, and are smaller than the unbudded cells of the control population (.....).

TABLE 6-8

**Mean Values of Fluorescence and Light Scatter Frequency Functions
for Cells Deprived of Biotin**

	Frequency Function Mean Values (from Figures 6-39 - 6-42)		
	Suspended Cells	Suspended Cells without Biotin	Immobilized Cells without Biotin
DNA fluorescence	51.5	39.6	38.2
RNA fluorescence	50.6	16.9	5.1
protein fluorescence	60.6	39.0	22.2
small-angle scatter	40.5	26.8	23.8

DNA compared with cells grown under optimal conditions (Table 6-8).

6.3.4.2 RNA

Results from the RNA measurements (Figure 6-40 and Table 6-8) indicate that, in suspended cells, biotin deficiency reduces the intracellular content of stable double-stranded RNA to a level approximately one-third that of cells grown in complete medium.

Biotin-starved immobilized cells suffer a severe reduction in RNA content. Only about 30% of the stable double-stranded RNA found in starved suspended cells is present in similarly treated immobilized cells attached to gelatin.

6.3.4.3 Protein

Protein levels in biotin-starved suspended cells are also reduced compared with cells provided with all nutrients. The protein content measured for the starved suspended cells (Figure 6-41 and Table 6-8) was 65% of that found in freely growing cells.

The starved immobilized cells contain roughly 55% of the protein present in biotin-deficient suspended cells.

6.3.4.4 Light Scatter

As indicated in Table 6-8 and Figure 6-42, biotin starvation reduces the average cell size.

The lack of budded cells in the biotin-free culture is evident in the shape of the light scatter histograms as well as in the scanning electron micrograph of Figure 6-34a. Assuming that the first peak of the control population frequency function in Figure 6-42 represents only G₁-phase cells, immobilized cell and suspended cell sizes after biotin starvation are smaller than for unbudded cells in the growing population. Light scatter levels for starved immobilized cells are about 10% lower than for starved suspended cells.

6.3.5 Discussion

The effects of biotin starvation to prevent growth of *Saccharomyces cerevisiae* are not apparent until after several generations of yeast are produced. Since *S. cerevisiae* cells are able to concentrate biotin internally at levels up to 1100 times those in the surrounding medium (Cooper, 1982), a long starvation time with growth may be required before sufficient dilution of the available biotin stores occurs.

In other studies of biotin deficiency in yeast (Ahmad, et al., 1961), complete cessation of growth occurred approximately 170 hours after removal of cells to a biotin-free environment. This restriction in biomass production was accompanied by a change in colour of the yeast from creamy white to pink. These effects were not observed in the present work, suggesting that a much longer starvation time is necessary before the cells are totally deprived of biotin.

Activity of immobilized and suspended cells was affected by biotin starvation, although there are indications that the metabolism of these cells was still in a state of transition. The presence of diminishing intracellular quantities of biotin allowed some increase in cell number. The oscillatory nature of ethanol production, with alternate periods of apparent synthesis and consumption, attests most strongly to the dynamic metabolic condition of the cells.

Although deficient in biotin, the suspended *S. cerevisiae* cells synthesized relatively large amounts of glycerol. Immobilized cells also produced glycerol, but not to the extent apparent with the suspended yeast. Similar shifts towards by-product formation under conditions of nitrogen starvation have been reported for both immobilized (Inloes, 1982) and suspended (Harrison and Graham, 1970) cells.

Classically, the glycerol pathway is utilized by yeast when alcohol dehydrogenase is inoperative. Under these conditions, regeneration of NAD^+ occurs by reduction of dihydroxyacetone phosphate to produce glycerol. The results for the biotin-starved suspended cells suggest that the upper glycolytic pathway, including the oxidative glyceraldehyde 3-phosphate dehydrogenase step, was functional, while the lower glycolytic enzymes were unable to produce the precursors required for reduction of NAD^+ by alcohol dehydrogenase.

Intracellular reserves of carbohydrate apparently provided the substrate for glycerol production by suspended cells, since glucose consumption from the

medium was not detectable. Glycerol production does not profit cells with either energy in the form of ATP or carbon compounds for biomass synthesis. Accordingly, continued cell growth in the experiment, although slow, is difficult to explain in terms of energy and mass balances. Although ethanol may serve as substrate for yeast and provide precursors for cellular components via the glyoxylate cycle, synthesis of the key enzymes of this pathway requires relatively high oxygen concentrations (Rogers and Stewart, 1973). This explanation for biomass synthesis seems unlikely in the present work, where anaerobiosis in the reactors was maintained by nitrogen sparging.

In the immobilized cell reactor, glucose consumption was evident, especially during the last hour of the 3 hour experiment. However, overall cell and glycerol yields from utilization of this substrate were low compared with the values measured for unstarved immobilized cells (Table 6-1). Cell yield was only 14% of that observed under optimal growth conditions, while the yield of glycerol was reduced by more than 75%. It is possible that substrate which did not appear as biomass or glycerol was utilized for synthesis of polysaccharides. If this were the case, then the response of immobilized cell metabolism to biotin starvation is very different compared with suspended cells, which apparently break down intracellular carbohydrates for glycerol production.

The scanning electron micrographs of biotin-starved immobilized yeast suggest that the strength of the cell wall is impaired by biotin deficiency. Ahmad and Rose (1962) have reported results for starved suspended cells which were shown to be less resistant to mechanical breakage than yeast grown in biotin-optimal medium. The cell wall composition of yeast is altered under conditions of biotin-deficiency (Dunwell, et al., 1961), and, in addition, the protein content of the cell wall is reduced (Ahmad and Rose, 1962). These changes may account for the unusual morphology pictured in Figure 6-34.

Levels of DNA, RNA and protein in starved immobilized and suspended cells are lower than in cells grown in the presence of biotin. Similar reductions in macromolecular contents were reported for starved suspended *Saccharomyces cerevisiae* by Ahmad, et al. (1961). Precursors of RNA and DNA synthesis may be excreted from cells cultured without biotin if inactivity of certain enzymes results in a build-up of synthetic intermediates (Ahmad, et al., 1961). If this occurred continuously during immobilized cell fermentation, then it is possible that some of the glucose unaccounted for by cell, glycerol or ethanol production is lost by the immobilized cells in the form of purine- and pyrimidine-containing compounds.

In contrast to hydroxyurea treatment, biotin deprivation effectively prevents overproduction of DNA in immobilized cells. However, the results from measurements of RNA and protein levels are consistent with those found for immobilized cells grown in complete medium and for hydroxyurea-inhibited immobilized cells. Under all conditions tested in this work, the immobilized cells contained severely reduced amounts of stable double-stranded RNA compared with suspended cells, while protein levels were not as adversely affected. The experiments with biotin starvation show that the trend towards reduced stable RNA content in immobilized cells is independent of the response which causes accumulation of DNA in the cells.

6.4 CONCAVALIN A - IMMOBILIZED CELLS

Saccharomyces cerevisiae cells were immobilized by lectin binding to Sepharose microspheres. Techniques using concanavalin A were developed in this work. The feasibility of applying this type of biocatalyst in studies of immobilized cell metabolism was tested.

6.4.1 Morphology

Concanavalin A-bound yeast cells are shown in the scanning electron micrographs of Figure 6-43. Figures 6-43a (x700) and 6-43b (x1400) illustrate the extent of loading of the Sepharose beads. Closer views of single cell attachment are provided in Figures 6-43c (x1700) and 6-43d (x1200).

In comparison with the photographs of Figure 6-1b and 6-1c, the interaction between concanavalin A and the cell walls of *S. cerevisiae* appears to involve a greater proportion of the cell surface than is the case with gelatin binding. Elongation of the cells is also evident.

6.4.2 Fermentation of Galactose

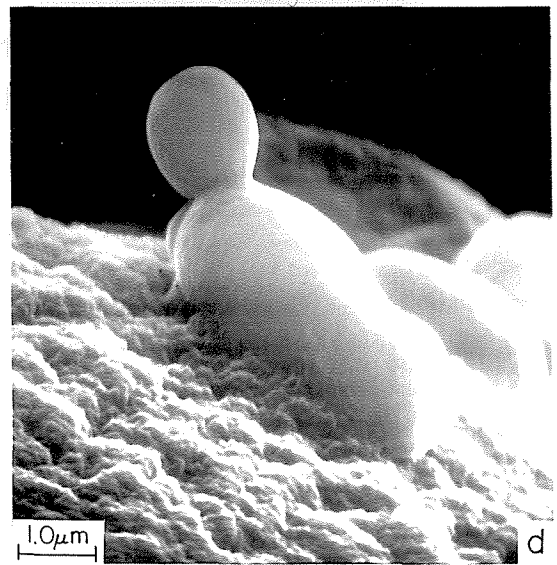
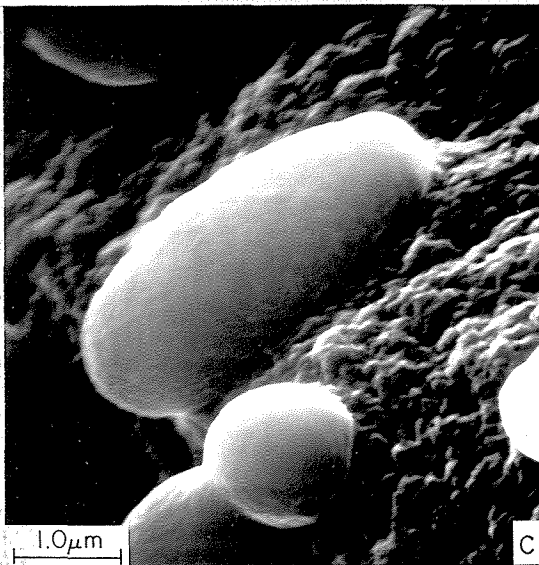
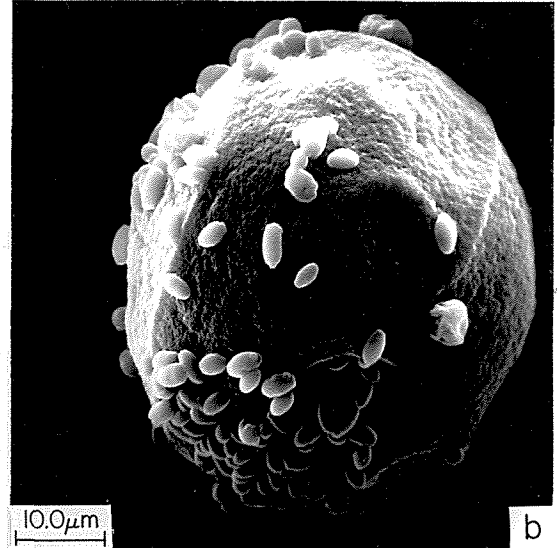
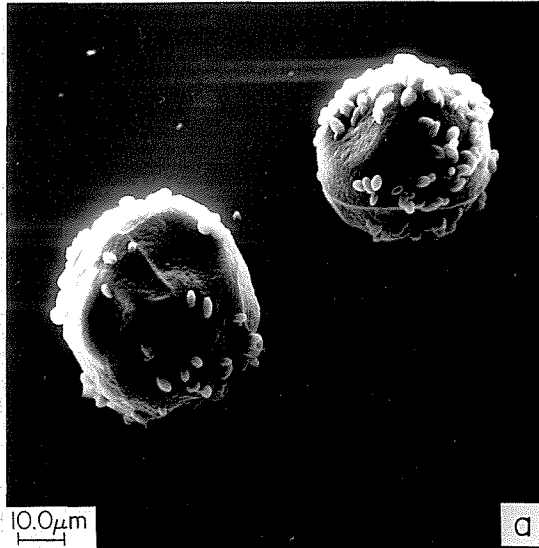
Once the yeast were immobilized, the Sepharose beads were added to the culture vessel described in Section 4.4.2.2 for fermentation studies. Since glucose interferes with the concanavalin A-cell wall bond, galactose was used as the carbon source for ethanol production.

Problems arose at this stage of the experiments due to detachment of cells from the microcarriers. Within six hours of exposure to the galactose medium, most yeast cells had been released into suspension.

A series of tests was carried out to determine the effects of medium pH on the reversal of cell immobilization. It was found that pH values between 4 and 5 allowed best retention of loading, although immobilized cell losses were still considerable. At $5.5 < \text{pH} < 7.5$, essentially complete removal of the yeast occurred.

Figure 6-43

**Scanning electron micrographs of immobilized yeast cells
bound to concanavalin A-activated Sepharose microspheres**
(a) x700; (b) x1400; (c) x1700; (d) x1200



6.4.3 Discussion

Biospecific attachment is not a common method for immobilization of microorganisms. In a previous study, bacteria and yeast were immobilized onto concanavalin A-activated magnetite (Horisberger, 1976). However, no fermentation experiments were reported with the lectin-bound cells. In view of the difficulties experienced in this work, the method may have limited applications unless desorption of cells in carbohydrate media can be controlled.

Possible explanations for detachment of the *S. cerevisiae* cells are discussed below:

- (i) Cells adhering to the microspheres were subjected to physical shear from the stirring action in the medium and from turbulence created as nitrogen gas was sparged through the liquid. These forces are known to be important factors in the surface culture of animal cells, which require very gentle handling (Sinskey, et al., 1981). Although yeast are not as delicate, mechanical factors may still play a role in rupturing the cell wall-concanavalin A bond.
- (ii) Commercial preparations of galactose always contain some quantity of glucose. Although specifications for the purified sugar used in this work indicated a glucose level of <0.01%, the effectiveness of small concentrations of glucose in eluting cells from concanavalin A is well established (Goldstein, et al., 1965).
- (iii) Even if residual amounts of glucose in the medium did not influence cell binding to concanavalin A, non-specific detachment of cells in the presence of galactose is known to occur (Kinzel, et al., 1976a).

Retention of immobilized yeast on the Sepharose beads is dependent on pH.

Improved adhesion below pH 5 is consistent with the known conformational properties of concanavalin A (Lis and Sharon, 1973). At pH values below 5.6, concanavalin A exists in solution as a single molecular species made up of two protomers. Saccharide binding is optimal at about pH 5. However, at pH 5.6, the protein forms tetramers, and at pH 7 and above, higher aggregates develop. Although the ease with which these changes occur may be affected by immobilization of the protein, conformational variations may be an important factor determining the success of concanavalin A-based immobilization procedures.

It is possible that changing environmental conditions may reduce problems with cell detachment. Concanavalin A-immobilization may be more successful in systems with organisms which are able to utilize other compounds besides saccharides as carbon substrates. In this way, competition between sugars in the nutrient medium and cell wall mannan could be eliminated.

6.5 GLYCOLYTIC OSCILLATIONS

Glycolytic oscillations in suspended yeast cells have been used in the past to monitor the regulation properties of enzymes in the pathway. The concentration of NADH is an effective indicator of the state of glycolysis in yeast under anaerobic conditions. In this work, changes in NADH levels in immobilized and suspended *Saccharomyces cerevisiae* were measured during starvation and after a step-up of nutrient supply. The cells were immobilized on the surface of crosslinked gelatin.

The experimental findings were analysed with reference to the mathematical model of glycolysis developed in Chapter 5. Details of the results are discussed below.

6.5.1 Experimental Observations

Immobilized and suspended cells were grown on complete glucose medium, harvested, and then starved in phosphate buffer. The same preculture conditions were followed for both cell types. Experimental conditions which were varied at the time of NADH measurement include:

- (i) starvation time;
- (ii) temperature; and
- (iii) concentration of glucose added.

6.5.1.1 *Suspended Cells*

A typical suspended cell transient response to glucose addition is shown in Figure 6-44. Potassium cyanide was added after the glucose to block respiration. A sugar concentration of 44.5 mM was used in the experiment of Figure 6-44.

Similar trains of damped sinusoidal oscillations were obtained at 25°C with suspended cells under a wide range of conditions. In all cases, addition of glucose at concentrations from 3.6 to 44.5 mM initiated the dynamic response. The starvation time necessary for oscillations to occur increased with decreasing sugar concentration and varied between 6 and 165 minutes.

The period of oscillation ranged from 0.83 to 1.04 minutes. No variation of period with starvation time was detected. The relationship between period of oscillation and glucose concentration is shown in Figure 6-45. Above about 10 mM glucose, higher concentrations of sugar increased the frequency of the glycolytic response until saturation of the transport system removed the dependence on sugar concentration.

Before addition of glucose to the suspended cells, the concentration of NADH

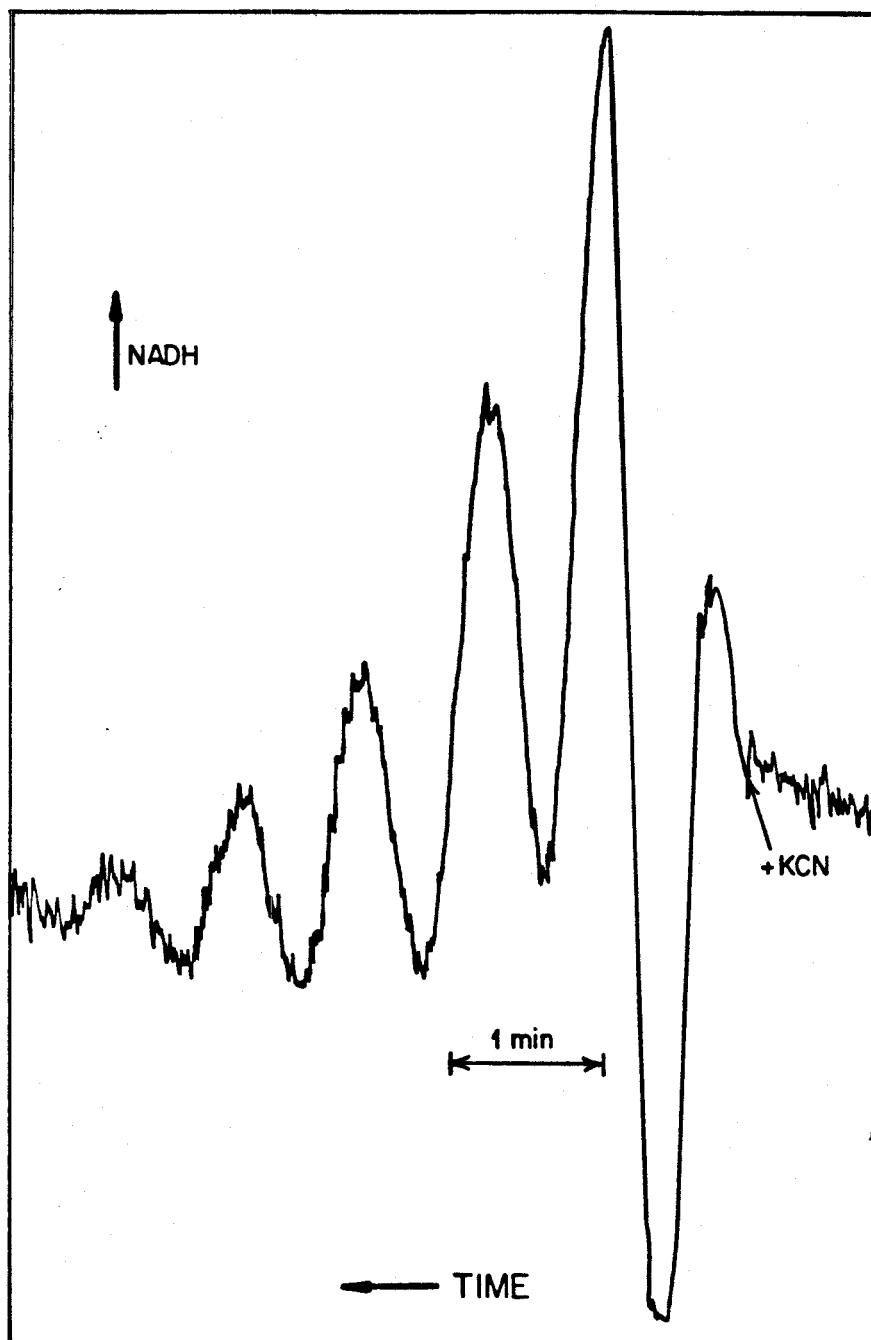


Figure 6-44

Typical NADH fluorescence trajectory for suspended cells. In this experiment, glucose was added to starved cells at a concentration of 44.5 mM.

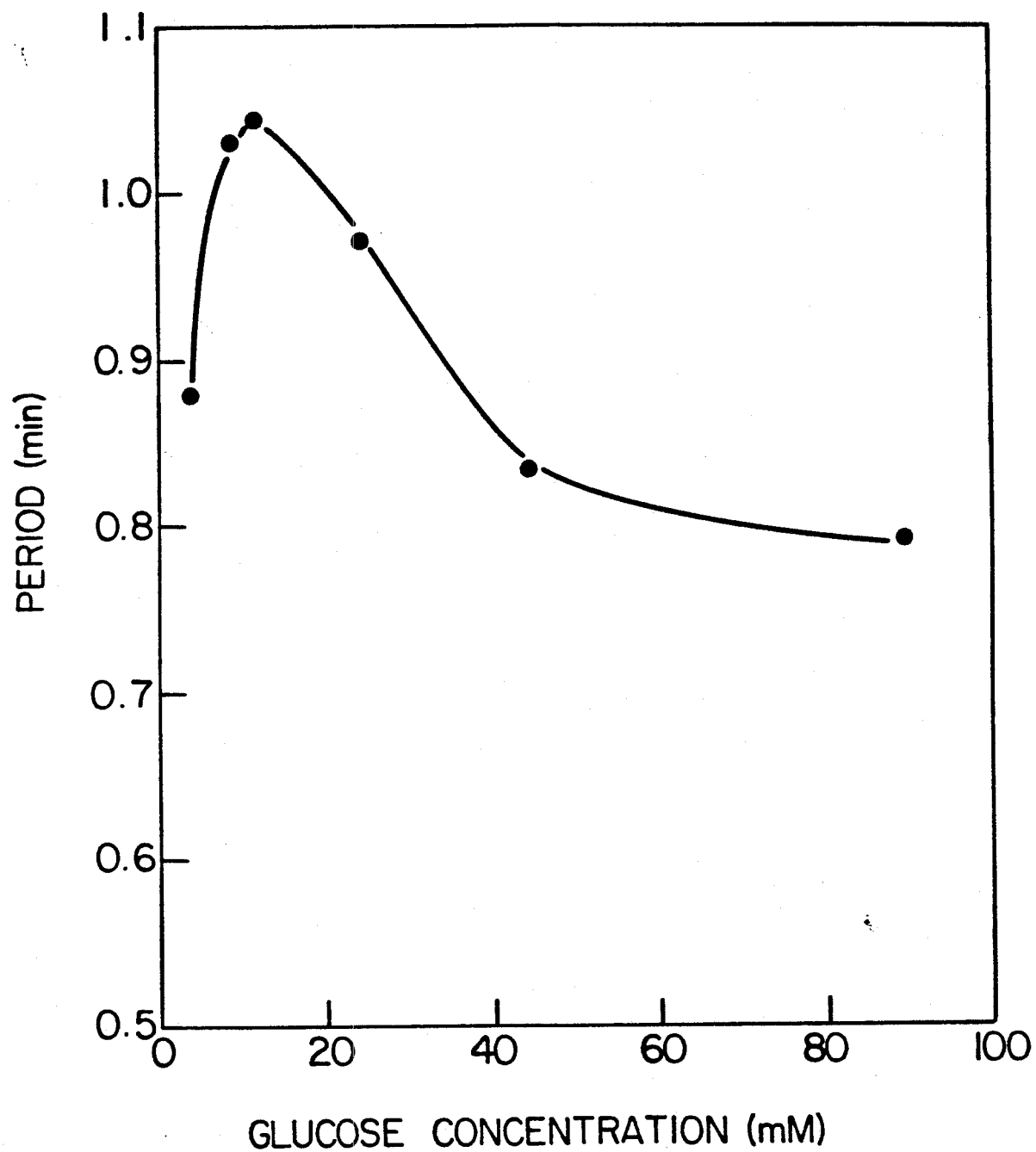


Figure 6-45

Relationship between glucose concentration and period of NADH oscillation for suspended cells.

was steady. NADH levels were not appreciably affected by endogenous utilization of carbohydrate reserves or by the lowering of glycolytic flux following glucose starvation.

6.5.1.2 Immobilized Cells

Response to the experimental conditions was altered in the case of immobilized cells, and was found to vary with temperature. In contrast to the suspended cell behaviour, periodic changes of NADH concentration were apparent before addition of glucose to the starved immobilized cells.

NADH oscillations for immobilized cells at 25°C are shown in Figures 6-46 and 6-47.

The periodic changes of Figure 6-46 were obtained from immobilized cells which had been starved in phosphate buffer for 62 minutes. No glucose was added. The fluctuations in fluorescence were not continuously damped, and the period of oscillation was approximately 1.1 minutes.

Similar non-sinusoidal waveforms were generated by immobilized cells after addition of 11.2 mM glucose (Figure 6-47). These cells had been starved for 141 minutes prior to the measurement in aerated phosphate buffer at 25°C. Again, the oscillations were not continuously damped.

Periodic behaviour of immobilized cells at 18°C is shown in Figures 6-48 and 6-49. In both cases, abrupt step-wise or saw-tooth fluorescence changes are superimposed on more gradual changes in NADH level.

The trajectory of Figure 6-48 was obtained with immobilized cells in phosphate buffer after 20 minutes of starvation. No glucose was required to elicit the response. The step-wise increases in NADH were part of a slow oscillatory cycle of period approximately 80 minutes. The period of the step changes decreased gradu-

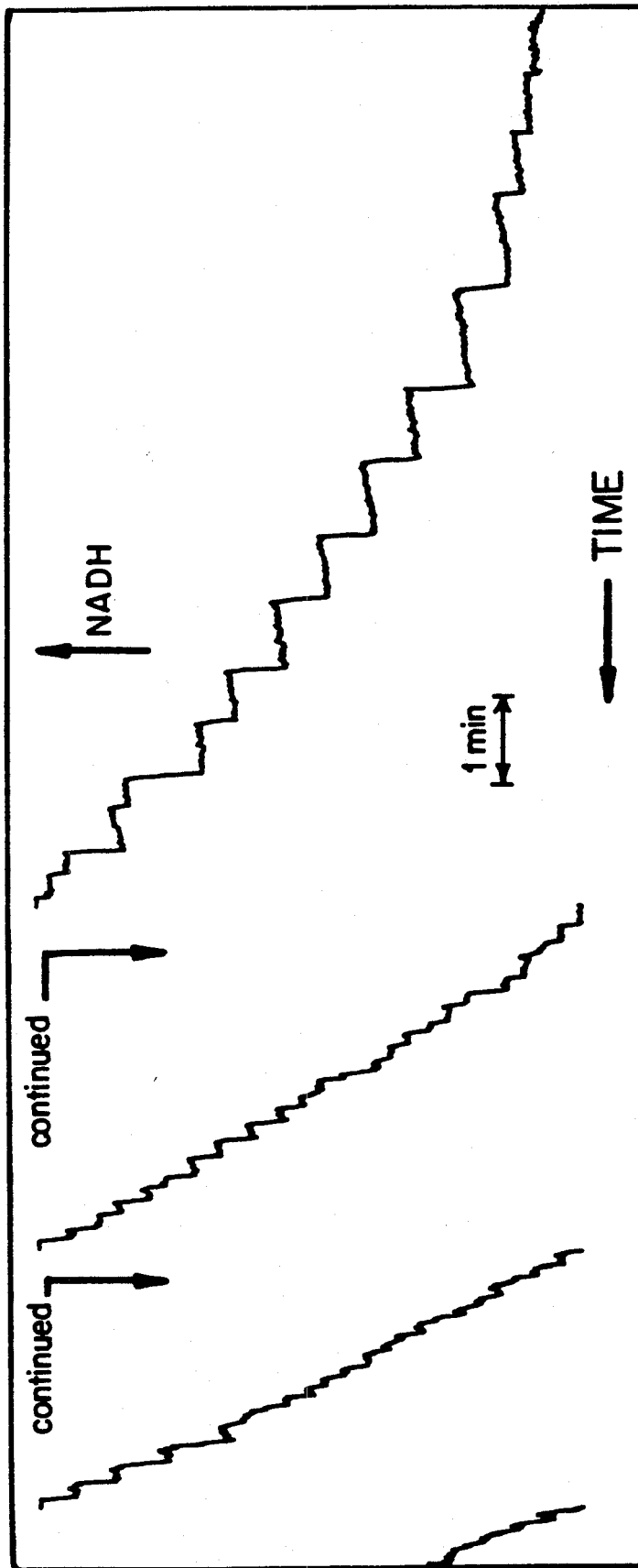


Figure 6-46
NADH fluorescence trajectory for immobilized cells in phosphate buffer at 25°C.
The cells were starved for 62 minutes. No glucose was added.

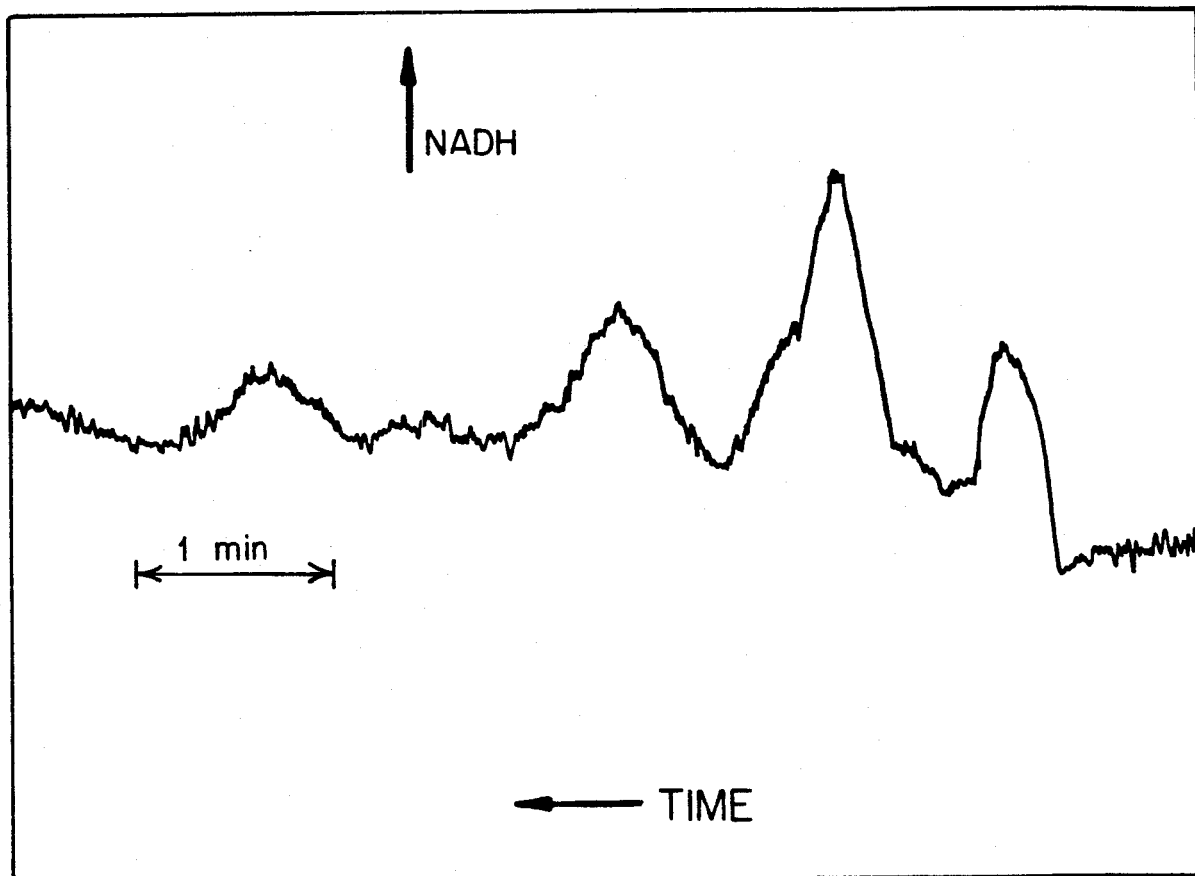


Figure 6-47

NADH fluorescence trajectory for immobilized cells at 25°C after addition of glucose. The glucose concentration was 11.2 mM.

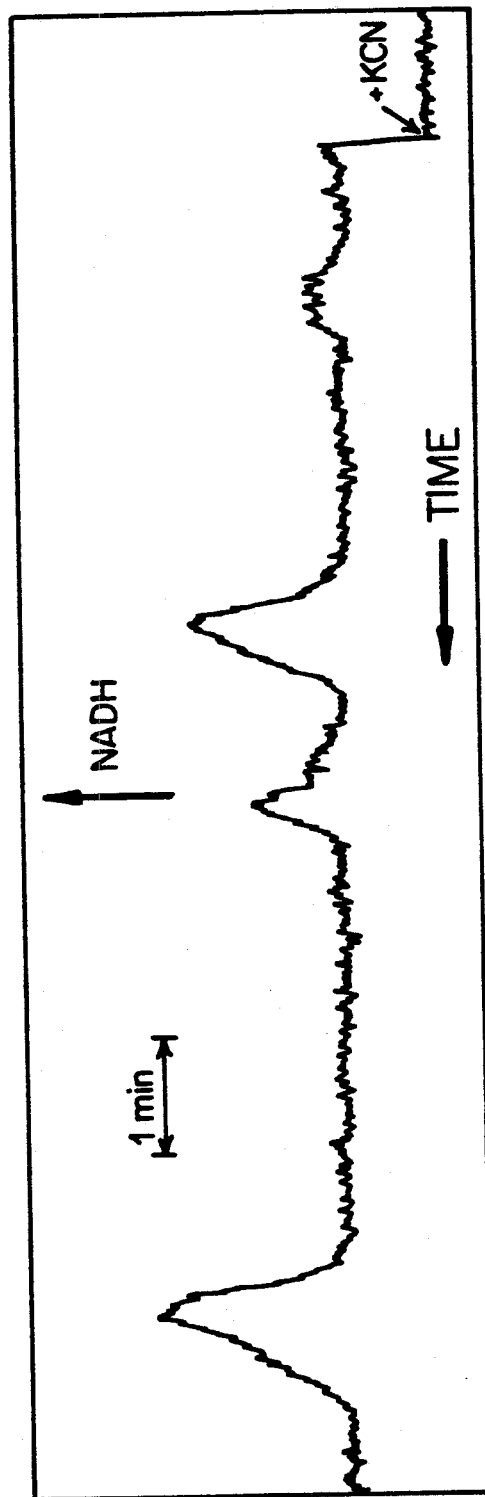


Figure 6-48
NADH fluorescence trajectory for immobilized cells in phosphate buffer at 18°C.
No glucose was added. The cells were starved for 20 minutes before fluorescence measurement.

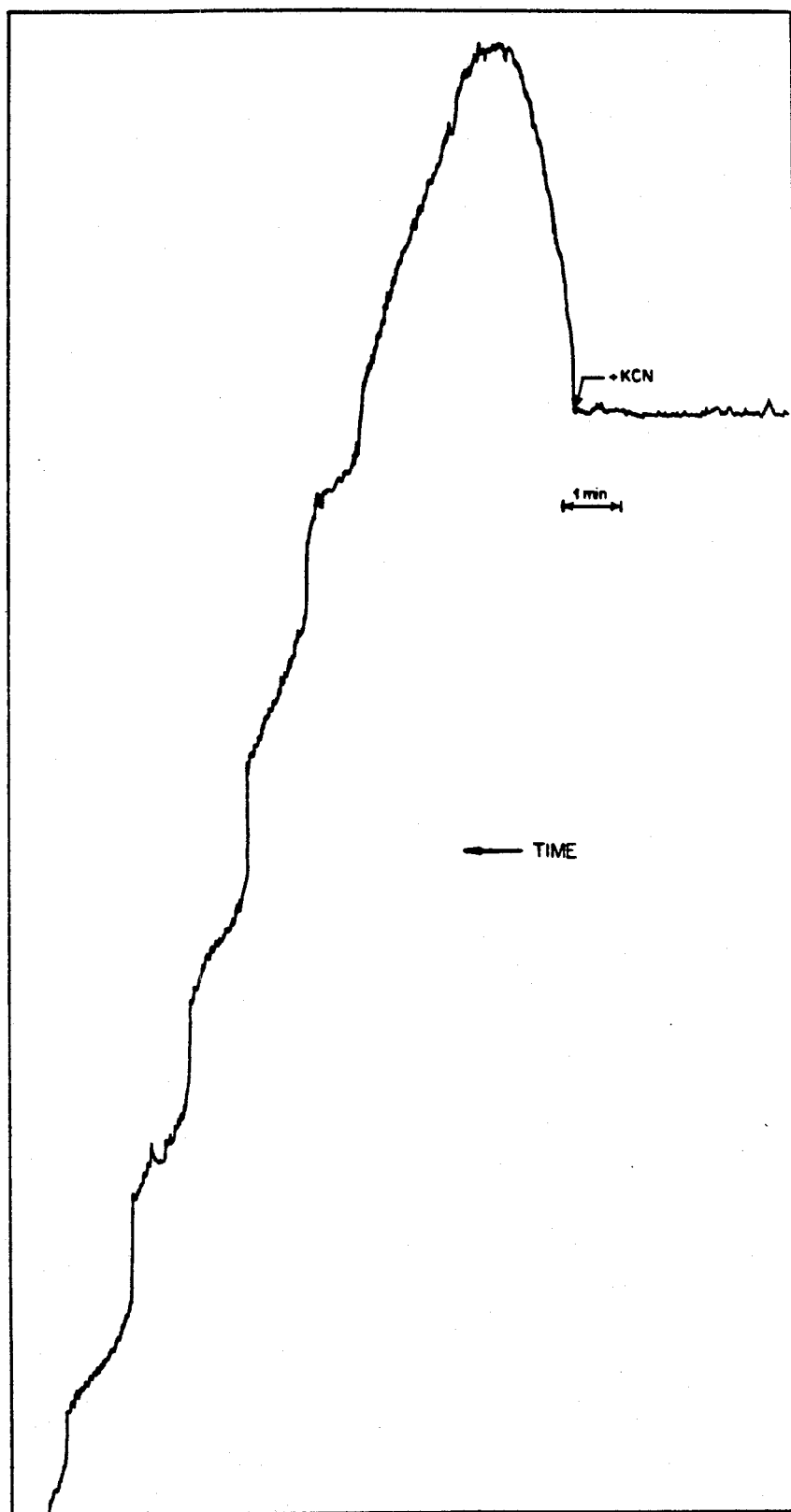


Figure 6-49
NADH fluorescence trajectory for immobilized cells at 18°C after addition of glucose. The concentration of glucose was 44.6 mM.

ally within 17 minutes from 55-60 seconds to less than 8 seconds.

Figure 6-49 shows the response of immobilized cells at 18°C to glucose addition. Cells starved for 18 minutes were exposed to 44.6 mM glucose. After an initial increase in NADH level, periodic step-wise drops in fluorescence occurred, approximately once every minute.

6.5.2 Mathematical Modelling

The model of glycolysis described in Section 5.2 was used to investigate the relationship between enzyme kinetics and the character and occurrence of NADH oscillations.

Steady state solutions of the model were generated. Local stabilities of the steady states admitted by the model were determined from the eigenvalues of the Jacobian matrix for the linearized set of equations.

Figures 6-50 - 6-52 show the results from the stability analysis. The bifurcation lines and domains for the different dynamic states of the system are presented as functions of the normalized substrate input rate, V_{in} , and the normalized output rate, $(V_{R,max})_{PK}$. In each figure, the lower line dividing stable and unstable states represents the locus of Hopf bifurcation points.

The different solution maps were generated by varying the values of other adjustable parameters in the model. The effects of an increase in the rate of ATP removal by ATPase is shown in Figure 6-50. The initial level of NADH present in the cell is changed in Figure 6-51, while Figure 6-52 shows the influence of the total concentration of adenosine phosphates on stability of the system. The destabilizing effects of an increased rate of ATP consumption and a decrease in steady state NADH level are evident in Figures 6-50 and 6-51.

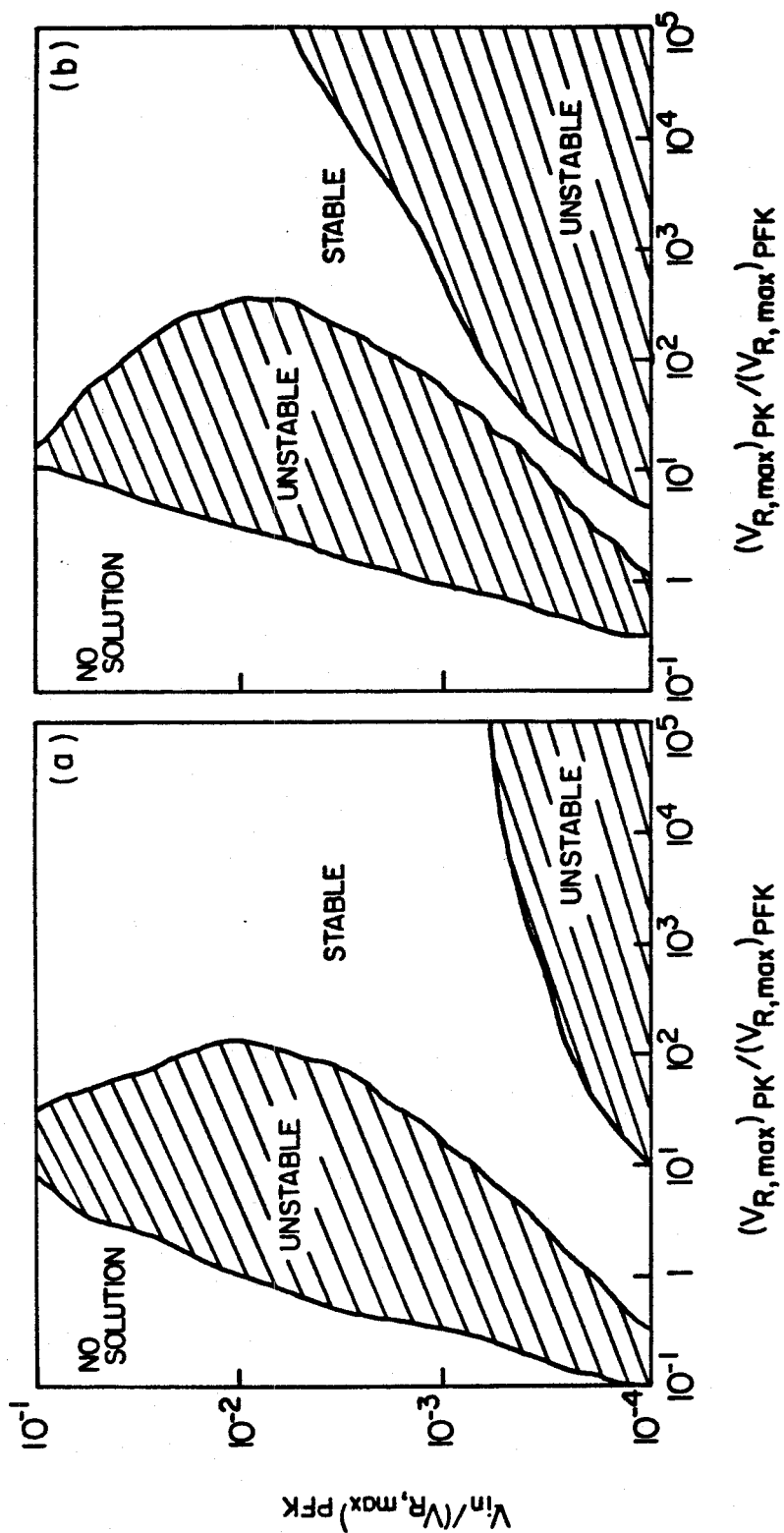


Figure 6-50

Domains of stability as a function of ATP consumption rate.

(a) $k_2 = 0.25 \text{ s}^{-1}$; (b) $k_2 = 2.5 \text{ s}^{-1}$.

The other adjustable parameters took the following values: $AP = 5 \text{ mM}$, $AN = 3 \text{ mM}$; $[NADH] = 0.03 \text{ mM}$.

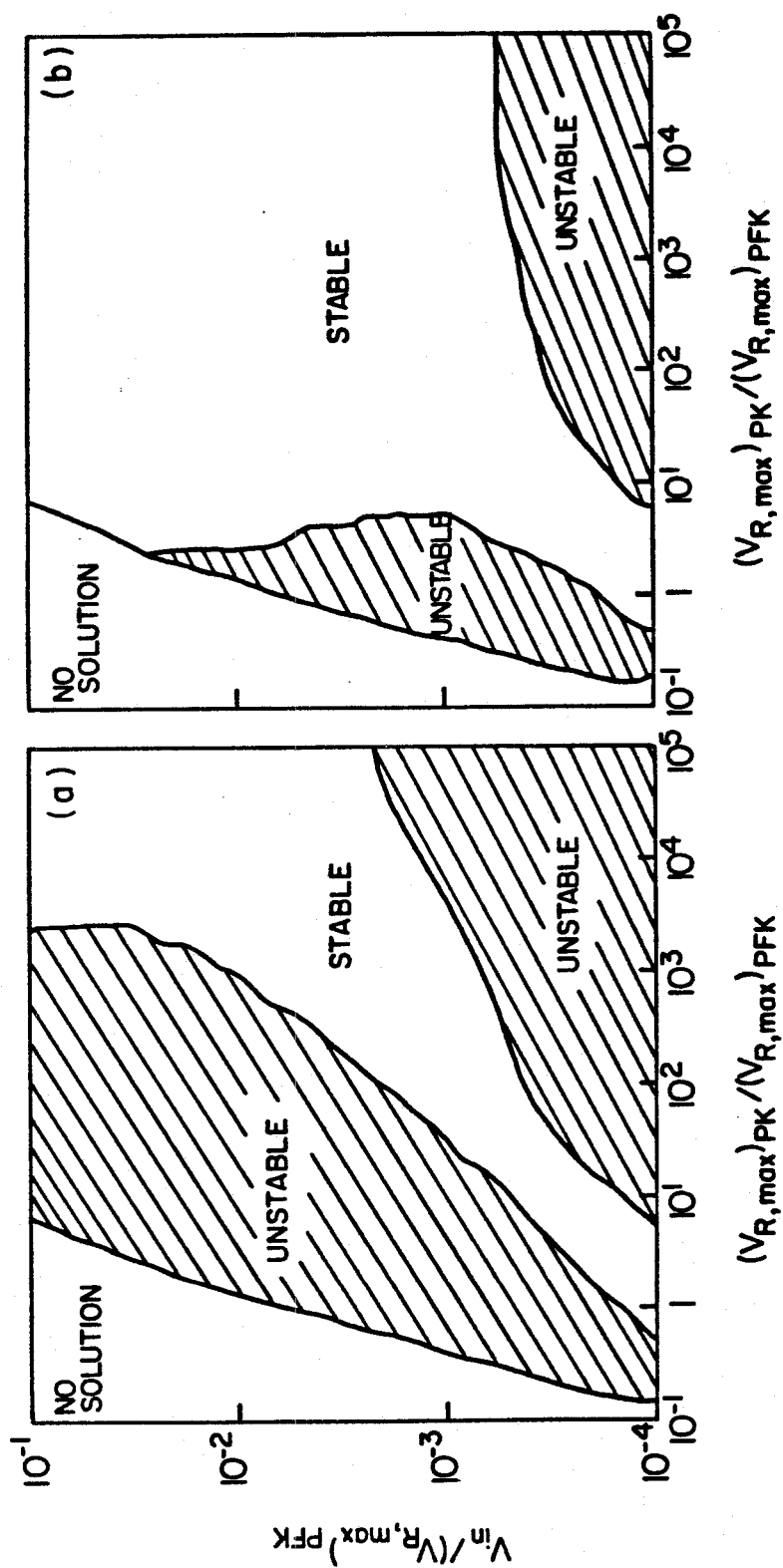


Figure 6-51

Domains of stability as a function of initial NADH concentration.

(a) $[NADH] = 0.06$ mM; (b) $[NADH] = 0.15$ mM.

The other adjustable parameters took the following values: $AP = 5$ mM, $AN = 3$ mM; $k_2 = 0.5$ s $^{-1}$

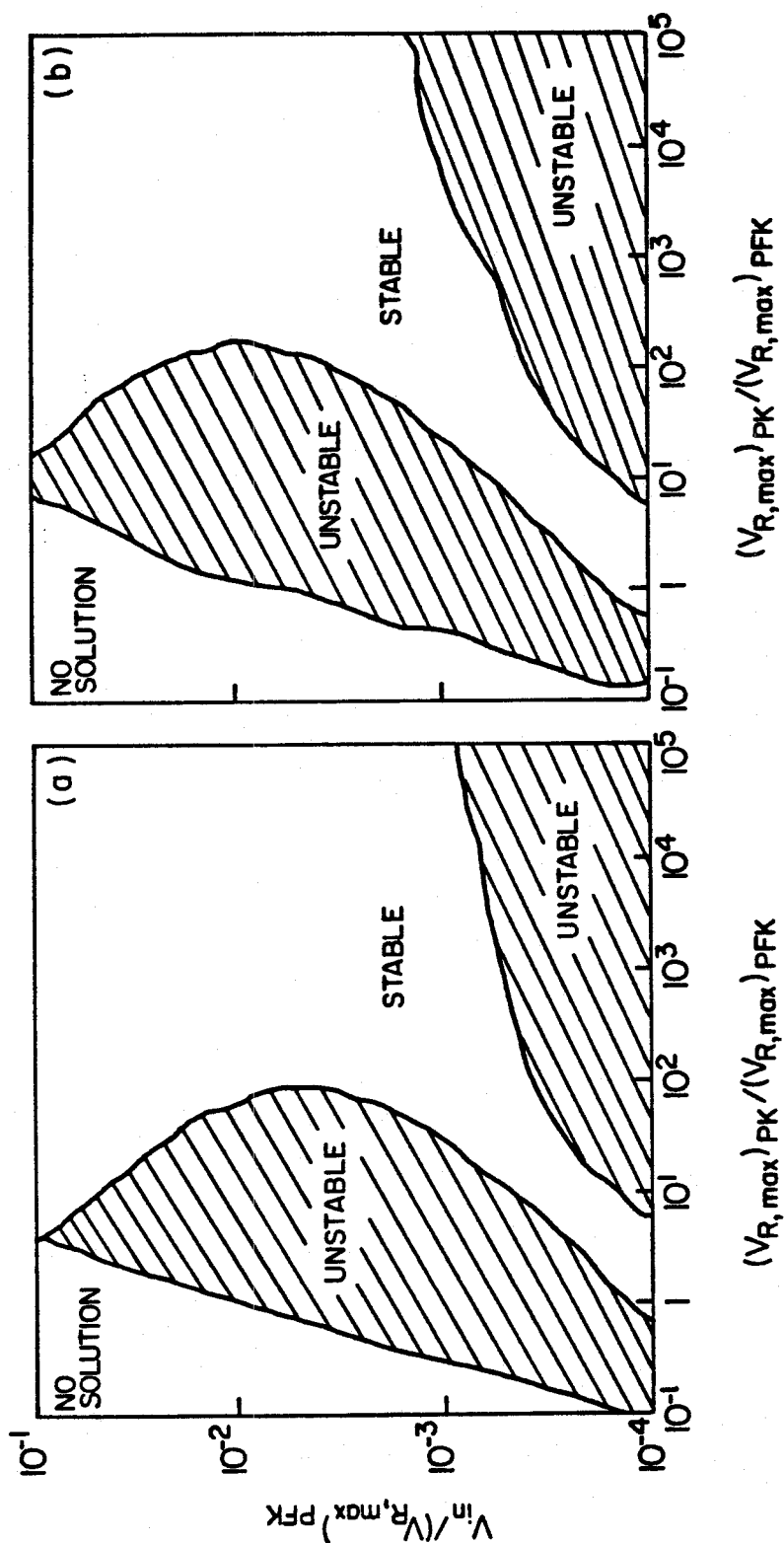


Figure 6-52

Domains of stability as a function of total adenosine phosphate concentration.

(a) $AP = 10 \text{ mM}$; (b) $AP = 5 \text{ mM}$.

The other adjustable parameters took the following values: $AN = 3 \text{ mM}$; $[NADH] = 0.03 \text{ mM}$; $k_2 = 0.5 \text{ s}^{-1}$

It is characteristic for this model that the region of stable steady states be, in part, surrounded by unstable states. For certain values of $(V_{R,max})_{PK}$, it is possible therefore, to leave one oscillatory domain and later enter another by increasing the rate of substrate entry into the system. The maximum value of $(V_{R,max})_{PK}/(V_{R,max})_{PFK}$ at which this can occur is generally of the order of 10^2 . However, the steady state NADH concentration is shown to markedly affect the limits of this transition in Figure 6-51.

The time evolution of NADH was investigated by perturbing the model system from several of the unsteady states indicated in the figures. Although both damped and undamped sinusoidal oscillations were obtained, none of the integrated trajectories showed relaxation characteristics. Further work is required to identify the parameter values which allow the model to produce oscillations of the type generated in experiments with immobilized yeast.

6.5.3 Discussion

Two significant differences in the dynamics of immobilized and suspended cells were revealed by measurements of NADH oscillations. First, endogenous metabolism by immobilized cells generated oscillations of the glycolytic intermediates, while starving suspended cells exhibited only stable behaviour. In addition, under conditions giving rise to sinusoidal variations of NADH level in suspended cells, immobilized cells produced relaxation oscillations.

The oscillations of glycolysis in the absence of external substrate (Figures 6-46 and 6-48) suggest that immobilized cells mobilize intracellular reserves of carbohydrate as a response to starvation conditions. This may follow from the fact that immobilized cells contain 5-6 times more storage material than suspended cells (Table 6-2). Suspended cells are known to use their reserves only sparingly in the

absence of external nutrient (Sols, et al., 1971); however, the rate of utilization appears to be enhanced for immobilized cells. In yeast, phosphorolytic degradation of glycogen occurs in response to rising levels of AMP as ATP pools become depleted. Since the adenosine phosphate balance is strongly implicated as an important controlling factor in glycolysis, it is possible that replenishment of ATP levels in immobilized cells moves the system into an oscillatory region of response to the incoming glucose 6-phosphate.

Identification of relaxation oscillations with immobilized cells is an important result of this work, and holds much promise in analysis of the regulatory processes in immobilized organisms. Relaxation behaviour is of special interest, since it provides a means for recognition of characteristic sections of the limit cycle.

In a dynamic system with widely varying time constants, motion of the system may proceed so that after initial relaxation of the rapid variables, only slow changes occur. However, if the rapid subsystem of events is not confined to regions of stable steady state, it is possible that during motion of the slow variables, regions of rapid variable instability may be reached. If this happens, the system jumps into another rapid variable steady state, and a discontinuous change, or catastrophe, occurs in the system.

Under relaxation conditions, the time courses of the oscillating reactants display two characteristic shapes. One may be described as a saw-tooth pattern, the other is a rectangular step or meander pattern. The NADH trajectories for immobilized yeast (Figures 6-46 - 6-49) provide evidence of both these features of metabolic catastrophe.

Relaxation oscillations of glycolysis have been reported previously by Hess and Boiteux (1968, 1973). In experiments with cell free extracts of yeast, continuous injections of fructose induced saw-tooth oscillations of NADH. Square wave

motions were detected in a reconstituted system of glycolytic enzymes after continuous addition of glucose or glucose 6-phosphate.

Although possessing all the characteristics of glycolysis, both these experimental systems lack the constraints imposed on intact cell metabolism due to presence of the cell membrane. In general, sugar uptake processes limit the rate at which cells can respond to changes in glycolytic flux (Betz and Moore, 1967; Pye, 1969). It is significant then, that relaxation kinetics were found only with immobilized cells in this study, since an enhanced capacity for substrate uptake was demonstrated in other experiments with the gelatin-attached yeast (Table 6-1).

Observations of relaxation kinetics provide the opportunity for identification of the rapid reactions of metabolism which are usually concealed during dynamic transitions. It is also possible from theory to recognize the points at which the system switches from one state to the other. However, the model of glycolysis developed in this work has not provided the information necessary in order to explain the mechanism of relaxation behaviour in immobilized cells. It is possible that choice of different parameter value combinations may give rise to this type of dynamic response.

Specific model requirements for relaxation oscillations are discussed by Dynnik, et al (1973), Sel'kov and Betz (1973) and Heinrich, et al. (1977). Oftentimes, the necessary interactions between elements of the model systems cannot be realized in the cell (Heinrich, et al., 1977). Many models which otherwise describe the dynamics of glycolysis may have limited potential in simulation of relaxation behaviour.

The emergence of rapid variable instability raises questions about the applicability of steady state approximations for rapid subsystems of biological processes. Under relaxation conditions, modelling of the fast subsystem of variables may

require consideration of those rapid reactions usually neglected in analysis of metabolism. It is possible that application of steady state approximations for these processes distorts the simulated stability properties of the rapid variable stationary states.

In view of the findings presented here, immobilized cell dynamics deserve further examination. Since the appearance of sudden catastrophes in metabolism are not common, their occurrence in immobilized cells suggests prior adaptation of the cells to specialized functions associated with rapid motions. This hypothesis is consistent with other results from this study which show enhanced rates of sugar consumption and energy metabolism in immobilized yeast. Identification of the differences between immobilized and suspended cell kinetics should be possible after careful mathematical analysis of the control features of glycolysis.

CHAPTER 7

CONCLUSIONS

This work shows that major shifts in metabolism can occur following immobilization of cells. Altered patterns of microbial growth, activity and regulation are clearly indicated. The results emphasize that information about cell properties and fermentation behaviour derived from studies of suspended microorganisms should be applied to immobilized cells with caution. The possibility of large changes in intrinsic kinetics and stoichiometry must be investigated when designing and applying immobilized cell biocatalysts.

The measured properties of immobilized *Saccharomyces cerevisiae* suggest that some of the differences between immobilized and suspended cells may stem from disturbance of normal cell cycle operation. Large amounts of DNA and cell wall polysaccharide were found in the immobilized cells, although growth was limited. It is postulated that cell-solid interactions interfere with bud initiation and development, while DNA and cell wall synthesis continues independently. This hypothesis of cell cycle disturbance remains largely speculative in the absence of more information about immobilized cell cycle operation.

A comparison of immobilized cell and suspended cell behaviour is provided by the results of this study. Conclusions drawn from the work are outlined below, together with recommendations for future investigation.

7.1 FERMENTATION CHARACTERISTICS AND CELL COMPOSITION

The following description of immobilized cells represents the outcome of a series of experiments with *Saccharomyces cerevisiae* immobilized on the surface of

glutaraldehyde-crosslinked gelatin-coated glass beads.

7.1.1 Fermentation of Complete Nutrient Medium

1. Immobilized cells consume glucose at twice the suspended cell rate.
2. Specific ethanol productivity is 40-50% higher with immobilized cells, although ethanol yields are lower.
3. The specific growth rate for immobilized cells is about one-half that for suspended cells.
4. Cell yield from glucose for immobilized cells is less than 30% that with suspended cells.
5. Immobilized cells contain elevated quantities of storage and structural polysaccharides.
6. The average immobilized cell size is smaller than the size of unbudded suspended cells.
7. Immobilized cells contain, on average, 3.8 times the DNA content of exponentially-growing suspended cells. A substantial proportion of immobilized cells appear to contain in excess of six copies of the genome.
8. Stable doubled-stranded RNA levels in immobilized cells are reduced to about 25% of the suspended cell content.
9. Average protein levels for immobilized cells are reduced to two-thirds those found in suspended cells.
10. The ability of immobilized cells to form colonies on agar is limited compared with suspended cells.

7.1.2 Fermentation with Hydroxyurea Inhibition

1. Immobilized cells are able to overcome hydroxyurea-induced inhibition of DNA replication more quickly than suspended cells.
2. The average DNA content for hydroxyurea-treated immobilized cells is over twice that for inhibited suspended cells.
3. After hydroxyurea-inhibition, the level of stable double-stranded RNA in immobilized cells is 25% that measured for suspended cells.
4. Immobilized cells synthesize protein on exposure to hydroxyurea to a greater extent than is found for suspended cells.
5. Hydroxyurea-inhibited immobilized cells contain approximately 15 times more polysaccharide than similarly treated suspended cells.
6. Immobilized cell ethanol productivity increases by an average of 24% after hydroxyurea treatment. For suspended cells, fermentation rates with hydroxyurea are up to three times higher than in the absence of inhibitor.
7. The increases in ethanol productivity for immobilized and suspended cells under the influence of hydroxyurea are paralleled by enhanced glucose utilization rates.

7.1.3 Fermentation with Biotin Starvation

1. The morphology of biotin-deficient immobilized cells is unusual in that localized swelling occurs on the surface of the cells.
2. Glycerol is produced at the expense of ethanol during fermentation by both immobilized and suspended cells in the absence of biotin.

3. Biotin-starved immobilized cells contain approximately the same quantity of DNA as starved suspended cells.
4. RNA and protein contents for biotin-starved immobilized cells are reduced compared with suspended cells also cultured without biotin.
5. Biotin-deficient immobilized and suspended cells are approximately the same size.

7.2 IMMOBILIZATION BY ATTACHMENT TO CONCAVALIN A

1. Yeast cells may be immobilized on Sepharose beads by concanavalin A linkage.
2. Desorption of cells is a problem in saccharide solutions.
3. Cell detachment is minimized at $\text{pH} < 5$.

7.3 GLYCOLYTIC OSCILLATIONS

1. Relaxation oscillations are produced by immobilized cells, while suspended cells generate sinusoidal waveforms under the same conditions.
2. NADH oscillations occur in starved immobilized cells without addition of glucose. Similar changes in NADH level are not apparent with resting suspended cells.
3. The character of immobilized cell oscillations changes with a decrease in temperature from 25°C to 18°C .

7.4 SUGGESTIONS FOR FUTURE WORK

Suspended cell properties should not be assumed when working with immobilized cell biocatalysts. All aspects of cell metabolism need to be reassessed in order to properly characterize immobilized cells. The results described here suggest that attention be directed towards the following aspects of cell functioning:

1. Substrate Uptake: Glucose consumption by immobilized cells occurred at a faster rate than with suspended cells. It is unclear whether a generalized increase in membrane permeability facilitated transport of substrate into the cell, or whether the activity of the carrier specific for glucose was enhanced. Experiments with other sugars which do not share the same permease, such as galactose, may reveal the mechanism for the observed rate improvements. Measurement of substrate uptake rate by immobilized and suspended cells can be made directly using radioactively labelled sugars.

2. Ploidy: Enhanced ethanol productivity and polysaccharide storage were coupled in immobilized cells with higher DNA contents. It is not known if ploidy directly affects glucose uptake rates and energy metabolism, or whether these properties of immobilized cells are unrelated. An extension of this work with immobilized polyploid strains of yeast may provide better understanding of the consequences of immobilization.

3. Cell Cycle: The cell cycles of immobilized cells need to be studied, and the influence of attachment on their normal operation determined. Evidence suggesting a decline in budding frequency was obtained in this work with immobilized yeast. Experiments using synchronized populations of immobilized cells to follow the time course of daughter cell production would provide more details of the differences between immobilized and suspended cell reproductive cycles.

4. *RNA Content:* Throughout all experiments with immobilized cells, stable double-stranded RNA levels were appreciably reduced compared with suspended cells. The experiments with biotin starvation show that this result is independent of the tendency of immobilized cells to accumulate DNA. At the present time, it is unclear whether RNA synthesis is impaired in immobilized cells or whether denaturation of RNA occurs. A study of the ribosomes of immobilized cells may provide the necessary information about RNA activity and protein synthesis to explain the measured effects of immobilization on RNA content.

5. *Glycolytic Oscillations:* The dynamic properties of immobilized cell metabolism warrant further investigation in view of the experimental findings described in this work. Continuation of the mathematical modelling started here is most likely to provide some insight into the occurrence of relaxation behaviour in immobilized cells. A detailed and more systematic experimental study may also show other important differences between immobilized and suspended cell dynamics.

APPENDIX I

Yeast Nitrogen Base Composition

The composition of Difco yeast nitrogen base without amino acids is shown in Table I-1 (Difco Laboratories, 1953). The values listed are the amounts of each component present in 6.7 g of yeast nitrogen base. This quantity of yeast nitrogen base is required to make up 1000 ml of medium.

TABLE I-1

Composition of Yeast Nitrogen Base without Amino Acids

<i>Component</i>	<i>Per 6.7 g</i>
<i>Nitrogen Source</i>	
Ammonium Sulphate	5 g
<i>Vitamins</i>	
Biotin	2 μ g
Calcium Pantothenate	400 μ g
Folic Acid	2 μ g
Inositol	2000 μ g
Niacin	400 μ g
<i>p</i> -Aminobenzoic Acid (Difco)	200 μ g
Pyridoxine Hydrochloride	400 μ g
Riboflavin	200 μ g
Thiamine Hydrochloride	400 μ g
<i>Trace Elements</i>	
Boric Acid	500 μ g
Copper Sulphate	40 μ g

Potassium Iodide	100 μ g
Ferric Chloride	200 μ g
Manganese Sulphate	400 μ g
Sodium Molybdate	200 μ g
Zinc Sulphate	400 μ g

Salts

Potassium Phosphate Monobasic	1 g
Magnesium Sulphate	0.5 g
Sodium Chloride	0.1 g
Calcium Chloride	0.1 g

APPENDIX II

Immobilized Cell Reactor Mixing Experiment

In the analysis of immobilized cell batch fermentation in this work, it is assumed that perfect mixing occurs within the packed column-stirred tank reactor shown in Figures 4-2 and 4-3. In order to verify that this system may be treated as an ideal CSTR, the residence time distribution was determined experimentally.

II.1 Tracer Experiment

Thymol blue was used as an inert tracer dye in the mixing tests. Its concentration in solution was determined by measuring absorbance (Bausch and Lomb Spectronic 21, Rochester, New York) at 430 nm. The linearity of the relationship between tracer concentration and absorbance is shown in Figure II-1.

To set up the experiment, the immobilized cell column was filled with beads and connected to the Bioflo fermenter (New Brunswick, Edison, New Jersey). Water was recirculated through the system at 550 ml/min. A level controller kept the volume of liquid in the reactor constant at 450 ml. A feed stream added water to the fermenter at a rate of 2.4 ml/min.

At time zero, the feed was changed from water to a solution of 0.0395 g/l thymol blue. Samples of effluent were collected every 12 minutes for the first 1.5 hours, and then less frequently for a total of 25 hours. Approximately 2 ml were used for measurement of solution absorbance.

II.2 Experimental Results

The absorbance results and concentrations derived from Figure II-1 are given in Table II-1.

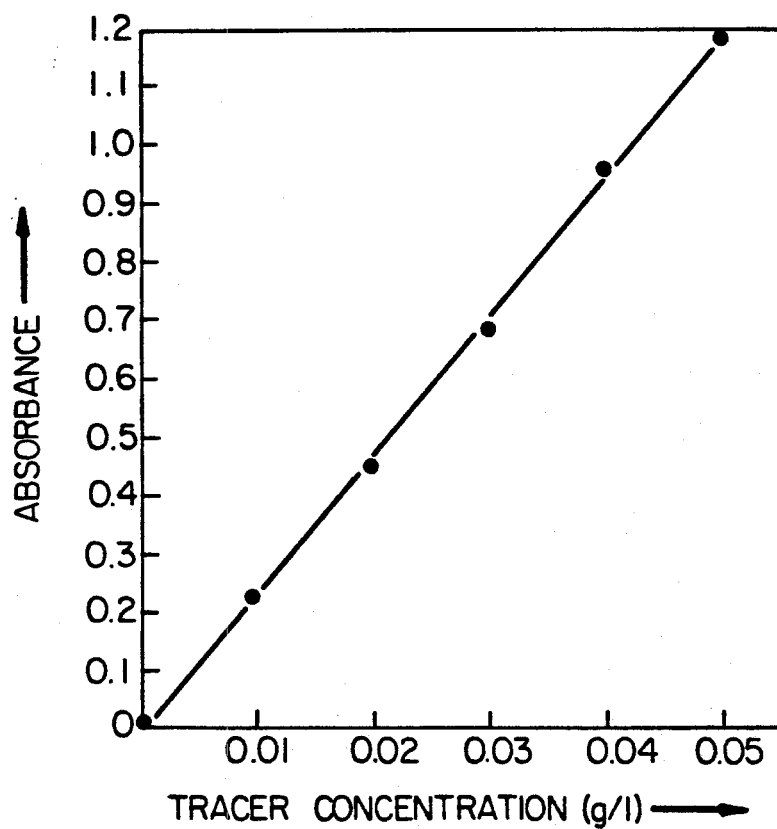


Figure IF-1
Absorbance of thymol blue solutions of different concentration

TABLE II-1

**Absorbance of Effluent from Immobilized Cell Fermenter System
Following Step Change of Tracer Concentration in the Feed**

Time, t (hr)	Absorbance	Concentration, C (g/l)
0	0	0
0.2	0.07	0.0030
0.4	0.08	0.0040
0.6	0.15	0.0068
0.8	0.16	0.0072
1.0	0.28	0.0123
1.2	0.31	0.0135
1.4	0.33	0.0144
1.8	0.40	0.0174
2.2	0.44	0.0190
2.6	0.51	0.0220
3.0	0.55	0.0236
3.4	0.58	0.0250
3.8	0.63	0.0270
4.2	0.69	0.0295
6.3	0.79	0.0337
25.0	0.93	0.0395

II.3 Residence Time Distribution Analysis

The residence time distribution, $E(t)$, is defined as:

$$E(t) = \frac{dF(t)}{dt} \quad (\text{II.3-1})$$

where $F(t)$ is the system response to a step change in tracer feed concentration.

The expression for $F(t)$ is:

$$F(t) = \frac{C(t)}{C^*} \quad (\text{II.3-2})$$

where

C is the concentration of tracer in the exit stream (g/l)

C^* is the feed concentration of tracer (g/l)

t is time (hr)

For a CSTR, a tracer mass balance gives:

$$\frac{dC}{dt} = \frac{F}{V}(C^* - C) \quad (\text{II.3-3})$$

where

F is the feed rate

V is the void volume of the reactor

For a step-up experiment where the initial tracer concentration is zero, integration gives:

$$C(t) = C^*(1 - e^{-(\frac{F}{V})t}) \quad (\text{II.3-4})$$

so that

$$F(t) = \frac{C(t)}{C^*} = 1 - e^{-\left(\frac{F}{V}\right)t} \quad (\text{II.3-5})$$

If the system is not thoroughly mixed due to by-pass or stagnation effects, the effective time constant of the system, $\frac{V}{F}$, is changed. Determination of $F(t)$, therefore, serves to indicate mixing characteristics.

If perfect CSTR mixing occurs, from equation (II.3-5), a plot of $\ln(1 - \frac{C(t)}{C^*})$ versus t , should give a straight line of slope $-(\frac{F}{V})$. Figure II-2 shows this relationship for the column-Bioflo reactor system. The slope is -0.31hr^{-1} . This compares well with the experimentally measured $-(\frac{F}{V})$ value of -0.32hr^{-1} .

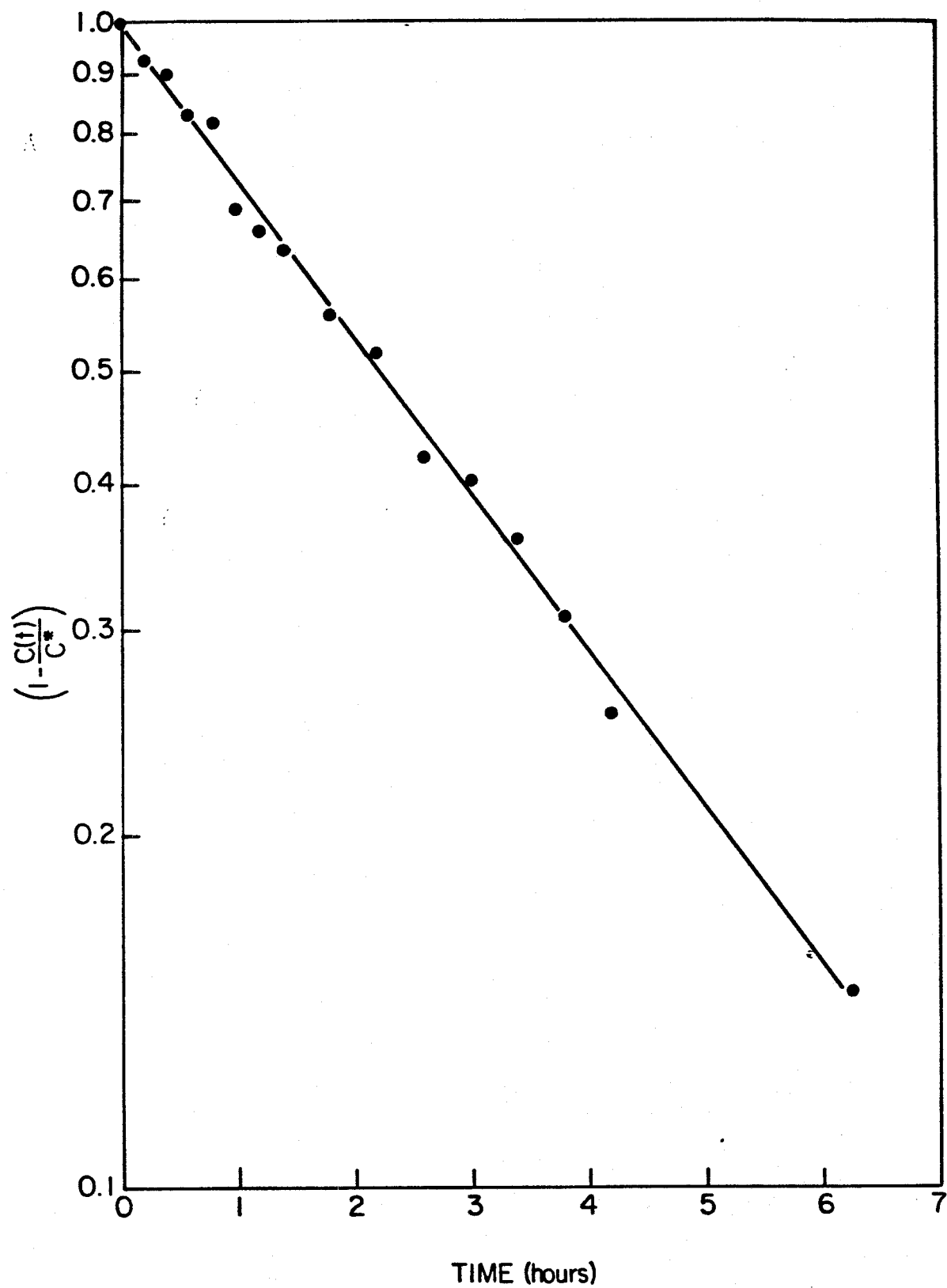


Figure II-2
Analysis of tracer concentrations from immobilized cell reactor mixing experiment

APPENDIX III

Determination of the Specific Rate of Cell Attachment onto Gelatin-Coated Beads

In development of the model for immobilized cell fermentation with complete nutrient medium (Section 5.1.2.2), it is argued that the rate of suspended cell attachment is negligible compared with the rate at which immobilized cell growth increases the loading of the support surface. In terms of equation (5.1-14):

$$\frac{dX_I}{dt} = [1 - \alpha(\frac{X_I}{X_F})]\mu_I X_I + \beta(1 - \frac{X_I}{X_F})X_S \quad (5.1-14)$$

this may be interpreted to mean that the value of $\beta \ll \mu_I$, since $X_I \sim X_S$ and $\alpha \sim 0$. To verify this, β was estimated from the results of a cell loading experiment. Crosslinked gelatin was used as the immobilization support.

III.1 *Experimental Conditions and Theory*

The conditions of the experiment were chosen so that loading of the beads due to proliferation of cells already immobilized could be ignored. The immobilization support was inoculated with a stationary-phase yeast culture which had depleted the supply of substrate in 100 g/l glucose medium. For these resting cells, μ_I and μ_S in equation (5.1-14) become equal to zero. It is assumed that the attachment characteristics of stationary-phase yeast do not differ considerably from those of exponential-phase cells.

A further simplification of equation (5.1-14) can be made, since a very high concentration of suspended cells was used in the inoculum culture. Under these conditions, the change in suspended cell concentration is negligible, even though some cells become attached to the surface of the support beads.

With these simplifications, equation (5.1-14) becomes:

$$\frac{dX_I}{dt} = \beta \left(1 - \frac{X_I}{X_F}\right) X_{sa} \quad (\text{III.1-1})$$

where X_{sa} represents an average X_S . Equation (III.1-1) describes the increase in immobilized cell concentration during loading from a non-growing population of suspended cells. Integration, with $X_I = 0$ at $t = 0$, gives:

$$X_I = X_F \left[1 - e^{\left(-\frac{\beta X_{sa}}{X_F} \right) t} \right] \quad (\text{III.1-2})$$

From equation (III.1-2), measurement of both X_I as a function of time and X_{sa} allows calculation of X_F and β . An experiment was performed by passing suspended cells through a packed bed of gelatin beads. The recirculation flowrate was 550 ml/min, and the temperature, pH and dissolved oxygen concentration in the medium were the same as in the immobilized cell batch fermentations. Samples of beads were removed from the reactor every 30 minutes for analysis of immobilized cell loading. The suspended cell concentration was also monitored.

III.2 Experimental Results

The cell concentrations measured during the loading experiment are listed in Table III-1. These values are also plotted in Figure III-1. As shown in the Figure, the concentration of suspended cells is virtually invariant, with $X_{sa} = 1.71 \times 10^{11}$ cells/l.

The data in Figure III-1 for immobilized cell loading were fitted by equation (III.1-2) using a Marquardt routine. The curve in Figure III-1 shows the best fit of the data. The results from this analysis are:

$$X_F = 8.82 \times 10^9 \text{ cells/l}$$

$$\beta = 0.00598 \text{ hr}^{-1}$$

TABLE III-1

Cell Concentrations during Loading Experiment

Time, t (hr)	X_I (cells/l $\times 10^{-9}$)	X_S (cells/l $\times 10^{-11}$)
0	-	1.77
0.5	0.46	-
1.0	0.95	-
1.5	1.31	-
2.0	1.59	-
2.5	-	1.69
3.0	2.85	-
4.5	3.42	1.62
5.5	5.80	-
6.5	4.44	-
7.5	5.08	-
8.0	-	1.70
8.5	5.30	-
9.5	3.96	-
10.0	-	1.73
10.5	6.07	-
11.5	7.19	-
12.5	7.26	-
13.5	6.96	-
14.5	6.45	-
15.5	7.71	1.74

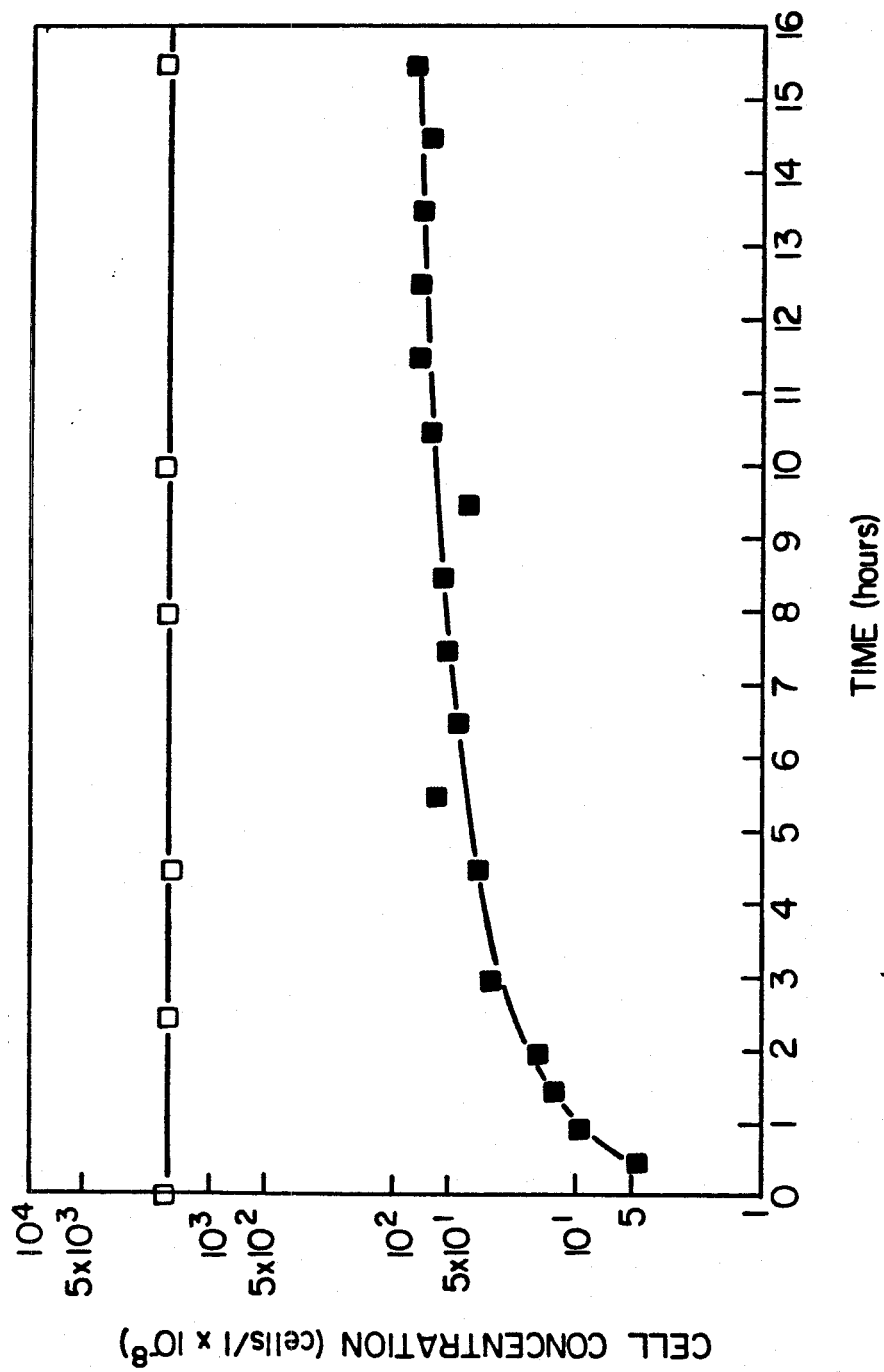


Figure IIF-1
Cell concentration data from immobilized cell loading experiment

III.3 Discussion

The average value for μ_1 measured in experiments with complete nutrient medium is 0.28hr^{-1} (Table 6-1, Section 6.1.2). Since this $\mu_1 \approx 47 \beta$, it is reasonable to assume that $\mu_1 \gg \beta$ under these conditions. The value of β , approximately 0.006hr^{-1} , is considered to be a general result which may be applied to analysis of other experimental data obtained under the same flow conditions.

The shape of the loading curve in Figure III-1 shows that there is a limitation on the number of cells which are able to attach to the support. This result verifies the assumption of Section 5.1.1 that a fully-loaded condition exists for the immobilization surface. When $X_1 = X_F$, the beads are considered to be fully loaded, even though microscopic examination may reveal areas of cell-free surface. It should be noted that the value of X_F is dependent on factors such as the surface characteristics of the support particles and their packing, and has been found to vary considerably from experiment to experiment.

APPENDIX IV

Data from Suspended Cell Batch Fermentations

The raw data from all suspended cell batch experiments discussed in Chapter 6 are listed in the following Tables:

Tables IV-1 - IV-3 Fermentation with complete nutrient medium

Tables IV-4 - IV-7 Fermentation with hydroxyurea

Table IV-8 Fermentation without biotin

TABLE IV-1

**BATCH FERMENTATION 1
SUSPENDED CELLS
COMPLETE NUTRIENT MEDIUM**

Time (hrs)	Concentration			
	Suspended Cell (cells/l x10 ⁻⁹)	Ethanol (g/l)	Glucose (g/l)	Glycerol (g/l)
0.00	2.07	0.743	19.75	0.076
0.45	2.63	0.780	20.01	0.074
0.82	3.25	0.628	-	0.072
1.25	3.91	0.762	20.05	0.085
1.63	4.82	0.800	20.03	0.085
2.08	6.06	0.880	19.90	0.090
2.55	7.66	1.001	19.57	0.112
3.18	11.16	1.144	19.15	-
3.62	12.31	1.205	19.06	0.139

TABLE IV-2
BATCH FERMENTATION 2
SUSPENDED CELLS
COMPLETE NUTRIENT MEDIUM

Time (hrs)	Concentration			
	Suspended Cell (cells/l x10 ⁻⁹)	Ethanol (g/l)	Glucose (g/l)	Glycerol (g/l)
0.00	1.00	0.465	18.54	-
0.17	1.05	0.476	18.77	-
0.35	1.13	0.497	18.68	-
0.55	1.31	0.470	18.63	-
0.73	1.48	0.504	18.65	-
1.00	1.51	0.496	18.61	-
1.18	1.78	0.504	18.53	-
1.60	2.51	0.504	18.47	-
1.90	2.95	0.542	18.35	-
2.27	3.05	0.595	18.43	-
2.60	-	0.568	18.43	-

TABLE IV-3
BATCH FERMENTATION 3
SUSPENDED CELLS
COMPLETE NUTRIENT MEDIUM

Time (hrs)	Concentration			
	Suspended Cell (cells/l x10 ⁻⁹)	Ethanol (g/l)	Glucose (g/l)	Glycerol (g/l)
0.00	1.46	0.541	18.92	0.040
0.17	1.61	0.531	18.97	-
0.33	1.68	0.535	18.86	0.048
0.50	1.88	0.537	18.98	-
0.83	2.23	0.548	18.90	-
1.00	2.38	0.531	18.70	0.055
1.17	2.57	0.569	18.63	-
1.50	3.09	0.589	18.53	-
1.83	3.60	0.604	18.54	0.058
2.17	4.37	0.663	18.49	0.060
2.55	5.10	0.699	18.42	-
2.83	5.85	0.749	18.40	0.072
3.33	7.45	0.783	18.44	0.086

TABLE IV-4

**BATCH FERMENTATION 4
SUSPENDED CELLS
COMPLETE NUTRIENT MEDIUM WITH HYDROXYUREA**

Time (hrs)	Concentration			
	Suspended Cell (cells/l x10 ⁻⁹)	Ethanol (g/l)	Glucose (g/l)	Glycerol (g/l)
0.00	9.87	0.634	18.41	0.036
0.17	10.1	0.640	18.67	-
0.33	9.32	0.693	18.36	0.058
0.50	10.2	0.746	18.49	-
0.67	10.2	0.813	-	-
0.83	10.7	0.853	18.35	-
1.00	11.9	0.859	18.29	0.108
1.17	11.7	1.051	17.45	0.118
1.42	13.4	1.093	17.44	-
1.67	12.9	1.220	16.73	0.162
2.00	14.9	1.440	15.40	-
2.33	14.2	1.604	15.68	0.236
2.67	14.1	1.854	14.62	-
3.17	14.5	2.254	14.64	0.361
3.67	15.5	2.762	12.99	0.442

TABLE IV-5
BATCH FERMENTATION 5
SUSPENDED CELLS
COMPLETE MEDIUM WITH HYDROXYUREA

Time (hrs)	Concentration			
	Suspended Cell (cells/l x10 ⁻⁹)	Ethanol (g/l)	Glucose (g/l)	Glycerol (g/l)
0.00	2.34	0.571	18.61	0.034
0.22	2.40	0.574	18.64	-
0.37	2.39	0.609	18.66	0.041
0.50	2.46	0.610	18.38	-
0.67	2.50	0.582	18.09	0.054
0.83	2.56	0.617	18.06	-
1.00	2.59	0.665	18.22	0.050
1.33	2.62	0.722	17.99	0.066
1.67	2.70	0.711	17.91	0.059
2.00	2.88	0.851	17.84	0.078
2.33	2.92	0.893	17.32	0.089
2.67	2.97	1.025	17.22	0.102
3.00	2.95	1.155	16.83	0.116
3.67	3.19	1.452	16.42	0.148

TABLE IV-6
BATCH FERMENTATION 6
SUSPENDED CELLS
COMPLETE MEDIUM WITH HYDROXYUREA

Time (hrs)	Concentration			
	Suspended Cell (cells/l x10 ⁻⁹)	Ethanol (g/l)	Glucose (g/l)	Glycerol (g/l)
0.00	1.15	0.396	20.05	0.027
0.25	1.19	0.406	19.79	0.029
0.50	1.07	0.441	20.13	0.024
0.75	0.95	0.461	19.39	-
1.00	1.10	0.488	20.17	0.026
1.25	1.13	0.491	19.32	0.028
1.50	1.32	0.496	19.91	0.033
1.75	1.32	0.518	19.61	0.037
2.00	1.22	0.552	19.16	0.045
2.25	1.36	0.565	19.36	0.051

TABLE IV-7
BATCH FERMENTATION 7
SUSPENDED CELLS
COMPLETE MEDIUM WITH HYDROXYUREA

Time (hrs)	Concentration			
	Suspended Cell (cells/l x10 ⁻⁹)	Ethanol (g/l)	Glucose (g/l)	Glycerol (g/l)
0.00	0.92	0.550	20.40	0.036
0.22	0.97	0.573	20.29	-
0.33	0.90	0.588	20.29	0.037
0.50	0.97	0.585	20.09	-
0.67	1.00	0.603	20.07	0.037
0.83	1.07	0.601	20.53	-
1.00	1.06	0.613	20.29	0.040
1.33	1.08	0.631	20.17	0.045
1.67	1.16	0.661	20.48	0.046
2.00	1.10	0.686	20.23	0.063
2.33	1.30	0.731	20.13	0.064
2.67	1.23	0.740	19.50	0.071
3.00	1.34	0.828	19.37	0.083
3.67	1.39	0.897	19.44	0.088

TABLE IV-8
BATCH FERMENTATION 8
SUSPENDED CELLS
BIOTIN-FREE MEDIUM

Time (hrs)	Concentration			
	Suspended Cell (cells/l x10 ⁻⁸)	Ethanol (g/l)	Glucose (g/l)	Glycerol (g/l)
0.00	0.92	0.293	18.11	0.024
0.17	1.25	0.460	17.40	0.017
0.33	1.18	0.469	17.45	0.025
0.50	1.33	0.286	18.52	0.019
0.67	1.16	0.521	19.84	0.024
0.83	1.29	0.280	19.39	0.027
1.00	1.19	0.276	18.31	0.031
1.33	1.22	0.349	17.09	0.036
1.67	1.36	0.390	19.13	0.039
2.00	1.33	0.545	18.26	0.048
2.33	1.48	0.327	18.87	0.061
2.67	1.56	0.312	17.80	0.071
3.00	1.57	0.536	18.11	0.080
3.67	1.58	0.281	19.74	0.110

APPENDIX V

Data from Immobilized Cell Batch Fermentations

The raw data from the immobilized cell batch experiments discussed in Chapter 6 are listed in the following Tables:

Tables V-1	Fermentation with complete nutrient medium
Tables V-3 - V-6	Fermentation with hydroxyurea
Table V-7	Fermentation without biotin

TABLE V-1
BATCH FERMENTATION 9
IMMOBILIZED CELLS
COMPLETE NUTRIENT MEDIUM

Time (hrs)	Concentration				
	Suspended Cell (cells/l x10 ⁻⁹)	Immobilized Cell (cells/l x10 ⁻⁹)	Ethanol (g/l)	Glucose (g/l)	Glycerol (g/l)
0.00	0.76	7.19	0.337	20.35	0.051
0.20	-	-	0.308	20.60	-
0.37	1.02	10.80	0.336	20.22	0.077
0.57	1.27	-	0.421	20.89	-
0.68	1.43	7.52	0.495	20.29	0.080
0.85	1.59	-	0.549	20.19	-
1.00	1.86	8.15	0.666	-	-
1.18	2.21	-	0.683	20.12	0.101
1.33	2.53	-	0.818	18.72	-
1.68	2.92	12.60	0.957	19.30	0.125
2.00	3.98	13.20	1.205	18.35	0.165
2.40	5.55	12.20	1.598	17.82	-
2.75	7.00	13.90	1.867	15.57	0.219
3.00	8.33	12.55	2.200	15.77	0.273

TABLE V-2
BATCH FERMENTATION 10
IMMOBILIZED CELLS
COMPLETE NUTRIENT MEDIUM

Time (hrs)	Concentration				
	Suspended Cell (cells/l x10 ⁻⁹)	Immobilized Cell (cells/l x10 ⁻⁹)	Ethanol (g/l)	Glucose (g/l)	Glycerol (g/l)
0.00	0.95	5.65	0.627	18.94	0.078
0.17	1.02	-	0.633	18.83	-
0.33	1.32	6.01	0.636	18.79	0.088
0.55	1.35	-	0.742	18.98	-
0.67	1.71	8.75	0.735	18.83	0.111
0.83	1.72	-	0.803	18.43	-
1.00	1.93	7.25	0.887	18.58	0.109
1.33	2.18	6.61	1.036	17.93	-
1.67	2.52	9.60	1.183	17.55	0.161
2.00	4.18	8.75	1.386	17.01	-
2.33	3.93	7.25	1.581	15.09	0.182
2.67	5.40	10.53	1.881	16.14	0.235
3.00	6.85	11.29	2.141	15.12	0.265

TABLE V-3
BATCH FERMENTATION 11
IMMOBILIZED CELLS
COMPLETE NUTRIENT MEDIUM WITH HYDROXYUREA

Time (hrs)	Concentration				
	Suspended Cell (cells/l x10 ⁻⁹)	Immobilized Cell (cells/l x10 ⁻⁹)	Ethanol (g/l)	Glucose (g/l)	Glycerol (g/l)
0.00	1.35	10.90	0.522	18.56	0.034
0.20	1.41	-	0.560	18.30	-
0.33	1.40	10.90	0.628	17.48	0.048
0.50	1.54	-	0.682	18.25	-
0.67	1.68	11.90	0.769	18.26	0.077
0.83	1.63	-	0.795	17.86	0.072
1.00	1.76	12.40	0.889	16.97	-
1.17	1.89	-	0.944	-	0.105
1.50	1.87	10.80	1.156	17.03	-
1.83	1.97	12.60	1.438	16.65	-
2.17	2.13	12.20	1.761	15.92	-
2.50	2.35	12.70	2.039	15.93	0.287
3.00	2.60	12.25	2.500	14.41	0.345

TABLE V-4
BATCH FERMENTATION 12
IMMOBILIZED CELLS
COMPLETE NUTRIENT MEDIUM WITH HYDROXYUREA

Time (hrs)	Concentration				
	Suspended Cell (cells/l x10 ⁻⁹)	Immobilized Cell (cells/l x10 ⁻⁹)	Ethanol (g/l)	Glucose (g/l)	Glycerol (g/l)
0.00	1.17	4.85	0.294	19.38	0.049
0.20	1.22	-	0.352	19.35	-
0.33	1.12	4.78	0.387	18.78	0.063
0.50	1.13	-	0.406	19.21	-
0.67	1.26	4.72	0.445	19.09	0.086
0.87	1.05	-	0.460	18.69	-
1.00	1.21	5.56	0.518	18.76	0.103
1.33	1.29	5.22	0.595	18.56	-
1.67	1.28	4.64	0.695	18.09	0.142
2.00	1.40	5.03	-	-	-
2.33	1.32	5.28	0.911	17.54	0.206
2.67	1.23	5.65	1.049	17.02	0.259
3.00	1.39	5.77	1.221	16.34	0.254

TABLE V-5

**BATCH FERMENTATION 13
IMMOBILIZED CELLS
COMPLETE NUTRIENT MEDIUM WITH HYDROXYUREA**

Time (hrs)	Concentration				
	Suspended Cell (cells/l $\times 10^{-9}$)	Immobilized Cell (cells/l $\times 10^{-9}$)	Ethanol (g/l)	Glucose (g/l)	Glycerol (g/l)
0.00	0.20	1.11	0.480	16.85	0.048
0.17	0.21	-	0.509	16.99	-
0.33	0.25	1.22	0.520	16.54	0.056
0.50	0.27	-	0.571	16.54	-
0.67	0.29	1.22	0.574	17.14	0.062
0.83	0.30	-	0.588	17.30	-
1.00	0.34	1.16	0.593	17.02	0.066
1.33	0.33	1.33	0.664	16.70	0.081
1.67	0.39	1.29	0.686	16.77	0.087
2.00	0.40	1.44	0.742	16.59	0.088
2.33	0.41	1.42	0.826	16.40	0.090
2.67	0.43	1.51	0.864	16.01	0.135
3.00	0.46	1.59	0.967	16.08	0.119

TABLE V-6

**BATCH FERMENTATION 14
IMMOBILIZED CELLS
COMPLETE NUTRIENT MEDIUM WITH HYDROXYUREA**

Time (hrs)	Concentration					Time (hrs)
	Suspended Cell (cells/l x10 ⁻⁹)	Immobilized Cell (cells/l x10 ⁻⁹)	Glucose (g/l)	Glycerol (g/l)	Ethanol (g/l)	
0.00	0.21	2.33	17.79	0.050	0.442	0.00
0.17	0.24	-	18.19	-	0.415	0.20
0.37	0.30	2.37	-	0.050	0.461	0.33
0.50	0.29	-	18.01	-	0.448	0.50
0.67	0.33	2.24	18.31	0.062	0.434	0.66
0.83	0.34	-	17.92	0.066	0.530	0.83
1.00	0.34	2.38	17.74	-	0.574	1.16
1.33	0.36	2.80	17.61	0.065	0.658	1.50
1.67	0.38	2.55	17.35	0.092	0.684	1.83
2.00	0.35	2.54	17.28	0.099	0.730	2.16
2.33	0.41	-	16.95	0.109	0.850	2.50
2.67	0.38	3.14	17.06	0.124	0.915	2.83
3.00	0.46	3.44	16.94	0.128	-	-

TABLE V-7

**BATCH FERMENTATION 15
IMMOBILIZED CELLS
BIOTIN-FREE MEDIUM**

Time (hrs)	Concentration				
	Suspended Cell (cells/l x10 ⁻⁹)	Immobilized Cell (cells/l x10 ⁻⁹)	Ethanol (g/l)	Glucose (g/l)	Glycerol (g/l)
0.00	0.92	11.39	0.324	19.43	0.017
0.22	0.90	-	0.345	18.82	0.017
0.37	0.96	8.83	0.329	19.02	0.019
0.50	1.30	-	0.345	19.43	0.024
0.67	1.17	11.82	0.287	20.04	0.019
0.83	1.36	-	0.332	18.67	0.020
1.00	1.36	9.72	0.271	20.35	0.025
1.33	1.50	11.92	0.293	19.48	0.029
1.67	1.65	8.76	0.360	20.55	0.022
2.00	1.89	9.29	0.257	19.18	0.020
2.33	1.78	9.74	0.280	19.23	0.024
2.67	1.97	11.85	0.339	18.82	0.022
3.00	1.91	8.06	0.287	17.04	0.034

APPENDIX VI

Flow Cytometry Results for Immobilized and Suspended Cells Grown on Complete Nutrient Medium

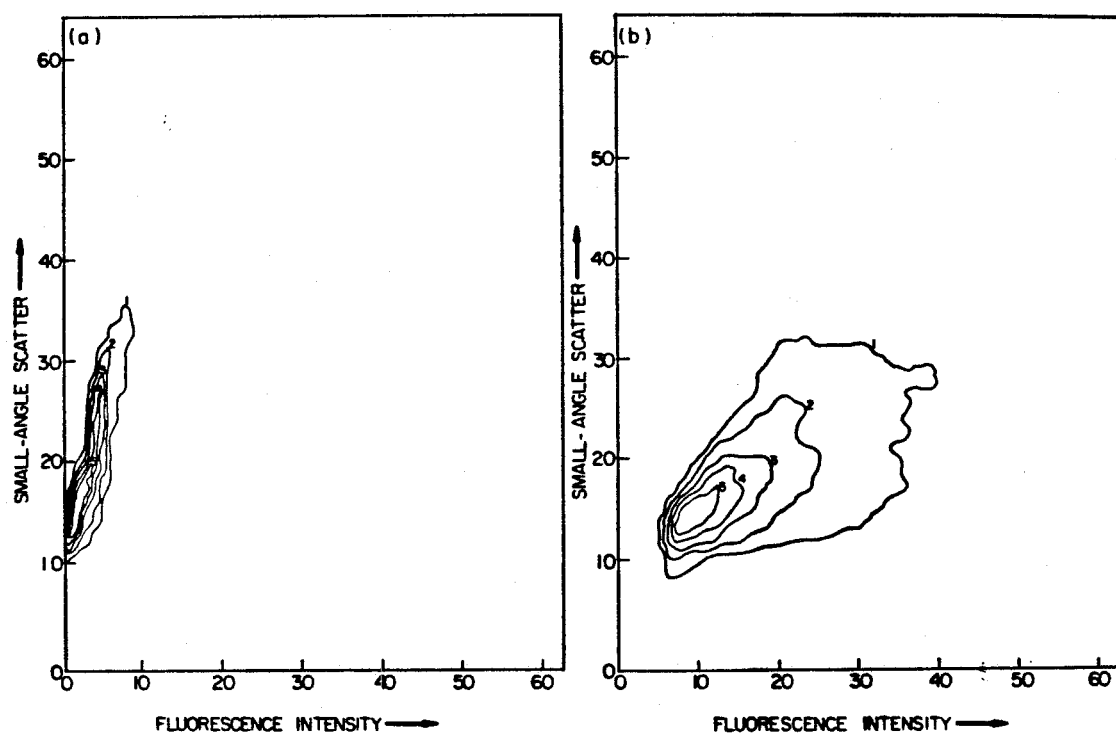


Figure VI-1
Relationship between DNA fluorescence and light scatter level for (a) suspended; and (b) immobilized cells grown on complete nutrient medium.

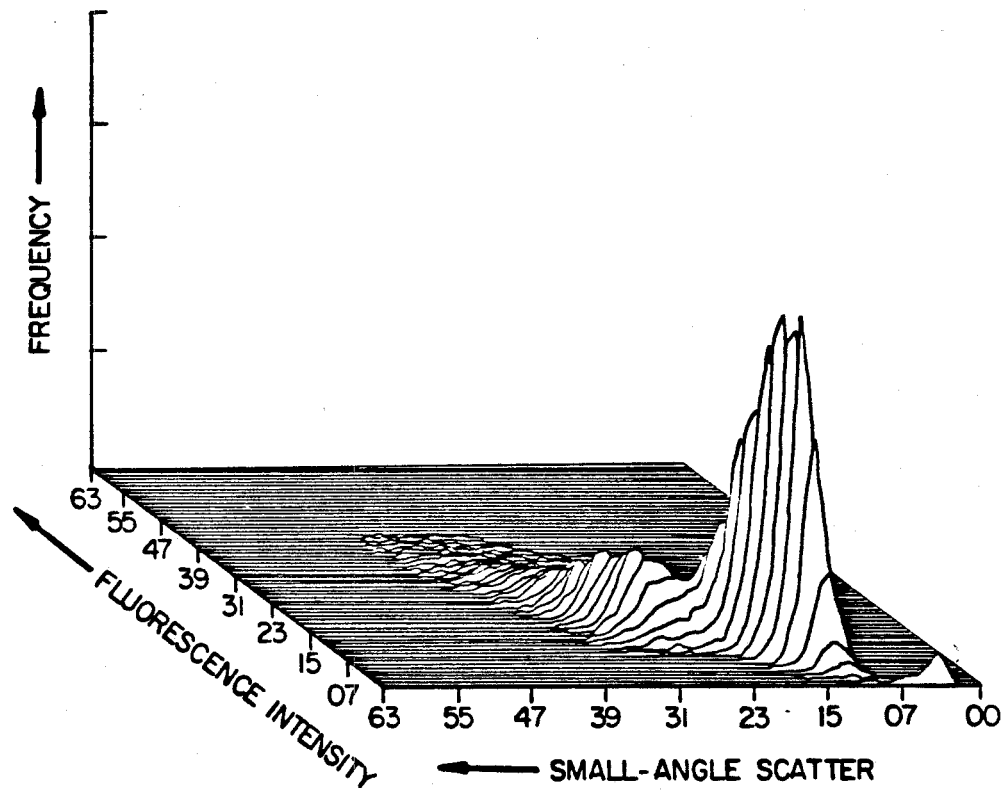


Figure VI-2
Single-cell stable double-stranded RNA frequency functions for suspended cells grown on complete nutrient medium.

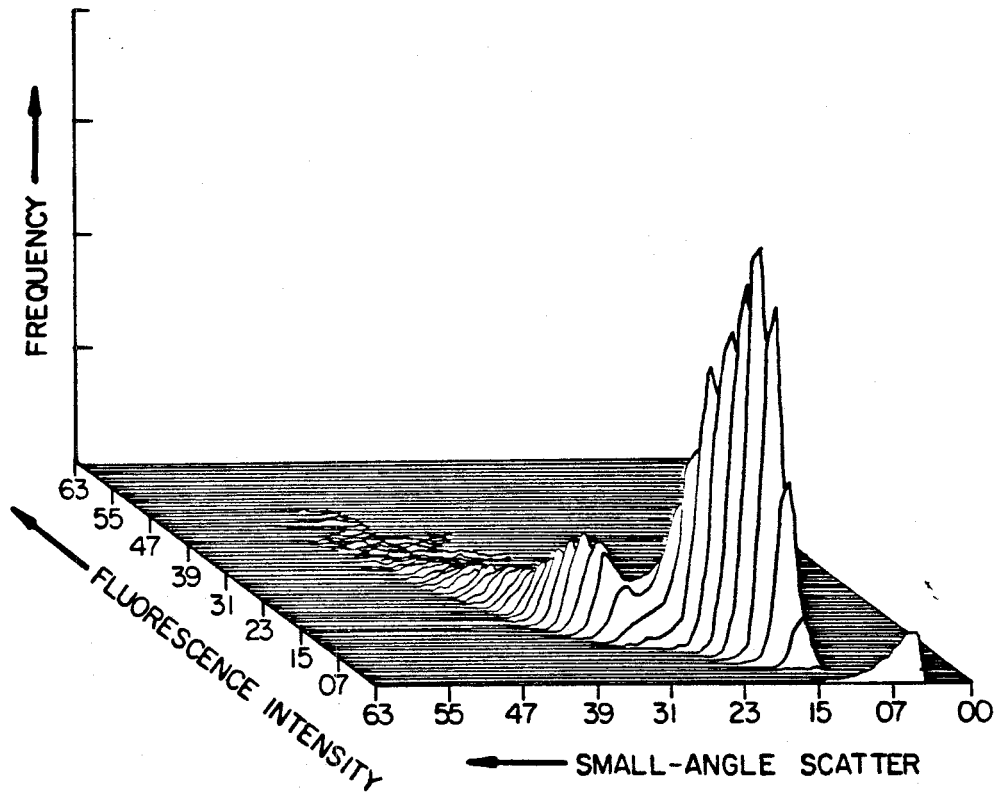


Figure VI-3
Single-cell protein frequency functions for suspended cells grown on complete nutrient medium.

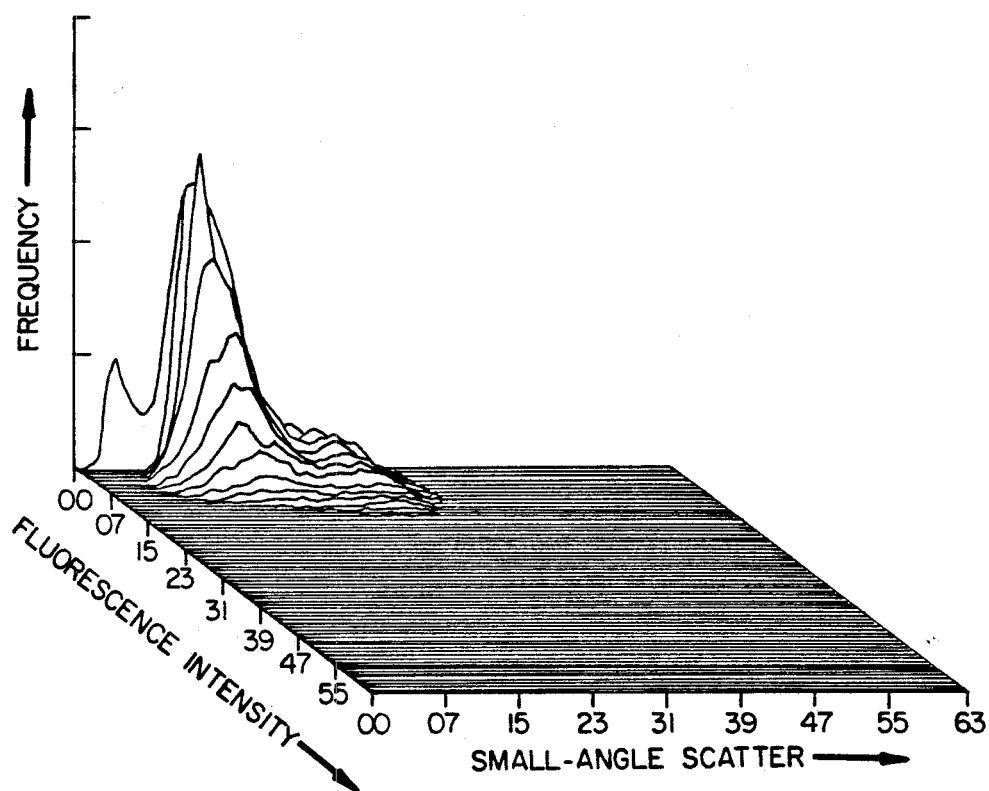


Figure VI-4

Single-cell stable double-stranded RNA frequency functions for immobilized cells grown on complete nutrient medium.

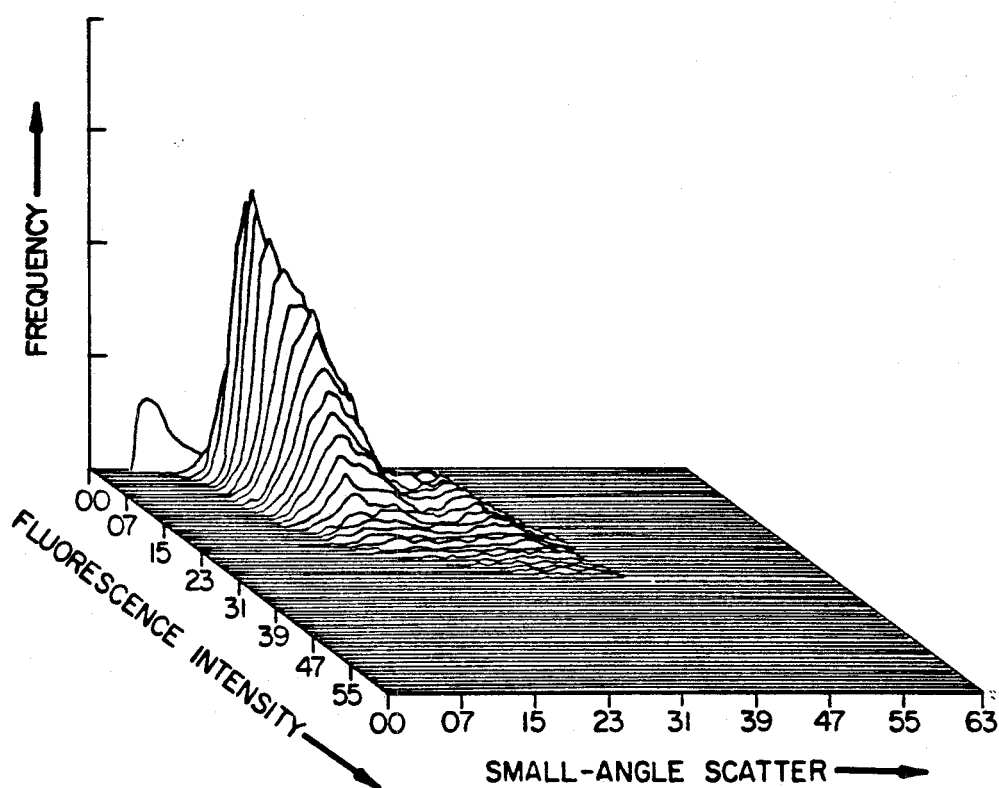


Figure VI-5

Single-cell protein frequency functions for immobilized cells grown on complete nutrient medium.

APPENDIX VII

Flow Cytometry Results for Hydroxyurea-Treated Immobilized and Suspended Cells

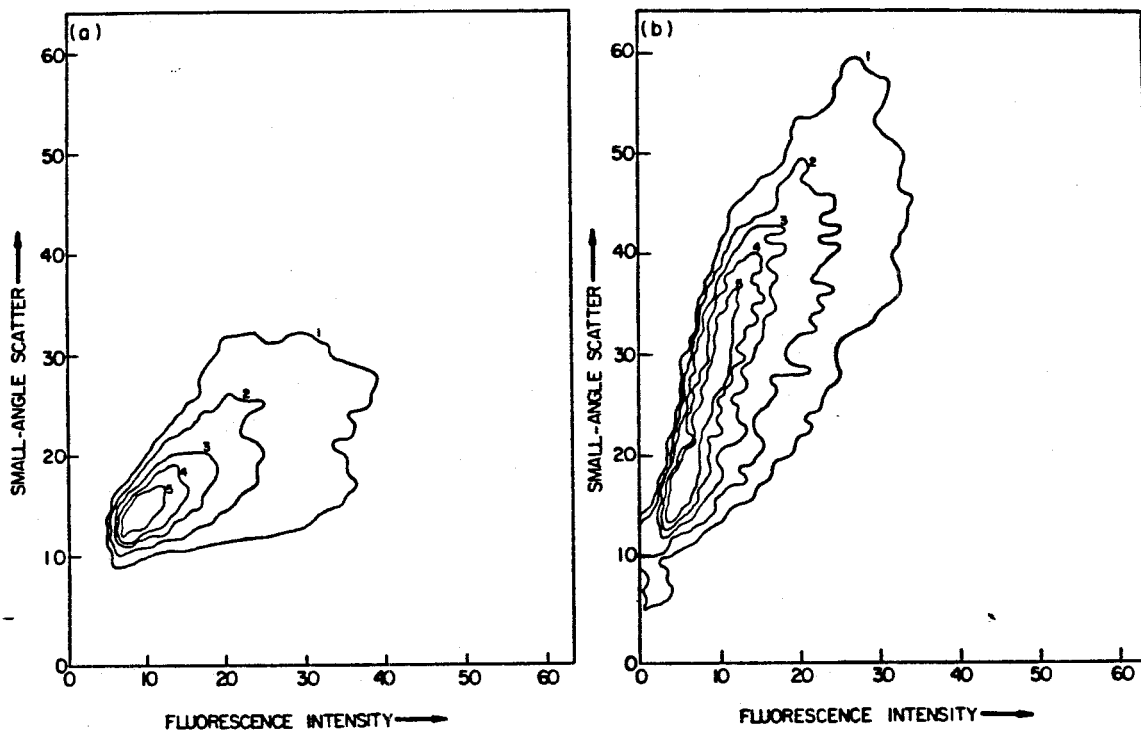


Figure VII-1
Relationship between DNA fluorescence and light scatter level for immobilized cells (a) without; and (b) with hydroxyurea.

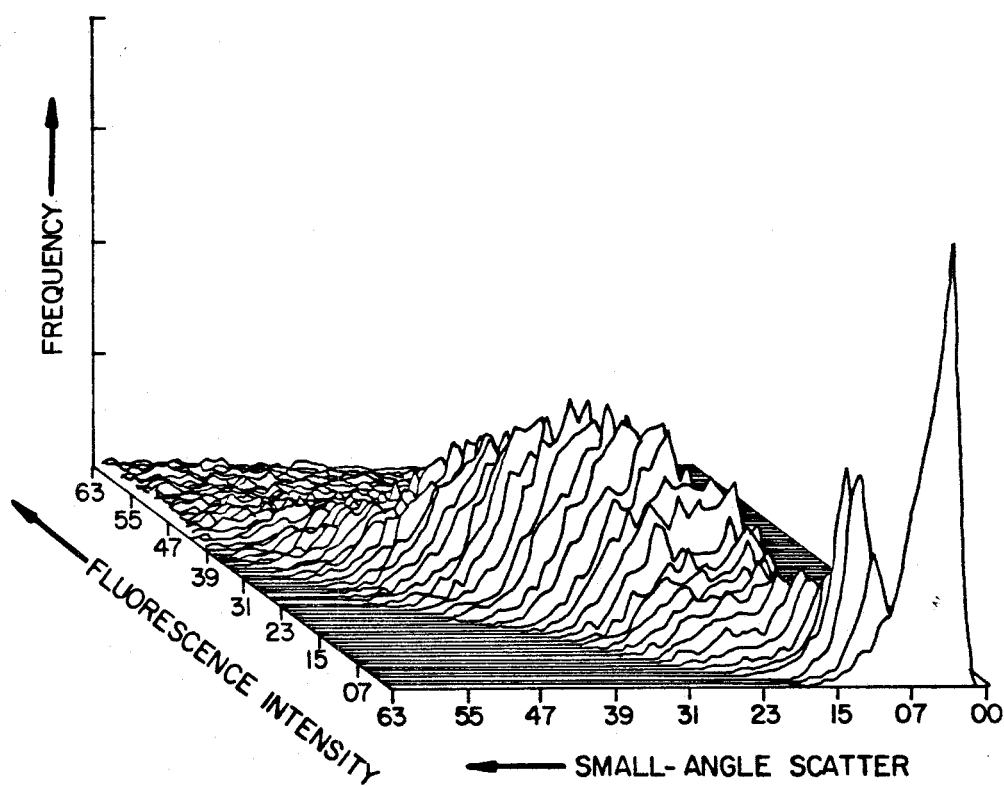


Figure VII-2
Single-cell stable double-stranded RNA frequency functions for suspended cells with hydroxyurea

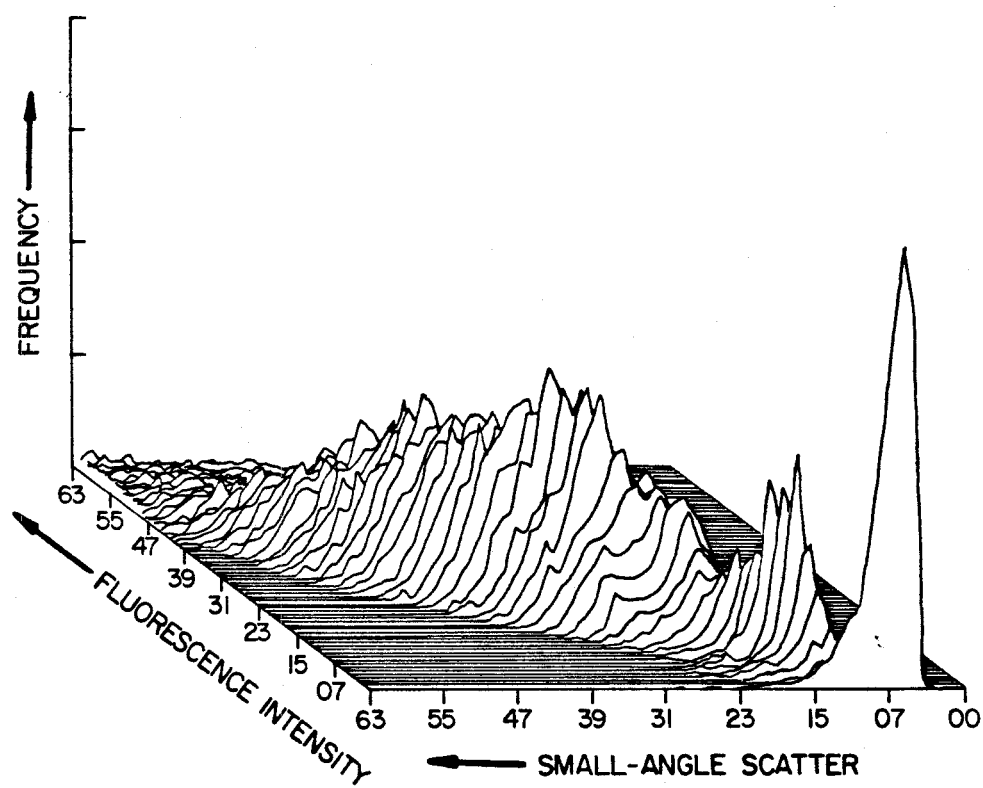


Figure VII-3
Single-cell protein frequency functions for suspended cells with hydroxyurea

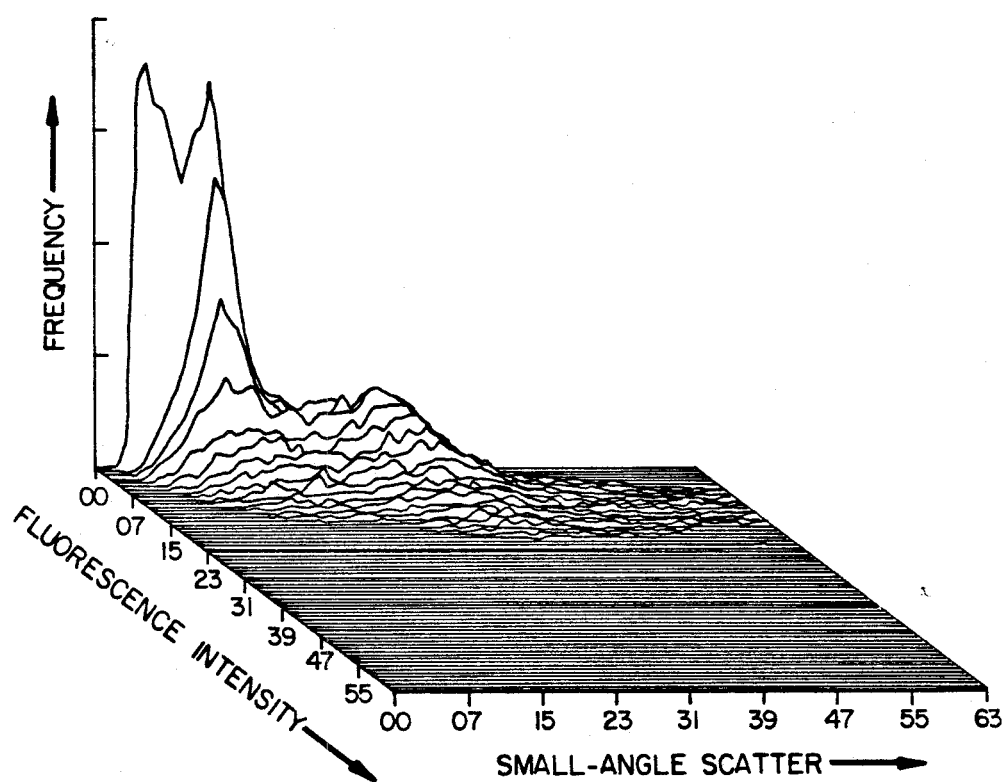


Figure VII-4
Single-cell stable double-stranded RNA frequency functions for immobilized cells with hydroxyurea

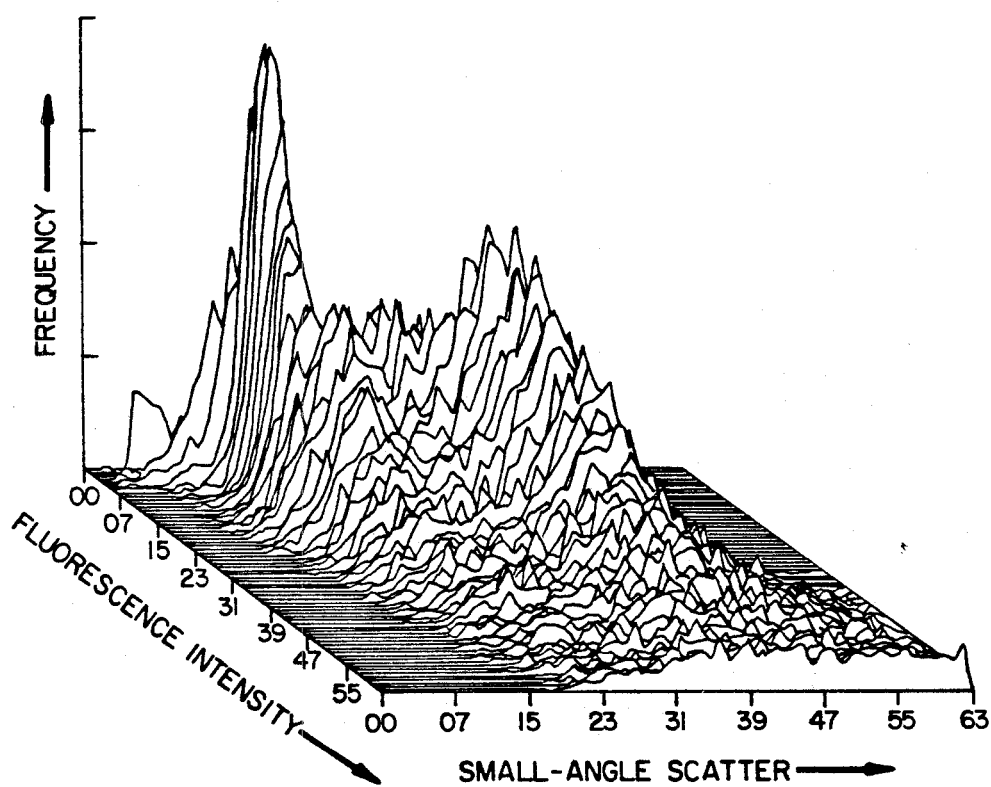


Figure VII-5
Single-cell protein frequency functions for immobilized cells with hydroxyurea

APPENDIX VIII

Flow Cytometry Results for Biotin-Starved Immobilized and Suspended Cells

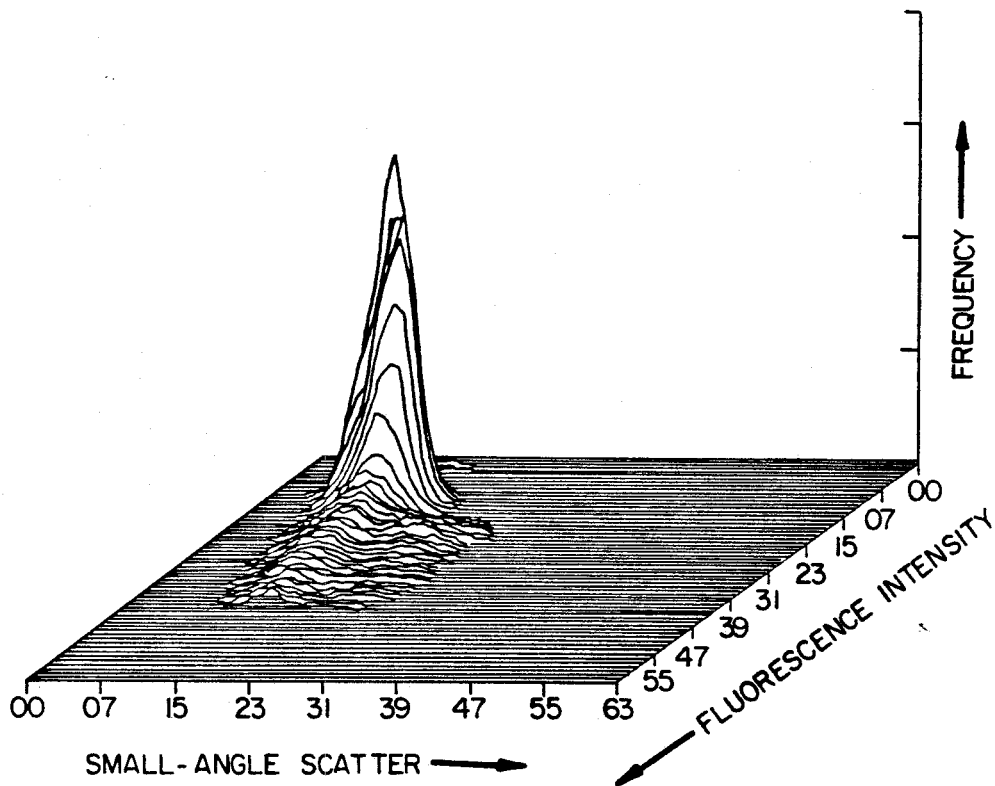


Figure VIII-1
Single-cell DNA frequency functions for biotin-starved suspended cells

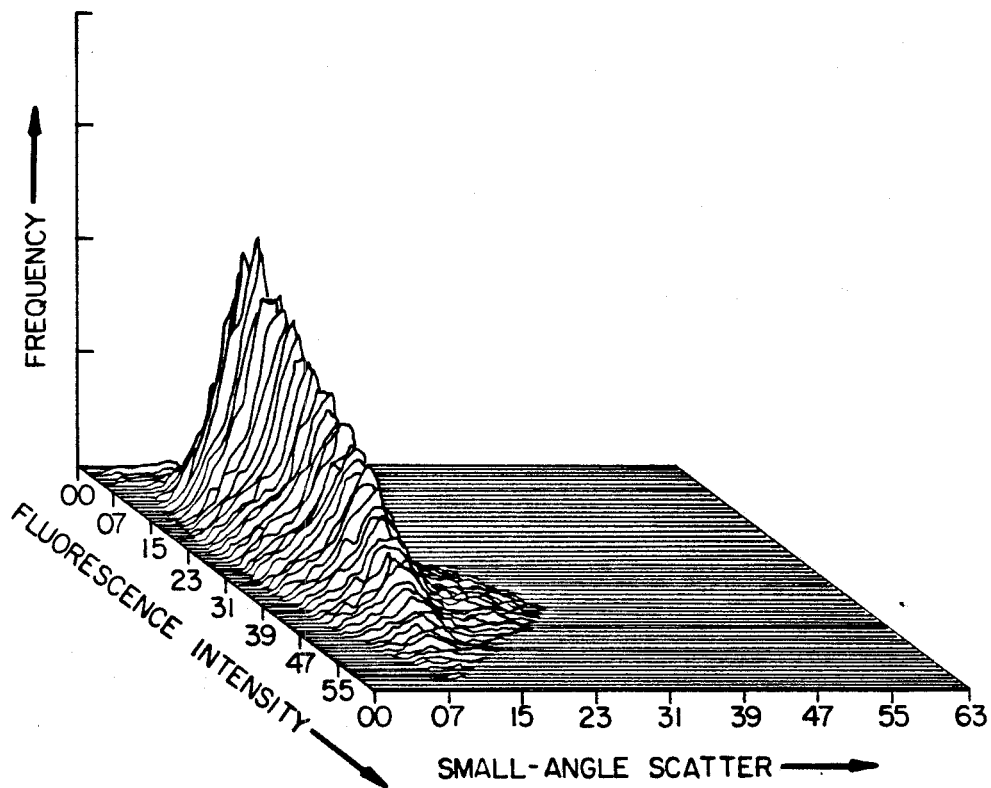


Figure VIII-2
Single-cell stable double-stranded RNA frequency functions for biotin-starved suspended cells

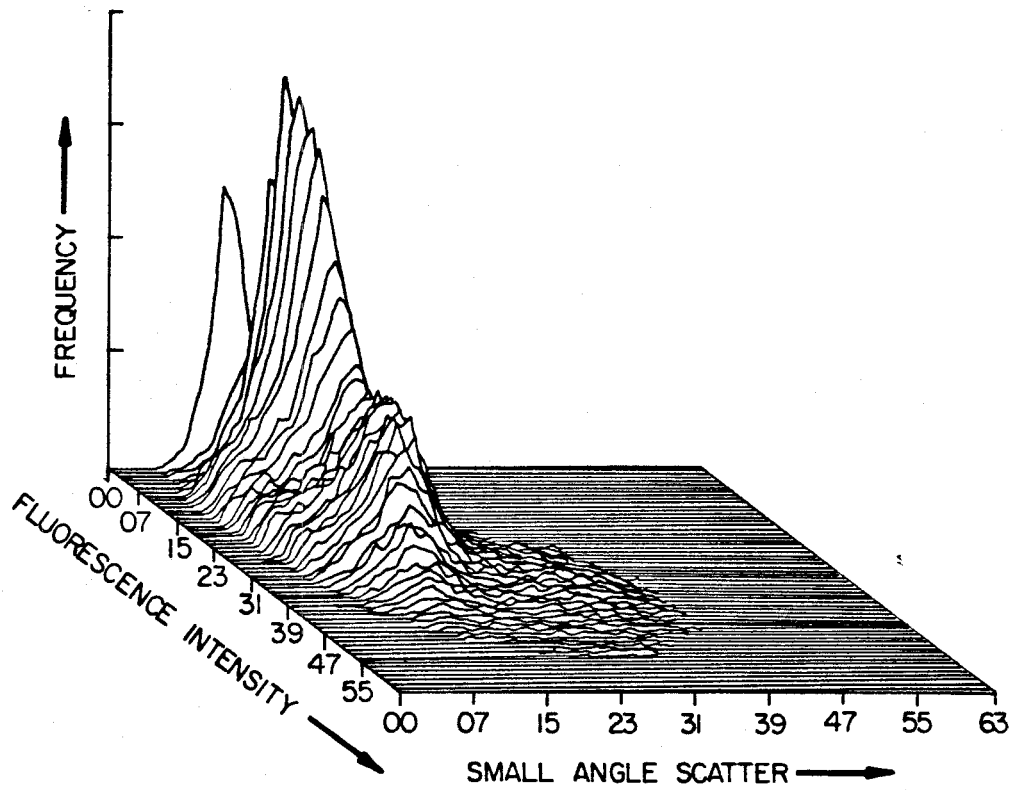


Figure VIII-3
Single-cell protein frequency functions for biotin-starved suspended cells

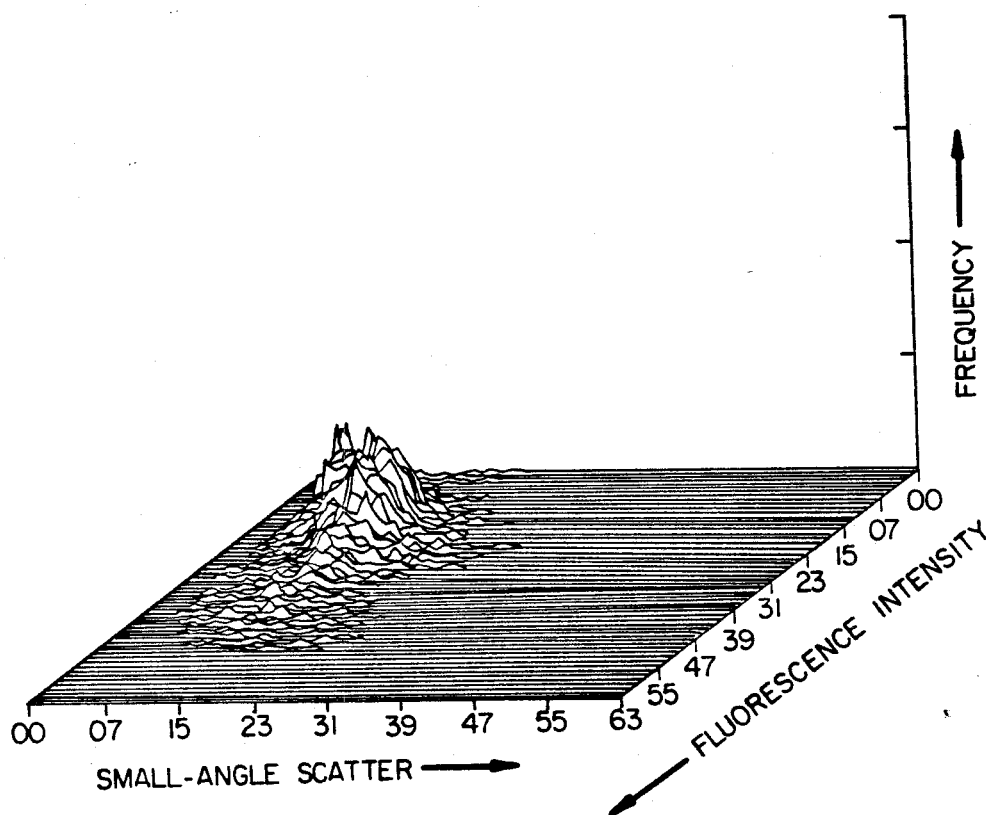


Figure VIII-4
Single-cell DNA frequency functions for biotin-starved immobilized cells

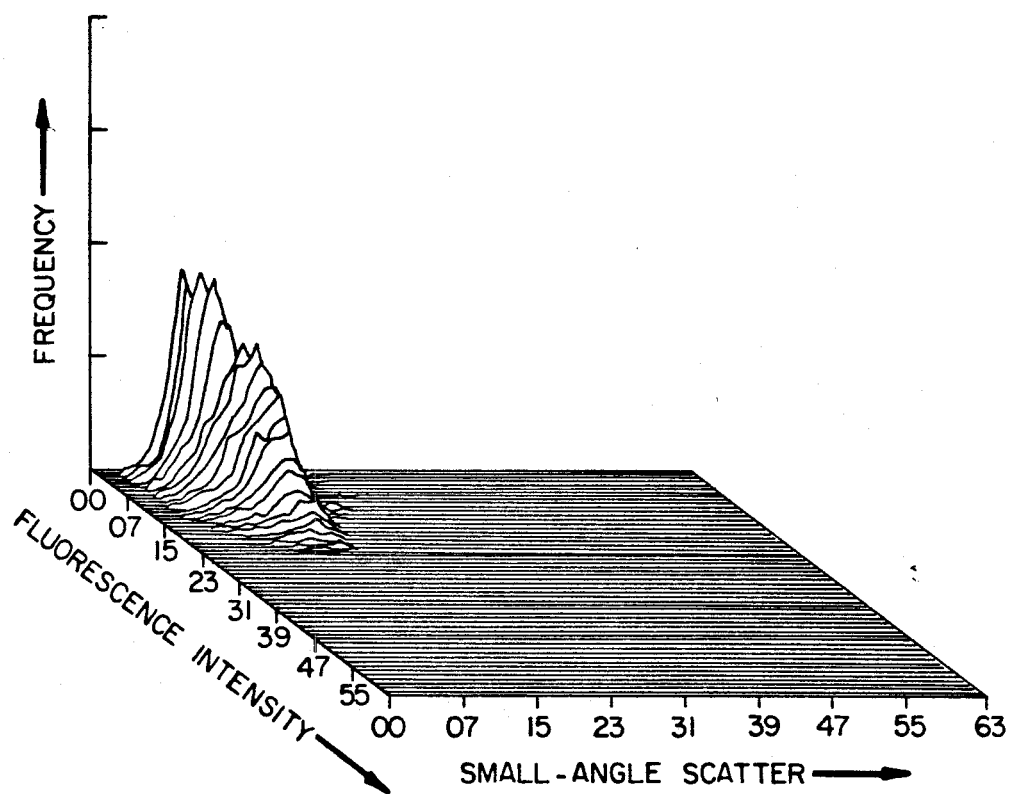


Figure VIII-5
Single-cell stable double-stranded RNA frequency functions for biotin-starved immobilized cells

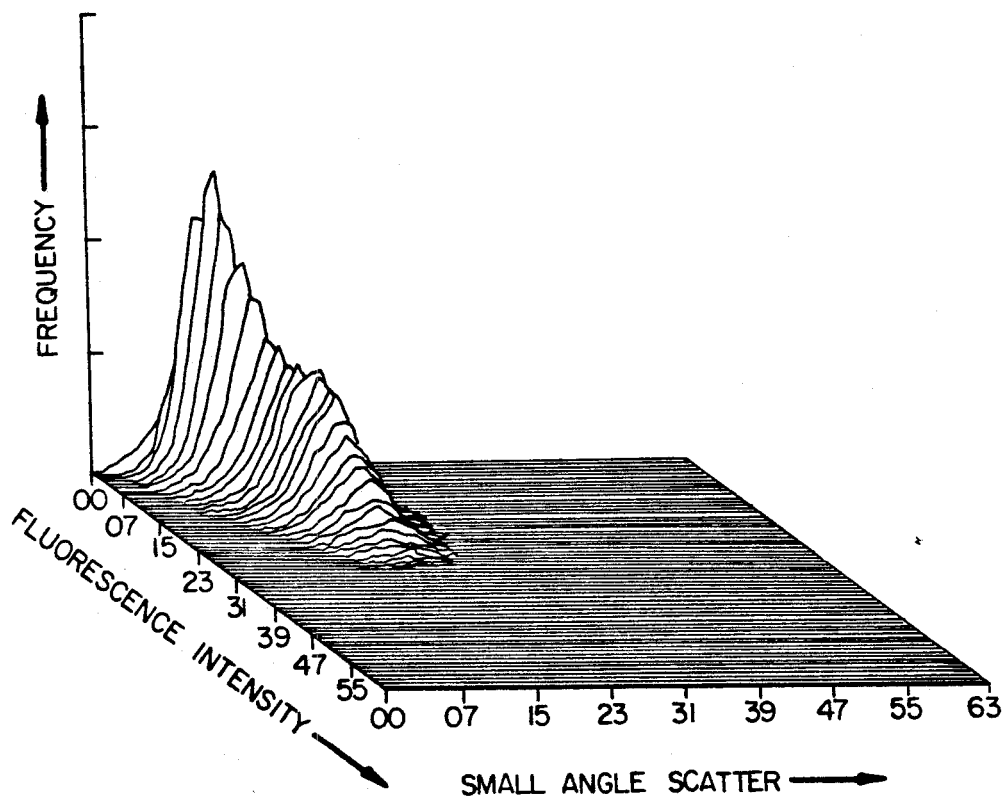


Figure VIII-6
Single-cell protein frequency functions for biotin-starved immobilized cells

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