JPL Publication 91-4

DOE/CS-66001-14
Distribution Categories
UC-61e & UC-61f

AICD

ADVANCED INDUSTRIAL CONCEPTS DIVISION

Catalysis and Biocatalysis Program Annual Report FY 1990

March 1991

Sponsored by

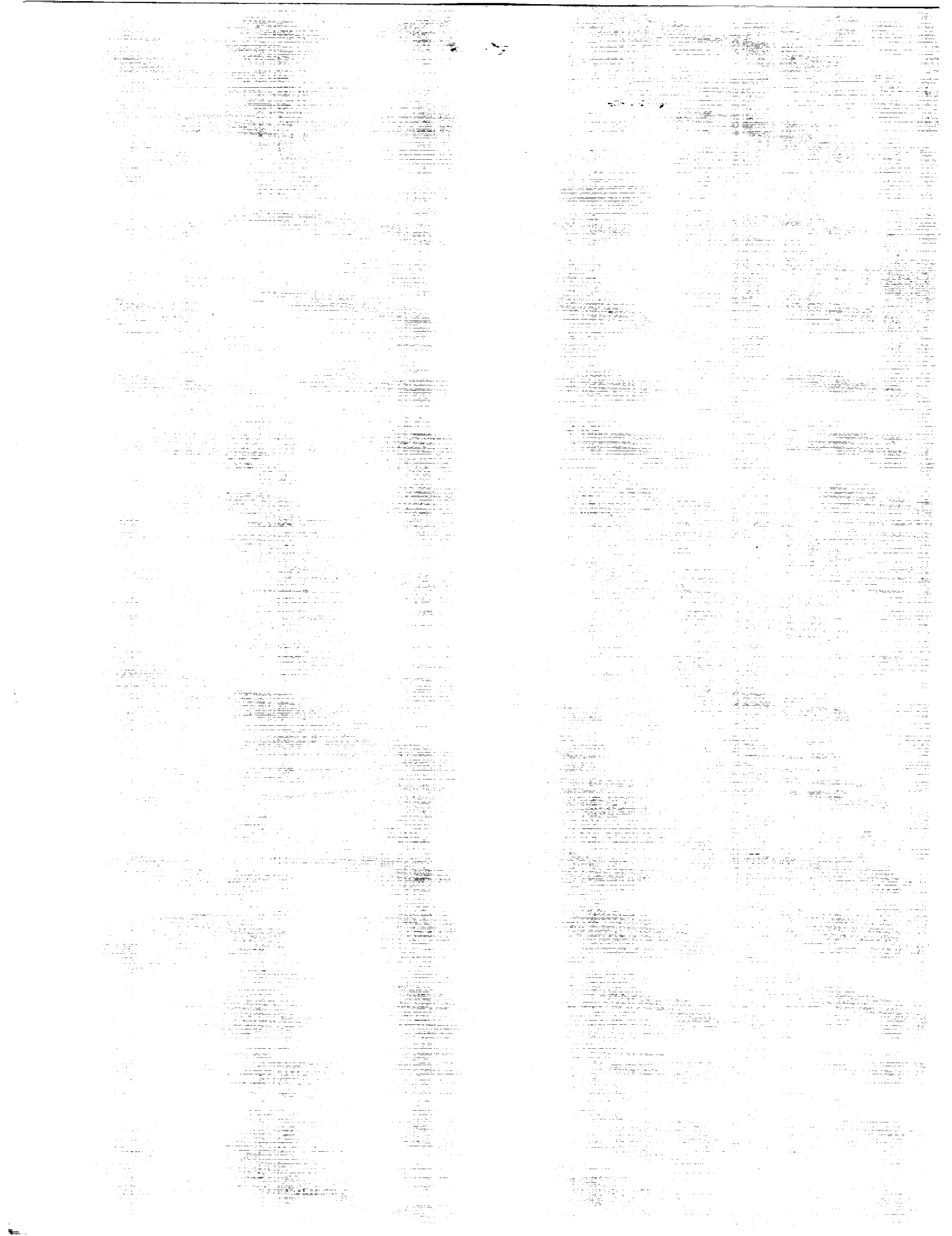
Advanced Industrial Concepts Division
Office of Industrial Technologies
U.S. Department of Energy

Through an agreement with

National Aeronautics and Space Administration

by

Jet Propulsion Laboratory
California Institute of Technology
Pasadena, California



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The Catalysis and Biocatalysis Program is under the direction of DOE Program Management with the Jet Propulsion Laboratory, California Institute of Technology serving as field manager for the United States Department of Energy through an agreement with the National Aeronautics and Space Administration (NASA Task RE-152, Amendment 307; DOE Interagency Agreement DE-A101-86CE90239).

The Catalysis and Biocatalysis Program focuses on resolving the major technical barriers that impede the potential use of biologically-facilitated continuous chemical production processes.

ABSTRACT

The Annual Report presents the fiscal year (FY) 1990 research activities and accomplishments for the Catalysis and Biocatalysis Program of the Advanced Industrial Concepts Division (AICD), Office of Industrial Technologies of the Department of Energy (DOE). The Catalysis and Biocatalysis Program was formerly under the Division of Energy Conversion and Utilization Technologies (ECUT) until the DOE reorganization in April 1990. The mission of the AICD is to create a balanced program of high-risk, long-term, directed interdisciplinary research and development that will improve energy efficiency and enhance fuel flexibility in the industrial sector. Under AICD, the DOE Catalysis and Biocatalysis Program sponsors research and development in furthering industrial biotechnology applications and promotes the integrated participation of universities, industrial companies and government research laboratories.

The Catalysis and Biocatalysis Program's technical activities were organized into five work elements:

The **Molecular Modeling and Catalysis-by-Design** element is designed to study the prediction of the structure of improved chemical and biological catalysts based upon theoretical considerations and to confirm predictions by experimentation.

The **Applied Microbiology and Genetics** element uses recombinant DNA technology to engineer metabolic pathways to improve yield and efficiency of chemicals production by biological systems.

The **Bioprocess Engineering** element examines novel bioreactors for improved operating characteristics and also studies the use of biocatalysts under unusual operating conditions such as organic solvent environments.

The **Separations and Novel Chemical Processes** element is directed toward solving the problems of product recovery from dilute aqueous solutions and the use of biological methods for the recovery of minerals.

The **Process Design and Analysis** element involves the integration of computerized systems for economic and energy assessments of complete bioprocesses, the development of user-friendly software for applications in industrial processes, and the initiation of technology transfer for advanced bioprocesses.

FOREWORD

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SECTION I

EXECUTIVE SUMMARY

The Catalysis and Biocatalysis Program is sponsored by the Office of Industrial Technologies which is within the U.S. Department of Energy's (DOE), Advanced Industrial Concepts Division (AICD). It was formerly under the Energy Conversion and Utilization Technologies (ECUT) Division until the DOE reorganization in April 1990. The Program is focused on applied interdisciplinary research to expand the knowledge base for the development of improved industrial energy conserving technologies, strengthen the foundation upon which innovative and advanced industrial concepts can evolve, and transfer to industry the emerging basic scientific and technical advances which address current technology needs. The Jet Propulsion Laboratory, California Institute of Technology, serves as a technical field manager for the DOE Catalysis and Biocatalysis Program.

The primary objective of the Catalysis and Biocatalysis Program is to resolve the critical technical constraints (e.g., poor productivity, high separation energetics, presence of aqueous medium, lack of design tools) that impede the utilization of biocatalysis for the production of chemicals and materials. To achieve this objective, the Program supports generic applied research which addresses the technological barriers to the commercial utilization of biochemical catalysis.

The Catalysis and Biocatalysis Program consists of five work elements, each addressing a key technical component of the required enabling technology base and defined by its scale of action. The **Molecular Modeling and Catalysis-by-Design** element focuses on the prediction of secondary and tertiary structures based on primary sequences. Such predictive capability will require the development of adequate theories, molecular modeling tools, and experimental verification of theoretical models. The ability to predict and design new biocatalysts would form the basis of new energy efficient technologies. The **Applied Microbiology and Genetics** element is directed towards using new techniques in the recombinant DNA field for the development of new microorganisms. Expected results include organisms that produce higher yields of desired products and which will overcome the problems of low yield and product inhibition, as well as structurally-modified enzymes whose predicted improved properties have been experimentally verified. The **Bioprocess Engineering** element emphasizes defining the basic engineering relationships between cellular scale events and macro-level parameters required for designing scaled-up biocatalyzed chemical production processes. Additionally, the technical feasibility of critical bioprocess control monitoring and control subsystems, and examining novel chemical and biological methods for chemical and

mineral production, separation, and recovery are investigated. The **Separations and Novel Chemical Processes** element focuses on the problems of product recovery from the dilute aqueous solutions common in biological processing such as the recovery of low molecular weight organics which usually requires energy intensive or relatively expensive chemical processes. Another important topic is the biological recovery of metals and other significant minerals. The **Process Design and Analysis** element focuses on developing user friendly computer programs which assess the energetics and economics of biocatalytic chemical production processes. Modular computer programs have been developed which provide capabilities for rapid assessment of energy expenditures and costs for unit operations within biocatalytic processes.

SECTION II

PROGRAM DESCRIPTION

A. THE CATALYSIS AND BIOCATALYSIS MISSION

The Catalysis and Biocatalysis Program seeks to:

(1) Monitor advances in basic scientific research and evaluate emerging technologies for applicability to energy conservation.

(2) Identify energy conservation research needs and develop programmatic directions for basic research.

(3) Develop and establish the feasibility of innovative, or revolutionary, conservation concepts.

(4) Effect technology transfer to DOE end-use conservation programs and private industry.

By accomplishing this mission, the Program transmits the power of basic research to the wheels of industry.

B. HISTORICAL BACKGROUND

In 1980, the Energy Conversion and Utilization Technologies (ECUT) Program established the Chemical Processes Project with the Jet Propulsion Laboratory as the lead center. At that time the Project had two work elements: (1) Catalysis (chemical catalysis and biocatalysis) and (2) Separation. Within these work elements several small contracts were completed that evaluated the energy efficiencies and potential applications of relatively new separation processes, such as supercritical extraction, membrane separation, and chromatography. Research was also started at the California Institute of Technology (Caltech) on chemical catalytic behavior models and kinetics of expression in recombinant-DNA microorganisms.

In 1982, the ECUT Program was reorganized and the Chemical Processes Project was renamed the Biocatalysis Project. As the name suggests, the Project emphasis was also changed to stress biocatalysis as the primary research focus. Its work elements were modified to include: (1) Molecular Modeling and Applied Genetics, (2) Bioprocess Engineering, and (3) Process Design and Analysis.

In 1989, interest increased in using knowledge obtained from biological systems to benefit chemical processing and recovery techniques, such as the removal of carbon dioxide and other "greenhouse gases" from exhaust streams and the environment. Therefore, in 1990 the project was renamed the Catalysis and

Biocatalysis Program. Due to a reorganization in the Office of Conservation and Renewable Energy in April 1990, the Catalysis and Biocatalysis Program was moved to the Advanced Industrial Concepts Division (AICD), Office of Industrial Technologies.

The Program now has five work elements: 1) Molecular Modeling and Catalysis-by-Design, 2) Applied Microbiology and Genetics, 3) Bioprocess Engineering, 4) Separations and Novel Chemical Processes, and 5) Process Design and Analysis. Each work element addresses a key technical component necessary for the development of more advanced and efficient chemical/biological processes. The elements also provide a systematic progression of understanding starting with the basic understanding of catalytic and biocatalytic mechanisms at the molecular level, to micro-level effects on process parameters in reactors (including effects of integrated separation processes), and finally to the development and assessment of new process concepts for technology transfer to the industrial sector. With this information the production of large-volume, low-cost, energy-intensive chemicals from renewable resources may be a practical alternative in the future.

The relationships between each of the work elements are defined by each element's scale of action. Research activities are conducted at various scales of action or specific size dimensions. Hence, Work Elements 1), 2) and some of 4), include research activities at a molecular and cellular level (i.e., a scale of 1 μm and smaller). To successfully exploit the findings at the molecular and cellular levels, Work Elements 3) and 4) involve research in the area of engineering kinetics and control, as well as novel concepts in reactor design. The scale in these work elements is generally at the 1 meter dimension, or at the reactor level. Finally, Work Element 5) focuses on activities that operate at the entire process level (hundreds of meters or more).

C. RELEVANCE

The U.S. Chemical Processing Industry (CPI) has encountered increasing challenges to its ability to compete in world markets, such as the growing production of bulk chemicals by oil-rich developing countries (most notably the OPEC nations), limited control of prices paid for foreign feedstocks, relatively high capital and labor costs, and increasing costs for environmental and pollution control. Compared to the years prior to 1981, export levels and growth are a concern to the CPI. Since 1984, the U.S. share of world chemical exports has fallen from 16.8 to 14.1%, and the slight recovery from 1988 to 1989 may be due mainly to changes in currency exchange rates.[1] Furthermore,

[1]Chemical and Engineering News, June 19, 1989, p. 76.

uncertainty about petroleum imports and prices suggests that increased utilization of renewable resources may become a critical factor in maintaining U.S. leadership in the production of industrial chemicals. Several conclusions can be made regarding the competitive status and future potential of the U.S. petrochemical industry:

(1) Many U.S. industries are losing their competitive edge as the global economy expands. This situation may worsen as Europe unites and competes collectively against the U.S. In addition, more natural resources will become less accessible, if not depleted, requiring more imports. This may adversely affect the balance of trade, and also make U.S. industry more vulnerable to shortages of strategic materials. The U.S. petrochemical industry is just one example of such an industry.

(2) To compete effectively in the future, the U.S. must take advantage of its scientific strengths, including basic research in molecular biology, biochemistry, chemistry, agricultural sciences, and transfer this technology to industry. Biotechnology is an example of scientific research whose application has tremendous potential for commercial use in the U.S. petrochemical industry. The U.S. is the indisputable leader in scientific research in most areas of chemistry and biology, but most basic or fundamental research is carried out in academic institutions and government laboratories. Relatively little is done by the private sector because it is high-risk, long-range research. However, this basic research data is available to foreign companies who often apply these data to solve practical problems and who are quick to market a new product or process. An example of this process is found in current patents issued. Over 50 percent of the biotechnology patents issued in the U.S. in 1988 were to foreign inventors, mainly the Japanese.

(3) Although biotechnology has been applied to the production of pharmaceuticals and a few specialty chemicals by the chemical industry, its application to the larger commodity chemical market is considered too high-risk and long-term to attract research and development dollars. In fact, many large chemical and petrochemical companies that were active in this area in the 1960s and 1970s, decided to drop out. Such decisions make sense for short-term economics since many problems must be overcome before commercial processes for biological production of bulk chemicals can be successful. Fortunately, many of these problems are common to most biocatalytic processes, and if

overcome would make the use of such processes much more feasible commercially.

D. GOAL AND OBJECTIVE

(1) Goal. The goal of the Catalysis and Biocatalysis Program is to (a) exploit the United States' competitive advantage in biotechnology by facilitating the production of chemicals efficiently from renewable energy sources via biocatalytic processes, and (b) develop a deeper understanding of the nature of catalytic processes so that improved chemical and biological catalysts can be designed and produced based upon predictive logic, leading to new or improved energy-efficient chemical processing.

The program supports high risk applied generic research and development which builds an enabling technology base that will allow the rational development and scale-up of large biocatalyst chemical production processes. The Catalysis and Biocatalysis Program does this by conducting exploratory development on, and by establishing the feasibility of, novel, innovative or revolutionary basic research advances in biotechnology.

(2) Objective. To meet this goal, the Catalysis and Biocatalysis Program focuses on developing predictive models and supporting novel bioprocessing concepts which can be utilized by commercial producers for large scale chemical production. In addition, the strategy of the Catalysis and Biocatalysis Program is to reduce production costs by increasing product yields, by increasing reactor productivity, and by decreasing energy requirements for production. These concerns are generic in a very large number of biocatalytic processes. Technology transfer is central to this Program.

E. PROGRAM STRUCTURE AND ORGANIZATION

The activities of the Catalysis and Biocatalysis Program have been incorporated into five major work elements each of which addresses a key technical component, and a Management Support Function. The five major work elements and their supporting tasks are:

(1) The **Molecular Modeling and Catalysis-by-Design** element is focused on defining optimal microscale parameters for biocatalysts and developing practical applications of basic molecular biology research findings. Activities include: a) modeling biological and chemical catalysts and developing rigorous models for the

prediction of three-dimensional (tertiary) structure of proteins from their amino acid sequence (primary structure) for the design of new or improved biocatalysts; b) providing a basis for the design of biomimetic chemical catalysts analogous to enzymes that will provide stable chemical process catalysts; c) developing tools for understanding the structure/function relationships of conventional chemical catalysts so that new or improved ones can be predicted and experimentally verified without extensive screening methods; d) providing methods and a data base for defining kinetic models of biocatalyst reactivity; e) developing engineering solutions to generic technical barriers that preclude widespread applications of biocatalysis. FY 1990 tasks included:

(a) Theory of Biocatalysis: Electron Transfer Proteins.

(b) Protein Engineering for Nonaqueous Solvents.

(c) Predictive Models and Effects of Structure on Catalytic Properties.

(d) Simulations on Calcium Regulated and Redox Biocatalytic Systems.

(2) The **Applied Microbiology and Genetics** element is directed towards using new techniques in the recombinant DNA field for the development of new microorganisms. Expected results include organisms that produce higher yields of desired products and which will overcome the problems of low yield and product inhibition, as well as structurally-modified enzymes whose predicted improved properties have been experimentally verified. FY 1990 tasks included:

(a) Metabolic Engineering: Effect of the Hemoglobin Gene on Metabolism.

(b) Chromosomal Amplification/Gene Fusion.

(c) Hyperproduction and Secretion of Polyphenol Oxidase.

(3) The **Bioprocess Engineering** element emphasizes defining the basic engineering relationships between molecular scale events and macro-level parameters required for designing scaled-up biocatalyzed chemical production processes. Additionally, the technical feasibility of critical bioprocess control monitoring and control subsystems, and examining novel chemical and biological

methods for chemical and mineral production, separation, and recovery are investigated. FY 1990 tasks included:

(a) Immobilized Cell System for Continuous, Efficient Biocatalyzed Processes.

(b) Multimembrane Bioreactor for Chemical Production.

(c) Biocatalyzed Hydroxylation in Organic Solvents.

(d) Study on an Integrated Biological-Chemical Process for Continuous Production of Methyl Ethyl Ketone and 1,3 Butadiene.

(e) Gas Phase Enzyme Biocatalytic Reactor.

(f) Enzyme Reactions in Reverse Micelles and Microcapsules.

(g) Immobilized Enzymes in Organic Solvents.

(4) The **Separations and Novel Chemical Processes** element focuses on the problems of product recovery from the dilute aqueous solutions common in biological processing such as the recovery of low molecular weight organics which usually requires energy intensive or relatively expensive chemical processes. Another important topic is the biological recovery of metals and other significant minerals. FY 1990 tasks included:

(a) Separation by Reversible Chemical Association.

(b) Biological Separation of Phosphate from Ore.

(c) Electrocatalytic Study of Ammonia Synthesis and Methane Dimerization in High Temperature Solid Electrolyte Cells.

(5) The **Process Design and Analysis** element activities include development of user-friendly software for biochemical and chemical process synthesis, integration and assessments; and conceptual process design and evaluation. Tasks for 1990 were:

(a) A Biological and Chemical Process Integration and Assessment Computer Program: BCPI.

(b) Bioreactor Kinetics and Yields for Growth-Associated Conversion of Substrate to Cells and Products: A Variable Yield-Coefficient Model.

(c) Conceptual Process Design: Evaluation of a Process for Conversion of Renewable Resources to Ethyl Acetate.

F. MANAGEMENT SUPPORT FUNCTION

The Management Support Function has three areas of responsibility: Task Management, Planning and Integration, and Industry Technology Transfer.

(1) The Task Management function administers and coordinates the various task elements of the Program. These responsibilities include contract management, preparation and implementation of the Annual Operating Plans (AOPs) and budgets, monitoring of program performance, and reporting of results and other pertinent information to DOE management and other interested parties. Certain tasks, namely those at Caltech, JPL, and a few continuing subcontracts are managed by the Catalysis and Biocatalysis Program Field Manager at JPL. Tasks carried out at the national laboratories and new contracts are managed directly from DOE Headquarters. Funding is therefore divided into that portion handled through JPL, and that handled directly by DOE Headquarters or through the national laboratories.

(2) The Planning and Integration task includes technical multi-year planning that leads to the initiation, modification, or termination of research tasks and contracts; monitoring and evaluating research progress; initiating and maintaining technical relationships with program and non-program individuals and organizations; and, assuring effective dissemination of research results to industry and other research scientists and engineers.

(3) The Industry Technology Transfer task ensures strong interactive relationships between the program and the end-use sector, in order to assist the transfer of enabling technology from the research groups to the private sector so that discoveries may be used for the production of chemicals via biocatalysis.

As a new approach to technology transfer, an initiative on "Catalysis-by-Design" was sent to the national Laboratories in 1990. This required industrial collaboration on mutually interesting research projects aimed at solving generic problems in catalyst design and evaluation. Prior to this, three workshops were held in 1989 to obtain industrial support for this initiative. Many large chemical and oil companies attended, and many have joined with one or more of the national laboratories in

submitting collaborative research proposals. In this way, the research will be end-use driven, since they will play a major role in carrying out the projects.

The Materials and Molecular Simulation Center (MSC) has been established at the Beckman Institute on the Caltech campus where theoretical computational tools based on molecular dynamics and quantum mechanics will be developed in conjunction with computer graphics capabilities. Academic, government and industrial researchers working together at the MSC will accelerate the exchange of information and problem solving. Tools to enhance molecular modeling capabilities and permit the rational design of useful materials are expected to result from these interactions. The long term effect of such interactions and development will be the ability to rationally design and synthesize materials with specified properties and predictable chemical interaction or transport characteristics, particularly for new, more efficient biocatalyst, catalyst, and related systems. DOE funding is being used to provide a portion of the necessary computational equipment and this will be augmented by direct financial support from industry. The resultant work will lead to strong industrial interactions and collaborations that can significantly contribute to technology transfer.

G. TASK DESCRIPTIONS

(1) The **Molecular Modeling and Catalysis-by-Design** work element consists of four research tasks: Theory of Biocatalysis: Electron Transfer Proteins, Protein Engineering for Nonaqueous Solvents, Predictive Models and Effects of Structure on Catalytic Properties, and Simulations on Calcium Regulated and Redox Biocatalytic Systems.

(a) Theory of Biocatalysis: Electron Transfer Proteins (D.N. Beratan, Jet Propulsion Laboratory). The purpose of this task is to obtain a more detailed understanding of the molecular processes in enzymatic reactions for the development of technological applications in catalysis and microelectronics. The design of enzymes poses unique challenges compared to small catalyst molecules. First, an understanding of enzyme catalytic mechanisms is fairly limited; secondly, it is not understood whether the surrounding protein superstructure is active or relatively passive in a particular enzyme-catalyzed reaction. The simplest biocatalytic reaction, electron transfer, has been selected for theoretical and experimental investigation because of the ubiquity of electron transfer in energy utilization and conversion pathways.

(b) Protein Engineering for Nonaqueous Solvents (F.H. Arnold, California Institute of Technology). The industrial applications of biocatalysts have been severely limited by constraints on the solvent environment of enzymes, which, being proteins, normally require an aqueous medium for effective stability and activity. Production of organic chemicals using enzymes would benefit from the use of enzymes in organic solvents because of the insolubility of organic reactants and products in aqueous systems. With the advent of convenient methods for altering the amino acid composition and sequence, and the synthesis of completely new proteins, it may be possible to engineer enzymes for use in nonaqueous solvents. However, the success of such protein engineering to construct enzymes for use in organic solvents requires a rational design procedure. This depends on an understanding of the relationships between amino acid sequence, secondary and tertiary structure of the protein, and activity and stability in different solvent systems.

Prior research in this area involved studies on the small protein crambin, a forty-six amino acid plant polypeptide. This polypeptide has been sequenced, and its X-ray crystallographic structure determined. It has a high solubility in organic solvents, and was chosen as a suitable molecule for structure/solubility studies. Structural modifications were carried out and their effect on solubility determined. This led to the hypothesis of a series of rules for predicting the effect of structural changes on the solubility of proteins in organic solvents.

These rules were used to predict certain structural changes which would stabilize the proteolytic enzyme Subtilisin in the presence of organic solvents. Experiments using site-specific mutagenesis to engineer the enzyme confirmed that single mutations increased the stability by an order of magnitude, and double mutations increased it by two orders of magnitude. A large biotechnology company has now offered similar engineered enzymes for commercial uses. This research will be continued with further application of the structure/stability rules to other enzymes.

Another reason for using enzymes in non-aqueous systems is to drive equilibrium reactions in favor

of synthesis rather than hydrolysis. Proteolytic enzymes are normally used to hydrolyze proteins or peptides to amino acids. But if water is removed, the reactions can be run in reverse to synthesize peptides. Since peptide synthesis by chemical methods requires blocking of reactive groups and subsequent removal of blocking groups, it is not an efficient method for peptide manufacture. Preliminary studies have shown that the engineered subtilisins will catalyze the synthesis of peptides in non-aqueous systems, and this application will be studied in some detail since it is of industrial value. Already the possibility of using this procedure to make aspartame has been discussed.

(c) Predictive Models and Effects of Structure on Catalytic Properties (W.A. Goddard, California Institute of Technology). A critical problem in the design of new biological catalysts is the limited current capability for the prediction of structure and properties of new protein systems. It is necessary to predict secondary and tertiary structures based on amino-acid sequences(primary structure), because physical and chemical properties depend on the 3-dimensional protein conformation. If the catalytic function and other properties of modified or synthetic enzymes (biocatalysts) could be predicted, it would be possible to design and synthesize new biocatalysts capable of performing specific desired or novel reactions or having certain desired properties of stability and specificity.

Prediction of protein structure is being investigated by three approaches that combine random fluctuations with molecular dynamics to allow molecular forces to guide optimization of local structure: (1) Full optimization, where a combination of molecular dynamics and random processes is used to search for energy minima of protein structures. Although only the amino-acid sequence is required, calculations are most complex and much method development is required. (2) Conformationally-constrained optimization where X-ray data place certain restraints on possible configurations. (3) NMR pair-constrained optimization, where nmr data further reduce the configurations that are possible.

A general approach to biocatalysis will be developed using the tools of molecular modeling to

design new biocatalysts and test the design validity by experimental verification. A goal is the design of catalysts for selective oxidation of particular organic substrates. Oxidative enzymes which have been well characterized will be used as the basis for models. Another area of interest is those enzymes which fix carbon dioxide, since the role of carbon dioxide in global warming is of increasing concern.

Although there are a number of difficult problems to solve in developing such biocatalysts, the design capabilities to be developed would provide a powerful tool for developing new and efficient chemical and biological catalysts.

(d) Simulations on Calcium Regulated and Redox Biocatalytic Systems (A. Redondo, P.J. Hay, and A.E. Garcia, Los Alamos National Laboratory). There are many biochemical reactions which are regulated by calcium. Calcium is intimately involved in cellular transport mechanisms as well as such phenomena as blood clotting. Many calcium-mediated enzyme reactions involve a non-enzymatic protein called calmodulin, which undergoes conformational changes in the presence of calcium and this calmodulin-calcium complex binds to a number of enzymes converting them to active forms. Models of calcium/protein binding and conformation changes have been carried out, and it has been found that they compare fairly closely with experimental results. Since the role of calcium is not actually involved in the catalytic action of the enzymes, it has been decided to terminate this research after completion of specific calculations on the molecular dynamics of water and on calcium ion relaxation and the rates of calcium capture by calmodulin.

Research efforts will then turn to another area of catalysis-by-design as part of the national laboratory/industry initiative. The new topic of research will concern the conversion of methanol to gasoline via 2C intermediate on zeolite catalysts. The industrial partner in this research is Amoco Oil Company.

This is clearly an important industrial process, especially at a time when the price and potential short supply of oil have become significant factors in the national economy. Theoretical

understanding of the mechanism of action of the catalyst, the relationship of catalyst structure to activity, and the structure of active sites could lead to improvements in catalyst activity, specificity, and stability, each of which would have considerable economic and energy-saving significance. A series of theoretical calculations, employing *ab initio* quantum chemistry and molecular dynamics, will be carried out on these catalytic reactions. Initial studies will be to: (1) determine the appropriate molecular clusters to be used in these quantum chemical calculations, and (2) initiate quantum chemical calculations of the structure of acid sites in zeolite ZSM-5.

(2) The Applied Microbiology and Genetics work element consists of three research tasks: Metabolic Engineering: Effect of the Hemoglobin Gene on Metabolism, Chromosomal Amplification/Gene Fusion, and Hyperproduction and Secretion of Polyphenol Oxidase. The focus is on using new techniques of recombinant DNA to overcome the problems of low yields and product inhibition.

(a) Metabolic Engineering: Effect of the Hemoglobin Gene on Metabolism (J.E. Bailey, California Institute of Technology). It has generally been accepted that the scale up and operation of aerobic fermentations at very high cell densities are limited by the ability to maintain sufficient dissolved oxygen in the system. The approach to solving this problem has been to find novel methods of aeration or novel bioreactor configurations, etc.

But many years ago, Marvin Johnson pointed out that there was also a problem with oxygen limitation within the bacterial cell itself, and that increased aeration did not overcome this problem.

The strategy in this work has been to increase oxygen transfer into bacteria and within the bacterial cell by inserting the bacterial hemoglobin gene into bacteria, thereby increasing the rate of oxygen uptake into the foreign bacteria and transfer of the oxygen to the sites of oxidation where it is required.

The hemoglobin gene from *Vitreoscilla* has been

inserted into *E. coli*, and it has been shown that the rate of oxygen uptake is doubled and that twice the dry weight of cell mass is produced when compared with the uninfected parental *E. coli*. The amount of glucose used to produce the increased dry cell mass is also reduced, showing that cell metabolism has increased in efficiency.

Other types of microorganisms have been transfected with the hemoglobin gene, and it has been shown that in certain antibiotic-producing organisms, the amount of antibiotic produced is increased, or the ratio of antibiotic produced is altered. In other organisms, increased amounts of enzymes are produced. This technology has been licensed to a new biotechnology company which already has a series of research agreements with companies in the American private sector.

Metabolic engineering refers to the directed rearrangement or augmentation of metabolic pathways, using recombinant DNA techniques, in order to provide overproduction of metabolites or metabolic derivatives.

The objectives of these experiments are to investigate the extent to which cellular metabolism is sensitive to amplification of individual activities in certain key positions in metabolism, to develop and demonstrate powerful experimental methods for investigation of metabolic perturbations (primarily ^{31}P in vivo NMR), to develop data for use in mathematical modeling studies of metabolic engineering, and to ascertain the effect of the *Vitreoscilla* hemoglobin gene on oxygen uptake, metabolism, and physiology in *E. coli*. Several different mathematical approaches to analysis of cell metabolism will be pursued as part of this research. A detailed single-cell kinetic model of recombinant *E. coli* will be enhanced to include more details of particular metabolic pathways, especially as pertaining to DHFR activity. In addition, global sensitivity analysis methods will be applied to explore those parameters in this model which are most important in simulated cell metabolism and growth kinetics. In addition, research will continue on mathematical frameworks for estimating *a priori* the overall cell response to metabolic engineering and for analyzing experimental data on altered metabolic pathway

flows in a systematic, powerful fashion.

(b) Chromosomal Amplification/Gene Fusion (G. Bertani, Jet Propulsion Laboratory). Although genetic engineering techniques can be used to increase micro-organism productivity, e.g., to produce larger amounts of a chemical in a fermentation process, the genetic information (that results in higher production) may be lost as the microorganism multiplies. The objective of this activity is to determine if the desired genetically-engineered component (plasmid or associated gene) can be inserted into the chromosome and amplified in place. Since the desired genetic information (recombinant trait) would then be in the microorganism chromosome, it could not be easily lost as the microorganism multiplies. Thus the problem of plasmid stability could be addressed. Direct physical (as opposed to genetic) evidence for the insertion of the plasmid into the bacterial chromosome has been obtained.

The stability of bacterial strains resulting from this work will be compared with the stability of strains created by conventional plasmid splicing techniques. While laboratory strains of *Escherichia coli* are being used in this research, the general principles involved could easily be applied to other types of bacteria.

(c) Hyperproduction and Secretion of Polyphenol Oxidase (W.V. Dashek and A.L. Williams, Atlanta University). The objectives of this research are: (1) to purify intracellular and extracellular polyphenol oxidase (PPO) from *C. versicolor* maintained in liquid culture; and (2) to determine if the extracellular enzyme is synthesized de novo or is activated from an intracellular precursor; and (3) clone the gene and express it in *E. coli*.

The interest in this enzyme is largely because of the potential application in lignin degradation. Lignin accounts for about 20% of the weight of lignocellulose and its presence poses many problems in the potential use of biomass as a major source of fuels and chemicals. Lignin is a highly cross-linked aromatic polymer composed largely of benzene rings with three-carbon side chains. If lignin could be broken down to small aromatic or aliphatic molecules, these would provide a low cost source of organic chemicals or

fuels. The technical problem is that ligninases and polyphenol oxidases (the enzymes which degrade lignin) are produced by very slow growing fungi which are difficult to grow in culture, and only produce or secrete low levels of enzyme.

1) Increasing secretion of the natural enzyme in the wood rot fungus by determining whether the intracellular enzyme is a precursor of the extracellular enzyme, and establishing methods for induction of increased enzyme secretion.

2) Using recombinant DNA technology to isolate the polyphenol oxidase gene and expressing it in a fast growing organism which can be readily grown in large scale fermentations for commercial production of the enzyme.

The first approach is to establish the time courses for the appearance of the two enzymes and to subsequently determine specific activities, and to establish the optimum growth conditions of the organism for maximum enzyme production, including any cofactor or inducer requirements.

The second approach is to isolate the DNA or the mRNA from the white rot fungus, use restriction endonucleases or cDNA methodologies to insert fungal genes into *E. coli*, and screen *E. coli* colonies for PPO production and secretion.

These approaches will then be applied to the other enzymes involved in biomass degradation, namely the ligninase and the cellulase.

(3) The **Bioprocess Engineering** work element consists of seven tasks: Immobilized Cell System for Continuous, Efficient, Biocatalyzed Processes, Multimembrane Bioreactor for Chemical Production, Biocatalyzed Hydroxylation in Organic Solvents, Study on an Integrated Biological-Chemical Process for Continuous Production of Methyl Ethyl Ketone and 1,3 Butadiene, Gas Phase Enzyme Biocatalytic Reactor, Enzyme Reactions in Reverse Micelles and Microcapsules, and Immobilized Enzymes in Organic Solvents. They are primarily concerned with the definition of basic engineering relationships between molecular and micro-scale events and macro-level parameters, required to design and scale-up bioprocesses.

(a) Immobilized Cell System for Continuous, Efficient, Biocatalyzed Processes (C.D. Scott, Oak Ridge

National Laboratory). The major problems holding back the successful industrial use of bioprocesses for the manufacture of bulk low cost chemicals are: (1) bioreactor productivity; (2) product yield; and (3) product concentration.

Bioreactor productivity (units of product/unit of time/unit of bioreactor volume) is low because in batch systems the microbial cells (the biocatalysts) go through a lag phase in their growth and eventually slow down as substrate is used up. In continuous systems, the biocatalyst is flushed out of the bioreactor with the product, and never reaches the high levels required for high productivity.

Product yield (weight of product actually produced/theoretical weight of product obtained/100) is lowered by the trade-off of lowered yield against improved bioreactor productivity, since the reactions slow down and increase the fermentation or retention time considerably if maximum yields are to be achieved.

Product concentration (weight of product/unit volume of fermentation broth) is usually low because of the low concentration of substrate in the fermentation feed and the toxicity of products such as ethanol, butanol, and acetic acid on the biocatalyst. This leads to the need for very large bioreactors, and high costs and energy requirements for the isolation of products from these dilute aqueous solutions.

As a result of the RFP for proposals for research on novel bioreactors, several highly efficient bioreactor systems have been developed. Two of these are undergoing further development as they show considerable promise at overcoming the three major problems discussed above.

Development of the ORNL immobilized cell bioreactor for the production of ethanol from corn and sugar by-products by *Saccharomyces* and *Zymomonas* has increased the bioreactor productivity by more than an order of magnitude and the yield of ethanol to about 98% of theoretical. It is a tubular continuous flow fluidized bed bioreactor with the biocatalyst trapped in beads which remain in the bioreactor while the substrate solution flows through it.

The multiphase bioreactor developed at Battelle Memorial Institute is based upon similar principles and was specifically designed for use in the acetone/butanol fermentation. Due to changes in staffing at Battelle, this work is being continued at ORNL. It is co-funded by the National Corn Growers Association, St. Louis, MO.

The problem now being addressed is that of product removal and recovery. Attempts will be made to integrate product removal methods developed at LBL with the fermentation process.

The use of dense product-absorbing beads in the continuous flow reactor has already been shown to meet the necessary physical requirements, namely that they can be added at the top of the column and will slowly pass through the bioreactor and can be collected at the bottom of the column.

Thus, they separate from the less dense cell-containing beads which are retained within the fluidized bed of the bioreactor.

In the case of the lactic acid fermentation, it has been shown that the lactic acid can be continuously removed, better pH control attained, and that the product can be removed from the beads and the beads can be reused.

Work will now concentrate on optimizing the removal of the products from the bioreactor by the beads, and the problems of product removal from the beads and bead recovery and reuse.

Work will also continue on the multi-phase fluidized bed reactor. The work on the multiphase fluidized bed bioreactor will concentrate on studying the continuous production of butanol by *C. acetobutylicum* with continuous product extraction by oleyl alcohol or trioctyl phosphine oxide, either in situ or in a side loop to the main system.

These may prove to be simple methods to overcome both the problem of product feedback inhibition and the high cost of product recovery from dilute aqueous solutions.

(b) Multimembrane Bioreactor for Chemical Production (M.L. Shuler, Cornell University). Immobilized cell reactors offer the potential to greatly

improve volumetric productivities for bioreactors. An important form of such reactors is that of entrapping cells between membranes. Scale-up of such units can lead to problems with the transfer of sparingly soluble gases (e.g., O_2 and CO_2). These gas transfer problems can result in a loss of productivity due to starvation for gaseous nutrients or in the physical disruption of the membrane reactor due to pressure increases caused by incomplete removal of by-product gas (e.g., CO_2). Although membrane reactors are satisfactory for the retention of cells, the true advantages of membranes are incompletely utilized in current configurations. The use of membrane selectivity for *in situ* product recovery offers the potential to simplify process design and increase reactor productivity by avoiding often encountered problems with product inhibition.

A multimembrane reactor concept is suggested. Four layers exist: one for cell entrapment, one for substrate flow, one for gas flow, and one for the flow of an extractant which selectively removes product. Task efforts include (i) development of a mathematical model relating the micro environment to reactor performance, (ii) development of a process simulator from the mathematical model, (iii) experiments to validate the model and to monitor the interrelationship of cell physiology to immobilization, and (iv) evaluation of long-term reactor operation.

(c) Biocatalyzed Hydroxylation in Organic Solvents (A.M. Klibanov, Massachusetts Institute of Technology). Enzymes (Biocatalysts) are proteins possessing catalytic activity. Since they are proteins, they are soluble in aqueous systems and generally not soluble in organic solvents. In fact, solvent precipitation was one of the methods used in enzyme purification. Since many enzyme substrates are more soluble in organic solvents than water, it would be useful to carry out enzyme reactions in organic solvents. However, it was generally found that enzymes were less active and less stable when water-miscible organic solvents were added to the aqueous solutions of enzymes. More recently it has however been found that if the level of water is reduced very low, enzymes are frequently both active and stable. This only occurs when the water level is reduced to levels required to solvate the protein and act as a

reactant where necessary.

This is because when the level of water is this low, it solvates the protein, but restricts its movements so that it cannot unravel and denature.

This phenomenon has been observed with noncatalytic proteins, and has been used to induce "molecular memory" into proteins. A protein is incubated with a low molecular weight ligand, and organic solvent is added to the aqueous solution. The protein configuration becomes frozen with the ligands bound to it. The ligand is then washed out of the protein which is still fixed in the organic solvent. This protein retains memory of the shape of the ligand because it is not free to change its configuration, and rebinds larger amounts of the ligand than protein similarly trapped in the organic solvent in the absence of bound ligand.

This technique will be applied to enzymes as a possible method for changing enzyme specificity. When incubated and precipitated in the presence of an inhibitor, which is later washed from the enzyme, the enzyme presumably remembers an active site conformation that favors interaction with substrates analogous to the inhibitor. Preliminary experiments indicate the validity of this hypothesis. This approach may lead to shorter reaction times, higher specificity, and more efficient biocatalysts to optimize energy utilization in chemical processes. It could also be used as a means of fixing cofactor analogues near the active site of an enzyme and possibly introducing new enzyme activities into proteins.

(d) Study on an Integrated Biological-Chemical Process for Continuous Production of Methyl Ethyl Ketone and 1,3-Butadiene (G.T. Tsao and P.B. Beronio, Purdue University). The objective of this task is to demonstrate the technical feasibility of an integrated Biological-Chemical process for the continuous production of two high volume/low value products: methyl ethyl ketone (MEK) and 1,3-butadiene from fermentable sugars. This conceptual bioprocess has been divided into two segments: production of 2,3-butanediol from fermentable sugars using an immobilized cell reactor containing living cells, and selective adsorption of 2,3-butanediol from the aqueous fermentation broth using a solid adsorbent column.

(e) Gas Phase Enzyme Biocatalytic Reactor (D.L. Wise, Northeastern University). The objective is to develop a dry or gas phase continuous enzyme reactor for the efficient conversion of gaseous substrates to useful products. Specifically the use of a bioreactor containing the enzyme alcohol oxidase for the conversion of ethanol to acetaldehyde will be demonstrated.

This technology could prove especially suitable for very low boiling substrates and substrates with limited solubility in aqueous systems, where the gas solubility limits the reaction rate. The approach is to develop a solid phase immobilized enzyme system and bioreactor, followed by investigation of the operating characteristics, reactor modeling and energy-economic analysis. Drs. Klibanov and Karel from Massachusetts Institute of Technology will assist in the transfer of their original gas-phase technology to the Principal Investigator at Northeastern University.

(f) Enzyme Reactions in Reverse Micelles and Microcapsules (H.W. Blanch, University of California, Berkeley). In order to use enzymes, in either free or immobilized form and to more broadly enter into the production of chemicals on a large scale, techniques for increasing the solubility and transport rate of organics at the site of enzyme action must be found. The approach proposed here is based on the use of a second organic liquid, in which the substrate or product is soluble to a much greater extent than in an aqueous system. The second organic liquid may be water miscible or immiscible. Such systems are used currently for the enzymatic transformation of steroids. A second aspect of the use of enzymes in nonaqueous environments is the potential to run many reactions "backwards". In cases where water is a reaction substrate, its high activity in aqueous solution generally results in a shift of equilibrium to favor the normal products of the reaction. In a non-aqueous system such reactions may be forced in the reverse direction, as water will be present at low concentrations. Examples of this type of reaction include esterification, peptide synthesis and dehydration reactions such as urea formation from $(NH_4)_2CO_3$.

The objective of this project is to investigate

and demonstrate extractive catalysis in two model systems: production of tryptophan in reverse micelles, and the oxidation of cholesterol by cholesterol oxidase in microcapsules.

The approach will be to: (1) Examine the conformation of the enzyme in reverse micelles and probe the structured nature of water within the micelles. (2) Determine transport and kinetic parameters within micelles by esr spectroscopy. (3) Investigate the role of substrate transport to the site of reaction in microcapsules. (4) Develop a fluidized bed bioreactor employing microcapsules and examine key parameters related to scale-up. (5) Investigate cofactor regeneration in a microcapsule system: e.g., NAH/NADH using steroid reactions, coupled with dehydrogenase enzymes for cofactor recycle using NMR and ESR spectroscopy.

(g) Immobilized Enzymes in Organic Solvents (H. Zemel, Allied Signal Research and Technology). Generally enzymes are used in aqueous solutions or environments. When a hydrolytic process is being catalyzed, the excess of water inevitably drives the system towards hydrolysis rather than condensation or synthesis due to the Law of Mass Action. To extend the practical use of enzymes to industrially feasible processes the use of immobilized enzymes in organic media will be studied for typical condensation reactions, i.e. lipase-catalyzed synthesis of esters. Already lipases have been shown to catalyze transesterification reactions in organic solvents. The general physicochemical principles on which this technique relies will be studied in detail; the role of the support geometry and surface, the influence of water activity in the organic phase, the influence of pH memory, and the effect of co-immobilized enzyme activators. It is anticipated that this approach towards a more detailed understanding of immobilized enzyme reactions will lead to a wider range of enzyme applications in organic syntheses.

(4) The Separations and Novel Chemical Processes work element consists of three tasks: Separation by Reversible Chemical Association, Biological Separation of Phosphate from Ore, and Electrocatalytic Study of Ammonia Synthesis and Methane Dimerization in High Temperature Solid Electrolyte Cells. The focus is directed toward the problem of product recovery from

dilute aqueous solutions.

(a) Separation by Reversible Chemical Association (C.J. King, University of California, Berkeley). One of the main problems holding back commercialization of bioprocessing is that of product recovery from dilute aqueous solutions. The objective of this task is to examine and evaluate the use of reversible chemical association, or complexation, with organic agents as a method for separating polar organic substances from dilute aqueous solutions, e.g., bioprocess product or waste streams. The goal is to obtain sufficient understanding of underlying chemical, equilibrium, and transport behavior to enable rational selection of separating agents, methods of regeneration, and methods of implementation, as well as rational conceptual design and economic evaluation.

Few organic acids or alcohols reach levels above 2-3% wt/v, because at higher levels they become growth inhibitory, and the cost and energy requirements for their recovery make bioprocesses uneconomical.

It has been shown that organic acids can be reversibly extracted into organic phases containing water-insoluble amines, and the organic acid subsequently recovered by thermal dissociation of the complex. Similar methods are being studied for the extraction of alcohols. Research will continue on the extraction of organic acids, alcohols, glycols, and carbonyl compounds from dilute aqueous solutions and their recovery from the organic phase.

(b) Biological Separation of Phosphate from Ore (R.D. Rogers, Idaho National Engineering Laboratory). The production of phosphate from phosphate ore requires large amounts of energy, the largest portion of which is in the actual extraction or solubilization of phosphate from the insoluble calcium phosphate. For example, FMC Inc., the largest phosphate producer in Idaho, is also the largest industrial user of electric power in the state of Idaho. It has been estimated that phosphate extraction uses about 0.5 Quads of electricity. The main problem is however that the high grade phosphate currently being extracted is being rapidly depleted and the lower grade ore is more expensive and requires more energy to

process. In fact, undersea mining has even been discussed. Biological solubilization of phosphate ore has long been known, but only recently has the possibility of the biological extraction of phosphate ore been examined as a possible solution to the above problems.

Research at INEL has shown that phosphate ore can be solubilized by a wide range of bacteria and fungi. Solubilization cannot be correlated with the production of any particular organic acids, but the presence of the living system is essential for high levels of solubilization. Two organisms, one bacterial and one fungal were selected for more detailed study.

Kinetic studies on ore solubilization will be completed, and laboratory scale experiments will be carried out to determine whether batch or continuous operation is most efficient. Determination will be made of the necessity for separate production of the microorganism. Initial designs of a process and a pilot plant will be made.

This work is co-funded by FMC, Inc., the largest phosphate producer and industrial electricity user in Idaho.

(c) Electrocatalytic Study of Ammonia Synthesis and Methane Dimerization in High Temperature Solid Electrolyte Cells (M. Stoukides, Tufts University). Work will continue on the electrocatalyzed fusion of nitrogen and hydrogen to form ammonia using a fused salt electrolyte system. In addition, work will continue on the electrocatalyzed dehydrogenation of methane to form ethane and/or ethylene. This method could lead to more energy efficient syntheses of ammonia and ethane or ethylene.

This work is co-funded by the National Science Foundation.

(5) The **Process Design and Analysis** work element includes development of user-friendly software for process synthesis, integration and assessments; and conceptual process design and evaluation. There were three research tasks for 1990: A Biological and Chemical Process Integration and Assessment Computer Program: BCPI, Bioreactor Kinetics and Yields for Growth-Associated Conversion of Substrate to Cells and

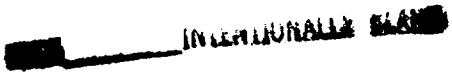
Products: A Variable Yield-Coefficient Model, and Conceptual Process Design: Evaluation of a Process for Conversion of Renewable Resources to Ethyl Acetate.

(a) A Biological and Chemical Process Integration and Assessment Computer Program: BCPI (J.D. Ingham, Jet Propulsion Laboratory). The objective is to develop an extensive computer program (designated BCPI) that can be used to rapidly determine the energy requirements and approximate product costs for chemicals that can be produced by various biological and chemical processes. The general process type to be evaluated is conversion of biomass to obtain dextrose sugars, followed in most cases by biocatalytic conversion to an intermediate, which would be chemically converted in one or more steps to an industrial organic chemical. The technical approach is to first define a series of conversion processes. Then relevant information is entered in the program. An interactive capability is included to provide new or more complete user data and to substitute routines, which will, respectively, improve, and provide greater flexibility for, assessment implementation. The phase 1 demonstration version is relatively easy to use, but the data base needed to evaluate a large number of different types of processes is not yet complete. It is planned that the completed program will consist of conventional menu displays for execution after starting from DOS, and will not require word processing or any other supplementary software.

(b) Bioreactor Kinetics and Yields for Growth-Associated Conversion of Substrate to Cells and Products: A Variable Yield-Coefficient Model (J.D. Ingham, Jet Propulsion Laboratory). When biocatalyzed processes are used to produce industrial organic chemicals, energy efficiency, economics and annual production rates and capacity will depend primarily on the kinetics of conversion of substrate to product. For most common growth-associated biochemical conversions the yield coefficients (ratios of amounts of chemical product or cells formed to total substrate converted) are nearly constant. Because cell growth and product formation are often inhibited at different rates as biotransformation proceeds, yield coefficients are not constant especially at the higher rates of conversion, where the actual product yields and rates of formation are most important. For example, cell

production may stop completely when the product concentration is about 90 g/l while product formation continues beyond 110 g/l. For both cell growth and product formation the rates decrease as the corresponding maximum product concentrations are approached. A variable yield-coefficient model has been developed to more satisfactorily reflect the kinetics of such bioprocesses for prediction of product yields for various types of bioreactor conditions and configurations that are applicable to large-scale production of chemicals by biocatalyzed processes. This work, including preparation of a publication to be submitted, has been completed.

(c) Conceptual Process Design: Evaluation of a Process for Conversion of Renewable Resources to Ethyl Acetate (N.K. Rohatgi and J.D. Ingham, Jet Propulsion Laboratory). The purpose of this task is to synthesize and evaluate a new energy-efficient process for producing ethyl acetate from renewable resources, such as ethanol. A complementary objective is to use the proposed process to evaluate alternative approaches so as to develop new process models. The process consists of a vacuum fermentation process to produce ethanol vapor, which is converted in the vapor phase to commercial ethyl acetate. A commercial process-simulator based on the ASPEN program developed at MIT for the Department of Energy is being used for process assessments. The results will be compared with results from models being developed as described previously.



SECTION III

FY90 TASK ACCOMPLISHMENTS

Technical discussions included in this section were provided by the investigators. Because of this, a unique perspective of the authors' work is obtained.

A. Molecular Modeling and Catalysis-by-Design

(1) Theory of Biocatalysis: Electron Transfer Proteins (D. N. Beratan, Jet Propulsion Laboratory). We have been investigating the mechanism of long range electron transport in native and semi-synthetic proteins including the cytochromes, blue copper, and iron-sulfur proteins, as well as the photosynthetic reaction center. The transport events involve quantum mechanical tunneling of the electron from donor to acceptor. As such, the chemical and structural details of the intervening protein are critical. This work has led to (a) protein design rules for obtaining modified protein with tailored electron delivery rates (b) a software implementation of the theoretical method, now in wide usage in experimental groups, for designing of new protein systems (c) suggestions of the crucial role played by protein secondary structure on protein electron transport and (d) a new theoretical method for calculating very weak electronic coupling in proteins.

The Tunneling Pathway Concept

Electron transfer reactions are central in the bioenergetic transduction schemes of plants and animals. These reactions must deliver electrons to appropriate physiological redox sites to power the organisms with minimal loss of free energy. It is also crucial that the electrons arrive at only the "proper" locations to avoid the production of highly reactive toxic species.

One physiological means of controlling these reactions is with distance; rates of electron transfer between weakly coupled donors and acceptors drop roughly exponentially with distance. The nature of this distance dependence has been the subject of intense interest for the last 10 years in small organic molecules. Only in the last several years has a series of experiments been reported that probes the role of the protein as a mediator of electronic coupling between donor and acceptor.

Based on our work on small model compounds, we

developed a relatively simple model for protein mediated electron transfer that takes into account the structural details of the protein and allows the prediction of relative rates as a function of the protein structure. We called this the "pathway model". The goal of the "pathway model" is to define a sequence of covalent, hydrogen, and van der Waals bonds between donor and acceptor that dominates the electronic coupling. The rapid fall off of the tunneling interaction with distance and the low degree of "interconnectivity" in proteins suggest that this will be an appropriate strategy. The goal of the theoretical work was first to make testable predictions of electron transfer rates as a function of protein structure and then to support the design of custom electron transfer systems if the preliminary work was successful.[2]

Software Development

Preliminary studies of protein electron transfer pathways "by hand" on transition metal labeled myoglobin proved encouraging.[3] This encouraged us to write a piece of software to systematically search proteins for the optimum tunneling pathway between two specified sites in the protein. The software development was initiated in 1988, and an improved version developed to run on our graphic computer workstations during the summer of 1990. A full description of the method appears in an October 1990 issue of the Journal of the American Chemical Society.

Cytochrome c

Our software has been used to interpret the rates of electron transfer in several redox labeled (ruthenium complex bound to surface histidine) proteins. Cytochrome c serves as the best case study since three different derivatives have been made and the family of rate measurements permits comparison with the theoretical predictions. The model does quite well to predict relative rates, whereas simpler models that neglect the chemical details of the bridging proteins do not adequately describe the coupling. With this success, our experimental collaborators are using the pathway software to design new heme and blue copper

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[2]J.N. Onuchic and D.N. Beratan, *J. Chem. Phys.* 92:722 (1990).

[3]J.A. Cowan, R.K. Upmacis, D.N. Beratan, J.N. Onuchic, and H.B. Gray, *Ann. New York Acad. Sci.* 550:68 (1989).

proteins with tailored rates.

Structure and Electron Tunneling

The covalent, hydrogen, and van der Waals bonds in the tunneling pathways determine the donor-acceptor coupling in proteins. However, secondary-structure (α-helix, β-sheet, etc.) defines how direct the average pathway in a given protein will be. Therefore, we have predicted different average distance dependences between proteins due to secondary-structure differences. To our knowledge, this is the first prediction of such a qualitative secondary structure effect in electron transfer proteins. We believe that it will lead to interesting test systems and will allow the rational design of systems with more or less "insulating" environments around the redox active group.

New Directions in Protein Electron Transfer

The pathway model that we described is approximate in that it neglects interactions between individual paths and simplifies certain chemical details of the bridging medium. We have recently developed a Green's Function technique that should allow more realistic electronic coupling calculations to be performed on proteins in the near future. The method is particularly promising because of its ability to determine a small electronic coupling for very large complex systems without the usual numerical problems associated with Schrodinger equation techniques. Future numerical work will focus on this new technique and on consideration of the role played by motion of the bridging protein on the transfer rates.

(2) Protein Engineering for Nonaqueous Solvents (F.H. Arnold, California Institute of Technology). The goal of this project is to demonstrate that enzymes can be engineered at the level of their amino acid sequences to improve their stability and catalytic activity in polar nonaqueous solvents. The ability to carry out biocatalysis in nonaqueous media greatly expands the potential scope and economic impact of biotechnology. For this reason, we are endeavoring to construct nonaqueous solvent-stable enzymes by site-directed and random mutagenesis of natural enzymes. By studying the stabilities and activities of these mutant enzymes in polar organic solvents, we hope to develop a general set of design criteria for engineering non-aqueous solvent-stable enzymes.

Proteases hydrolyze peptide bonds in aqueous solution. Proteases will also synthesize new peptide bonds from peptides or amino acids, and the efficiency with which they do so depends strongly on the amount of water in the reaction mixture. These synthetic reactions can conceivably be carried out with the high specificities and stereoselectivities exhibited in aqueous media, provided that the enzyme is active in the organic solvent. As practical models for engineering nonaqueous solvent-stable enzymes, we have chosen two bacterial proteases, subtilisin E and α-lytic protease, both of which might be used in peptide and polymer syntheses.

In an earlier phase of this project, several "design rules" for protein stability in nonaqueous solvents were proposed, based on our understanding of solvent effects on the forces that contribute to the stability of folded proteins in solution and our studies of crambin, a natural protein that is stable and soluble in a wide variety of polar organic solvents. We have been testing these design rules by making selected (site-directed) alterations in the amino acid sequences of the proteases. In addition to testing the design rules by site-directed mutagenesis, we have also begun random mutagenesis experiments combined with screening of the resulting mutants for improved stability. These random mutagenesis studies will serve to test the proposed design criteria as well as help us to identify additional mechanisms, not considered in our earlier work, by which stability and improved activity in polar organic solvents can be achieved.

Using various mutagenic techniques we have constructed a number of subtilisin E and α-lytic protease mutants which exhibit improved stability in nonaqueous solvents. These experiments were primarily designed to test the hypothesis that altering charged residues on the enzyme surface would influence stability in organic solvents. Many water-soluble proteins have highly hydrophilic surfaces with numerous charged residues that are solvated by water molecules. Transfer to a medium that is less effective in solvating charges could lead to unfavorable protein-protein interactions, a result of deionization, protein aggregation, or misfolding. It was proposed that the replacement of noncritical surface charges by uncharged residues would stabilize a protein for use in non-aqueous solvents by making the protein surface less hydrophilic and presumably less dependent on solvation by water for proper folding and stability. This strategy for protein stabilization in non-aqueous solvents is

attractive since proteins are particularly tolerant of amino acid substitutions at surface positions.

A strategy for creating a non-aqueous solvent-stable enzyme that includes rendering its surface more hydrophobic is also reasonable when one considers natural proteins whose native environments are largely non-aqueous. Crambin, which is soluble and stable in solvents such as dimethylformamide (DMF) and glacial acetic acid, has very few free charges on its surface, while its water-soluble homologs exhibit multiple charged Arginine (Arg) and Lysine (Lys) residues. Similarly, membrane proteins have surfaces which reflect their surroundings; the membrane-exposed surfaces of transmembrane proteins are significantly more hydrophobic than the surfaces of water-soluble proteins. In contrast, the interiors of water-soluble and membrane proteins are similar, a reflection of the very high degree of packing and complementary interactions that proteins have been able to achieve.

To test this hypothesis, we have replaced Aspartic acid (Asp) 248 and Arg 186 in subtilisin E with uncharged amino acids. Substitution of Asp 248 by Alanine (Ala), Leucine (Leu) and Asparagine (Asn) caused a slight decrease in stability of the enzyme in 40% DMF. We attribute this result to unfavorable charge-charge interactions in the mutated enzyme. Substitution of Arg 186, on the other hand, leads to a dramatic improvement in subtilisin stability in 40% DMF. To further study the effects of surface amino acid substitutions, we randomly altered the sequence of α-lytic protease at two surface charges, Arg 45 and Arg 78. At each position we have found five variants whose stability is greater than that of the original enzyme in 40% DMF. In eight of the ten variants which exhibit improved stability, the charge has been replaced by a neutral or hydrophobic amino acid.

The second approach that we have suggested for improving enzyme stability in non-aqueous solvents is to incorporate stabilizing interior crosslinks in the form of disulfide bridges, hydrogen bonds, or other electrostatic interactions. Hydrogen bonding should play a particularly important role in protein stability in non-aqueous solvents. In aqueous solution, a fully-extended polypeptide chain can satisfy its hydrogen bonding potential through interactions with the solvent. Similarly, folded proteins utilize a combination of internal protein-protein hydrogen bonds and interactions with the solvent to fulfill a very large portion of their hydrogen bonding potential. In a

non-aqueous solvent, unable to form hydrogen or weak bonds with its surroundings, the protein is forced to form these bonds internally upon folding in order to stabilize its structure. Mutations which improve internal (protein-protein) hydrogen bonding would be expected to increase protein stability in organic solvents.

The substitution of Asn by Serine (Ser) at position 218, originally discovered by another group using random mutagenesis and screening for thermostable variants, improves several internal hydrogen bonds and increases the stability of subtilisin BPN. We have introduced this substitution into subtilisin E in an effort to determine the extent to which improved internal hydrogen bonding will stabilize the enzyme in the presence of organic solvents. The Ser 218 variant is two times more stable than the original enzyme in 40% DMF.

Based on the experience of numerous groups, including ours, single mutations which result in improved stability in organic solvents can be combined, yielding combination variants in which the effects on stability are additive. The effects of combining mutations at positions 218 and 248 in subtilisin E, for example, are additive. Additivity is generally observed when the individual mutations do not interact with one another. Mutations in surface residues are not expected to interact with one another. We plan to combine the most promising single mutations at each surface charge position into a single variant.

This year, we have also begun to tackle the problem of improving enzyme activity in nonaqueous solvents. Due to our relatively poor understanding of the effects of amino acid substitutions on activity, we have chosen a random mutagenesis approach for this work. Random mutagenesis of subtilisin E by PCR techniques, combined with an efficient screening procedure, has allowed us to identify a mutation that improves by more than a factor of two the activity of subtilisin E in DMF/water mixtures. This result, obtained in only a few weeks, demonstrates the efficacy of this approach in generating enzyme variants that exhibit improved activity in nonaqueous media. Sequencing and characterization of these mutants will aid us in determining the important factors influencing activity in nonaqueous solvents. The mutation identified occurs in the substrate binding pocket and apparently improves interactions with the substrate. We hope that in the long term this research will provide us with clearly

defined strategies for selecting enzymes that are suitable for use in polar organic solvents and for engineering enzymes to exhibit improved stability and activity in these solvents.

Honors and Awards

Dr. Arnold is the recipient of a David and Lucile Fellowship in Science and Engineering awarded by the David and Lucile Packard Foundation. Dr. Arnold will receive $100,000 per year for five years and was among 20 outstanding young university professors nationwide chosen in the second year of the fellowship program. The funding provides for research expenses such as scientific equipment, research supplies, and scholarship support for graduate students.

Dr. Arnold joined the Caltech faculty in 1986 as a visiting associate, and became assistant professor in 1987. She received her BS from Princeton in 1979 and her PhD from UC Berkeley in 1985. Dr. Arnold's research uses molecular engineering techniques to design proteins with useful new properties, such as the ability to bind metals. Engineered metal binding sites can be used in the purification of recombinant proteins or to develop protein based techniques for removing toxic metals such as lead and mercury from wastewater. Another area Dr. Arnold is researching is the modification of enzymes to make them stable and active in solvents other than water. Dr. Arnold was named an Office of Naval Research Young Investigator in 1988, and a Presidential Young Investigator in 1989.

(3) Predictive Models and Effects of Structure on Catalytic Properties (W. Goddard, California Institute of Technology).

The Biocatalysis Design and Biotechnology Simulations Center

The DOE Center for Biocatalysis Design and Biotechnology Simulations (DOE-CBD) was established as part of the Materials and Molecular Simulation Center (MSC) of the Beckman Institute at Caltech. The goals of the MSC are to:

a. Focus *theoretical research* on the key bottlenecks that obstruct application to the important industrial problems.

b. Facilitate the *technology transfer* of advances in molecular and materials simulations from university research labs to industrial practice.

The premise in establishing the center is that the advances in quantum chemistry and molecular dynamics (collectively referred to as molecular simulations) have progressed to the point where there are

opportunities for application to a number of important industrial problems involving biotechnology. Indeed many industrial leaders in the United States, Europe, and Japan have the general perception that theory is becoming an important industrial tool and have begun to develop theory thrusts in their industrial research laboratories. Despite this obvious potential, straightforward use of theory for the most important industrial biotechnology problems is often impossible.

Thus, finesse is a critical component of most advances. The scientist with a thorough understanding of both the basic theory and the application makes simplifications in the theory to make the calculations practical, while ensuring that the simulation adequately describes the application of interest. Consequently, progress in this area is most effectively made by coupling the theorists at the cutting edge of theory and simulation with the chemists, biotechnologists, or material scientists driven to design new or improved materials, catalysts, processes, etc.

There is a difficulty in achieving this coupling. The basic theoretical research is concentrated in universities, and these groups often have little direct knowledge of the most important industrial applications. In addition, the theoretical efforts tend to be fragmented with no central location where a variety of theoretical efforts are coordinated to focus on the objective of providing the breakthroughs needed for real applications. On the other hand, even the largest industrial organization usually has at most only a handful of theorists, far too few to keep up with the research advances in the various fundamental areas of theory or with the applications to various fields.

For this reason, we have made a major emphasis in the DOE-CBD the establishment of strong collaborative efforts with leading industrial laboratories. Each industrial collaborator funds, or partially funds, a project in the DOE-CBD (or in other parts of the MSC) and assigns specific scientists to spend time at the DOE-CBD on the collaborative project. During 1990, the MSC industrial collaborators included: Allied Chemical (Dr. Willis Hammond), BP America (Dr. John Kerins), Dow Chemical (Dr. Ed Sanders), Failure Analysis (Dr. Alan Donaldson), General Electric (Dr. Randy Steward, Dr. Ken Smith, Dr. Herman Finkbeiner), Xerox Canada (Dr. Tom Kavassalis), ICI (Dr. Elizabeth Colbourn and Dr. Dave Clark), Nippon Steel (Dr. Atsushi Nogami), and Asabi Blass (Dr. Takashige Maekawa and Dr. Hiroshi

Yamamoto). All of these companies made financial contributions, provided collaborators, and spent between 1 week and 1 year at the MSC working on the collaborative projects. In 1991 Eastman-Kodak and MMM have agreed to join and several other companies have expressed strong interest. The MSC has space and computer workstation facilities for up to 10 visitors at any one time.

In addition to the intense interactions of the collaborations, the DOE-CBD and MSC will have several workshops each year, especially organized for industrial MSC members. Two or three workshops per year will be aimed at newcomers to simulations and modeling. Typical participants here would be industrial scientists trained as experimentalists who want to begin using simulations and modeling in their research. Such workshops will be for about three days with a combination of lectures and hands-on practice (with one-on-one assistance from MSC tutors). In addition, we plan one or two one-day workshops aimed at industrial managers. These would focus on case studies to illustrate successes (or failures) in simulation plus a window into new developments and new opportunities. In addition, there will be two or three workshops (2 or 3 days) focussing on advanced topics in specialized areas.

Our first MSC workshop was held from July 9 to 13, 1990. This workshop considered a range of molecular modeling techniques appropriate for polymers, biomolecules, and crystal surfaces. It was aimed more at newcomers to modeling and managers and was designed for industrial collaborators. The first two days were ideal for managers, with discussions of the basics and of recent advances in modeling. This was followed by three days of hands-on practice sessions (restricted to 10 participants) by the participants, who worked closely with DOE-CBD and MSC staff on strategies for solving real problems. Each participant had his own Silicon Graphics 4D/25 super workstation (attached to a network containing a 4D/220 server) and a personal MSC tutor for the hands-on sessions.

Research Summary

Our focus is to develop and validate the tools for a new approach to biocatalysis and biotechnology using the techniques of macromolecular simulation a) to *design completely new biocatalysts*, b) to redesign old systems so as to be stable at higher temperature or in new solvents or on new supports, c) to test the design

concepts through computer simulation, and d) to provide data that would facilitate validation of the design through experimental tests (infrared and Raman spectra, NOE couplings, structures in various media).

A major objective of this work is to provide case studies of simulation/design on various systems to illustrate for industrial scientists and others how such approaches might be used for their own proprietary systems. Also, we want to establish the reliability of various approaches (force fields, type of dynamics, structure prediction algorithms, etc.) so that experimentalists using simulation techniques would be better able to select appropriate methods and to judge the results.

In order to help obtain these objectives we are using cytochrome P-450 as a prototype system for de-novo design of new biocatalysts. These studies use the results from quantum chemistry calculations to quantify the relation of force field parameters with oxidation state, spin state, and character of axial ligands for Fe-porphyrin complexes. The goal is to design systems selective for partial oxidation of new substrates.

Methodology

The focus of our research is to develop and validate atomic-level simulation tools needed to design, develop, and characterize new optimized systems for biotechnology.

In atomic-level simulations, one starts with a general description of the energy as a function of geometry (a force field).

$$E(\mathbf{R}_1, \ldots, \mathbf{R}_N), \tag{1}$$

calculates the forces by taking derivatives,

$$\mathbf{F}_I = -\nabla_I E, \tag{2}$$

and calculates the dynamics (trajectory of correlation and velocities as a function of time) by solving Newton's equations.

$$M_I \frac{d^2\mathbf{R}_I}{dt^2} = \mathbf{F}_I \tag{3}$$

Such calculations can include the effect of solvent and are practical for systems with several thousand atoms.

Biotechnology applications requiring these methods include, a) predicting how the structure and activity of an enzyme are modified by placing the enzyme in a hydrophobic medium (which allows products to be more easily separated) and redesigning the solvent-accessible regions to stabilize the active form of the enzyme in a hydrophobic medium, b) redesigning the active site of an enzyme[4] with recombinant DNA techniques so that it will be less sensitive to the presence of product of other inhibitors, or so that it operates on a new substrate or so that it is stable at a higher temperature, c) predicting the three-dimensional structure[5] for use in developing better inhibitors or for use in designing modified enzymes, and d) characterizing the three-dimensional structure of an enzyme in solution by combining distance information from two-dimensional NMR-NOE studies with structure predictions from force fields to obtain the three-dimensional structure[6].

Critical to simulations is *accuracy* of the **force field** used to predict structure and dynamics. The force fields are described in terms of short-range valence (bonded) interactions (E_{val}) expressed in terms of two-body (E_{bond}), three-body (E_{angle}), and four-body ($E_{torsion}$, $E_{inversion}$) terms.

$$E_{val} = E_{bond} + E_{angle} + E_{torsion} + E_{inversion} \quad (4)$$

and long-range nonbonded interactions (E_{nb}) composed of van der Waals (E_{vdw}) and electrostatic (E_Q) terms

$$E_{nb} = E_{vdw} + E_Q. \quad (5)$$

A critical problem in the design of new biological systems (biopolymers, biocatalysts), is the **prediction of the tertiary structure** and properties of the new system. Thus, given a specific primary sequence of amino acids, we want to predict the secondary and tertiary structures (folding) of the functional protein

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[4]A.M. Naylor and W.A. Goddard III, In: ACS Symposium Series No. 392, Biocatalysis and Biomimetics, Eds. J.D. Burrington and D.S. Clark (American Chemical Society, Washington, DC, 1989), Chapter 6, pp. 65-87.

[5]K.W. Plaxco, A.M. Mathiowetz, and W.A. Goddard III. Proc. Nat. Acad. Sci. USA, 86:9841 (1989).

[6]K.T. Lim, A.M. Mathiowetz, and W.A. Goddard III. "Predictions of Structure Using NOESY Plus Full Relaxation Matrix Evaluation of NOE Intensities: Application to CRO", to be submitted.

complex. The problem is that the conformation is determined by weak forces (van der Waals, hydrogen bonding, electrostatic), with the result that an enormous number of conformations give similar energies. A further complication is that the time scale of a system refolding from one conformation to another is slow (maybe seconds). Thus, in a molecular dynamics simulation, the system may oscillate about one conformational minimum of 10^9 to 10^{15} time steps before it begins to unfold and refold to a better conformation. As a result, it is not yet possible to predict the conformation (tertiary structure) of even small proteins (say, 50 amino acids or 500 atoms). However, to design new biochemical systems, it is essential that we be able to predict the structure so that the concomitant properties can be properly predicted.

Because of these difficulties we are pursuing several strategies to structure prediction.

a. Torque Mechanics With C_α — Constraints (TM/C_α). Many interesting proteins belong to homologous classes, probably linked by evolution, where the overall folding can be expected to be similar despite insertions and deletions at various places and despite nonhomologous regions. With this in mind, we have developed the following approach.[7]

i. We start with the primary sequence of the protein with all structures in the optimum configuration for separate residues and map them onto a given C_α structure in such a way that no bond lengths or angles are changed and such that the RMS error in the C_α positions is a minimum. This is done by sequential addition of residues with local optimization of backbone and sidechain torsions (torque minimization). This minimization is carried out in the presence of harmonic constraints to bias the C_α positions to match the input C_α coordinates.

ii. The sidechain positions are optimized by minimizing the torques for all sidechain torsions (mainchain atoms fixed).

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[7]A.M. Mathiowetz and W.A. Goddard III, "Torque Mechanics with C_α Constraints. An Approach to Structure Predictions for Homologous Proteins", to be published.

iii. Solvent is added, followed by full minimization of protein/solvent system with mainchain atoms fixed.

iv. All restrictions are relaxed and the structure from iii is reoptimized.

In order to estimate the position of C_α's to be deleted or inserted, we use the following approach. Considering *only* the C_α's, we include harmonic bond length, angle bend, and torsions by using equilibrium values corresponding to the known reference structure. Deleting and adding a residue leads then to deleting or adding C_α's in this pseudo-structural calculation, and we reminimize the C_α positions with these modifications. It is this C_α structure that is used to initiate step *i*. above.

A recent success for this approach was the prediction[5] of the DNA-protein complex HIX/HIN 52 (where HIN 52 is the DNA-binding part of HIN recombinase and HIX is the 13 base-pair binding site). The HIN 52mer is somewhat homologous to λ CRO, and an x-ray structure of λ CRO (without DNA) has been completed, but only C_α coordinates are available. Thus the first step was to complete the structure of λ CRO using TM/C_α. The validity of the structure was tested by docking the predicted λ CRO to DNA in the conformation previously suggested by the x-ray crystallographers. All interactions were reasonable. We then built the HIN 52mer onto the λ CRO C_α framework and carried out TM/C_α. At this point we carried out an extensive series of studies docking the predicted HIN 52mer to the HIX site of DNA and using various data from footprinting and genetics studies to guide the search. We carried out the minimization on several candidate structures and eventually obtained a structure for HIX/HIN52 that satisfies all current experimental data. This was used to suggest a number of experiments that could give detailed tests of the predictions of the effect on binding of changes in protein and DNA. A number of these experiments are in progress; so far the model has passed all tests.[8]

[8]K.W. Plaxco and M. Simon, private communication.

b. **Optimal Loops.** The most variable parts of homologous proteins are the loops connecting various α-helices and/or β-sheet regions. Often these loops are less than 20 amino acids. We have found that torque dynamics or torque minimization with loop end points (TD/LE or TM/LE) is an effective approach to predictions of these structures. Thus, to determine the structure for the 15 amino acid sequence connecting helices B and C, we start with the distance between the terminal C_α positions of B and C, and (with this distance fixed) optimize all atoms of the 15 amino acids using torque dynamics. This is implemented by calculating all forces in cartesian space and then transforming to torques about all dihedrals and applying these torques to the cartesian structure. After finding the low energy structure from this fixed endpoint calculation, we include the atoms of the B and C helices (still kept fixed) and reoptimize. At this point the solvent can be added and the full structure optimized.

c. **Full Prediction of Tertiary Structure.** Because of the vast size of the configuration space, we believe that first principles predictions of tertiary structure will involve stochastic sampling of configuration space to obtain overall conformations followed by molecular dynamics to optimize short-range structures. To this end we have explored many approaches with Monte Carlo simulated annealing[9] and related approaches. A simple variation we have found useful is using Brownian Torsions, where random pulses in the torques are used to heat the torsion space and allow rapid sampling of conformational space, while keeping the remainder of the structure at rest. Although our goal is to find general approaches to the folding problem, our focus currently is upon various constrained approaches, with an emphasis on techniques that have a chance to be extended to full optimizations of protein-sized systems (>40 amino acids).

d. **Three-Dimensional Structure From MD and 2D-NMR NOE.** A major problem in designing and developing new enzymes and other biological materials is to determine the three-dimensional structure of the folded protein. X-ray crystallography requires a large amount of protein, the ability to

[9]N. Karasawa and W.A. Goddard III, J. Phys. Chem., 92:5828 (1988).

crystallize good quality crystals, and considerable effort in extracting the structure from the data. Theoretical prediction of tertiary structure from primary sequence is a noble goal, but practical techniques are not yet on the horizon. However, a practical method does not exist for combining experimental and theoretical studies to obtain three-dimensional structures of proteins and nuclei acids in solution.

The two-dimensional nuclear Overhauser effect (NOE) spectrum of a protein contains information about the molecule's structure, primarily in the form of distances between cross-relaxing protons. The observed NOE intensities have the form

$$\mathbf{A} = \mathbf{A}_o e^{-\mathbf{R}t_m} \qquad (6)$$

where **A** is the matrix of peak intensities for all pairs of protons, $\mathbf{A}_o$ contains the magnetization at zero mixing time, **R** is the relaxation rate matrix (where the off-diagonal element *ij* is directly proportional to R_{ij}^{-6}), and t_m is the mixing time. The A_o scaling factors may be obtained either from extrapolation of multiple mixing experiments or from one-dimensional relaxation measurements. A motional model (typically isotropic tumbling with no internal motions), must be assumed. Internal motions are generally neglected, leading to likely errors of around ± 0.2 Å.[10]

Most commonly used to obtain structure from NOE data is the distance-geometry approach (NOE/DG) that uses the observed peak intensities to suggest a range of distances for each pair of protons and then searches for a three-dimensional structure satisfying all the pairwise distance constraints. This has led to useful results but is limited to cases where most peak assignments have been made. In addition, it generally does not provide information on the location of various protein domains with respect to each other. An alternative approach is to use the NOE distances as constraints in molecular mechanics minimization calculations. This allows the NOESY data to bias the calculations toward the desired structure. Such molecular mechanics minimization (MM) with

[10]A.N. Lane, J. Magn. Reson. 78:425-439 (1988).

NOE distance constants (DC) or MM/DC/NOE has the significant advantage over distance geometry (NOE/DG) of being able to take into account all van der Waals, electrostatic, and solvent effects. For proteins, these are especially important determinants of tertiary structure. This MM/DC/NOE approach has been successfully applied to model problems but predictions of unknown structures from MM/DC/NOE have not yet been reported.

Our approach is instead to calculate the NOE *intensities* directly from the geometry using (6) so that all spin diffusion pathways are included in the relaxation terms. We then constrain the dynamics with these NOE intensities, MD/INT/NOE. Thus, for a given structure, the difference between the calculated and observed peak intensities provides a force that is used in conjunction with the force field to refine the structure. This simulation process is computationally expensive since it must be applied repeatedly as the structure is improved. On the other hand, it has the advantages of taking into account all possible spin diffusion pathways and of being able to use partial peak volume information. In particular, it is possible to handle overlapping peaks by comparing sums of calculated intensities with integrated volumes. We believe that this approach will allow accurate prediction of crystal structures. A major advantage of dynamics over minimization methods is that it can sample large portions of the available conformation space because of the thermal energy in the system. It is thus less vulnerable to being trapped in false minima. This method of combining molecular dynamics with NOE simulation should be a significant advance over current techniques.

The force field energy expression includes

$$\sum_{i>j} \left(A_{ij}^{calc} - A_{ij}^{obs} \right)^2 \qquad (7)$$

deviations in NOE intensities as defined in equation (6). This allows interactive calculations within our molecular dynamics program.

Currently we have found that a truncated power series of between 12 and 20 terms adequately defines the NOE intensity equation (6) and yet reduces computational time when compared with the standard eigenvalue method of Keepers and James.[11] Further optimizations taking advantage of the highly sparse nature of the relaxation rate matrix reduce the required time even more. This algorithm, when combined with the powerful multiprocessing computers with vector hardware now available makes it feasible to execute many NOE simulations over the course of a dynamics run. The sum of the squares of the deviations of the calculated intensities from the observed ones, scaled by an appropriate amount, is added to the energy calculated from the force field. For methyl protons, indistinguishable protons in rapidly rotating rings, and protons producing overlapping peaks, the sum of the intensities calculated for the protons and the sum of the observed intensities is compared. This has been tested using published experimental data from Crambin and for λ CRO. In the case of Crambin, there is an X-ray structure for assessing accuracy. For λ CRO, only the C_α coordinates from X-rays are available, and we used the structure predicted by the TM/C_α procedure.

We see this MD/INT/NOE technique becoming a major tool in biotechnology over the next few years. One of our goals during the next year or so will be to develop this into a robust methodology that could be used rather routinely by the biotechnology community.

Biocatalyst Design

In order to provide a case study of how to use simulation in developing biocatalysts, we have decided to focus on a system capable of selective oxygenations with the goal of converting alkanes or alkenes into specific oxygenates. There are several organisms that metabolize alkanes effectively,[12,13] and our prototype enzyme is cytochrome P-450, known to play a role in

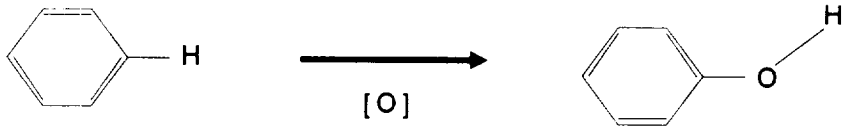
[11]J.W. Keepers and T.J. James, J. Magn. Reson. 57:404-426 (1984).

[12]S. Fukui and A. Tanuaki, Advan. Biochem. Engr., 19:217 (1981).

[13]H.J. Rehm and I. Reiff, Advan. Biochem. Engr., 19:175 (1981).

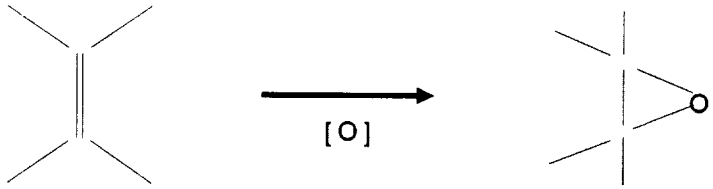
partial oxidation of long-chain alkanes and selective epoxidation of alkenes.[13,14] There is potential for use of this enzyme in biotechnology since methods of induction[15] and of cloning[16] the structural gene for (mouse) cytochrome P-450 are known.

Enzymes of the cytochrome P-450 class serve as highly selective monoxygenases, either in hydroxylation



(8)

or epoxidation



(9)

and we propose to design biocatalysts capable of carrying out these reactions on particular substrates (alkanes, alkenes, aromatics, alcohols). A great deal is known about various cytochrome P-450 enzymes.[14,17] Each involves an Fe-heme unit at the active site (Figure 3.1) and the Fe changes through a number of oxidation and spin states as it (a) is activated by presence of substrate, (b) activates O_2 and (c) hydroxylates or epoxidizes substrate. Indeed, we would want to incorporate control procedures similar to those in P-450:

a. In the resting state, the Fe is six-coordinate, low spin Fe(III) (with several H_2O in the sixth coordination site).

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[14]Cytochrome P-450: Structure, Mechanism, and Biochemistry, Ed. P. R. Ortiz de Montecellano (Plenum Press, New York, 1986).

[15]R.V. Smith and P.J. Davis, Advan. Biochem. Engr., 14:61 (1980).

[16]Y-J. Chen, M. Negishi, and D.W. Nebert, DNA 1:231 (1982).

[17]For example, see Cytochrome P-450. Eds. R. Sato and T. Omura (Academic Press, New York, 1978).

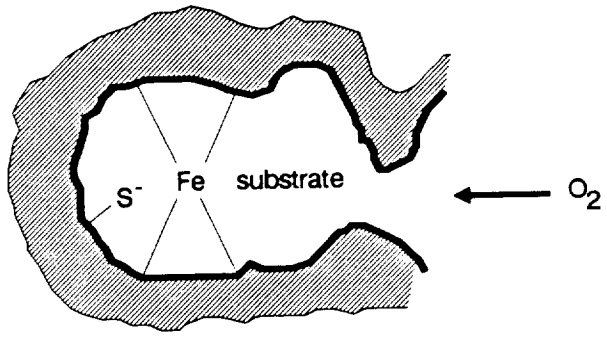


Figure 3.1 Active Site of P-450.

b. Binding of substrate displaces the ligands at the sixth coordination site, leading to high spin Fe(III), which has a redox potential that allows the Fe(III) to be reduced (by cofactor) to high spin Fe(II).

c. High spin Fe(II) binds O_2 to form an Fe(II)—O—O complex[18] (spin singlet) similar to that in myoglobin or hemoglobin [however, high spin Fe(III) cannot bind O_2].

d. The redox potential for the Fe(II)—O—O complex[18] changes sufficiently so that the complex is reduced (by cofactor) to form an Fe(II)—O—O^- complex.[18]

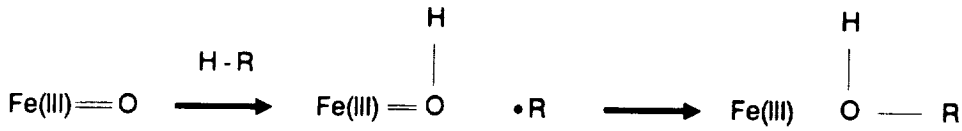
e. Very rapidly, this complex is protonated [probably going through an Fe(II)—O—OH intermediate] to yield[18] an Fe oxo intermediate, Fe(III)=O, which may lead to a porphyrin π radical with an[18] Fe(II)=O active site.

f. This Fe(III)=O or Fe(II)=O unit reacts with substrate to form epoxide or alcohol in a fast two step (cage) process[18]

g. Product leaves the hydrophobic cavity and water enters at the sixth ligand Fe(III) site, leading to the original low spin six-coordinate Fe(III) we

[18]We use a formalism in which covalent bonds to a metal are shown with solid lines and the oxidation state refers to the d configuration remaining on the metal [i.e., Fe(II) implies d^6; Fe(III) implies d^5]. To convert to the normal oxidation state formalism, increase the oxidation state of the metal by one unit per covalent bond. Thus, F(II)—O—O would normally be considered in terms of an Fe(III) center, and the Fe of Fe(III)=O would normally be considered as Fe(V).

started with in a.



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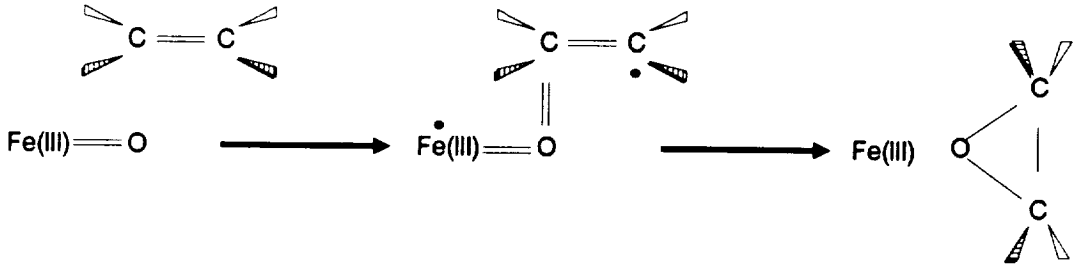
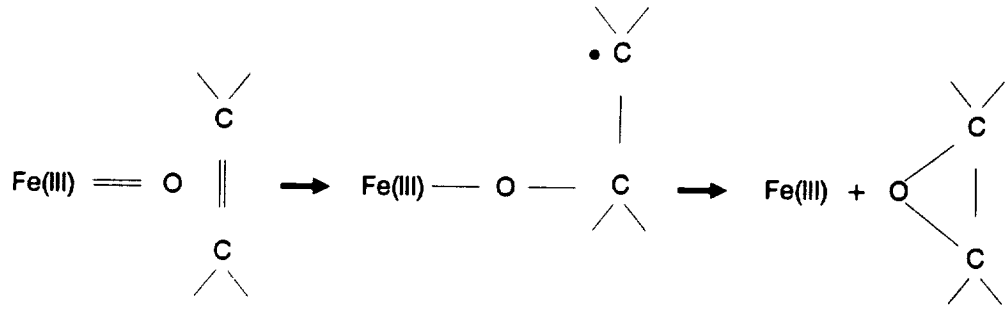


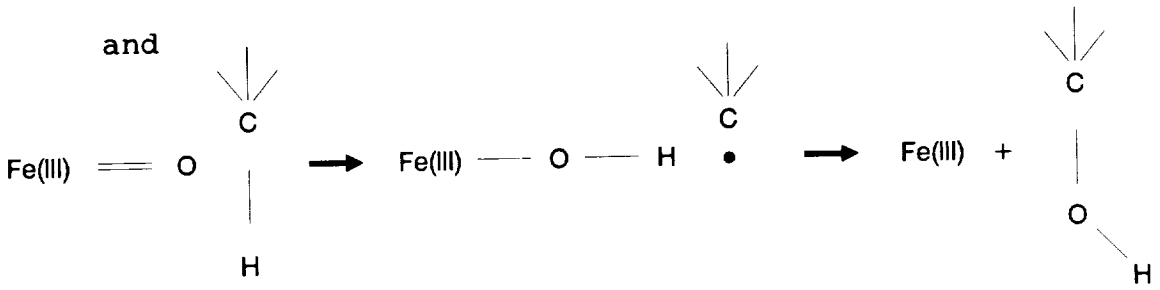
(11)

As the first step in this project, we have carried out accurate quantum chemical calculations on model systems for Fe-heme complexes without and with axial ligands (O or H_2O plus Cl^- to simulate cysteine). These studies included Fe in either ferrous or ferric oxidation states [including Fe(III)=O and Fe(II)=O] and considered either high spin, intermediate spin, or low spin.[19] In each case we have obtained potential surfaces for moving Fe and axial ligands and for the porphyrin. This provides the force field data that, combined with more standard force fields for proteins, can be used to simulate the *chemistry* of P-450 active sites.

As part of this project, we will extend this work to additional axial ligands, including O_2 and OOH. In addition, we will examine the potential surfaces for the actual reaction



[19]C. Park and W.A. Goddard III, "Molecular Description for the Active Site of P-450cam", J. Am. Chem. Soc., to be published.



With this force field, we will study first the P-450cam system, including either substrate or H_2O clusters in the active site and with either O or O_2 attached to Fe. We hope to clarify issues relative to mechanism for this system, including the pathways by which substrate, O_2, H_2O, and product diffuse between active site and environment and the pathways for electron transfer.

Later studies will involve other P-450 systems for which we will use techniques to predict the structure (starting with the experimental folding for P-450cam). As we obtain a good understanding of the P-450 system, we will attempt to build the same chemistry into soluble heme-containing systems whose structures are known (cytochrome c, myoglobin, hemoglobin). Guillochon[20] has shown that immobilized hemoglobin does hydroxylate substrates in a fashion quite similar to cytochrome P-450 (of liver microsomes) and hence this might be a good system to start design activities.

The goal here is to develop an approach by which we could design catalysts for selective oxidation of particular organic substrates. We will start with enzymes for which there are abundant experimental data on structure and kinetics (e.g., such selective biological oxidases as cytochrome P-450). As the project progresses to the point where designs are confirmed with both simulation and experimental studies, we can proceed to entirely new systems that may be small enough to be synthesized in more direct ways.

Major goals of the studies will involve:

a. Designing modified pockets in available enzymes for selective binding of simple chemical substrates (alkenes, alkanes, aromatics, alcohols, etc.). The selectivity of the new bonding pocket can be tested both with simulation and experiment.

[20]D. Guillochon *et al.*, Enzyme Microbiol. Tech., 4:96 (1962).

b. Developing strategies for covalent attachment of modified enzymes in particular configurations on appropriate supports. The efficacy of these strategies can be tested with experiments.

c. Developing approaches for eliminating the bulk of the protein to achieve a stable active site complex having the same (or better) activity. This complex would be covalently attached to a support.

d. Designing a semiconductor support that with proper doping could serve to reduce (or oxidize) the active site at appropriate steps in the reaction sequence. Control would then be provided by appropriately adjusting the external potential to the support.

e. Designing a catalyst monitor system using a related, modified enzyme; it could, for example, be covalently attached to the gage for a Field Effect Transistor (FET) to develop a sensitive monitor for charge transfer steps in catalyst operation.

There are a number of tough problems to solve in successfully designing such biocatalysts. However, such design capabilities would provide a powerful means for developing new specialized biocatalysts and could form the basis of new energy-efficient technologies.

Immunological Response to Chemicals

As part of a general project in predicting biological response to chemicals, we are focusing on predicting the structures of the antigen-binding sites of immunoglobulins. As indicated in Figure 3.2, six loops of polypeptide constitute the hypervariable regions. The three from the variable domain of the light chain (VL) are denoted L1, L2 and L3. The three from the variable domain of the heavy chain (VH) are denoted H1, H2, and H3. Within each domain these loops are connected to a β-sheet framework whose structure is conserved.

By analyzing several hundred antibody sequences (from Human and Mouse) and 10 crystal structures Chothia *et*

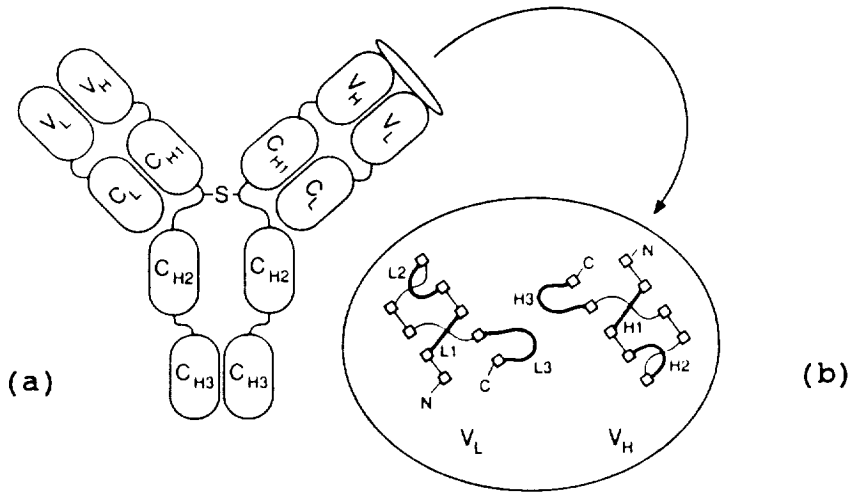


Figure 3.2 Antibody (a) and antibody site binding (b).

al.[21] were able to classify most antibody loop sequences into a small number of sequence types which determine the three-dimensional structures. This analysis applies to between 50% to 95% of the various L1, L2, L3, H1, and H2 loops. However it does not apply to H3 nor does it apply to other types of proteins or to antibody loops not corresponding to the canonical structures.

Our goal is to develop a general method for predicting all loops in all systems. Thus we wish to predict the structures of H3 loops as well as L1-3 and H1-2. The method is also applicable to loop-predictions in other types of proteins. Our method currently works as follows:

a. Build the loop as a straight-chain (using the peptide builder).

b. Side chains are taken to have their most likely conformation positions (based on prior analysis of the BKV data base).

c. The Monte Carlo method is used to modify the loop one residue at a time. At each step, one residue is chosen and its conformation angles (phi and psi) are changed to a phi and psi chosen from a grid (5-degree, 10-degree, 15-, 30-, or 60-) representing the probability that a given residue will have a given phi/psi pair. These probabilities have been determined from the BKV data base. This process is repeated until the

[21]C. Chothia *et al.*, Nature 342:877 (1989).

loop can be closed (using the loop-closing algorithm of Go and Scheraga).

d. Given this initial conformation of the loop, we optimize the side-chains using our Torque Minimization Methods.

e. All structures created are saved and ordered by energy.

We are in the process of testing this procedure for the known crystal structures. After completion, we will then apply it to several catalytic antibodies. For example, there are catalytic antibodies which catalyze the hydrolysis of choline carbonates and esters. These have sequences similar to McPC603 (one of the crystallized antibodies).

Collaborative Projects

A major goal of the DOE-ECUT projects in biocatalysis is to develop the capability for design of catalysts through experimental characterization and theoretical modeling of catalytic systems. The idea is to utilize graphics/energy optimization software in the design of biocatalysts for conversion of low-molecule weight hydrocarbons to oxychemicals.

Collaborative projects under this program, using the DOE-CBD facilities, include:

Theory of Biocatalysis: Electron Transfer Proteins. (D. Beratan). The purpose of this project is to obtain a more detailed understanding of molecular processes in enzymatic reactions for the development of technological applications in catalysis and microelectronics. The design of biocatalysts, or enzymes, poses unique challenges compared to design of small molecular catalysts. First, an understanding of the molecular mechanisms of biochemical reactions is more limited than for small molecule chemistry. Second, it is not yet clear *a priori* whether the surrounding protein super-structure is active or relatively passive in a particular biocatalytic reaction, and its role is generally not clearly defined. In this project, the simplest biocatalytic reaction, electron transfer, has been selected for theoretical and experimental investigation because of: a) the ubiquity of electron transfer in biocatalytic energy harvesting and conversion pathways, and b) the unusually large potential for taking advantage of such reactions to develop catalysts based on qualitatively new design principles.

Protein Engineering for Nonaqueous Solvents. (F. Arnold). The success of a rational design procedure for constructing proteins to use in organic solvents depends on understanding relationships among various factors: amino acid sequence, secondary and tertiary protein structure, and activity and stability in nonaqueous solvents. The goal of this research is to begin to define these

relationships, by implementing an integral and iterative protein engineering approach based on the model hydrophobic protein Crambin. The unusual stability and solubility of Crambin in a wide range of nonaqueous solvents will be investigated. The structures, stabilities, and solubilities of Crambin mutants will be measured and correlated with specific alterations in the protein amino acid sequence. This research includes studies of the properties of Crambin, design of new mutant Crambins by computer/molecular graphics, production of new mutants by site-directed mutagenesis, and detailed NMR studies of Crambin stability and response to nonaqueous solvents. By means of a combination of genetic engineering and chemical modifications of Crambin, a new semisynthetic enzyme that will be stable in organic solvents will be developed. The results of this work will be used to formulate a set of criteria and methods for rational design and production of proteins to be used in the presence of nonaqueous solvents.

Phosphophoryn. (J.S. Evans and S.I. Chan). Phosphophoryn is a polyelectrolyte mineral matrix protein which binds $C\alpha^{2+}$ and regulates the formation of the solid phase mineral, calcium hydroxyapatite $[C\alpha_{10}(PO_4)_6(OH)_2]$, from solution. There are several important questions, concerning these proteins, including

a. What conformations do these proteins assume in the presence of monovalent counterion ($N\alpha^{+}$) and divalent counterion ($C\alpha^{2+}$)?

b. What are optimal distances between amino acid sidechain oxygen atoms (bearing lone pairs) and counterions with charges of +1 and +2?

c. How do these idealized structures compare to spectroscopic data obtained for phosphophoryn and synthetically-prepared peptides?

The answers to these questions should be useful in understanding both biomineralization and the properties of polyelectrolyte polymers. The specific theoretical studies focus on molecular mechanics (energy minimization), molecular dynamics; and Monte Carlo (torque dynamics) studies of the conformation of n-acetyl C-methyl ester peptides $(Asp)_{10}$, $(Asp)_{20}$, $(Pser)_{20}$, $(Pser-Asp)_5$, and $(Pser-Asp)_{10}$. These peptides represent domains which are located in phosphophoryn. Complementary NMR studies of the systems are also in progress.

Modeling of Carbohydrates (G. Richards). The possibility of modeling three dimensional structure of polysaccharides is being investigated based on the K63 monomer (D-galactose-a-3-D-galacto-uronic acid-o-3-l-fucose) for application to drag reduction of Navy vehicles (funded by ONR/DARPA). Along with Richards, K.T. Lim (of the MSC) is working on the use of molecular simulation techniques to predict structures and properties for oligomers of this and related systems. They are generating an improved force field for polysaccharides, by modifying the Dreiding force field to predict the exact conformations and relative energetics of simple sugars, comparing X-ray structures in the Cambridge Crystallographic Database and HF quantum chemical calculations. They will then use this force field to predict structures of various trisaccharides thought to have good drag reduction properties. Two-dimensional NMR-NOE (nuclear magnetic resonance -

nuclear Overhauser effect) studies of such systems will also be investigated and will use NOE intensity-constrained molecular dynamics (MD/INT/NOE) to refine the structures based on the force field plus experimental data. We will also predict structures and properties of variant monomers (e.g., acetylated) and oligomers in order to design optimal materials.

Modification of Superoxide Dismutase (SOD) (J. Valentine). Dr. Valentine of UCLA is a leading expert in chemistry of SOD systems and has carried out numerous studies of the effect of metal replacement and of mutations upon the chemistry of SOD systems. Over the last year there has been a joint project involving a graduate student in Professor Valentine's group and a graduate student in Professor Goddard's group to predict the structures of SOD systems using structural data for homologous SOD's for which structural data exist. These structure predictions are being used to design experiments.

Characterizations of Iron-Sulfur Proteins (T. Spiro). Dr. Spiro of Princeton is a leading expert in the spectroscopy of iron-sulfur moieties, both inorganic and biological. Over the last year a postdoctoral fellow in Professor Spiro's laboratory has been collaborating with a postdoctoral fellow in Professor Goddard's laboratory to develop a force field adequate for describing the infrared and Raman spectroscopy of Fe-S units in various ferrodoxins. With a good force field we could predict the correlation between shifts in characteristic vibrational states with changes in three-dimensional structure and use real-time observation of the shifts to infer real-time changes in the three-dimensional structures.

Structure of Crambin in Various Solvents (F. Arnold). Dr. Arnold of Caltech has funding from DOE to develop new or modified enzymes that will be soluble and active in hydrophobic media (allowing easier extraction of products). An early step in this program is to explore how the structure changes in various media and later to see how the structure changes for modified proteins. To this end her group has used their high-field NMR system to obtain NOE data as a function of mixing times, and they have assigned nearly all peaks for a form of Crambin. Using these data to obtain the A_o matrix of peak intensities, the Goddard group will use these constraints with MD/INT/NOE to predict the structures in solution. The X-ray structure is known for this first system so that one can test the validity of the predictions (of course, some changes in structure are expected between solution and crystal).

After completion of this first system, we should be able to quickly examine the perturbations due to different solvents, different temperatures, and mutations. This is the first step of a vigorous collaboration in which simulation will play a pivotal role in allowing the results of the experiments to be interpreted, which will in turn allow the design to be iterated and improved.

Structure of the BAND 3 Protein in a Membrane (S. Chan). Dr. Chan of Caltech is a leading expert in characterizing mechanistic aspects of BAND 3 protein. Two graduate students from his group in collaboration with a graduate student in Professor Goddard's group have succeeded in establishing a three-dimensional structure

for the ~500 amino acid region involving the membrane.[22,23] They used all available experimental data to assess the likely fragments embedded in the membrane, docking of the 13 trans-membrane regions to form a most plausible active site, building the connecting loops onto these regions, and reorienting to minimize strain. The result was the first atomic level mechanism[23] to explain the known characteristics of BAND 3. Refinement of this structure is continuing.[24]

Dihydrofolate Reductase (DHFR) (S. Benkovic). Dr. Benkovic of Pennsylvania State has led a vigorous program involving selective mutations in the active site for DHFR (*E. coli*), followed by very detailed kinetic studies of modified enzymes.[25] This research has been in collaboration with the Goddard group (Adel Naylor) where computer simulation was used to predict the changes in microscopic structure and mechanism for the various mutated enzymes.[4,25] This project was quite successful, with the simulation providing a number of insights that helped design and interpret new experiments. This collaboration will continue now that Dr. Naylor is in Pennsylvania (Merck, West Point, Pennsylvania), but will no longer involve the Caltech group.

Dendrimers (D. Tomalia). Another successful collaboration involves a new class of polymers, the starburst Dendrimers, developed by Dr. Don Tomalia and co-workers at Dow Chemical.[26] These polymers show promise as guest/host complexes for drug delivery and other applications; however, there has been no experimental method for obtaining microscopic structural information. The Goddard group initiated the first simulations on such systems and was able to characterize the structures, including internal cavities.[4,22] This work led to predictions of systems for encapsulating a molecule (dopamine) and delivering it to dopamine receptor sites.[4] We have now completed a comprehensive review of dendrimer systems using the insights from simulations to suggest designs of new materials.[27] This area is ripe for exploration, and the Goddard research group will collaborate with interested experimental groups.

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[22]C.B. Musgrave, S. Dasgupta, and W.A. Goddard III, "A New Force Field for Silicon-Hydrogen Systems", to be published.

[23]N. Vogelaar, Ph.D. Thesis, California Institute of Technology (1988); N. Vogelaar, S.I. Chan, and W.A. Goddard III, to be published.

[24]K. Kanes, A.M. Mathiowetz, and W.A. Goddard III, unpublished work.

[25]S.J. Benkovic, J.A. Adams, C.A. Fierke, and A.M. Naylor, Pteridines, in press (1989); S.J. Benkovic, C.A. Fierke, and A.M. Naylor, Science 239:1105 (1988).

[26]D.A. Tomalia *et al.*, Macromolecules 19:2466 (1986); D.A. Tomalia, M. Hall, and D.M. Hedstrand, J. Am. Chem. Soc., 109:1601 (1987).

[27]D. Tomalia, A.M. Naylor, and W.A. Goddard III, Angew. Chem. Int. Engl. 29:138 (1990).

(4) Simulations on Calcium Regulated and Redox Biocatalytic Systems (A. Redondo, P.J. Hay, and A.E. Garcia, Los Alamos National Laboratory). In this project we have undertaken theoretical calculations along two parallel paths. In the first approach, we have recently developed a method to carry out simulations for the efficient computation of reaction rates for biomacromolecules in solution. The calculation of the reaction rates is accomplished by solving a three-dimensional partial differential equation whose solution is the time-dependent probability of finding one molecule or atom at any point surrounding a specified biomacromolecule. In the second set of calculations we have studied the interactions of the mutual effects between a biopolymer and the solvent that surrounds it. These simulations have been performed using the methods of molecular dynamics.

Calculation of reaction rates

The calculation of the reaction rates between a biopolymer and other molecules has been hampered by the magnitude of the computational process. Even the simplest processes, such as the binding of an atom or ion to a molecule require astronomical amounts of computer time if the atoms have nonvanishing interactions. In principle, it is possible to calculate reaction rates using molecular dynamics. In this approach, one would carry out a large number of trajectory computations for the motion of the ion around the molecule, each one starting with different initial conditions. Then, one would use the trajectories to compute the reaction rates by carrying out statistical mechanical averages.

Unfortunately, the direct simulation of reaction processes via molecular dynamics is hindered by the large amounts of computer time necessary to obtain meaningful results. These systems are characterized by the "rare event" nature of their dynamical evolution from reactant to product states. Because there is a "bottleneck" in phase space through which the system must pass to change states, direct integration of the equations of motion may require many years of computer time before a single reactive event is observed.

For such systems it is necessary to develop alternative

approaches to direct molecular dynamics.[28] Accordingly, we have developed a procedure based on a particular version of the Fokker-Planck equation known as the Smoluchowski equation.[29] The idea of this procedure is as follows.

For definiteness, let us consider the following system. Calmodulin is a calcium binding protein that has a number of charged amino acids that lead to electrostatic interactions with its surrounding environment. Any calcium ions diffusing in the solution feel this electric field and may eventually be captured by calmodulin. The protein, after binding up to four calcium ions, is thought to undergo conformational changes that allow it to activate enzymatic processes. Our goal is to calculate the reaction rate for the binding of calcium ions to the protein. For this purpose, we set out to calculate the probability $w(\mathbf{r},t)$ of finding a calcium ion at time t at a point $\mathbf{r}$ in the surroundings of the protein. Once this probability is known for sufficiently long times, one can easily calculate the reaction rate by properly identifying those sites in the biopolymer that bind the ions.

If the calcium ion did not interact with the protein, the probability $w(\mathbf{r},t)$ could be found by solving the diffusion equation

$$\frac{\partial w(r,t)}{\partial} = D\nabla^2 w(r,t)$$

where D is the diffusion constant of calcium ions in aqueous solution. This, however, is never the case for biopolymers because they interact with the ion via the nonbonded forces, such as the Coulomb attraction and repulsion. Therefore, it is necessary to determine $w(\mathbf{r},t)$ using an equation that takes into account the fact that the ion diffuses in the solution in the

[28]One such approach has been proposed by Chandler and coworkers [D. Chandler, *J. Chem. Phys.* 68:2959 (1978); J.A. Montgomery, Jr., D. Chandler, and B.J. Beme, *J. Chem. Phys.* 70:4056 (1979)] and by Voter and Doll [A.F. Voter and J.D. Doll, *J. Chem. Phys.* 80:5832 (1984); ibid 82:80 (1985); A.F. Voter, idem 82:1890 (1985)]. However for the study of biomacromolecules, this procedure still requires the calculation of large numbers of molecular dynamics trajectories.

[29]S. Chandrasekhar, *Revs. Mod. Phys.* 15:1 (1943).

presence of a potential V(r). This equation is the Smoluchowski form of the Fokker-Planck equation.

$$\frac{\partial w(r,t)}{\partial} = D\nabla^2 w(r,t) + \gamma\nabla[w(r,t)\,V(r)]\,,$$

where γ is a friction coefficient.

Because the Smoluchowski equation in three dimensions is considerably more complicated than the diffusion equation, it must be solved numerically. To that effect, we have developed an efficient algorithm, based on the alternating-direction-implicit method,[30] that solves the equation in a three-dimensional mesh of points. An advantage of our method is that it can be applied to systems other than biological molecules. In fact, it is our intention to use it in a study of the catalysis of methanol by zeolites that is currently under way in our laboratory.

Before we can find a solution to the Smoluchowski equation, however, we must decide what the potential V(**r**) looks like. This is not a trivial point because both the biomacromolecule and the ion are immersed in an aqueous solution with a non-neutral pH. Indeed, the charges on the protein are going to be screened by the ions and molecules of the solvent. We must, therefore, use a potential that takes this effect into account. To do this, we have employed a potential obtained by solving the Poisson-Boltzmann equation.[31] This approximation assumes that the ions and molecules of the solvent are arranged around the biopolymer in a manner consistent with the Boltzmann distribution and the Poisson equation of electrostatics. Although this approximation fails at very small distances, it appropriately describes the screened fields at distances of the order of the size of the biomacromolecule, where the calcium ion is diffusing. In Figure 3-3 we show a 3D surface plot of the Poisson-Boltzmann potential for the molecule calmodulin corresponding to an equipotential surface potential of -1 Kelvin/electron.

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[30]R.D. Richtmeyer and K.W. Morton, Difference Methods for Initial Value Problems, 2nd Ed. (Interscience, New York, 1967), p. 211.

[31]I. Klapper, R. Hagstrom, R. Fine, K. Sharp, and B. Honig, *Proteins* 1:47 (1986).

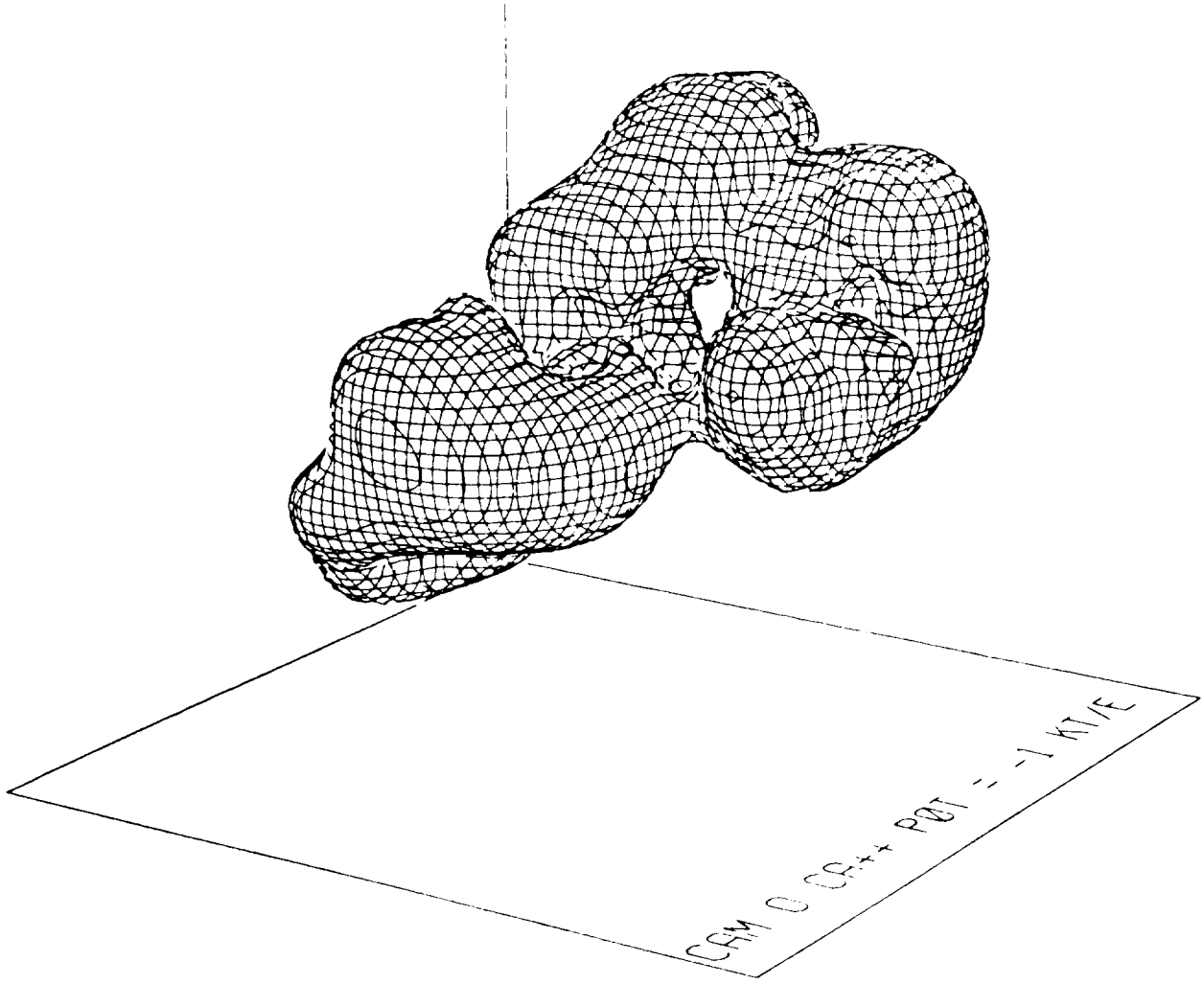

Figure 3-3. 3D equipotential surface plot for calmodulin.

Preliminary calculations using our algorithm for solving the Smoluchowski equation indicate that the rate of capture of calcium ions by calmodulin is several tens of times larger than one would expect by pure diffusion without the aid of the electrostatic field. These calculations also show that the algorithm is sufficiently efficient to be able to carry out many of the calculations in modestly priced computers, such as those based on RISC architectures. We are currently carrying out a more extensive set of calculations for calcium binding to four sites in calmodulin. These results will be reported in future publications.

Molecular dynamics studies

In the course of our studies of the interactions of calmodulin with ions and solvent molecules we came to the conclusion that it is necessary to assay in a systematic manner the effect of the solvent on the protein and vice versa. These effects have been sparsely treated in the literature, but are essential to the understanding of biocatalytic effects. In order to carry out such systematic studies we decided to carry out molecular dynamics studies of the interaction between water and the relatively small (46 amino acid residues) protein crambin.[32] The crystal structure of this protein is known[33] and was used as a starting point for our calculations.

We performed molecular dynamics simulations lasting for 240 picoseconds in steps of 0.05 ps. These calculations produced more than 640 Mbytes of atomic trajectories. With these atomic positions and velocities of all the atoms present in the simulation, we have carried out statistical mechanical analyses of the interaction of the molecule with the solvent molecules.

One important aspect of any molecular dynamics simulation is the stability of the molecule throughout the simulation. This is an aspect related to the quality of the potentials used in the computations. It is possible that the potentials are such that the proteins, in the presence of solvent molecules, unfold as the simulation progresses in time. This effect, which is not uncommon, is more easily observed in long time simulations, such as ours. In our calculations the protein maintains its structural stability throughout the simulation. This is easily seen from the Ramachandran plots of the N-C_α rotation angle ϕ vs the rotation angle ψ around the C_α-C′ bond for each amino acid residue of the molecule. As an example of the stability of the protein, we show in Figure 3-4 a Ramachandran plot of the valine found in position 8 of the amino acid sequence of crambin. The plot shows that, as one would expect, for valine the angles ϕ and ψ stay around the values -60 and -45 without transitions into anomalous regions of the ϕ - ψ space. Similar results are observed for the other residues of

[32]C.H. Van Etten, H.C. Nielsen, and J.E. Peters, *Phytochem.* 4:467 (1965).

[33]W.A. Hendrickson and M.N. Teeter, *Nature* 290:107 (1981).

crambin.

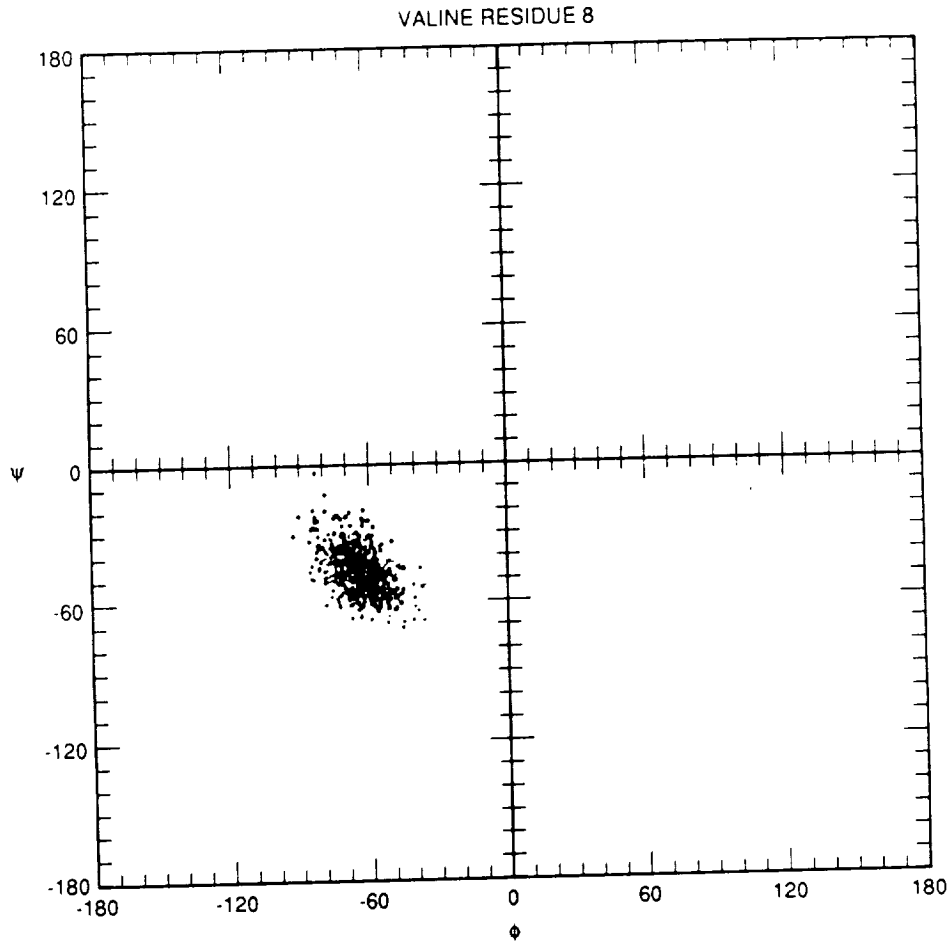

Figure 3-4. Ramachandran plot of position 8 valine of crambin.

Preliminary analysis of the time dependence of the N-C_α-C′ angles for different amino acids strongly suggests nonlinear effects in the dynamics of the molecules. This is expected for residues such as glycine but we were surprised to find similar effects in cysteines. These effects merit further theoretical analysis. A study of the correlation functions for angles among atoms along the backbone of the molecule confirms these observations. Figure 3-5 shows the correlation function for the angle among the C_α-C_β-S_γ atoms of the cysteine-4 residue. This plot exhibits a long range decay tail whose origin we do not yet

understand. This is clearly not a harmonic effect because the time scale of vibrational motions is of the order of 10 picoseconds.

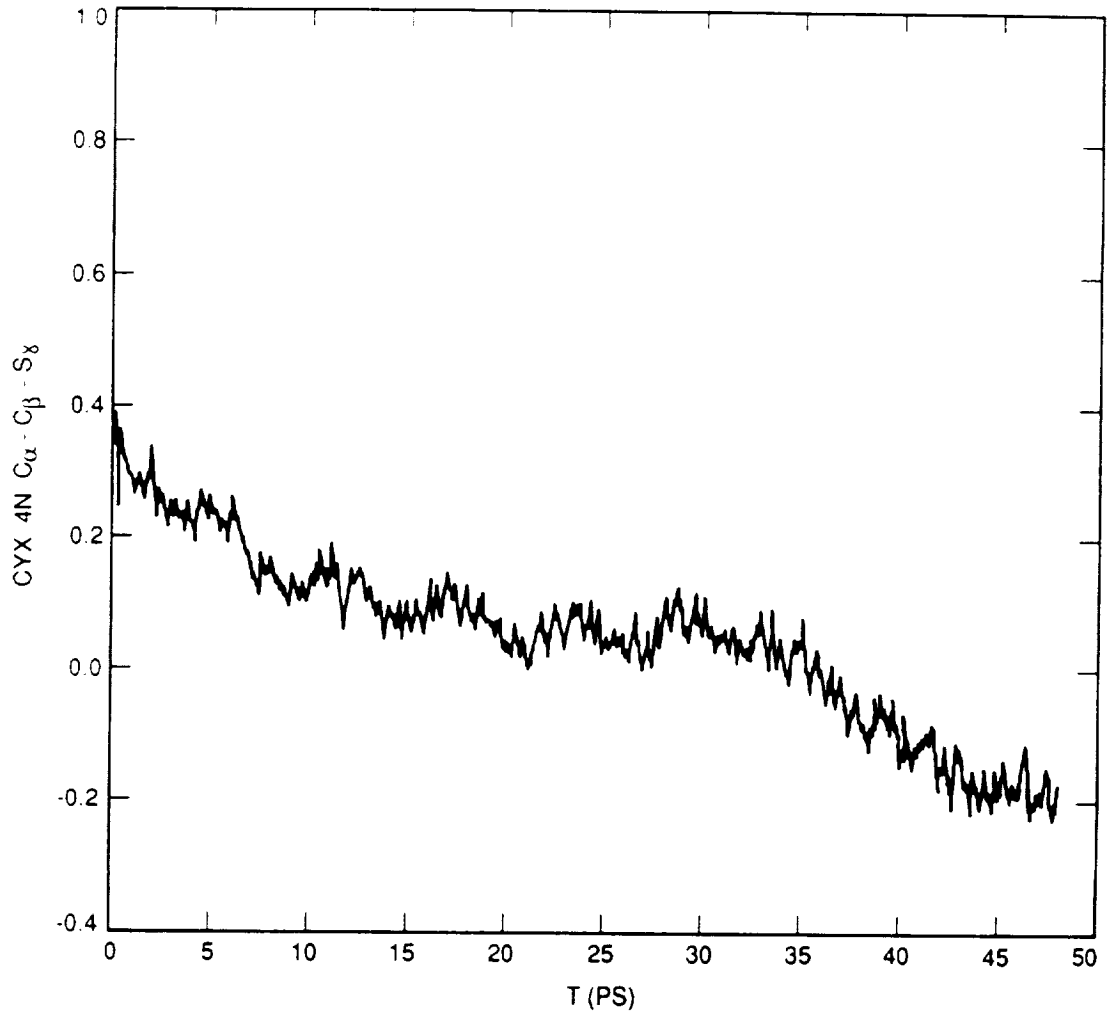

Figure 3-5. Correlation function for the C_α-C_β-S_γ atoms of the cysteine-4 residue.

Another aspect of these simulations, and the main reason for this study, is the interaction of water molecules with the charged residues of the molecule. Pair-correlation functions of oxygen and hydrogen atoms in the water with respect to fixed points in the charged residues, such as aspartic acid, show that water molecules bind at least as strongly as they do to negatively charged ions in solution, such as chlorine. In fact, our results show that the first hydration shell of water around aspartic acid is approximately

10% closer to the negative charge value than it is in a similar calculation for chlorine ions in water.

These effects are also observed in studies of the relaxation times of water molecules about selected amino acids in crambin. For example, Figure 3-6 shows a plot of the relaxation time for the first and second hydration shells about the THR2 residue. To illustrate the meaning of the relaxation time, suppose that at a given time a number of water molecules form the first hydration shell of a specific residue. The relaxation time then measures the time that *those same* water molecules stay in this hydration shell. Figure 3-5 demonstrates that this time can be as much as 10 picoseconds.

Our preliminary results of the binding of water to the charged residues in the protein emphasize an important point for simulations in biocatalysis. In many instances, researchers choose to carry out simulations of reactions in which only the reacting molecules are included in the calculations. It is usually argued that the effects of the solvent are secondary because the water molecules are exchanged in times shorter than the reaction times. Our results indicate that this view should be carefully analyzed. In fact, our analysis suggests that solvent molecules should be included in simulations involving active sites with or near charged residues.

The analysis of the data generated by these simulations is currently under way. We expect to submit a number of publications reporting different aspects of these studies in the near future.

B. Applied Microbiology and Genetics

(1) Metabolic Engineering: Effect of the Hemoglobin Gene on Metabolism (J. Bailey, California Institute of Technology). Research was focused primarily on investigating the influence of cloned *Vitreoscilla* hemoglobin on the metabolism of *Escherichia coli* and yeast. Additional research has been done to develop methods and models useful for metabolic engineering both in the context of cloned hemoglobin and also in a more general setting. In particular, measurements and models of *E. coli* membrane activities have been conducted, cellular measurement systems have been refined, and metabolic control models have been formulated and applied to the production of polyhydroxybutyrate production by the bacterium *Alcaligenes eutrophus*.

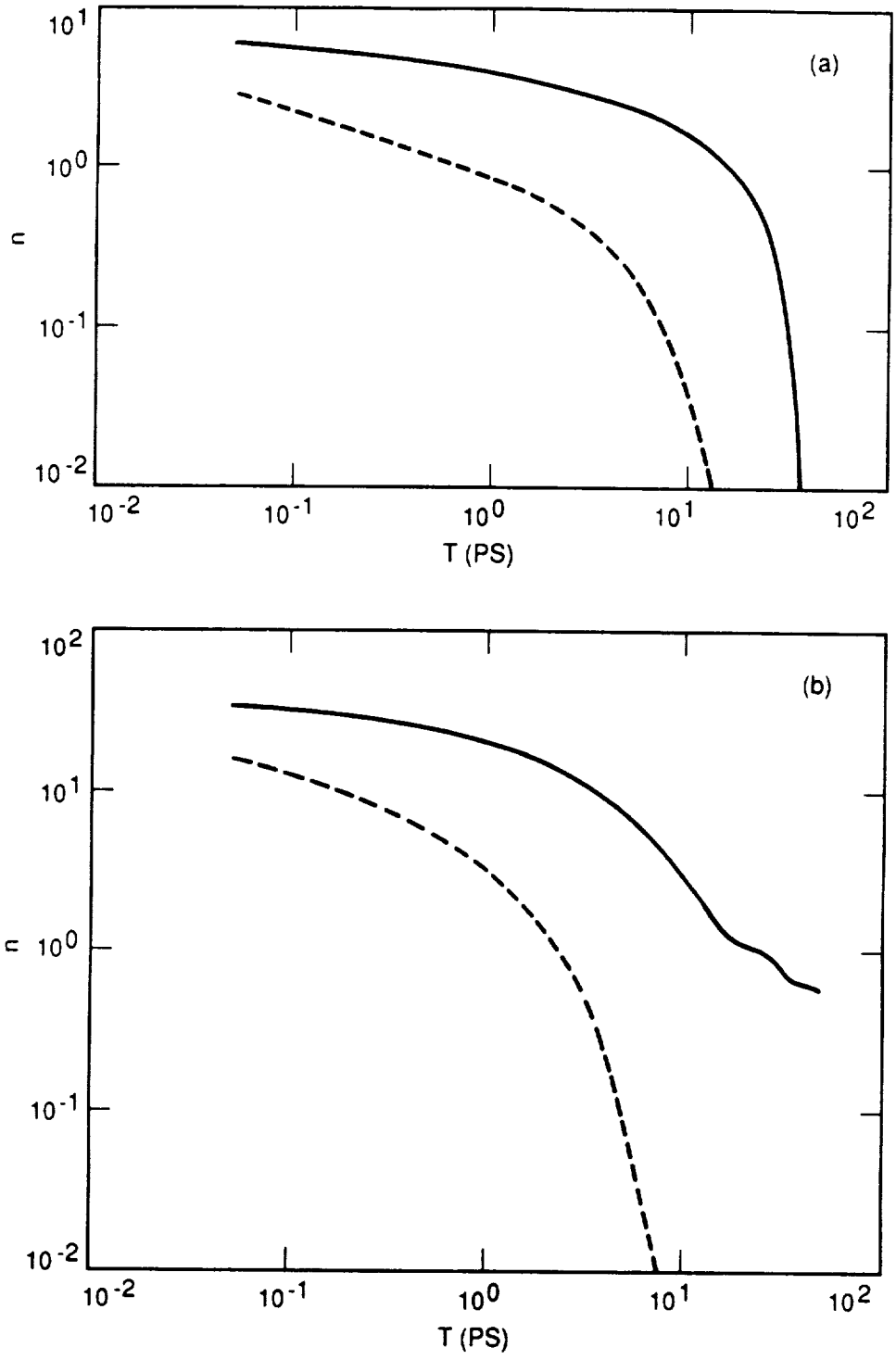

Figure 3-6. Relaxation time for the (a) first and (b) second hydration shells of the second threonine residue.

The central theme of this project is the beneficial influence of cloned *Vitreoscilla* hemoglobin on oxygen-limited metabolic activities of *E. coli*. In an effort to elucidate the basis for these benefits, which is important to understand in order to identify other opportunities for beneficial use of cloned hemoglobin, fed-batch fermentations with monitoring of a number of process variables were conducted with hemoglobin-expressing strains and hemoglobin-free controls. These experiments indicate that hemoglobin increases the efficiency of protein synthesis, and has the influence of increasing both protein per cell and the amount of a cloned gene product per cell. Analysis of the process data using a rudimentary metabolic model indicates that hemoglobin serves to improve the stoichiometry of ATP production by the respiratory chain or to reduce the net utilization of ATP for energizing the cytoplasmic membrane of *E. coli*. Preliminary investigation of the hypothesis that hemoglobin functions by direct interaction with the respiratory chain was conducted by examining the influence of cloned hemoglobin in two different strains, one with a mutant in the cytochrome d terminal oxidase and the other with a mutation in the cytochrome o terminal oxidase. Hemoglobin enhanced growth of both mutants comparably under microaerobic conditions, suggesting that the locus of hemoglobin action is neither of these terminal oxidases.

Investigation of cloned hemoglobin in the yeast *Saccharomyces cerevisiae* indicates that under poorly aerated conditions glucose uptake is increased with concomitant increase in production of ethanol. Further research is in progress to further analyze this finding through a combination of genetic and physiological investigations.

Nuclear magnetic resonance measurements have been conducted to characterize the transport of lactate and acetate across the charged cytoplasmic membrane of *E. coli*. The results indicate that acid permeates the membrane in charged and uncharged forms and that a metabolically coupled transport system for lactate exists. Data analysis was accomplished in part using a new theoretical description of protein-mediated membrane transport developed as part of this research. These findings are important in view of the major influence of membrane energetic state on overall energetics in *E. coli* and other cells. Efforts to implement on-line nuclear magnetic resonance spectroscopy measurements of growing cell cultures are progressing.

A metabolic pathway model has been formulated to describe carbon flow and its regulation in the process for synthesis of biopolymer (poly-β-hydroxybutyrate; PHB) by the bacterium *A. eutrophus*. Extensive experimental data have been shown to be consistent with a model which estimated intracellular fluxes through key metabolic pathways based upon external assays of fructose, cell mass, PHB, CO_2 production, and oxygen utilization. An initial simulation model which enables calculation of batch reactor performance under nitrogen-limited growth conditions has been formulated and found to provide results qualitatively similar to those found by experiment.

(2) Chromosomal Amplification/Gene Fusion (G. Bertani, Jet Propulsion Laboratory). The aim of this task is to study methods for the transfer of foreign genes directly to the bacterial chromosome, and for their amplification and stabilization thereon, as an alternative to the standard use of multicopy cytoplasmic plasmids.

The initial carrier of the foreign gene is a vector plasmid. Our approach consisted in exploiting the capability of certain bacterial viruses to integrate their DNA at specific places on the bacterial chromosome, and particularly that of bacteriophage P2, which is extremely stable once integrated as prophage. The viral genes necessary for the integration were inserted into the plasmid.

Our first plan was based on the external control of plasmid replication by means of a temperature sensitive mutation in the polymerase I gene of the host bacterium, an enzyme that is necessary for autonomous replication of the plasmid, as a method for the elimination of non-integrated copies of the plasmid. This approach was very successful in placing a single copy of the plasmid onto the bacterial chromosome through an easily reproducible procedure. The constructs obtained are extremely stable and require no selection for their maintenance. We also succeeded in isolating amplifications (i.e. tandem duplications followed by recombinational "polyplication" under a selection regime) of these plasmids while on the chromosome. Unfortunately, a method we considered for the stabilization of these structures against the effects of recombination in the absence of selection, did not work under conditions of external control.

We have then turned to two other alternatives. (a) The

polymerase mutation is the source of the problems because it introduces a recombination block. Unfortunately, using strains having normal polymerase I, where plasmid replication is self-regulated, makes much more laborious a step in the protocol, i.e. the elimination of extrachromosomal copies of the plasmid. This approach, as described in last year's report, gave new, scientifically interesting results, but negative from the point of view of the task purpose. Through persistence, however, we have now succeeded in isolating a strain that carries the plasmid stably integrated in the bacterial chromosome at the appropriate site, in the absence of any extrachromosomal copies and of any other detectable plasmid DNA sequences (we started with a normal polymerase I strain). We plan to analyze further this strain and to submit it to the previously tried methods for amplification and recombinational stabilization. (b) Recently a method has been described[34] that allows construction in vitro of tandemly amplified DNA sequences, including a foreign gene, and these can then be inserted in plasmid pSK3. The latter however offers only ampicillin resistance as a selective marker. In principle, it is possible to introduce the genes for chromosomal integration into pSK3: it was necessary to show first that ampicillin resistance can be used in the isolation of chromosomally integrated plasmids. We have then constructed a derivative of our plasmid pEE240, in which a short sequence containing little more than the integration site is inserted in the tetracycline resistance gene, ampicillin resistance being used for recognition. Ampicillin resistance is much less sensitive to gene dosage effects, since it is caused by an enzyme that degrades the antibiotic. Nevertheless we succeeded in obtaining strains that carry such a plasmid in single copy on the chromosome in the standard P2 location, as demonstrated earlier for pEE240. It is now only a matter of routine manipulations to use the above methods for introducing in vitro tandemly amplified foreign genes into the chromosome of a recombination-defective strain.

Transformation (i.e. the introduction of DNA into a cell) is a very basic tool in genetic engineering. As far as plasmid DNA is concerned, transformation works as well in recombination-deficient as in normal bacteria. An exception is the plasmid that was mentioned in last year's report, pEE502. Its efficiency of transformation is at least 100X lower in

[34]Kim and Szybalski, *Gene* 71:1 (1988).

recA⁻ bacteria than in the wild type. This plasmid was constructed from pEE240 by moving the genes for chromosomal integration from the ampicillin to the tetracycline resistance gene. We have investigated in which way this new property of pEE502 is correlated with its structure. We find that the effect is eliminated if the direction of the inserted sequences is inverted. The effect is also eliminated if DNA sequences neighboring the attachment site are deleted. This is confirmed by the fact that in those rare transformed bacteria that can be recovered after exposure to pEE502 DNA, the plasmids carried have undergone deletion of part of the inserted sequences. Finally, if another, related plasmid already resides in the same bacterium, it can help pEE502 establish itself. While the situation is still incompletely elucidated, it suggests previously unsuspected effects of the recA gene on transformation, effects that may involve replication of plasmid DNA or transcription at the tetracycline resistance gene, and the complex DNA/protein structures that are known to be formed around the chromosomal attachment site during preparation of the highly site-specific integration process.

(3) Hyperproduction and Secretion of Polyphenol Oxidase (W.V. Dashek and A.L. Williams, Atlanta University). During the past year, we continued efforts to over-produce Polyphenol Oxidase (PPO) from *Coriolus versicolor* by utilizing recombinant DNA protocols. Specifically, both DNA shotgun and cDNA approaches were employed in this study to clone the PPO gene(s).

From these cloning protocols, an array of recombinant vectors was generated, carrying genomic DNA inserts (via pBR325) and cDNA sequences (via λgt-11). Subsequent to transformation or transfection, putative PPO clones were selected, purified and retested for antibiotic resistance and/or reporter gene activity (i.e., β-galactosidase). Using cDNA probes several positive clones harboring PPO DNA inserts were identified. One cloned insert (~ 1.0 kb) has been recovered from the pBR325 vector by use of selective restriction enzymes. Using other restriction enzymes, a restriction map of this insert has been constructed.

Additionally, PPO activity was detected in "crude" extracts of *E. coli* HB101 transformants. That is, most of the activity appeared to be found in the supernatant fraction as compared to the pellet. Currently, aliquots of these extracts are being applied to SDS-PAGE analysis which will be probed with

PPO-"tagged" antibodies (i.e., Western blots). These Western blots should serve to confirm whether PPO is indeed being expressed in *E. coli* cells. Subsequently, experiments will be designed to modify or truncate certain cloned PPO insert(s) for enhanced expression capabilities with respect to PPO production.

Lastly, we have received a New Brunswick fermenter via Oak Ridge National Laboratory. With this acquisition, we plan to develop and optimize fermentation processes conducive to the expression of recombinant PPO proteins in microbes. Furthermore, these cloned inserts will be subjected to *in vitro* mutagenesis, recloned into expression vectors and screened for their abilities to overexpress *C. versicolor* PPO gene(s) in keeping with the mission of mass production of PPO.

During the past year, we also continued efforts to over-produce and enhance the secretion of *C. versicolor's* PPO without the aid of recombinant DNA technologies.

Over-production

Preliminary experiments centering about the time-dependent addition of catechol (PPO's substrate) to liquid cultures revealed that the o-diphenol could regulate the *in vitro* growth of *C. versicolor* as well as affect hyphal ultrastructure. These observations have paved the way for utilizing o-diphenol to attempt substrate induction of PPO in culture. Thus, this possibility is being thoroughly investigated through the time-dependent addition of suitable diphenols to liquid culture medium and subsequent assay of both intracellular and extracellular PPO.

Enhanced Secretion

During the past year, advances were achieved with regard to "mapping" the route of PPO secretion through intracellular hyphal organelles. Whereas Scanning Electron Microscopy (SEM) revealed the growing hyphal tip's wall to be smooth-surfaced and the non-growing region's to be convoluted, Transmission Electron Microscopy (TEM) of the non-growing region's cytoplasm of glutaraldehyde pre-fixed and OsO_4 post-fixed hyphae demonstrated structures such as mitochondria, endoplasmic reticulum, lipid, ribosomes, and electron lucent vesicles containing both electron dense material and electron lucent amorphous masses. In contrast, the growing tip's cytoplasm appeared to lack these

structures, except that it had abundant electron masses and very few electron dense vesicles. In addition, PPO was detected within homogenates of hyphae "pre-fixed" with cacodylate-buffered glutaraldehyde thereby enabling the TEM substrate localization of PPO.

As for the distribution of PPO within subcellular organelles centrifuged from hyphal homogenates, time-dependent (0-16 culture days) alterations in PPO specific activity (spc. act.) indicated that the enzyme was particulate rather than cytosolic. Endomembrane components mediating PPO secretion are being identified using both TEM and marker enzyme analysis of pellets obtained after centrifugation at 10,000, 40,000, and 105,000 x g.

At the present time, antibodies to commercial fungal PPO are being produced in order to localize intracellular hyphal PPO by "gold tagged" antibody via immunoelectron microscopy. This effort is being supported by the substrate localization of PPO within hyphae through the utilization of ultrastructural cytochemistry. Concomitant with these activities are continued attempts to purify both intracellular and extracellular PPO via sequential dialysis → $(NH_4)_2SO_4$ fractionation → gel filtration → hydrophobic interaction and hydroxylapatite chromatographies in order to derive purified enzyme (as judged by SDS-PAGE) for antibody production. Antibodies directed toward purified intra and extracellular PPO proteins will be "tagged" with colloidal gold and used in localization of PPO by immunoelectron microscopy.

Concurrent with these activities are efforts to regulate secretion by separating PPO synthesis from secretion via the employment of traditional respiration inhibitors and diethylthiocarbamate. Thus, it is anticipated that enhanced PPO synthesis and secretion can be obtained by substrate induction combined with the use of inhibitors to regulate secretion in "batch" *C. versicolor* cultures. In this connection, methods are being developed for the rapid isolation and purification of PPO from batch cultures. This approach may provide an alternative to recombinant DNA technologies to over-produce PPO via *E. coli* transformants harboring an appropriate *C. versicolor* DNA insert.

Finally, we have begun to extend these investigations to cellulases and ligninases, enzymes of value to the paper-pulp industry and the agricultural community.

C. Bioprocess Engineering

(1) Immobilized Cell System for Continuous, Efficient, Biocatalyzed Processes (C.D. Scott, Oak Ridge National Laboratory, ORNL). Within the Biocatalysis Program, ORNL seeks to increase fundamental and practical knowledge in the areas of advanced bioreactor concepts and efficient immobilized biocatalyst systems. The use of high productivity fluidized-bed bioreactors (FBRs) is being extended to the production of organic acids and neutral solvents in multiphase systems with simultaneous fermentation and separation. This biparticle reactor has been demonstrated with lactic acid production. A modeling effort for the production of ethanol in FBRs was also completed.

Integrated Fermentation and Separation

FBRs have the advantage that additional solid phases can be added to perform useful functions and that these phases will separate or stratify based on density or size. We have added a phase with extractive properties for the desired product both in order to enhance the productivity and accomplish a separation. This phase could be either a solid or an immiscible liquid. A solid adsorbent and the production of lactic acid from glucose by *Lactobacillus delbreuckii* were used to test this concept. In this case, both the product, lactic acid, and the low pH resulting from the acidogenic fermentation are inhibitory to the bacteria.

Biparticle FBR - The biparticle FBR consists of two hydrodynamically separable solids - one containing the immobilized cells and the other an adsorbent for the fermentation product. We confirmed the hydrodynamic feasibility of this design using beads of 4% κ-carrageenan gel with added inorganic oxides to control the density. Both counter-current and cocurrent operation were confirmed.

Screening tests were performed on several candidate adsorbents. Filtrasorb 100, an activated carbon, was chosen as the most promising initial candidate and later was replaced by the Reillex 425 resin, a polyvinyl pyridine (PVP). Filtrasorb 100 was found to have a capacity near 0.12 g lactic acid/g of adsorbent at pH 3 and a lactic acid concentration of 5 g/L. Its capacity, however, decreased to near 0.02 g lactic acid/g adsorbent when the pH was raised above pH 6. This is explained by different adsorption capacities for the protonated lactic acid and for the lactate ion.

(The pK_a of lactic acid is pH 3.8.) The capacity of Reillex 425 for lactic acid was measured at 0.10 g lactic acid/g adsorbent and was independent of the pH. Activated carbon had a small, but measurable, capacity for glucose, the substrate; Reillex 425 has no measurable capacity for glucose. Initial tests have also been performed on the regeneration of the sorbent. Reillex 425 was loaded with lactic acid. Less than half of the acid can be back-extracted from the sorbent by placing the sorbent into water. Contacting with 1 N NaOH removed all the lactate. Methanol will recover 60% of the loaded lactic acid from the resin. This partial capacity is then reusable to absorb more lactate.

The ability of the adsorbent to control pH was tested in batch fermentation with *L. delbreuckii*. The optimum for the lactic acid fermentation is above pH 5. Without any pH control, the reaction halted due to the low pH. The addition of sorbent to the reaction removed some lactic acid, raised the pH, and allowed the conversion of more glucose into lactic acid. The fermentations were performed in several configurations with the adsorbents. One test is described in detail below.

Lactobacilli were immobilized into gel beads and placed into a tapered FBR. One set of tests was made with complete recycle of the effluent and only an initial glucose feed of 5 g/L. In a test without the addition of the resin adsorbent, the pH rapidly fell to a value of 3.3, and the rate of glucose conversion significantly decreased (Figure 3-7). Ultimately, most but not all of the glucose was removed after more than 45 h with essentially complete conversion to lactic acid at a rate of about 1 g/h/g biomass. In a second test, similar conditions were used except that Reillex 425 was added to the top of the column and recovered at the bottom of the column at a rate of about 0.8 g/min. Glucose was completely consumed in less than 14 h, and the pH was maintained at higher levels throughout the test (Figure 3-8). There was again essentially complete conversion to lactic acid at a rate of 4 g/h/g biomass which is substantially greater than that in the test without the adsorbent. The overall yield in these runs was 1 g lactic acid/g glucose, the stoichiometric limit.

The biparticle FBR has been shown to be feasible. With proper design of the system and particles, the continuous counter-current addition and removal of

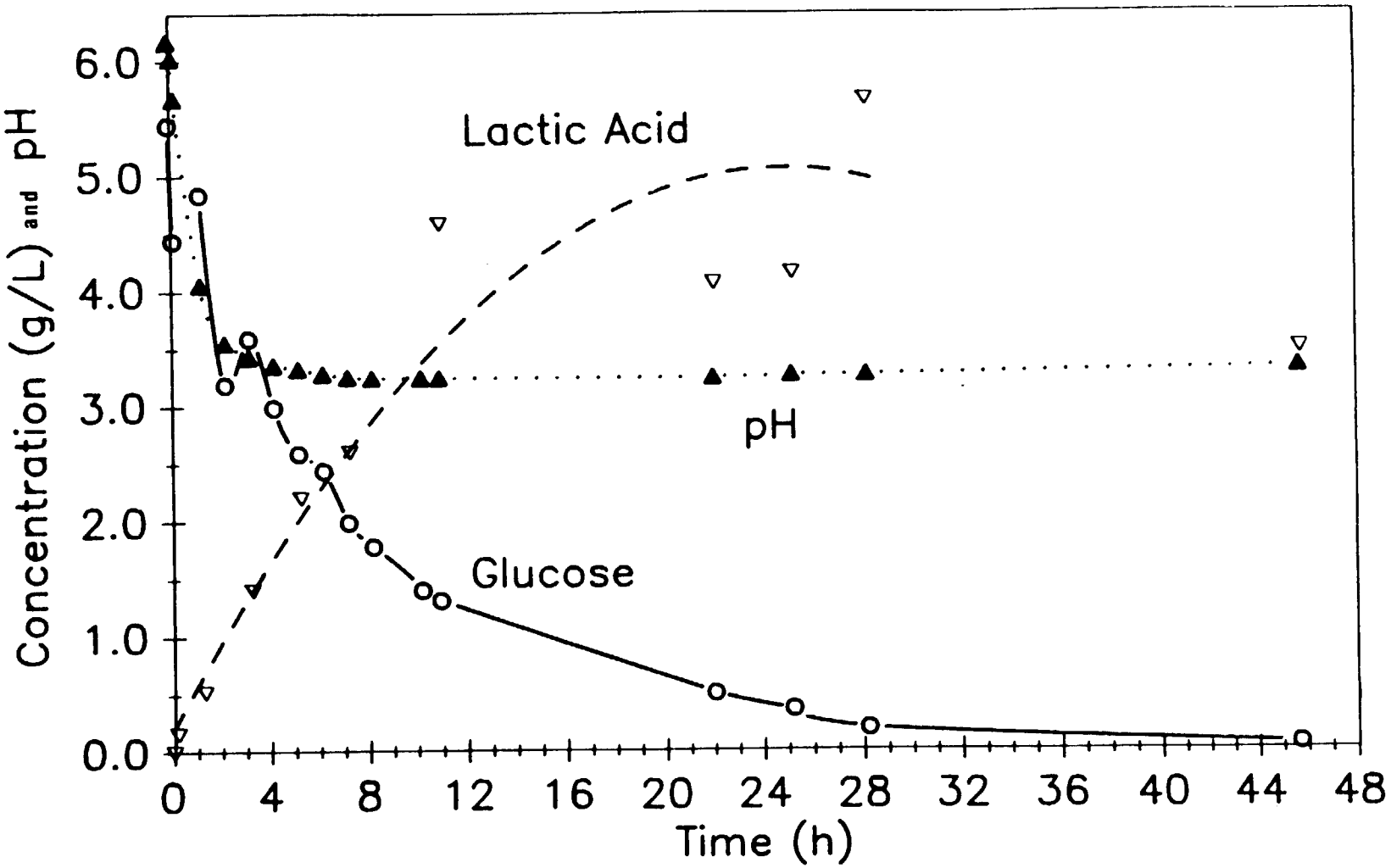

Figure 3-7. Lactic acid production in biparticle FBR with no resin added.

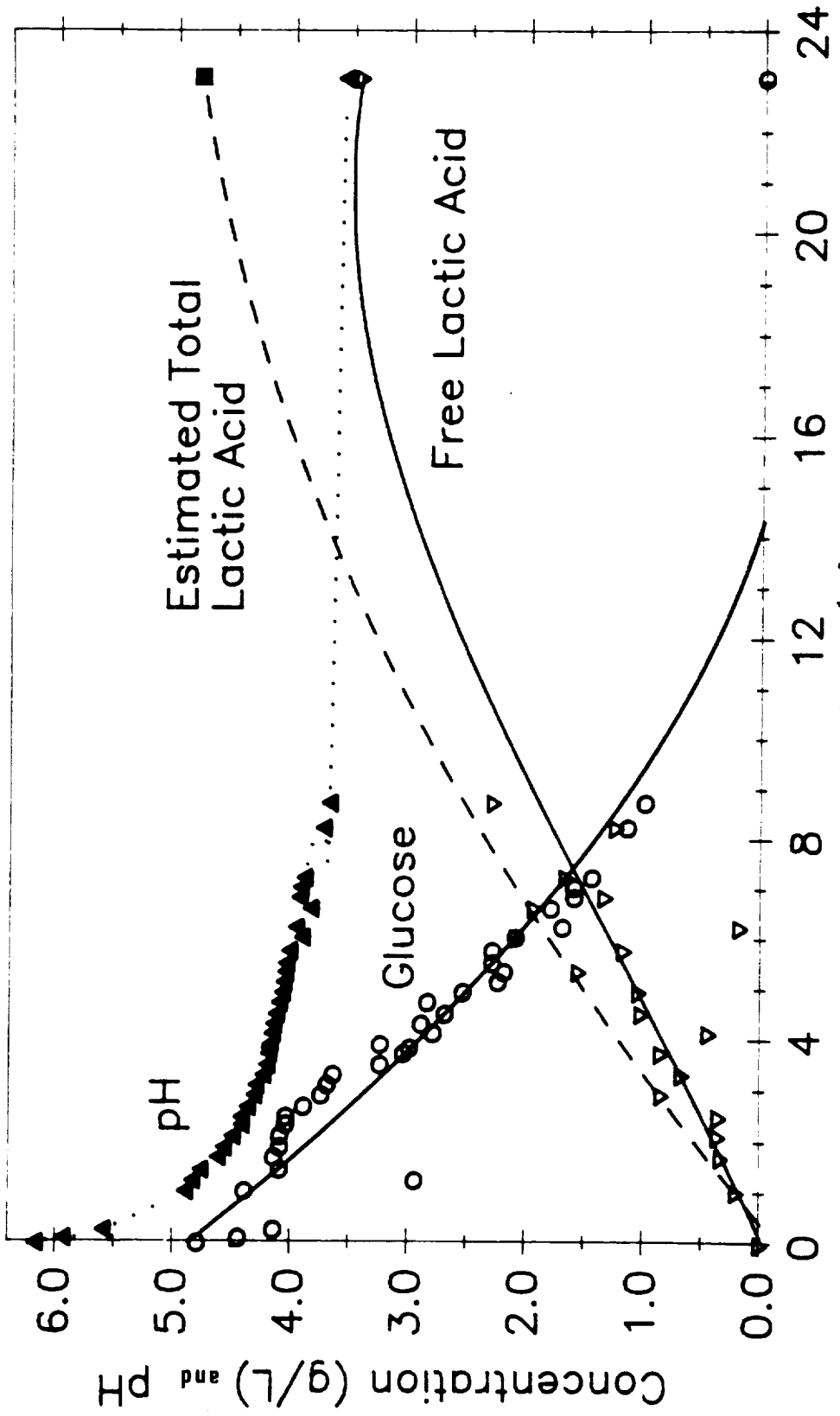

Figure 3-8. Lactic acid production in biparticle FBR with resin present.

desired particles to a stable fluidized bed of other biocatalysts can be achieved. The particle sizes and densities can be adjusted to change the system hydrodynamics and residence times. Various sorbents for lactic acid have been tested and two candidates explored in detail. The system was shown to enhance the production of lactic acid in an FBR.

Solvent Extractive Fermentation for Acetone/Butanol

Another approach to simultaneous fermentation and separation is to use an immiscible extractive solvent instead of an adsorbent particle. We are examining the acetone-butanol fermentation with *Clostridium acetobutylicum* in an immobilized-cell fluidized-bed reactor with continuous introduction of a solvent for the extraction and removal of the products. A small columnar reactor was assembled to test the basic operation of the system. These tests indicated that there was good phase mixing within the fluidized bed with subsequent phase separation at the top of the reactor column.

Initial toxicity tests on the growth of *C. acetobutylicum* were made with three candidate extraction solvents in which toxicity was reported to be minimal. These solvents were oleyl alcohol, dibutyl phthalate and a solvent mixture, Cyanex 923. The bacteria remained viable in the presence of all the solvents. However, the presence of Cyanex 923 and dibutyl phthalate deleteriously affected the glucose consumption rate when compared to a control. Oleyl alcohol had a minimal effect. Oleyl alcohol is the least toxic solvent of these tested.

FBR Hydrodynamics and Modeling

The study of the hydrodynamics and kinetics of the three-phase FBR is necessary in order to understand, model, and scale-up systems with low density solids. This model was developed to compare with experimental data from the high-productivity ethanol FBR. A mathematical model of a three-phase, tapered, fluidized-bed bioreactor was developed. This model includes the effects of the tapered bed, variation in the dispersion coefficient, and the concentration profile inside the biocatalyst bead on the reaction rate within the bed. Three parameters in this model were adjusted, within a realistic range, for an optimal fit between the values predicted by the model and those obtained experimentally. The model was found to predict additional experimentally obtained

concentration profiles quite accurately. It also demonstrates the need to include the effects of variable dispersion in three-phase systems where the gas phase is being generated inside the reactor, as the dispersion coefficient varied by more than an order of magnitude across the fluidized-bed.

(2) Multimembrane Bioreactor for Chemical Production (M.L. Shuler, Cornell University). The primary objective of this project was to test the technical and economic potential of a multimembrane reactor system for the production of chemicals from microbial sources when the chemical accumulates to high enough levels to inhibit its own production. The model system chosen for demonstration purposes was ethanol production from the yeast, *Saccharomyces cerevisiae*.

The reactor concept involves dividing the reactor volume into four compartments: (i) gas layer, (ii) cell layer, (iii) nutrient layer, and (iv) solvent layer. In the original design these layers are separated from each other by selective membranes. The purpose of the gas layer is to supply gaseous nutrients and to provide effective removal of gaseous by-products such as CO_2. The build-up of CO_2, and its disruption of immobilizing matrices, has been a problem with some immobilized cell reactors. The cell layer retains cells at high concentrations and catalyzes the conversion of sugar (e.g. glucose) into ethanol and CO_2. The nutrient layer provides sugar and other nutrients to maintain cells in an active state. The solvent layer extracts the ethanol from the nutrient layer. The removal of ethanol from the aqueous phase reduces product inhibition and promotes more rapid reaction rates. Further, because the solvent has a high boiling point, the recovery of ethanol is simplified.

Experiments have confirmed that such a system is functional, and with suitable modifications, the system can sustain high reaction rates for extended periods while operating continuously (at least 3,000h). In our model system we used the solvent tri-normalbutyl phosphate (TBP). Computer models of the process have allowed assessment of key operating parameters and will be useful in design. Preliminary economic evaluation suggests that the reactor system is economically comparable with traditional systems for plants with a production capacity of 10^8 L/yr of 95% ethanol while reducing the input of external energy by approximately 60%. At lower levels of production (e.g. 10^7 L/yr) the multimembrane reactor becomes increasingly economically

attractive compared to the traditional system, which may be important in processing inexpensive waste materials on site to avoid collection and transportation costs. Decreases in membrane costs or the use of a solvent with an increased distribution coefficient for ethanol would significantly improve the economic attractiveness of the multimembrane reactor system.

This project has led to two patents (both of which have won NASA Tech Brief Awards), eleven papers in refereed publications, three presented papers at national or international meetings and two Ph.D. theses.

Final Results

A prototype multimembrane reactor was constructed and tested.[35] The most important result of this work was the development of the concept of "physical" or "phase" toxicity and the demonstration that the multimembrane reactor system could circumvent this problem. Prior to our work, solvents, such as TBP, were found to be inhibitory to yeast growth and ethanol production, and this lack of biocompatibility had strongly limited the possibilities for *in situ* product extraction. Our work demonstrated that the toxicity of TBP was due to the direct interaction of cells with emulsified solvent droplets. The dissolved component of TBP exerted no toxic effect. The use of the multimembrane reactor with a hydrophobic membrane and the correct application of pressure (nutrient side pressure higher than the solvent, but less than the critical entry pressure) prevents TBP emulsification. Operated in this manner, *in situ* extraction without toxicity was possible. A further implication was that we could routinely operate at 35°C with the multimembrane reactor which was impossible with a traditional system. The yeast cells become more sensitive to ethanol at higher temperatures, so *in situ* ethanol extraction extends the range of effective operating temperatures.

The multimembrane reactor, however, gave reaction rates that were lower than desirable. This low activity was primarily due to mass transfer limitations within the cell layer. This problem was solved by initiating a pressure-cycle operation. By alternately cycling gas phase pressure so that it was less than and then greater than the nutrient pressure, a convective flux

[35]T. Cho and M.L. Shuler, *Biotechnol. Prog. 2:53-60 (1986).*

of liquid in and out of the cell layer was obtained.[36,37] The pore size of the membrane separating the cell and nutrient layers is important. For pore sizes less than 0.45 μm the membrane resistance to flow is important. With 0.22 μm pore membranes *S. cerevisiae* was completely retained. With a 0.45 μm pore size membrane the reaction rate improved at least two-fold, but cell leakage through the membrane was observed. This leakage was at a low rate and would likely be of little consequence in a commercial scale system with single pass continuous flow but was unacceptable in our experimental apparatus, since it was operated in a batch-recycle mode.

With pressure cycling the multimembrane reactor was successfully operated for up to 800 hours. However, instabilities in the fill and empty cycles occurred due to pressure changes resulting from the wetting of the hydrophobic membrane separating the gas from cell layer. To alleviate this problem the reactor was modified to allow effective long term continuous operation by the substitution of a liquid-level controller for the gas/cell layer membrane and by reinforcement of the cell/nutrient layer membrane to prevent stretching and tearing.[38] This reactor system gave stable, relatively high-level, rates of ethanol production for over 3,000h; operation was terminated voluntarily, and appeared to be sustainable indefinitely. Membrane fouling due to microbial metabolism was not a significant operational constraint.

This work was done with a glucose-based medium. A molasses-based medium could not work because of membrane fouling. A medium using a corn starch hydrolysate gave performance comparable to the glucose-based medium. Attempts to use *Zymomonas mobilis* in place of *S. cerevisiae* failed. Under the culture condition *Z. mobilis* filamented, and the resulting culture mass impeded the flow of nutrients into the cell layer and the flow of ethanol out of the cell layer during pressure cycling.

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[36]G.S. Efthymiou and M.L. Shuler, U.S. Patent 4861483, Apparatus and Process to Eliminate Diffusional Limitations in a Membrane Biological Reactor by Pressure Cycling.

[37]G.S. Efthymiou and M.L. Shuler, *Biotechnol. Prog.* 3:259-264 (1987).

[38]D.E. Steinmeyer and M.L. Shuler, *Biotechnol. Prog.* in press (1990).

Because these experiments require extended periods to complete and the number of potential parameters is large, the experimental optimization of the process is impracticable. Consequently, we sought to construct a computer model that would aid in identifying optimal reaction conditions. The heart of any such model must be the model of the culture itself.

We have developed a detailed model for the growth of *S. cerevisiae* at 35°C.[39,40] This model has been validated by comparing predictions of dynamic behavior of a culture against experimental data. We believe that this model is the best existing model for *S. cerevisiae*.

The cell model has been embedded in an overall computer model of the multimembrane bioreactor.[41] The reactor model accurately predicts the dynamic behavior of the system based on comparison of predicted with observed behavior. Combinations of changes in membrane structure, solvent characteristics, ratio of extractive to reactive membrane area, were investigated. A four fold improvement in productivity over the base case (similar to our long term continuous case) appears possible.

We also note at this point a strategic mistake, which in retrospect we would have done differently. All of our modeling has been at 35°C. With extractive fermentation we can operate effectively at that temperature. However, the increased cellular sensitivity to ethanol reduces the ultimate concentration of ethanol in the TBP. An economic process will require higher ethanol concentrations in the solvent, and this is probably achievable only at a lower fermentation temperature. The parameters in the cell model would have to be reevaluated to allow the application of the reactor model to lower temperatures. But the basic structure of the model is independent of temperature.

An economic evaluation of the multimembrane reactor

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[39]D. Steinmeyer, T. Cho, G.S. Efthymiou, and M.L. Shuler, *Chem. Tech.* 18:680-685 (1988).

[40]D. Steinmeyer and M.L. Shuler, *Chem. Eng. Sci.* 44:2017-2030 (1989).

[41]D.Steinmeyer and M.L. Shuler, *Biotechnol. Prog.* (accepted).

system has been completed.[42] At a production level of 7×10^7 L of 95% ethanol/yr the costs of a TBP-based multimembrane reactor are the same as a traditional batch fermentation system. At larger sizes the traditional unit is cheaper. At 1×10^7 L/yr the ethanol produced is 10 to 15% cheaper when produced with a TBP-based multimembrane system. Thus, the multi-membrane reactor system would be particularly well suited to cases where the collection costs of the substrate restricted the amount of substrate available.

Further, the multimembrane reactor system reduces energy inputs by about 60%, which would be a significant advantage in the event of another energy crisis.

The costs for the multimembrane reactor are high and insensitive to process scale due to the high cost of membrane. Further improvements in membrane module production with a resulting reduction in cost are anticipated and this expectation is justified by recent progress in this area.

The other factor which could greatly increase the economic competitiveness of a multimembrane reactor is the use of a solvent with a higher distribution coefficient. Preliminary experiments and calculations with a solvent mixture of trialkyl phosphine oxides (Cyanex 923 from American Cyanamid Corp.) suggest a 9% reduction in costs compared to a TBP-based multimembrane reactor system. Other, even more effective, solvent mixtures may exist.

This study has demonstrated that the multimembrane reactor concept is technically feasible. Tools to allow its commercial development have been built (e.g. prototypic reactors and computer simulators). The economic potential for development exists under special circumstances but is probably impracticable at current membrane costs and with the potential solvents identified so far.

(3) Biocatalyzed Hydroxylation in Organic Solvents (A.M. Klibanov, Massachusetts Institute of Technology). We have developed a novel approach to the design of artificial receptor molecules in organic solvents. It is based on the phenomenon of molecular imprinting, demonstrated for several unrelated proteins and

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[42]M.L. Shuler, D. Steinmeyer, A.P. Togna, S. Gordon, P. Cheng, and S.J. Letai, *Appl. Biochem. Biotechnol.* (accepted).

ligands, which forms selective receptors from common proteins by utilizing their high conformational rigidity in anhydrous media (compared to water). By lyophilizing a protein in the presence of a ligand for which an affinity is desired, followed by that ligand's extraction with an anhydrous solvent, protein preparations are obtained which exhibit a marked affinity for the original ligand in anhydrous media. During the past year, our efforts have focused on exploring the fundamentals and generality of this phenomenon. To that end, we have examined the specificity of ligand binding to imprinted protein, the dependence of this imprinting effect on the nature of the protein, the solvent's effect on binding, as well as potential applications of these artificial receptors. Our research accomplishments to date are outlined below.

The imprinting phenomenon has been demonstrated for a variety of proteins including lysozyme, chymotrypsinogen, ovalbumin, hemoglobin and bovine serum albumin (BSA). For example, BSA imprinted with malic acid binds 27 mole-equivalents of this ligand in ethyl acetate as compared to just 3 mole-equivalents for the non-imprinted protein (and even less for both proteins in water). When imprinted under identical conditions, the proteins exhibit a net binding (i.e., the difference in the binding capacity of the imprinted protein and that of its non-imprinted predecessor) that is proportional to their molecular volume. For example, when the aforementioned proteins are imprinted with 0.5M malic acid at pH 2.0, they exhibit a net binding of 4, 9, 16, 24, and 24 mole-equivalents of the ligand, respectively, in ethyl acetate. During imprinting, some template molecules become permanently entrapped within the protein, thereby rendering further evidence that cavity formation is occurring throughout the protein's volume.

In addition, we have examined how modifications in the structure of the proreceptor protein affect its ability to be imprinted. For example, cleavage of BSA with cyanogen bromide prior to imprinting has little effect on the binding capacity: the imprinted, cleaved BSA binds 22 mole-equivalents of malic acid compared to 24 mole-equivalents for the imprinted, intact protein. However, complete digestion of the peptide backbone via acid hydrolysis destroys the ability to be imprinted. Thus, when the acid hydrolyzate of BSA is imprinted with malic acid, it shows no appreciable binding of this ligand in anhydrous ethyl acetate. Moreover, neither oxidized glutathione (a hexapeptide) imprinted

with malic acid, nor poly-L-lysine imprinted with p-hydroxybenzoic acid, exhibits any affinity for its respective ligand in anhydrous media. Therefore, a tertiary structure, lacking in these molecules, seems to be required for the competent proreceptor protein.

Protein preparations obtained by our imprinting methodology have proven to be very stable. In a dry state, the imprinted powders may be stored at 8°C under vacuum for periods as long as a month without any loss of "memory". However, contact with water erases the imprinting effect: when BSA imprinted with malic acid is re-lyophilized from water in the absence of template, its ability to subsequently bind ligand in ethyl acetate is negligible, i.e., identical to that of the non-imprinted protein. Addition to the solvent of compounds enhancing protein flexibility results in significant reductions in the amount of ligand bound. For example, in ethyl acetate containing 0.5% formamide, the net binding is only 16 mole-equivalents of malic acid compared to 24 in the absence of formamide; at 2% formamide, the net binding drops to 7 mole-equivalents.

It has been found that the specificity of ligand binding to a given imprinted protein is determined by the number and nature of the functional groups on the ligand molecule. In our model system of BSA imprinted with malic acid, molecular recognition likely occurs via hydrogen bond formation. Consequently, addition or removal of the ligand's functional groups which participate in hydrogen bonding markedly alters the binding observed. For example, addition of a second hydroxyl group to malic acid (to yield tartaric acid), increases the net binding of BSA imprinted with malic acid from 24 to 33 mole-equivalents. Conversely, removal of the hydroxyl (to yield succinic acid) reduces the net binding to 11 mole-equivalents. The hydroxyl group may be substituted with another moiety capable of engaging in hydrogen bonds with little consequence. For example, malic acid imprinted BSA binds comparable quantities of malic and N-acetyl-L-aspartic acid. In addition, the carboxyl group may be replaced with an amide (to yield succinamic acid) with little change in binding capacity, but esterification of even one of the carboxyl groups (to yield mono-methyl succinate) results in the loss of all appreciable binding.

The ligand's size, shape and rigidity also influence its binding to imprinted protein. Thus, upon the introduction of a double bond into the carbon backbone

of the ligand, the net binding significantly increases: while imprinted BSA binds only 11 mole-equivalents of succinic acid, it binds 17 and 36 mole-equivalents of fumaric and maleic acids, respectively, under identical conditions. This effect may be attributable to the greater rigidity of the unsaturated ligands and hence, the smaller entropic losses upon binding. Additionally, increasing the size of the ligand, either by introducing a cyclohexane or phenyl ring, greatly reduces the observed net binding -- approximately 7 mole-equivalents of the ligands *cis* and *trans* 1,2-cyclohexanedicarboxylic acids and phenylsuccinic acid are bound by the imprinted BSA.

In the case of BSA imprinted with L-malic acid, the binding of ligand to the imprinted protein has been found to proceed with essentially no enantioselectivity. Thus, in ethyl acetate containing racemic malic acid, the L-malic acid imprinted BSA binds 12 mole-equivalents of the L-isomer and 14 mole-equivalents of the D-isomer at equilibrium.

In the presence of two distinct ligands for which the imprinted BSA exhibits comparable binding capacities, the strength of this binding appears to be similar as well. For example, in ethyl acetate containing an equalmolar mixture of L-malic and N-acetyl-L-aspartic acids, BSA imprinted with malic acid binds equal amounts of each ligand.

The ability of imprinted protein to bind ligand has been shown to be very sensitive to the nature of the organic solvent employed in the binding assay. Whereas significant net binding is observed in anhydrous ethyl acetate, hexyl acetate, and 2-butanone (24, 21, and 14 mole-equivalents, respectively), no binding is observed in anhydrous tetrahydrofuran, pyridine and dimethylformamide. Since the latter group of solvents has been demonstrated not to erase the imprinted protein's memory, presumably they interfere with the protein-ligand interaction. A mechanistic investigation of this phenomenon is in progress.

The ability to imprint from protein-dissolving, nonaqueous solvents instead of water has also been studied. Although such imprinted proteins do exhibit a "ligand memory", much lower binding capacities are observed due to the lower template concentrations attainable during imprinting. For example, when chymotrypsinogen is freeze-dried from dimethyl sulfoxide containing N-acetyl-L-aspartic acid, the imprinted protein binds only 3 mole-equivalents of this

ligand in ethyl acetate, compared to 1 mole-equivalent bound by the non-imprinted protein. Presently we are exploring conditions which will enhance the magnitude of this effect, including freeze-drying from other protein-dissolving solvents, such as formamide and ethylene glycol.

The generality of the observed imprinting phenomenon was further examined using macromolecules other than globular proteins. We have readily converted gelatin, dextran, diethylaminoethyl(DEAE)-dextran, and carboxymethyl-dextran to artificial receptors. For example, when DEAE-dextran (500 k Daltons) was imprinted with malic acid in the same manner as BSA, it bound 168 mole-equivalents of this ligand in ethyl acetate compared to only 16 mole-equivalents for the non-imprinted polymer.

Potential applications of artificial receptors may include their use for separations and purifications. We have demonstrated that imprinted protein can be successfully employed in a chromatographic separation of mixtures of structurally related compounds. Using imprinted BSA, we completely separated an equalmolar mixture of acrylic and maleic acids in ethyl acetate, with recoveries of 95% and 90%, respectively. In addition, we are currently exploring the feasibility of the use of molecular imprinting as a strategy for the design of new biocatalysts. For instance, by creating binding sites which will strongly interact with transition states for useful synthetic reactions (as is naturally the case of catalytic antibodies), it may be possible to design new catalysts from these receptor-mimicking precursors.

(4) Study on an Integrated Biological-Chemical Process for Continuous Production of Methyl Ethyl Ketone and 1, 3-Butadiene (G.T. Tsao and P.B. Beronio, Purdue University). Concepts of microbial energetics, fermentation kinetics and bioprocess control were synthesized into an oxygen transfer rate control strategy which was able to improve the productivity of batch culture production of 2,3-butanediol by up to 30%. This was characterized by the energetic model of Jansen[43] which was developed to provide equations describing culture kinetics as a function of qO_2 only. The validity of these relationships was demonstrated in oxygen-limited batch and continuous culture. Also, these kinetic studies provided basic kinetic parameters

[43]N.B. Jansen and G.T. Tsao, Biotechnol. Bioeng., 27:573 (1984).

required for the analysis of system productivity. A model based on the energetic equations and kinetic parameters indicated that by controlling qO_2 at a set point, an enhancement of butanediol productivity, Π_{BDL}, relative to the case where qO_2 continually falls, could be expected. A control mechanism was developed, based on the modulation of the oxygen transfer rate by the partial pressure of oxygen in the feed gas, PO_2^{IN}. The control system was implemented and as expected resulted in increased Π_{BDL} at the higher set point values. This is the first demonstration of oxygen transfer rate control in the microbial production of 2,3-butanediol.

Energetic Model

Pyruvate is the central branch point in the catabolism of glucose to all observed end-products. Thus, upon application of energy, redox, and material balances, various relationships among the flows were derived. The relationship describing the average degree of oxidation of the end-product flow, η, mathematically described the fact that as oxygen becomes more limited, the end-product flow becomes less oxidized. The energy balance, together with the substrate balance and the observation that the specific substrate uptake rate is constant under oxygen-limited conditions, allowed derivation of an energetic relationship which accounts for the observation that growth and oxygen supply are tightly coupled under oxygen-limited conditions. This relationship also provides a new way to calculate the energetic constants Y^{mx}_{ATP} and mc. Furthermore, this coupling indicates that qO_2 is the sole independent variable describing the system.

Although this model formulation could account for individual product formation rates, only pairs of pathways could be treated. However, the batch and especially continuous culture data indicated that at least three and as many as five products could be simultaneously produced. This then is the area of energetic modeling which deserves the most attention. If this technique is to become more versatile, such a limitation should be removed; however, this is not an easy task. For example, calorimetry and the enthalpy balance may provide another energetic relationship. However, for each additional pathway, another such technique must be demonstrated. Metabolic mechanisms, such as a description of the acetoin/2,3-butanediol equilibrium through 2,3-butanediol dehydrogenase may also be of use in this regard.

Oxygen-Limited Batch Culture

The pattern of 2,3-butanediol and co-product formation was studied in batch culture. In general, all end-products were produced simultaneously, except acetic acid, which was produced during the aerobic phase and strongly during the early oxygen-limited phase. There was some modulation in product formation: acetoin was produced most strongly early in the oxygen-limited regime, ethanol produced most strongly at the end, and 2,3-butanediol produced throughout at a relatively constant fraction of the total end-product flow. Specific substrate uptake rate was constant and cell growth was linear and proportional to the oxygen transfer rate. This regime was termed energy-coupled growth. However, once qO_2 fell below a minimal value of about 15% of qO_2^{mx}, cell growth came to an almost stationary phase and the substrate uptake and product formation rates fell substantially. This was termed energy-uncoupled growth.

The energetic model was successfully applied to the energy-coupled growth regime. The energetic parameters Y^{mx}_{ATP} and mc were determined to be 14.8 g dry weight (g dw)/mole and 0.023 mole/g dw-hr, respectively. These values, although somewhat high with respect to Jansen's, fall within the ranges reported elsewhere. For these experiments, Φ_{ST} averaged 0.27 mole/mole-hr. Cell density, substrate uptake, and cell mass yield profiles agreed well with those expected from the energetic model.

The analysis of product formation required that the TCA cycle rate be back-calculated from the redox balance. Reasonable trends for Φ_{TCA} were obtained. Also, general agreement between the observed and expected total product formation rate was obtained.

Oxygen-Limited Continuous Culture

Several oxygen-limited continuous culture experiments were performed in order to study steady state growth and product formation kinetics and energetics. Cell density, as expected, fell with dilution rate, with OTR constant. The cell mass yield was proportional to dilution rate for $D < 0.06\ hr^{-1}$. A constant Y_{XS} of 0.25 g dw/g was observed for $D > 0.60\ hr^{-1}$. It is apparent, given this trend and also those of decreasing RQ and increasing η, that the continuous culture becomes more aerobic at the higher dilution rates.

Strong energetic coupling between growth and oxygen supply was observed. The energetic parameters Y^{mx}_{ATP} and mc were determined to be 23.5 g dw/mole ATP and 0.040 mole ATP/g dw-hr, respectively. Both these values are significantly higher than those determined in oxygen-limited batch culture.

Specific product formation rates in general exhibited trends with respect to qO_2 that are consistent with the idea that the more oxygen-limited the system is, the less oxidized the end-product flow will be. Optimal production of 2,3-butanediol, from both a specific rate and fractional yield basis, was observed at the lowest dilution rate ($0.16hr^{-1}$).

Wall growth is a concern in any bacterial continuous culture. A method was proposed and was used to demonstrate that wall growth, under the experimental conditions used here, was not significant.

Oxygen Transfer Rate Controlled Batch Culture

It is evident from the batch and continuous culture experiments presented herein and from the energetic and kinetic analysis of these experiments, that the degree of oxygen limitation is the controlling variable for the system. On a specific basis, optimal 2,3-butanediol productivity is realized within the energy-coupled growth regime, as opposed to the energy-uncoupled regime (Table 3-1).

Table 3-1. Comparison of Energy-Coupled and Energy-Uncoupled Growth

	Coupled Growth $\mu = 0.30$	Coupled Growth $\mu = 0.23$	Uncoupled Growth $\mu \cong 0$
Φ_{ST} (mole/mole-hr)	0.27	0.27	0.18
Φ_{BDL} (mole/mole-hr)	0.20	0.23	0.16
f_{BDL}	0.80	0.80	0.90

We therefore decided to maintain the batch culture at a constant degree of oxygen limitation by manipulating the OTR continuously during the oxygen-limited regime.

$$OTR^{SET}(t) = qO_2^{sct} X_c \exp[\mu^{sct}(t=\tau_c)]$$

A direct effect of such control is that the cell density increases in an exponential manner, as opposed to the linear growth usually exhibited in oxygen-limited batch culture. As a result, the fermentation is completed faster. This effect, coupled with an increase in 2,3-butanediol plus acetoin concentration, results in a significant increase in Π_{BDL}, Table 3-2.

Table 3-2. Summary of Effects of OTR^{SET} vs. Batch Culture Conditions

Batch Conditions	Φ_{BDL} (mole/mole-hr)	Y_{xs} (g dw/g)	BDL + ACN (g/l)	τ_f (hr)	Π_{BDL} (g/l-hr)
NONE	0.14-0.18	0.1-0.15	23.4	9.0	2.0
μ^{SET}=0.23	0.14-0.18	0.1-0.16	27.3	9.3	2.3
μ^{SET}=0.3	0.14-0.22	0.1-0.15	26.3	7.2	2.7

In addition to this performance enhancement, several physiological effects of the control are noteworthy. Balanced states of growth, in terms of μ, and of substrate catabolism, in terms of RQ, are imposed by the control. The formation of the co-product ethanol is repressed, while the formation of acetoin is increased. Thus, the product flow under controlled conditions is more oxidized than in uncontrolled culture.

Several features which should improve system performance such as agitation control have been tested. Results indicate that these alterations should be developed in conjunction with PO_2^{IN} control. This will preclude the need to increase PO_2^{IN} beyond 21%, which apparently had severe toxicological effects on the culture.

The most significant improvement in Π_{BDL} will be realized when the type of oxygen transfer rate control developed here is combined with a mechanism which allows on-line system monitoring. The control objective pursued in this work was to maintain a given degree of oxygen limitation. We know that this in turn would modulate the relative flows of substrate to biosynthesis and energy, or to product formation, and further would modulate the extent of product oxidation. However, the most obvious control objective would be to optimize Φ_{BDL} or Π_{BDL} directly, and not indirectly as was accomplished. Systems such as on-line mass

spectrometry[44] could play a deciding role. Research associates of the author are now attempting to develop this more sophisticated control approach.

We envision a procedure by which, rather than controlling the reactor after μ falls to μ^{set}, as was done here, the system would be allowed to proceed into the oxygen-limited regime where some index of 2,3-butanediol production approaches an optimal value. Then, perhaps the type of set point control on qO_2 discussed herein would be initiated. Furthermore, if the system began to drift from the optimal value of 2,3-butanediol production, a policy of adaptive control would be employed, where qO_2^{set} would either be decreased or increased, depending upon which direction the degree of oxidation of pyruvate was moving[45]. Such a policy would close the control loop on the end-product, rather than on the degree of oxygen limitation.

NOMENCLATURE

η	Average degree of oxidation of pyruvate flow
μ	Specific growth rate, hr^{-1}
μ^{set}	Set point for μ, hr^{-1}
Π_{BDL}	Overall butanediol productivity, mole butanediol/l-hr
τ_c	Time into oxygen limitation when control is initiated, hr
τ_f	Time of batch completion, hr
Φ_{BDL}	Specific butanediol productivity
Φ_{ST}	Total specific substrate uptake rate, mole S/mole X-hr
Φ_{TCA}	Specific TCA productivity
ACN	Acetoin
ATP	Adenosine triphosphate
BDL	Butanediol
D	dilution rate, hr^{-1}
f_{BDL}	Fraction of product formed which is butanediol
mc	Maintenance coefficient, mole ATP/mole X-hr
mx	maximum
OTR	Oxygen transfer rate, mole O_2/l-hr
PO_2^{IN}	Partial pressure of oxygen in feed gas, %
qO_2	Specific oxygen uptake rate, mole O_2/mole X-hr
RQ	Respiratory quotient
SET	Set point
t	time
TCA	Tricarboxylic acid

[44]M. Hayward, *et al.*, Anal. Chem., 62:1798 (1990).

[45]G.D. Austin, *et al.*, Proc. Control & Quality, in press (1990).

X_c Cell density concentration at instant of initiation of control

Y^{mx}_{ATP} Maximum cell mass yield on ATP, mole X/mole ATP

Y_{XS} Cell mass yield on substrate, mole X/mole S

(5) Enzyme Reactions in Reverse Micelles and Microcapsules (H.W. Blanch, University of California, Berkeley). Reverse micelles provide an aqueous environment for enzymes in an organic medium, making it possible to perform enzyme-catalyzed reactions involving hydrophobic substrates and/or products. The goal of this work is to study the enzyme-reverse micelle system in order to gain a better understanding at the molecular level of enzyme solubilization and reactions in reverse micelles. The model systems under investigation consist of the enzymes α-chymotrypsin and horse liver alcohol dehydrogenase (LADH). α-chymotrypsin, a globular protein of molecular weight 24,800, is a protease which may be used for dipeptide synthesis. LADH, a prolate ellipsoid of molecular weight 80,000, is an oxidoreductase which can catalyze the synthesis of flavor and fragrance compounds. The anionic surfactant Aerosol-OT (AOT) and the cationic surfactant CTAB are solubilized in iso-octane and iso-octane/chloroform (1:1), respectively.

The manner in which proteins are solubilized in reverse micelles is an important consideration when studying enzymatic catalysis in these systems. There are three main methods for solubilizing proteins in reverse micelles: injection of an aqueous protein solution into a surfactant-containing organic phase; addition of dry protein powder to a reverse micelle solution; and transfer of protein from an aqueous phase to an organic phase. Figure 3-9 shows these three methods schematically. The total amount of protein which can be solubilized in reverse micelles is dependent upon the method of protein solubilization. Through comparison of the solubilization characteristics of different enzymes in reverse micelles using the three protein-addition techniques, one can postulate possible mechanisms for protein solubilization in reverse

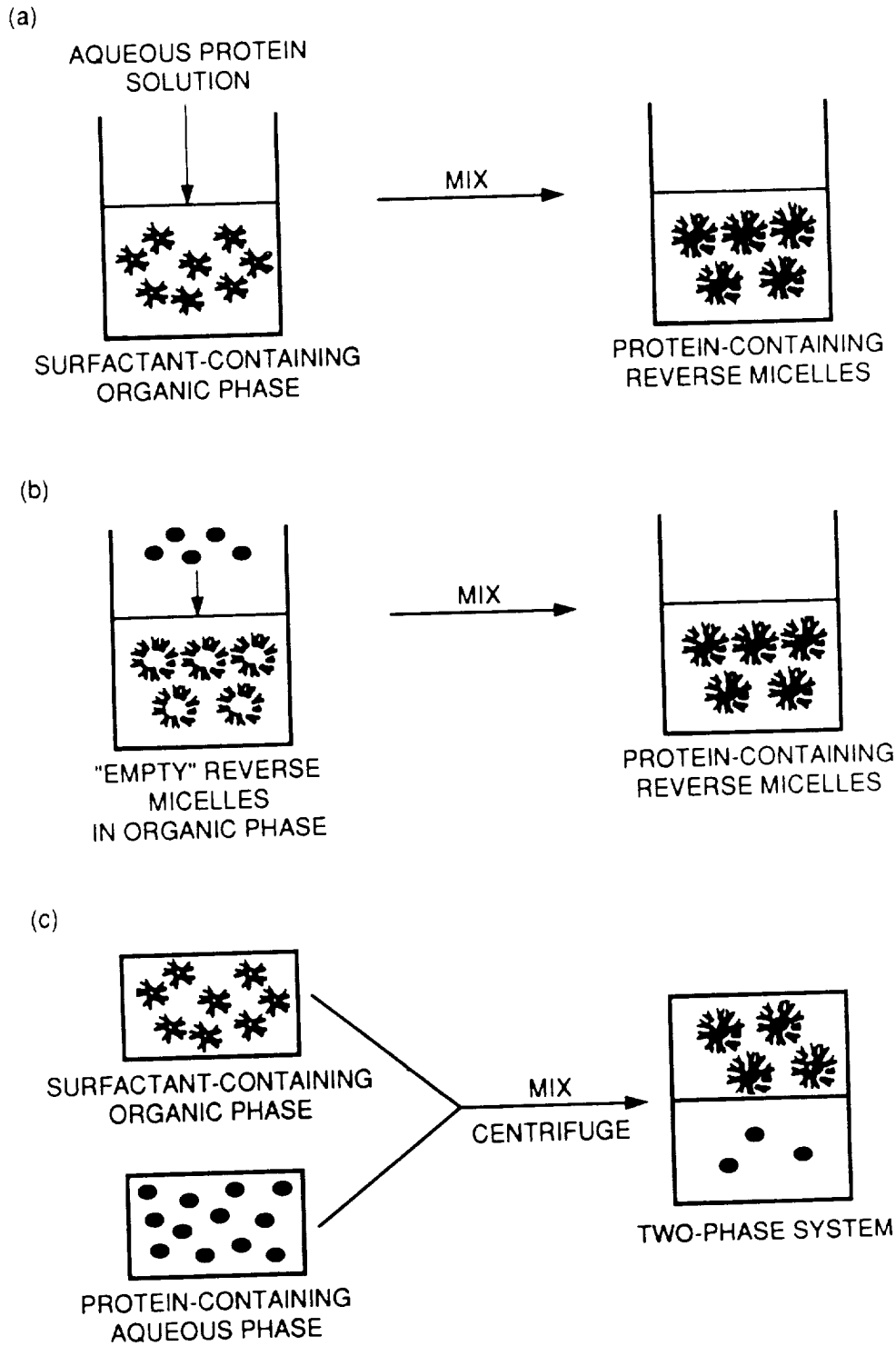
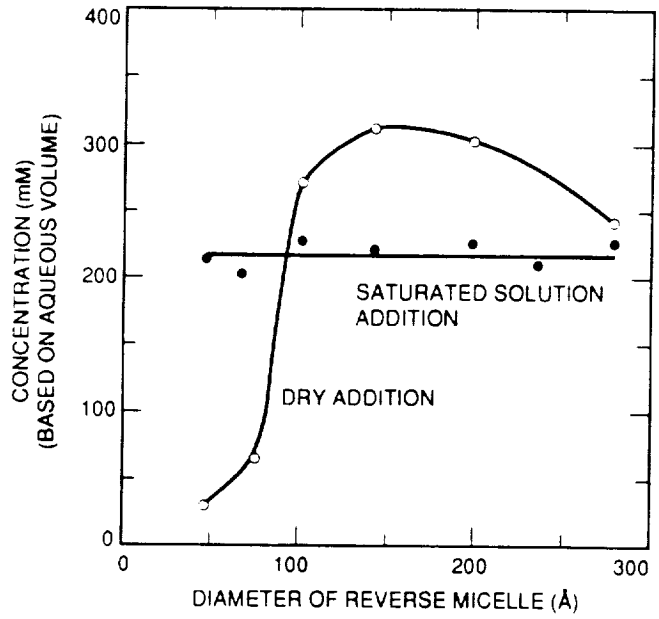
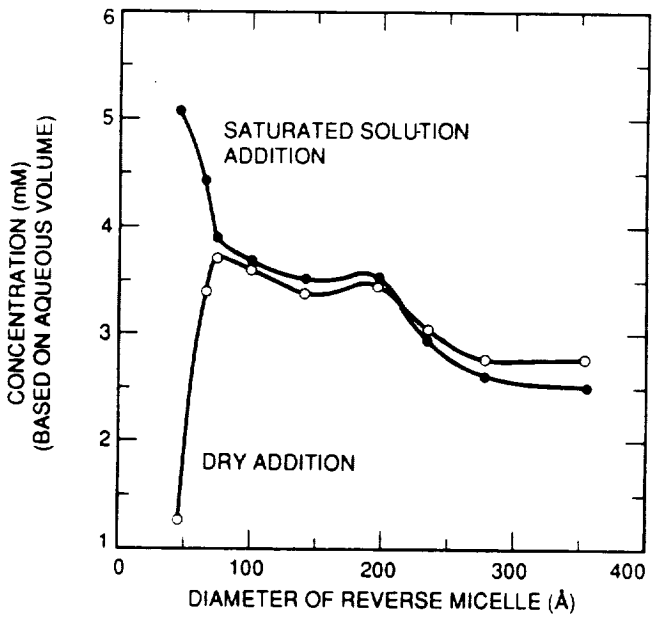


Figure 3-9. Methods of protein solubilization in reverse micelles. a) Direct injection of an aqueous protein solution. b) Addition of a dry enzyme powder. c) Phase-transfer.

micelles and ascertain the driving forces which affect this solubilization. Figure 3-10 shows the solubilities of LADH and α-chymotrypsin as a function



(a)



(b)

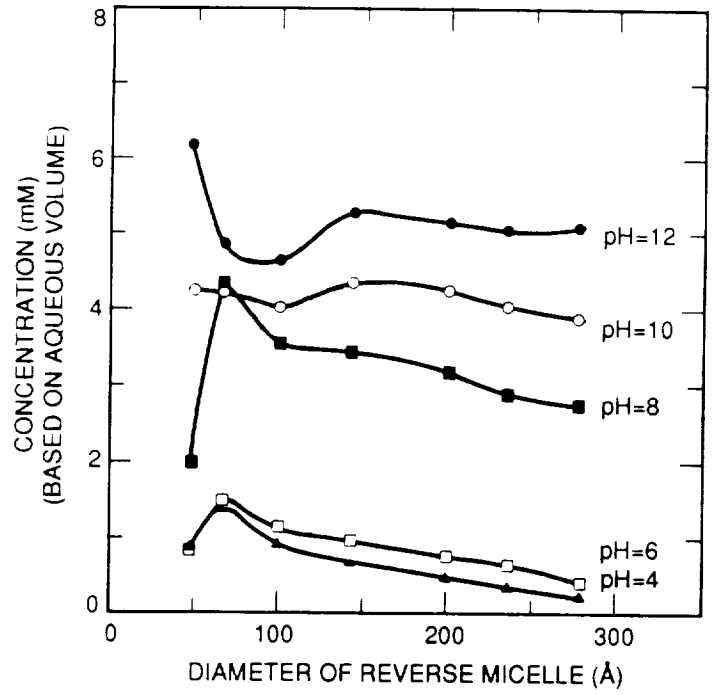
Figure 3-10. Solubility of LADH (a) and α-chymotrypsin (b) in reverse micelles.

C-2

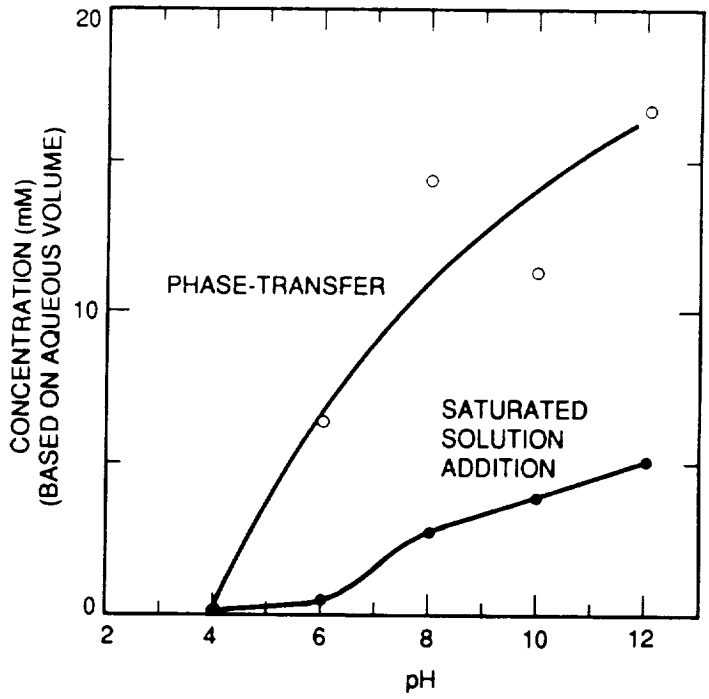
of micellar size using different protein-addition techniques. For the dry-addition method, the protein is not appreciably solubilized until the diameter of the micelle is similar in size to that of the enzyme. For the saturated-solution addition method, the protein solubility is not a strong function of micellar size, especially in the case of LADH. This observation might be expected since in the injection case, the micelles are forced to form with the protein already inside. For the case of the dry-addition solubilization of LADH the reverse-micelle system seems to enhance the solubility of LADH above that which is attainable in a bulk aqueous phase. This phenomenon would most likely indicate that LADH interacts with the micellar interface.

The pH of the aqueous micro-phase also has a profound effect on the solubilization of proteins in reverse micelles. Figure 3-11 shows the solubility of α-chymotrypsin in reverse micelles as a function of micellar diameter and pH. The surfactant used in this study was AOT which is an anionic surfactant. As the pH is increased past the iso-electric point of the protein (pI=8), the protein becomes negatively charged. The solubility of the protein increases with increasing pH which would not be expected if the driving force for solubilization is the charge-charge interaction of the surfactant head group with the protein.

The phase-transfer technique is the third possibility for protein solubilization. As shown in Figure 3-9, the reverse-micelle phase is in equilibrium with a bulk aqueous phase. The anionic surfactant, AOT, was also used in these studies. As the pH of the aqueous phase was increased, the amount of protein which was transferred to the organic phase increased. This trend is similar to that observed in the injection case. Figure 3-11 shows a comparison of the maximum attainable organic-phase protein concentration using the phase-transfer technique and the injection technique. At the high pH range, more protein can be



(a)



(b)

Figure 3-11. Effect of pH (a) and protein addition method (b) on α-chymotrypsin solubility in reverse micelles.

solubilized using the phase-transfer technique, which indicates that a different solubilization mechanism is occurring in this case.

Additional phase-transfer experiments employing positively-charged and non-ionic surfactants will be performed to isolate the specific driving forces responsible for solubilization in these systems.

In addition to the above solubilization experiments, the properties of water in the reverse micelles are also being studied. Experiments are currently under way utilizing NMR spectroscopy to study water motion in reverse micelles as a function of aqueous-phase pH. To gain a better understanding of kinetic results for the two enzymes studied, active-site titrations were performed in aqueous surfactant and reverse micellar solutions. Figure 3-12 shows the results for LADH indicating that AOT quickly decreases the number of active sites. LADH has a cationic zinc at the active site which may attract the negatively charged AOT thus inactivating the enzyme. CTAB has much less effect on the active site concentration. The α-chymotrypsin results shown in Figure 3-13 are less easy to interpret. AOT has little effect on the active site concentration while the number of active sites in CTAB reverse micelles decreases with increasing reverse micelle size (as indicated by w_o).

Kinetic experiments for α-chymotrypsin in AOT/iso-octane reverse micelles are now under way with the substrate N-glutaryl-L-phenylalanine-p-nitro-anilide, an amino acid derivative. The effect of reverse micelle size on enzyme activity will be examined for this system. α-chymotrypsin kinetic experiments will also be performed for another amino acid derivative, N-formyl-L-phenylalanine-p-nitro-anilide. This substrate is not commercially available so it was necessary to synthesize it. The synthesis has now been completed.

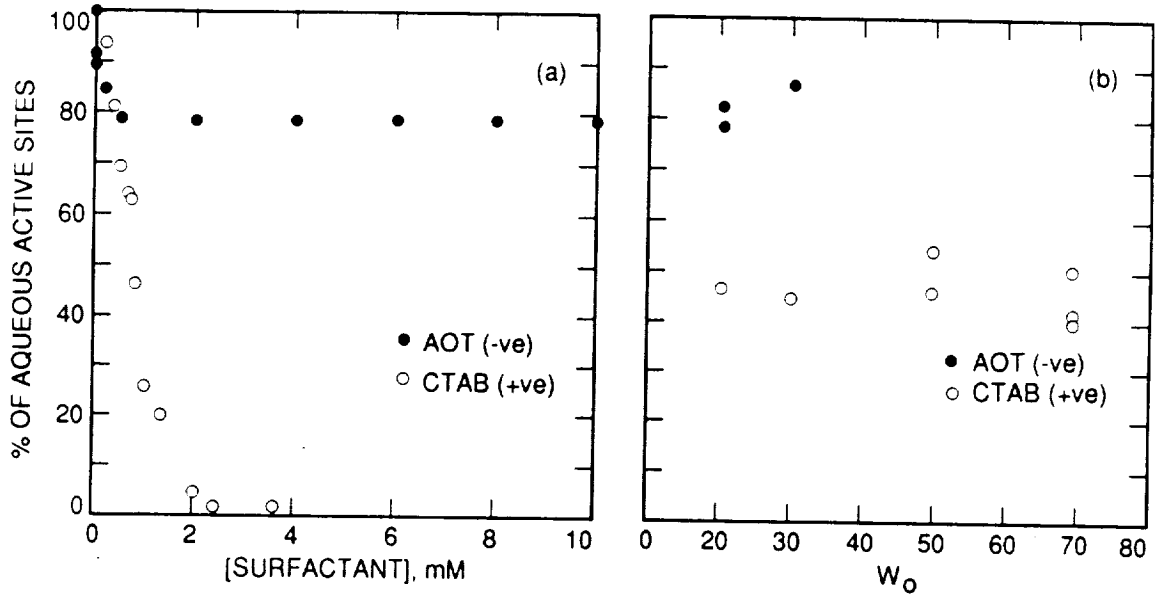


Figure 3-12. LADH active site titrations in (a) aqueous surfactant solutions and (b) reverse micelle solutions.

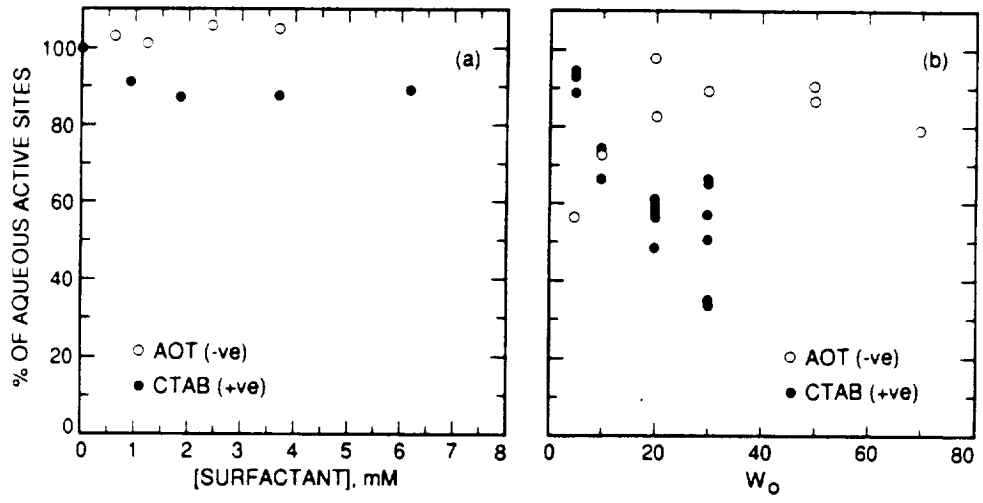


Figure 3-13. α-chymotrypsin active site titrations in (a) aqueous surfactant solutions and (b) reverse micelle solutions.

EPR experiments have been completed for LADH. This enzyme has been labelled both at the active site and at lysine residues on the enzyme surface (Figure 3-14). The EPR spectra of the active site label indicate increasing mobility at the enzyme active site with increasing water content. The EPR spectra of the surface label show little change in the line shape in going from a bulk aqueous solution to a reverse micelle solution except at low water content. This may indicate that the enzyme is located in the aqueous center of the reverse micelle.

EPR experiments will be performed with α-chymotrypsin using the same spin labels - an active site label and a surface label. These results will be used in conjunction with the kinetic results to obtain structure-function relationships.

(6) Gas Phase Enzyme Biocatalytic Reactor (D.L. Wise, Northeastern University). In recent years, the traditional method of using enzymes in water-based environments has been changed with the discovery of enzyme reactions in non-aqueous environments. Enzymes at "reduced hydrations" have been shown to maintain activity when suspended in organic solvents or supercritical fluids. These demonstrations have expanded the range of reactions which can be considered for enzyme-based biocatalysts.

In an important application of these non-aqueous enzyme systems, the modification of gaseous substrates by dehydrated ("dry") enzymes has emerged as an exciting biotechnological concept with potential applications in analysis and novel gas-solid bioreactions. A specific example of this type of gas-phase enzyme bioprocessing is that for the enzyme catalyzed reaction of ethanol to acetaldehyde, as defined by Barzana.[46] The enzyme specific for this reaction, alcohol oxidase, is well

[46]E. Barzana, A.M. Klibanov and M. Karel. *Appl. Biochem. Biotechnol.* 15:25-34 (1987).

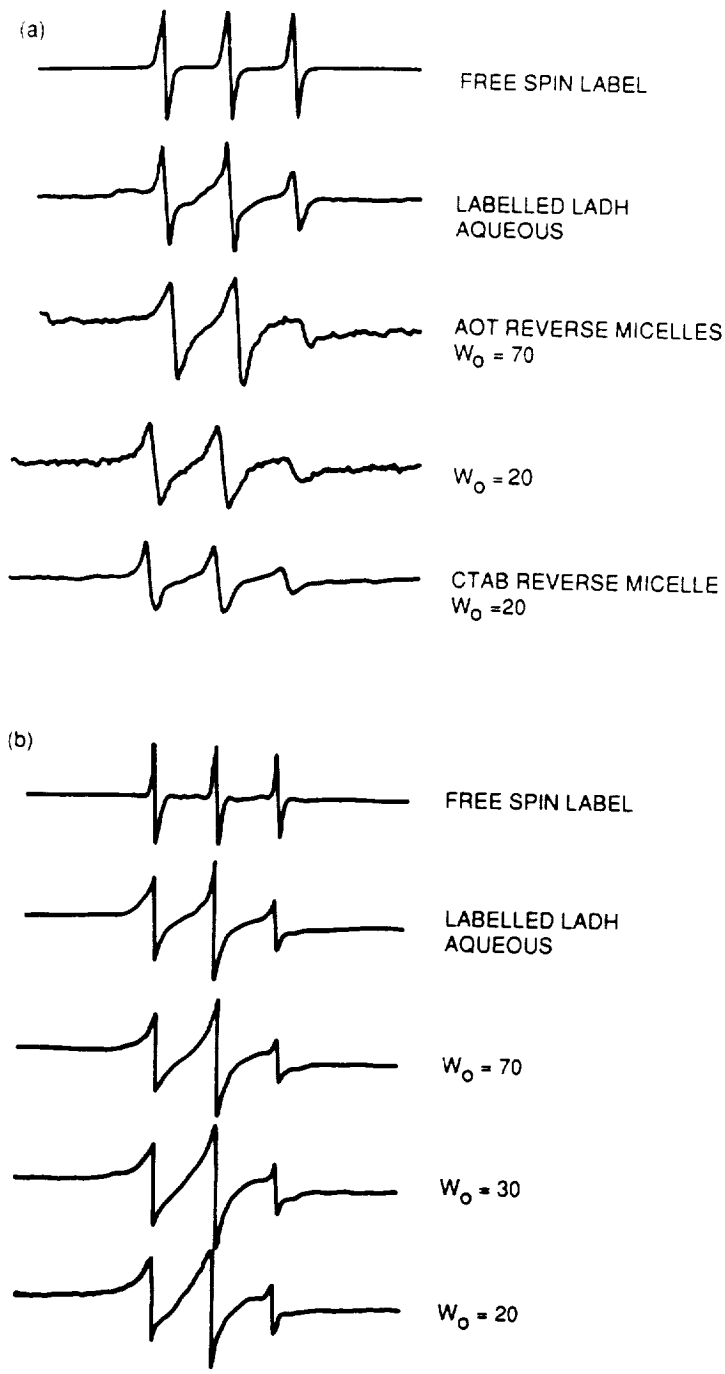


Figure 3-14. **Active site-labelled LADH in aqueous and reverse micelle solutions (a) (label: 4-(2-Iodoacetamido)-2,2,6,6-tetramethyl-1-piperidinyloxy, free radical) and lysine-labelled LADH in aqueous and AOT reverse micelle solutions (b) (label: 2,2,5,5-tetramethyl-3-pyrrolin-1-oxyl-3-carboxylic acid N-hydroxysuccinimide ester).**

defined and has been studied in many respects. Thus, the biocatalytic "gas-phase" conversion of ethanol to acetaldehyde was chosen as the model for this investigation. Building upon the work of Barzana, this work specifically focuses on gas-phase biocatalysis in a continuous reactor format, an important prelude to the broader application of "dry" enzymes in bioprocessing.

Designed in the current state-of-the-art of biotechnology process and materials development, a continuous flow bench-scale experimental model of this gas-phase enzymatic biocatalytic reactor (in this case, a fixed bed reactor) forms the basis of this study.

A description of the project's work is presented as follows:

Solid-Phase Support-System Development

The objective of this task is to develop and validate the design of a solid-phase support system that will operate in a "dry" enzyme reactor. Using developed assay techniques, preliminary evaluations are being made to determine the reactivity of the resulting biocatalyst on solid-phase supports, i.e., column packing material. A parametric experimental design is being used to compare the performance of the immobilized biocatalyst under different conditions and studies first carried out as batch experiments.

Model Biocatalytic Reactor Design, Fabrication and Operation

The overall objective of the work will be to evaluate the model biocatalyst system in a continuous flow reactor simulating actual process conditions, so that realistic engineering projections may be made. To this end, preliminary physical and analytical modeling studies are being carried out to establish design parameters for the solid-phase support system.

Preliminary design studies were conducted early in the program to develop geometrical parameters for the solid-phase support system. An experimental model was used to perform these studies. Samples of solid-phase support materials (no biological activity) were tested. This provides mechanical design input to the solid-phase support development and a firm basis for the subsequent design and use of the functional laboratory reactor.

Biochemical Process Modelling and Economic Analysis

The objective of this task is to provide a better understanding of the basic kinetics and mass transfer relationships operating at the micro-scale and to develop a process working model to aid in interpreting results in terms of potential commercial economics. Early modeling tasks are planned to interpret the experimental data. The process model will provide a basis for subsequent decisions to proceed beyond this proposed work and to identify sensitive processing parameters for further investigations.

In the first quarter of this project, the focus of the laboratory work was on the preparation and preliminary characterization of the biocatalyst. For the biocatalytic reaction, it was first necessary to prepare the immobilized enzyme. There are two different methods for enzyme immobilization. A static contacting method and a dynamic contacting method. Static contacting was investigated first, and DEAE cellulose was used as the solid-phase support for the immobilization of alcohol oxidase. It was found that, in this case, the distribution of the enzymes within the support material (DEAE cellulose) was uniform and the enzymes were active compared with the nonuniform enzymes. After the immobilization of alcohol oxidase on DEAE cellulose, the enzymes were dried by the air flow method using pre-dried air. Further work showed, however, that, while DEAE cellulose was a good immobilization material, its mechanical properties

limited its uses in an operating bioreactor.

In the further development of the continuous gas phase reactor, optimization of the biocatalyst itself was identified as the focal research point. In this aspect of the work, two methods were used for the preparation of the biocatalyst: (a) physical adsorption and, (b) ionic binding. In the biocatalyst studies, an optimum immobilization time of 2.5 hours was determined based on the apparent enzyme activity retained on various supports. Throughout this study, the immobilized enzyme (biocatalyst) was kept moderately hydrated using air saturated with water vapor. One step of biocatalyst preparation, i.e., drying, was omitted in the overall bioreactor studies because initial work using a rigorously dried biocatalyst indicated the necessity for strict control of hydration levels.

The continuous gas phase reaction was performed and it was found that the product acetaldehyde was produced from the continuous gas phase reaction. Our present work addresses the optimization of the various bioreactor operations, with a major emphasis of the work still being biocatalyst preparation. Of particular interest in the overall development are substrate manipulation, catalyst efficiencies, flow controls, and product recovery. This information will then be utilized in developing an appropriate process model for this, and, more importantly, other gas-phase bioreactor applications.

(7) Immobilized Enzymes in Organic Solvents (H. Zemel, Allied Signal Research and Technology). The synthetic application of enzymes to organic solvents is an emerging technology with tremendous promise for the chemical industry, as it renders biocatalysis more compatible with the existing industrial environment. The bottleneck in the application of this technology is the low activity and stability of biocatalysts in non-aqueous media. Although considerable progress has been made in this area in recent years, the mechanism

of enzyme deactivation, as well as the quantitative aspects of the parameters limiting overall productivity, are not fully understood. The objective of this project has been to investigate the physical chemistry of immobilized enzymes operating in organic solvents, and improve the efficiency of an enzyme reactor performing trans- or interesterifications in organic media. The model system chosen for the study is the production of low cost alternatives to Cocoa Butter, a specialty fat used extensively by the confectionary industry, through the enzymatic interesterification of inexpensive Palm Oil triglycerides.

In 1989 we laid the foundations for the project. Analytical methodology was developed using gas chromatography to follow the rate of enzymatic triglyceride interesterification. Tripalmitin was selected as a model substrate, rather than Palm Oil, in order to afford a more interpretable kinetic scheme. Amano Lipase P from *Pseudomonas cepacia* was found to be the most active one for our transformation, and it was non-covalently immobilized on celite, the support exhibiting the least enzyme inactivation. Immobilization was achieved by adding a concentrated, buffered lipase solution to the dry support in a sufficient quantity to wet the support. The wet, lipase-impregnated support was then lyophilized. The pH of the enzyme solution used for immobilization was optimized for maximum activity. Enzymatic incorporation of Stearic Acid into Tripalmitin was carried out in petroleum ether, a food grade solvent. The ratio of substrate to enzyme, and the overall concentrations were selected to provide an easily measurable reaction rate.

Having established the experimental conditions, we set out to examine the various parameters that affect the activity and stability of the lipase in the non-aqueous environment. Reports in the literature suggest that the water concentration is by far the most important

factor controlling the rate and efficiency of such an enzymatic process. Increase in the water concentration seems to improve the enzyme activity. We were interested in the quantitative aspects of this relationship, and its meaning in terms of the enzyme state and conformation. With the lipase immobilized on celite 560, we varied the amount of water added to the reaction mixture from 0 to 0.5%, and measured the initial rate of stearate incorporation into the triglyceride as a function of the added water. The amount of water added was above the solubility of water in petroleum ether. Using Karl-Fischer titrations of the solvent and the supported enzyme, we have determined that practically all the added water (>98%) resides on the support. At most of these water concentrations the support was free flowing rather than wet and sticky. As the water content was raised from zero to an intermediate value, the activity increased dramatically, then reached a maximum and started to decline slowly as more water was added. Similar qualitative behavior was observed with two other celite supports that have similar chemical composition but different morphology. The enzyme activity initially rose as water content was increased, then reached a maximum and decreased slowly with additional water. However, the quantitative aspects of this behavior were different for the three supports. The optimal lipase activity was obtained at different water concentrations. The enzyme activity at the three different optimal water concentrations, however, was similar. The amount of water needed to obtain the maximal activity, $C(w)_{max}$ correlated neither with the amount of enzyme or support, which were the same for the three supports, nor with the surface of the supports. The amount of water at maximal activity did show a very nice linear correlation with the pore volume of the particles: activity started to decline when half of the pore volume was filled up with water. This finding suggests that from this point, mass transfer limitations become the major factor controlling the interesterification rate. As long as

the water layer on the particle is thin and does not cause blockage of the pore's opening, the solvent can enter all the pores. The water insoluble substrates and products have thus easy access to the lipase in the water phase. When the amount of water increases to the point of filling up some pores, more and more of the enzyme becomes isolated from the substrates. The rate of mass transfer becomes the dominating factor reducing the overall interesterification rate.

Since all the added water resides on the hydrophilic support, the water has to form a separate phase, or a layer, between the particle surface and the organic solvent. The question then arises, as to whether the enzyme is partially or completely dissolved in this aqueous microenvironment. The lipase we have been using (Amano Lipase P) is a very crude preparation containing only about 1% lipase. In order to determine the solubility of the lipase, we have followed the total protein dissolved in the appropriate buffer as well as the esterase activity of that supernatant at various hydration levels. Very good correlation between the two measurements was obtained. The major protein component of the crude material had esterase activity and high solubility. A minor protein component (<10%) with very low solubility showed no esterase activity, suggesting it is not a lipase. The solubility data imply that the lipase deposited on the support is being dissolved as we add water to the system. The enzyme is completely in solution at a water level that is slightly lower, or is the same as the level needed for maximal activity, $C(w)_{max}$ depending on the support. This implication relies on the assumption that all the added water is bulk water available to the enzyme. In reality, some of that water is tightly bound to the inorganic support and is not available for enzyme solubilization. Thus the immobilized lipase might need slightly more water than the free enzyme to completely dissolve. These considerations lead to the conclusion that the dramatic increase in interesterification rate as the water

content rises from 0 to $C(w)_{max}$, results mainly from the solid enzyme being dissolved. In other words the active conformation of the lipase is the one it attains when dissolved in aqueous buffer and not the solid form.

The picture emerging from these findings is rather simple. Although the overall water concentration in this system is minute, less than 0.5%, the lipase is actually operating in its natural milieu, an aqueous buffered microenvironment. The amount of water needed to allow the highest enzyme activity is a compromise between two opposing forces. Too little water will not dissolve all the immobilized enzyme, and too much water will clog the support pores causing an apparent decrease in catalysis rate. Our results actually afford a way to predict the optimal water requirements for an enzyme with known water solubility and a support with a given pore volume.

In many studies described in the literature, the percent water reported is the overall one. The actual water concentration at the enzyme's vicinity could have been as high as in our system. The conclusions of our work can shed light on many reported observations. For example, the pH memory in similar systems can simply be attributed to reconstituting the buffer in the aqueous microenvironment to provide optimal enzyme activity in solution.[47] The results are also in agreement with reports in the literature[48] that enzymes operating in organic solvents do not exhibit any change in their conformation from that in aqueous media. The results might also provide an explanation as to why in some enzyme/organic solvent systems the observed activity is of the order of aqueous enzyme activity, while in other cases the activity is orders of magnitude lower. More

[47]P.A. Burke, S.O. Smith, W.W. Bachovchin and A.M. Klibanov. *J. Amer. Chem. Soc.* 111:8290 (1989).

[48]D.S. Clark, L. Creagh, P. Skerker, M. Guinn, J. Prausnitz, and H. Blanch, *ACS Symp. Ser., Biocatalysis & Biomimetics* 392:104 (1988).

work in this direction is in progress.

D. Separations and Novel Chemical Processes

(1) Separation by Reversible Chemical Association (C.J. King, University of California, Berkeley). The objective is to investigate and evaluate the use of reversible chemical association, or complexation, as a method for separating polar organic substances from dilute aqueous solution. Solutes of particular interest include fermentation chemicals such as carboxylic acids (acetic, propionic, etc.), alcohols (ethanol, butanol, etc.), and multifunctional compounds involving these groups (dicarboxylic acids, hydroxycarboxylic acids, glycols, etc.). The project also has widespread application to energy-consumptive separations in the chemical industry, including manufacture of alcohols, glycols and carboxylic acids from petrochemical sources. The research is also applicable to removal and recovery of these and related substances (e.g., sugars and starches) from waste streams.

In our work separations are implemented through solvent extraction by organic liquids and sorption by polymeric solids, but they can also be implemented in a number of other ways.

The goals are: (a) to obtain sufficient understanding of underlying chemical, thermodynamic, and transport phenomena to enable rational selection of separating agents and methods of implementation and regeneration, as well as rational conceptual design and economic evaluation, and (b) to devise improved processing technology, especially for regeneration.

Extraction of Carboxylic Acids and Regeneration of Extracts by Precipitation of the Acid.

Measurements of the solubilities of succinic, adipic

and fumaric acids in various solvents have revealed that in some solvents there is a large increase in solubility due to the presence of co-extracted water. For ketone solvents (cyclohexanone, methylcyclohexanone and methyl isobutyl ketone) solubilities in the presence of co-extracted water are as much as eight times greater than those in anhydrous systems. The number of moles of water co-extracted with each mole of additional extracted acid is roughly the same for all three acids, being 4.4 and 5.5 moles of water per mole of acid for methylcyclohexanone and cyclohexanone, respectively.

This phenomenon leads to a simple and energy-efficient method for regenerating extracts resulting from the extraction of carboxylic acids from aqueous solutions into various organic solvents. The approach is to remove co-extracted water selectively, with the result that most of the carboxylic acid will precipitate and can thereby be recovered as product. For the three ketone solvents mentioned above, and for many other organic solvents, the relative volatility of water in the solvent is high, with the result that the co-extracted water can be selectively removed by a simple stripping process.

Exploratory calculations have been made of the energy requirements for a process in which a non-volatile carboxylic acid is extracted from aqueous solution and then precipitated from the organic extract through partial evaporation of the extract. The comparison case was simple dewatering in a five-effect, evaporator system. For 95% recovery of the acid and aqueous feed concentrations of 2.5 to 4.5 wt. %, the five-effect simple evaporation process requires 3.0 to 6.6 times as much energy as the extraction/evaporative precipitation process.

Extraction equilibria for extraction of fumaric, adipic and succinic acids from water into methylcyclohexanone have been compared at 25°C and 45°C. Comparison of the

extraction-equilibrium data at the two temperatures reveals that the transfer of acid from the aqueous phase to the organic phase is exothermic and occurs with a decrease in entropy. The enthalpy for transfer of fumaric acid is about twice as large as for succinic and adipic acids. It appears that at low concentrations the transfer of water is entropy-driven and energetically unfavorable, while at higher acid concentrations water transfer is energy-driven and entropically unfavorable. These results support the concept of acid-water complexation in the organic phase.

Regeneration of Carboxylic Acid Extracts with Strong, Volatile Aqueous Bases.

In earlier work we used low-molecular-weight tertiary amines in aqueous solution as regenerants for recovering carboxylic acids from high-molecular-weight tertiary amine extractants. In particular, we demonstrated that aqueous trimethylamine (TMA) is effective for recovery of lactic, succinic and fumaric acids from Alamine 336 extracts. There are two advantages to this method of regeneration: a) TMA is volatile, and therefore the resulting trimethylammonium carboxylate salt can be decomposed thermally, giving the product acid as well as TMA vapor which can be condensed for recycle and reuse. This method of back-extraction therefore neither consumes chemicals nor produces a waste salt stream, as does the classical calcium precipitation method. b) In aqueous solution, TMA is a very strong, ionizing base. It therefore can extract carboxylic acids effectively from strongly basic extractants or adsorbents. A well chosen strongly basic extractant, adsorbent or anion exchanger can recover a carboxylic acid from an aqueous solution with pH above the pK_a of the acid, and still be regenerated by leaching with aqueous TMA (pK_a is the value of pH at which an acid is half dissociated). This property is advantageous, since many fermentations producing carboxylic acids operate best at pH above the

pK_a of the acid.

A theoretical model has been developed to describe leaching of acid-laden sorbent with aqueous trialkylamines. This model uses the ideal-exchange sorption model and data of Garcia and King[49], and combines them with a description of the aqueous phase through the pK_b of the trialkylamine. The model thereby contains no fitted parameters. The model was used to predict acid recovery as a function of the molar ratio of aqueous trimethylamine (TMA) to acid, with the complexation strength of the sorbent as a parameter. The results indicate that leaching with TMA should remain effective for sorbents of much greater basic strength than have heretofore been used. This substantiates the expectation that regeneration with aqueous TMA should allow operation at pH substantially above the pK_a of the acid.

Sorption isotherms have been measured for the uptake of succinic acid at natural pH from aqueous solution by means of strong-base sorbent Bio-Rad AG3-X4. The capacity is about 0.4 g succinic acid per g dry resin, with a water uptake of about 1 g/g. Substantial capacity is retained two or more pH units above pK_a (4.2) of succinic acid. Capacities of 0.25 and 0.10 g/g were obtained at pH 6 and 8.8, respectively.

Recovery and Fractionation of Glycols.

We are investigating the use of glycol-boronate complexes for recovery of glycols from aqueous solution and for fractionation among different glycols. Boronate complexation is used in analytical chromatography for analysis of glycols, sugars and related substances. It is one of the few methods of complexation effective for glycols. Using boronate complexation on a production scale requires overcoming problems of (a) matching the pK_a of the boronate to

[49]Garcia and King, *Ind. Eng. Chem. Res.* 28:204-212 (1989).

that of the solution, (b) enabling the boronate anion to be present in an extraction or adsorption process, and (c) regeneration. We are investigating those areas.

3-Nitrophenylboronic acid (NPBA) exhibits much lower values of pK_a than do other common boronates. It can therefore complex effectively with glycols in solutions with pH as low as 5, whereas most other boronates ionize and complex only at much higher pH. The nitro and phenyl groups also serve to reduce the aqueous solubility of the boronic acid. We have measured apparent stoichiometries and stability constants for NPBA with 1,2- and 1,3-propanediol at temperatures ranging from 25°C to 45°C. The complexes formed with 1,2-propanediol are substantially stronger than those formed with 1,3-propanediol, because the five-member ring formed in the complex with 1,2-propanediol is less strained than is the six-member ring formed with 1,3-propanediol.

(2) Biological Separation of Phosphate from Ore (R.D. Rogers, Idaho National Engineering Laboratory). Applying biotechnology to the process of solubilizing phosphate ore has been updated into the following five areas: (a) modifying the current bioprocessing system in order to maximize the peculiar phosphate solubilizing biochemistry of selected microorganisms; (b) continued collaboration and cost sharing with industry in the development and integration of methodology into their phosphate extraction process stream and to recover phosphate from low grade ore which is presently treated as waste; (c) develop biomechanisms for the concentration/separation of phosphate from process solutions; (d) calculate kinetics of the solubilization process; and (e) enhance the ability of the solubilizing microorganisms by manipulation of external/physical/chemical factors and manipulation of genetic composition.

This report represents a compilation of data from those

studies conducted during the last 12 months. Technical highlights for this report are reported chronologically rather than in the sequential order listed above.

The effects of mineral and organic acids on rock phosphate (RP) solubilization were determined in a series of abiotic studies. Normalized data from both abiotic as well as biotic studies indicated that the mechanism of RP solubilization includes more than just the presence of organic acids or acidic conditions. In biotic studies in which the microorganism selected as the preferred solubilizer (the bacterium E37) was present there was almost twice the amount of RP solubilized as compared to those abiotic studies which simulated the biotic conditions of solution pH and organic acid content. These data strongly supported what we had only been able to allude to in past work, that is, that although the process of RP solubilization involves physical/chemical reactions the action of the microorganism is required. The bacteria used in our studies apparently promote mechanism(s) - in addition to acidification and calcium chelation - which enhance(s) the solubilization process.

In order to learn more about the effect that organic acids have on the solubilization process, cooperative work was initiated with the TVA National Fertilizer Center. Their staff used the MINEQL model to provide data on the ability of organic acids to chelate calcium as well as promote the solubilization of apatite. It was obvious from the model's data that at the working conditions of the biological solutions used in the solubilization studies, little or no chelation of calcium would occur. In addition, the model predicted that the presence of organic acid does not promote the solubilization of apatite. Previously discussed laboratory work, together with these data, provides compelling evidence that the mechanism which promotes RP solubilization involves the chemical action of hydrogen ion and some other process associated with the intimate contact of the bacteria.

During the year, work has been ongoing in the development and testing of a continuous bioprocessing system to help maximize the peculiar biochemistry involved in the RP solubilizing process. A prototype system consisting of a 2-L stirred-tank laboratory fermenter connected to a series of 125-mL secondary biocontact cells was developed during the previous year. Because of encouraging results, modifications have been made in this system to increase both the volume of RP processed as well as the rate of solubilization. Both the 2-L fermenter and a new 10-L fermenter are now being used to produce microbial extractant. New secondary-biocontact-reactor designs include a "V" trough, packed columns, fluidized bed, and a stirred tank. Problems associated with the scale-up procedure are being addressed. Results from the use of these reactors show that the rate of solubilization can be increased in the scaled-up processes. Studies with the packed-bed columns demonstrate that the process could be adapted for "heap leaching" purposes. If this is proven to be true, given the rate of solubilization and the passive nature of the process, heap leaching could provide a viable option for the recovery of phosphate from low grade RP now considered waste.

Three subcontracts have been ongoing during the year. One is now complete and two are still active. The completed one was with the University of Wyoming to determine if phosphate can be biologically reduced to phosphine (a volatile form of phosphorus). Such a process could be used as a method to remove and then concentrate soluble phosphate. Data from this work showed that phosphine is being produced in naturally occurring samples. These results were encouraging and this area of research will be continued if adequate funding can be found. The two continuing subcontracts involved determining the changes which occur in the organic matter of RP as it is being bioprocessed, and work at Cal State Los Angeles in the area of genetic analysis of the E37 bacterium. Results from the

organic matter work have shown that there are substantial changes occurring in RP organic material as a result of bioprocessing. There appear to be modifications occurring which give rise to the possibility that the organic fraction could serve as a carbon source for solubilization and, in addition, there appear to be changes occurring which make the recalcitrant material more amenable for the extraction of usable oil. This could have important spin-offs for the processing of both highly organic RP and of oil shale for the purpose of energy-saving oil recovery. Finally, work designed to provide an understanding of the genetic mechanism of solubilization is progressing. Such an understanding will lead to an enhancement to the process.

The FMC Corporation, Phosphate Chemicals Division was a direct contributor to this work both intellectually and monetarily through a technology transfer agreement.

(3) Electrocatalytic Study of Ammonia Synthesis and Methane Dimerization in High Temperature Solid Electrolyte Cells (M. Stoukides, Tufts University). High temperature proton conductors are solid state materials that predominantly exhibit H^+ conductivity at 400-1000°C.[50] These materials can be used in heterogeneous catalysis to electrochemically modify the catalytic rates of hydro- and dehydrogenation reactions. To this end, the catalytic reaction is studied in an H^+ conducting solid electrolyte cell. The anode of the cell is exposed to a hydrogen-rich gas and serves as the catalyst under study. The cathode is exposed to an inert or oxygen-rich gas stream.

Useful chemicals and electrical energy can be simultaneously generated if the cell is operated as a fuel cell.[50] In this case, hydrogen spontaneously flows through the electrolyte in a closed-circuit where the driving force is the chemical potential difference

[50]M. Stoukides, *Indus. Eng. Chem. Res.* 27:1745 (1988).

between the two electrodes. The cell can also be utilized as an electrochemical hydrogen pump where hydrogen can be controlled and removed from a gaseous mixture.[50] This method can selectively control reactions where dehydrogenation products can form. The two proposed applications of the H^+ conducting cell have been used in the past year - the first of a three-year project - to study the dimerization of methane at 800-1000°C and 1 atm and the synthesis of ammonia over Fe-based catalytic electrodes at 450-550°C.

The technical highlights of the past year can be classified into several categories which are:

APPARATUS

Complete assembly of two nearly identical sets of apparati for this project, one of which is already being used to study methane dimerization, has been completed. An apparatus consists of the gas feed system, the reactor, and the analytical system. Reactants and products are analyzed by on-line gas chromatography. A dual CH_4-NH_3 infrared analyzer and a H_2 analyzer monitor the effluent stream. The reactor cell is shown in Figure 3-15.

ELECTROLYTE PREPARATION

The technically difficult task of assembling a reactor with a satisfactory electrolyte has been accomplished. The $SrCe_{0.95}Yb_{0.05}O_3$ electrolyte disk made in our laboratory is prepared using a method similar to that of Iwahara.[51] A disk is prepared by mixing powders of

[51]H. Iwahara, T. Esaka, H. Uchida, and N. Maeda, *Solid State Ionics* 314:359 (1981).

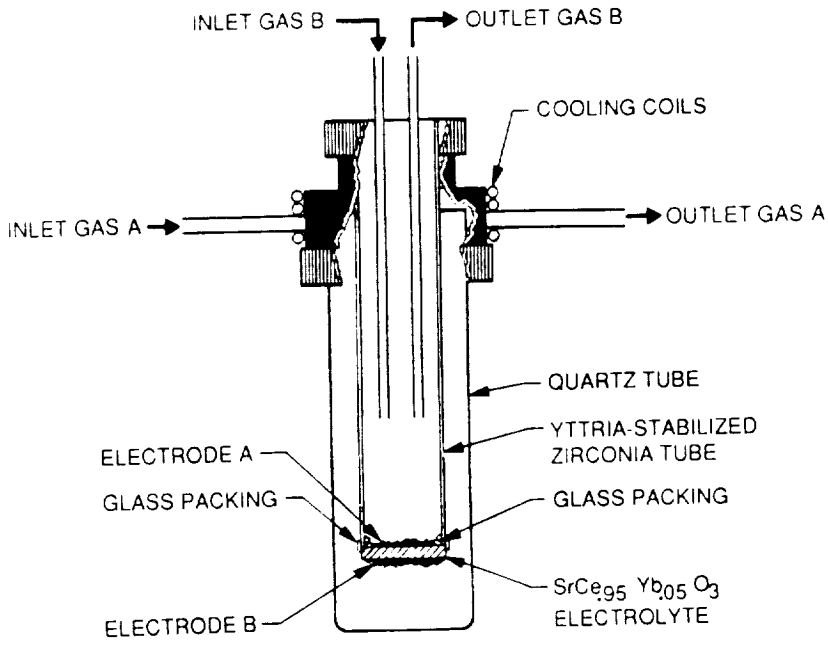


Figure 3-15. High temperature solid electrolyte reactor cell.

strontium carbonate, cerium dioxide, and ytterbium oxide. The mixed powders are slowly heated up to 1450°C and calcined for 6 hours. The calcined oxides are grounded and pressure-molded into a thin (0.3-1.0 mm) disk of 25 mm diameter. The disk is reheated to 1350-1400°C for 6 hours in air to produce a stable H^+ conducting ceramic. The disk is cut to a 19 mm diameter and is sealed to an open-ended yttria-stabilized zirconia tube. The seal was acceptably gas-tight since at 20 cc/min, the air leak does not exceed 20 ppm.

ELECTROLYTE PERFORMANCE

Experiments were run at 900-1000°C with Pt, Au, and Fe electrodes to determine the electrolyte conductivity. Methane and hydrogen mixtures in helium were passed over the catalyst-electrode. It was determined that the $SrCe_{0.95}$ $Yb_{0.05}O_3$ electrolyte did conduct protons. The

current efficiency (i. e., the amount of current that is due to ionic conductivity) is, however, typically 40% with a maximum of 60%. We have initially reported on the efficiencies.[52] There are current attempts to improve these efficiencies. The maximum total current passed through the electrolyte as a hydrogen pump at 1000°C was about 250 mA over a Pt electrode superficial area of 2 cm^2. At this high temperature and current, the electrolyte life is less than 48 hours since a) the ceramic seal sometimes reacts with the electrolyte and b) carbon deposits, which form from CH_4 decomposition at high CH_4 partial pressures, poison and apparently alter the electrolyte composition. With both problems, the electrolyte's ionic conductivity is greatly reduced after 48 hours.

CATALYTIC PERFORMANCES

Over the past three years, the partial oxidation of methane has been studied in our laboratory.[50,53,54,55 & 56] The most recent oxygen pumping experiments were done with Pt and Au-Y_2O_3/ZrO_2 catalysts of which the latter was found to be active for coupling (yields up to 15.4% at 900°C). A systematic search of materials that are electronically conductive and catalytically active has resulted in the following catalysts thus far tested: Ag, Pt, Au, Fe, Cu, Pd, Au-Li/MgO, and Au-Y_2O_3/ZrO_2. The most conductive catalysts were Pt (125 mA/cm^2 at 1000°C) and Fe (100 mA/cm^2 at 900°C). The most catalytically active were again Pt and Fe (70% methane conversion at 900°C). At high methane concentrations,

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[52]D. Eng, P. H. Chiang, and M. Stoukides, paper #83 in 3B Symposium, presented at the 1989 International Chemical Congress of Pacific Basin Societies, Honolulu, December 1989.

[53]S. Seimanides and M. Stoukides, *J. Electrochem. Soc.* 133:1807 (1986).

[54]D. Eng, Ph.D. Thesis, Tufts University, 1990.

[55]D. Eng, *J. Electrochem. Soc.* 137:215C (1990).

[56]D. Eng and M. Stoukides, paper #22c presented at the 17th Australasian Chemical Engineering Conference, Gold Coast, Queensland, August 1989.

carbonaceous deposits were observed to form, resulting in poisoning of the catalysts and lowering of the C_2 selectivity. The maximum selectivity found over Fe was 23% to almost all C_2H_4.

E. Process Design and Analysis

(1) A Biological and Chemical Process Integration and Assessment Computer Program: BCPI (J.D. Ingham, Jet Propulsion Laboratory). The objective is to develop an extensive computer program (designated BCPI) that can be used to rapidly determine the energy requirements and approximate product costs for chemicals that can be produced by various biological and chemical processes. At the present time ethanol is the only chemical that is made in very large amounts by fermentation, primarily because it can be easily produced at higher product concentration and rates (relative to other fermentation products) from corn starch or other renewable substrates. The processes used are competitive with production from petroleum-derived ethylene because feedstock and processing energy requirements are much lower than for ethylene processes. Unfortunately, there are serious problems in the biocatalyzed production of other chemicals such as 2,3-butanediol, n-butanol, isopropanol, acetone, and acetic acid. Product concentrations are usually less than 30 g/l, and the rates are at least an order of magnitude lower than for ethanol. The latter can be used as a primary intermediate to make many organic chemicals, including acetaldehyde, acetic acid, acetic anhydride, many esters (e.g., ethyl acetate, acrylate or butyrate), acetamide, acetonitrile, butadiene and its derivatives, vinyl acetate, ethylene oxide, ethyl benzene, etc. Ethylene is the organic chemical intermediate produced in the largest amounts in the US, and all of its derivatives can also be derived from ethanol. Although the recent estimated cost of producing ethylene from bioprocess ethanol has been prohibitive (about twice the cost of petrochemical ethylene) any current difference could easily decrease

significantly in the future.

The main advantage of large-scale industrial production of chemicals by biocatalyzed processes is that renewable feedstocks can be used to make a chemical product, which can then be used as an intermediate to be converted to other desired chemicals. As an example, 1,3-butadiene (1,3-BDn, which is used for synthetic rubber, nylon and other plastics production) can be made from bioprocess ethanol, EtOH, or 2,3-butanediol, 2,3-BDl. In either case, the first intermediate (EtOH or 2,3-BDl) could be made by fermentation and then converted to 1,3-BDn. For some products, the second conversion to the desired chemical product could also be biocatalytic, e.g., enzymatic conversion of alcohol to aldehyde, or to an ester, but such conversions are not yet economically competitive. The complete general process is conversion of biomass to obtain dextrose sugars, followed in most cases by biocatalytic conversion to ethanol, which would be chemically converted in one or more steps to an industrial organic chemical. There are several chemical methods for converting ethanol to butadiene that may involve other intermediates, such as acetaldehyde.[57 & 58] Because of minimization of selling costs, market restraints, energy integration and other factors, resources and capital required are less when a series of conversions are combined than when the intermediates and product are made by independent processes. The program being developed will be applicable to these kinds of biochemical-chemical processes.

Program Development

There are several commercial computer-aided design

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[57]C.R. Noller. Chemistry of Organic Compounds, W.B. Saunders Co., Philadelphia, PA (1955).

[58]D.F. Rudd, S. Fathi-Afshar, A.A. Trevino, and M.A. Stadherr. Petrochemical Technology Assessment, John Wiley & Sons, New York, NY (1981).

(CAD) programs available that can be used for detailed process assessments; however, the primary purpose of these is to develop detailed process designs for specific chemical plants. When plant cost information is included, the claimed accuracy of cost estimates is low. A complete study estimate of plant costs, accurate to within 25%, can cost more than $20,000.[59] Because of the resources required, including study and computer time, and the emphasis on design, CAD programs are not always appropriate for general use when the objective is to compare process advantages. It was decided that the computer program to be developed would be based primarily on existing plants and processes. Although the program can be used as a starting point for preliminary design, its primary purpose is for energy-economic evaluations. Since the basis for each assessment is completely defined, the user can objectively judge the usefulness and reliability of the results. Also, every attempt has been made to compare the results with reasonably well established alternatives to clearly define expected advantages. Unless technological advantages strongly indicate that a proposed chemical plant would be profitable, it would probably not be financed and constructed on the basis of design estimates, regardless of the predicted cost or expected accuracy.

The technical approach is to first define a series of conversion processes. Then relevant information is entered in the program. An interactive capability is included to provide new or more complete user data and to substitute routines, which will improve, and provide greater flexibility for, assessment implementation. The phase 1 demonstration version is relatively easy to use, but the data base needed to evaluate a large number of different types of processes is not complete. It is based primarily on currently operational plants, with modifications and supplementary information introduced where appropriate. At the current stage of

[59]A. Pikulik and H.E. Diaz. *Chem. Eng.* 84:106 (1977).

development the input files are created in a word processing program. After any needed editing, one command is typed to execute the program and obtain the printed results. The displayed list of input files provides a menu for process selection and definition, but it is planned that the completed program will consist of conventional menu displays for immediate execution; it will not require word processing, or any other supplementary software.

(2) Bioreactor Kinetics and Yields for Growth-Associated Conversion of Substrate to Cells and Products: A Variable Yield-Coefficient Model (J.D. Ingham, Jet Propulsion Laboratory). When biocatalyzed processes are used to produce industrial organic chemicals, energy efficiency, economics and annual production rates and capacity will depend primarily on the kinetics of conversion. For most growth-associated biochemical conversions the yield coefficients ($Y_{p/s}$, $Y_{x/s}$) (ratios of amounts of chemical product or cells formed to total substrate converted) are nearly constant. Because cell growth and product formation are often inhibited at different rates as biotransformation proceeds, yield coefficients are not constant at the higher rates of conversion where the actual product yields and rates of formation are most important. Cell production may stop completely when the product concentration is, e.g., 90 g/l while product formation continues beyond 110 g/l. For both cell growth and product formation the rates decrease as the corresponding maximum product concentrations are approached. A variable yield-coefficient model has been developed to more satisfactorily reflect the kinetics and product yields of such bioprocesses for various types of bioreactor conditions and configurations that are applicable to large-scale production of chemicals by biocatalyzed processes.

Model Description

When inhibition caused by substrate is not the same for

cells and product, it can be described by defining two terms, sc and sp, and substituting them in the equations for cell growth and product formation that also contain factors for cell and product inhibition:[60,61, & 62]

$$sc = s+k_{sc}+s^2/k_{sc}\omega x \quad (1)$$

$$sp = s+k_{sp}+s^2/k_{sp}\omega p \quad (2)$$

$$(dx/dt)/x = \mu_{max}(1-p/pmc)^{nc}(1-x/xmc)^{ncx}(s/sc) \quad (3)$$

$$(dp/dt)/x = \nu_{max}(1-p/pmp)^{np}(1-x/xmp)^{npx}(s/sp) \quad (4)$$

Then (3) is divided by (4):

$$dx/dp = (\mu_{max}/\nu_{max})(1-p/pmc)^{nc}/(1-p/pmp)^{np}$$

$$(1-x/xmc)^{ncx}/(1-x/xmp)^{npx}(sp/sc) \quad (5)$$

Equation (5) is used to calculate the concentration of cells, x, for any concentration of product, p, with a computer by numerical integration, along with (4) to determine the time dependency. (Model computer programs will be described in more detailed publications).

For most biocatalyzed processes the product concentration is usually <50 g/l, but it has been shown[63] that modern genetic engineering techniques can be used to develop and isolate yeast strains that can tolerate alcohol concentrations >160 g/l. Productivity, pd, has the dimensions, g/l-h, and is

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[60]T.K. Ghose and R.D. Tyagi, *Biotechn. Bioeng.* 21:1387 (1979).

[61]J.M. Lee, J.F. Pollard and G.A. Coulman. *Biotechn. Bioeng.* 25:497 (1983).

[62]O. Levenspiel, *Biotechn. Bioeng.* 22:1671 (1980).

[63]T. Seki, S. Myoga, S. Limtong, S. Uedono, J. Kumnuanta, and H. Taguchi, *Biotechn. Lett.* 5:35 (1983).

numerically equal to the number of g of product that can be made in 1 L of reactor volume in 1 h. As product concentration is increased, productivity decreases because of rate inhibition by product. For example, as shown in Table 3-3 (which was compiled from excerpts of a calculation of a curve as in Figure 3-16, where the dashed lines were calculated from the model), productivity increases as the cells grow and substrate inhibition decreases, but then as product inhibition increases, the rate, dp/dt, decreases and the productivity, which is the integral of the rate up to any particular value of p, begins to decrease. It can be seen that productivity is relatively low and is about the same as for any ethanol batch process. Higher cell concentrations can be used in a continuous or fed-batch reactor to obtain productivities >30 g/l-h.

The model demonstrated in Table 3-3 would also apply to an ideal plug flow reactor without back mixing, where each time element, dt is equivalent to a corresponding increment of a cross sectional element, dz. In this case inlet cell concentration would be near zero; however, in a practical continuous reactor, cells are usually produced in a separate process and are maintained either as a flocculent or immobilized cell mass, which can be modeled as a fixed cell concentration over the length of the reactor. In fixed-bed, plate, spiral or honeycomb immobilized cell bioreactors[64, 65, & 66] the cell concentration is essentially

[64]J.F. Bourassa and A. LeDuy, *Biotechn. Bioeng.* 29:1127 (1987).

[65]A.J. Daugulis and D.E. Swaine, *Biotechn. Bioeng.* 29:639 (1987).

[66]G. Oda, H. Samejima and T. Yamada, Continuous Alcohol Fermentation Technologies Using Immobilized Yeast Cells, Biotech '83 Proceedings, International Conference on Commercial Applications and Implications of Biotechnology, Tokyo, Japan (1983).

Table 3-3. Summary of Calculated Kinetics for a Genetically-Modified Yeast*

s, g/l	p, g/l	x, g/l	t, h	dp/dt, g/l-h	(dp/DT)/x, h-1	dx/dt, g/l-h	pd, g/l-h
345.3	5	1.78	5.59	1.268	0.712	0.268	0.853
333.5	10	2.82	8.70	1.966	0.697	0.397	1.149
310.1	20	4.73	12.60	3.175	0.671	0.573	1.587
287.2	30	6.40	15.34	4.120	0.644	0.637	1.956
264.8	40	7.80	17.57	4.783	0.613	0.590	2.277
243.1	50	8.84	19.57	5.147	0.582	0.440	2.555
222.3	60	9.47	21.50	5.194	0.548	0.203	2.791
202.4	70	9.61	23.47	4.924	0.512	0	2.983
182.8	80	9.61	25.58	4.559	0.474	0	3.128
163.3	90	9.61	27.87	4.172	0.434	0	3.229
143.7	100	9.61	30.40	3.763	0.392	0	3.289
124.1	110	9.61	33.22	3.325	0.346	0	3.311
104.6	120	9.61	36.46	2.860	0.298	0	3.291
85.0	130	9.61	40.31	2.363	0.246	0	3.225
65.4	140	9.61	45.11	1.832	0.191	0	3.103
45.9	150	9.61	51.66	1.262	0.131	0	2.904
26.3	160	9.61	62.53	0.650	0.068	0	2.559
20.4	163	9.61	68.06	0.458	0.048	0	2.395

* Variable values: pmc = 67, pmp = 170, xm = 10 and px = 163, ωx,ωp = 1120, μ_{max} = 0.39, ν_{max} =1.25, k_{sc} = .22, k_{sp} = .44

constant, but will vary for a fluidized bed or flocculent microorganism in a tower reactor. The model can be easily modified if the cell concentration profile is available. For known constant x, the model

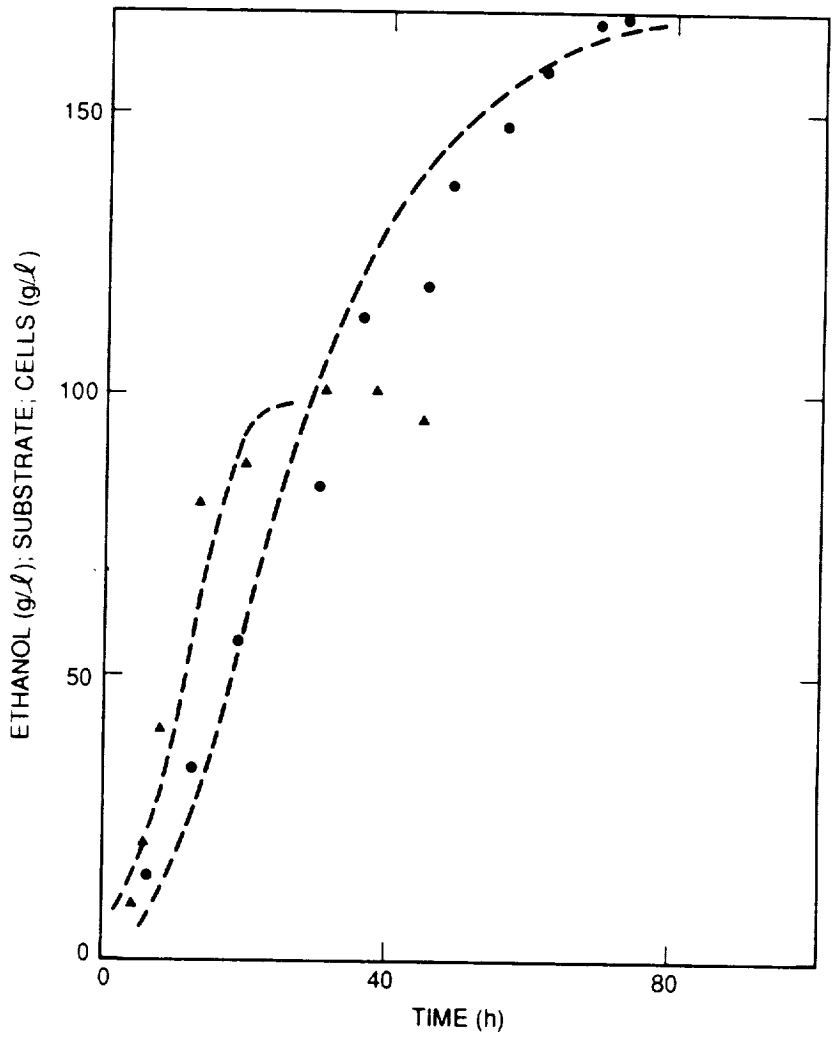

Figure 3-16. Comparison of experimental points for cells (▲) and product (●) with the kinetics model (---) for biocatalyzed production of ethanol.

calculations are as for the lower part of Table 3-3 (where x has reached its constant maximum value). For a continuous stirred tank reactor, cstr, product and other concentrations in the effluent are constant and productivity would be reflected by the constant, relatively low rate under such conditions. For example, if the effluent concentration in a cstr was 163 g/l, the productivity would not be equal to the integrated productivity (during the residence time of a

tubular or batch reactor as shown in the last column). Instead, it would equal the equilibrium rate, as 0.46 g/l-h (fifth column) instead of 2.4 g/l. Of course if the time for cleaning the batch reactor was 12 h, then 12 h has to be added to the total time for reaction to give a productivity of only 2.0 when p = 163 g/l. (Productivities shown were calculated from pd = p/t.)

Model calculations of this type are extremely useful for preliminary optimization of reactor and bioprocess design features, and to provide information needed to assess the relative advantages of bioprocess conceptual designs. The model can be employed for the critical evaluation of different bioreactor types for process synthesis and assessments, and for the evaluation and comparison of various new bioconversion concepts, including continuous, immobilized cell processes, and will be used for bioprocess assessments as discussed above. This technical effort (2) is considered to be completed. Future research will be devoted to (1), the process assessment and integration program (BCPI) and relevant program applications.

Nomenclature

p,s,x	product, substrate, cell concentration (g/l)
xm	maximum cell concentration observed or expected for specific reactor conditions (g/l)
xmc	cell concentration where cell growth stops (g/l)
xmp	cell concentration where product formation stops (g/l)
ncx	exponent in the cell growth equation for inhibition by cells
ncp	exponent in the product rate equation for inhibition by cells
px	maximum product concentration observed or expected for specific reactor conditions
pmc	product concentration where cell growth stops (g/l)
pmp	product concentration where product formation stops (g/l)
nc	exponent in the cell growth equation; inhibition by product
np	exponent in the rate equation for product; inhibition by product
$\omega x, \omega p$	substrate inhibition constants for cell growth, product
pd	productivity, mass of product formed per unit reactor volume/hour (g/l-h)
μ_{max}	rate constant or maximum specific growth rate (h^{-1})

ν_{max} rate constant or maximum specific rate of product formation (h^{-1})

k_{sc} saturation constant; substrate concentration when specific growth rate is one half the maximum (g/l)

k_{sp} saturation constant; substrate concentration when specific rate for product is one half the maximum (g/l)

(3) Conceptual Process Design: Evaluation of a Process for Conversion of Renewable Resources to Ethyl Acetate (N.K. Rohatgi and J.D. Ingham, Jet Propulsion Laboratory). The primary objective of this work is to investigate potential energy or cost advantages when fermentation ethanol (6 to 10 wt. % purity) is converted directly into ethyl acetate instead of being recovered by distillation units to obtain 93 wt. % (190 proof) ethanol. The ASPEN process simulation software was used to generate an optimized flowsheet as shown in Figure 3-17 for ethanol distillation (to produce 93 or 95 wt. % ethanol). The flowsheet for ethyl acetate production is shown in Figure 3-18. The principal accomplishments during FY'90 were:

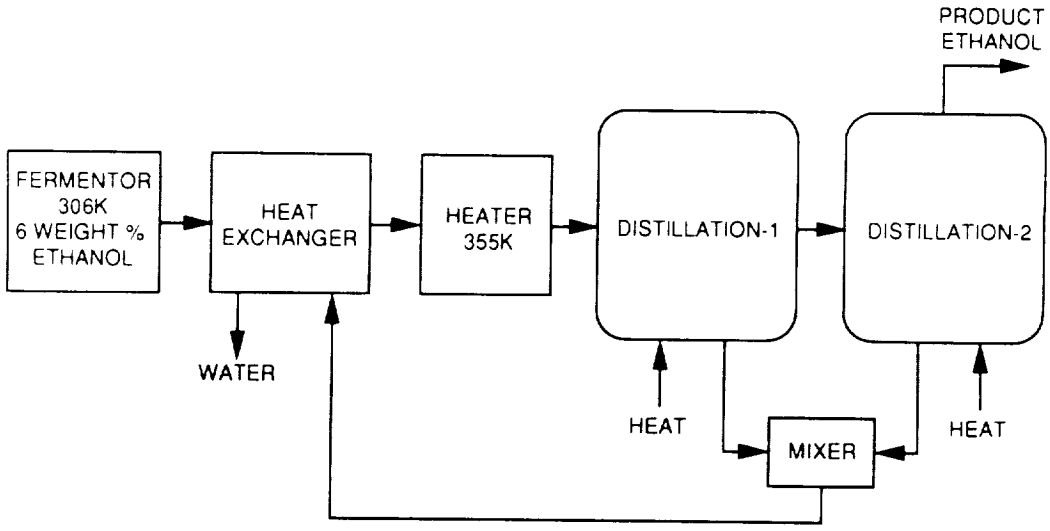

Figure 3-17. ASPEN simulated process scheme for distillation of fermented ethanol.

- Prepared input computer codes (Basic language) to generate an optimized process flowsheet shown in Figure 3-17 and also completed energy and mass

balance calculations. The energy consumption to produce 93 and 95 wt. % ethanol from 6 wt. % fermentation ethanol is 4,018 and 7,829 btu/lb of ethanol produced, respectively.

- o Prepared input computer codes for the process flowsheet shown in Figure 3-18 for ethyl acetate synthesis. The energy consumption for this process scheme is 1,881 btu/lb of ethyl acetate produced.

- o Designed most of the process equipment associated with flowsheets shown in Figures 3-17 and 3-18 for economic assessments.

- o The authors received awards for this work, which is to be published as a NASA Tech Brief.

Currently a LOTUS 123 spreadsheet is being developed to complete economic assessments for these process schemes at various production rates.

F. Management Support Function

(1) Technology Transfer (J.S. Tuan, Bernard Wolnak and Associates). Bernard Wolnak and Associates (BWA) has carried out a program to enhance awareness and promote technology transfer for ECUT sponsored research in Biocatalysis in Nonaqueous Media. In May 1990, BWA held a two and a half day international conference on "The Industrial Use of Enzymes: Technical and Economic Barriers" in Chicago, IL. A total of 74 organizations: 67 industrial firms (46 U.S., 10 Japanese, 7 European, 2 Mexican, 1 each from Canada and Australia), 2 U.S. government agencies, 5 academic institutions (2 Japanese, 3 U.S.) were represented by 110 attendees and speakers. A half-day session entitled "By-passing Barriers, Industrial Possibilities of Nonaqueous

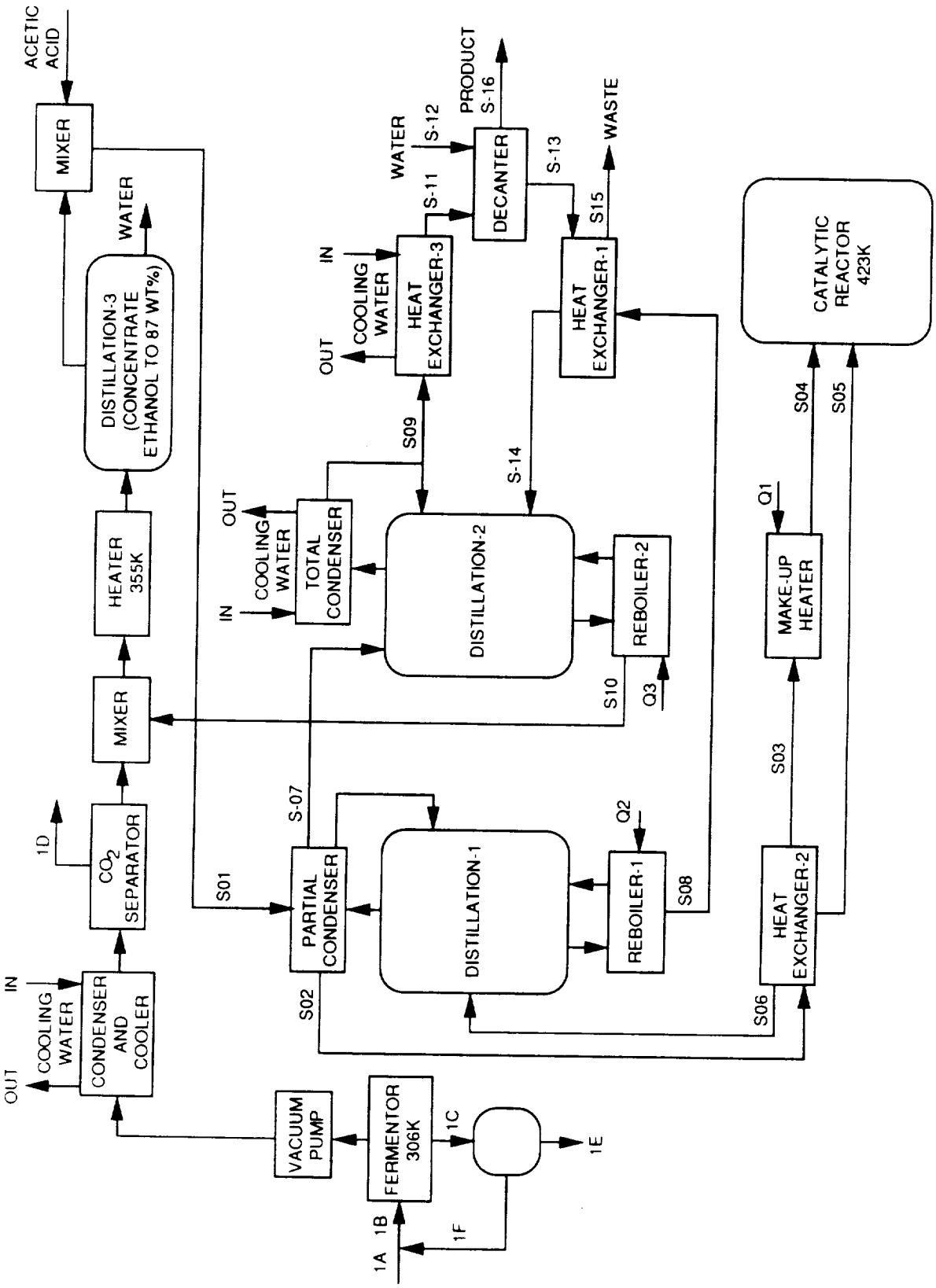

Figure 3-18. ASPEN simulated process scheme for ethyl acetate synthesis.

Solvents" was presented. This session was chaired by Dr. Gene Petersen of the Jet Propulsion Laboratory and included four speakers, two of whom were ECUT sponsored researchers. A 200 page volume of the conference proceedings will be published.

In the area of technology transfer, BWA has focused on three ECUT sponsored research programs that we believe have near term potentials for industrial applications. In addition to visiting over 12 companies in person, direct contacts were made to over 220 individuals at more than 125 firms in the United States with a letter briefly describing the scope of the research programs and DOE's intent to promote technology transfer. These included firms in the diagnostic, pharmaceutical, chemical, energy, separation, and enzyme industries. Fourteen companies responded positively to the letter and expressed an interest in one or more of these programs. These companies include 3M, Abbott Laboratories, Amano International Enzyme Co., Beckman Instruments, BioDesign Inc.*, BioProbe International Inc.*, Catalytica*, Ensys Inc., Genex, Mallinckrodt Specialty Chemical Co., Pharmacia LKB Biotechnology*, Rohm & Haas, Sepragen*, Terrapin Technologies. BWA then prepared, in consultation with the three ECUT sponsored Principal Investigators, descriptive program summaries of their research projects for distribution to interested parties. Five firms (* firms above) indicated that they may be interested in an organized information exchange such as a symposium to discuss details of these research programs. BWA will continue to facilitate these kinds of interactions and discussions and assist in the establishment of direct dialogues between the companies and the Principal Investigators.

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SECTION IV

PATENTS

Patents Based on Research Supported by Biocatalysis,

A. Dr. M.L. Shuler

Use of Inhibitory Solvents in Multi-Membrane Bioreactor (T. Cho and M.L. Shuler, Assigned to Cornell Research Foundation, Inc.), U.S. Patent 4940547 (July 10, 1990).

Apparatus and Process to Eliminate Diffusional Limitations in a Membrane Biological Reactor by Pressure Cycling (G.S. Efthymiou and M.L. Shuler; Assigned to Cornell Research Foundation, Inc.), U.S. Patent 4861483 (August 29, 1989).

B. C.D. Scctt

Production Techniques for High Productivity Biocatalyst Beads. DOE Docket #S-62,550 (January 1985). Applied for C-I-P extension.

Biocatalyst Beads with Incorporated Adsorbent. DOE Docket #S-63,668 (September 1985).

Advanced System for Production of Biocatalyst Beads. DOE Docket #S-63,677 (February 1986).

Biosorption Beads for Removal of Dissolved Metals from Aqueous Streams. DOE Docket #S-64,986 (October 1986).

Gel Bead Composition for Metal Adsorption. DOE Docket #S-68,102 (February 1989).

A New Gel Material for Bioreagent Immobilization. (submitted March 1988).

A Biparticle Fluidized-Bed Reactor (submitted April 1989).

C. C.J. King

Regeneration of carboxylic acid extracts by selective removal of co-extracted water with concomitant precipitation of the acid (submitted 1990).

D. D.N. Beratan

Molecular Implementation of a Molecular Shift Register based on Electron Transfer, NASA Tech Briefs 14, 55 (1990); patent filed 8/89 by NASA.

All Optical Photochromic Spatial Light Modulators based on Photoinduced Electron Transfer in Rigid Matrices, NASA Tech Briefs 13, 44 (1989); patent filed by NASA.

A FORTRAN program to determine electron tunneling pathways in proteins, submitted to COSMIC, the NASA software center 12/89.

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TECHNICAL REPORT STANDARD TITLE PAGE

1. Report No. 91-4	2. Government Accession No.	3. Recipient's Catalog No.
4. Title and Subtitle AICD Advanced Industrial Concepts Division Catalysis and Biocatalysis Program Annual Report FY 1990		5. Report Date March 1991 6. Performing Organization Code
7. Author(s)		8. Performing Organization Report No.
9. Performing Organization Name and Address JET PROPULSION LABORATORY California Institute of Technology 4800 Oak Grove Drive Pasadena, California 91109		10. Work Unit No. 11. Contract or Grant No. NAS7-918 13. Type of Report and Period Covered JPL Publication
12. Sponsoring Agency Name and Address NATIONAL AERONAUTICS AND SPACE ADMINISTRATION Washington, D.C. 20546		14. Sponsoring Agency Code RE 152 PL-778-19-25-03-07

15. Supplementary Notes

16. Abstract

The Annual Report presents the fiscal year (FY) 1990 research activities and accomplishments for the Catalysis and Biocatalysis Program of the Advanced Industrial Concepts Division (AICD), Office of Industrial Technologies of the Department of Energy (DOE). The Catalysis and Biocatalysis Program was formerly under the Division of Energy Conversion and Utilization Technologies (ECUT) until the DOE reorganization in April 1990. The mission of the AICD is to create a balanced program of high-risk, long-term, directed interdisciplinary research and development that will improve energy efficiency and enhance fuel flexibility in the industrial sector. Under AICD, the DOE Catalysis and Biocatalysis Program sponsors research and development in furthering industrial biotechnology applications and promotes the integrated participation of universities, industrial companies and government research laboratories.

17. Key Words (Selected by Author(s))	18. Distribution Statement
Biological and Medical Sciences (General) Chemistry (General) Energy (General) Materials (General)	Unclassified; unlimited

19. Security Classif. (of this report)	20. Security Classif. (of this page)	21. No. of Pages	22. Price
Unclassified	Unclassified	164	

JPL 0184 R 9/83