The Biological and Chemical Technologies Research Program is managed under the direction of Department of Energy Headquarters Program Management with technical support from the National Renewable Energy Laboratory through an agreement (Field Work Proposal EEWIP0B-93) between the National Renewable Energy Laboratory and the Department of Energy.

The Biological and Chemical Technologies Research Program focuses on resolving the major technical barriers that impede the potential use of biologically-facilitated continuous chemical production processes and that restrict the design of new chemical catalysts.

ABSTRACT

The annual summary report presents the fiscal year (FY) 1993 research activities and accomplishments for the United States Department of Energy (DOE) Biological and Chemical Technologies Research (BCTR) Program of the Advanced Industrial Concepts Division (AICD). This AICD program resides within the Office of Industrial Technologies (OIT) of the Office of Energy Efficiency and Renewable Energy (EE). The annual summary report for 1993 (ASR 93) contains the following: A program description (including BCTR program mission statement, historical background, relevance, goals and objectives), program structure and organization, selected technical and programmatic highlights for 1993, detailed descriptions of individual projects, a listing of program output, including a bibliography of published work, patents, and awards arising from work supported by BCTR.

FOREWORD

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1.0 INTRODUCTION

This document contains a summary of the projects undertaken by the Biological and Chemical Technologies Research (BCTR) Program in 1993. The BCTR program is a program within the U.S. Department of Energy's (DOE) Advanced Industrial Concepts (AICD) Division within the Office of Industrial Technologies (OIT).

The mission of the DOE BCTR Program is **to provide evidence of the technical and economic feasibility of advanced chemical and biological concepts that can improve the energy utilization, operation efficiency, and environmental soundness of U.S. industry process operations**. The program combines **science with technology** to develop novel and revolutionary process concepts, to improve conventional process approaches, and, if the economics and market needs warrant, to facilitate the **introduction** of these technological advances in the chemicals, petroleum refining, pulp and paper, other renewable, and emerging biotechnological industries.

Together, these advanced technologies foster U.S. competitiveness through new product and market expansion, cost-effective and environmentally safe processes, reliable domestic energy and feedstock supplies, and lower dependence on foreign energy sources. The use of renewables for fuels, chemicals, and electric power also supports the National Energy Strategy (NES). The BCTR program serves a dual role in meeting the needs of industry and other DOE programs and channeling technology into these two areas.

The overall goal of the BCTR program is to stimulate and nurture the development, testing, and commercial deployment of advanced chemical and biological technologies. The achievement of this goal is gauged by the primary energy use that could potentially be impacted by the successful commercial deployment of BCTR sponsored innovations and which is estimated to be 2.4 to 3.5 eJ in savings for petroleum displacement by 2030.

Toward this goal, the program objectives are to:

1. Pursue the development of technologies aimed at overcoming the limitations of the use of indigenous resources in the chemicals industry

2. Facilitate the introduction of biotechnology into the chemicals industry

3. Investigate innovative chemical and biological process routes that offer the potential to reduce energy use and mitigate environmental impact within the chemicals and petroleum refining industries.

4. Communicate the results of the program research efforts to OIT end-use programs and/or the industrial sector for continued development towards commercial deployment.

Meeting program goals and objectives involves two strategies and is based on the energy-supply/demand issues of process operations. Strategy 1 - **Boost Energy Supply** by replacing petroleum feedstocks with a) indigenous resources, and b) waste material from the industrial, commercial, utility, and residential sectors. Strategy 2 - **Cut Energy Demand** by developing and activating technologies that reduce energy demands for chemicals and materials processing.

The Program pursues two basic approaches in implementing its program objectives. First, it builds upon the findings of basic science to enhance technology applications within industry and end-use programs. Second, it applies BCTR developed technology in response to the R&D needs of industry and other OIT programs. These approaches are displayed in in Exhibit 1.1.

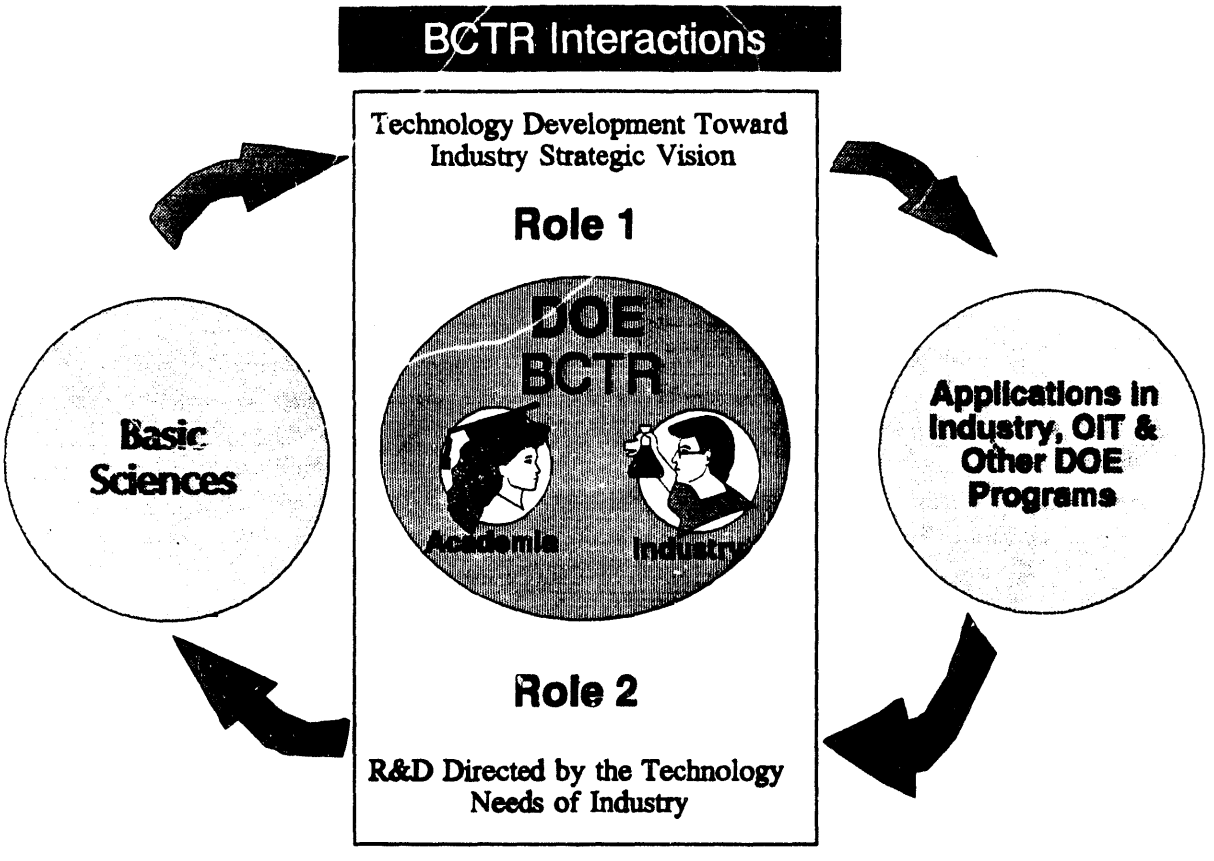

Exhibit 1.1

These two approaches are embodied in a program structure containing elements that address the industry needs and vision of the future (see section 2.0). The five elements are described below including programmatic objectives and goals.

Computer Aided Catalyst Design (CACD) **Objective:** Create tools to aid in the design of chemical and biological catalysts for chemicals and materials production. **Sub-activities:** Chemical CACD and Biological CACD. **Goals:** Chemical and Biological CACD sub-model applications by 1996 and model applications by 2008.

Advanced Bioprocess Systems (ABS) **Objective:** Develop and integrate biological processes and schemes into chemicals and allied products industries. **Sub-activities:** Bioprocesses for commodity chemicals and integrating bioprocesses for chemicals processing. **Goals:** Novel aqueous bioreactor system in commercial operation by 1996; other aqueous and organic-phase bioprocesses in operation by 2005.

Feedstocks/Process Interaction (FPI) **Objective:** Develop base technology for biological and/or chemical approaches to expand renewable and recoverable materials use in conventional process operations. **Sub-activities:** Renewables/Lignocellulosics and Recoverables/Inorganics. **Goal:** Commercial use of renewables, including CO_2, as feedstocks for chemicals production by 2015.

Novel Process Development (NPD) **Objective:** Exploratory screening of novel biological and chemical processes and syntheses developed with support from Basic Energy Sciences, National Science Foundation, and industry for the evaluation of energy efficiency, productivity, and environmental soundness in industrial process operations. **Goal:** Transfer three novel methods to EE end-users and industry by 2015 for continued engineering development.

Process Control and Systems Analysis (PCSA) **Objective:** Develop process models and simulation capabilities that researchers can use to guide the engineering development of industry process systems. **Goals:** Industrial applications of a process simulation model by 1997; a process control(s) for advanced systems by 2010.

BCTR projects are specifically designed to evaluate high risk opportunities for industrial applications that lead to investment grade technology. BCTR projects stop short of developing technology that is meant to provide immediate commercial deployment by industry. Also, BCTR projects are not the high risk, broad reward efforts typically found in Basic Energy Sciences.

The program has a number of significant achievements through 13-plus years of exploratory research and development which include:

- **Computer Aided Design of Catalysts**

  Monsanto, General Motors and DuPont are using catalyst design tools developed at Pacific Northwest Laboratories and Ames to characterize the surface properties of catalysts and for determining the chemical composition of multimetallic automobile catalysts.

  Catalytic antibody technology, partly developed under BCTR support, is being employed at Affymax, a small biotechnology firm specializing in pharmaceutical and specialty chemical applications, to investigate chemical catalysis opportunities.

- **Advanced Bioprocessing Systems**

  The DOE Alcohol Fuels and Alternative Feedstocks programs are employing bioreactor systems developed at Oak Ridge National Laboratory that provide continuous operation with high productivities and yields in process scale-up for ethanol and succinic acid production, respectively.

  Allied-Signal Corp. is employing enzyme technology developed at Allied-Signal and MIT for the production of unique chemicals using immobilized enzyme systems operating in organic solvents.

- **Novel Processing Systems Development**

  A novel bioprocessing system to extract phosphate from ores, developed at the Idaho National Engineering Laboratory, is being scaled-up to pilot-plant operation in collaboration with J.R. Simplot, Inc.

  Advanced, low-energy complexation/separation technologies for isolating dilute organic acids produced by fermentation developed at Lawrence Berkeley Laboratory are being employed in the commercial production of polymer precursors by a major agriprocessor.

  The ability to improve aerobic fermentations for more efficient productivity and energy use by engineering microorganisms has been demonstrated and is being commercialized by a spin-off small business, Exogene, which started as a direct result of BCTR funding.

- **Industry/Academic/Government Research Center**

  A state-of-the-art, hands-on research center that promotes and facilitates government-industry-university interaction and collaboration on computer-aided catalyst design was established at the California Institute of Technology in 1991 with DOE, the Department of Defense (DOD), and industry funding. It currently operates with 8-10 industrial partners who are able to evaluate and develop modeling tools for the study of their own catalysis and biocatalysis problems.

The **economic benefits** from BCTR supported projects has most directly been evidenced by the formation of small businesses. The following is a list of the five small businesses that have begun partly or wholly as a result of DOE BCTR funding. They continue to operate today, employ over 400 individuals, and return over $60 M to the economy in salaries and benefits.

Molecular Simulations, Inc. Integrated modeling software company,
Exogene - R&D in chemicals and pharmaceuticals,
Affymax - Biotechnology and pharmaceuticals R&D,
Alkermes, Inc. - Value-added products for pharmaceutical and food industries, and
Optifood Ingredients,Inc. - R&D in the food processing industry.

Industrial Involvement and Impacts

It is noteworthy that the program has an industrial constituency of 34 industrial partners. At least 20 other potential industrial partners have been identified by program participants. A compilation of industrial contributions showed that for the year 1993 industry provided matching resources, both contractual and non-contractual that lead to completion of work, that amounted to **67%** of the total BCTR program budget (**$3,384K industrial cost-share** and $5,000K in DOE resources). Additionally, a total of **52.8** full-time equivalent personnel were employed directly as a result of BCTR funds and industry employed about **29** individuals who supported BCTR related projects.

Program output - Publications, Patents and Awards

Publications and patents are one of the major means of communicating this technology to industry. For the ASR 93 reporting period, program participants continued to publish many noteworthy articles and papers in the scientific literature. Eighty-two (82) peer reviewed articles, chapters in books, DOE reports, etc. were published. As a measure of the technology transfer occurring in the program, one patent was filed by program participants in FY93 (note that eight were filed in FY92). Several program participants received individual awards and honors. **Dr. Alex Klibanov** was elected to the prestigious National Academy of Engineering for "Research in Enzyme and Protein Technology and Contributions to the Field of Biocatalysis in Nonaqueous Solvents." **Dr. F. H. Arnold** presented the first Biennial Lilly Biocatalysis Symposium in Indianapolis. **Dr. Alex Klibanov** was honored by the 1993 Arthur C. Cope Scholar Award from the American Chemical Society. The work of **Dr. A. Hess and M. Thompson** and co-workers of Pacific Northwest Laboratory was featured on the cover and in an article of the Chemical Design Automation News. **Dr. C. Judson King** was the first recipient of the Clarence G. Gerhold Award in Separations Technology presented by the American Institute of Chemical Engineers. **Dr. King** also received a Centennial Medallion (one of 200 given) from the American Society for Engineering Education.

2.0 BIOLOGICAL AND CHEMICAL TECHNOLOGIES RESEARCH PROGRAM

2.1 Introduction

The Department of Energy has five core businesses of which the BCTR program directly supports two: Supporting industrial competitiveness via science and technology, and Promoting diverse energy supply and demand through technology development and deployment. The BCTR program mission, goals, objectives, and strategies support DOE core business priorities by managing science and technology for results through the interaction and use of DOE's organizational systems.

2.2 Program Description

The Advanced Industrial Concepts Division (AICD) Biological and Chemical Technologies Research (BCTR) Program evolved from the Energy Conversion and Utilization Technologies (ECUT) projects/programs in the Chemical Processes, Catalysis and Biocatalysis areas which originated in 1981. In 1983 only the biocatalysis component was continued as the Biocatalysis Research Activity. From 1984-1993 the BCTR Program (and its predecessors) has been funded through a budget line item. In 1990, with an increased interest in novel chemical processing, the program was renamed Catalysis/Biocatalysis. In April of 1990, a reorganization of the Office of Conservation and Renewable Energy moved this program to the AICD under the Office of Industrial Technologies and the Office of Industrial Processes (OIP). The program is now titled Biological and Chemical Technologies Research and is in the Energy Efficiency and Renewable Energy Office. The operational model for the BCTR program is depicted in Exhibit 2.1

This operational model allows BCTR to evaluate the industrial applicability of fundamental findings and to respond to DOE end-use programs and industry needs that can be filled with existing or previous BCTR developed technology. BCTR technology development falls short of large-scale pilot plant operations but is intended to demonstrate the applicability of technologies for industrial uses. Often, some fundamental research is required in the course of demonstrating proofs of concept, but the general scope of BCTR work is not fundamental research.

2.3 Industry Drivers/Vision and BCTR Response

The chemical, by-products, and petroleum industries will continue to influence U.S. energy, economy and environmental matters in the 21st Century. The renewables industry is expected to increase its role in the U.S. economy, initially as a response to environmental requirements, but ultimately as a reliable feedstock serving a diverse energy market. Exhibit 2.1 displays the current drivers of these three industries and the vision they are forming for the future.

Exhibit 2.1 Industry technology drivers and vision for three related industries

Industry	Technology Drivers	Technology Visions
Petroleum Refining	Emissions regulations and changing product specifications Lower quality crude oils containing higher impurities (S, gravity, etc.) Natural gas use	Become environmentally benign Enlarge heavy crude conversion technologies Use syngas and convert natural gas to liquid products
Chemicals	Emission regulations and reducing wastes New products for market expansions Produce commodities near source Maintain competitiveness with current products	No waste processes or minimized wastes from processes Environmentally friendly biotechnological products High value-added products through bioprocessing Processes that offer feedstock flexibility
Renewables	Effective land use - eliminate farm subsidies Higher profit margins Stabilize rural America	Use the 40-60 M acres of set-aside land for productive farming Increase level of biobased products in the market Site biobased products near sources Employ novel biorefinery/bioprocessing concepts

Economic/market competitiveness: The following are examples of how economics and market forces are changing the face of these three industries.

- The value of products derived from catalysts is about 17% of the U.S. gross national product (GNP), but the development of catalysts is highly empirical and expensive. Catalyst R&D is losing favor in the U.S. while growing in importance in Europe.

- Although the U.S. chemical processing industry has an enviable record in world trade, exports of commodity chemicals is decreasing as Asian nations and other third world countries are building their own capacity. This moves jobs off U.S. shores. Forces affecting the market include dwindling oil supply, poor quality feedstocks, exchange rate shifts, political upheavals, and environmental controls. Competitiveness in United States industries will require the capability to be flexible in feedstock use and product swing.

- The North American Free Trade Agreement will solidify the strong export market to Canada and Mexico and help maintain commodity chemicals production. Overall, the exports of large volume and large dollar chemical categories, organics and synthetic resins, are showing slow growth, while low-volume, low-dollar chemical categories are showing larger growth. This trend is expected to continue as other nations build their own capacity, especially in Asia.

Energy: These industries are major energy users. Improved process efficiency will become important as energy costs rise. Using alternative feedstocks lowers the need for petroleum imports and may be less energy demanding.

Environment: Environmental laws and more stringent solid, liquid, and air emission controls mandate industry to reduce wastes, use dilute feedstock streams and combustion gases, and to develop novel processes to effect zero-discharge processes. Capital investments for environmental compliance or divestiture of processes will continue into the next decade. This will be followed by a life extension program to maintain the capital investments and the search for "clean processes." Ultimately, new processes will be required to meet public demands for environmentally clean and energy efficient processes.

The research activities within BCTR apply across much of the industrial sector. However, the focus of the program is toward the chemical and allied products (SIC 28) and petroleum refining (SIC 29) industries. Exhibit 2.1 gives the broad vision needs of the three constituent industries that the BCTR program seeks to support. In Exhibit 2.2 additional items are added that show the technical areas that the BCTR program addresses and that meet the needs of the industry vision. These are noted in bold type.

Exhibit 2.2 BCTR response to industry technology drivers and vision for three related industries

Industry	Industry Technology Visions	BCTR Technology Meets Industry Vision
Petroleum Refining	Become environmentally benign Enlarge heavy crude conversion technologies Use syngas and convert natural gas to liquid products	**Highly specific catalysts** **More robust catalysts** **New catalysts and processes**
Chemicals	No waste processes or minimized wastes from processes Environmentally friendly biotech products Processes that offer feedstock flexibility	**High specificity catalysts** **Value-added products through bioprocessing** **Integration of bioprocessing into typical chemical processes**
Renewables	Use the 40-60 M acres of set-aside land for productive farming Increase level of biobased products in the market Site biobased products near sources Employ novel biorefinery/bioprocessing concepts	**Helping alternative feedstocks become part of agricultural product slate** **Develop novel processes that employ biomass** **Biomass refineries or Renewable Energy Parks** **R&D in chemical and biological processes**

The program structure described in section 1.0 of this report is designed to organize the efforts of the BCTR program in response to the needs of industry. The following displays the structure in diagram form including the subactivities, where applicable.

PROGRAM ELEMENTS

Computer-Aided Catalyst Design	Advanced Bioprocess Systems	Feedstock/Process Interactions
Chemical CACD	Bioprocessing for commodity chemicals	Renewables/Lignocellulosics
Biological CACD	Chemical industry integration	Recoverables and Inorganics

Novel Process Development	Process Control/Systems Analysis

2.4 BCTR projects in FY93

Listed below are the specific projects undertaken in 1993 which support each of the program elements.

Computer-Aided Catalysis Design (CACD)

Chemical CACD

Periodic *Ab initio* Hartree-Fock theory/site isolation in selective alkane oxidations *(PNL)*

Design characteristics and testing of multimetallic catalysts for pollution control *(AMES)*

Theoretical studies of hydrocarbon catalysis on zeolites *(LANL)*

Theory-assisted design of metal and zeolite catalysts *(LBL)*

Biological CACD

Genetic engineering of methanogenic bacteria for industrial/chemical applications *(JPL)*

Chemistry, immunology, and modeling as tools for the rational design of enzymes *(LBL)*

Theory of biocatalysis-electron transfer reactions *(U. Pittsburgh)*

Predictive models and effects of structure on catalytic properties and materials and molecular simulation center *(CIT)*

Mimetics

Computer aided molecular design of biomimetic CO_2 activation catalysts *(SNLA)*

Advanced Bioprocessing Systems

Bioprocessing for Commodity Chemicals

Engineering enzymes for stability and activity in organic solvents *(CIT)*

Bioprocess engineering: Immobilized cell systems for continuous, efficient biocatalyzed processes *(ORNL)*

Integration into Chemicals Industry

Designing and improving enzymes for use in organic solvents *(MIT)*

Immobilized enzymes in organic solvents *(Allied-Signal)*

Biological separation of phosphate from ores *(INEL)*

Feedstock/Process Interactions

The carbon dioxide bioreactor: Conversion of industrial waste carbon dioxide into polyhydroxybutyrate (PHB) *(JPL)*

Photobiological conversion of syngas into microbial polyester *(NREL)*

CO_2 photoreceptor research *(ORNL)*

Levogucosan polymers *(NREL)*

Clean fractionation process *(NREL)*

Novel Process Development

Overproduction and enhanced secretion of enzymes (polyphenol oxidase) *(Howard University and Clark-Atlanta University)*

Separations by reversible chemical association *(LBL)*

Metabolic engineering *(CIT)*

Electroreduction of lipids *(Tulane)*

Product recovery in supercritcal solvents *(U. Hawaii)*

Process Control/Systems Analysis

Bioengineering Simulation Technology (BEST) development *(NREL)*

A Biological and Chemical Process Integration and Assessment Computer Model: (BCP1) *(JPL)*

Program support to BCTR computer aided catalyst design program *(PNL)*

BCTR program planning and support *(PNL)*

3.0 SELECTED HIGHLIGHTS FOR FY 1993

These highlights are abbreviated descriptions of noteworthy efforts within the BCTR program. They are not listed in any prioritized order and the absence of a project description is not intended to imply that work was less important than any other.

3.1 Lawrence Berkeley Laboratory

Separation technology applied in industry; theoretical modelling applied to industrial needs; unique chemical transformation performed by biocatalyst

Chemical complexation technology that has been under evaluation for several years at LBL is being applied to proprietary isolation processes in the production of organic acids by Cargill, a large agriprocessor. The process allows for simple separation and recovery from the process stream, which eliminates the typical energy-intensive water evaporation step. The product can also be easily removed and recovered for concentration and purification. The projected energy savings of this approach, if fully deployed in applicable markets (such as citric acid, lactic acid, etc.), is estimated at 0.016 quad/year.

The validation of theoretical models for various aspects of the acid sites for the commercially important catalyst, ZSM-5 has been shown: 1) Acid sites based on a 34 atom cluster embedded in the electrostatic field were modelled and predictions of bonded geometry within this cluster were in good agreement with available evidence from solid state NMR and IR spectroscopy; and 2) The two molecular dynamic techniques developed for predicting transport diffusivities in zeolites provided predictions that were in good agreement with experimental evidence.

The remarkable specificity of an antibody has been used to accomplish highly selective functional group transformations not readily attainable by current chemical methods. An antibody raised against N-oxide hapten catalyzes the regiospecific reduction of a diketone to a hydroxyketone with greater than 75:1 selectivity for one of two nearly equivalent ketone moities. Moreover, the antibody-catalyzed reduction is highly stereospecific, affording the hydroxyketone in high enantiomeric excess. The simple strategy presented herein may find general applicability to the regio- and stereoselective production of a broad range of compounds.

3.2 Idaho National Engineering Laboratory

CRADA work begun in scaling of laboratory processes

J.R. Simplot, Inc. and Idaho National Engineering Laboratory are teaming up to further evaluate and the biosolubilization of phosphate ores work that has been ongoing at INEL for the past few years under a CRADA arrangement. The desired end-point will be sufficient data to allow scaling of the process for evaluation of commercial feasibility.

3.3 Sandia National Laboratory, Albuquerque

Synthesis of advanced catalyst designs completed and testing was begun; CO_2 binding studies completed; structural characterization and validation of CAMD methods verified

A series of fluorinated derivatives of cobalt dodecaphenylporphyrin (CoDPP) catalysts have been synthesized will be tested using a newly designed and built electrochemical catalyst testing and characterization apparatus. NMR studies of substrate binding to the octa*alkyl*-tetraphenylporphyrin (OATPP) catalysts, Fe(III)OmeTTP and Fe(III)OEtTPP-F_{20}, have been completed. Binding energies are low and will require the design of improved catalysts. Predicted catalyst structures were verified by X-ray crystallography, resonance Raman spectroscopy, and NMR spectroscopic studies. New collaborations with W. Goddard of Caltech will allow improvements in treatment of metals.

3.4 University of Pittsburgh

Enzyme biocatalyst electron transfer reaction models refined

Progress towards computer-aided design of enzyme biocatalysts has continued steadily. Accomplishments in 1993 include: 1) Development of software to compute the role of multiple coupling pathways in mediating electron transfer in proteins: 2) development of numerical and graphical software for performing reduction of complex chemical structures to represent their "essential" connectivity with respect to electronic coupling mediation; 3) determination of the mechanism and base pair dependence of electron coupling in DNA bridged donor-acceptor systems; and 4) introduction of the new concept of tunneling pathway phase transitions in disordered materials and investigation of their importance in the design of organic polymer based electron transfer systems.

3.5 California Institute of Technology

New tools developed for atomic scale modeling and simulation of biological systems; Protein engineering renders enzymes highly stable and allows synthesis of unnatural chiral polymers

Protein stitchery for biochemical synthesis and design. A strategy for designing proteins to recognize particular base-pair sequences of DNA was developed and verified experimentally for a 10 base pair (bp) site a 16 bp site. This is the first time a regulatory protein has been successfully designed. *Molecular dynamics perturbation theory (MD/PT).* Using MD/PT, the free energy cost in having the wrong base pair at the active site in DNA for the protein netropsin was shown. This is the first time that such a quantity has been accurately determined, theoretically or experimentally. *Patent filed.* A patent was filed on the new Cell Multipole Method (CMM) technology developed with partial support from DOE-AICD. This technology allows atomistic simulation on 1 million particle systems (such as a complete rhinovirus).

Enzyme Stabilization. Use of a substitution-inert metal, ruthenium, forms essentially covalent crosslinks in proteins with engineered chelating sites leading to an exceptionally large degree of stabilization. Cytochrome c's melting point was raised by 23°C and conformational stability was increased by 5.5 kcal mol^{-1} (vvG). *Enzyme activity increased.* A variant of subtilisin was isolated

that hydrolyzes a peptide substrate up to 256 times more efficiently than wild type subtilisn over a wide range of concentrations of a polar organic solvent, dimethylformamide (DMF). This variants and others can also catalyze the ligation of several nonnatural amino acids such as pentenyl glycine, into chiral polymers.

3.6 Pacific Northwest Laboratories

VPO catalysts better defined, modeling work highlighted in national publications, software being tested

Theoretical model of CPO catalyst. Progress has been made in experimental and theoretical work involving vanadyl pyrophosphate (VPO) catalysts. Solid-state structural models developed by PNL have clarified a long standing structural controversy for VPO catalysts and a hypothesis for the structure for the active site has been developed (validation is being conducted by their industrial partner, Monsanto). *Article selected.* One of the studies on solid state catalysts titled "An Examination of the Electrostatic Potential in Silicalite using Periodic Hartree-Fock Theory" was selected by the Journal of Physical Chemistry to be featured in the journal's first color issue. *Software under valuation.* The graphical interface and crystallography codes are currently in the ß-release mode at 20 test sites throughout the United States and initial feedback has been very positive.

3.7 Ames Laboratory

General Motors and DuPont using bimetallic catalyst model developed at Ames Laboratory.

Researchers at Ames Laboratory have developed a model (named "DeCal") that has the capability to predict the surface chemical composition of bimetallic catalysts from bulk compositions. The model was developed from catalyst systems composed of platinum-group metals and has been shown to explain the performance of the various catalyst compositions. The model is now being used by General Motors, and is under evaluation at DuPont in collaboration with the Ames researchers.

3.8 Los Alamos National Laboratory

BCTR program highlighted at North American Catalysis Society Meeting, modeling work progresses

Los Alamos National Laboratory and the BCTR program managers hosted an open house at the semi-annual meeting of the North American Catalysis Society Meeting in Pittsburgh. Several BCTR participants in the Computer Aided Catalysis Design program subelement gave presentations. A new and novel technique for carrying out molecular dynamics simulations using quantum mechanical forces was developed to study chemical reactions including hydrocarbon cracking reactions in acid zeolites.

3.9 Oak Ridge National Laboratory

Biparticle, Fluidized Bed Reactor improved; gas phase reactor built and operated; joint efforts with the DOE Office of Transportation Technologies begun; national meeting supported by ORNL

Biparticle, Fluidized Bed Reactor improved. Specific applications of the advanced bioreactor system, the biparticle, fluidized bed reactor (FBR) have been demonstrated including production of lactic acid, acetic acid, and butanol. These demonstrations included advanced separation techniques using both solid and liquid adsorbents. *Gas phase reactor built and operated.* A columnar, liquid-continuous gas-phase bioreactor for the conversion of the gaseous substrates pentane and isobutane has been continuously operated for over 20 months. Conversion rates will need to be improved for commercial application. *Joint efforts with the DOE Office of Transportation Technologies begun.* Based on laboratory- and bench-scale results for ethanol production that show 10-fold productivity increases and near theoretical maximum yields, the Office of Transportation Technologies, through the National Renewables Energy Laboratory, has entered into a joint research effort with BCTR & Oak Ridge National Laboratory for continued engineering development of the bioreactors. The collaboration will cost-share the engineering scale-up and model development efforts of the Oak Ridge fluidized bed bioreactor for ethanol production toward field demonstration and commercial deployment. *National meeting supported by ORNL.* Oak Ridge National Laboratory was a cosponsor of the 15th Symposium on Biotechnology for Fuels and Chemicals held in Colorado Springs in May, 1993.

3.10 Massachusetts Institute of Technology

Selection of organic solvents for maximal enzymatic activity defined and lyoprotectant mechanism better defined

Research has established, contrary to suggestions in the literature, that a solvent's immiscibility with water and apolarity by themselves are not relevant to enzymatic activity in the solvent and that a solvent's hydrophobicity is the key pertinent parameter. In addition, the chemoselectivity of enzymes is strongly dependent upon the solvent. The mechanism by which lyoprotectants (non-reducing sugars) enhance enzymatic performance in organic solvents appears to be based on the ability of lyoprotectants to alleviate reversible denaturation of enzymes upon lyophilization.

3.11 Jet Propulsion Laboratory

Analysis of feedstock and process energy of top 50 chemicals begun

As part of an overall strategy to focus BCTR research efforts on industry relevant problems, an analysis of the process energy and feedstock energy associated with the production of top 50 chemicals produced in the United States was compiled by Jet Propulsion Laboratory (JPL). This will form the basis for a systems level study of potential applications of BCTR derived technology and provide better guidance for applying research technology to problems that will give the greatest impact for reducing energy usage by the chemicals and petroleum refining industry. The systems level study will be carried out in 1994 by JPL, Pacific Northwest Laboratories, National Renewable Energy Laboratory, Los Alamos National Laboratory, and DOE HQ personnel.

3.12 BCTR industrial panel convened.

In conjunction with the North American Catalysis Society Meeting held in Pittsburgh, the guidance and evaluation panel met again to review progress from the November 1992 meeting and to attend the principal investigator review for CACD work. Progress on recommendations were made and the panel was charged with providing reviews of the technical merit of the CACD work. The panel also reviewed the energy audit of the top 50 chemicals and provided further guidance on use of this information.

4.0 FY 1993 TECHNICAL PROJECT DESCRIPTIONS

The following includes detailed descriptions of the FY 1993 projects. The projects have been categorized according to the five program elements discussed in Section 2.5. Each project summary contains a brief description of the effort including the names of the investigators and the site of the research, a summary of the major 1993 accomplishments, a description (where applicable) of the anticipated 1994 efforts, and a brief discussion about industrial involvement and motivation. A more in-depth discussion of 1993 research results is also included.

4.1 Computer Aided Catalyst Design

4.1.1 Chemical

1. **Project Title:** Periodic *Ab Initio* Hartree-Fock Theory/Site Isolation in Selective Alkane Oxidation.

Principal Investigators: A. C. Hess and M. R. Thompson

Project Site: Pacific Northwest Laboratory

Description:

The development of experimental and theoretical tools to study heterogeneous catalysis has been the focus of this effort. Research focuses on the development of a novel solid-state formalism, its application to zeolites and clays, and experimental validation of molecular-level processes in commercial catalytic reactions. For the study of the molecular-level aspects of homogeneous catalysis, there exists a relatively large tool kit which can be used to probe the catalyst system for information about structure, dynamics, and reaction mechanisms, including vibrational spectroscopy, molecular quantum chemistry, and NMR. However, when studying the molecular-level events in a heterogeneous system with an external or internal surface, the number of useful tools is considerably diminished due to the complexity of the physics. As part of the CACD program of the BCTR program, we are pursuing the development of several tools aimed at facilitating the study of solid-state structure in heterogeneous catalysts. These include the development of periodic *ab initio* Hartree-Fock theory and its application to aluminosilicates and metal oxides, and the formulation of a model to describe the phenomena of "site isolation" in selective oxidation catalysts. Improvements are sought that are clearly revolutionary relative to current practice or understanding and not just incremental.

1993 Accomplishments:

<u>VPO Catalysts:</u>

Progress has been made both relative to experimental and theoretical work involving the VPO catalyst system in FY 93. We have previously reported the structures for two polytypes of vanadyl pyrophosphate (an active/selective commercial catalyst for the conversion of butane to maleic anhydride, or through an alternate process to furan). This work has significantly clarified a long-standing controversy concerning the solid-state structure of this material. In addition, we have presented a hypothesis for the structure of the active-site responsible for the activation of oxygen and alkane. In FY 93, the PI's and our industrial collaborators at Monsanto Company were invited to contribute to a special issue of Catalysis Today

dedicated to the structure and chemistry of the vanadyl pyrophosphate catalysts. This work has been widely accepted and highly complimented by our peers. However, this hypothesis must be validated.

Solid Acid Catalysts and Theory Development:

In addition to the VPO catalyst work several studies using the periodic Hartree-Fock method (PHF) were completed on zeolites and on the surface chemistry of metal oxides. These studies essentially demonstrated the feasibility and value in using this theory to study systems of catalytic interest. In fact, one of the studies titled "An Examination of the Electrostatic Potential in Silicalite using Periodic Hartree-Fock Theory" was selected by the editorial staff of the Journal of Physical Chemistry to be featured in the Journals first color issue. Figure 4 of that article, which depicted the spatial minimum in the electrostatic potential (useful for predicting the location of charge particles in the lattice) of Silica ZSM-5 was used on the front cover of the August 21 issue. The article focused on the electrostatics of the lattice including the magnitude of the electric fields inside the cavities of the zeolite. This study represented one of the largest of all electron ab initio studies done to date and is a good example of the promise held by state-of-the-art methodologies. During 1993 we also began to benchmark the accuracy and utility of applying the Periodic Hartree-Fock theory to the study of the external surface chemistry of metal oxides. These studies have shown that the surface structure of simple oxides such as MgO, ZnO and Al_2O_3 are in good agreement with experimental data. In the second phase of the surface studies, adsorption and dissociation processes were investigated for a wide range of small molecules (CI_2, CO, H_2, H_20, etc.) on the surfaces of these materials. The surface science/catalysis community is currently trying to understand the atomic level details of heterogeneous catalysis that occurs on these simple surfaces. We are engaged in several joint studies with experimental groups in the U.S. for the purpose of performing detailed comparisons of experimental and theoretical results on precisely the same systems. The goal of those studies is to test both the experiment and the theory for level of accuracy and agreement and to understand the initial steps in the chemical reactions which occur on the surfaces of these compounds. It is our opinion that the past year's work using PHF theory has gone a long way in demonstrating the utility of the method to the catalysis community.

1994 Planned Activities:

Our general plans for FY 94 are centered around four areas: a) the experimental validation of VPO theory and crystallography studies; b) the chemistry of solid acid catalysis (primarily zeolites) c) surface catalysis of metal oxide surfaces; d) the continued development of the gaussian basis density functional theory and enhancements to the periodic Hartree-Fock code.

Calculations on vanadium ($^{+4}$) and vanadium ($^{+5}$) phases will continue in order to explain, as fully as possible, the nature of the electronic structures and physical properties for these transition metal systems. VPO phases under study include: polytypes a_1- and a_2-$VOPO_4$, and the polymorph b-$VOPO_4$, half-cell and full-cell structures of polytypical vanadyl pyrophosphate. Experimental validation of the active site hypothesis will include the structural relationship between epitaxial $VOPO_4$ phases on vanadyl pyrophosphate surfaces and their role in providing active oxygen. In addition, we propose to study the reactivity of a series of cyclic hydrocarbons with vanadyl pyrophosphate catalysts to empirically determine the size/shape selectivity of the active site for alkane and functionalized substrates (Temporal Analysis of Products, Gleaves/Ebner/Thompson).

In addition we will continue to validate the PHF code on zeolites and metal oxide surfaces of materials such as TiO_2, Al_2O_3, MgO, and ZnO. In addition, metal overlayers (such as Cu on ZnO) will also be initiated to begin to understand the role of the support/metal interactions. Chemical reactions involving small organic species will be selected as test cases. We will also continue the parameterization of molecular dynamics potentials from the *ab initio* data generated by the PHF theory for use in the treatment

and study of zeolites. The primary development effort will focus upon the periodic 3- and 2- dimensional gaussian basis density functional program and the stabilization of the next release of the crystallography/interface package.

Annual Technical Summary Report:

VPO Catalysts:

Two fundamental questions relative to the solid-state structure are under investigation. (1) There are many potential polytypes of this material, why are only two observed (as large single crystals)? (2) Is there a one-to-one structural relationship between single crystals and catalyst powders? Relative to the first question, theoretical results from FY 93 indicate that the solid state structures exhibited by single crystals of vanadyl pyrophosphate represent the lowest energy polytypes of this material, lying as much as 300 Kcal below the highest energy conformers. These results are based on periodic *ab initio* Hartree-Fock calculations on half-cell structures (higher symmetry polytypes) of vanadyl pyrophosphates. From these calculations it is apparent that many structures with differing symmetry are thermodynamically accessible under butane oxidation reaction conditions. *Ibis* observation may lend some insight into the long equilibration times necessary to achieve optimum catalyst performance in the VPO system. Relative to the second question, there are many differences, both glaring and subtle, between the experimental structure factors (diffraction studies) of single crystal and microcrystalline catalysts. In particular, it is apparent that several classes of reflections suffer 10-fold broadening in powder diffraction studies of catalysts. These effects can be traced back to the pyrophosphate network within the solid by virtue of the peculiar symmetry of vanadyl pyrophosphate. Theoretical calculations on an idealized crystal structure indicate that significant changes in the symmetry of the pyrophosphate network can occur with relatively small differences in total energy.

Solid Acid Catalysts and Theory Development:

The theory development activities which occurred in 1993 have progressed faster than we had initially anticipated. The initial parallelization of the PBF code (a replicated data scheme) was carried out by our European colleges. The next generation of parallel versions of CRYSTAL will be conducted as a joint effort between Daresbury Laboratory and PNL. The goal is to take the current replicated data algorithms and establish a fully distributed version of the code. The contributions of the Europeans enabled us to focus our attention on the next generation of software which will eventually replace the current generation of periodic Hartree-Fock codes. The new algorithms are based upon density functional theory using gaussian basis sets for the expansion of relevant quantities. This method has the advantage over Hartree-Fock theory in that it has a worst case scaling of N^3 (where N is the number of basis functions) as opposed to N^4 in conventional Hartree-Fock. The projected scaling of the completed density function code (including screening, use of multipoles, avoidance of explicit diagonalizations, etc) is less than N^3 were the best achieved to data with PHF is in the range of $N^{2.6}$. The new density functional theory has progressed very rapidly, since January we have written and tested approximately 45,000 new lines of FORTAN which constitute the molecular version of the program. We have recently completed the mathematical formalism for the 3-dimensional (crystalline) materials and have begun to program the new equations. In order to solve the next generations of catalysis problems new theoretical tools which are faster than those we currently have will be necessary. Our approach is to reduce the order of operations in the theory as low as possible (thus the switch to density functional theory) and to optimize all algorithms from the start to run on massively parallel computer architectures. The combined effect will allow the next generation of theoretical methods to directly probe more complex issues concerning the chemistry of the catalytic process.

The graphical interface and crystallography codes are currently in a β-release mode at 20 tests sites throughout the U.S. Initial feedback has been very positive, there seems to be particular interests from educational institutions (which we didn't really anticipate) who would like to use the software to teach solid state and crystallography courses.

2. **Project Title:** Design, Characterization, and Testing of Multimetallic Catalysts for Pollution Control.

Principal Investigators: T. S. King, A. E. DePristo and M. Pruski

Project Site: Ames Laboratory

Description:

The research goals are to develop and implement sophisticated methods of modeling small, bimetallic particles composed of on the order of 100 to 1000 atoms; to predict morphology and surface properties as a function of composition, reactive environment, and support material using these methods; and to test and validate the prediction experimentally using traditional techniques as well as unique, solid state NMR studies of metals and adsorbates.

The use of supported bimetallic catalysts in industry is extensive in spite of the general lack of fundamental understanding of how the small metal particles interact with the reacting species. The present activity focuses on one very important application, automobile exhausts catalysts, with the intent of generating the scientific basis needed to design the next generation of catalysts in an efficient manner. Consequently the proposed work encompasses basic and applied research that is heavily dependent on a close relationship with researchers at General Motors Research Laboratories. The major challenge for this program, and the reason for the close industrial collaboration, is the application of the modeling and experimental technique developed at the Ames Laboratory to more complex catalysts.

The exhaust from internal combustion engines contains small concentrations of hydrocarbons and CO from incomplete combustion of the fuel, and of nitrogen oxides, NO_x, from nitrogen fixation at the high temperatures of combustion. These contaminants are major contributors to air pollution. Federal legislation enacted in 1981 set the maximum allowable levels of pollutants for new cars and trucks (in grams per mile) as 0.41 for hydrocarbons, 3.4 for CO, and 0.4 for NO_x. These figures were set on a gradually diminishing scale, and have been modified as the technical difficulties and the costs to remove the pollutants have become better understood. The original figure set for NO_x has been particularly difficult to achieve, and was modified in 1982 to 1.0 g/mile. The original figure still remains a research goal. Catalysts used for oxidation of CO and hydrocarbons are based on Pt or Pt in the presence of small amounts of Pd. Pt is more active for oxidation of paraffins, Pd for oxidation of CO and possibly unsaturated compounds. In an oxygen-rich atmosphere Pt on alumina support sinters more readily than does Pd; Pt is, however, more resistant to lead poisoning than is Pd. Pd exists on the catalyst as the oxide, and Pt is more active as the metal, upon which oxygen is chemisorbed.

NO_x must be removed by reduction to nitrogen gas. The most active catalyst is Pt, but some NO_x are reduced to ammonia, which is not desired. The most effective catalyst for the reduction of NO_x to nitrogen is one in which some Rh is added to Pt. Rh is not mined anywhere separately; Rh is obtained as a byproduct of production of Pt and Pd, the ratio being about 0.06 to 0.04 Rh to Pt in South African ores. Thus the optimum catalyst composition will be markedly influenced by market economics which set the price of a byproduct of strictly limited availability.

The potential impact of this activity on technology is large. The driving forces behind the development of improved automobile exhaust catalysts are both economic (i.e. the current catalysts use expensive platinum and rhodium) and legal (i.e. improved performance of emission abatement technology is mandated by the Clean Air Act.) There will likely be applications to other technologies that employ supported metal catalysts, particularly in the petroleum and petrochemical industries.

Theoretical (molecular dynamic) calculations have predicted that for both systems, Rh tends to accumulate at the particle surfaces. Experimental verification is obtained by proton NMR spectroscopy. Hydrogen is adsorbed on the particles, and the relative field shifts of various particle compositions are used to infer the surface compositions. Experimental testing of the particles, using gases of various compositions, is being conducted at GM Research Labs (Warren, MI).

1993 Accomplishments:

The development of the MD/MC-CEM and CEM models for isolated mixed metal clusters of Al, Ni, Cu, Rh, Pd, Ag, Ir, Pt and Au has proceeded on schedule with calculations being completed for all 36 pairs for clusters of size 201 and 1289 atoms[3]. Work has been initiated on the incorporation of clusters in "reducing" environments with the first system to be treated being H/Pt/Rh. Work has also been initiated on incorporation of supports such as SiO_2.

The CEM method has been used to parametrize the thermodynamic BOS model for Al, Ni, Cu, Rh, Pd, Ag, Ir, Pt and Au using clusters of size 201, 586, 1289 and 2406 metal atoms. The parametrized values were found to be rather insensitive to the cluster size, indicating that the values can be used for the entire range of cluster sizes.

Surface segregation behavior of clean and oxygen covered Au_3Cu alloy surfaces was modelled by the tight-binding formalism[4].

The experimental portion of this program has progressed in two major areas. Considerable effort has been invested in the development of a microcalorimeter system to empirically determine the energetics associated with the adsorption of small molecules such as hydrogen. We have nearly completed construction of a sensitive microcalorimeter to measure the enthalpy of adsorption from 10^{-6} to about 1000 torr. Preliminary experiments on silica-supported Ru have yielded heats of adsorption of hydrogen that range from about 80 kJ/mol to 10 kJ/mol depending on coverage. The effect of the gaseous environment on a catalyst's morphology and composition must be taken into account in any procedure that endeavors to describe a "working" catalyst.

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A. M. Schoeb, T. J. Raeker, L. Yang, X. Wu, T. King and A. E. DePristo, Surf. Sci. Lett. 278, L125 (1992).
L. Yang, T. J. Raeker and A. E. DePristo, Surf. Sci. 290, 195 (1993).
L. Yang, T. J. Raeker, A. M. Schoeb, T. S. King, and A. E. DePristo, Theoretical and computational description of bimetallic clusters, J. Cluster Sci., invited review, (in preparation).

[4] B. C. Khanra and T. S. King, Surface Composition of Clean and Oxygen Covered Au_3Cu Alloy, Phys. Rev. B47, 16494 (1993).

We have used ^{1}H NMR to determine surface compositions for relatively simple systems such as Ru-Cu. The theoretical basis of the Knight shift dependence that allows us to determine surface compositions of this and other systems was recently studied in greater detail[5]. We have now extended this approach to the more complicated Pt-Rh bimetallic system where hydrogen adsorption modifies the segregation behavior[6].

1994 Planned Activities:

During this period work will involve the extension of both the theoretical predictive capabilities and the NMR characterization method. In addition, the experimental validation studies coupled with standard catalytic characterization methods will continue. The focus of this work will be to assimilate the previous experience and extend it to the study of gaseous environments, support and promoter effects. This approach will serve to close the "gap" between understanding obtained by model studies and experience with commercial catalysts.

We plan to continue the experimental validation program and establish the viability of the ^{129}Xe NMR approach for the determination of surface composition. At this time it is not known whether this approach will be successful, however, of all the potential techniques suggested for this application, it holds the most promise. The determination of the surface composition of an oxygen-covered supported bimetallic particle may also be determined via ^{129}Xe NMR.

Several important advances in the ability to simulate and predict catalysts under operating conditions can be made. Clearly, the influence of adsorbates resulting from chemisorption of reactants and products is an important factor in catalyst surface properties. This circular and synergetic effect makes simulations complicated. The surface perturbs adsorbing species in a manner than causes them to react, and the adsorbate changes the surface properties of the catalyst. Simulations are difficult, especially for adsorbates such as oxygen, due to the level of sophistication needed in the theoretical procedures to model highly directional bonding and the transition from covalent to ionic bonding.

The energetic effects of chemisorption must be determined for input into the SMPP model. The microcalorimetric studies initiated in FY 1993 will serve as an experimental basis for the initial input parameters. However, this approach lacks adsorption-site specificity and the determined values of the heat of adsorption tend to be averaged over the entire surface. In principle, initial values of the isosteric heats of adsorption would give the values associated with the strongest binding sites, but due to the nature of the highly porous catalysts we cannot eliminate chromatographic effects entirely and we cannot unequivocally identify the adsorption site. Consequently, it is proposed that companion theoretical approaches also be developed to estimate the energetics of adsorption for specific surface sites. We plan to incorporate into the models the capability to simulate the effects of simple adsorbates such as hydrogen. The CEM method can treat H adsorption quite accurately on metals and thus will be used to parametrize the partial bond energy model and also to test the MD/MC-CEM method. Either of the faster methods will be used to investigate structures of H/Pt-Rh clusters. More complex adsorbates such as oxygen are too computationally demanding at this time but are suitable subjects for future work.

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5 B. C. Khanra and T. S. King, J. Phys. Chem. 97, 4164 (1993).
B. C. Khanra and T. S. King, Knight Shift of Adsorbed Hydrogen on Ru-Cu Bimetallics, J. Phys. Chem. (submitted).

6 B. C. Khanra, A. M. Schoeb, X. Wu, B. C. Gerstein, and T. S. King, The Effect of Hydrogen on Surface Segregation in Supported Pt-Rh Bimetallic Catalysts, J. Catalysis, (in preparation).

One very important goal currently being pursued is to combine three levels of modeling capability into one software package. The software, operating on a desktop workstation, would be able to produce a very fast simulation using the bond order simulation/Monte Carlo method or a slightly more computationally demanding simulation using the MD/MC-CEM approach. The former will contain parameters determined from the CEM theory while the latter will allow for size effects that can distort the cluster away from an fcc lattice when the sizes of the atoms are significantly different. Both of these methods will be fast enough to use in real time testing of various combinations of metals. We shall also build in the full CEM simulation capabilities but these are so much more computationally demanding that they will provide answers mainly to check the simpler approaches. CEM simulations will not be feasible in real time. The purchase of a workstation for this effort is critical to the success of the project, and this was a special request in FY 1993. Our intent is to have a working, user-friendly software implementation of the CEMBOS ("chem boss") model for the DECAL (design of catalysts at Ames Lab) project by the end of FY 1994.

The ^{1}H NMR used previously will be applied to promoted catalyst systems to infer the degree of contact between the metals and the promoter and also to probe the effect of the promoter on the electronic structure of the metal particles. This latter effect will be manifested via the Knight shift of chemisorbed hydrogen. The magnitude of the Knight shift is very sensitive to the electron density of H-metal bonding states at the fermi level. The theoretical development is being actively pursued in this project.

We will also continue work with ^{129}Xe NMR characterization of supported metal catalysts. The high polarizability of Xe results in large chemical shifts from only weak interactions (collisions) with surface atoms and, therefore, does not perturb the surface morphology. This approach is expected to be applicable to adsorbate-covered (e.g. oxygen) systems as well.

Annual Technical Summary Report:

During this year we have devoted considerable effort to increasing the range of applicability of the theoretical models and testing them against the small amount of available experimental data. In addition, we have focused on the experimental characterization of supported bimetallic catalysts using ^{129}Xe NMR and on the experimental determination of the energetics of adsorption of hydrogen on supported metal catalysts.

A major advancement has been our ability to produce more accurate parametrizations of the partial bond energy model with the CEM method using actual simulations on model clusters instead of on extended surface planes. This has been accomplished for the metals Ni, Cu, Rh, Pd, Ag, Ir, Pt and Au. The results of partial bond energy simulations are in much better agreement with those of the full CEM values on small clusters than when the parameters were determined from extended surfaces.

Using the various levels of theory inherent in the CEMBOS model, we have investigated a number of systems over the past year, comparing to experiment at every opportunity. A number of interesting features of small bimetallic clusters have been discovered. First, for the Rh-Pt system, we have validated the CEM predictions of both the heat of formation in Rh_xPt_{1-x} alloys and the surface segregation behavior in $Rh_{0.9}Pt_{0.1}(111)$. (The latter was measured by the collaborators at GM.) A very important point followed from the fact that the CEM calculated cohesive energy of Rh is slightly smaller than that of Pt but the surface energy of Rh is significantly larger. In the small clusters and on the single crystal plane, Pt segregates to the surface and thus the driving force for surface segregation is the relative surface energies not the relative cohesive energies. In general, we showed that one can predict surface energy differences by properly accounting for the variation of the bond energy with coordination, indeed this is done in the thermodynamic model by using CEM calculated results.

Second, in computer simulations of Rh_xPd_{1-x}, Rh_xNi_{1-x} and Ni_xPd_{1-x} clusters with $0.1 \leq x \leq 0.9$ containing 201 and 1289 atoms at 200 K, 600 K, and 1000 K, we showed that the metal with lower surface energy segregates first to the catalytically important edge-corner sites, then to other surface sites. Increasing the temperature decreased the degree of segregation at edge-corner sites more strongly than on the entire surface. Third, for other 201- and 1289-atom 50%-50% bimetallic clusters at 600 K selected from Rh, Ni, Pd, Au, and Ag mixtures, we showed that large atom-size mismatch can invalidate the simple geometrical constraints on surface segregation that hold for other bimetallic clusters.

Considerable effort has been expended in developing methods to experimentally validate the theoretical models. It must be noted that a major motivation in pursuing the theoretical studies is that the experimental determination of those surface properties that govern catalytic processes is difficult at best. For example, surface composition of highly dispersed bimetallic particles has been determined reliably in only a few specialized cases. More complicated properties such as surface micromixing have never been determined experimentally.

We have used ^1H NMR to determine surface compositions for relatively simple systems such as Ru-Cu. The theoretical basis of the Knight shift dependence that allows us to determine surface compositions of this and other systems was studied in greater detail. Comparison of the calculated and experimentally determined Knight shifts for Ru-Cu suggests that the resonance is dominated mainly by the adsorbed hydrogen atoms experiencing a hyperfine interaction with three Ru first nearest neighbors. In the case of Pt-Cu, the Knight shift results from a hyperfine interaction of hydrogen with perhaps as few as one Pt first nearest neighbors. Using this understanding to calculate the surface composition for the Pt-Cu bimetallic system indicated that the surface composition determined via NMR was approximately the same as that determined via SMPP. The energetic influence of the adsorbed hydrogen present in the NMR experiment is the likely cause of the difference.

The fact that adsorbate probes such as hydrogen perturb the surface composition of bimetallic particles led us to pursue other experimental methods of surface composition determination. We have initiated studies using ^{129}Xe as a probe of surface composition for two reasons: 1) it binds only weakly (physical adsorption) to metal surfaces and therefore will not greatly influence the relative surface composition; and 2) it has a large number of easily polarized electrons that produce large shifts in the NMR experiment. Even though ^{129}Xe NMR has been used for surface characterization of zeolites, for example, it has not been used for the purposes intended in this program. To date we have successfully built a xenon probe and obtained preliminary spectra on a simple, zeolite system that does not contain metal particles. The analysis of supported pure metal systems is just starting.

The effect of the gaseous environment on a catalyst's morphology and composition must be taken into account in any procedure that endeavors to describe a "working" catalyst. In preparation for theoretical studies briefly described in the next section we have begun to develop the experimental capability to measure the energetics of adsorption for simple molecules (e.g., H_2) on supported metal catalysts. We have begun construction of a sensitive microcalorimeter to measure the enthalpy of adsorption from 10^{-6} to about 700 torr. Preliminary experiments on silica-supported Ru have yielded heats of adsorption of hydrogen that range from about 90 kJ/mol to 12 kJ/mol depending on the coverage.

3. **Project Title**: Theoretical Studies of Hydrocarbon Catalysis on Zeolites.

Principal Investigators: A. Redondo and P. J. Hay

Project Site: Los Alamos National Laboratory

Description:

The original goal of this project was to develop computational tools to model chemical transformations of small hydrocarbon molecules within zeolite catalysts. The project has been expanded, and now includes AICD Computer Aided Catalyst Design (CACD) coordination activities of inorganic catalysts, together with an experimental program to provide verified, generic modeling tools for catalysts of interest to industry.

In this project, catalyst research has focused on the details of carbon-carbon bond formation within the pentasil zeolite, ZSM-5. This catalyst has special significance for energy applications; for example, it is used in the "methanol-to-gasoline" process, and has many uses in conventional petroleum cracking. Pentasil zeolites provide unmatched shape selectivity for hydrocarbon molecules; hydrocarbon molecular weight produced within zeolite cannot exceed the limit of light hydrocarbons of gasoline fractions.

Many of the details of the catalytic processes are unclear, however, and warrant fundamental investigations if the yield and selectivity are to be improved, and consequently energy to be saved in these processes. These details include the nature of the acid sites responsible for catalysis, and transport properties such as absolute diffusivities. Models of the adsorption and bond formation processes within zeolites will be derived by classical (molecular-dynamics), quantum, and quantum-statistical methods.

Catalyst research will also include modeling and experimental studies of metal oxide oxidations of hydrocarbons. These case studies were suggested by industrial collaborators of the project. Specific project technical objectives include: 1) predict the chemical state and the role of alumina in zeolite catalysis, and verify the predictions by X-ray absorption; 2) use solid-state ionic conducting ceramics to study hydrocarbon oxidations, using new experimental techniques to control the surface free energy. Specific CACD coordination activities include: 1) establish industrial/DOE collaboration programs and workshops; 2) integrate computational tools developed at LANL into other DOE-sponsored projects, to transfer the computational tools to industry; and 3) set experimental standards and protocols for experimental verification of the models.

1993 Accomplishments:

Zeolite catalysis. From results of semiempirical calculations on the structures and relative energies of the acid sites in zeolite ZSM-5, the acidities of these sites have been correlated with local geometrical properties of the Al-OH-Si linkage at the 12 different T sites where Al substitutes for Si in the lattice. Calculated proton affinities of the sites are used as a measure of acidity, where high acidity corresponds to low proton affinity. The least acid sites are found to have small Al-O-Si bond angles around 120 degrees while the most acid sites are found to correspond to larger angles around 145 degrees.

Extensions of these calculations have been carried out on the zeolite faujasite which has only one distinct T site and four corresponding O atom sites. The preferred O atom sites for the protons at the active site have been determined using both semiempirical and density functional techniques and the results have been compared with experiment from site occupancies measured using neutron diffraction.

Metal oxide catalysis. A series of molybdenum-bismuth oxides have been synthesized as candidates for studies of selective hydrocarbon oxidation catalysis such as propylene to propylene oxide using both conventional and electrochemical reactors. Calculations on two model metal oxide systems for selective oxidation of C-H bonds have identified products and transition states for radical abstraction types of processes. These studies represent the initial part of a systematic study of the chemistry involved in different types of M-O linkages in potential metal oxide catalysts.

1994 Planned Activities:

Zeolites. Having studied the acid site properties of several types of zeolites using different theoretical methods, we will begin exploring chemical reactions occurring at these acid sites. Initially the focus will be on acid site cracking of hydrocarbons in which the proton at the acid site attacks the hydrocarbon which subsequently breaks into smaller fragments. Studies on smaller hydrocarbons up to propane or butane interacting with acid sites will compare the thermochemistries and reaction barriers from *ab initio* calculations with results from semiempirical methods and with available experimental information. The analysis of the findings for these smaller systems will determine the approaches used for modeling cracking processes of larger hydrocarbons.

We will continue an ongoing collaboration with the group of Prof. Anthony K. Cheetham at the University of California at Santa Barbara and Biosym Technologies, of San Diego, California to study hydrocarbon interactions in zeolites, particularly faujasite and ferrierite. These studies aim at understanding the diffusional properties of reactant and product hydrocarbons in zeolitic catalysts for applications in petroleum cracking and other petrochemical processes.

During the summer months we have implemented a novel technique for carrying out molecular dynamics simulations using quantum mechanical forces. In contrast with the traditional molecular dynamics method (i.e., employing predetermined force fields that describe the interactions between the particles in the system) this approach is ideally suited for the study of chemical reactions. We intend to employ this new technique in the study of hydrocarbon cracking reactions in acid zeolites.

Metal oxides. We intend to continue our collaboration with the group of Professor Allan Jacobson of the University of Houston and ARCO Chemical Company on metal oxide catalysis. The aim of this project is to develop new materials for the selective partial oxidation of low molecular weight hydrocarbons. The initial processes that we are targeting are the oxidation of methane and propylene to produce propylene oxide, MTBE and TBA, which are gasoline additives. Our plan is to scope a series of materials with the general formula Bi_2O_2–MO_4, where M can be Mo, V, or Mn. We are particularly interested in materials with high ionic conductivities because part of the project seeks to use the electrochemical properties of the catalysts to try to enhance selectivity and/or efficiency.

The alkane reactivity studies on Cr and Fe model complexes will continue in order to understand the type of M-O linkage necessary for selective oxidation. The previous work at the Hartree-Fock level will be extended to include electron correlation effects to see if the transition state structures are affected and whether CH_3 is evolved along the reaction coordinate from transition state to products. In addition, work will be initiated on the O_2 activation step, since an efficient oxidation process must cleave the O-O bond so that both O atoms can be incorporated in product and not be lost as H_2O.

1. Zeolite catalysis

a. Acid site properties. A. Redondo, P. J. Hay (LANL) The catalytic activity of zeolites is determined primarily by the properties of the acid sites in the zeolite lattice where Al (or other metal) has been substituted for Si to create Al-OH-Si linkages. Extensive semiempirical calculations using the MNDO method have been carried out on cluster models that represent the different Bronsted acid sites in the zeolites ZSM-5 and faujasite. Using clusters containing typically 100 to 120 atoms the geometries of the atoms in the immediate vicinity of the acid site are determined while the positions of atoms farther from the acid site are fixed at their lattice positions as obtained from X-ray or neutron diffraction studies on the zeolite containing only small amounts of Al. From these studies the preferred location of Al and H atoms in the lattice and the relative energies can be computed.

The acidities of the 48 distinct T site locations in zeolite ZSM-5, as measured by the calculated proton affinity of each site, have been found to correlate directly with the Al-OH-Si bond angle as shown in Fig. 1. Sites with small angles of 120 degrees have the highest proton affinity and hence lowest acidity while sites with larger angles of 145 degrees exhibit protons with the lowest proton affinity. An additional distinction can be made between "pore" sites where the protons are located in the larger pore regions and "framework" sites where the protons lie within the confines of the zeolite framework. The latter have larger bond angles and higher acidity but are probably physically inaccessible in the zeolite.

A more direct comparison with experiment is possible for faujasite where there is only one distinct T site for Al substitution and four possibilities for proton sites at neighboring O atoms. Neutron diffraction studies have measured the relative occupancies of these sites. From cluster calculations using the MNDO method on an $AlSi_{22}O_{57}H_{23}$ cluster the relative energies is found to be $O_1 < O_4 < O_3 < O_2$ with respective relative energies of 0, 1.5, 7.0 and 9.0 Kcal/mole (i.e., O_1 is most stable with the highest proton affinity). The crystallographic O_1 is thus found to be the most favorable location, as is observed experimentally. However, the neutron diffraction results indicate the ordering $O_1 < O_3 < O_2 < O_4$ for the other sites. To investigate this further, calculations on a smaller $AlSi_7O_{22}H_{13}$ cluster were carried out using both MNDO and density functional DGauss techniques. Similar results were obtained for MNDO but much less dispersion (only 1-2 kcal/mole) was found between the sites using density functional theory. The effects of other Al atoms are being studied to account for these discrepancies since the faujasite (zeolite Y) contained 40 percent Al content.

b. Adsorption/ desorption phenomena P. MacDougall, P. J. Hay, A. Redondo (LANL) One widely used method to measure acidity in zeolites involves the introduction and subsequent desorption of ammonia, pyridine and other bases--as measured by temperature-programmed desorption (TPD) or microcalorimetry experiments. This can often be correlated with other measures of acidity such as relative cracking rates of hydrocarbons. We have been pursuing *ab initio* electronic structure studies of ammonia adsorbed at acid sites using small cluster models.. In these studies, one issue concerns the relative stability of the hydrogen bonded form NH_3 -- [Al-OH-Si] and the protonated form NH_4+ -- [Al-O-Si]$^-$. In the initial studies using small clusters the hydrogen bonded form was calculated to be more stable. Experimental evidence such as IR and NMR spectra of amines in zeolites indicates the protonated form is the dominant species. For the case of NH_3, we have subsequently found that the protonated species is the more stable form if the NH_4+ can form two hydrogen bonds to neighboring O atoms as in NH_4^+ -- [-Si-O-Al-O-Si-]$^-$.

c. Molecular dynamics simulations N. Henson, A. Cheetham (UCSB); A. Redondo, B. Holian (LANL), J. Newsam (Biosym) Molecular dynamics techniques are being implemented for carrying out simulations of the motion of hydrocarbons in zeolite lattices. Initial calculations have been carried out to determine the diffusion constant of benzene in zeolite Y using existing literature potentials for the zeolite and adding

benzene-zeolite interactions. Since Na^+ ions are also present, we are also developing potentials for Na-benzene interactions from quantum mechanical calculations using the Møller-Plesset approximation.

Preliminary studies indicate excellent agreement between the calculated adsorption sites for benzene in zeolite Y and the neutron scattering experiments carried out in Prof. Cheetham's group.

d. Quantum molecular dynamics. A. Redondo and B. Calef (LANL). Within the last month we have implemented a novel technique for molecular dynamics simulations that employs quantum mechanical forces. This method is ideally suited for the study of the dynamics of chemical reactions in real time. In it, the forces on every atom in the system in question—for example, reactant molecules at an active site of a catalyst—are calculated at every time step from a solution of Schrödinger's equation using the MNDO approximation. This set of programs has already been tested for the problem of electrophilic substitution in benzene. These results show how a proton is incorporated as a full bonding partner into the benzene ring to form a cyclohexadienyl ion, and how it can be abstracted by a nucleophilic agent, such as a bromide ion. Our plan is to use these codes to study hydrocarbon cracking at acid sites of zeolitic catalysts. These studies will help us elucidate relationships such as that between acidity and cracking efficiency.

2. Metal oxide catalysis

a. Selective oxidation of hydrocarbons. Y. Yang, T. H. Lee, A. Jacobson (U. of Houston); E. Ong, I. Raistrick (LANL); A. Gaffney (ARCO). The aim of this project is enhance the selectivity of chemical processes leading to the partial oxidation of hydrocarbons. There were five initial objectives to the work: (i) design and construction of an electrochemical reactor, (ii) design and construction of a chemical microreactor, (iii) synthesis of a first series of catalytic materials, (iv) characterization of ionic properties, and (v) initial scoping of catalytic properties. Items (i) and (ii) are well in hand and initial tests of the reactor systems have already commenced. Particular care had to be placed in the design of the electrochemical reactor because a gas recirculating process had to be incorporated to track products that would be produced in small amounts. This is particularly important for those studies in which one tries to use the electrochemical properties of the materials to enhance selectivity. We have also synthesized a number of catalysts containing Bi and a metal and are currently studying their ionic and catalytic properties. In particular, we have examined the following three series of mixed oxides: $Bi_2O_2(Mo_xV_{1-x})O_{4-x/2}$, $Bi_2O_2(Mo_{1-x}Ni_x)O_{4-x/2}$, and $Bi_2O_2(V_{1-x}Mn_x)O_{3.5-x/2}$. They show excellent polycrystalline properties and high ionic conductivities when compared to other ionic conductors, such as $(Bi,Er)_2O_3$. Their catalytic properties are currently under study.

b. Modeling of selective oxidation of C-H bonds. A. K. Rappe, (Colo. State Univ.) Theoretical studies of reactions of model metal oxide systems with C-H bonds are being carried out using quantum chemistry approaches. From an understanding of the thermochemistry of these reactions and the transition states involved in these pathways, one can begin to develop a strategy for selective oxidation of C-H bonds. To develop a systematic understanding of the reactivity of methane with M-O linkages to form methanol, for example, one must react methane with the different bonding modes accessible to M-O linkages found in inorganic systems. We began by picking two metal complexes with terminal M-O linkages: (1) $(OH)_2CrO_2$--a model of a MO_3 (111) surface containing a formaldehyde-like M-O double bond, and (2) a dinuclear Fe(III) complex with a terminal oxo ligand--a model of the (100) surface of hematite (Fe_2O_3). For the first system, there is indirect experimental evidence that it reacts with alkanes as though it were a radical. Characterization of the reaction coordinate for this reaction has shown that the alkyl product $(OH)_2CrOH(CH_3)$ is unstable to loss of CH_3OH or CH_3 at the Hartree-Fock (HF) level and that the transition state is rather product-like, indicating the reaction is endothermic.

The geometry has been optimized for the second system, and the ground state of the dinuclear iron complex has been found to be a singlet state with other states of intermediate spin ranging up to high spin (S=5) states arising from the common underlying electronic structure. This different type of bonding resembling O_2 should prevent this unit from acting like a radical abstracting center, but the reaction of this system with methane needs to be carried out to verify this hypothesis and compare to the reaction of O_2 with methane. Mononuclear Fe complexes such as $[(H_2O)_4Fe(OH)O]$ have been constructed to see if they would retain the O_2-like bonding in the dimer; similar bonding has been observed but differences arise in the nature of low-lying excited states. The influence of the trans ligand on the Fe-O bond is being explored prior to studies of the reaction with methane to learn about influencing the properties of catalysts through changes of the trans ligand or changing the coordination environment of the trans ligand.

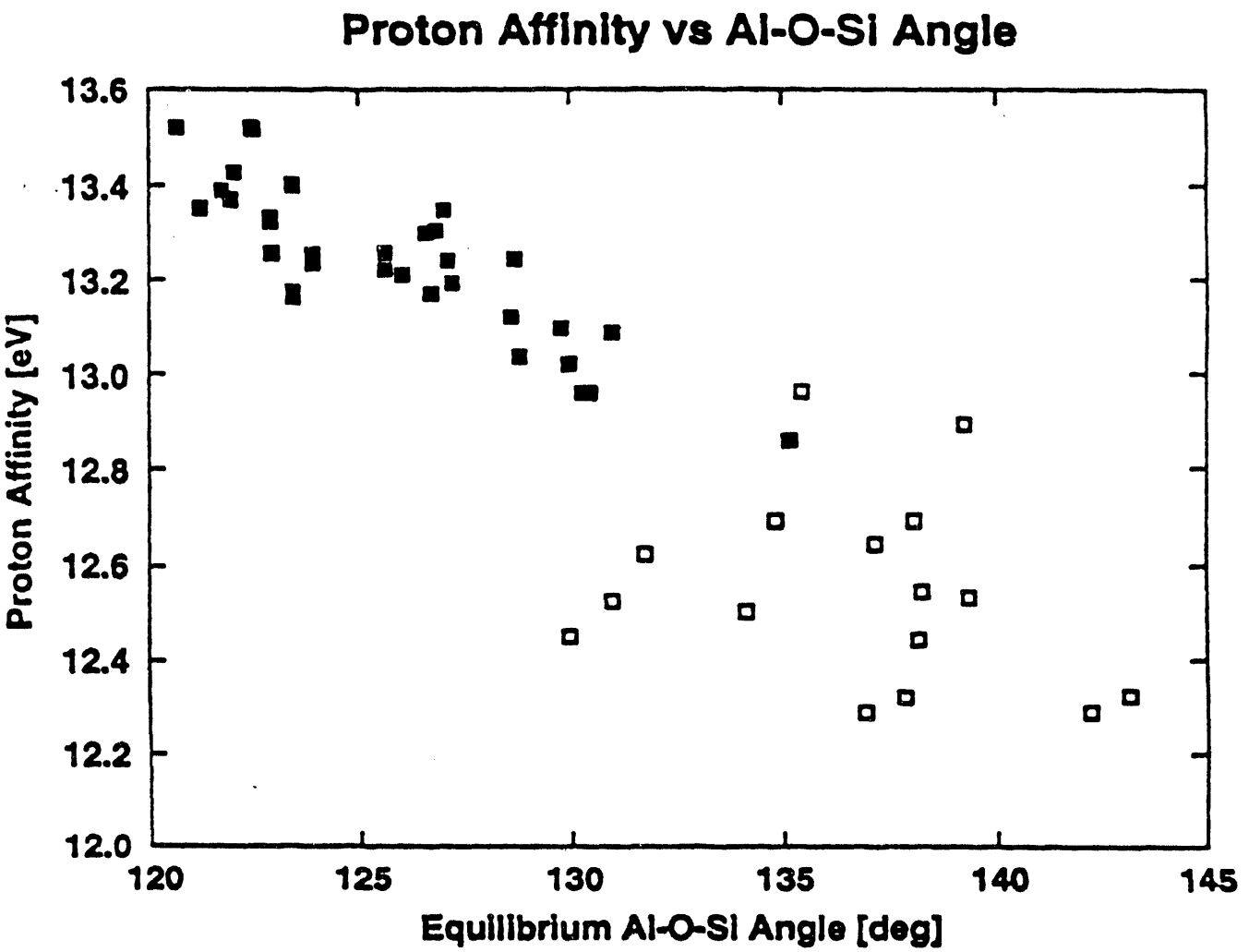

Figure 1. Calculated proton affinities of the 48 distinct acid sites in zeolite ZSM-5 as a function of Al-OH-Si bridging angle. Solid squares indicate acid sites where the protons are located in the large pore regions of the zeolite, and open squares show acid sites where the protons are located in the physically less accessible framework regions of the zeolite.

4. **Project Title:** Theory-Assisted Design of Metal and Zeolite Catalysts.

Principal Investigators: A. T. Bell and M. Van Hove

Project Site: Lawrence Berkeley Laboratory

Description:

The project will develop and validate a hierarchy of theoretical models that could be used by industry for the design and development of catalysts, especially zeolites and metals. Catalyst properties (enthalpies and entropies of adsorption and formation and other thermodynamic parameters) will be calculated and compared with known experimental values. The overall goal is a complete description of various industrially relevant heterogeneous catalyst systems, with models of increasing degrees of sophistication. These models will allow increasingly better prediction of industrially important phenomena, which can be used for the design of these two classes of catalysts.

1993 Accomplishments:

Calculation of the Structure and Electronic Properties of an Acid Site in H-ZSM-5. We have employed quantum mechanical local density functional theory to characterize the structure and electronic properties of the Brönsted acid site in the zeolite H-ZSM-5, responsible for industrially important reactions such as the conversion of methanol to gasoline, alkylation of benzene, isomerization of xylenes, and the cracking of petroleum. The calculations were performed on a 34 atom cluster embedded in the electrostatic field generated by the zeolite framework. Our predictions for the bonded geometry around the aluminum atom and for the location and vibrational frequency of the proton are in very good agreement with available evidence from solid-state NMR and IR spectroscopy. The nature of Brönsted acidity was characterized using the hard/soft acid-base principle and perturbation molecular orbital theory.

Sorption Thermodynamics of Aromatic Molecules in Silicalite. The prediction of sorption isotherms for bulky molecules that experience a tight fit within zeolites pores is impossible with conventional molecular simulation techniques, as the insertion moves these techniques employ have negligible probability of being accepted. From the point of view of applications, however, bulky molecules are the most interesting, as they are most affected by the molecular sieving action of the host lattice. We have developed a new energy- and cavity-biased Grand Canonical Monte Carlo (GCMC) strategy that enables prediction of isotherms for such molecules. Applying this algorithm to aromatics in the zeolite silicalite, we have correctly predicted the emergence of non-Langmuir isotherm shapes at intermediate and lower temperatures and the strong occupancy-dependence of the heat of sorption observed calorimetrically. We are exploring ways of coarse-graining our atomistic description of the zeolite/sorbate system into a lattice model that would allow predicting the isotherms with even smaller computational effort.

Prediction of Transport Diffusivities in Zeolites. We have been developing molecular simulation techniques that can predict the diffusion rates of organic molecules in a zeolite from an atomistic-level description of the host crystal and its interactions with guest molecules. Past dynamic simulation efforts have focused on predicting the self-diffusivity through equilibrium molecular dynamics (MD) simulations. Of greater practical importance, however, is the transport diffusivity, i.e., the proportionality constant relating flux to concentration gradient. We have designed two nonequilibrium MD techniques to predict the transport diffusivity and tested them on the system methane/silicalite. Our predictions are in excellent agreement with experimental evidence and provide a rigorous interpretation of the Darken equation, often used to correlate the concentration dependence of the transport diffusivity.

Ethylene Hydrogenation on Pt Surfaces. As a starting point towards mapping the mechanism of ethylene hydrogenation, we have computed various energy versus site curves for ethylidyne ($-CCH_3$) on the Pt(111) surface through extended Hückel calculations. Our results are in favorable agreement with surface crystallographic findings.

1994 Planned Activities:

Density Functional Theory Calculations of Specific Interactions Between Zeolitic Acid Sites and Polar Sorbate Molecules. Ammonia will be used as a test sorbate. The geometry and energetics of binding will be quantified and the rate of proton transfer from the zeolite acid site to the ammonia molecule will be estimated. A potential hypersurface will be developed that can be used as input for dynamic simulations of the polar sorbate in the zeolite.

Prediction of the Diffusivities of Long Chain Molecules in ZSM-5. The rate of diffusion of C_8-C_{16} alkanes in ZSM-5 is of great importance for understanding the extent to which zeolite crystals are used in fluid catalytic cracking. The characteristic times for diffusion in these systems exceed by far the time scales that can be addressed with conventional MD techniques. We will develop a coarse-grained approach, based on Brownian dynamics and transition state theory with parameters extracted directly from atomic-level information, to predict diffusivity and at the same time elucidate the configurations of these molecules in the sorbed state.

Transition State Theory of Aromatics Diffusing in ZSM-5. The diffusivities of aromatics in ZSM-5 are a key to the shape-selectivity observed in alkylaromatic transformations. Predicting the diffusivity of these tightly fitting molecules is a frontier problem for molecular simulation. We will attack this problem with multidimensional transition state theory, building upon methodology we have developed in past years.

Ethylamine Decomposition on Pt Surfaces. The mechanism of ethylamine decomposition on Pt(111) and Pt(100) surfaces will be studied through extended Hückel calculations.

Annual Technical Summary Report:

A. Metals:

Extended Hückel Calculations of the Energetics of Ethylene Dehydrogenation

We have pursued our studies of the dehydrogenation process of ethylene on two crystal faces of platinum: molecular modeling of ethylene decomposition on both Pt(111) and Pt(100) has been performed. We now use a modified version of the extended Hückel theory within the tight-binding formalism to investigate the hydrogenation of ethylene on platinum surfaces. This version of the original programs, modified by Calzaferri, Forss and Kamber, employs a distance-dependent extended Hueckel constant and also calculates two-body repulsions to allow a more reliable investigation of the intermediate surface species present in the hydrogenation process, an important aspect of the project. We intend to describe the energetics of each intermediate step of interest in the process, and trace other factors that govern the structure; the contributions of bonding and electronic factors, steric influences and reactivities, and the influence of adsorption sites will be thoroughly examined.

Initially, we have concentrated on a set of carbonaceous species which are possibly present as intermediates on the Pt(111) and Pt(100) crystal surfaces during the process of ethylene hydrogenation. We restricted ourselves to the ones which include a C-C bond, namely acetylene, vinylidene, ethylidyne, vinyl, di-sigma- and pi-bonded ethylene, and ethylidene. We made calculations in order to verify which is the

most stably bound on the two platinum surfaces. To that end, we used the following definition of the binding energy (B.E.) of an adsorbate species (A) on a metal surface:

> B.E. = energy of composite (surface + A) - energy of clean surface - energy of A in a hypothetical gaseous state.

We thereby first confirmed earlier theoretical and experimental results, according to which ethylidyne (CCH_3) is the most stable species, by at least 2.5 eV on each surface.

Next, we studied this most stable ethylidyne species on the two platinum crystal faces. We started by putting it on the "fcc" 3-fold site of Pt(lll), normal to the surface, in the exact geometry that was determined from experiment (by automated tensor LEED in this same research group). Accordingly, the C-C and Pt-C bond lengths were fixed at 1.49 Å and 2.01 Å, respectively. We ran a charge iteration on the valence-state ionization energies (Hii) of platinum and determined better, refined parameter values that we should use for this composite system. In this manner we reduced the excessive electron flow of electrons in the metal slab, one of the drawbacks of the non-self-consistent extended Hueckel method. And thus we calculated the amount of charge shifted to the platinum surface to be 0.215 of an electron charge, for ethylidyne located at the fcc hollow site as described above. Such a charge transfer is consistent with experimental data.

Then we examined the relative stability of ethylidyne on different sites of Pt(111): the fcc hollow (3-fold coordinated), the bridge (2-fold), the hcp hollow (3-fold), and the on-top (1-fold) sites. In each case, at least one Pt-C bond length was kept at 2.01 Å and the C-C bond length at 1.49 Å. Also, the C-C axis was held perpendicular to the surface, and the methyl group of ethylidyne was kept rigid, with the C-C-H angle at 109.5° and each C-H bond length at 1.10 Å. This confirmed that the most stable sites for ethylidyne are the fcc and the hcp hollows, with very little difference between them. The bridge site ranks third, destabilized by 0.85 eV with respect to the fcc hollow, and last the on -top site, 2.47 eV above the binding energy of the fcc hollow position.

We also optimized the Pt-C bond length at every site, keeping the internal geometry of ethylidyne constant. We found the following values for each site:

fcc hollow:	1.84 Å
hcp hollow:	1.84 Å
bridge:	1.74 Å
on-top:	1.51 Å

It is not surprising that our method is underestimating the Pt-C distance, relative to experiment: this is due to the underestimation in the calculated core-core repulsion energies, a familiar feature of extended Hueckel theory, which is always kept in mind when discussing bond lengths.

We proceeded to construct the potential energy curve that characterizes the rotation of the methyl group ($-CH_3$,) of ethylidyne around the C-C axis, when ethylidyne stands in the fcc hollow, at a distance of 1.21 Å from the platinum surface. We calculated an energy barrier of 0.032 eV for rotation of the methyl group, with a local maximum corresponding to the orientation of the methyl group in an eclipsed fashion with respect to the 3-fold arrangement of the three nearest platinum atoms on Pt(lll). This barrier is equal to about 3kT at 90K, and implies rather large angles of rotational vibrations, on the order of 2° of rotation or 0.3 Å hydrogen displacement. This is compatible with our surface crystallography findings of quite large hydrogen vibrations at 90K.

We similarly calculated the potential energy curve that describes the tilt of ethylidyne towards and away from the bridge site. The resulting curve is parabolic near the minimum but no longer symmetric for tilt angles beyond about 20°. The energy increase for a 10° tilt is 0.033 eV, implying a thermally-induced average tilt angle of about 6° at 90K, close to the experimental value of about 7°.

At this point, we started working on enhancing our extended Hückel program with a conjugate direction set minimization routine, based on Powell's method. This would allow us to fully optimize molecular adsorption geometries with respect to energy. This is necessary as we intend to energetically and geometrically characterize the diffusion barriers and pathways of ethylidyne and other species on the metal surface.

With this enhancement, we are currently calculating the lowest energy structure of ethylidyne at a variety of distinct positions on the Pt(111) surface, ranging from highly symmetric to totally unsymmetric ones. At every position we allow the entire ethylidyne structure to relax. Using symmetry considerations of the platinum surface, we will be able to extract the potential energy surface of this adsorbate-substrate system.

B. Zeolites:

Transport Diffusivity Through Nonequilibrium Molecular Dynamics Simulations

Past dynamic simulation work on zeolites, including our own, has focused exclusively on the prediction of the self-diffusivity D_s that characterizes the exchange of labelled and unlabelled molecules in an equilibrium system. Experimental techniques for measuring the self-diffusivity include pulsed field gradient NMR and quasielastic neutron scattering. In most applications, however, diffusion takes place under the influence of concentration gradients. Under these conditions, the quantity of interest is the transport diffusivity D_t, defined as the proportionality constant between flux and concentration gradient in Fick's law.

We have designed and performed non-equilibrium molecular dynamics (NEMD) simulations to study dynamical processes in the sorbate phase in the presence of concentration gradients. Two different computational methods were developed. The first (Gradient Relaxation MD) consists in setting up a periodic concentration profile throughout a model zeolite crystal and tracking the relaxation of that profile to equilibrium through isothermal MD. By fitting the temporal evolution of the profile to the solution of the corresponding continuum diffusion problem, the transport diffusivity is extracted. The concentration dependence of D_t is obtained by repeating the computer experiment at different average concentrations. The second method (color field NEMD) determines the intracrystalline mobility by imposing a weak homogeneous force field on the sorbate molecules and monitoring the steady-state flux induced by the field. From the mobility and the equilibrium sorption isotherm, calculated via GCMC simulation, an estimate of D_t can be obtained based on linear response theory.

We have employed the simple system methane/silicalite to test these ideas. Phenomenologically, the Darken equation has been used to describe the concentration dependence of the transport diffusivity. In this equation, D_t is set equal to a "corrected diffusivity" D_0. times a thermodynamic correction factor (derivative of the logarithm of the fugacity with respect to the logarithm of the intracrystalline concentration at constant temperature) that is obtainable from the sorption isotherm. Despite the widespread use of the Darken equation, there was very little fundamental molecular evidence to support its correctness. An important result from our NEMD work is that, for the system we investigated, the Darken equation provides a good description of the concentration dependence of D_t, with the corrected diffusivity D_0 being practically concentration-independent and equal to the self-diffusivity D_s in the limit of very low occupancy. Our simulations have provided a microscopic understanding of why the self-diffusivity and the transport diffusivity exhibit different concentration dependencies, the former falling and

the latter rising with increasing concentration. The difference arises from the thermodynamic correction term in the transport diffusivity, which is a strongly increasing function of concentration at high occupancies. Of the two non-equilibrium MD techniques we employed, color field NEMD had distinct computational advantages. We have identified the limits to the force fields or concentration gradients that can be used in NEMD without exiting the linear response regime.

The importance of this work lies in that it introduced, for the first time, a concrete framework for predicting transport diffusivity in zeolite systems through molecular simulations; also, in that it provided molecular-level insight into the phenomenological approaches used to relate self- and transport- diffusivity.

Prediction of Sorption Thermodynamics of Aromatic Molecules in Silicalite With a New Grand Canonical Monte Carlo Algorithm

The behavior of aromatic molecules in zeolites of the ZSM-5 family is of critical importance in many catalytic and separations applications. From the point of view of molecular simulation, predicting the sorption isotherms of aromatics in silicalite is a frontier problem. The close fit experienced by aromatics in the zeolite pores makes their motion extremely sluggish, and thus precludes the use of molecular dynamics. On the other hand, the large size of the molecules makes conventional Monte Carlo (MC) methods based on random insertion of sorbate molecules inapplicable, as sorbate/zeolite and sorbate/sorbate overlaps are extremely likely.

We have developed a novel energy-biased and cavity-biased grand canonical MC (GCMC) algorithm for the prediction of sorption isotherms. The energy bias component of the algorithm inserts molecules preferentially in positions and orientations that result in favorable interaction with the zeolite framework. The cavity bias component tracks open spaces within the framework, in which spaces it inserts molecules preferentially; its role is critical at high occupancies. Both types of bias are removed by appropriate design of the selection criteria, so that the MC algorithm correctly samples the probability density of the grand canonical ensemble. Our development of this new MC algorithm has made it possible to predict full isotherms for benzene and *p*-xylene over a variety of temperatures, a feat that is absolutely impossible with conventional GCMC algorithms.

Experimentally, the sorption isotherms of benzene in the temperature interval 0 to 100°C exhibit characteristic "steps" from an occupancy of roughly 4 molecules per unit cell to roughly 8 molecules per unit cell. On the other hand, X-ray diffraction measurements on the silicalite/*p*-xylene system indicate that the zeolite framework undergoes a transformation from Puma (ORTHO-symmetry) to $P2_12_12_1$ (PARA-symmetry). By simulating sorption equilibria in both the ORTHO and PARA frameworks, we were able to show unequivocally that the observed steps in the isotherms are due to this framework transformation, which is induced by the aromatic sorbate molecules at high loadings. Remarkably, the subtle differences between ORTHO- and PARA- frameworks are sufficient to cause significant differences in the siting preferences of aromatic molecules towards the three types of environments present in silicalite and in the maximal occupancies that can be achieved. Our predictions for the isotherms and heats of sorption are in very good agreement with available experimental evidence. Furthermore, our simulations provide a detailed picture of the siting and orientation of the molecules at a variety of occupancies, which helps interpret recent NMR and diffraction data.

The importance of this work lies in the development of a new, powerful GCMC approach that can address the thermodynamics of tightly fitting molecules in zeolites; such molecules are of most interest from the point of view of shape-selective catalytic and separations applications. Application of this approach to aromatics in silicalite systems has helped resolve some puzzles concerning the sorption behavior of these molecules.

Quantum Mechanical Study of an Acid Site in H-ZSM-5 and its Interactions with Ammonia

A major goal of our efforts is to develop an understanding of the sorption and diffusion of organic molecules in real zeolites that contain acidic sites. Our interest in these systems is motivated by the fact that these are the systems that are used in industrial applications involving catalytic conversion and separation processes. Developing an understanding of the thermodynamic and transport characteristics of organic molecules and ions in pragmatically relevant zeolites also presents significant scientific challenges that must be confronted and overcome. In order to take steps toward this goal, it is imperative that we first develop an understanding of the acidic sites in a real zeolite. In FY 93 we have completed such a study of the thermodynamically most stable acid site in the zeolite H-ZSM-5. In addition to the fact that our results compare well with experimental studies, they also serve to elucidate features of the acid site that were unresolved from an experimental viewpoint. In FY 93 we have also initiated a study of the interaction energy hypersurface that characterizes the interactions of ammonia with the acid site in H-ZSM-5 that we have already investigated. In the following we provide the highlights of our results characterizing the acid site, and outline the studies relevant to ammonia adsorption that are currently in progress.

We have characterized the structural and electronic features of the acid site in H-ZSM-5 that is created by substituting aluminum at the T_{12} tetrahedral site. Our results were obtained by performing quantum mechanical calculations within the framework of density functional theory. Specifically, we have performed Kohn-Sham calculations using a code of our own design that invokes the local density approximation. We have studied pentameric clusters (34 atoms) that are embedded in a classical electrostatic field that represents the zeolite framework. Full structural relaxation was allowed in our studies. Our results pertaining to structural features of the acid site (e.g., Al-O distances, proton position) are in remarkable agreement with experiment. One interesting feature pertaining to structure is that the acidic proton is found to be out of the Al-O-Si plane. This counterintuitive result has since been observed experimentally at another laboratory.

We have characterized the electronic features of the acid site in terms of partial atomic charges, polarizability of the electron density distribution in the region surrounding the acid site, and its relative hardness (in the Pearson sense). One interesting feature of these results is that the acidic proton seems to have two possible locations; a strongly bound state and a loosely bound state. This loosely bound state has not been considered heretofore, and its existence may rationalize some existing experimental data. This result also suggests some interesting experiments that could serve to shed further light on the acidic properties of H-ZSM-5.

We have embarked upon characterizing the nature of the interactions of ammonia with the T, acidic site in H-ZSM-5. These studies aim to elucidate how ammonia adsorbs to the acidic center, and to develop an empirical force field for the interaction in question. These studies involve calculating the energy of interaction as a function of various degrees of freedom; e.g., the distance and orientation of the ammonia molecule from the acidic center, and its internal configuration. Thus, we must perform many quantum mechanical calculations involving the embedded cluster and the ammonia molecule in order to map out the energy hypersurface. In order to make possible the routine calculation of these energy hypersurfaces, in FY 93 we directed considerable effort toward improving the efficiency of our computer code. Several changes were incorporated that have led to a reduction of computer time by a factor of roughly five. Using this improved code we are now performing calculations to determine the energy hypersurface for the interactions of ammonia with H-ZSM-5. Our early results suggest that a bidentate adsorbed structure will be favored.

4.1.2 Biological

5. Project Title: Genetic Engineering of Methanogenic Bacteria for Industrial Chemical Applications.

Principal Investigator: L. Baresi

Project Site: Jet Propulsion Laboratory and
University of California, Los Angeles.

Description:

New breakthroughs in genetic engineering and knowledge of fundamental synthetic pathways in biological systems have made feasible the controlled methanogenesis (decarboxylation of acetic acid and higher molecular weight acids to methane) for industrial applications. To that end, this project seeks to induce anaerobic bacteria to operate under abnormal toxic conditions (viz., in the presence of oxygen) by genetic engineering, using a "shuttle vector" to produce this new property. A shuttle vector is any cloning vehicle that can replicate in more than one organism. This shuttle vector (usually a plasmid) introduces DNA into the host organism; it functions to move genetic information from one organism to another. Plasmids are small molecules of circular double-stranded DNA that replicate independently of the chromosomal DNA, and are found in some bacteria. The project will use the model anaerobic organisms *Methanobrevibacter smithii* and *E. coli.*

The project will develop strategies for genetic engineering of methanogens for commercial purposes. It will employ the recent software package Metabolic Pathways Systems (MPS) developed by Professor James Bailey at the California Institute of Technology for biocatalytic pathways within a cell (intracellular). The project will expand the use of this artificial intelligence program to reach a solution for complex strategies on intercellular metabolism. The approach is coupled with genetic manipulation to favor specific pathways described above. This simulation strategy will greatly aid the understanding of the microbial population interactions and the selection of target products for combined ensemble of microbial communities (before and after genetic manipulation) towards a high activity, necessary for the use of the pathway in the purposeful production of chemicals. The project will enunciate potentially useful pathways in a time- and cost-effective way, avoiding time-consuming empirical experimentation.

1993 Accomplishments:

Work on the generation of a phasmid for use as a shuttle vector has started. Work on getting the MPS program operational is continuing. A data base is being compiled which includes both pathway information from *E. coli* and methanogens in general.

1994 Planned Activities:

The MPS program will be employed to generate new data bases containing information on organisms, enzymes, and substrates. Work on generating a shuttle vector will continue by the introduction of phage DNA into the tetracycline site of the pBR 328 vector keeping the original *ORI* site intact. The puromycin gene (gene marker for methanogenic bacteria) will be introduced into these methnophage-pBR 328 composites and each tested for transformation of *M. smithii* and *E. coli.*

Annual Technical Report:

Work on the generation of phasmid has started. The *E. coli* vector, pBR 328 has been modified eliminating its *ORI* site (*ORI*$^-$), this is the region of the plasmid that initiates replication. DNA from the methanophage has been ligated into the pBR 328 (*ORI*$^-$) DNA at the missing replication site and tested for its ability to transform *E. coli*. Thus far these DNA composites have not been able to transform the host *E. coli* cells. There are several possible reasons for this. For example, the ORI site from the methanophage might be present but can't be used to initiate replication in *E. coli* or the methanophage ORI site has not been secured in the first place or the methanophage ORI site is located at several points in the original DNA and the complete segment is not being recovered. Because of this a new strategy is being employed where the methanophage DNA is being introduced into a second site of the original pBR 328 vector. Because the original ORI site is present, these newly constructed phasmids should successfully transform the host *E. coli*. After obtaining a methanophage library a gene used for selection of the phasmid in the methanogen will be introduced and these new phasmids will be used to transform *M. smithii*. Success will insure a vector that can replicate both in *E. coli* and *M. smithii*.

Work on getting the MPS program operational is continuing. A data base is being compiled which includes both pathway information from *E. coli* and methanogens in general.

6. **Project Title:** Chemistry, Immunology, and Modeling as Tools for the Rational Design of Enzymes.

Principal Investigators: P. G. Schultz and M. Levitt

Project Site: Lawrence Berkeley Laboratory

Description:

Recently it has been shown that the diversity and specificity of the immune system can be tapped to produce highly selective catalysts. Because antibodies[7] can be generated that selectively bind almost any molecule of interest, this new technology offers the possibility of generating tailor-made catalysts for applications in chemistry and biology. The specific objective is to develop methods for generating antibodies that bind substrate and a reactive cofactor[8] in a catalytically productive fashion. The project engineers antibodies that contain metal binding sites in order to carry out redox and hydrolytic reactions with antibodies. A combination of theoretical modeling and recombinant DNA methodology is being used to design and introduce the metal binding site. A second class of cofactors of interest are reducing agents such as metal hydrides which are involved in industrially important reactions such as carbonyl reductions. Antibodies are being generated to catalyze metal hydride-dependent stereospecific carbonyl reduction. Molecular modeling is used to understand the structural and electronic features of hapten and antibody required for enzymatic activity.

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[7] Antibody: an immunoglobulin present in the serum of an animal and synthesized by plasma cells in response to the invasion by an antigen, conferring immunity against later infection by the same antigen.

[8] Cofactor: a small, non-protein molecule, that associates with the protein portion (apozyme) of an enzyme and is essential for the enzyme to function.

1993 Accomplishments:

The remarkable specificity of an antibody has been used to accomplish highly selective functional group transformations not readily attainable by current chemical methods. An antibody raised against an N-oxide hapten catalyzes the regiospecific reduction of a diketone to a hydroxyketone with greater than 75:1 selectivity for one of two nearly equivalent ketone moieties. Moreover, the antibody-catalyzed reduction is highly stereospecific, affording the hydroxyketone in high enantiomeric excess. The simple strategy presented herein may find general applicability to the regio-and steroselective reduction of a broad range of compounds. We are currently extending this chemistry to simple aliphatic ketones and heteroatom containing substrates.

1994 Planned Activities:

(i) Characterize ability of antibodies generated against aliphatic N-oxide to catalyze the asymmetric reduction of simple dialkyl ketones.

(ii) Characterize ability of antibodies generated against aliphatic sulfoxide to catalyze the asymmetric reduction of simple dialkyl ketones.

(iii) Characterize kinetics, specificity and mechanism of antibodies that catalyze sulfide oxidation.

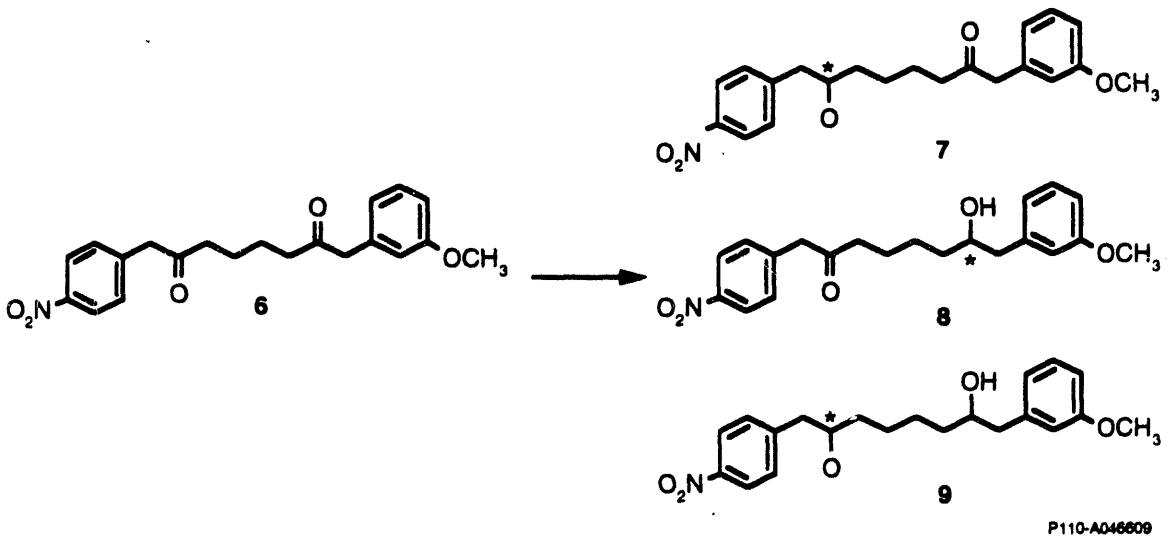
(iv) Complete cloning of McPC603 metal binding antibody and generate transgenic mouse.

(v) Generate antibodies against five and six-membered ring cyclic amine haptens and characterize the glycosidase activity of the resulting antibodies.

Annual Technical Summary Report:

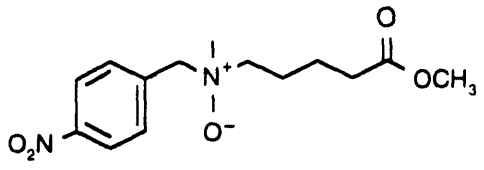
Regio- and stereoselective reductions

Reactions which involve both regio- and stereoselective control, for example, the selective reduction of diketone **6** to a single enantiomer of hydroxyketone **7** are difficult or impossible to carry out via known chemistry. The similar chemical environments of the two carbonyl moieties in the substrate (distinguishable only by methoxy and nitro groups five and six atoms away) render this transformation extremely difficult to achieve by known chemical methods. In order to catalyze the regio- and stereoselective reduction of **6**, antibodies were raised against N-oxide hapten **10**.



P110-A046609

These antibodies should not only stabilize the tetrahedral transition state resulting from nucleophilic attack of hydride on the carbonyl group, but also direct regioselective addition of hydride to the nitrobenzyl substituted carbonyl group of substrate **6**. Moreover, the chiral environment of an antibody combining site induced by one of the two enantiomers of hapten **10** should discriminate between the enantiotopic faces of a prochiral substrate, affording a highly stereoselective reduction. In order to avoid the need for cofactor recycling, the inexpensive reductant sodium cyanborohydride ($NaBH_3CH$) was chosen as the hydride donor for the antibody-catalyzed reaction.



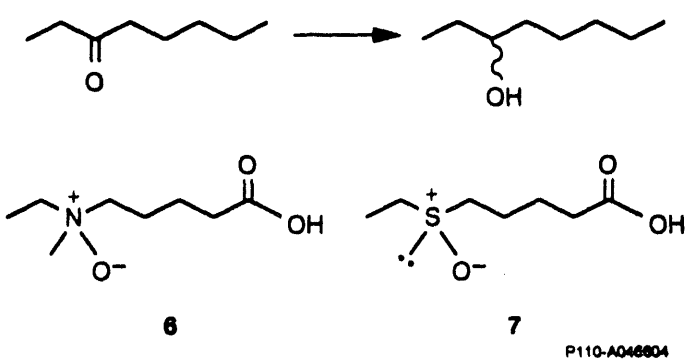
10

P110-A046601

A number of antibodies generated to N-oxide **10** catalyzed the $NaBH_3CN$ dependent reduction of ketone **6**. One antibody, 37B.39.3, catalyzed the reduction regioselectively with greater than 75:1 selectivity for one of the two nearly equivalent ketone moieties. Moreover, the reaction was highly stereoselective affording the (S) enantiomer of the hydroxyketone in 96.3% enantiomeric excess. In contrast, the nitrobenzyl carbonyl group was reduced more slowly than the methoxybenzyl carbonyl group in the uncatalyzed reaction (V_{rel} = 0.74). The overall yield of hydroxyketone (2) -7 was 94%, which is significant in light of the fact that the background reaction produces eight products.

Antibody 37B39.3 also catalyzed the stereoselective reduction of a number of substituted benzylketones including 1-ρ-nitrophenylbutan-2-one **11** (96% ee) and 1-ρ-nitrophenyl- 3-phenylpropan-2-one **12** (87% ee), in which the carbonyl substituents are distinguishable only by the *para* nitro group. The second order rate constant, $k_{cat,app}/K_{m,app}$, for the antibody catalyzed reduction of **11** was 1.9×10^3 $min^{-1}M^{-1}$, a considerable acceleration over the uncatalyzed reaction, $k_{uncat} = 1.1 \times 10^{-3}$ min^{-1} M^{-1}.

We are now targeting the enantioselective reduction of 3-octanone. Since the catalyst must discriminate between ethyl and pentyl alkyl side chains, this reaction is difficult to accomplish using conventional chemical reagents. Amine-oxide hapten **6** was synthesized by reacting monomethyl glutarate with N-ethylmethylamine in the presence of EDC to form the amido-ester. Reduction of the amide with borane



6 **7**

P110-A046604

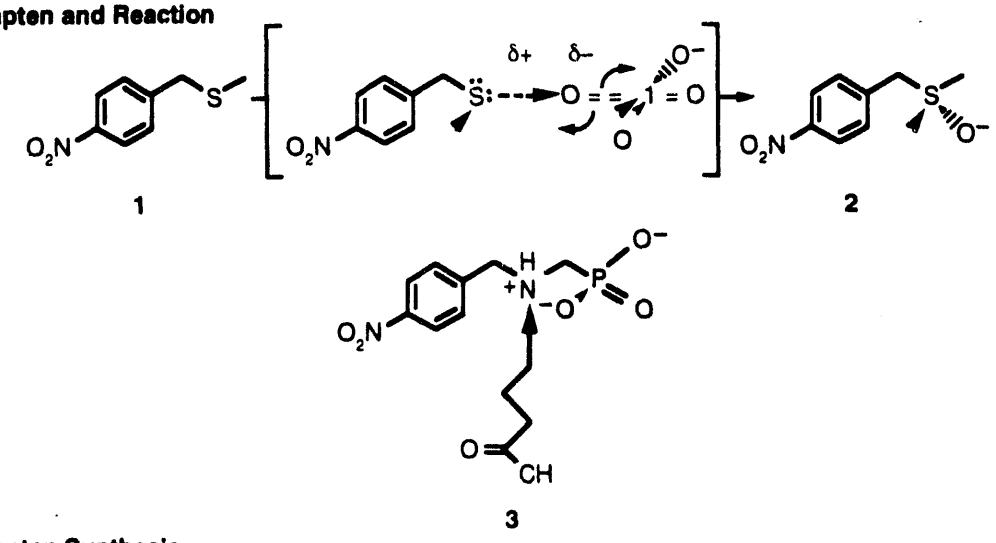
gave the amino-ester, and saponification of the ester followed by mCPBA oxidation gave hapten **6**. Since hapten **6** was synthesized as a mixture of enantiomers, both (R)- and (S)- reductases are expected. Twenty-four antibodies have been generated and are currently being purified by protein A affinity chromatography. These antibodies are being assayed at pH 5.0 in the presence of $NaCNBH_3$ or $NaBH_4$

by gas chromatography (GC). Concurrently, twenty-two monoclonal antibodies specific for sulfoxide hapten **7**, an isoelectronic analog of hapten **6**, have been elicited and will be assayed for the same reaction.

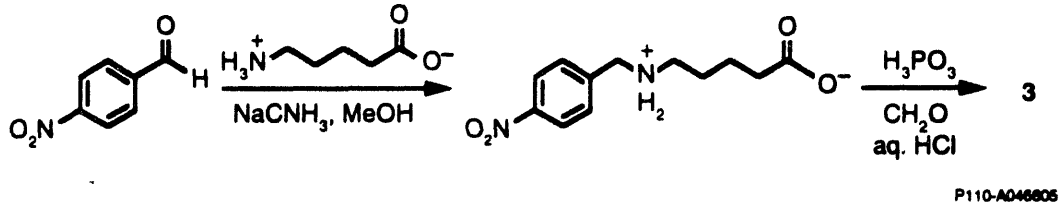
Asymmetric heteroatom oxidation

Given the programmable nature of the binding energy and specificity of the immune system, we asked whether simple yet general strategies exist for generating antibodies that catalyze stereoselective oxidative reactions of heteroatoms. Such reactions are important in many industrial chemical processes. As a model system we chose the oxidation of p-nitrobenzylmethyl sulfide (**1**) to sulfoxide **2** using sodium periodate ($NaIO_4$). This reaction has been proposed to proceed through an S_N2-like transition state resulting in the electrophilic oxygen-transfer from the periodate anion to the bivalent sulfur atom. Hapten **3** is expected to mimic the transition state for the reaction since it incorporates a positively charged amine to mimic the incipient positive charge on sulfur and a phosphoric acid moiety to accommodate a periodate molecule in the antibody combining site. The hapten was readily synthesized by the reductive amination of p-nitrobenzaldehyde with 5-aminovalerate in the presence of sodium cyanoborohydride followed by reaction with phosphorous acid in the presence of formaldehyde and aqueous hydrochloric acid.

Hapten and Reaction



Hapten Synthesis



P110-A046605

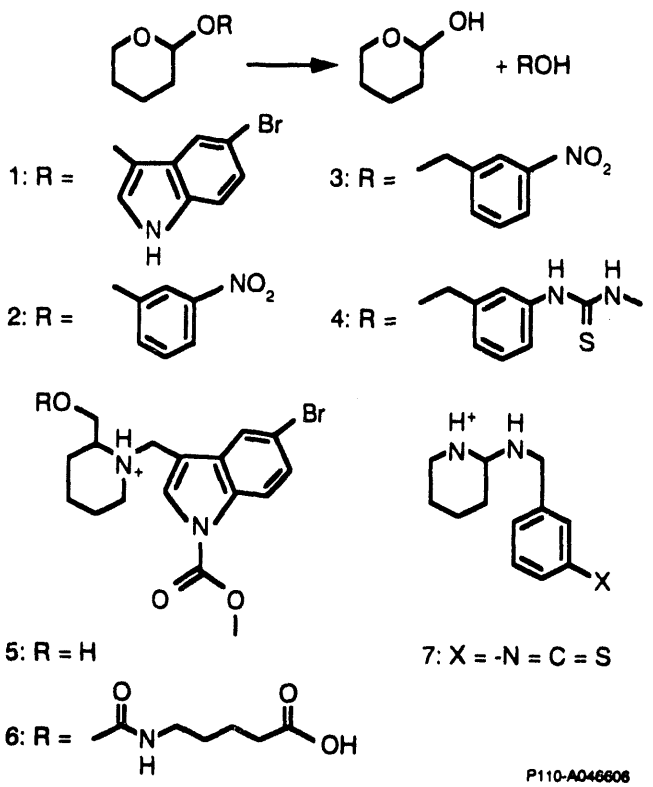
Forty-five monoclonal antibodies specific for hapten **3** have been elicited and are currently being screened for sulfide oxidation in aqueous buffer at pH 5.5, 7.1, and 8.0 containing $NaIO_4$ and 5% (v/v) ethanol. Product formation is monitored using high-performance liquid chromatography. One of the five antibodies screened thus far accelerates the oxidation of substrate **1**. This antibody exhibits $NaIO_4$-dependence and is inhibited by free hapten. After screening the remainder of the antibodies, the enantioselectively of these antibody-catalyzed reactions will be characterized.

Approaches toward the generation of antibodies with glycosidase activity

The development of antibodies that catalyzed the sequence specific cleavage of oligosaccharides would greatly facilitate the characterization and manipulation of carbohydrates. One mechanism for the

enzymatic hydrolysis of oligosaccharides is thought to involve the general acid-catalyzed expulsion of the leaving group to form a half-chair oxocarbenium ion that is stabilized by an active site carboxylation anion. In an effort to generate antibodies with glycosidase activity, monoclonal antibodies were generated against transition states (mimics) for the hydrolysis of the model cyclic acetals **1-4**.

The design of hapten **6** is based on known cyclic amine glycosidase inhibitors such as nojirimycin and castanospermine. The positively-charged ammonium ion is expected to induce groups in the antibody combining site that stabilize the charge delocalized transition state involved in the hydrolysis of substrate **1**. The 5-bromoindoyl leaving group should provide a simple chromogenic assay of cell culture supernatants for catalytic activity and a recognition element for substrate. N-substitution is expected to place the indoyl group of the hapten in the same relative position as that of the indoyl group in the more labile axial conformer of substrate **1**. Antibodies were also generated against a second hapten **7** in an effort to catalyze the hydrolysis of substrates **2-4**. This hapten is based on the potent amidine containing glucosidase inhibitors which are thought to mimic both the half chair conformation as well as the positive charge in the high energy oxocarbenium ion intermediate. In this case, the metasubstituted phenyl group provides the chromogenic assay and a substrate recognition element. The benzylic leaving group insures protonation of the amidine group (and possible represents an "exploded" transition state analogue for the hydrolysis of substrate **2**).

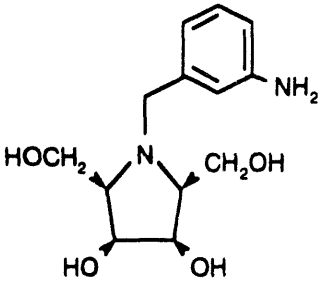


P110-A046606

In order to synthesize hapten **6**, a formyl group was introduced into the 3-position of 5-bromoindole ($POCl_3$/DMF) followed by protection of the indole ring with dimethylpyrocarbonate. The formyl group was reduced to the alcohol ($NaBH_4$), converted to the bromide (PPh_3Br_2), and reacted with 2-hydroxymethyl piperidine to give **5**. Treatment of **5** with methyl-5-isocyanovalerate followed by saponification afforded hapten **6**. Hapten **6** was linked to carrier protein via an N-hydroxysuccinimide ester. Hapten **7** was synthesized by treatment of δ-valerothiolactam with 3-nitrobenzylamine under anhydrous conditions. Acidification followed by hydrogenation over $Pd(OH)_2$ and subsequent reaction with thiophosgene afforded the isothiocyanate. Balb/c mice were immunized with the KLH (keyhole limpet hemocyanin) conjugates of haptens **6** and **7**.

Cell culture supernatants and crude ascites from cell lines specific for hapten **6** were directly assayed for hydrolytic activity using substrate **1**. Ten cell lines showed activity in cell culture supernatants and eight of these had activity ascites fluid. The antibody AA71.17 was characterized further after purification by protein A affinity chromatography and mono Q ion exchange chromatography. Hydrolysis of substrate **1** by antibody AA71.17 was performed in 10 mM MES [2-(N-morpholine)ethanesulfonic acid] or EPPS [N-2-hydroxyethyl)piperazine-N'-3(3-propanesulfonic acid)] buffer at pH 5.5, 7.0 or 8.5 with the ionic strength adjusted to 100 mM NaCl. The antibody-catalyzed hydrolysis showed saturation kinetics with k_{cat} = 0.904 h^{-1} and K_M = 324 μM (pH 5.5) (Fig. 1). The second-order rate constant for the background hydrolysis of substrate **1** by the general acid, AcOH, was found to be 1.3 × 10^{-4} $mM^{-1}h^{-1}$. The reaction was inhibited by hapten **6** with a K_i = 35 μM as determined from a Dixon plot. The k_{cat} values at pH 7.0 and 8.5 were 0.0913 h^{-1} and 1.00 h^{-1}, respectively, showing that the antibody reaction is also general base-catalyzed. The second-order rate constant for the hydrolysis of substrate **1** by AcO^- was measured to be less than 1.61 × 10^{-5} $mM^{-1}h^{-1}$.

Antibodies specific for hapten **7** were purified by protein A and mono S ion exchange chromatography and assayed for their ability to catalyze the hydrolysis of substrates **2, 3,** or **4** in 50 mM MES, 50 mM NaCl, 0.02% NaN_3 buffer, pH 5.5. Substrate **4** was also assayed in 10 mM borate, 100 mM NaCl buffer at pH 8.25. Surprisingly, no catalytically active antibodies were found. One possible explanation is that the planar nature of the amidinium ion in the hapten may not allow a favorable axial geometry for the leaving group in the substrate. We have now initiated the generation and characterization of antibodies



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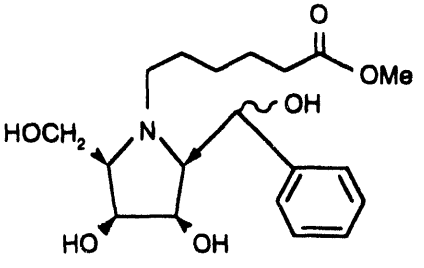
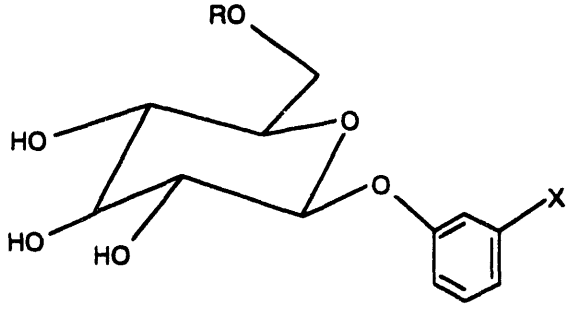


11

P110-A046602

specific for haptens **10** and **11** for their ability to hydrolyze β-glycoside **12**. If successfully, we will incorporate these design elements into a polymeric cellulose-like hapten.



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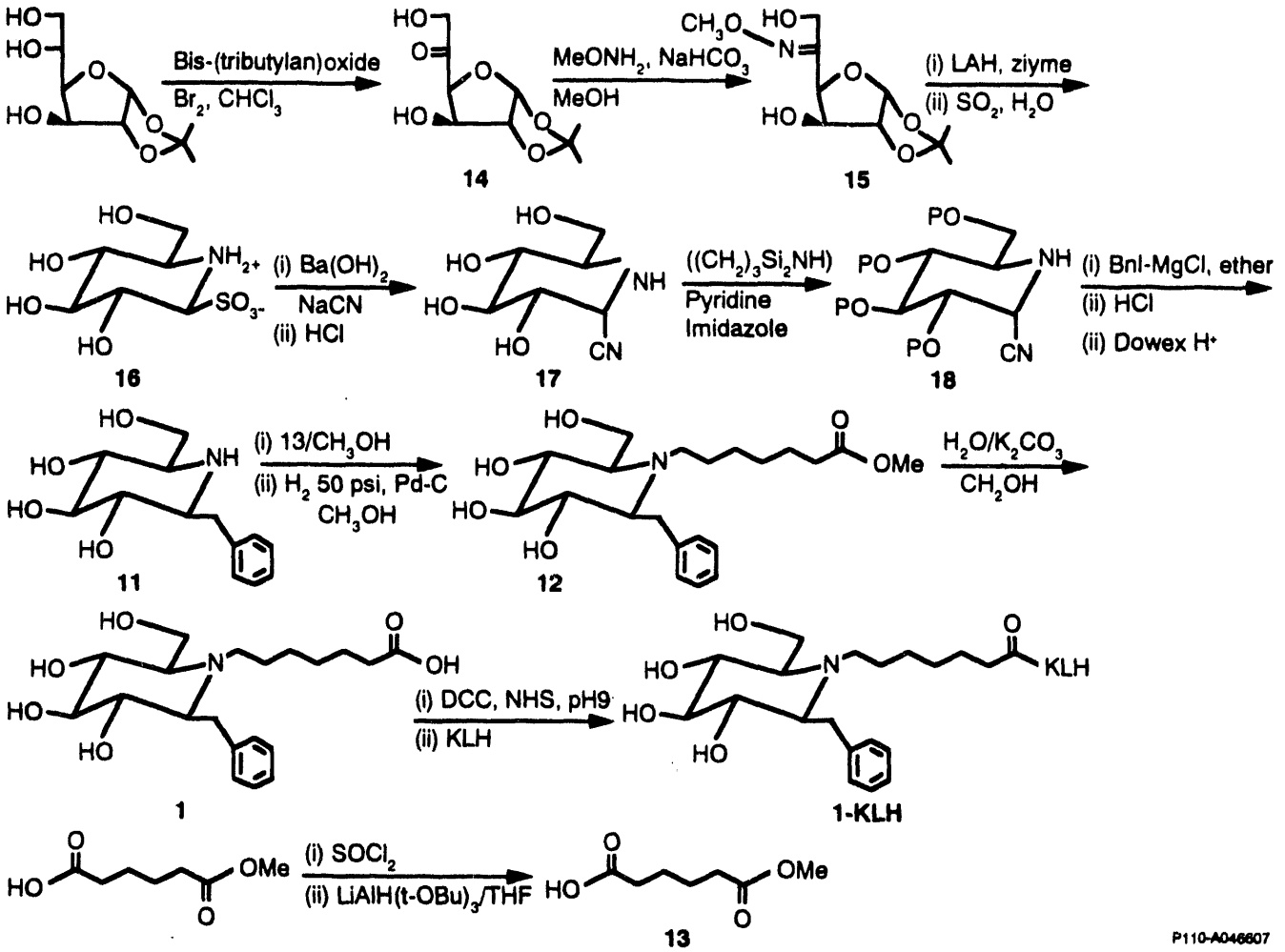
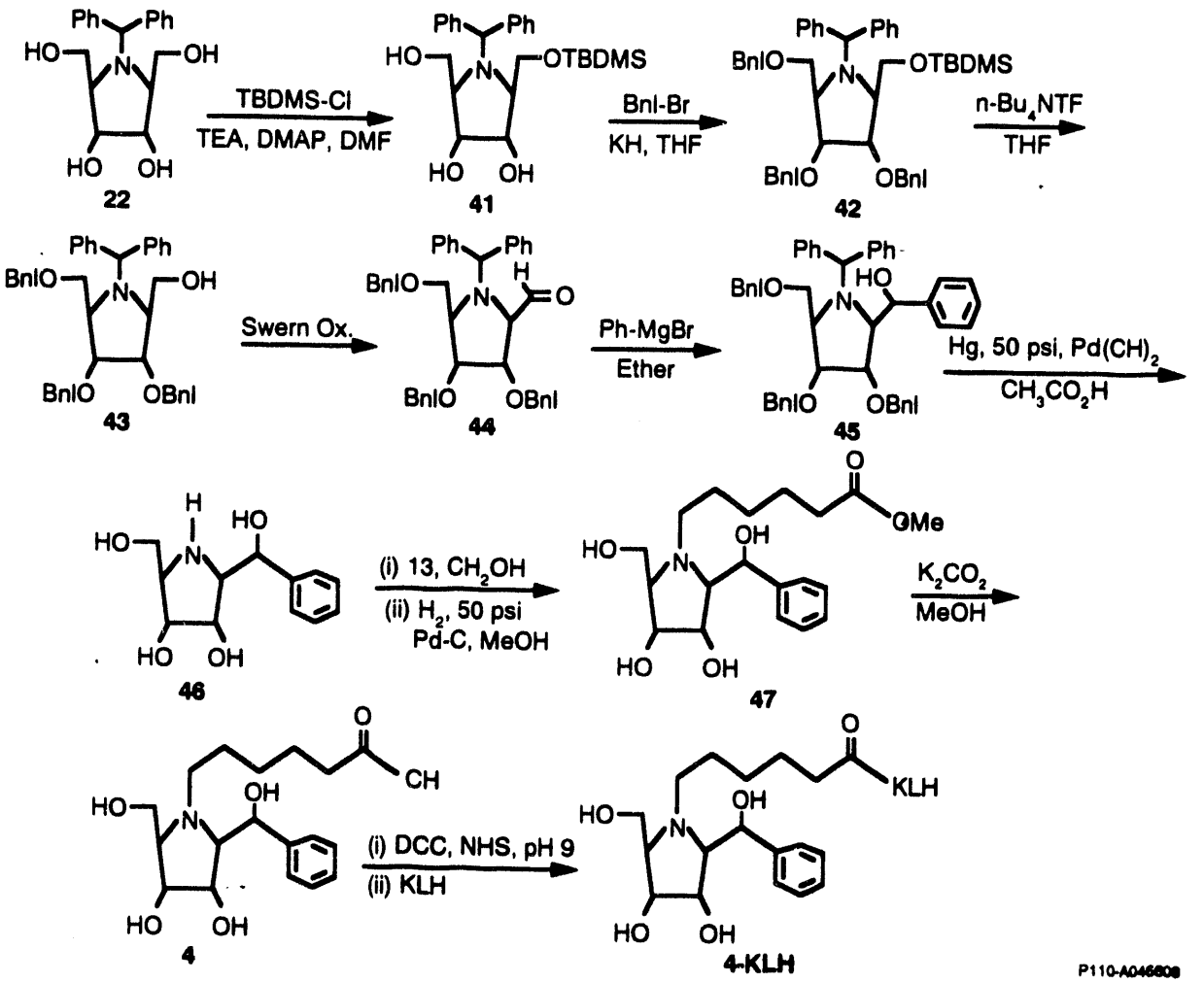
Haptens **10** and **11** have been synthesized, conjugated to the carrier proteins KLH and BSA and hybridoma production is in progress.



Metal Binding Antibodies

In collaboration with the Scripps groups a metal binding light chain has been cloned into a suitable expression vector for production of transgenic mice expressing metallo-binding antibodies. Fertilized mouse embryos were microinjected with this clone and subsequently implanted into foster mothers. DNA and serum κ chains of the offspring have been shown to express the metallo light chain in active B-cells in the spleen. We are currently immunizing with a variety of haptens in order to elicit an immune response. Theoretically, the antibodies generated should contain the transgenic light chain and a hapten specific rearranged heavy chain, directly affording a metallo catalytic antibody. Metallo antibodies of this sort may find use in catalysts in many energy intensive processes including hydrolytic reactions of polysaccharrides (cellulose).

In addition, a number of Zn^{2+} binding McPC603 light chains have been designed based on the antiparallel β-sheet motif of the metalloenzyme carbonic anhydrase. The metal ion has been placed at three different sites in CDR3 of the light chain. We are continuing our cloning efforts on this project.


Ph Ph
HO OH
HO OH
22
TBDMS-Cl
TEA, DMAP, DMF
OTBDMS
41
Bnl-Br
KH, THF
BnlO
42
n-Bu4NTF
THF
43
Swern Ox.
H
O
44
Ph-MgBr
Ether
HO
45
Hg, 50 psi, Pd(CH)2
CH3CO2H
46
(i) 13, CH2OH
(ii) H2, 50 psi
Pd-C, MeOH
OMe
47
K2CO2
MeOH
CH
(i) DCC, NHS, pH 9
(ii) KLH
4
KLH
4-KLH
P110-A046608

7. **Project Title:** Theory of Biocatalysis - Electron Transfer Reactions

Principal Investigator: D. N. Beratan

Project Site: University of Pittsburgh

Description:

The principal project goal is the computer-aided design of enzyme biocatalysts with tailored catalytic rates. The focus of this project has been on electron transfer enzymes, a major class of biological enzymes. In this project, the theory of *protein electron tunneling pathway* has been developed, implemented as computer models, which connect enzyme electronic structures and their reaction rates.

Specific project objectives include: 1) develop algorithms to map the key residues in proteins between electron transfer sites that mediate electronic coupling and allow electron transfer reaction to proceed with great speed and specificity: 2) identify "hot" and "cold" spots with respect to electron transfer in native and modified proteins; 3) develop an understanding of primary, secondary, tertiary, and quaternary structural effects on electron transfer rates; 4) use knowledge gained to stabilize energetic charge separated states and to enable the development of semisynthetic/modified protein energy conversion systems.

1993 Accomplishments:

In order for the potential applications of biological electron transfer reactions to become of use in biocatalytic applications, one needs to develop a quantitative understanding of the molecular structure/property relationships for these materials. With this goal in mind, we are developing theoretical methods to (a) map out the electron transport routes ("tunneling pathways") in enzymatic proteins, DNA, and other macromolecules; (b) develop strategies to harness the stored energy of the separated charge by manipulation of the pathways; (c) test related methods on less well organized chemical systems to determine their applicability to relatively simple electron transfer based systems.

The last year has been extremely productive. Specific accomplishments include (1) development of software to compute the role of multiple coupling pathways in mediating electron transfer in proteins; (2) development of numerical and graphical software for performing reduction of complex chemical structures to represent their "essential" connectivity with respect to electronic coupling mediation; (3) determination of the mechanism and base pair dependence of electronic coupling in DNA bridged donor-acceptor systems; (4) introduction of the new concept of tunneling pathway phase transitions in disordered materials and investigation of their importance in the design of organic polymer based electron transfer systems.

1994 Planned Activities:

Our plans for the next year will include three major thrusts. First, we will continue our calculations that probe the role of multiple coupling pathways in protein and DNA. This work is absolutely critical from the standpoint of understanding the success of our simple tunneling pathway models and for understanding how the model might be limited. Second, we will investigate specific protein and DNA structures to map the bridge dependence of the electronic coupling using both pathways and green's function (multi-pathway) models for electronic coupling. In proteins, the success or failure of the pathway model depends on secondary and tertiary structure. DNA is more nearly a periodic "one-dimensional" material. We plan to determine just how the base sequence, presence of water in the major and minor grooves, location of donors and acceptors (whether intercalated or attached to the ribose-phosphate backbone), and distortion from the idealized geometry influences the coupling. We will dissect the coupling mechanism into sigma

and pi-electron components. Third, we will continue the new direction (initiated this year) of electron transfer in disorganized materials like organic polymers and starburst dendrimers. There is increasing experimental interest in systems like this, and we plan to work closely with our experimental collaborators on this project.

Annual Technical Summary Report:

In the first phase of this year's research we focused on two new types of macromolecule electron transfer systems, nucleic acids and starburst dendrimers. These were chosen to broaden the understanding of electron transfer in macromolecules that was gained from our earlier studies of proteins. I am especially excited about the idea of exploiting electron transfer reactions to probe subtle intra- and inter-molecular interactions in these novel materials. For example, I believe that electron transfer reactions in polymer and dendrimer films will eventually be used to reveal information about the materials' structure and dynamics. And such information will aid our ability to design biocatalytic systems. Similarly, I believe that electron transfer rates in nucleic acids, and in their complexes with proteins, will be diagnostic of base sequence and the nature of intermolecular interactions. The possibility of using the simple "one-dimensional" nucleic acids as frameworks for charge separation is being considered for the first time at present. The area of DNA electron transfer experiments is about to expand drastically (from 2 or 3 groups with uninspiring preliminary data, to about 6 active groups with important new experiments), and there is essentially no theoretical work in this area going on outside of my own research group. Our preliminary results indicate important dependence of electron transfer rates on the sequence of intervening bases and on the disposition of the major and minor grooves of the double helix with respect to electron donors and acceptors. These results are being prepared for publication.

Having completed our exploratory research on electron transfer in DNA, our focus turned to starburst dendrimers. Starburst dendrimers have the connectivity of Bethe lattices or Cayley trees. These species are of particular interest because they are partially ordered. That is, their backbones consist of identical repeating units (just like in linear polymers), but the materials fold into somewhat disordered 3D structures. Because starburst dendrimers have three or more branches coming from each repeat unit (conventional linear polymers have only two such units), the electronic coupling characteristics of these materials are unique.

We have found that because of the branching in these structures, the wave function amplitude splits at each branch leading to more rapid decay of coupling with distance than in the corresponding linear materials. This effect could be used to control the degree of charge localization in macromolecule based electron transfer processes. This localization is even seen in the "band" states of the materials, which in the corresponding idealized 1D linear polymers would correspond to fully delocalized Bloch states. These results are being prepared for publication.

With preliminary calculations on DNA and starburst dendrimers completed, we have returned to our work on protein electron transfer coupling calculations. Here our goal is to investigate the importance of secondary structure effects and quantum interference on protein electron transfer reactions. We demonstrated that a full 5,000 orbital (all valence orbitals, independent electron level) is accessible using modern supercomputers (Pittsburgh Supercomputer Center Cray YMP-C-90). These new methods allow the calculation of long-range electronic couplings (and rates) in proteins the size of cytochrome *c*. With improved independent electron parameters (coming from other quantum chemistry groups), reliable calculations of electronic couplings involving *all* pathways and summations of "scattering" interactions to all orders should become accessible in the near future. Preliminary results suggest the nature of quantum interference effects in protein electron transfer will be qualitatively different in α-helix vs. β-sheet structures. This effect could be used to control the directionality and specificity of electron transfer in biocatalytic systems. These results are being prepared for publication.

8. **Project Title:** Predictive Models and Effects of Structure on Catalytic Properties

Principal Investigator: W. A. Goddard III

Project Site: California Institute of Technology

Description:

Project is directed to the development of tools for atomic scale modeling and simulation of biological systems, and the prediction of critical parameters for experimental validation of the models. Project will also assess the critical impediments to the widespread application of biotechnology in industry, and will examine possible approaches to overcoming the barriers, using the tools developed at the Materials and Molecular Simulations Center (MMSC). The MMSC was established as a place where academic, government, and industrial researchers can work together on theoretical studies related to catalyst design and structure using molecular dynamics, quantum mechanics, and computer graphics capabilities. The MMSC is a means of technology transfer between the researcher and the end user. The needs of industry therefore direct the project goals and orientation.

Methods and techniques to permit the prediction of the structure and hence function of proteins are under investigation. Project goals include the design of proteins that recognize specific DNA sequences. These proteins would be used to develop regulatory proteins for controlling biocatalysts. In particular, the pseudo-spectral generalized valence bond (GVB) techniques will be used. The current chemical mechanical calculations, the first step in molecular mechanics simulations, are extremely CPU intensive. Consequently, these calculations are restricted to molecular systems that are small (10-20 heavy atoms). However, most industrial applications of interest are much larger in number of atoms of interest and are thus much more computer intensive (in terms of method of calculation) than is practically feasible. Recent developments in the pseudo-spectral GVB techniques, which use ideas originally developed to solve fluid dynamics simulations, have made it more feasible to calculate systems of 100 atoms, which would facilitate substantially *ab initio* calculations on crystals and systems with periodic boundary conditions. Massively parallel computing capabilities will be necessary to accomplish this goal and to expedite these calculations into reasonable cost/time operations.

1993 Accomplishments:

1. Protein Stitchery for Biochemical Synthesis and Design. We developed a strategy for designing proteins to recognize particular base-pair sequences of DNA and proved it experimentally for a 10 base pair (bp) site and a 16 bp site. This is the first time that a regulatory protein has been successfully designed.
2. The Repressor of Primer (ROP) Based Framework for Biochemical Design. We designed a four-helix bundle system (based on the ROP protein) that should be ideal for designing stable framework molecules for catalytic and electron transfer applications.
3. MD//PT. We developed Molecular Dynamics Perturbation Theory (MD//PT) computer software and optimized it for use on the Kendall Square Research parallel supercomputer.
4. Using MD//PT, we showed that the free energy cost in having the wrong base pair at the active site decreases the free energy of binding of netropsin to DNA by 2.9 to 3.1 kcal/mol depending on which of the four base pairs in the active sites is modified. This is the first time that such a quantity has been accurately determined (theory or experiment).
5. Biomineralization. Models for Polyelectrolyte Mineral Matrix Protein (PMMP), which stereospecifically coordinates divalent cations and counterions and initiates directional growth of inorganic crystals, and showed that:

a. Asp_n forms supercoiled conformers, with a 2:1 ratio of β-turn to β-strand dihedral angles;
b. $(PSer)_n$ and $(PSerAsp)_n$, both exhibit a 1:2 ratio of turn-to-strand preference, this results in the formation of hairpin conformers for $PSer_n$, and spiral ("distorted" hairpin) conformers for $(PSerAsp)_n$.

6. Two additional companies BF Goodrich (Polymers) and Vestar (vesicles for drug delivery) became collaborators of the Materials and Molecular Simulation Center (initiated with DOE-AICD funding) in the Beckman Institute at Caltech.
7. A patent was filed on the new Cell Multipole Method (CMM) technology developed with partial funding from DOE-AICD. This technology allows atomistic simulation on 1 million particle systems (e.g., a complete rhinovirus).

1994 Planned Activities:

The objective of our program is to improve the technology base for providing enhanced chemically and biologically based processes for energy production and conservation.

Thus we will continue:

- developing and validating the computer-aided tools required for molecular modeling of enzymatic biocatalytic processes useful for industrial and DOE end-use applications.
- illustrating the strategies and techniques in using these tools, and
- developing specific models by tackling the applied problems of general importance to industry or of specific interest to other AICD supported projects.

This involves:

- developing necessary tools for atomistic modeling and simulation,
- validating the tools by application to properties and systems amenable to experimental verification,
- illustrating the strategies and techniques in using these tools by applying them to design of biocatalysts and to other relevant problems,
- developing solutions to critical bottlenecks for industrial applications of such biocatalysts,
- assist other DOE-AICD biotechnology projects with support and guidance on theory, modeling, and simulation.

Specific projects to be emphasized include:

- The Hierarchical Protein Folding Strategy (HPFS). We continue to develop and apply the HPFS to first principle calculations of protein conformations (tertiary structure) from primary sequence.
- Protein Stitchery for Biochemical Design. We will expand this approach to the design of proteins from 22 base pair (bp) sequences and develop a general library for applications.
- The ROP Framework for Biochemical Design. We will work with Professor Peter Schultz of Berkeley to carry out the gene expression and cloning and the protein expansion, purification and characterization required for synthesizing the variants of ROP. Tests of the design will be done the first year.
- We will further optimize the MD//PT program for parallelized molecular dynamics perturbation thermodynamics. We will make this more user friendly by building a graphic interface.

Annual Technical Summary Report:

Protein Stitchery

Based on modeling studies carried out with previous DOE-AICD support, we developed a basic strategy, called *Protein Stitchery*, for designing *new* proteins to recognize new specific sites of DNA. All previous studies in the literature have focused on systems provided by nature. Our first paper on Protein Stitchery designed a new protein for a new DNA sequence. We synthesized the protein and target oligonucleotides and used gel retardation assays to show that the protein binds to the target DNA site with a binding constant of about 1 mm but does not bind to other similar bases. From DNAase I footprinting we showed that the predicted binding site is indeed protected by the new protein, proving the design. *This was the first successful demonstration of the design of a new protein to recognize a specific site of DNA.*

The basic idea in Protein Stitchery is to use knowledge from experiment of which protein sequence selectively recognizes particular DNA segments and to combine these to stitch together a long protein that recognizes specific long DNA sequences.

Using Protein Stitchery we have now synthesized a 97 amino acid peptide (p-CC-NC), which we had designed to recognize a new 16 base pair site of DNA (o-CC-NC). This synthesis was achieve by stitching together three 31 amino acid segments as illustrated in Figure 2. We then carried out gel retardation assays (Figure 3) and DNAse I footprinting (Figure 4). These results show that the 16 bp site is perfectly protected by the protein, proving that the new protein bonds exactly to the target site.

Atomistic Simulations of Superlarge Macromolecules

Earlier developments (partially supported by DOE-AICD) involved developing:

- the CMM method (Cell Multipole Method) for rapid and accurate calculation of nonbond interactions for superlarge systems (it has been successfully used for systems with over 1 million atoms) and
- the NEIMO method (Newton-Euler Inverse Mass Operator) which allows rapid solutions of the dynamics in terms of internal coordinates (e.g., torsions).

For large molecules, these new methods are several orders of magnitude faster than previous methods, opening up the opportunity for modeling and designing of realistic biocatalysis systems.

The CMM methodology was prototyped on a 3D workstation (Silicon Graphics) where it was used to model the half million atom protein coat for tomato busy stunt virus, polio virus, and rhino virus. We have now developed a parallelized MD/CMM program for production calculations on systems with up to 1.2 million atoms.

These developments will provide the capabilities of realistic atomistics simulations on any system for which plausible starting conformations can be predicted.

MD/PT. A Tool for Predicting Accurate Energies for Binding and Solvation

In order to calculate the free energy of binding of macromolecular complexes in solution, it is essential to properly include both the enthalpy and entropy contribution of all of the components of the system, including solvent, over a set of momenta and conformations that properly represent a canonical ensemble for the system. The free energy differences of interest are often as small as ~ 1 kcal/mol, and hence extreme accuracy is required.

The methods for doing this are based on Molecular Dynamics with Perturbation Thermodynamics (MD/PT) and we have developed very fast, accurate parallelized software (MD//PT). It uses constant temperature dynamics and has been converted to the KSR parallel supercomputer for production studies.

Our first project with MD//PT was to determine how strongly the drug molecule (netropsin) recognized DNA. We applied MD//PT to the case of netropsin interacting with its binding site (AAAA/TTTT) of DNA. The goal here was to determine the change in binding due to G/C replacement at each A/T site. The calculations were carried out using a periodic unit cell with 10 base pairs of DNA, netropsin, plus over 1500 water molecules in each cell.

The results (Figure 1) show a selectivity for recognition of A/T versus G/C of

$$\Delta G_{net}(AT \rightarrow GC) = 2.84 \text{ to } 3.11 \text{ kcal/mol} \tag{10}$$

depending on which of the four sites is involved.

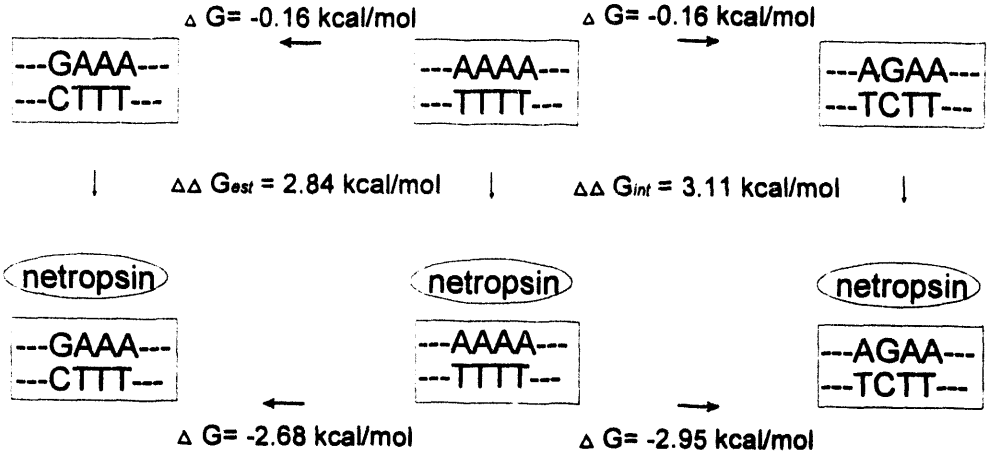


Figure 1. The free energy profiles for various MD/PT transformations. The degree of convergence is indicated by the small degree of hysterisis.
(a) the transformation from [$dA_{10}\cdot dT_{10}$] to [$dA_6(dap)A_3\cdot dT_{10}$] and back;
(b) the transformation from [$dA_{10}\cdot dT_{10}$:netropsin] to [$dA_5(dap)A_4\cdot dT_{10}$:netropsin] and back;
(c) the transformation from [$dA_{10}\cdot dT_{10}$:netropsin] to [$dA_6$9dap)$A_3\cdot dT_{10}$:netropsin] and back.

Experimental data suggest that the total selectivity for each of the internal pairs is ~ 3.0 ± 0.5 kcal/mol, in excellent agreement with theory.

Protein Folding

A critical problem in the design of new biological systems (biopolymers, biocatalysts) is the prediction of the structure and properties of the new proteins. Thus, given a specific primary sequence of amino acids, we want to predict the secondary and tertiary structures (folding) of the functional protein 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 for 10^9 to 10^{15} timesteps 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; thus we have focused on protein folding as a central research problem and with DOE-AICD support we have developed a strategy that we believe will be successful. Our strategy is hierarchical with four levels of refinement:

1. **Lattice** - At the coarsest level each amino acid is replaced with a single effective center (the C_α position) and constrained to be on a lattice point (face centered cubic). This allows an exhaustive search through all possible conformations to determine a collection of low energy conformations which should be tested at the next level.
2. **Off Lattice** - To remove the geometrical constraints of the lattice while retaining the simplification of considering only the C_α positions, we have developed a force field suitable for describing each amino acid with a single C_α position (the C_α Force Field, $C_\alpha FF$). The parameters in the $C_\alpha FF$ were determined by fitting to the accurate crystal structures of the protein data base. Starting with each structure selected from level *a*, we use the $C_\alpha FF$ to optimize the structure and select the lower energy conformations to be tested at the next level.
3. **Protein Creation** - We have developed a very effective Monte Carlo/Molecular Dynamics procedure for growing the protein from the C_α positions. This determines the main chain and side chains atoms but the C_α positions are fixed. This is used on each structure from level *b* to determine the collection of structures to be tested at the next level.
4. **Full Protein Optimization** - For each structure selected from level *c*, we optimize the full structure including effective interactions with the solvent environment.

This Hierarchical Protein Folding Strategy (HPFS) is designed to consider proteins with 40-50 amino acids, allowing us to test systems for which there is good structural data. The HPFS allows either the thermodynamic approach (lowest energy structure) or the kinetic approach (grow the protein from the *N* terminus without relaxing to the global minimum). Our selection procedure must ensure that the best structures for the final structure are in the subsets selected at each level, *a, b, c*. This requires a high accuracy in assessing the energetics of the structures at each level. To be practical we believe that it is necessary to have sufficient accuracy that we can select the best 0.01% of the conformations from each level. We believe that this hierarchical strategy is the correct one. However, our current methodology is not yet adequate. We expect significant progress over the next two years.

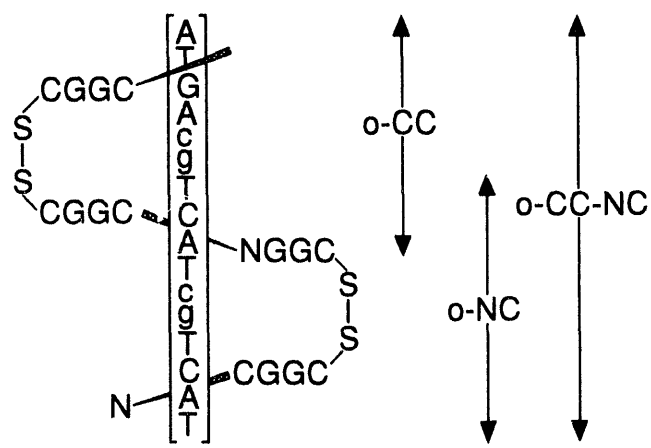


Figure 2. The o-CC-NC DNA site for which p-CC-NC was designed.

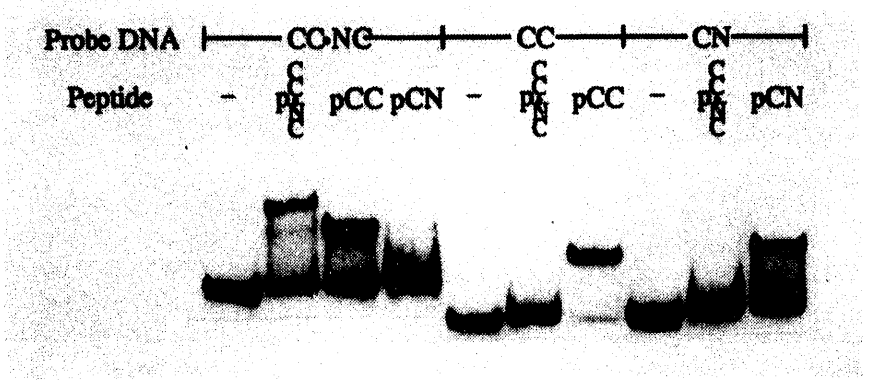


Figure 3. Gel retardation assays for p-CC-NC and other protein binding to 0-CC-NC and to other oligonucleotides.

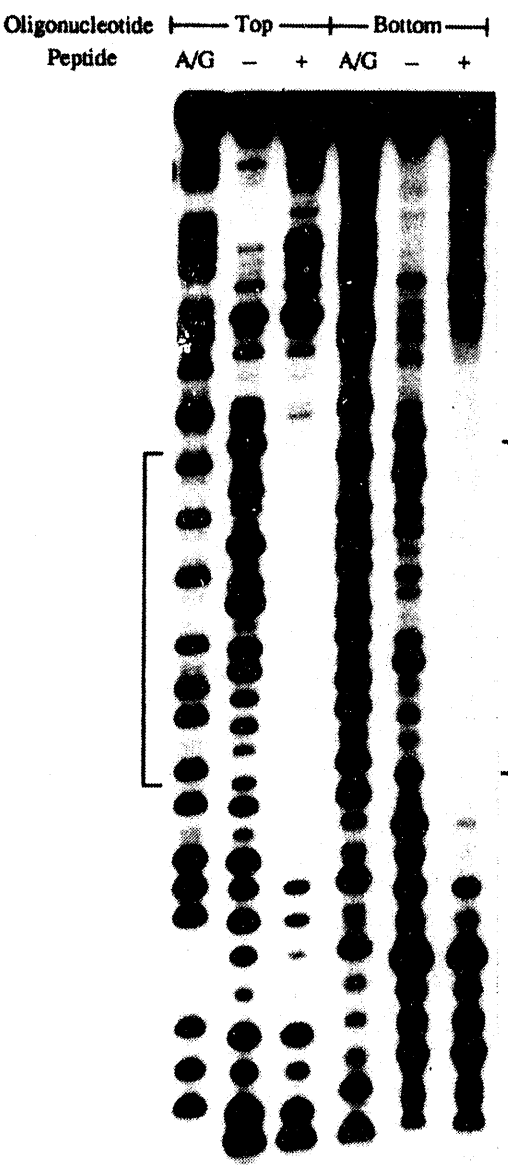


Figure 4. DNAase I footprint showing that p-CC-NC binds selectivity to the target site of o-CC-NC.

4.1.3 Mimetics

9. **Project Title:** Computer Aided Molecular Design of Biomimetic CO_2 Activation Catalysts

Principal Investigator: J. C. Shelnutt

Project Site: Sandia National Laboratories

Description:

The goal of the research is to develop computer-aided molecular design (CAMD) methodologies using classical- and quantum-mechanical molecular modeling techniques and apply them to design synthetic analogs of CO_2-activating enzymes. Particular emphasis has been placed on the biomimetic activation of CO_2 to produce methanol, methane, and other products, using sunlight as the energy source for the chemical reaction. The understanding of the biochemistry of carbon dioxide conversion has only recently reached a sufficient level for designing catalysts that mimic the C_1 catalysis of methanogens and other CO_2 metabolizing bacteria.

The activation energy barrier to produce an excited state of CO_2 and the large free energy increase that must accompany reduction of CO_2 to useful products have made the investigation of biomimetic synthetic routes attractive. To use CO_2 as a feedstock, energy must be used in the most efficient manner possible, as occurs in the biological use of sunlight as the energy source for the reaction. Specific elements of the research are: (1) the development of a photoredox cycle to provide the required chemical reducing agent, (2) studies of the enzymes that catalyze the activation and conversion of CO_2, (3) computer-aided molecular design of catalysts that mimic the active sites of these enzymes, (4) development of improved molecular modeling techniques, (5) synthesis, structural characterization, and testing of the designed CO_2-activation catalysts, and (6) integration of the system components into a solar-driven process for CO_2 reduction to high value products.

Several biomimetic catalysts systems have been examined within the scope of this project. Particular emphasis has been placed on the metalloporphyrins and related organometallic complexes. These molecules are known to catalyze CO_2 conversion and their activity can be improved by modifying their molecular structure to mimic the structural features of CO_2-activating enzymes. The specific objective is to design metalloporphyrins with the desired structural features and, subsequently, synthesize and test the most promising of the designed catalysts.

1993 Accomplishments:

Our accomplishments for FY93 are primarily in the areas of synthesis of advanced catalyst designs, studies of CO_2 binding to the substrate binding cavity of the catalysts, structural characterization and validation of the CAMD methods, development of the photocatalyst required to drive the reaction, and testing of the designed catalysts.

Synthesis of advanced catalyst designs. One of our design goals is to be able to control the reduction potential of the metal at the active center of the cobalt-porphyrin catalysts. Toward this goal we have synthesized a series of fluorinated derivatives of cobalt dodecaphenylporphyrin (CoDPP) catalysts. We have demonstrated the Co(II)/Co(I) reduction potential can be varied over a wide range by varying the degree of fluorine substitution from 0 to 36 fluorine substituents. Recently we synthesized several members of the octa*acid*-tetraphenylporphyrins (O*A*TPPs). This class of porphyrins is of interest because

they are water soluble and they can be made soluble in organic solvents by forming the octamethylesters. Only two other classes of water soluble porphyrins are known. Also, the carboxylate groups of these porphyrins can be used to attach the catalysts to support materials such as alumina. And finally, some of these porphyrins possess a potential binding pocket for small molecules like CO_2. Other potential CO_2-conversion catalysts were designed and synthesized.

CO_2-Binding Studies. NMR studies of substrate binding to the octa*alkyl*-tetraphenylporphyrin (OATPP) catalysts, Fe(III)OMeTPP and Fe(III)OEtTPP-F_{20}, have been completed. The binding of CO_2 to the latter molecule, which has a binding cavity designed for CO_2, is about the same as similar sized molecule like dichloromethane; however, the binding energies of larger molecules like chloroform and benzene are reduced relative to CO_2. The binding energies for all substrates however are apparently low (< 1 kcal/mol) in solution, so we are now designing improved catalysts.

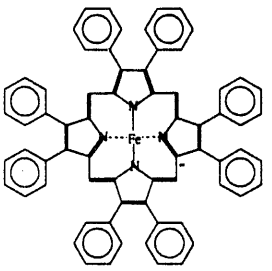
Structural Characterization and Validation of the CAMD Methods. Predicted catalyst structures were verified by X-ray crystallography, resonance Raman spectroscopy, and NMR spectroscopic studies. Several manuscripts which verify the use of our molecular models for predicting metalloporphyrin structure have been published in *Journal of the American Chemical Society, Inorganic Chemistry,* and *Journal of Raman Spectroscopy*. These manuscripts complete the initial phase of model validation for the designed catalysts. A new collaboration with Prof. Bill Goddard will allow us to improve our treatment of metals in POLYGRAF/BIOGRAF.

Catalyst Testing. We have designed and implemented an electrochemical catalyst testing and characterization apparatus. We have reproduced some published catalytic CO_2 reduction results with nickel-cyclam catalysts related to the nickel porphyrins and begun applying these electrochemical testing methods to the designed Co-porphyrin catalysts.

Photocatalytic System Development. A system composed of an electron donor, a photocatalyst (supported tin porphyrin), CO_2, and solvent is being developed for a photochemical process for production of CO and H_2, using our designed cobalt-porphyrin CO_2-activation catalysts.

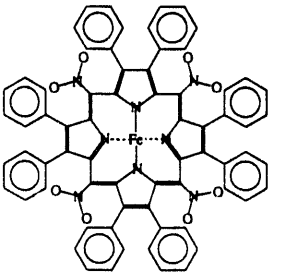
1994 Planned Activities:

Synthesis of Advanced Catalyst Designs. We will complete the synthesis of octaacetic acid tetraphenylporphyrins and analogous porphyrins with lipid, carborane, and amino substituents. These water soluble porphyrins have uses as immobilized catalysts on alumina and other heterogeneous materials and electrodes. The halogenated and nitrated octaphenyl porphyrins will be synthesized. Several of these OPP derivatives are shown in Figure 12. Many of these porphyrins have a cavity suitable for binding CO_2. The meso-nitro substituents will be converted to the amines to provide for hydrogen bonding to the bound CO_2. Several octa-isopropyl porphyrins will be synthesized to attempt to construct a tighter cavity. Isotopically labeled nitro porphyrins will be synthesized for spectroscopic studies. Many other electrocatalysts will be synthesized as promising new designs are discovered in testing.



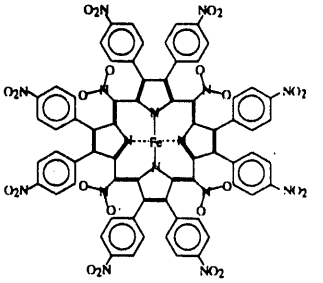
Fe octaphenylporphyrin (FeOPP)

$\Sigma\sigma_p = -0.08$ $\Sigma\sigma_m = 0.48$



$Fe(P)_8(NO_2)_4P$

$\Sigma\sigma_p = 3.04$ $\Sigma\sigma_m = 3.32$



$Fe(p\text{-}NO_2P)_8(NO_2)_4P$

$\Sigma\sigma_p = 4.96$

Figure 1. Octaphenylporphyrin and derivatives with high Fe(III)/Fe(II) and Co(II)/Co(I) reduction potentials.

We will also design catalysts with CO_2 hydrogen bonding functionality. Finally, we will attempt to synthesize advanced hyper-hindered catalysts like octa-neopentyl-tetraphenylporphyrin using the synthetic methods developed to synthesize octaisopropylporphyrins.

Characterization of Advanced Catalyst Designs. We will complete the structural characterization of metal derivatives of fluorinated DPPs, octaphenylporphyrins, and octaisopropylporphyrins with resonance Raman spectroscopy, NMR spectroscopy, UV-visible absorption spectroscopy, and X-ray crystallography. These studies should determine the effect of electron withdrawing substituents without complications resulting from conformational differences. We will complete our structural studies of the nickel derivatives of the nitroporphyrin series OAN_nP, where n = 1, 2, 3, and 4. Finally, we will characterize the octa-acid-tetraphenylporphyrins with regards to their interaction with electron donors like methylviologen and with surfaces of heterogeneous materials.

Development of New Modeling Methods. The periodic metal potential currently in POLYGRAF will be modified to include a second term that will allow the curvature and barrier height to be varied independently. The present functional form underestimates the barrier height when reasonable force constants are used. The SHAPES potential will be implemented in the commercial version of the DISCOVER code in the December 1993 release. We will develop metal parameters for Biosym's SHAPES potential and for POLYGRAF's modified periodic potential. We will calculate electronic structure of the catalysts using DMOL density functional methods and compare results with our previous and new INDO/CI method results. We will calculate normal modes for the optimum geometry using the ZINDO method. The normal mode obtained from the semi-empirical methods will be compared to those modes obtained from classical molecular mechanics calculations using DISCOVER or the VIBRATE module of POLYGRAF. The goal of the latter work is a spectroscopically accurate force field for metalloporphyrins.

CO_2-Binding Studies and Modeling of CO_2-Cavity Interactions. A new ^{13}C-NMR method developed for measuring substrate binding affinity for the catalysts will be used to determine the relative affinities of methane, benzene, chloroform, dichloromethane, and CO_2 for fluorinated FeDPPs (Figure 2). If a suitable amino porphyrin can be synthesized with hydrogen bonding functionality, then CO_2 and solvent binding energies will be determined. We will use the results of these experimental studies of substrate binding to catalyst to verify and improve modeling methods for substrate binding calculations. We will also carry out *free energy* calculations to compare with results of the NMR binding studies. We will use Nose cononical molecular dynamics to calculate the length of time various substrate molecules remain bound to the cavity. Binding studies will be carried out with the new massively parallel MD code and conventional canonical MD calculations with the new forcefield and metal potentials.

Catalyst Testing. We will complete the development of the electrochemical apparatus for testing the catalysts and test the Co-porphyrin CO_2 activation catalysts, including the halogenated DPPs and water-soluble octa-acid porphyrins. Most of our effort in the coming year will be devoted to this task.

Photocatalytic System Development. We will develop new photocatalyst materials for CO_2 reduction by supporting new Sn porphyrins on preparations of alumina substrate with the goal of improving the performance of the supported SnP. We have recently successfully prepared nanoclusters of titania for this application and hope to use these synthetic methods to prepare alumina and magnesia nanoclusters as supports for our porphyrin photocatalysts.

Annual Technical Summary Report:

Synthesis of Halogenated Dodecaphenylporphyrins. The synthesis of the iron derivatives of the fluorinated dodecaphenylporphyrins (DPP) has been completed. The compounds are: DPP, DPP-F_{20}, DPP-F28, and DPP-F_{36}. The latter compound, the perfluro-dodecaphenylporphyrin (DPP-F_{60}) or "teflon" porphyrin, could not be synthesized by the present methods. The halogenated porphyrins are of interest because of their high stability to oxidation and demetalation and their novel electronic and structural properties. The Fe(III)Cl derivatives of the four fluorinated DPPs have been synthesized and subjected to electrochemical measurements in preparation for electrocatalytic CO_2 reduction studies. Figure 1 shows one of the Fe derivatives of the fluorinated DPP series that was synthesized.

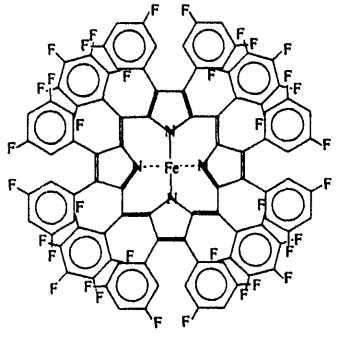


Figure 1. Fe(III)Dodecaphenylporphyrin-F_{36} (FeDPP-F_{36} The sum of the Hammett substituent constants (para and meta) give a measure of electron withdrawing capability of the substituents.) ($\Sigma\sigma_p$ = **2.84** *(est. 0.15)* $\Sigma\sigma_m$ = **2.96** *(est. 0.20))*

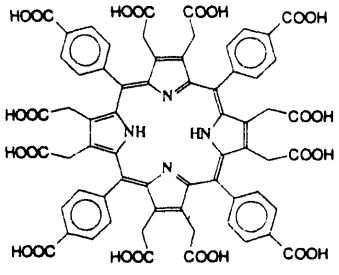


Figure 2. Dodeca-acid-tetraphenyl-porphyrin.

Figure 3 shows a plot of the potential of the Fe(III)/Fe(II) redox couple for FeDPP-F_{28} and FeDPP-F_{20} and several other Fe porphyrin derivatives. The plot illustrates the effect of electron withdrawal by the peripheral substituents on the redox potential. We plan to use this effect to tune the redox properties of the cobalt porphyrin catalysts for CO_2 reduction. The synthesis of other metal derivatives of the DPPs, including the cobalt derivatives, is progressing.

The redox potential of the metal is important in determining the electrode potential at which CO_2 reduction takes place and, thus, the efficiency of the electrocatalytic reaction. For the cobalt catalyts, the important reduction is the Co(II)/Co(I) couple. From Figure 4 we see that this reduction potential of the Co couple also becomes more positive as the electron withdrawing power of the substituents becomes stronger. Based on the slopes of the lines in Figures 2 and 3, we should be able to lower the potential for the Co(II)/Co(I) couple to less than approximately -0.5 V using the fluorinated DPP series. (The potentials for other ring oxidations and reductions show either no trends or the potentials measured are unreliable because of overlapping processes.)

We are continuing to characterize the metal derivatives of the octa-acid porphyrins and their octamethylesters, including octa-methanol-tetraphenylporphyrin and octa-acetic acid-tetra(*p*-nitrophenyl)porphyrin, with resonance Raman spectroscopy and molecular modeling. Most recently, Dr. Miura at BNL has synthesized the dodeca-acid porphyrin in Figure 2. The - $C_{20}H_{41}$ ester of this porphyrin has novel solubility (in hexane) and was engineered for preparation of Langmuir-Blodgett films on electrodes.

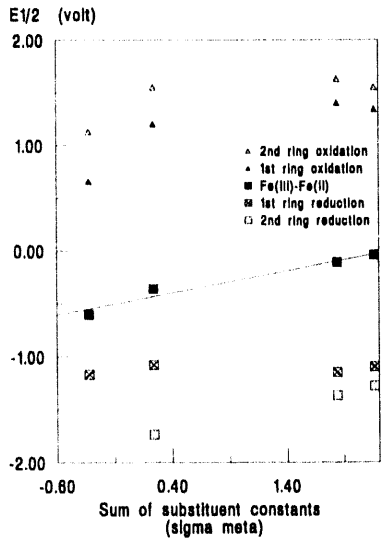


Figure 3. Dependence of various reduction potentials of iron porphyrins as a function of the electron-withdrawing capacity of the peripheral substituents. Least-squares fit for Fe(III)/Fe(II) couple is shown. Iron porphyrins: FeOETPPCl, FeTPPCl, $FeDPPF_{20}Cl$, and $FeDPPF_{28}Cl$.

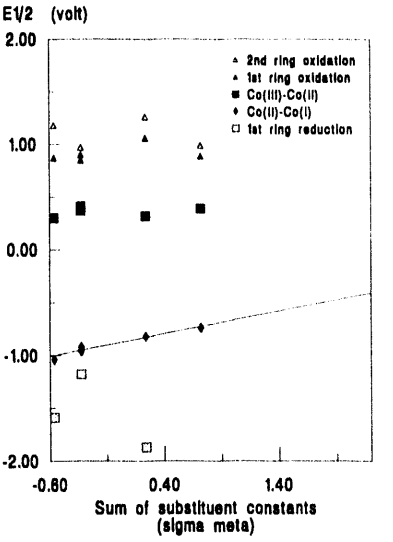


Figure 4. Dependence of various reduction potentials of cobalt porphyrins as a function of the electron-withdrawing capacity of the peripheral substituents. Least-squares fit for Co(II)/Co(I) couple is shown. Cobalt porphyrins: CoOMTPP, CoOETPP, $CoTC_6TPP$, CoDPP, CoTPP, and CoOEP.

Another class of porphyrins being synthesized is the group of octaalkyl-nitro-porphyrins (OAN_nPs, n = 1, 2, 3; 4). These porphyrins and some of their analogs have been synthesized by Prof. Martin Quirke and larger quantities of OAN_4P have been synthesized by Medforth. The importance of these porphyrin derivatives for our catalyst design goals are: (1) They are highly substituted and therefore, at least for the tetra-nitro derivative on the right, have the saddle shaped structure required to form a CO_2 binding pocket. (2) Further, they possess the highly electron withdrawing nitro substituents at the bridging carbons of the porphyrin ring. The nitro groups give a more oxidatively stable porphyrin while retaining the alkyl groups that form the substrate binding pocket. And, (3) the use of either the nitro or phenyl groups allow us to electronically influence the catalytic activity of the metal. The nitro substituents can also be converted to amines to provide hydrogen bonding to CO_2.

Earlier, we calculated the CO_2 binding energies for several catalyst in a vacuum, but we measured much smaller relative binding energies in solution. Consequently, a new porphyrin catalyst which incorporates the possibility of hydrogen bonding to CO_2 within the cavity has been designed and the binding energy has been calculated. (See Figure 6.) The catalyst has amino groups adjacent to the CO_2 binding cavity, permitting the CO_2 molecule to hydrogen bond to a pair of amino groups at the end of the cavity. As expected the binding energy is significantly enhanced by the hydrogen bonding interactions. Preliminary estimates give over 20 kcal/mole for the binding energy in vacuum which should be compared to less than 9 kcal/mole for a catalyst analog without the hydrogen bonding functionality. The hydrogen bonding adds to the binding energy for CO_2, but not for organic solvents. Design and modeling of other catalysts with CO_2 coordinating groups is underway.

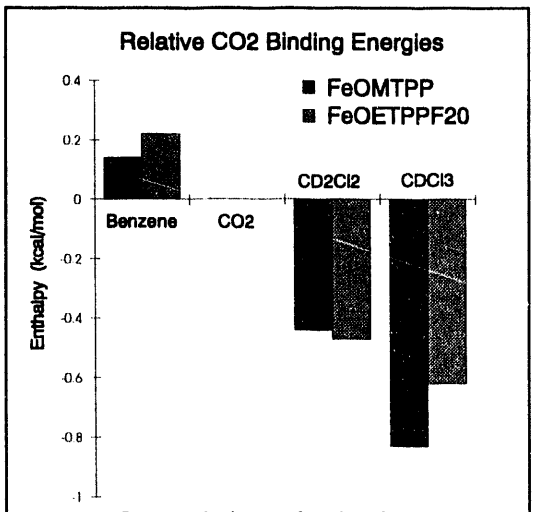


Figure 5. Enthalpies for binding various substrates relative to CO_2 obtained from ^{13}C-NMR paramagnetic relaxation rates as a function of temperature.

Characterization of Advanced Catalyst Designs. Complete characterization studies for many of the catalysts can be found in the published papers listed in the Project Output section below.

CO_2-Binding Studies. X-ray crystallography has shown that a dichloromethane molecule is located in the binding cavities in crystals of CuOETPP and CoOETPP, and collaborators at BNL have observed solvent molecules in the cavities of NiOETPP and $NiTC_6TPP$. The dichloromethane-porphyrin complex is consistent with our calculations which show several possible orientations for the bound molecule. Solution NMR studies of substrate binding to the octa*alkyl*-tetraphenylporphyrin (OATPP) catalysts have now been completed. The relative binding energies are plotted in Figure 5 for Fe(III)OMTPP and Fe(III)OETPP-F_{20}, which has a more well defined binding site for CO_2. The binding of CO_2 to the molecule with a binding cavity designed for CO_2 relative to a similar sized molecule like dichloromethane is about the same; however, the binding energies of larger molecules like chloroform and benzene are reduced relative to CO_2. The binding energies for all substrates are apparently low (< 1 kcal/mol) in solution, so we are now designing improved catalysts (*vide infra*). Two possible improvements are: (1) tightening of the binding cavity would improve binding affinity for CO_2 while lowering the affinity for larger substrates, and (2) incorporation of porphyrin substituents that are in a position to hydrogen bond to CO_2 would raise its affinity but leave unchanged the affinity of substrates without hydrogen bond acceptors, possibly stabilizing a CO_2 reaction intermediate.

We have also completed a study of CO_2 binding to the cavity of the designed catalysts (NiOETPP) using Nose-Hoover canonical molecular dynamics. We found that CO_2 is released from the cavity sooner than for conventional microcanonical dynamics calculations (125 ps *versus* >200 ps).

Another NMR method to determine the solution conformation of the cobalt-porphyrin catalysts has been developed. The method predicts the proton-NMR spectrum of Co porphyrins based on either our calculated structures; predicted NMR spectra are then compared with experimental spectra. Experimental agreement with only one of the calculated conformers is usually the case, allowing determination of the solution conformer. Finally, we have discovered that axial ligands are oriented in the binding cavity. Catalysts lacking the binding pocket show low or no barrier to the rotation of axial ligands bound to the metal.

Development of New Modeling Methods. One goal of the modeling work is to improve the current Sandia force field for porphyrin catalysts by utilizing the vibrational analysis capabilities and thermochemical calculations that have just become available as add-on modules for MSI's BIOGRAF and POLYGRAF. The modules, VIBRATE and THERMO, have been ordered. Already the improved forcefield is capable of predicting the relative energies of various energy-optimized conformers as work completed in this quarter demonstrates. New experimental information concerning the relative energies of conformers of metalloporphyrin catalysts has shown that the previous forcefield, while giving highly accurate structures for porphyrin conformers, failed to predict which conformer was the lowest in energy and, therefore, which conformer would be observed experimentally. The improved forcefield now completed does not suffer from this limitation. The most important modifications were the use of an exponential-6 functional form for the van der Waals potential and inclusion of the electrostatic contributions to the energy. Several other refinements have been made including modification of some force constants and van der Waals parameters and introduction of the large interaction force constants that are known from the normal coordinate analysis of nickel octaethylporphyrin. The new forcefield successfully predicted the lowest energy conformer for a number of different porphyrins, including the lowest energy conformer of nickel octaethyl-tetranitroporphyrin as shown by a recent crystal structure reported in the Chinese literature. Also, we have also succeeded in converting the Sandia forcefield for porphyrin catalysts from an AMBER format to Biosym's *cvff* format through the collaboration with Biosym and carried out the first free energy calculations of substrate binding to our designed catalysts.

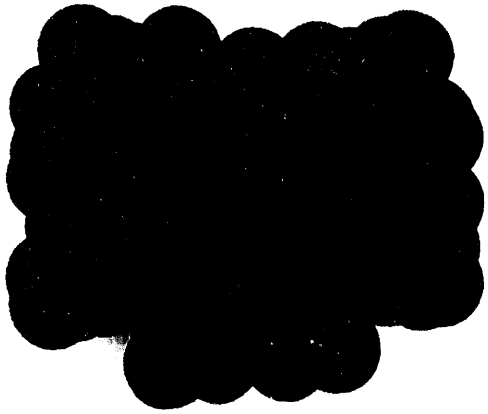


Figure 6. Structure of the CO_2-Ni(II) octaethyl-tetra(2,6-diaminophenyl)porphyrin complex.

ZINDO/s Electronic Structure Calculations. We have now obtained a HYPERCHEM software upgrade which allows us to more efficiently carry out INDO/CI molecular orbital calculations of the optical spectra of metalloporphyrins. We found that accounting for either the macrocycle conformation or the peripheral substitution alone is not sufficient to be able to calculate accurate transition energies. When both effects are included however accurate excited state energies are predicted by the ZINDO calculations. We need the new program to investigate the spectra of the nitro-octaalkyl-porphyrins, identifying new spectral features resulting from the nitro substituents.

Catalyst Testing. We have designed and implemented an electrochemical catalyst testing and characterization apparatus and electrochemical measurements on the iron and cobalt catalysts are underway. We have observed CO_2 reduction with the related nickel-cyclam catalysts using the new electrochemical methods and are now testing the designed Co-porphyrin catalysts, which cover a wide range of redox potentials for metal reduction.

4.2 Advanced Bioprocess Systems

4.2.1 Bioprocessing for Commodity Chemicals

10. Project Title: Engineering Enzymes for Stability and Activity in Organic Solvents.

Principal Investigator: F. H. Arnold

Project Site: California Institute of Technology

Description:

Improving understanding of the molecular basis of protein stability and enzyme catalysis, combined with the ability to create large quantities of proteins of virtually any amino acid sequence, gives us the ability to redesign natural proteins to fit the requirements of industrial applications. As a result, biotechnologists no longer have to limit themselves to designing processes around natural biocatalysts; designing a biocatalyst to fit the process is gradually becoming an achievable goal. The ability to carry out biochemical syntheses in organic solvents, where solubilities are greatly enhanced and new chemistries are available, greatly expands the scope and potential applications of biocatalysis in the chemical industry. Unfortunately, most enzymes respond unfavorably to transfer to polar organic solvents; they are highly destabilized and their catalytic activities are often reduced by orders of magnitude. The overriding goal of this project is to develop design rules for engineering enzymes at the level of their amino acid sequences to improve stability and catalytic activity in polar nonaqueous solvents. The strategy employed is to use both random and site-directed mutagenesis techniques to alter the amino acid sequence of Subtilisin E, a serine protease with numerous potential applications in organic synthesis and preparation of novel polymers. This research will provide general tools and design rules for engineering stable and efficient biological catalysts. This project seeks to understand and enhance engineering enzyme stability and reactivity in nonpolar and nonaqueous solvents, using the prototype enzymes. Design tools will be provided to guide the selection and efficiency of macromolecular catalysts.

1993 Accomplishments:

This year we published two impressive demonstrations of enzyme engineering to obtain industrially-useful properties. We have shown that a substitution-inert metal, ruthenium, forms essentially covalent crosslinks in proteins with engineered chelating sites, leading to an exceptionally large degree of stabilization[7]. Crosslinking two histidines on opposite strands of a β-sheet in cytochrome *c* increased the protein's melting temperature by 23 C and more than doubled the protein's conformational stability ($\Delta\Delta G$ = 5.5 kcal mol^{-1}). This stabilization approach should be widely applicable to industrial enzymes.

Using random mutagenesis and screening, we have developed a subtilisin variant (called PC3) that hydrolyzes a peptide substrate up to 256 times more efficiently than wild-type subtilisin over a wide range

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[7] "Ru^{II}-mediated protein crosslinking and stabilization," A. Muheim, R. J. Todd, D. R. Casimiro, H. R. Gray and F. H. Arnold, *J. Am. Chem. Soc.*, **115**, 5312-5313 (1993).

of concentrations of a polar organic solvent, dimethylformamide (DMF)[8]. PC3 subtilisin E and other variants containing different combinations of amino acid substitutions are effective catalysts for important synthetic reactions such as transesterifications and peptide synthesis in a variety of organic solvents. We have demonstrated that these variants can catalyze the ligation of several nonnatural amino acids, such as pentenyl glycine, into (chiral) polymers.

1994 Planned Activities:

The random mutagenesis approach we have applied with great success to enzymes in polar organic solvents will be applied to further improve activity as well as substrate specificity. To obtain enzymes capable of producing polymers of nonnatural amino acids and other substrates, we will be developing powerful protocols to screen directly for polymerization efficiency. In particular, we are developing an assay that will allow us to directly identify protease-catalyzed ligation reactions (as opposed to hydrolysis) in the presence of polar organic solvents. We will also be optimizing the reactions conditions for subtilisin-catalyzed polymerization of nonnatural amino acids and characterizing the resulting polymers (MW, size distribution, optical and mechanical properties).

This next year we plan to extend our engineering efforts to other enzymes of potential industrial importance. We have nearly completed negotiations with Eli Lilly & Co. to engineer a specific esterase enzyme for use in antibiotics synthesis. This collaborative research project will involve random mutagenesis to optimize the enzyme for activity under the conditions of the actual industrial application. We also intend to begin work on the cloning and expression of oxynitrilase, an enzyme with applications in organic synthesis.

Annual Technical Summary Report:

Proteins are surprisingly tolerant of amino acid substitutions--only a fraction of the amino acids in a protein are critical for function, folding, or stability. This sequence flexibility can be exploited to create enzyme catalysts with features critical for industrial application.

Protein Stabilization

A successful approach to stabilizing enzymes must provide stabilization while minimizing the probability that existing favorable interactions will be disrupted. Furthermore, a convenient approach is general and can be applied without detailed knowledge of the enzyme's structure. We have developed stabilization strategies that fulfill these requirements. One strategy involves replacement of surface charged residues and was described in a previous report. A second strategy involves metal binding by simple chelating sites engineered into the protein surface. Several years ago we showed that as few as two properly positioned coordinating ligands (e.g., His-X_3-His in an α-helix) can form sites with reasonably high affinities for metal binding. Kinetically labile metal ions (e.g., Cu^{2+}, Zn^{2+}) stabilize these engineered metal-binding proteins by binding preferentially to the folded state. The secondary structure provides the precise geometry and the rigidity required for strong binding, such that kinetically labile metals (e.g., Cu^{II}, Zn^{II}) bind preferentially and stabilize the folded protein. Cu^{II} binding to di-histidine chelating sites in *S. cerevisiae* cytochrome *c* adds up to 2.7 kcal mol^{-1} to the protein's conformational stability.

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8 "Tuning the activity of an enzyme for unusual environments: sequential random mutagenesis of subtilisin E for catalysis in dimethylformamide," K. Chen and F. H. Arnold, *Proc. Natl. Acad. Sci. USA* **90**, 5618-5622 (1993).

An even more impressive result was obtained this year with a substitution-inert metal, ruthenium, which will form essentially covalent crosslinks in proteins with engineered chelating sites [7]. Crosslinking two histidines on opposite strands of a β-sheet (His 39 and His 58) in cytochrome *c* with a Ru^{II} complex increases the protein's melting temperature by a very large amount, 23 C, and more than doubled the protein's conformational stability ($\Delta\Delta G$ = 5.5 kcal mol^{-1}).

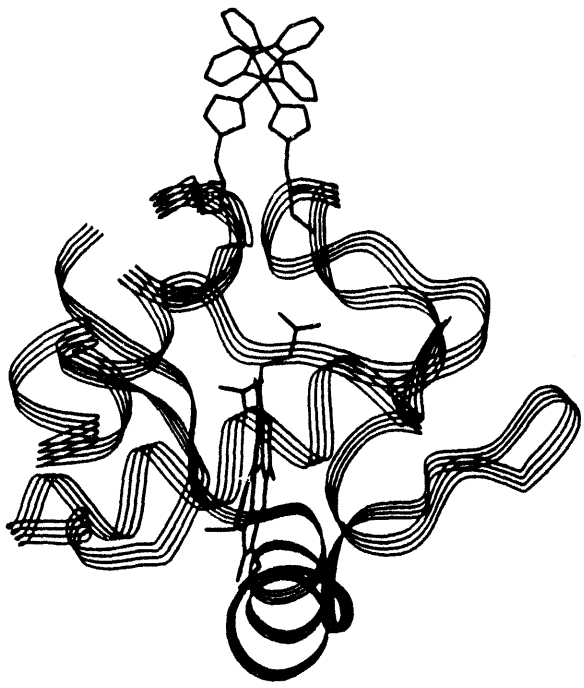


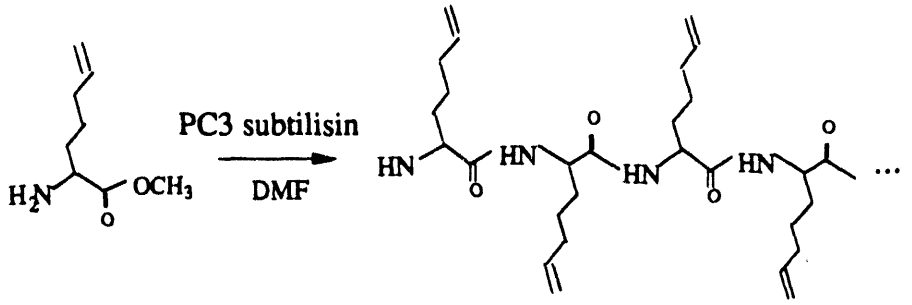
Figure 1. Energy-minimized model of $Ru^{II}(bpy)_2$-$H_{39}H_{58}$ cytochrome *c*. Two histidines crosslinking the β-sheet provide an appropriate geometry for metal chelation, forming an essentially covalent crosslink with minimal changes in the backbone structure.

This stabilization approach can be applied to industrial enzymes. Because α-helices and β-sheets are common to virtually all proteins, it should be possible to insert simple metal-chelating sites into many enzymes with a minimal number of mutations and little or no disruption of the catalytic function. Since stabilizing mutations often provide cumulative benefits when combined, it may be possible to engineer multiple metal-binding sites to achieve additional stability. Furthermore, surface metal-binding sites can serve additional purposes: as "affinity handles" for single-step purification by metal-affinity chromatography and for immobilization, as sites for incorporation of spectroscopic probes, or as possible nucleation sites for crystallization. Our future work with enzymes of potential commercial importance will make use of this simple and effective approach to stabilization.

Random mutagenesis: enhancing enzyme activity in organic solvents

Using random mutagenesis and screening, we have developed a subtilisin variant (called PC3) that hydrolyzes a peptide substrate up to 256 times more efficiently than wild-type subtilisin over a wide range of concentrations of a polar organic solvent, dimethylformamide (DMF) [5]. PC3 subtilisin E and other variants containing different combinations of amino acid substitutions are effective catalysts for important synthetic reactions such as transesterifications and peptide synthesis in a variety of organic solvents. PC3

subtilisin E can catalyze the ligation of a series of nonnatural amino acids such as pentenyl glycine into polymers in DMF, as shown in Fig. 2. An optically active polymer is produced, starting from a racemic mixture of the amino acid ester monomers. These polymers show considerable promise for production of biodegradable and biocompatible materials with very interesting structural and mechanical properties. This result is particularly significant because extensive efforts on the part of our collaborators (Prof. Harvey Blanch and Prof. Doug Clark at UC Berkeley) to identify a natural enzyme that could catalyze this reaction had failed. Thus this reaction indicates the potential for engineering enzymes with unique capabilities not found in naturally-occurring systems.



(D,L) pentenylglycine methyl ester poly (pentenylglycine)

A major effort this year was to optimize the subtilisin mutagenesis and expression systems so that we would be able to efficiently extend our random mutagenesis methods and studies. A *Bacillus-E coli* shuttle vector was constructed to allow efficient transfer from cloning in *E. coli* to expression in *B. subtilis*. Significantly larger libraries of random mutants can be generated with this system, which is also easier to use than the previous pKWC vector.

Random mutagenesis is being carried out to extend subtilisin activity to even higher concentrations of organic solvents.

11. Project Title: Bioprocess Engineering: Immobilized Cell Systems for Continuous, Efficient, Biocatalyzed Processes

Principal Investigators: C. D. Scott and B. H. Davison

Project Site: Oak Ridge National Laboratory

Description:

This project will advance practical and fundamental knowledge of bioreactor dynamics and immobilized biocatalyst systems. The goals of the project are as follows: development and testing of new bioreactor configurations; understanding and modeling of the kinetic properties of biocatalyst particles and the dynamics of bioreactor systems; and development of enhanced bioreactors for continuous production of industrial products (enzymes, chemicals, etc.).

1993 Accomplishments:

A primary goal of this program is the development and improvement of advanced bioreactor systems that significantly increase the productivity and yield of bioconversion processes. Several of these concepts under investigation utilize biocatalysts which are microorganisms immobilized into or onto particles. These biocatalysts can be effectively used in fluidized-bed reactors or in other columnar systems.

Processing concepts with immobilized cells are being studied for simultaneous fermentation and product separation. The biparticle, fluidized-bed bioreactor (FBR) utilizes microorganisms immobilized in gel beads and adsorbent particles. A system for the continuous addition and removal of the adsorbent resin from the biparticle FBR has been assembled and initial tests performed for lactic acid production by *Lactobacillus*. While resin screening continued, the current best sorbent is Reillex 425. Reillex 425 polyvinyl pyridine resin was assayed for repetitive lactic acid adsorption and regeneration with continued capacity, potential for recovery, and for concentrating the product stream. Regeneration methods include methanol, vacuum, and preferably hot water. Acetic acid production was also examined with successful immobilization of *Clostridium thermoaceticum* and resin screening. Another system using an extractive immiscible solvent, oleyl alcohol, to remove the inhibitory butanol from the immobilized-cell fermentation in an FBR was summarized in a publication. A predictive modeling program for the multiphase ethanol FBR is being developed in collaboration with Washington State University.

Another columnar reactor is being tested for the conversion of gaseous substrates. The microorganisms are able to remove hydrocarbons (pentane and isobutane) from air by biological action. The liquid-continuous gas-phase bioreactor has enriched a stable microbial population which continues to actively convert the gaseous hydrocarbons as their sole carbon and energy source for over 20 months.

Programmatic milestones were met by preparing technical papers on the gas-phase bioreactor system and on the predictive modeling of the FBR ethanol system. In addition, Fluor Daniel has signed a subcontract to evaluate the advanced ethanol column by comparison with current technology.

Subcontract research programs at UC Berkeley, Northeastern, Clark/Atlanta, and Howard Universities were administered and concluded. The Symposium on Biotechnology for Fuels and Chemicals was partially supported.

1994 Planned Activities:

The development of advanced bioreactor concepts will continue to focus on the use of immobilized-cell FBRs. The biparticle FBR for simultaneous fermentation and separation will be tested in continuous bench-scale operation for lactic acid production. A key emphasis will be on the resin handling and regeneration. The *Lactobacillus* converts glucose stoichiometrically into lactic acid. The use of adsorbents will be examined in fermentations of other organic acids. A small effort will continue to test the solvent extractive FBR for butanol production.

The predictive model for the high-productivity ethanol FBR will be continued to support a scaleup demonstration in another program. Economic evaluations on this system will be performed by an outside subcontract. Work will continue on extending the predictive model being developed for the ethanol FBR into a model for the systems with simultaneous separation and fermentation.

Work will begin in biocatalyst testing and development as part of a CRADA with DOW Chemicals on modification and treatment of halogenated compounds.

The use of columnar bioreactors with microbes for the removal of volatile organic hydrocarbons will be continued. A gas-continuous system has been assembled to improve mass transfer, and degradation and growth kinetic experiments will provide increased biomass in order to experimentally evaluate the this proof-of-principle system to determine feasibility.

Initial work will begin on the use of biocatalysts in biphasic liquid systems. Biocatalysts which reside in an aqueous phase will be investigated for use in removal/reaction of molecules contained in an immiscible organic media.

In most cases, there will be at least five elements in the development of each new bioprocessing concept, some of which can be carried out concurrently: (1) evaluation of the primary mechanisms; (2) integrated study of the new concept on a bench scale; (3) development of a predictive mathematical model; (4) establish technical feasibility at an adequate scale; and (5) transfer of the new technological concept to a more applied DOE program or to the industrial sector. Each of these projects is at a different stage of this approach. Several subcontracts for related work at universities will be administered and support for the Symposium continued.

Annual Technical Summary Report:

Advanced bioreactors can greatly increase the productivities and yields of microbial fermentations and enzymatic reactions for useful products such as chemicals and fuels. ORNL's approach is to use fundamental concepts as the basis for developing these advanced processing systems. After the technical feasibility of the approach is demonstrated, transfer of the technology is established through industrial interactions and publications. Recently we have emphasized the use of columnar bioreactors, in particular FBRs with immobilized cells.

Biparticle Fluidized-bed Bioreactor for Fermentation and Adsorption—An additional advantage of the multiphase FBR is the potential to add new phases to perform additional functions beyond the usual three (solid catalyst, liquid media, and gas coproduct). The new approach is to add a fourth phase with extractive capability for the desired product. This will serve to enhance the fermentation by moderating reactor pH, while simultaneously accomplishing product separation. The bioreactor consists of a columnar fluidized bed of immobilized microorganisms. A stream of more dense sorbent particles is added to the top of the fluidized bed. These particles progress downward through the fluidized biocatalysts, adsorb the inhibitory product, and are removed from the base of the columnar reactor.

A variety of adsorbents have been screened for the properties required for use in the continuous fermentation and separation of lactic acid in a biparticle FBR. These properties include capacity, specificity, ease of regeneration, stability, regenerant product concentration, adsorption/desorption kinetics and residence time in the FBR. No adsorbent exhibited all of the desired properties and a small effort in resin screening will continue as initial continuous biparticle FBR use the most promising resin to date, Reillex 425. Of the resins tested, only R425 possesses stable capacity with repeated regeneration in hot water. However, at the desired reactor media concentrations R425 exhibits only 0.01 g/g capacity for lactic acid due to the neutral pH and a slight (0.004 g/g) capacity for glucose. The kinetics are rapid occurring within 10 minutes. The impact of this on the operating system will be examined. Ongoing research has focused upon the design and demonstration of a biparticle FBR with continuous resin addition and product recovery. A system has been assembled for continuous solid and liquid addition with sequential resin regeneration. Continuing experiments are demonstrating the process components in both mock and actual fermentation trials using immobilized *Lactobacillus delbreuckii.*

Current efforts have emphasized production of lactic acid. However, also in this year, we successfully immobilized the anaerobe *Clostridium thermoaceticum* and placed the beads in a FBR for an initial test of acetic acid production.

Gas-Phase Bioreactor Systems—Bioreactor concepts that provide for interaction with gaseous substrates such as volatile organics are being investigated. The removal of hydrocarbon gases from effluent gas streams is of particular interest with the primary emphasis on *n*-pentane and isobutane. These gases are sparingly soluble in water, therefore good mixing and high-surface contact area between the gas and liquid phases is required. The bioreactors contain microorganisms that are able to remove and degrade the dilute gaseous hydrocarbons (*n*-pentane and isobutane) from effluent airstreams by biological action. Columnar bioreactors, including a liquid-continuous packed column, were continuously operated for over 20 months with sustained degradation of the gaseous hydrocarbons. The reactor was operated in a liquid continuous mode with gas recirculation and a slow addition of the organic-containing air. In these systems, the hydrocarbons, pentane and isobutane, are the sole carbon and energy sources for the microbes. The microbes were shown to be able to degrade these gaseous hydrocarbons completely in a closed bioreactor without any additional nutrients, though other nutrients may limit the total biomass production. The hydrocarbon removal rates measured between 1 and 2 $g\ h^{-1}\ m^{-3}$. The overall conversion rate was doubled by operating at higher gas flow rates. This indicates the importance of improving mass transfer and gas contact in these systems.

Ethanol FBR: Economics, Modeling, and Hydrodynamics—A planned project will be the demonstration at a larger scale of the high productivity ethanol fermentation using immobilized *Zymomonas mobilis* in a FBR. A proposal has been submitted through NREL in order to perform this work. A subcontract has been signed by Fluor Daniel, Inc. to perform an economic and engineering assessment of the ethanol FBR system. This has delayed this assessment.

J. N. Petersen of Washington State University is assisting in the modification of an existing computer model to be used for scaleup of the ethanol FBR through a subcontract. A fully predictive mathematical model of a three-phase, tapered, FBR with immobilized microorganism has been developed. This model includes the effects of the tapered bed, variable dispersion coefficient, and variable solid holdup upon the concentration profiles developed in the bed. In addition, the effect of the concentration profile which is developed inside the biocatalyst bead is included by means of an effectiveness factor calculation. Using accepted correlations for the dispersion coefficient and for the liquid, gas and solid holdup in the bed, the model is fully predictive. The model was found to adequately predict experimentally obtained concentration profiles.

Both ORNL and the Autonomous University of Barcelona have used successfully FBRs for high-productivity yields of ethanol. In collaboration, we found that different regimes of operation appeared which were related to the physical properties (density, viscosity, surface tension) of the phases and, in particular, the low density of the solid particles. A map of these parameters was prepared which separated regions of satisfactory and unsatisfactory fluidization of two different FBRs: a immobilized-yeast FBR fluidized with air and a immobilized *Zymomonas mobilis* FBR that was liquid fluidized.

Biocatalysis in Nonaqueous Media—Planning efforts in this area were emphasized. In particular, the development of a CRADA for biocatalyst improvement with DOW Chemical Co. may lead to such research and the use of biocatalysts in biphasic media will be examined.

4.2.2 Integration into Chemicals Industry

12. Project Title: Designing and Improving Enzymes for Use in Organic Solvents

Principal Investigator: A. M. Klibanov

Project Site: Massachusetts Institute of Technology

Description:

This project seeks to elucidate the mechanisms of enzyme stability in organic solvents and to apply the knowledge to processes of industrial importance. The project will also investigate gas-phase biocatalysis. The phenomenon of "molecular memory" of enzymes will be explored. Rigid protein configurations in enzymes retain the conformation (imprint) induced by the original ligand/stability factor. The result of this molecular memory is an enzyme with altered catalytic characteristics or a protein with new binding site, which might be able to perform new chemical reactions.

1993 Accomplishments:

The overall goal of this project is to rationally improve and control the catalytic performance of enzymes (proteases and lipases) in neat organic solvents. The following are our principal accomplishments in this project over the last year.

(i) We have unequivocally established that, in contrast to suggestions frequently made in the literature, solvent's immiscibility with water and apolarity by themselves are irrelevant to enzymatic activity in this solvent. Rather, solvent's hydrophobicity is the key pertinent parameter. This knowledge should be quite useful in selecting organic solvents conducive to maximal enzymatic activity.

(ii) We have discovered that lyophilization of enzymes from aqueous solutions containing lyoprotectants (e.g., non-reducing sugars) dramatically increases the subsequent enzymatic activity in organic solvents compared to that of enzymes lyophilized in the absence of such excipients. This and other findings made by us point to a mechanism based on the ability of lyoprotectants to alleviate reversible denaturation of enzymes upon lyophilization.

(iii) We have discovered that chemoselectivity of enzymes is strongly dependent on (and sometimes even reserved by) the solvent. A mechanistic rationale for this phenomenon has been elaborated.

1994 Planned Activities:

(i) Using direct biophysical methods, we will examine the conformation of lyophilized enzymes suspended in organic solvents. Specifically, using high-resolution NMR spectroscopy following hydrogen isotope exchange in organic solvents, we will test the hypothesis that, unless lyophilized in the presence of lyoprotectants, most enzyme molecules in a lyophilized powder are present in a reversibly denatured state.

(ii) A new approach will be explored to a chemoenzymatic synthesis of sugar-based polyacrylates. The proposed methodology is based on complexation of sugars with substituted boronic acids, thereby solubilizing the sugars even in hydrophobic solvents. The resultant complexes are then regioselectively acryloylated enzymatically in such solvents, followed by chemical polymerization and removal of the boronic moiety.

(iii) Semiempirical thermodynamic calculations will be employed to explain the discovery by us of marked solvent dependence of enzymatic substrate specificity. The goal is to develop

mathematical equations correctly predicting enzyme's substrate specificity as a function of fundamental interactions between the solvent and the substrate.

(iv) In order to maximize the enzymatic activity in organic solvents, we will systematically investigate its dependence on the way the enzyme is recovered from water. The methods of enzyme recovery will include lyophilization, crystallization, solvent precipitation, and spray-drying.

Annual Technical Summary Report:

The overall goal of this project is to rationally improve and control the catalytic performance of enzymes in neat organic solvents. To this end, we have investigated the dependence of enzymatic activity of several model hydrolases (lipases and proteases) in nonaqueous solvents on (i) physico-chemical properties of the solvent, and (ii) the presence of excipients in enzyme aqueous solutions prior to lyophilization and subsequent placement of the enzyme into the organic solvent.

With respect to (i), the question of whether the solvent's water-immiscibility is relevant to enzymatic activity was addressed by assaying four different hydrolases (three lipases and one protease) in nine anhydrous solvents of similar hydrophobicities of which four were infinitely miscible with water and five were not. For no enzyme was a jump in activity observed upon a transition from water-miscible to water-immiscible solvents. The relevance of solvent apolarity to enzymatic efficiency was also examined. To this end, three groups of isomeric anhydrous solvents were selected where within each group one solvent was apolar (i.e., lacked a permanent dipole moment). For none of the four enzymes studied was activity significantly higher in apolar solvents than in their polar counterparts. Thus we conclude that often-cited solvent's immiscibility with water and apolarity by themselves are irrelevant to enzymatic activity and solvent's hydrophobicity is the key pertinent characteristic.

With respect to (ii), we discovered that when seven different hydrolytic enzymes (four proteases and three lipases) were lyophilized from aqueous solution containing a ligand, *N*-Ac-L-Phe-NH_2, their catalytic activity in anhydrous solvents was far greater (one to two orders of magnitude) than that of the enzymes lyophilized without the ligand. This ligand-induced activation was expressed regardless of whether the substrate employed in organic solvents structurally resembled the ligand. Furthermore, non-ligand lyoprotectants [sorbitol, other sugars, and poly(ethylene glycol)] also dramatically enhanced enzymatic activity in anhydrous solvents when present in enzyme aqueous solution prior to lyophilization. The effects of the ligand and of the lyoprotectants were non-additive, suggesting the same mechanism of action. Excipient-activated and non-activated enzymes exhibited identical activities in water. Also, addition of the excipients directly to suspensions of non-activated enzymes in organic solvents had no appreciable effect of catalytic activity. These observations indicate that the mechanism of the excipient-induced activation is based on the ability of the excipients to alleviate reversible denaturation of enzymes upon lyophilization. Activity enhancement induced by the excipients is displayed even after their removal by washing enzymes with anhydrous solvents. Subtilisin Carlsberg, lyophilized with sorbitol, was found to be a much more efficient practical catalyst than its "regular" counterpart.

In addition, we addressed the important question of the possibility of rationally manipulating enzyme chemoselectivity by the solvent. To this end, kinetics of monoacylation of different aminoalcohols with trifluoroethyl butyrate catalyzed by a dozen of proteases and lipases were investigated in a variety of anhydrous solvents. Chemoselectivity of these transformations (defined as the ratio of the rate of *O*-butyrylation to the rate of *N*-butyrylation, v_O/v_N) was found to be markedly dependent on the solvent under otherwise identical conditions. For example, with *N*-α-benzoyl-L-lysinol as the aminoalcohol, although *Pseudomonas* sp. lipoprotein lipase preferred the OH group and *Mucor meheii* lipase the NH_2 group in all solvents tested, the extent of this preference varied up to 20 fold depending on the solvent. Moreover, for porcine pancreatic lipase, a solvent-induced inversion of chemoselectivity was observed. A

mechanistic rationale was proposed which related the v_o/v_N values to physico-chemical properties of the solvent.

13. **Project Title:** Immobilized Enzymes in Organic Solvents

Principal Investigator: H. Zemel

Project Site: Allied Signal Research & Technology, Des Plaines, IL

Description:

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 inter-esterifications in organic media. We have studied the effect of water content on the enzymatic interesterification of tripalmitin with stearic acid in a non-polar solvent, petroleum ether. The enzyme, Amino P lipase, was immobilized non-covalently on a porous inorganic support. In this system, we have been successful in demonstrating that 98% of the water added to the solvent (less than 1% *v/v*) resides on the support particles, and forms a fully aqueous microenvironment. We have shown that in this aqueous microenvironment, the fraction of the enzyme that is dissolved operates essentially as in monophasic pure water. With no water added and no dissolved enzyme, no significant activity has been observed. The second major parameter affecting the rate of reaction is mass-transfer limitations between the two phases: as the thickness of the water layer increases, and the particle pores fill up with water. These two factors, namely, the fraction of dissolved enzyme and the particle morphology, which determines when mass-transfer between the two phases becomes rate limiting, were sufficient to explain all our findings. Thus, we have been able to formulate a uniform theory which: 1) Provides simple explanations for most observations in systems with low or high water content 2) Affords the prediction of water requirements for optimal operation of supported enzymes in organic solvents 3) Provides a clearer view of future research needed in order to transform the phenomenon from an academic curiosity to a viable commercial process technology.

The application of enzymes to organic synthesis in non-aqueous media is an emerging technology with tremendous promise for the chemical industry. It might very well be the key to legitimizing biocatalysis and transforming it into a widely practiced commercial chemical processing technology. The need for this transformation is becoming increasingly obvious, as environmental issues of toxic waste generation are forcing the industry to take action aimed at reducing the production of pollutants. Enzymes, in their specificity and efficiency can contribute immensely towards cleaner and safer chemical production processes. One of the obstacles to utilizing enzymes in a commercial setting is the fact that most industrial chemicals are insoluble in the enzymes natural aqueous environment. Thus the adaptation of enzymes to organic media is a major requirement. Moreover, the use of organic solvents as the enzymatic reaction's medium offers many other advantages as well. For example, the need to covalently immobilize the enzymes is eliminated as they are insoluble in the organics. So are many enzyme cofactors; these will be retained with the enzyme on the surface of a support in contact with the solvent, and will not wash off. Such consideration will provide a simpler and less costly bioreactor. Other advantages of enzymes/organic-solvents systems include: reduced product and substrate inhibition, simplified separation and reversal of thermodynamic equilibria.

The potential of the technology is great. What is needed in order to realize this potential is the understanding of the phenomenon on a molecular basis so that the parameters, which affect the efficiency and stability of such systems, can be intelligently manipulated to afford the practical scale-up of enzyme-catalyzed chemical production processes in non-aqueous media. 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.

1993 Accomplishments:

Our project's objective is to study the critical factors that limit commercial scale applications of enzymes in organic solvents, in particular, the effect of added water. The ultimate product of this project will be a process for production of a precursor to optically active polymers. The first 12 months of the project were dedicated to the complete understanding of systems which have a microaqueous phase supported on porous particles.

Using triglyceride inter-esterification as a model reaction, we have been able to describe the overall rate dependence on water content in terms on two regions: 1. a minimal water region, where enzyme solubility and enzyme kinetics play a role, and 2. a mass transfer/support dependent regime.

In the minimal water regime, we have established a broad correlation between enzyme solubility and activity in the system. We have also advanced significantly towards determining the distribution of the enzyme between the bulk aqueous microphase and its interfaces with the support and solvent.

We have formulated a model for triglyceride depletion, and establish that mass transfer across the solvent/water interface is a major factor controlling the overall rate; diffusion of reactants through the solvent within the porous particle in not rate limiting. We have demonstrated that the water content of the non-aqueous system affects the rate through both intra-particle effects as well as inter-particle aggregation.

We have designed a support, that will alleviate the mass transfer problem, and maintain an aqueous phase sufficient for enzyme dissolution. It relies on in-house proprietary technology. The support will afford control of pore geometry and hydrophilicity, and thus provide a means for achieving maximal bioreactor efficiency.

We have identified an Allied Signal product, which is produced through a chemical trans-esterification reaction, and suffers from residual catalyst problem. We will be evaluating an enzymatic synthesis in non-aqueous media to replace the current process.

1994 Planned Activities:

In the next year we will complete our investigation of low water systems (Task I), complete the mass transfer modelling studies (Task II), and put most efforts into characterizing and scaling up the anhydrous trans-esterification reaction (Task III). The details are as follows:

Task 1 Low Water System (completion in April 1994)

Demonstrate the applicability of the Biphasic theory on a new low-water system: pure enzyme, immobilized vs. suspended enzyme, covalent vs non-covalent immobilization.

We have already confirmed the past observations with a pure enzyme on various supports. In the process of completing this task, we will compare Klibanov's method of using free suspended enzyme vs support immobilization in terms of overall performance and in particular the water dependence of the enzyme activity. We will also address the issue of enzyme configuration in the system: whether it is adsorbed to the support surface, dissolved in the bulk aqueous microphase, or concentrated at the aqueous/organic solvent interface. Part of the work towards this specific goal has already been performed. The results

obtained so far provide guidance in the selection of enzyme support. We will test some proprietary supports that should provide optimal enzyme performance.

Task 2 Mass-Transfer (completion in October 1994)

Investigate the mass transfer contributions to the observed kinetics experimentally and theoretically.

The modelling of mass transfer in the low water system will be completed, and results utilized in the selection and design of better enzyme supports.

Task 3 "Dry" Systems (completion in October 1995)

Study the water dependence in "dry" systems with water content lower than the solubility in solvent.

Work has already begun on a trans-esterification of naphthyl ethanol with vinyl acrylate. This reaction has been studied by A. Klibanov of MIT, and he is providing us with initial reaction conditions and analytical methods. We will study the water dependence of this "dry" reaction and the effect of enzyme immobilization. The immobilized enzyme reactor will then be optimized for production of a chiral precursor to a polyacrylate polymer. The understanding of this dry reaction will then be used to investigate the applicability of anhydrous enzymology for production of another Allied Signal proprietary monomer. If Successful, the remainder of the time will be devoted to scaling up this particular trans-esterification reaction, which is of utmost importance in the manufacturing of a newly launched product.

Annual Technical Summary Report:

The overall goals of our DOE-funded project are to investigate the critical factors that limit commercial scale applications of enzymes in organic solvents, and to scale-up a process for the production of a precursor to optically active polymers. Initially, we are attempting to quantify the effect of intra-particle dynamics on overall process performance. This involves examination of coupled enzyme kinetics, solubility, wetting and mass transfer effects toward support selection, reactor design/scheme and scale-up. For this study we selected a pure enzyme preparation: a lipase from *C. Viscosum*. The enzyme has been immobilized on celite and the catalyzed interesterification kinetics in petroleum ether and its dependence on water concentration have been determined. The results are very similar to those obtained in the past with crude *Pseudomonas Cepacia.* So far we have formulated a model for tripalmitin depletion, based on taking mass balances. A parameter has been included which accounts for the effect of water content inside the celite particle. The initial rate of depletion can be described by Monod-like kinetics. Our experimental data, at 0.003 mg H_2O per cm^2, fit a model and parameters presented in the literature. Because of the small size of the support, the relatively low amount of water, and the low reaction rates, our calculations predict that the lipase system at 0.003-0.01 mgH_2O/cm^2 is reaction rate limited. The mass transfer through the organic phase within the support particles is not limiting. In order to test the model, we studied the affect of particle morphology on the interesterification kinetics. Fully dense particles with varying diameter but the same total surface area have been tested and compared to the porous celite. The water content dependence with the fully dense particles suggest that mass transfer limitations due to inter-particle aggregation are the main cause of slowed down kinetics beyond a water level of 0.01 mgH_2O/cm^2. With porous supports such as celite, the onset of rate decrease occurs at a higher water content (0.03 mgH_2O/cm^2 for celite 560) as the inner volume of the pore accommodates more water, thus preventing it from accumulating on the outer particle surface and causing aggregation. The implications of these findings for reactor design are discussed.

The overall goals of our DOE-funded project are to investigate the critical factors that limit commercial scale applications of enzymes in organic solvents, and to scale-up a process for the production of a precursor to optically active polymers. The first 12 months of the project were to be dedicated to the complete understanding of low water systems and the mass transfer modelling with initial experiments on "dry" systems:

Task 1 Low Water System (completion in 18 months)

Demonstrate the applicability of the Biphasic theory on a new low-water system: pure enzyme, immobilized vs. suspended enzyme, covalent vs non-covalent immobilization.

Task 2 Mass-Transfer (completion in 24 months)

Investigate the mass transfer contributions to the observed kinetics experimentally and theoretically.

Task 3 "Dry" Systems (completion in 30 months)

Study the water dependence in "dry" systems with water content lower than the solubility in solvent.

After working on the project for eight months we are ahead of schedule in both Task 1 and 2, and on schedule on Task 3.

In the past we have studied the effect of water content on the enzymatic interesterification of tripalmitin with stearic acid in a non-polar solvent, petroleum ether. A crude enzyme preparation, Amino P lipase, was immobilized non-covalently on a porous inorganic support. These studies yielded a correlation between the water dependent peak activity and the support particle's pore volume. The system was, in fact, a biphasic system with a large organic phase and a minute aqueous phase, in which we attributed the decline in enzyme activity at the higher water levels to mass transfer limitations. As this correlation was demonstrated for one crude enzyme, we felt that in order to support our case, we need to demonstrate that the same conclusions apply to other enzymes, to pure enzyme and to various enzyme configurations (Task 1). Also included in the initial target of this phase of the project has been to model mass transfer in the system and quantify the effect of inter- and intra-particle dynamics on overall process performance (Task 2). This involves examination of coupled enzyme kinetics, solubility, wetting and mass transfer effects toward support selection, reactor design/scheme and scale-up. A preliminary model of tripalmitin depletion has been formulated and shown to fit our experimental data, at very low water levels. In parallel, we have tested the interesterification rate dependence on water content with a pure lipase immobilized on non-porous particles. The results shed more light on the major factors controlling the overall reaction rate, and thus provide guidance towards reactor designs with maximal efficiency.

Materials and Experimental Methods

Enzyme immobilization

Immobilization of the lipase onto the support was accomplished by deposition from a buffered solution. Typically, 5 mg lipase from *C. Viscosum* was dissolved in 1-2.5 ml of 0.005M potassium phosphate buffer. The volume of the enzyme solution depended on the support's pore volume, less was needed for non-porous supports. The enzyme solution was added to 500mg of 35-80 mesh support particles. A polystyrene petrie dish was utilized for preparation of the enzyme/support slurry, since in a glass container some of the enzyme adsorbs to the glass rather than the support. The mixture was air-dried in the refrigerator and then lyophilized, to give a dry support with 1% w/w enzyme loading (a different loading was used with sand as the support). This immobilization procedure was accurate within about 20%.

Variation in the loading could be significant from one batch to another especially for supports which did not adsorb the crude enzyme as well as the better adsorbers.

Interesterification rate measurements

The rate of tripalmitin interesterification with stearic acid was monitored by Gas Chromatography, using a RSL-300 fused silica capillary column from Alltech, and a Perkin Elmer 8500 Gas Chromatograph equipped with a flame ionization detector, and PE Nelson 2600 Chromatography Software. All kinetic measurements were conducted with samples containing 4ml of petroleum ether, 10mg of supported lipase, 0.03M tripalmitin, 0.08M stearic acid, and 20µl of tetradecane or pentadecane, an internal standard for the GC analysis. All of the components were placed in a teflon/silicon capped vial at room temperature. At that temperature the tripalmitin and the stearic acid were insoluble in the organic solvent. The reaction was initiated by warming the mixture up to 45°C which caused the substrates to go into solution, and then adding the water. The water was injected into the sample and placed on the vial wall above the support particles. The mixture was then swirled gently to allow the water to migrate homogeneously into the support. The support particles never clumped in the process. The vials were placed on a shaker at 45 degrees Celsius between sampling times. Liquid samples were removed from the reaction mixture by capillary action at appropriate time intervals using approximately 2.4 cm sections of non-heparinized microhematocrit tubes. Each sample filled tube was immediately placed in a GC autosampler vial. The sample was then derivatized by first adding 780 µl dry chloroform and 10 µl N-methyl-N-(trimethylsilyl)-trifluoracetamide to the autosampler vial. Next, the vial was sealed, vortexed to mix its contents, and allowed to incubate at room temperature for 20 minutes. The derivatizing agent reacted with the mono and di-glycerides tc form their silyl ethers, and with palmitic and stearic acid to form their silyl esters. Each component was quantified using the appropriate standard. All triglyceride and diglyceride standards were obtained from Sigma. In one or two cases, where a standard could not be obtained or it was too contaminated to use, a response factor of a similar component was applied. The rate of stearate incorporation was calculated as the rate of change of the ratio of stearate moles incorporated into the di- and tri-glycerides, divided by the total moles of stearate and palmitate in the di- and tri-glycerides. Each chromatogram provided a value of S/Glycerides for a specific time.

Spectroscopic kinetic assays

Kinetic measurements of the lipase's esterase activity with p-nitrophenol-caproate (PNP-caproate) were performed spectrophotometrically in aqueous buffer, using Hewlett Packard's 8451A Diode-Array spectrophotometer. Release of p-nitrophenol was monitored at 400nm.

Low Water Systems: Water Dependence Studies

Task 1

Pure Lipase Kinetics

We have studied the effect of water content on the enzymatic interesterification of tripalmitin with stearic acid in a non-polar solvent, petroleum ether. For this study we selected a pure enzyme preparation: a lipase from *C. Viscosum*. The enzyme was non-covalently immobilized on celite 560 by mixing the support with a buffered solution of concentrated enzyme, and drying it in air, followed by lyophilization. The loading level was 1% by weight. The interesterification kinetics of this enzyme in petroleum ether and its dependence on water concentration has been determined. The results are very similar to those obtained in the past with crude *Pseudomonas Cepacia*, confirming the generality of the phenomenon. The plots of the initial rate of stearate incorporation into the glycerides vs. water content for the two enzymes immobilized (deposited) on celite 560, are given in Figures 1 and 2. The maximal rate in both cases is obtained at a water level that reflects half filled pores. The differences in absolute rates are due to different loadings.

FIGURE 1

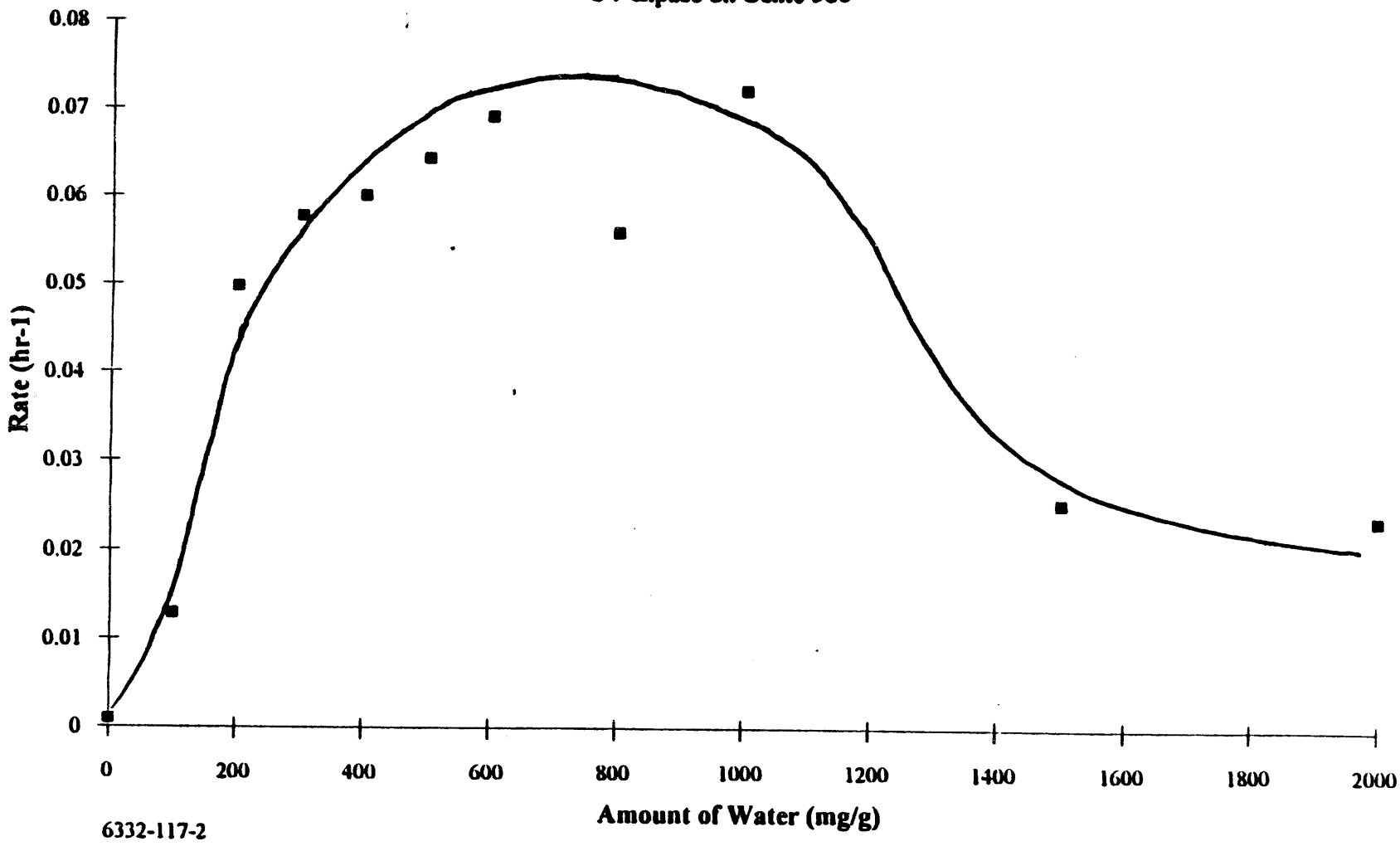
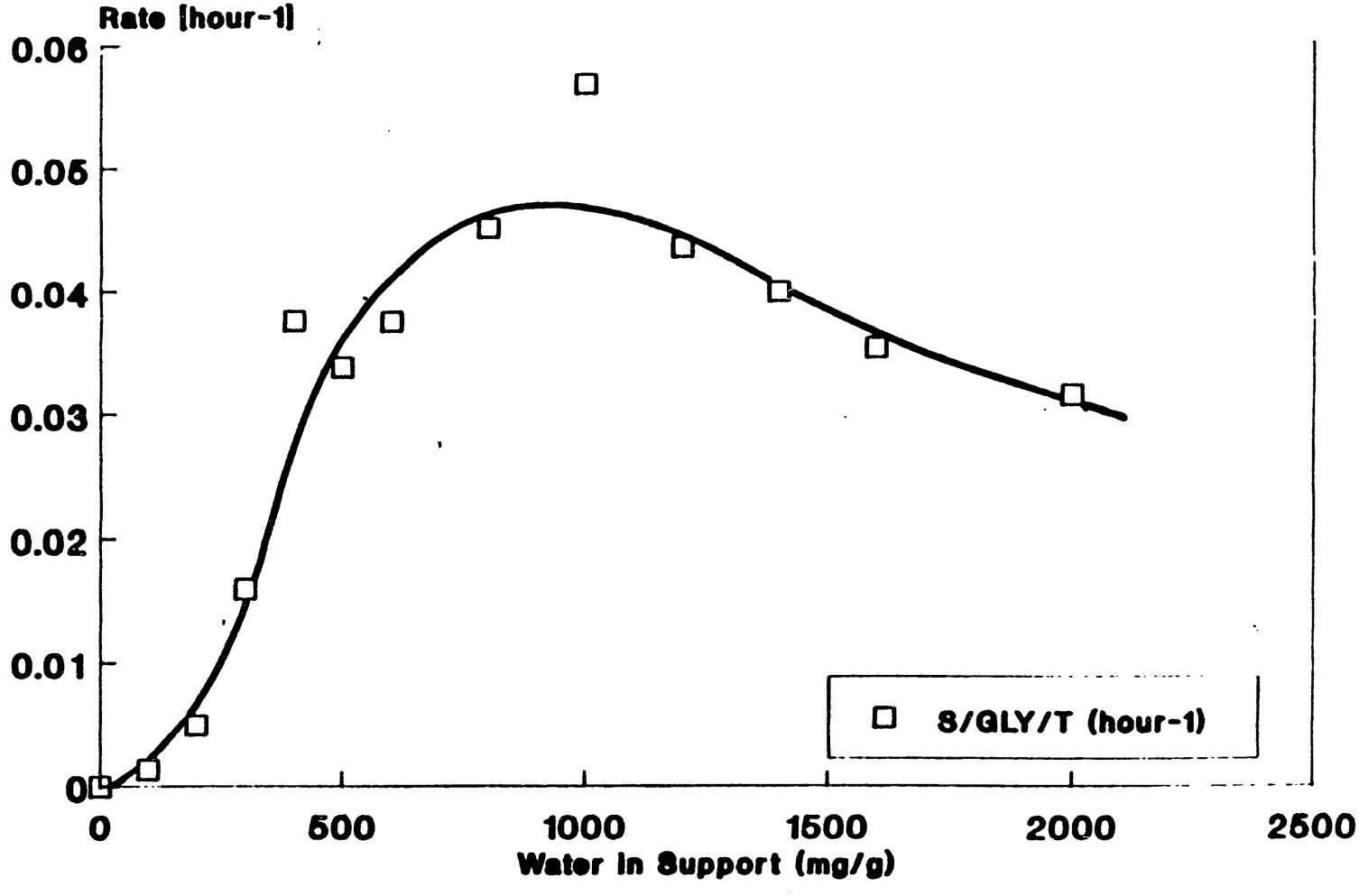

Intersesterifaction Rate vs. Water Content
CV Lipase on Celite 560
Rate (hr-1)
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
0
200
400
600
800
1000
1200
1400
1600
1800
2000
Amount of Water (mg/g)
6332-117-2

FIGURE 2

INTERESTERIFICATION RATE VS. WATER CONC.

10mg Celite supported P.F. Lipase, 10/89



Controls

The interesterification reaction was attempted with all ingredients present except for the enzyme. No reaction was detected.

Some glycerol could be generated in the process as a by-product of hydrolysis. Theoretically, a significant concentration of glycerol in the water might increase the solubility of the glycerides and fatty acids in the aqueous phase, and thus affect the reaction rate. We have determined that no significant change of solubility is occurring in the presence of up to 60% glycerol, a concentration which is orders of magnitude higher than experimentally suggested.

Enzyme Adsorption to the Support

For the mathematical modeling of the mass transfer it is important to know where the enzyme is; whether it is adsorbed on the support side of the aqueous phase, dispersed (dissolved) in the bulk water or concentrated at the aqueous/organic solvent interface. We have so far concluded that under conditions which represent the kinetic experiments, namely, completely hydrated supported enzyme, no significant adsorption of lipase to the support surface occurs. This was determined by incubating various amounts of celite with a concentrated enzyme solution, removing the supernatant, and testing it for protein content and esterase activity. Both tests showed no change from the original enzyme concentration, even as the celite amount was high enough to make a very thick slurry, Figures 3 and 4.

FIGURE 3

Enzyme Concentration vs. Amount Celite560

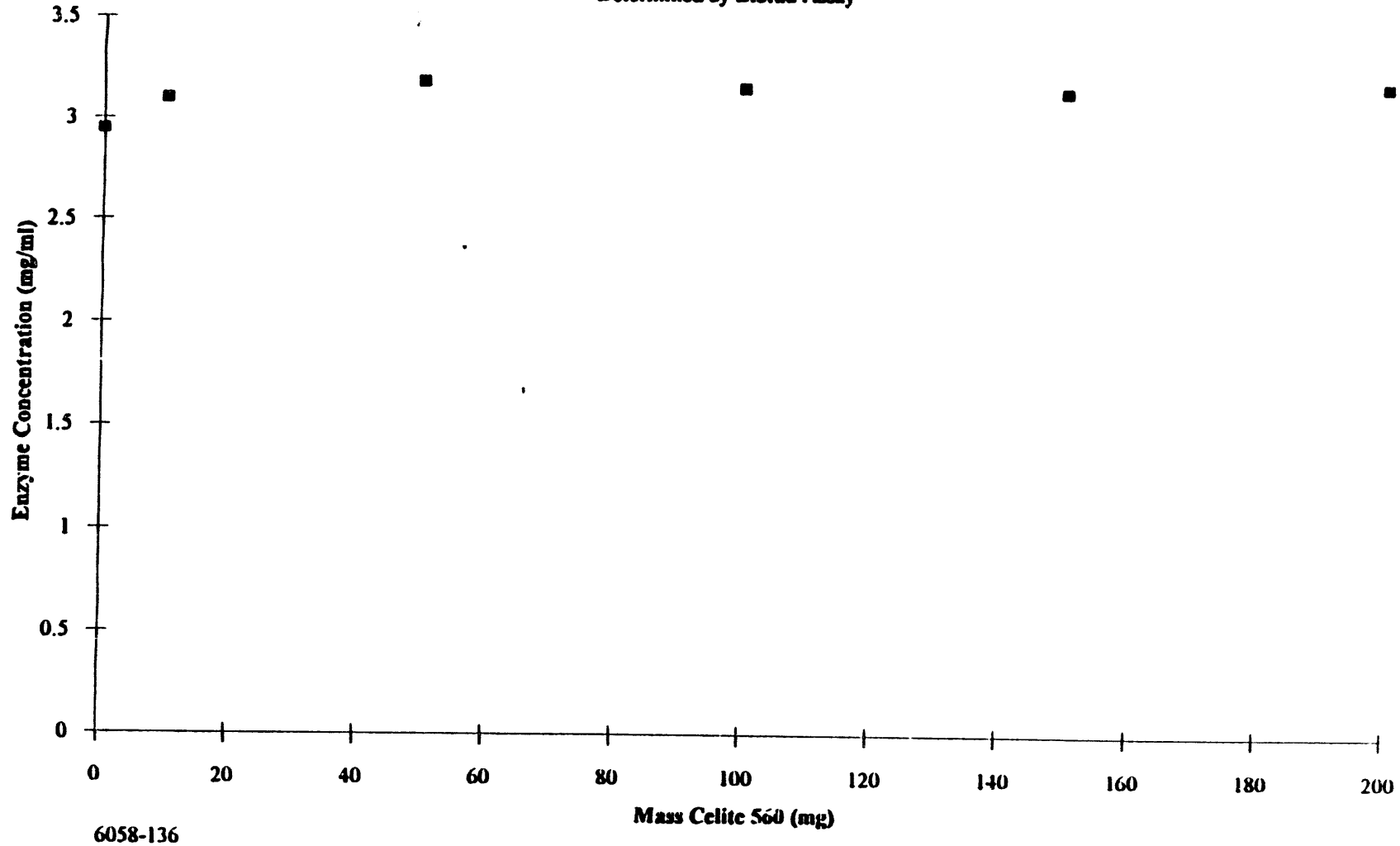
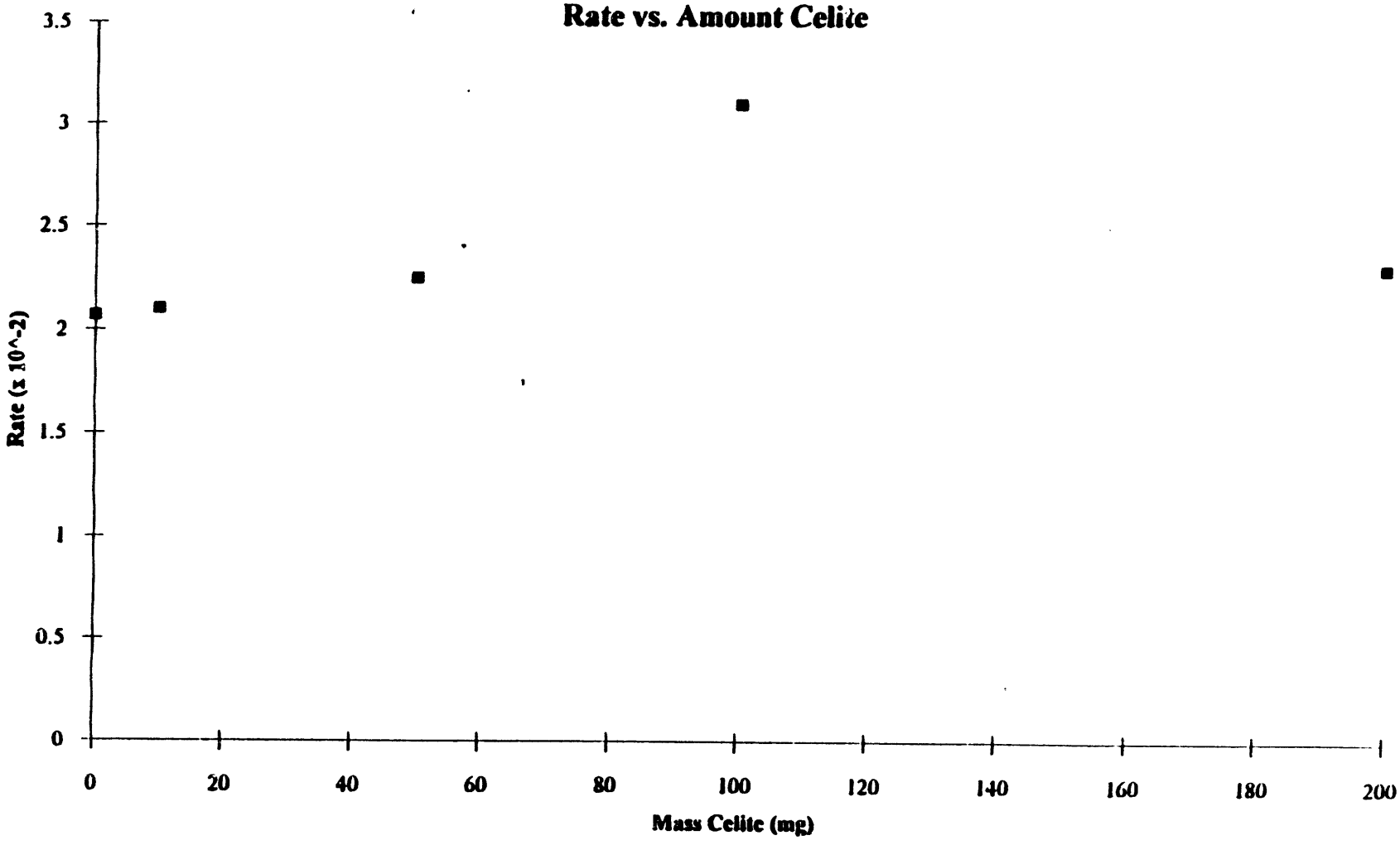


FIGURE 4

PNPC Assay Determination of Celite Absorption of CV Lipase



Morphology Effects on Interesterification Rates

Initially, we had planned to try variations in the support particle size distribution and pore size distribution as a means for estimating the importance of microscopic geometry. Hence, mercury intrusion and SEM analysis have been performed on the celite 560 originally used as the microaqueous environment support. The results suggested that the pore size and shape distributions are very inhomogeneous. In order to simplify the analysis and at the same time separate intra-particle from inter-particle effects, non-porous particles were tested. We employed two supports: proprietary monodisperse silica microspheres with 1μ diameter, and sand with average diameter of 250μ. The weight of support used for kinetic experiments was chosen so that it will provide the same surface area as provided by 10 mg of celite 560. Surface area was estimated from the average particle diameter rather than measured, and therefore, might contain significant error. The CV lipase was immobilized on these supports with loadings that resulted in the same absolute enzyme concentration as in the celite experiments. Different techniques had to be developed for each of these supports. The 1μ microspheres do not settle and stay in solution in a very fine suspension. Thus they had to be dispersed and hydrated while sonicating. The heavy sand particles needed a special mixing arrangement to make sure they disperse properly. In both cases, despite the mixing, aggregation could be easily observed starting at a rather low water level: approximately 0.012 mgH_2O/cm^2, and resulting in a slurry above 0.03 mgH_2O/cm^2. This is not surprising since all the water added is on the outer surface. Initial rates of stearate incorporation into the glycerides was measured as a function of added water. The results are presented in Figures 5 and 6. The water content is expressed in weight per unit surface: mg/cm^2. Figure 7 presents the celite data replotted on the same scale for comparison. It is clear that there is a direct correlation between the aggregation and the decrease in rate. The results clearly demonstrate, that with the fully dense particles, mass transfer limitations due to inter-particle aggregation are the main cause of slowed down kinetics beyond a water level of 0.01 mgH_20/cm^2. With porous supports such as celite, the onset of rate decrease occurs at a higher water content (0.03 mgH_2O/cm^2 for celite 560) as the inner volume of the pore accommodates more water, thus preventing its accumulation on the outer particle surface and delaying aggregation. Intra-particle mass transfer through the aqueous phase is also an important contributor to the delayed decrease in interesterification rate in the porous particle case.

FIGURE 5

Intersesterifacation Rate vs. Water Concentration

1% CV lipase loaded 1μ silica spheres

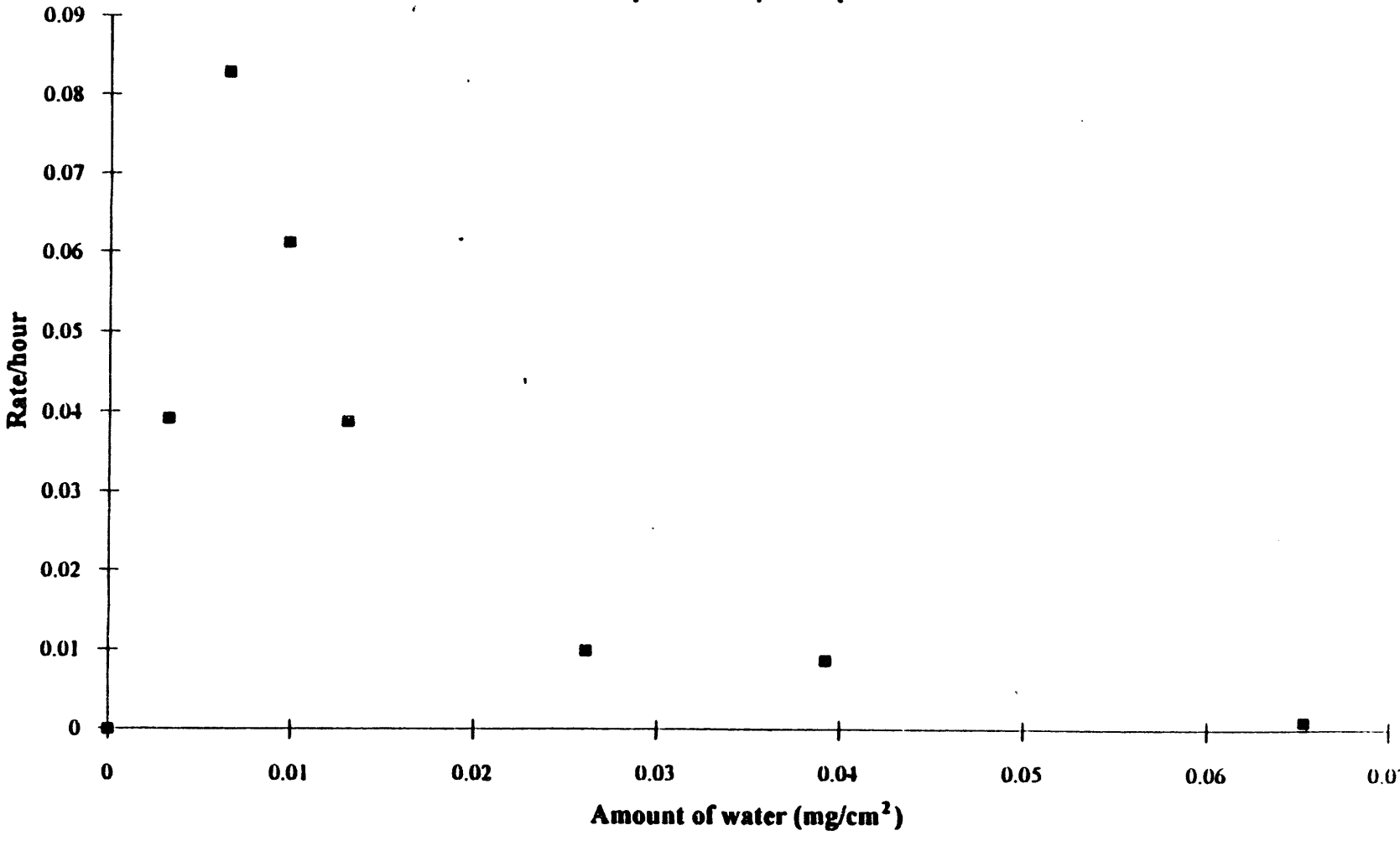


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FIGURE 6

Interesterifaction Rate vs. Water Concentration

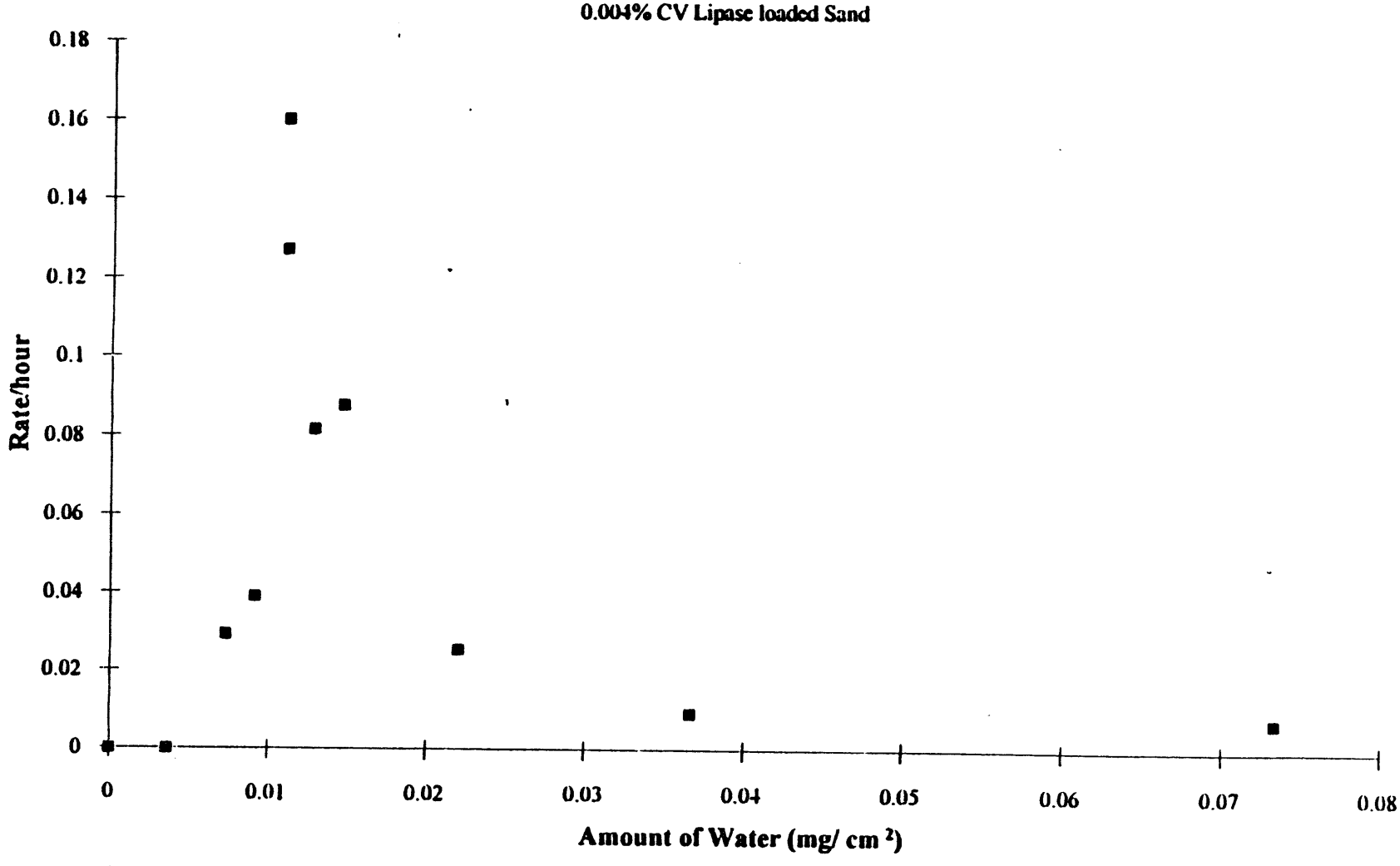

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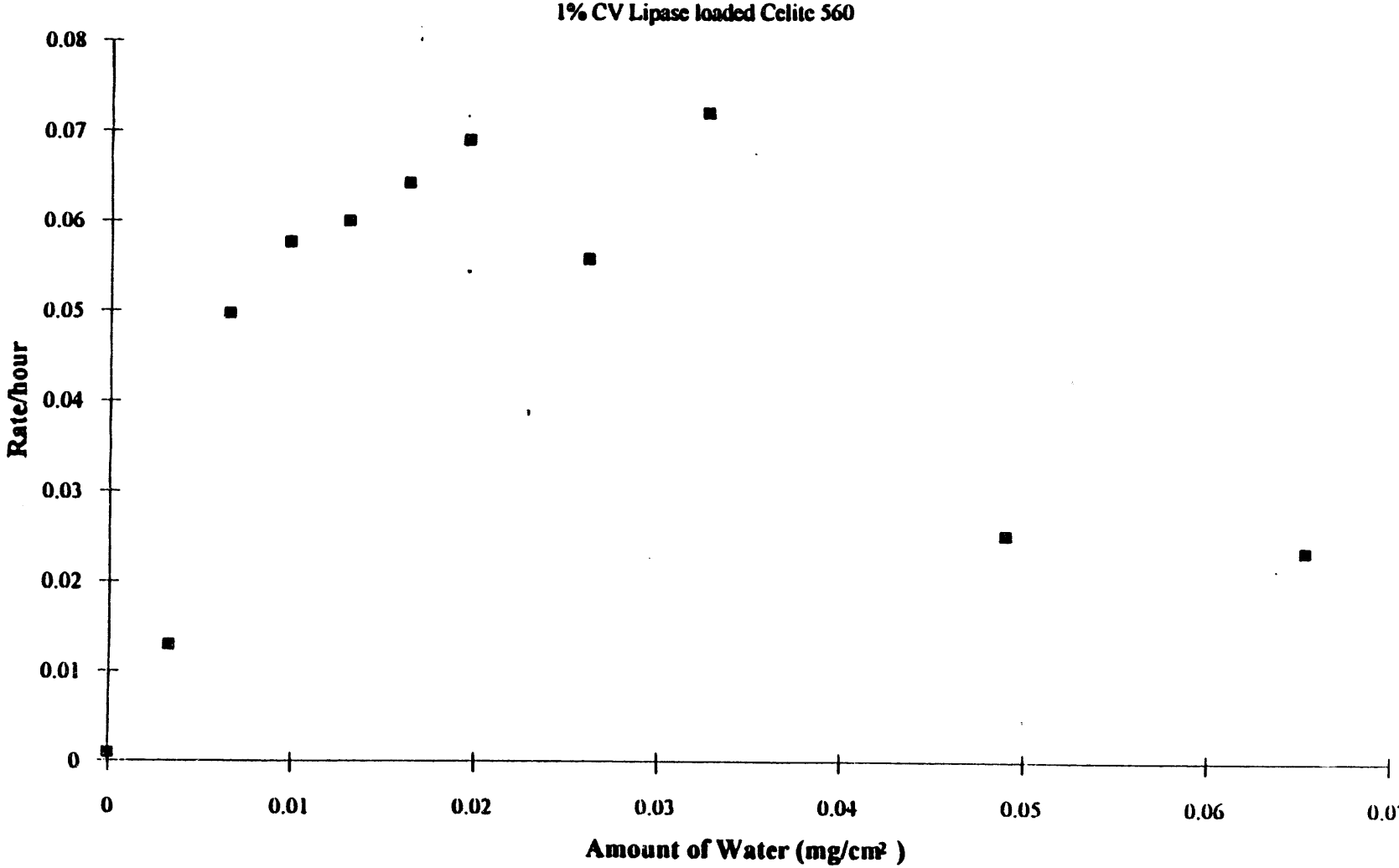
FIGURE 7

Interesterication Rate vs. Water Concentration



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Modeling Kinetics and Mass Transfer Effects
Task 2
Mass Balance

Mathematical models can be used to predict the reaction yields and selectivity, and optimize reaction conditions. We will take an evolutionary approach to model building: We will start with simple models then add more detail if required for a more complete description. As a first step, we considered the mass balance of tripalmitin. The depletion of tripalmitin is an approximate indicator of the incorporation of stearic acid by lipase, especially in the initial stages of the reaction.

To demonstrate the mass balance approach to modeling, consider a balance on tripalmitin (TP). TP must travel from the bulk phase to the enzyme-containing celite. Assume that the organic bulk phase is well-mixed throughout the reaction period. Therefore, for a batch system, the accumulation of TP is equal to the depletion:

(1)

$$\frac{dC_p}{dt} = -v_{obs}$$

where C_p = concentration of tripalmitin (M)
t = time (min)
v_{obs} = observed reaction rate (M/min)

Each of the celite beads are surrounded by a static film of the organic phase. Since the beads themselves act as a sink for TP (through reaction), the concentration difference of TP in the bulk phase and at the support surface is the driving force for the mass transfer of TP through this static film:

(2)

$$v_{obs} = k_1 a (C_p - C_p^s)$$

where k_1 = mass transfer coefficient (cm^2/min)
a = external surface area of support (cm^2)
C_p^s = concentration of tripalmitin at surface (M)

Diffusion is the dominant mechanism of mass transfer through the organic phase inside the celite beads. The concentration profile of TP is a function of radial distance from the center, as well as time. Assume a uniform distribution of enzymes inside the support. The accumulation of TP inside the support then equals the diffusion of TP in the support minus the depletion of TP:

(3)

The "active" surface area, a_{sw}, accounts for the effect of water content inside the celite support. When no water is present, the enzyme is inactive and a_{sw} is zero. The enzymes become more active as water wets the pores, eventually reaching a maximum (corresponding to a maximum a_{sw}). "Activation" by water may be due to the solubilizing of the enzyme. The water-insoluble substrates cannot diffuse into the water-filled pores. So as water fills the pores in higher than optimal concentrations, less enzyme becomes accessible

$$\frac{\partial C_{ps}}{\partial t} = \frac{1}{r^2}\frac{\partial}{\partial r}\left(D_e r^2 \frac{\partial C_{p_e}}{\partial r}\right) - v^1 a_{sw}$$

where

C_{ps} = concentration of tripalmitin inside support
r = radial distance center of support (cm)
D_e = diffusion coefficient (cm^2/min)
v' = reaction rate of TP inside support per active (M/min/cm^2)
a_{sw} = active surface area (cm^2)

to the substrate, thereby lowering the observable enzyme activity. Also, since water-filled pores reduce the area of the organic/water interface, fewer enzymes will be available at the interface.

The active surface can be estimated experimentally. Over time, a_{sw} depends on pore size distribution, pore shapes, wettability and surface energy of celite; and enzyme activity, diffusion, and solubility. To obtain a theoretical expression for a_{sw}, a better understanding is needed of how the enzyme is distributed in the two liquid phases, at the liquid interface, and on the solid surface.

The effectiveness factor (η) is defined as ratio of the observed reaction rate to the reaction rate without mass transfer limitations. Use of η allows us to relate the intrinsic reaction rate with the observed reaction rate:

$$v' = \frac{V_{obs}}{\eta a_{sw}} \tag{4}$$

To complete the mass balance of tripalmitin (equations 1-4), we need to develop an expression for the intrinsic rate (v') in terms of concentrations and rate constants.

Reaction Rate Model

Miller et al. (1991) studied the kinetics of an unsupported lipase in cyclohexane plus 0.05% water to interesterify trilaurin with lauric acid. They found that esterification is three times faster than hydrolysis. After applying the appropriate corrections, the model and parameters of Miller et al. can be used to estimate the reaction rate for our tripalmitin/stearic acid system. The following equations represent the hydrolysis step:

$$E + TP \underset{K_s}{\rightleftharpoons} E\text{-}TP \xrightarrow{k_2} E\text{-}P + 1,2PP$$

$$E\text{-}P + H_2O \xrightarrow{K_3} E + P$$

where E = *enzyme*
TP = *tripalmitin*
$E\text{-}P$ = *acyl-enzyme*
P = *palmitic acid*
$1,2PP$ = *dipalmitin*

Before equilibrium is reached, all reactions proceed from left to right. Miller derived the rate for these reactions as

$$V = \frac{V_{max}[TP]}{K_m + [TP]} \quad (5)$$

where

$$v = -\frac{d[TP]}{dt} \quad (6)$$

or the rate of tripalmitin disappearance. The square brackets represent concentrations (in M). Also:

$$V_{max} = \frac{[E_0]\, k_2 k_3}{K_2 + k_3} \quad (7)$$

where $[E_0]$ *is the initial enzyme concentration. And*

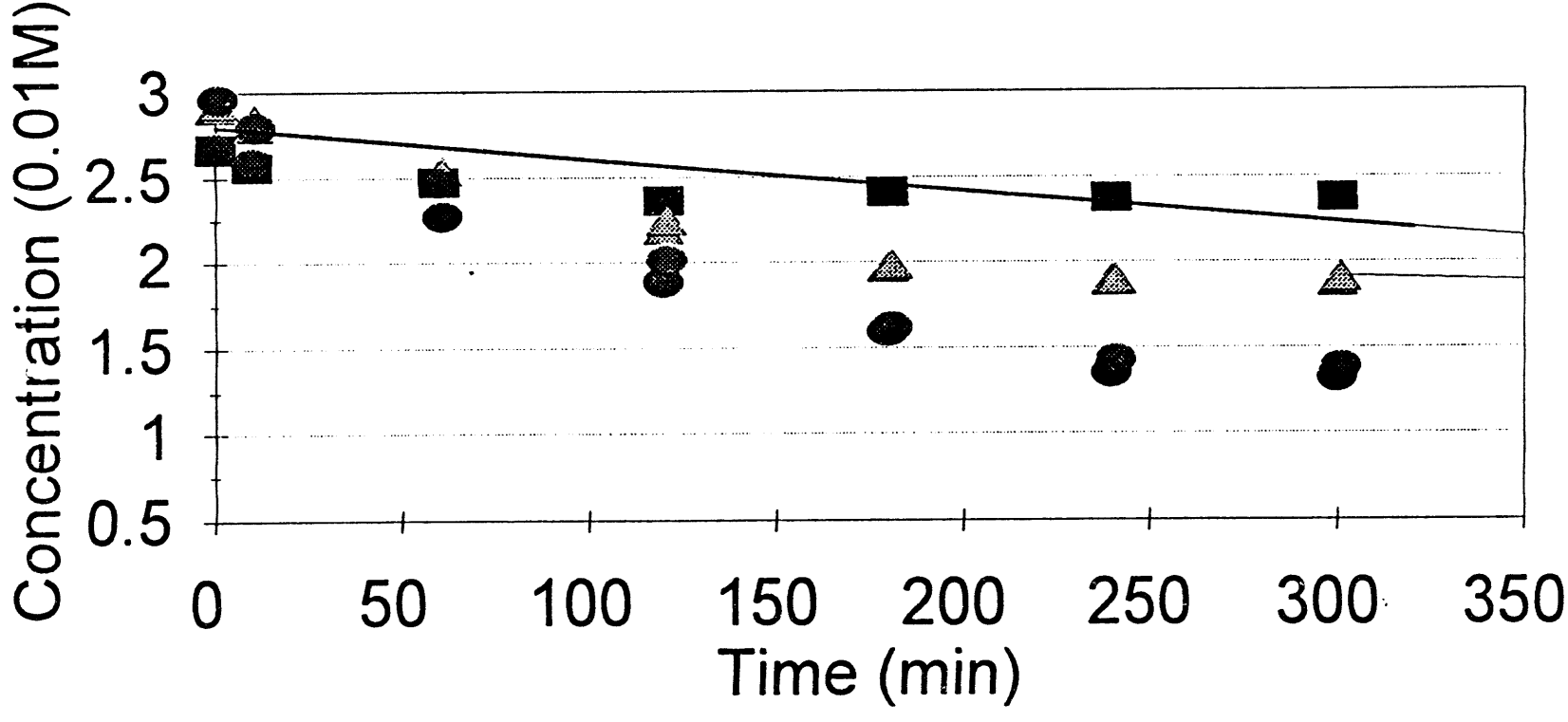
$$K_m = \frac{K_s k_3}{k_2 + k_3} \quad (8)$$

Adjusting for the different temperature, and for the different enzyme quantity and activity, the constants of Miller et al. become $K_m = 7.8 \times 10^{-10}$ M, and $v_{max} = 2.46 \times 10^{-5}$ M/min. Figure 8 compares the experimental tripalmitin profile with the model prediction. Our tripalmitin profile fits the model for 10% hydration. Higher hydrations have faster rates. The Miller model describes unsupported enzymes containing only 0.06% water. Water may wet celite surfaces not containing enzymes. Therefore, we expect enzyme solvation by water to be more efficient in an unsupported system such as Miller et al.'s. We will modify the model to account for these water effects.

FIGURE 8

Lipase on Celite in Hexane

Tripalmitin Profile



Hydration (g water per g support, %):

- 10
- 20
- 30
- 0.06 (Miller et al.)

Mass Transfer versus Reaction Rate Limited

Bailey and Ollis (1977) derived an "observable" Thiele modulus:

$$\Phi_{obs} = \frac{V_{obs}}{D_s S_0}\left(\frac{V_p}{A_p}\right)^2 \tag{9}$$

where v_{obs} = *observed reaction rate (M/min)*
D_s = *substrate diffusivity in the support (cm²/min)*
S_0 = *initial tripalmitin concentration (M)*
V_p = *volume of single support pellet (cm³)*
A_p = *external surface area of support particle (cm²).*

Φ_{obs} is a measure of reaction rate versus diffusion rate. High values of Φ_{obs} indicate diffusion limitations. The substrate diffusivity in hexane inside the support (D_s) depends on the porosity, which is controlled by the water content. For a liquid, the pore diffusivity in the support (D_s) and the free diffusion coefficient (D) are related by (Vermeulen, et al.):

$$D_s = \frac{D X_{eff}}{\tau} \tag{10}$$

where X_{eff} = *the effective internal porosity (pore volume filled with organic phase/support volume)*
τ = *tortuosity.*

The tortuosity can be approximated as $1/X_{eff}$, giving

$$D_s = D X_{eff}^2 \tag{11}$$

The effective porosity depends on the hydration as follows:

$$X_{eff} = X - h\rho_s \tag{12}$$

where X = *porosity (pore volume/support volume)*
h = *hydration (g water/g support)*
ρs = *piece density of support (g support/support volume)*

The piece density of celite 560 is 0.87 g/cc and the porosity is 62%. At 30% hydration (h = 0.3), the effective porosity is 0.36. Please note that when the celite pores are completely filled with water, h = 0.71 g/g, and the effective porosity becomes zero. This analysis is valid only when most of the reaction occurs inside the support, and not at the surface.

Assuming that trilaurin diffusivity in cyclohexane is on the order of tripalmitin in hexane, we can adjust the Miller et al. value for temperature to obtain $D = 4.76 \times 10^{-6}$ cm^2/sec. At 30% hydration, $v_{obs} = 6.59 \times 10^{-5}$ M/min when $S_0 = 0.028$ M. For 30-80 mesh Celite, the average particle size is 0.0192 cm. These numbers give us a value of $\Phi_{obs} = 0.00262$. Such a low Thiele modulus means that the effectiveness factor is very close to one. Therefore, the observed reaction rate is nearly equal to the intrinsic reaction rate.

Because of the small size of the support, the relatively low amount of water, and the low reaction rates, our calculations predict that the lipase system at 10-30% hydration is reaction rate limited. The mass transfer through the organic phase is not limiting.

Conclusions

A Monod-type model for tripalmitin describes the initial reaction rate well. For longer times, the accumulation of other compounds must be considered. This can be accomplished by taking a mass balance on all of the significant products. The effect of water on enzyme activity and solubility must also be determined.

Discussion

The above mathematical model deals with intra particle diffusion through the organic phase. It describes the kinetics for tripalmitin depletion, based on taking mass balances. A parameter has been included which accounts for the effect of water content inside the celite particle. The initial rate of depletion can be described by Monod-like kinetics. Our experimental data, at 0.003 mg H_2O per cm^2, fit a model and parameters presented in the literature. Because of the small size of the support, the relatively low amount of water, and the low reaction rates, our calculations predict that at low water levels, 0.003-0.01 mgH_2O/cm^2, interesterification will be hydrolysis reaction rate limited and not mass transfer limited. The mass transfer through the organic phase within the support particles is not rate limiting. Thus one expects to see no difference in the rate behavior between the porous and the non-porous supports at a water level below 0.01 mgH_2O/cm^2. Within experimental error the data at this water level is probably in agreement with the model's prediction. At higher water contents it is clear that there is a large difference between the porous and the non-porous supports. The experimental observation of aggregation in this region for the fully dense particles clearly accounts for this difference, and identifies the inter-particle aqueous pools as the cause for mass transfer limitations. With porous supports such as celite, the onset of rate decrease occurs at a higher water content (0.03 mgH_2O/cm^2 for celite 560 as the inner volume of the pore accommodates more water, thus preventing its accumulation on the outer particle surface and delaying aggregation. The various porous supports that we have tested in the past showed a correlation of the onset of rate decrease (or rate maximum) with the pore volume of the support. The higher the pore volume, the more water can be accommodated within the pores as compared to the outer surface, and the larger is the delay in rate decrease. In addition, as the water fills up the pores, intra-particle mass transfer effects are also expected.

A similar water dependence has been reported for suspended enzyme (Affleck, 1992). It is very probable that the same aggregation phenomenon due to added water is responsible for the rate decrease under those conditions as well. We will be testing free suspended enzyme in our system shortly.

The aggregation causes a major decrease in the interesterification rate. For practical applications, this could be overcome by appropriate reactor design. We will be examining various reactor designs that should eliminate this problem, i.e. various configurations in which the hydrophilic support particles are isolated from one another, either physically, or by effective fluidization.

14. **Project Title:** Biological Separation of Phosphate from Ores.

Principal Investigator: R. D. Rogers

Project Site: Idaho National Engineering Laboratory

Description:

The project is aimed at bioseparation of insoluble phosphate complexes from ore matrix. To achieve this, selection and culturing of microorganisms capable of dissolving and concentrating insoluble phosphate forms have been made. The mechanism by which microorganisms dissolve the phosphate complexes are being investigated. Promising bioreactor configurations will be tested at the bench scale.

1993 Accomplishments:

Accomplishments for the past 12 month period include the continuance of the CRADA with the Simplot Fertilizer Co., completion of an engineering assessment of the bioseparation process, and physical, chemical, and molecular genetic work designed to increase the amount of soluble phosphate in solution.

The effort with Simplot has been one of technology transfer. Using the biosolubilization phosphate work as a nucleus they have been developing a biotechnology capability. They have acquired sufficient staff and equipment to allow them to use our biosolubilization technology. Their organization has been using a variety of bioreactor systems including 1 L, 10 L, and 200 L. Most of the work has been with the 10 L system. Using that system, tests have been conducted to elucidate clarifier and exchange column designs. Attempts has been made to start the 100-200 L reactor system. Experience with the 100-200 L system will provide Simplot personnel with the know how to manage larger reactor operations.

The engineering assessment of the process was published as an official EG&G document under the title "Research and Engineering Assessment of Biological Solubilization of Phosphate". Using design and process parameters which had proven successful for phosphate solubilization during laboratory studies, the assessment was used to develop a conceptual bioprocess for the purpose of processing a substantial quantity of ore. System modifications and plant economics were evaluated in light of system scale up.

Both cation (CER) and anion (AER) exchange resins were placed in-line in the system to studied the ability of CER to remove excess calcium and then collect and concentrate soluble phosphate with AER. Initial indications are that these systems could help improve the concentration of phosphate in the working lixiviant.

Work with the Mineral Phosphate Solubilization (Mps) gene and the mechanism of solubilization continue to progress. Data show that the enhanced mineral phosphate solubilization trait exhibited by our microorganism is the result of extracellular glucose being oxidized to gluconic acid by a quinoprotein glucose dehydrogenase enzyme system. Data such as these are providing the basis for development of a metabolically engineered process.

1994 Planned Activities:

Ongoing work will be developed around the four critical hurdles which were defined in the document "Research and Engineering Assessment of Biological Solubilization of Phosphate". Research and engineering efforts will be used to overcome these hurdles in order to commercialize a viable biosolubilization process. The critical hurdles are:

- Development of methods to obtain soluble phosphate concentrations which will satisfy manufacturing and marketing requirements.

- Reduce the number of separation steps required during processing in order to decrease both capital equipment costs and energy consumption.

- Continue testing the effect of ore particle size on the rate of phosphate release.

- Development of methods to maintain a pure microbial culture without the need for extensive sterilization of process streams.

Annual Technical Summary Report:

In response to an AICD request an engineering assessment of the project was undertaken. This major milestone was completed and a report entitled the "Research and Engineering Assessment of Biological Solubilization of Phosphate" was published. Using design and process parameters which had proven successful for phosphate solubilization during laboratory studies, the assessment was used to develop a conceptual bioprocess for the purpose of processing 2 million tons of phosphate ore per year. A mass balance calculation for this conceptual bioprocess found that, as initially conceptualized, a large amount of water would be necessary to operate the process. The water requirement was directly linked to the quantity of soluble phosphate which the biologically produced lixiviant was able to maintain in solution. Since previous project data had demonstrated that soluble phosphate concentration could be increased through the management of accumulated calcium ion, an optimized bioprocess was conceptualized that increased soluble phosphate in the process streams from 200 mg/L to that of 5,00 to 20,000 mg/L. Mass balance calculations showed that such an optimized bioprocess would substantially reduce water requirement. If water requirements were reduced, then it appeared there would also be a substantial change in the handling and holding requirements. When combined, these changes would make the process economically viable. For the optimized bioprocess, capital equipment costs were estimated to be approximately $69,000K. The total fixed capital cost for a bioprocessing plant was estimated to be roughly $318,000K. Further, the bioprocess was estimated to use nearly the same amount of energy as the existing wet acid process but would produce an upgraded phosphate product of increased economic value to the producer. It was shown that the bioprocess was considerably more efficient than the oxidation method currently being used to produce upgraded phosphate products.

Economic, energy, and environmental data were encouraging to the CRADA partner, J. R. Simplot Co. Since initially recognizing the significance of the work, Simplot has invested a significant amount of their resources in facility upgrades and personnel to begin capitalizing on the bioprocess.

Work on the Mineral Phosphate Solubilization (Mps) gene and the mechanism of solubilization continued to progress. Data was obtained that supported evidence for the hypothesis that the enhanced Mps+ trait exhibited by the bacterium E-37 (the microbe currently being used in the bioprocess) is the result of extracellular glucose oxidation. In this process the glucose is being oxidized to gluconic acid by a glucose dehydrogenase (GDH) enzyme system. This system was thought to be located on the outer face of the bacterium's cytoplasmic membrane.

Additional work demonstrated that the GDH enzyme has a prosthetic quinoprotein group whose presence is required for activation of the enzyme. This group is a pyrolo-quinoline quinone (PQQ). Control experiments using mineral phosphate medium supplemented with PQQ confirmed that the extracellular, nonphosphorylating, direct oxidation pathway provides the metabolic basis for the Mps trait in gram negative bacteria (E-37 is gram negative). It was speculated that the Mps gene may be a regulatory gene for the PQQ operon in these organisms. Based on these data, it would appear that there is a genetic and

biochemical bases for metabolic engineering of the solubilization system. The development of a metabolic engineering strategy for the production of E-37 strains, with a highly enhanced biotransformation capability for mineral phosphates, appears to be possible. Using these data it may be possible to develop a "super solubilizing" bacterium.

Increasing the rate of the solubilization reaction continued to be pursued. One of the basic solubilization hypotheses proposed in the literature has been that organic acids are important to the solubilization process because they act as chelators for removal of solubilized calcium. Removal of calcium from the reaction would tend to force the equilibrium between insoluble and soluble phosphate to the right, and thus favor increased phosphate production. While we have shown in our studies that organic acids do not chelate calcium during solubilization, the idea of a calcium chelation system is still attractive.

Work progressed on the use of cation exchange resin (CER) as a method for calcium removal. Results with CER in continuous processing continued to be encouraging. In addition, efforts designed to evaluate the ability of in-line placement of anion exchange resin (AER) to separate and concentrate soluble phosphate from the process lixiviant were conducted. Studies using the two types of resins (CER and AER) placed in tandem in the process lixiviant stream were conducted. Three utility bioreactors were used for the studies. The first reactor was setup to run at ambient pH (no pH control) while the pH of the contents of the second and third reactors were maintained near the mid 3's through the continuous additions of NaOH. After initial start up, both the second and third reactors had CER columns placed in-line between the contact cell (where phosphate ore is exposed to biologically produced lixiviant) and spent lixiviant collection vessels. After placement of the initial CER column, the third reactor had an AER column placed in-line down stream from the CER column. Once the AER was in place and functioning, a closed-loop recycle of a portion of the lixiviant was recirculated to the contact cell. With further process improvements it is intended that this split flow will be returned to the bioreactor for utilization of any residual carbon.

Results of the work showed once again that calcium can be removed from the processing lixiviant by using CER. In addition, there was a good indication that the AER was able to remove and concentrate soluble phosphate from the lixiviant. Follow on studies will be required to modify the system to allow the stripped lixiviant to be fed back into the process bioreactor for efficient lixiviant utilization.

The project continued to benefit from peer involvement. It was possible to enhance the work through the use of subcontracts to those who were experts in related fields. For example, much of the molecular level understanding of the mechanism of biosolubilization of phosphate was a result of cooperative work with Dr. Alan Goldstein, California State University, Los Angeles.

4.3 Feedstock/Process Interactions

15. Project Title: The Carbon Dioxide Bioreactor: Conversion of Industrial Waste Carbon Dioxide into Polyhydroxybutyrate (PHB).

Principal Investigators: R. Kern

Project Site: Jet Propulsion Laboratory

Description:

The long term goal of this project is to construct CO_2 fixation plasmid genes to convert CO_2 and hydrogen to biodegradable polymers such as the polyesters (PHB) from the bacterium, *Alcaligenes eutrophus* or polysaccharides from a variety of other microbial sources. These polyester plastic and polysaccharide materials have a wide variety of applications, including the food and cosmetic industries, rheology modifiers, enhanced oil recovery, chromatography industry, the paper and metals finishing industries, and packaging industries. Currently many of these products are derived from plant sources outside the United States. It is estimated that the use of microbes for polymer synthesis can reduce the costs of manufacture from four to six times over that of conventional chemical synthesis. However, the cost of isolation of this product from bioreactors is still too high for the product to compete with larger volume film/packaging applications. ICI has the bulk of the patents in this area in the production of PHB and copolymers of PHB and polyhydroxyvalerate from glucose. Bacteria such as *A. eutrophus* grow rapidly (2-3 h generation time) in the presence of carbon dioxide as the sole carbon source, hydrogen for energy from some source, and oxygen as electron acceptor. These generation times are comparable to those in the biological production of commercial aminoacids. Nutrient requirements appear minimal (including nitrogen). High cell densities are achieved and about 70-80 wt% of polymer granules are produced intracellularly from a glucose-utilizing mutant. After cell disruption, the polymer is extracted with an organic solvent. For other systems, the polysaccharide is excreted making the broth extremely viscous and limiting the access of cells to substrates, especially CO_2. In the short term, this project has two overall goals. One, examine ways to deviscofy media broths and enhance the utilization of CO_2 by microbes. Second, begin genetically modifying *A. eutrophus* to use CO_2 directly in the production of polyester synthesis. These two short range goals will be done sequentially.

1993 Accomplishments:

A large collection of Xanthobacter autotrophicum strains where obtained and evaluated for production of both extracellular polysaccharide and the intracellular reserve material polyhyroxybutyrate. Strains capable of growth on sucrose, a common fermentation feedstock, were identified. Of the collection of 64 strains 40 were selected for initial screening based on the criteria of relatively rapid, robust growth. To date 24 of these strains have been quantitatively evaluated for production of exopolysaccharide and polyhydroxybutyrate under both heterotrophic and autotrophic growth condition. A loose reciprocal correlation was found between the level of polyhydroxybutyrate and polysaccharide production. One strain in particular, X. a. 9 was found to give extremely high levels of polysaccharide production, apparently in excess of 90% in some heterotrophic batch culture conditions. This atypical strain is being further characterized, and reconfirmed to be Xanthobacter autotrophicum prior to publication of this finding. Polymer yields with few exceptions were found to be higher under heterotrophic conditions. The exceptions however suggest that mutations may be possible to permit autotrophic levels of polymers to match those obtained under heterotrophic conditions.

Yield enhancement by mutation has already been demonstrated. A spontaneous non-exopolysaccharide variant of type strain 7C was compared to wild type exopolysaccharide producer. The blockage of

exopolysaccharide synthesis resulted in a two fold increase in the polyhydroxybutyrate yield per cell under heterotrophic conditions. This increased the production level from 32% to 71%. In addition to yield enhancement by mutagenesis the second year's effort plans to employ recombinant DNA constructs to enhance yield. With the aid of the project advisor a gene library of alcaligenes eutrophus, perhaps the most robust and rapidly growing of the chemoautotrophs, has been obtained to serve as a source of genes and regulatory regions involved in growth on CO_2/H2/O_2.

A comparative study of polysaccharide producing and nonproducing variants of the same strain have shown that production of polysaccharide reduces the growth rate and decreases the reproduciblity of batch culture yield of both biopolymer and whole cells. The model system used for these experiments was the World Health Organizations reference strain for the production of Klebsiella pneumonia capsular material K63, an extremely viscous water soluble polymer. Further experiments with this system are planned to determine if this result was due to intracellular shunting of carbon and energy or simply due to the blockage of physical diffusion of oxygen.

1994 Planned Activities:

During the upcoming year this effort will focus on the genetic enhancement of polymer yields and rates of production. However, the characterization of the Xanthobacter strain collection for yield and quality will continue as time and resources permit. This will include evaluation of exopolysaccharide for rheological performance as well as characterization of the polyhydroxybutyrate produced.

Prior to further genetic work the Xanthobacter collection will be screened to identify those strains which can serve as recipient for pVK102 hybrid plasmids. Transfer and expression of the NAD-reducing soluble hydrogenase of Alcaligenes eutrophus to Xanthobacter strains is a major goal. While in theory a pVK102 plasmid bearing the hoxS locus, which encodes for this enzyme, could be isolated from in house gene libraries; such a construct has requested from the laboratory of Barbel Friedrich at the Institut fur Pflanzenphysiologie und Mikrobiologie der Frein Universitat Berlin, Germany. This particular construct has already been demonstrated to confer soluble hydrogenase activity on its host, which has similar physiology to Xanthobacter, resulting in an increased rate of growth. Our goal is to confer soluble hydrogenase activity on biopolymer producing strains and characterize its effect on biomass, polyhydroxybutrate and polysaccharide production.

In order to give A. eutrophus the potential to convert CO_2 to a wide variety of organic chemicals for direct application or use as a feedstock, a broad host range hydrogen chemoautotrophic expression vector has been designed by project advisor Kjell Andersen. It will be constructed during the upcoming year. This should permit the expression of biosynthetic pathways of compounds of interest under growth conditions where CO_2 conditions. The pursuit of this alternative approach will leverage the experience gained during the hydrogenase gene transfer and expression work thus broadening the range of biochemicals produced by carbon dioxide fixation via hydrogen chemoautotrophs.

Annual Technical Summary Report

Along with plants several types of microorganisms have the ability to convert carbon dioxide into biomass. In some cases these microbes use sources of energy other than sunlight. The hydrogen oxidizing chemoautotrophs, often referred to as hydrogen bacteria, use the oxidation of hydrogen as a source of energy for the reduction of carbon dioxide. Among the potential products from this process are the biopolymers polyhydroxybutyrate and polysaccharides as well as lower molecular weight organic molecules.

Because of the recognized utility of polyhydroxybutyrate as a biodegradable thermoplastic with properties otherwise similar to polypropylene emphasis has been placed on hydrogen bacteria producing this material from CO_2. Alcaligenes eutrophus has been a focus of study because many of the genes involved have been both directly and indirectly cloned and sequenced. It is also the bacterium currently used to produce polyhydroxybutyrate commercially, utilizing glucose as a carbon source, by Imperial Chemical Industries (ICI). Xanthobacter autotrophicum is also being studied because of its ability to produce not only polyhydroxybutyrate but also a series of strain specific polysaccharides with rheological properties of interest to industry. Among the applications of direct interest to DOE should be those Xanthobacter strains producing pseudoplastic thickening polymers which retain their properties over a wide range of temperature, pH, and salt concentration.

The basic strategy of the currently funded phase of research can be described in the following sequence of three steps. At this point step one is completed, step two is two thirds complete and step three is in the planning stage.

Step 1: Strains have been collected for a survey of the range of naturally occurring biopolymer production from two species X. autotrophicum (64 isolates) and A. eutrophus (8 isolates). In addition two A. eutrohus gene libraries based on the broad host range vector pVK102 have been obtained from project advisor K. Andersen and previous work by the R. Kern the Principal Investigator.

Step 2: The strain collections are currently being characterized to identify the natural range genetic variation within the species to assess the need for and type of genetic manipulation. It has been demonstrated that A. eutrophus strains, while producing significant amounts of polyhydroxybutyrate, 43% to 65% of the whole cell weight do not excrete polysaccharide into the media. A survey of 24 strains of X. autotrophicum under standardized batch culture conditions has demonstrated a wide range of both polyhydroxybutyrate (less than 5% to greater than 90%) and polysaccharide (less than 5% to over 460%) relative to whole cell dry weight.

This study further revealed a loose inverse correlation between the amount of polyhydroxybutyrate and polysaccharide produced. The collection will soon be screened to identify those strains with the ability to act as a recipients of the pVK102 broad host range plasmid, a preliminary step towards autotrophic gene transfer experiments.

Step 3: Genetic manipulation of these strains isfocused on enhancing both the yield and rate of biopolymer production. Based on the observation that polyhydroxybutyrate levels were usually higher in those strains that produced relatively low quantities of exopolysaccharide, a mutant of the type strain 7C was selected which excretes no detectable polysaccharide. Comparison of the mutant with the naturally occurring form of the strain showed that polymer production more than doubled from 32% to 71% under some growth conditions.

Those strains identified in the X. autotrophicum collection as suitable recipients for the broad host range vector pVK102 will be targets for the transfer and expression of the genes encoding for the soluble hydrogenase of A. eutrophus. It has already been demonstrated by others that these genes enhance the efficiency of hydrogen utilization during carbon dioxide assimilation. Furthermore, when transferred and expressed, the rate of cell growth. Our effort will focus on transfer and expression of these genes and measurement of their effect on increased growth rate and on polyhydroxybutyrate and polysaccharide production.

Looking beyond the one year time frame of the current contract two other activities are planned. First, during the course of this basic study a search for specific companies who may have an interest in either hydrogen chemoautotrophic bacteria for the production of biopolymers or other organic chemicals will

be conducted. Second, it is anticipated that an expression vector will be constructed suitable for mobilizing biosynthetic genes for a wide range of small molecular weight organic molecules into A. eutrophus. This should broaden the potential impact of this study.

16. **Project Title:** Photobiological Conversion of Synthesis Gas into Bioplastics

Principal Investigator: P. Weaver

Project Site: National Renewable Energy Laboratory

Description:

This effort addresses one phase of an overall larger project which involves the conversion of lignocellulosics into syngas which is then converted by a photobacterial system into a bacterial polyester, polyhydroxybutyrate (PHB). By manipulation of the culture, the production of PHB valerate can also be achieved. These bioplastics can be produced to simulate polystyrene or polyethylene-like plastics. One of the major processing hurdles that prevents this work from being considered in industrial applications is good process engineering data that allows us to design an overall process. In particular, the data on mass tranfer of gases, a critical processing step, needs to be determined. This effort is designed to obtain that data. Future work would be required to complete the total development of this project. Currently, the strains in use only produce about 33% of their biomass as the bioplastic. Future efforts should include engineering strains to increase their production of bioplastic.

1993 Accomplishments:

Two integrated research projects were initiated in April 1993 on the photobiological conversion of synthesis gas into polyhydroxyalkanoate (PHA) thermoplastics. An internal NREL-supported project investigated microbiological, biochemical, and genetic components of the research and a BCTR project focused on inexpensive solar bioreactor designs that promote enhanced mass transfer of the gaseous substrates. The two projects are highly interactive, with the NREL project leading to strain selection and growth conditions that can be incorporated into a reactor design. Reactor design also dictates what is feasible for growth parameters.

We have obtained essentially 100% conversion of the H_2 and CO components of synthesis gas into new bacterial cell mass, driven by solar energy. Required solar (i.e., day/night) cycles enhanced the PHA content of about 10 strains examined so far by a factor of two in most cases. This is interpreted to be a result of fermentation of endogenous polysaccharides into the organic acid precursors to PHA during the nighttime hours. Day/night cycles incorporated with nutrient limitation have yielded bacterial cell mass containing up to 32% PHAs.

Six different bioreactors have been designed at 100–200 liter scale. Three have been constructed. The others are first being examined at laboratory scale. Outdoor trial runs were performed on two of the bioreactors — a bubble tower design and a sprinkler design — using artificial synthesis gas and solar energy. The reactors were maintained at constant temperature. Photosynthetic bacteria capable of utilizing synthesis gas were inoculated into the non-sterile bioreactors. Contaminating microbes (methanogens, acetogens, sulfate-reducers, and algae) could be detected when ammonia was added as the nitrogen source, but never more than about 10–20% of the cell mass. When N_2 was the nitrogen source, however, contaminant levels were reduced to less than 2% (mostly algae or cyanobacteria). Use of N_2 as the sole source of nitrogen provides an easy method to grow the photosynthetic bacteria readily without the need for sterile conditions.

Bioreactor design is greatly simplified by the fact that synthesis gas is nearly totally consumed (ethane and methane may be exceptions). The bacteria do not produce any waste gases that require separation. Also, there are no soluble waste products excreted by the bacteria into the aqueous phase. The microbes could be harvested and the water recycled for successive batch cultures. This property also could be of potential importance for the economics of the process.

1994 Plans:

The major factor in determining the economic and technical feasibility of utilizing photosynthetic bacteria, synthesis gas, and sunlight to produce PHAs remains a cost effective and efficient bioreactor that enhances the mass transfer of the gaseous substrates into the aqueous realm of the microbes. The bioreactor designs with the lowest energy inputs utilize floating platforms, chips, or beads, which allow, in principle, development of microbial cell mass and PHA synthesis at a solar irradiated interface between aqueous and gaseous phases. These will receive the primary focus. Low-density polyamide, polybutadiene and polyallomar surfaces will be examined for cell adhesion and wetability. Surfaces derivatized with cationic functionalities are commercially available and will ionicly bind the net anionic surface of bacteria.

All of the different bioreactor designs will be monitored for long-term productivity of cell mass from synthesis gas. Comparisons between reactors will be correlated with solar energy and electrical energy inputs. Since an accurate mass determination is difficult with some of the reactor designs, consumption of the artificial synthesis gas will be used to measure productivity. Losses from gas permeation or leaks will be determined on uninoculated reactors.

Laboratory research is directed towards bacterial strains that exhibit high levels of syngas consumption in light and high rates of fermentation of endogenous polysaccharides in darkness, especially those producing acetic and propionic acids. They will be added to the outdoor bioreactors and monitored for survivability.

Annual Technical Summary Report:

On an ongoing basis, we have isolated about 400 distinct strains of a unique subclass of photosynthetic bacteria that are able to utilize raw synthesis gas (primarily CO and H_2) from thermally gasified biomass. In darkness, the bacteria quantitatively convert the CO component of synthesis gas into additional H_2 and CO_2. In light or light/dark cycles the bacteria quantitatively assimilate both the CO and H_2 components into new bacterial cell mass containing up to 65% protein if N_2 or a fixed source of nitrogen is present. Trace levels of H_2S and NH_3 are also photoassimilated, as is excess CO_2, dependent on redox balance. Other than essential minerals and occasional B vitamins, no other nutrients are necessary for growth. If nitrogen or some essential mineral is present at insufficient levels for protein synthesis and cell division, however, the bacterial strains continue to photoassimilate the synthesis gas, but only synthesize polysaccharide or polyhydroxyalkanoate (PHA) products. PHAs are linear, long-chain polyesters that have physical properties similar to polypropylene or polyethylene thermoplastics. An important difference is that the chiral bioplastics are completely degradable. Different photosynthetic bacterial strains and different growth conditions produce varying amounts and types of PNAs. At present, the best bacterial strain and culture conditions photoconvert 100% of the CO and H_2 into new cell mass, which is up to 32% polyhydroxybutyrate/hydroxy valerate (70/30) copolymer. Addition of supplemental acetate (a precursor to PHAs) yields cell mass that is up to 80–90% PHA and establishes a research goal to match from synthesis gas alone.

The microbiological and, to a lesser extent, biochemical research on photobiological conversion of synthesis gas into PHAs is currently being supported by an internal NREL Director's Development Fund (DDF) award for the latter half of FY 93. Observations indicated that mass doubling times on liquid

cultures growing on H_2 and CO were approximately 7 hours, whereas doubling times on readily soluble substrates were about 90 minutes, thereby indicating that mass transfer of the gaseous substrates was limiting productivity. In order to prove the feasibility of the incipient technology for practical applications, it was deemed necessary to test simple, solar-driven bioreactor designs that would promote enhanced mass transfer with minimal expenditure of energy. This work was initiated in May 1993 with support from the BCTR program.

17. Project Title: CO_2 Photoreceptor Research

Principal Investigator: E. Greenbaum

Project Site: Oak Ridge National Laboratory

Description:

This project focuses on the fundamental aspects of photobiological reactor research and development with the goal of utilizing photobiological and photobiochemical systems for the synthesis of chemicals and fuels from renewable resources such as carbon dioxide. A new photophysical reaction has been discovered for the direct photoconversion of lignocellulosic substrates. The photoconversion occurs in the structured environment of the lignocellulosic matrix of wood, agricultural residues, etc. Fig. 1 is a schematic illustration of the photoconversion reaction. A second aspect of this project is focused on the optical reaction cross-section problem of photosynthesis. This is the problem associated with limitation of photosynthetic productivity and the light saturation curve of photosynthesis.

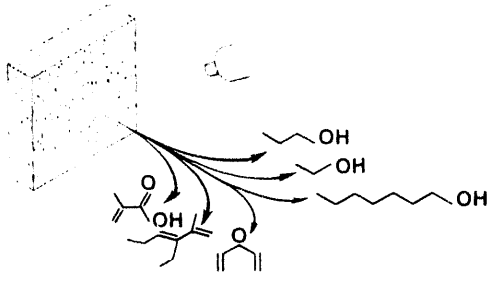


Fig. 1 Illustration of the basic concept of direct photoconversion of solid lignocellulosic substrates to volatile hydrocarbons. The photosensitizer is implanted into the wood matrix with a high pressure cell. Upon irradiation volatile hydrocarbons and molecular oxygen are produced.

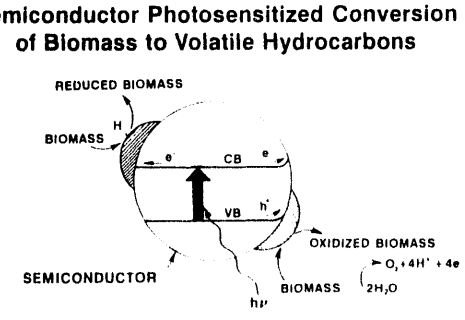


Fig. 2 The semiconducting oxides such as ANO, TiO_2, WO_3, etc form a special class of photosensitizers. As illustrated in the diagram absorption of a photon that exceeds the band gap of the semiconductor leads to the spatial separation of electrons and holes. Redox reactions can occur on the surface of the particle.

1993 Accomplishments:

We have discovered a new photophysical reaction when selected photosensitizers are implanted into lignocellulosic matrices such as poplar. For example, when a solution of $FeCl_3$ is forced into a solid piece of poplar, the implanted ferric ions photosensitize it making possible an intriguing UV photoreaction, the

simultaneous photoevolution of volatile hydrocarbons and molecular oxygen. Small-particle semiconductor photosensitizers also work. Fig. 2 is a schematic illustration of the way in which these small particles effect the absorption of light, undergo a spatial charge separation via the generation of electrons and holes, and achieve simultaneous oxidation and reduction reactions at the surface of the particles. An example of data obtained with one such sensitizer, ZnO, is illustrated in Table 1. These data illustrate an interesting principle of photoconversion reactions in sensitized lignocellulosic substrates: the product distribution depends on the nature of the photosensitizer that is implanted into the wood matrix. It is also important to note that the photoevolution of molecular oxygen and hydrocarbons indicates we have discovered an endergonic photoreaction which is a mimetic analog of normal biological photosynthesis. Moreover, these reactions occurred in a helium atmosphere indicating that either water or the lignocellulosic substrate served as the source of reductant for the generation of the hydrocarbons.

Table 1. Photoproduction of Hydrocarbons from ZnO-Photosensitized Poplar

Compound identity	Peak time (min)	Formula
2-butanol	13.80	$C_4H_{10}O$
hexanal	19.50	$C_5H_{11}CHO$
2-pentyl-furan	23.74	$C_9H_{14}O$
c_3-cyclopentane	23.85	C_8H_{16}
2-octyldodecan-1-ol	29.57	$C_{20}H_{41}OH$
heptadecane	31.65	$C_{17}H_{36}$
1-heptadeconol	33.90	$C_{17}H_{35}OH$
pentadecane	34.12	$C_{15}H_{32}$
1-heptadecanol	36.89	$C_{17}H_{35}OH$

1994 Planned Activities:

The research planned for FY 1994 follows logically from the promising results that have been obtained thus far. New sensitizers will be studied with respect to improved efficiencies of photoconversion and product distribution. There is one photocatalyst in particular that suggests itself. This is the implantation of $[PtCl_6]^{2-}$ ions into lignocellulosic matrices. The reason this catalytic system might be especially interesting, either alone or in conjunction with photosensitizers co-implanted with it, is that it is capable of undergoing an *in situ* conversion to colloidal platinum via the four-step reduction $[PtCl_6]^{2-} + 4e^- \rightarrow Pt\downarrow + 6Cl^-$. It will be interesting to note if catalytic platinum can be implanted into the lignocellulosic matrix and what reactions it is capable of catalyzing, either alone or in cooperation with a co-implanted photosensitizer. The objective of these new experiments is to understand the nature of the implanted photocatalysts and the product distribution they achieve. These results may be of use to the catalyst by design researchers in this program by providing an experimental model system for an endergonic reaction associated with a specific product distribution and photocatalyst.

Annual Technical Report:

The inorganic semiconducting oxides such as zinc oxide, titanium dioxide, or strontium titanate are a particularly intriguing class of photosensitizers. These materials can be formulated as very small particles, small enough to show quantum effects on their band structures, and impressed into the fibers of the wood.

The band gap of these semiconductors corresponds to the ultraviolet. Upon absorption with ultraviolet light, electron transfer from the valence band to the conduction band occurs, resulting in the formation of mobile electrons and holes. Table 1 presents data obtained with the photosensitized zinc oxide system.

Technical progress has been made in two areas: (1) working with the particulate semiconductor zinc oxide as the sensitizer, volatile hydrocarbons, different from the ones obtained with previous sensitizers, have been obtained; (2) in related work, additional progress has been made in photobiological reactor design and development in the application of vacuum technology to the creation of anaerobiosis and active mass transport in hydrogen production by light-activated microalgal water splitting.

The key objective of this mission-oriented research program is the development of new physicochemical techniques for utilization of CO_2 in the production of high-value chemicals and avoiding possible environmental penalties for CO_2 emissions. The organizing principle of this work is focused on a general process approach to convert feedstock material into products: FEEDSTOCK → PROCESS → PRODUCTS. The theme of this research program, based on a discovery in the Chemical Technology Division, Oak Ridge National Laboratory, is direct photoconversion of lignocellulosic materials to high-value-added fuels and chemicals.

A new endergonic (energy-conserving) photoreaction involving the photocatalytic activation of pure microcrystalline cellulose and wood has been discovered. Although the most abundant product of photosynthesis, cellulose is not a photoactive material. We have discovered that ordinary wood, such as poplar or pine, can be made photoactive by high-pressure insertion of specific photosensitizers into it. Examples of these sensitizers are quantum dot colloids such as ZnO and photoredox active ions such as ferric ions. Insertion of the sensitizers was achieved with a hydraulic pressure cell operated at 20,000 psi. Upon irradiation with near-UV light, the simultaneous photoevolution of molecular oxygen and volatile hydrocarbons were observed. It is important to note that the above reactions occurred in a *helium* atmosphere. This suggests a novel type of photochemistry in which a disproportionation reaction enables the lignocellulose to serve as both source of reductant and reduced product. These photoreactions can be thought of as completing the work of photosynthesis, which formally stops at the carbohydrate level:

$$CO_2 + H_2O + \text{light} \rightarrow CH_2O + O_2.$$

Schematic illustrations of the experimental technique used for detection and analysis are illustrated in Figs. 3 and 4. Volatile aliphatic, aromatic, and oxygenated hydrocarbons have been identified in the gaseous samples collected from several biomass photoconversion reactions. The analysis has been carried out by combined thermal desorption, gas chromatography, and mass spectrometry (TD/GC/MS). In each of these photoconversion experiments, a triple sorbent (TST) trap was placed at the outlet end of the photocell to collect the evolved volatile organic components. The TST traps were thermally desorbed, and the desorbed material was quantitatively transferred to a cryogenic focusing loop (which was connected to the inlet end of a gas chromatographic column) for subsequent GC/MS analysis.

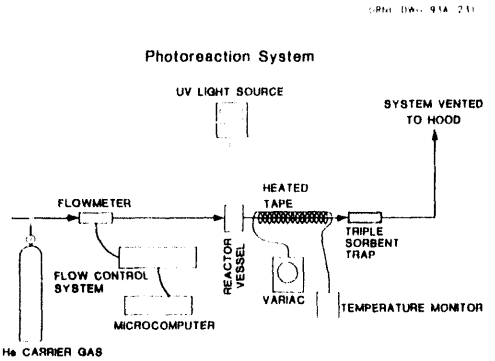


Fig. 3 The photoreaction system for irradiation and trapping samples of volatile hydrocarbons is illustrated. The triple sorbent trap (TST) collects the photoproducts from the reactor vessel. The helium carrier gas is vented. The heated tape assures that heavier hydrocarbons do not condense between the reactor and TST. The TST trap is then transferred to the MS/GC system.

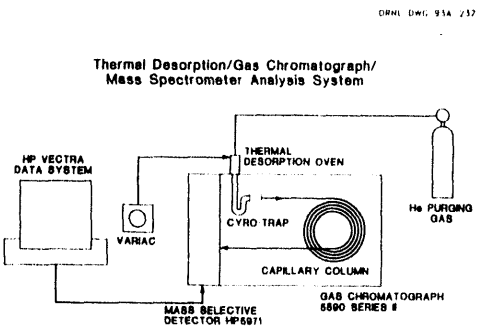


Fig. 4 The thermal desorption-gas chromatograph-mass spectrometer analysis system is illustrated. The TST trap is placed in the thermal desorption oven. The volatile hydrocarbons are desorbed from the TST trap and re-trapped in the cryo trap. Release from the cryo trap injects the sample into the capillary column and mass selective detector.

Results obtained from electron impact mass spectral analysis were used to tentatively identify the components that were generated from photoconversion reactions. During this reporting period, reactions have been carried out using pine and poplar as sources of microcrystalline cellulose. Each wood sample was treated with zinc oxide or ferric nitrate as photosensitizer. Photoproducts generated from these experiments were fairly similar. Low-molecular-weight hydrocarbons, including alkanes, cyclic alkanes, and alkenes, were found to be in abundance. Low-molecular-weight aromatic hydrocarbons with one or two rings, as well as their alkylated species, were also detected. One of the intriguing observations was that a fair amount of oxygenated hydrocarbons, such as alcohols, esters and furans, was detected.

18. Project Title: Levoglucosan Polymers

Principal Investigator: Luc Moens

Project Site: National Renewable Energy Laboratory

Description :

Levoglucosan, a monosaccharide that is the 1,6-anhydro form of D-glucose, is presently attracting much attention as a major product of the thermochemical degradation of cellulose and cellulosic feedstocks under pyrolytic conditions.

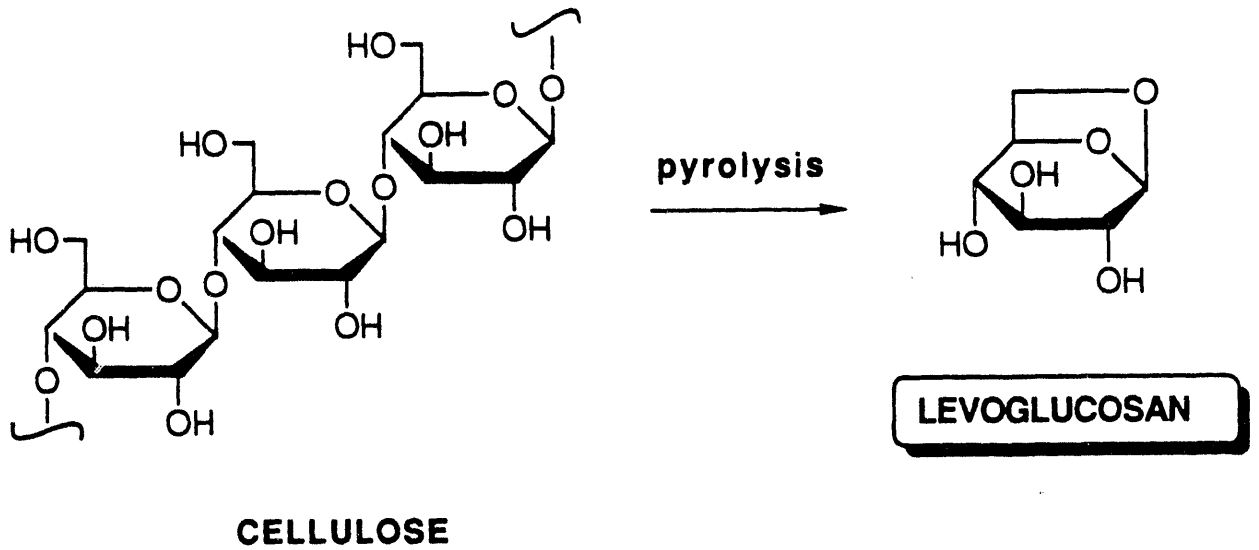


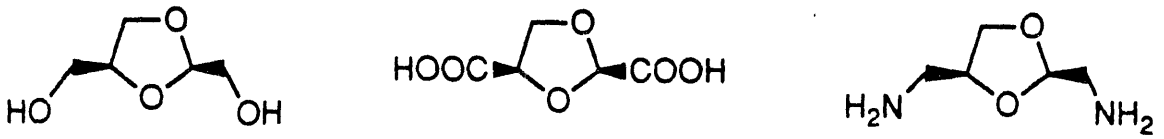
Figure 1

The preparation of levoglucosan through this process continues to be the focus of much research because it cannot be readily synthesized using more conventional technologies that start from petrochemicals. This monosaccharide is considered to be a potentially very useful starting material for a variety of chemical reactions and processes but its unavailability and extreme cost make further R&D work on large scale virtually impossible. However, recent research efforts in many laboratories worldwide, but mainly in our laboratories at NREL, have led to new methods for the isolation of levoglucosan from (ligno)cellulose-derived pyrolysis oils. This is expected to stimulate the development of new applications for levoglucosan as a chemical feedstock.

The objective of this subtask is to prove the concept that levoglucosan can be converted into new types of polymers that have several interesting features. First, the polymers will be designed such that they will be enantiomerically (chirally) pure and thus optically active. The synthesis of chiral polymers continues to attract much attention since they can be used as support materials in enantioselective chromatography that allows for the preparation of enantiomerically pure chemicals. Also, several examples are known of catalysts that contain polymer-bound asymmetric centers. Asymmetric catalysis with such optically active polymers also allows for further modification of the conformational rigidity around the asymmetric center depending on the chemical nature of the polymer backbone. Secondly, the optical properties of these polymers could make them useful as building blocks for liquid crystals. A third important feature is a highly oxygenated polymer backbone that is expected to promote biodegradability and/or acid sensitivity.

1993 Accomplishments:

The work that was carried out under this program covered the period of March to August 1993 only. During this period, all the work dealt with the exploration and development of new routes for the chemical conversion of levoglucosan into the monomeric building blocks that will be used for polymerization reactions. The synthesis of two monomers has been accomplished, namely that of diol **1** and dicarboxylic acid **2**, while that of diamine **3** is still under investigation.



1 2 3

Figure 2

1994 Plans:

As mentioned earlier, these monomers will be used in polymerization reactions that should lead to an interesting new class of polymers that contain the 1,3-dioxolane ring in the backbone. The following general structures illustrate the multitude of possible polymer structures that can be prepared from these monomers.

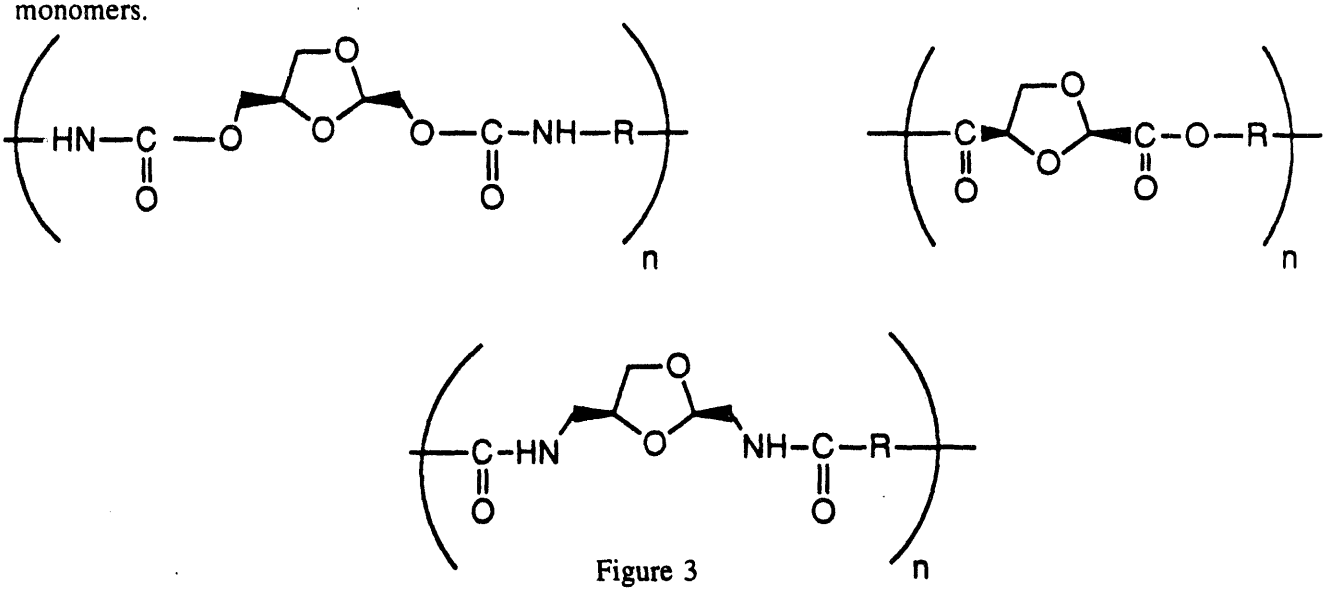


Figure 3

The first polymerization experiments are underway at this time and during the next year the work will focus on the preparation of a few selected polymers (with different R groups) in such a way that they can be compared to analogous polymers that are known in the literature, i. e. polymers that have an alicyclic unit in their backbone. The influence of the dioxolane backbone on the physico-chemical properties can then be evaluated with respect to further possible applications. The synthesis of those few polymers will also afford data regarding the molecular weights as a function of the reaction conditions. The lessons learned from these investigations will then be used for the further development of an extensive range of 1,3-dioxolane-based polymers that can be tailored towards specific applications.

Annual Technical Summary Report

Levoglucosan was prepared on multigram scale through pyrolysis of cellulose and fractionation of the resulting pyrolysis oil. The glycolic bonds (C2-C3 and C3-C4) of the anhydrosugar were then cleaved oxidatively using sodium periodate in water. The resulting *cis*-1,3-dioxolane-2,4-dialdehyde (4) was isolated in its hydrated form as a syrupy material. This hydrate is primarily a hemialdal ring structure of which the exact stereochemistry has not yet been established because ^{1}H- and ^{13}C-NMR spectra revealed that a complex equilibrium mixture of several hydrated forms is present.

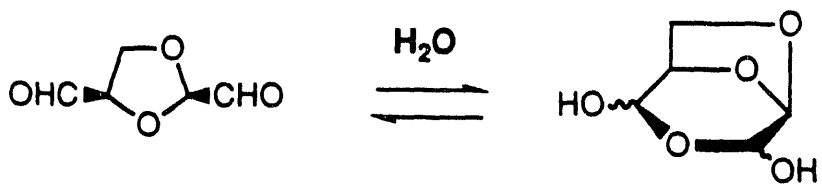


Figure 4

Intramolecular as well as intermolecular acetal formation is possible, and is strongly dependent on the choice of solvent (alcohol) used in the work-up. The existence of an equilibrium between the dialdehyde and its hydrated forms was shown by the complete reaction of this product mixture with nitromethane and benzylamine with formation of 3-nitro-2,4-dibenzylamino-2,3,4-tridesoxy-β-D-idosan (Figure 5). This confirmed that the ill-defined equilibrium mixture could regenerate the desired *cis*-1,3-dioxolane-2,4-dialdehyde in a further reaction.

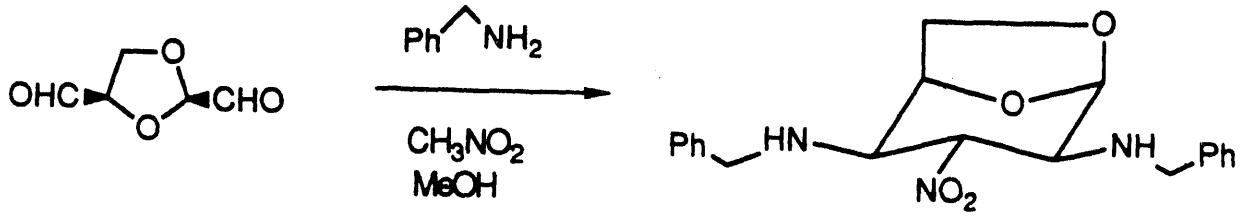


Figure 5

The reduction of dialdehyde (Figure 4) to diol (Figure 1) was accomplished with sodium borohydride and multigram quantities of this optically active diol have been prepared. It is now being tested in polymerization reactions. The oxidation of dialdehyde (Figure 4) to dicarboxylic acid (Figure 2) was achieved with a simple bromine oxidation. Milder oxidation methods such as the use of hypochlorite will be tested in the near future.

A multigram quantity of (Figure 4) was converted in nearly quantitative yields into its dioxime form using hydroxylamine hydrochloride salt. Several reduction methods to convert this dioxime into a diamine are presently under investigation. These methods include the use of several hydrides and also catalytic hydrogenation. The reactions with hydrides result in complex mixtures of compounds that are difficult to separate. No significant reaction was observed during catalytic hydrogenation of the dioxime over Pd / graphite, presumably due to poisoning of the catalyst by small amounts of the desired diamine. The use of Rh / graphite catalyst afforded low yields of the diamine and experiments using slightly acidic media are being carried out to obtain better yields. Nevertheless, the low reactivity with the Rh catalyst is surprising since several examples of oxime reductions are known in the literature that show this to be the preferred catalyst.

19. **Project Title:** Clean Fractionation Process

Principal Investigator: David Johnson

Site: National Renewable Energy Laboratory

Accomplishments:

Work on the clean fractionation process during the past year has succeeded in showing that it is possible to fractionate several biomass feedstocks into three product streams derived from the three major components of biomass, cellulose, hemicellulose and lignin, with high selectivity. Biomass feedstocks including aspen, hybrid poplar, black locust, and switchgrass have been successfully fractionated. The selectivity of the fractionation is very high with 90-95% of the cellulose in the feedstocks ending up in the cellulosic product stream (pulp), and about 80% of the lignin in the feedstocks ending up in the lignin product stream. The lignin product streams were of very high purity containing about 95% pure lignin. The cellulosic pulps were of high purity also containing 80-90% glucan, about 6% xylan and the remainder mostly lignin. This organosolv pulping system appears well suited as a basis for an investigation into organosolv pulping processes in general.

A technoeconomic assessment of the clean fractionation process has indicated that it would be suitable for producing carbohydrates at a cost of $0.035/lb and lignin at $0.082/lb. This assessment was based on an in-depth study of organosolv pretreatment (detailed in a report entitled, *Evaluation of Pretreatments of Biomass for Enzymatic Hydrolysis of Cellulose, H. L. Chum et al, Oct. 1985, SERI/TR-231-2183*) adapted for the clean fractionation process by A. J. Power of A. J. Power and Associates. The assessment was performed primarily to compare the clean fractionation process to dilute acid pretreatment. Consequently, the economics were calculated assigning the differential cost of using the organic solvents for clean fractionation to the lignin product, allowing the carbohydrates to be produced at the same cost as they would from dilute acid pretreatment. There are several applications that could use a lignin costing $0.082/lb including its use as an intermediate in anthraquinone (AQ) manufacture, and as an intermediate in the production of surfactants for enhanced oil recovery.

1994 Plans:

Plans for 1994 should the program wish us to persevere with this project, are to conduct a systematic study of how solvent composition and properties correlate with the extent of delignification and the characteristics of the cellulose, lignin and hemicellulose product fractions. It is known that the extent and rate of delignification of biomass is influenced by solvent strength, we intend to quantify these solvent effects by correlating solubility parameters, and hydrogen bonding capacity of solvent systems compatible with clean fractionation, with the various stages critical to effective delignification. This study will require the measurement of solubility parameters and hydrogen bonding capacity for several solvent systems. The effectiveness of these solvent systems in swelling biomass will need to be measured as swelling is the first critical step in what is believed to be the process of delignification. Swelling of the biomass is necessary for effective penetration of the biomass macro structure prior to hydrolysis of the bonds between carbohydrates and lignin. Hydrolysis reactions permit solubilization of the hemicelluloses. Solvent does not appear to effect this step strongly unless insufficient water is available to dissolve the xylan. For the lignin, depolymerization reactions as well as solubilization must occur before delignification takes place. The solvent should obviously have a large role in lignin solubilization. Previous work has shown that lignins are soluble in solvents having a solubility parameter of about 11 and hydrogen bonding capacity greater than about 0.1. The solubility of lignin in various solvent systems will be measured to verify that the solvent systems of interest to clean fractionation fit with previously developed models relating solvent properties to lignin solubility. The effect of solvent parameters on the chemical and physical structure of

the cellulose and lignin products will also be studied. This work will involve characterization of the physical properties of the cellulosic pulp including molecular weight distribution, and fiber properties. Solid state NMR will also be used to assess changes in the relative amounts of crystalline and amorphous cellulose in the pulp. These analyses will permit us to assess the level of degradation of the cellulosic fibers occurring during organosolv pulping. Normally acid catalyzed pulping of wood produces pulp fibers that have lower strength. A goal of this work will be to examine whether solvent properties can be manipulated to preserve the fiber structure and maintain their high strength. Characterization of the lignins chemical structure will also be performed to assess the level of depolymerization and the extent to which the various chemical interunit linkages have been cleaved during pulping. Lignin characterization will involve determination of molecular weight distribution, various types of NMR characterization, and functional group analyses. A model of the effect of solvent parameters on organosolv fractionation will be developed based on the data obtained. By correlating solvent properties with swelling effectiveness, degree of delignification, removal of hemicelluloses, and the polymer characteristics of the lignin and cellulose products we expect to increase our understanding of how solvent composition effects fractionation selectivity, and determine what are the critical molecular level interactions involved. It is expected that the fractionation model will allow us to tune process conditions to give products for specific applications, i.e., pulp and paper, cellulose derivatives, glucose, quinones, phenolics, etc.

Annual Technical Summary Report:

The clean fractionation process is a form of organosolv pulping employing novel combinations of solvents that permit fractionation of biomass feedstocks into their three main components, cellulose, hemicellulose and lignin. These three components are obtained as three separate product streams, with high selectivity through a simple manipulation of the solvents. Development of this process at NREL has been able to show that highly selective fractionation is possible using aspen wood chips. Recent work has explored the effectiveness of clean fractionation with other feedstocks, both woody and herbaceous, that are more relevant to commercial utilization, including hybrid poplar, black locust, and switchgrass. Evaluation of the product streams from these feedstocks is currently ongoing.

Initial exploration of process parameters has been conducted using an experimental set up of six small reactors. A larger reactor with the capability for continuous solvent flow is now in use to verify results obtained in the small reactors, to produce sufficient material for characterization of the products, and to obtain design data for the process. The products are subjected to extensive compositional analysis so that complete material balances can be performed on all components of the feedstock. The lignin product is being further analyzed to determine its chemical functionality and physical characteristics so that it may be evaluated for a variety of co-product uses. Cellulosic and hemicellulosic products are being evaluated for their suitability for ethanol production by simultaneous saccharification and fermentation (SSF), and C5 fermentation, respectively. The cellulosic product is also being evaluated for its bleachability. Acetylation of bleached and unbleached pulps has also been performed to assess the suitability of the cellulosic pulp as a feedstock for cellulose derivatives.

Experiments conducted in the last year have demonstrated that clean fractionation of biomass into lignin, cellulose and hemicellulose is possible. Bark and knot free aspen wood chips were initially treated with a range of solvent compositions and a standard set of organosolv pulping process conditions. After pulping, a high purity (90% pure) cellulosic pulp was obtained, containing more than 90% of the C6 sugars originally in the wood. The hemicellulose components and lignin were dissolved in the liquor. The lignin product was separated from the dissolved sugars using our proprietary method in very high purity, containing only small amounts (less than 4%) of carbohydrates. The lignin product contained most (80%) of the lignin originally in the wood. The hemicellulose product was also obtained very selectively. Degradation of C5 and C6 sugars to furfural and hydroxymethyl furfural was very low (less than 2% and

0.2%, respectively). Five solvent compositions were studied, with similar results being obtained for the three intermediate compositions. The two extreme compositions resulted in lower selectivity fractionation.

Clean aspen wood chips, however, are an idealized feedstock, consequently recent research has concentrated on the use of feedstocks of greater commercial interest. The feedstocks used included switchgrass, hybrid poplar and black locust (both of the latter were not debarked). From preliminary experiments with these whole wood feedstocks and the herbaceous material it is clear that process conditions optimized for one feedstock will not necessarily be optimal for all feedstocks. The presence of minor components such as ash, extractives, and protein (particularly in the herbaceous feedstocks) significantly effects the optimization of the fractionation.

Hybrid poplar was fractionated with similar selectivity to that seen for aspen wood. The cellulosic pulp from hybrid poplar contained 95% of the cellulose originally in the wood but was not as pure as that from aspen (only 80% pure). The lignin product was again essentially pure lignin containing almost 80% of the lignin originally in the wood.

With switchgrass a cellulosic pulp was produced that also contained almost all (90% yield) of the cellulose originally in the feedstock, although again its purity (80%) still needs improvement. The lignin product contained almost all (more than 90%) of the lignin in the wood and as before very little carbohydrate. With switchgrass the lignin analysis is more difficult than with most woody species because of the presence of protein that condenses in the traditional klason analysis. Another analytical problem with switchgrass is the presence of relatively large amounts (about 15%) of non-structural carbohydrate. Complete material balances on these materials require additional analytical work.

Recently operation of the larger scale flow-through reactor has been initiated. This reactor is capable of treating up to 180g of oven-dried equivalent biomass. Preliminary assessment of the fermentability of the carbohydrate product streams is being made and characterization of the lignin begun. Further work with the small reactors will focus on mapping out the effect of solvent composition on the rate and extent of delignification.

A literature survey has uncovered a very wide range of solvent conditions that have been used for organosolv pulping. The solvents all have moderate to high hydrogen bonding capacity with solubility parameters in the range of 9-14. These solvents are all good to excellent lignin solvents and are moderate to strong swelling solvents for wood. Figure 1 shows the relationship mapped out by Scheurch (JACS, 74, 5061-7 (1952)) between solubility parameter (δ), hydrogen bonding capacity ($\Delta\mu$) and kraft lignin solubility for a wide range of solvents. The solvents that have been used for organosolv pulping include the alcohols (methanol, ethanol, and on up to octanol), glycols, phenols, ketones and esters (particularly ethyl acetate in mixtures with acetic acid). The solvent normally contains some water often as much as 50%. The solvent can also contain an acidic catalyst usually no more than 2% if a strong acid is used, but larger amounts if a weaker acid is employed. Alkaline solvents have also been used by adding a base, most often sodium hydroxide. Pulping solvents have also been employed that contain no added acid or base, but then much higher temperatures are applied, around 200 °C. In the presence of acid or base, pulping temperatures are in the range of 130-190 °C, with times of 1-2 hours. Generally softwoods require more severe pulping conditions than hardwoods, and in some solvent systems effective pulping of softwoods has not been achieved.

A preliminary economic assessment has been made of the clean fractionation process relative to dilute acid pretreatment assigning the differential cost of using and recycling the organic solvent to the lignin co-product assuming that the fermentable carbohydrate products are obtained for the same cost ($0.035/lb) as from dilute acid pretreatment. The cost of the lignin under these assumptions was $0.082/lb. This assessment is simplified since it did not include the effects of downstream processing which could be

considerable, principally for enzyme recycle. The clean fractionation process would allow simultaneous saccharification and fermentation of the cellulose to be performed without the presence of the lignin component, which can absorb/adsorb enzymes. These downstream effects on the cost of the overall ethanol production need to be assessed. However, the Clean Fractionation Process appears a very promising route for ethanol coproduction with chemicals.

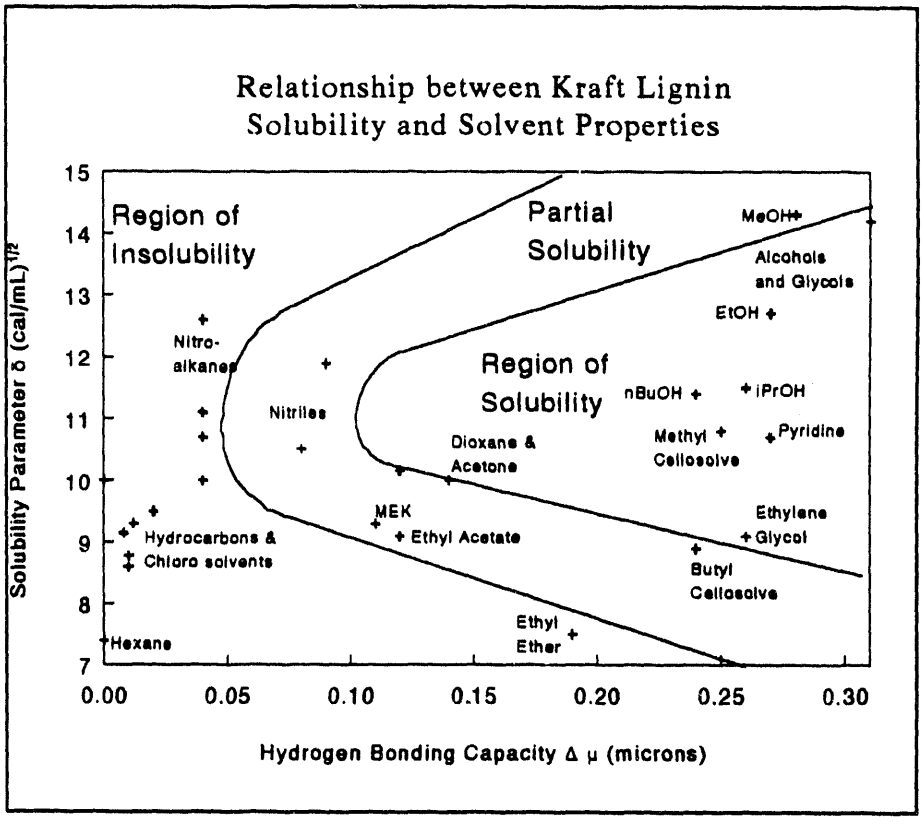
If this lignin is to be used for making products then what materials could be made from a lignin costing $0.082/lb. One of the most valuable products that lignin could be upgraded to is anthraquinone (AQ). It has been shown that organosolv lignin can be converted to an AQ type pulping catalyst. Technoeconomic assessment of this process indicates that from lignin costing $0.016-$0.18/lb, AQ can be made for about $0.82-$1.04/lb. The current price for AQ is about $4.50/lb and so is used to only a limited extent in paper pulp manufacture. There is some indication that a reduction in price to $2/lb could be sufficient to see a majority of pulp producers change to AQ pulping. The current US sulfate/soda wood pulp market is about 50 million short tons coming from about 100 million short tons of wood. One estimate has placed the most economic level of AQ addition at 0.067%, giving a potential AQ market of 133.4 million lbs. The yield of AQ from lignin used in the technoeconomic assessment was 20%, indicating a potential lignin market of 667 million lbs. Although this potential use of lignin can accommodate its relatively high cost, the market is quite small. This market could be expanded with other uses for the AQ derivatives that have not yet been assessed.

One of the major uses of lignins today is in lignosulfonates which are used as chemicals in oil fields and a variety of other areas. In 1989 US production of lignosulfonates was 1.28 billion lbs. Prices for lignosulfonates vary from $0.054/lb to $0.26/lb. The potential usage of surfactants in enhanced oil recovery is unclear with projections ranging from 65 million lbs (currently) to 15,000 million lbs. The lignin derived surfactants that could be used range from lignosulfonates to lignin surfactants made by reducing lignin at high temperatures and pressures, as proposed by Texaco researchers. The latter surfactants would compete with petroleum-derived surfactants which cost about $0.95/lb.

Pilot scale organosolv pulping is progressing worldwide. With assistance from the Canadian federal government in early 1989, Repap Enterprises Corp. started a 33 tpd demonstration plant for its Alcell process at Miramichi (N.B.), Canada. This process uses ethanol/water and hardwood chips. A 250 tpd facility is in the planning stages and may be funded soon. The pilot scale facilities were derived from a subcontract that SERI let to GE, which then spun off as a company in Valley Forge (PA), that was subsequently purchased by the Canadian company Repap Enterprises. At Valley Forge there is a complete pilot scale facility, which could be used for a CRADA or other type of agreement for the assessment of this technology as soon as the larger scale tests in the NREL laboratories provide a range of optimum conditions. A very inexpensive test at 33 tpd can be envisioned with this company.

There are other sources of organosolv lignins beginning commercialization. Germany has a 5 tpd MD Papier Organocell process using methanol for softwoods, which is being expanded to 85 tpd with additional funding from the EEC. Another process is AQ catalyzed in the Alkali-Sulfite-Anthraquinone-Methanol (ASAM) process, which produces pulps with similar strength as kraft and greater yield (2-10%). With chlorine-free bleaching a very high brightness pulp can be achieved. With the more severe environmental constraints in Europe, and particularly, in Germany, these organosolv processes are likely to continue development. Of particular importance is the availability of low cost AQ to make this process more attractive. NREL technology could serve as a basis for international ventures in countries which are more severely constrained by environmental pressures to their development. The package of Clean Fractionation/AQ production could be the basis for international trade in AQ and expansion in EEC/Brazil, since bagasse lignin is an excellent source for chemicals.

Considering that we are in the preliminary stages of development of this technology, results to-date are very encouraging, particularly for co-production of a variety of products that can be selected on the basis of market and economic opportunities. A better understanding of how solvent properties effect the yields and properties of the products will allow us to learn how to tune process conditions to give products for specific applications. The same capital equipment would then have the flexibility to manufacture a variety of products.



4.4 Novel Process Development

20. Project Title: Overproduction and Enhanced Secretion of Enzymes.

Principal Investigator: A. K. Williams

Project Site: Howard University

Description:

The project seeks to understand the biological and chemical processes involved in the secretion of the enzyme polyphenol oxidase (PPO) by the hyphae, the basic unit of the filamentous fungus *Coriolus versicolor*. These studies are made to determine rational strategies for enhanced secretion of PPO, both with the use of recombinant DNA techniques (Howard University), and without (Clark-Atlanta). This effort is done in concert with work at Clark-Atlanta University but focuses on recombinant DNA techniques to enhance enzyme production.

1993 Accomplishments:

During 1993 (02/18/93 to 08/31/93), we devoted efforts to the over production of lignocellulosic enzymes of Coriolus versicolor by genetic engineering methodology. Specifically, we batch-cultured Coriolus versicolor in a defined medium and established a synchronized growth curve over a period of 15 days. Enzymatic profiles of lignocellulosic enzymes (e.g., C1 cellulase, lignin peroxidase, polyphenol oxidase) were determined yielding peak activity between days 6-9. Recombinant clones harboring lignocellulosic genes were generated and probed with specific antibodies. Additionally, the polyphenol oxidase (PPO) 'model system' continued to provide the fundamental basis for our study of the lignocellulosic enzymes. Also, the employment of immunopreciptation appeared to render increased detection and activity of PPO as well as lignocellulases in batch cultures.

1994 Planned Activities:

1. To continue with the perfection of the PPO 'model' system for global industrial applications;

2. To fully characterize the expression of cloned DNA inserts harboring C1 cellulase and lignin peroxidase (ligninase) genes;

3. To mass produce C1 cellulase and lignin peroxidase by genetic and cultural manipulations;

4. To provide large scale production of cellulases and ligninases via microbial fermentation coupled with recombinant DNA technology; and

5. To publish a series of papers pertaining to the cloning, expression, and production of polyphenol oxidase (PPO), C1 cellulase, and lignin peroxidase from Coriolus versicolor.

Annual Technical Summary Report:

During the past several months (02/18/93 - 07/18/93), we have devoted efforts to the over production of lignocellulosic enzymes of Coriolus versicolor by genetic engineering methodology.

Specifically, we initially grew C. versicolor on minimal agar plates (Figure 1A) prior to being batch cultured in a defined liquid medium (Kirk and Kelman, 1965). Aliquots of cultural filtrate were removed

at different time intervals over a growth period of 15 days. After which, a growth curve (Figure 1B) was constructed from measuring the dry weight of mycelial samples as a function of time (i.e., every three days). Next, the activity of lignocellulosic enzymes (e.g. C1 cellulase, lignin peroxidase, polyphenol oxidase) was measured from the above cultural filtrates (Tables 1,2,3). As shown in these tables, all three enzymes appeared in the external milieu between days 6-9.

Figure 1A - Growth of Coriolus versicolor on Agar Plate (Day 6)

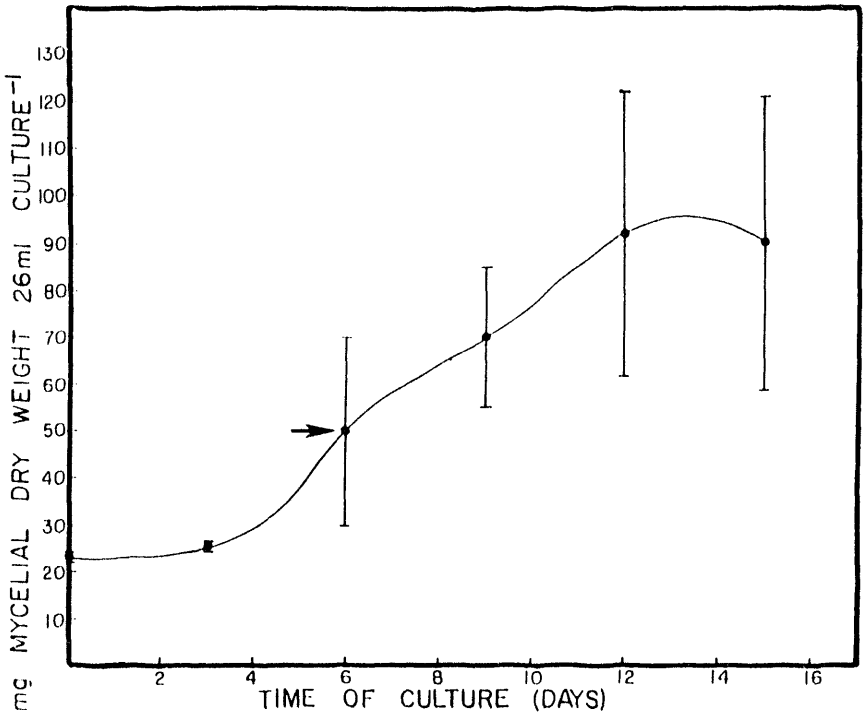


Figure 1B - Growth Curve of Coriolus versicolor in Liquid Medium

Table 1. Measurement of Ligninase Activity in Coriolus versicolor Cultural Filtrates

Culture	Ligninase Units	Total Protein Conc. mg/ml
Day 0	0	0
Day 3	0	0
Day 6	51.92	.03
Day 9	11.65	.14
Day 12	2.20	.86
Day 15	1.99	1.07

Table 2. Measurement of C1 Cellulase Activity in Coriolus versicolor Cultural Filtrates

Culture	C1 Cellulase Units	Total Protein Conc. mg/ml
Day 0	0	0
Day 3	1.5	0
Day 6	183	.03
Day 9	101	.14
Day 12	17	.86
Day 15	0	1.07

Table 3. Measurement of Polyphenol Oxidase (PPO) Activity in Coriolus versicolor Cultural Filtrates

Culture	Polyphenol Oxidase Units	Total Protein Conc. mg/ml
Day 0	0	0
Day 3	60	0
Day 6	800	.03
Day 9	107	.14
Day 12	143	.86
Day 15	1272	1.07

Subsequent to the determination of the enzyme profiles, recombinant DNA techniques were employed to generate clones harboring lignocellulosic genes. (Figure 2).

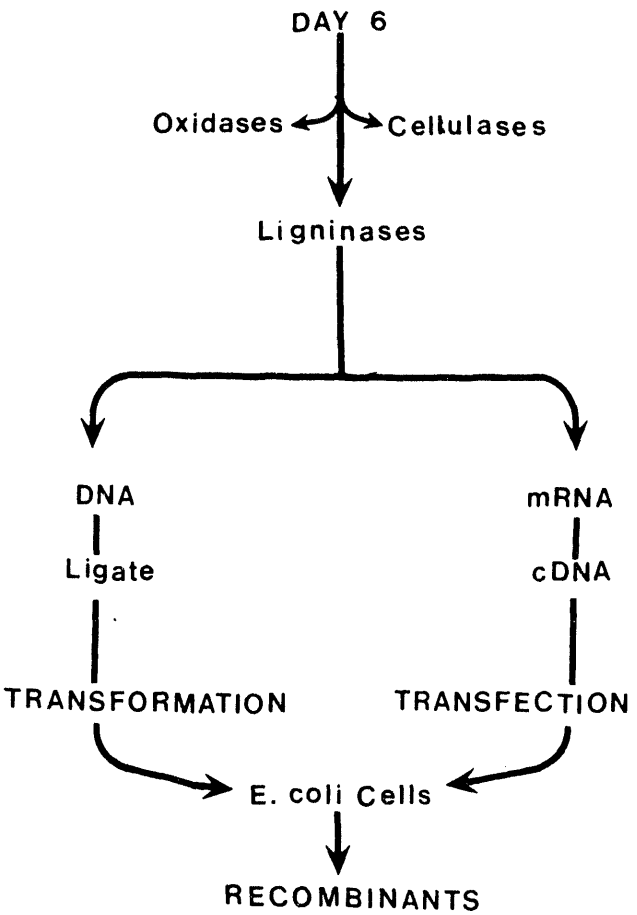


Figure 2 - Plan of Study: A Molecular Approach

First, the **shotgun** approach was employed (Figure 3) to clone lignocellulosic genes of C. versicolor.

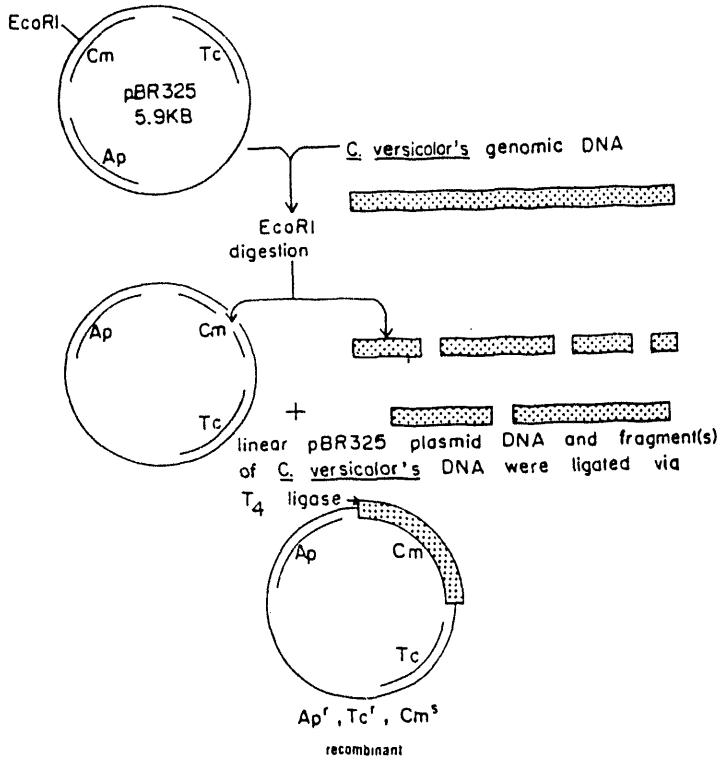


Figure 3 - Cloning Scheme #1

Figure 4 shows an agarose gel electrophoretic pattern of recombinant plasmid (pCV1) harboring a genomic insert (0.6 kb) of C. versicolor. Numerous clones were generated carrying an array of different sizes of genomic DNA inserts.

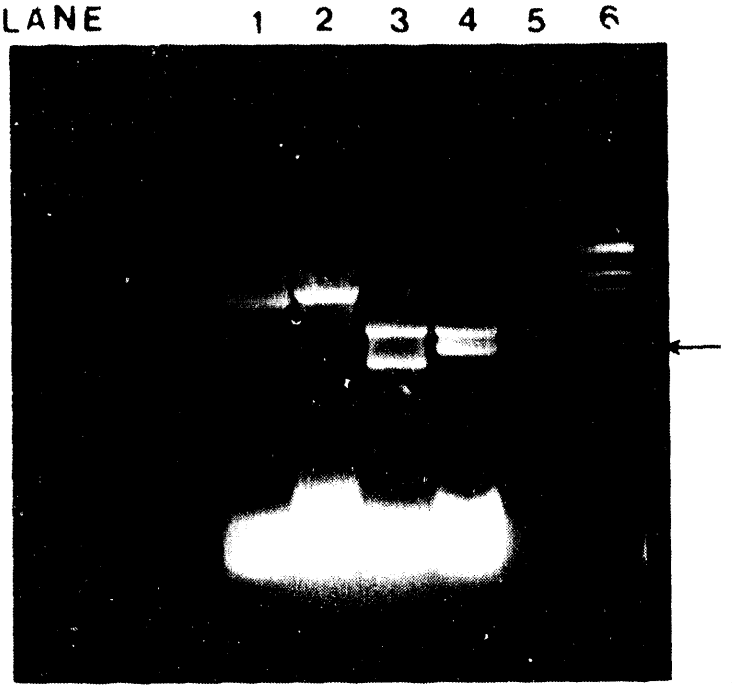


Figure 4 - Agarose Gel Electrophoresis of Recombinant Plasmid (pCV1) DNA (Lane 2) and 0.6 kb Genomic Gragment (Lane 5) of C. versicolor. Lanes: (1) pBR325; (3) pBR325/PstI-HindIII; (4) pCV1/PstI-HindIII; (6) Lambda HindIII marker.

Secondly, the **cDNA** approach was utilized to clone lignocellulosic genes as stated. Bulk RNA was isolated and purified from 6 day hyphae of C. versicolor. This RNA was fractionated into poly (A)+ and poly (A)- species via oligo (dT)-cellulose chromatography (Figure 5).

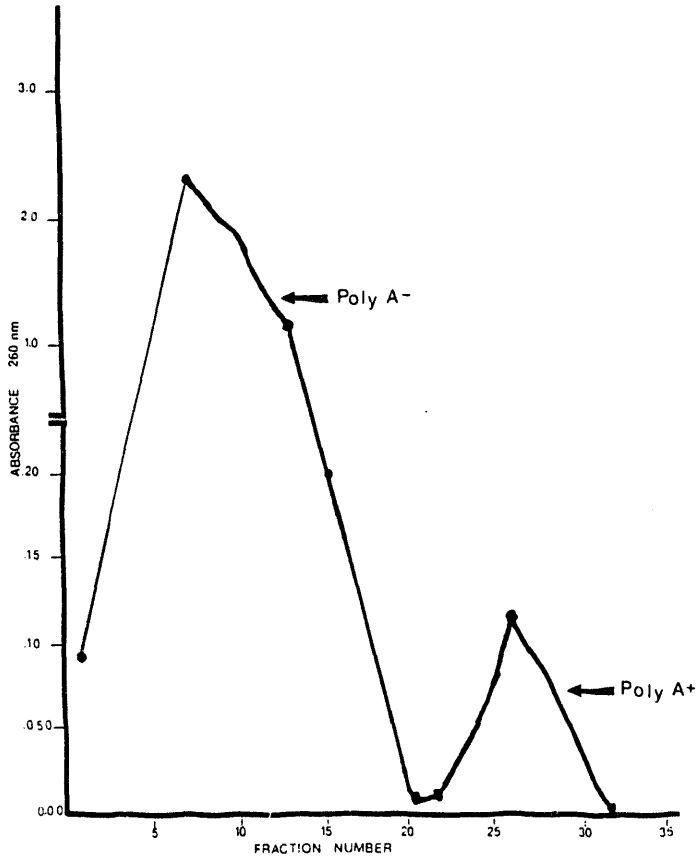


Figure 5 - Fractionation of Purified FNA via Oligo (dT) - Cellulose Chromatography

Individual fractions of poly (A)+ and poly (A)- RNA were subjected to agarose (formaldehyde) gel electrophoresis (Figure 6) prior to being employed in the in vitro translation protocols. The identity of certain fractions was ascertained by antibody probing of translational product(s).

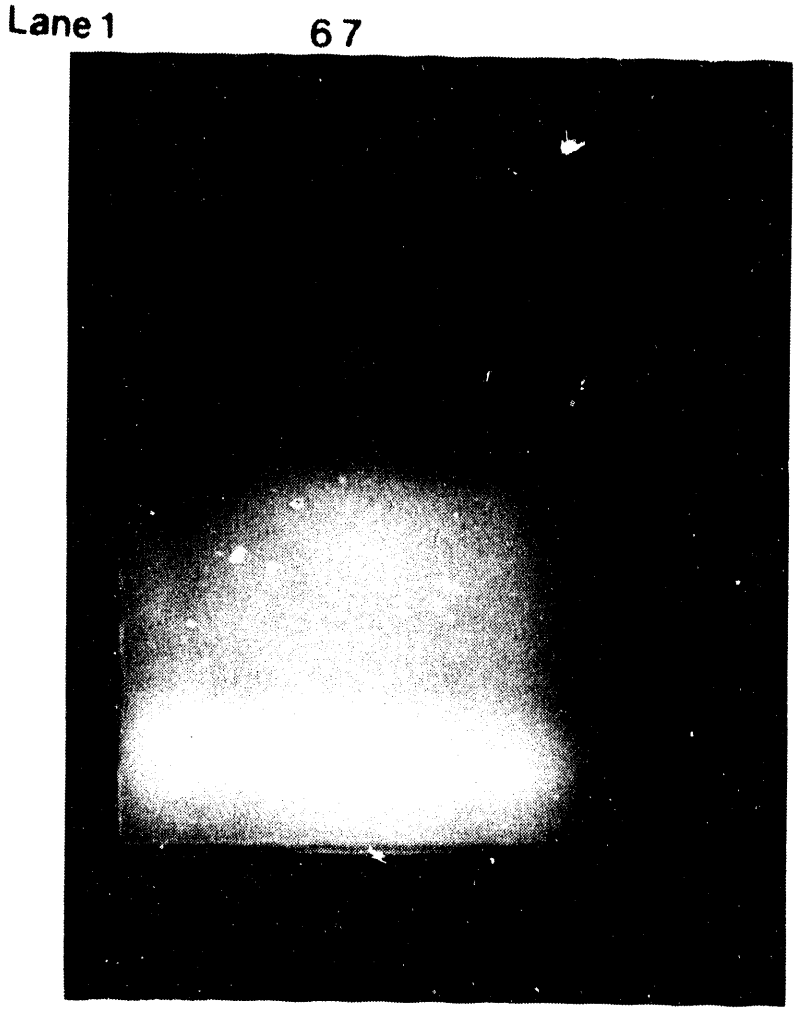


Figure 6 Agarose (Formaldehyde) Gel Electrophoresis of mRNA from c. versicolor. Lanes (1) Marker; (6) Poly (A) + RNA; (7) Poly (A) - RNA

The RNA from the positive samples (e.g., lignocellulases) were employed in cDNA synthesis for the C1 cellulase or lignin peroxidase (ligninase) gene (Figure 7).

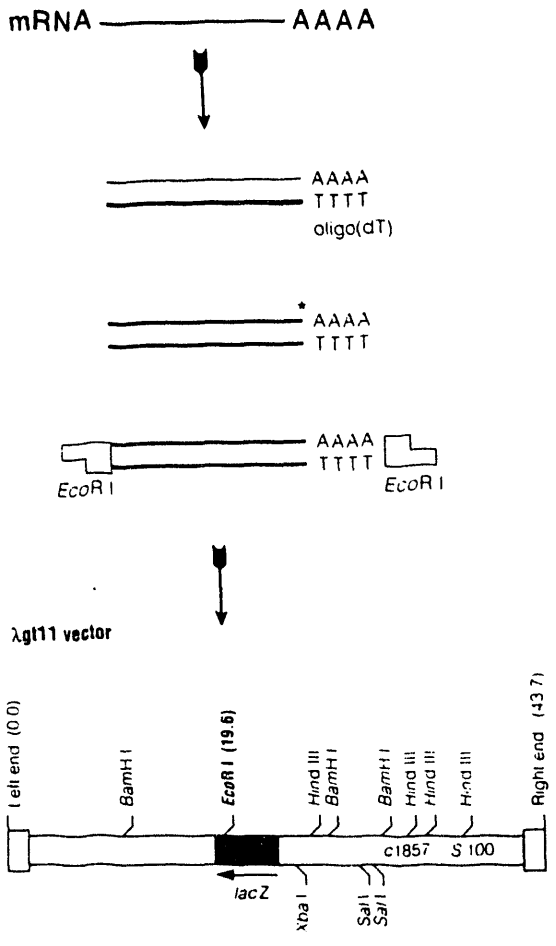
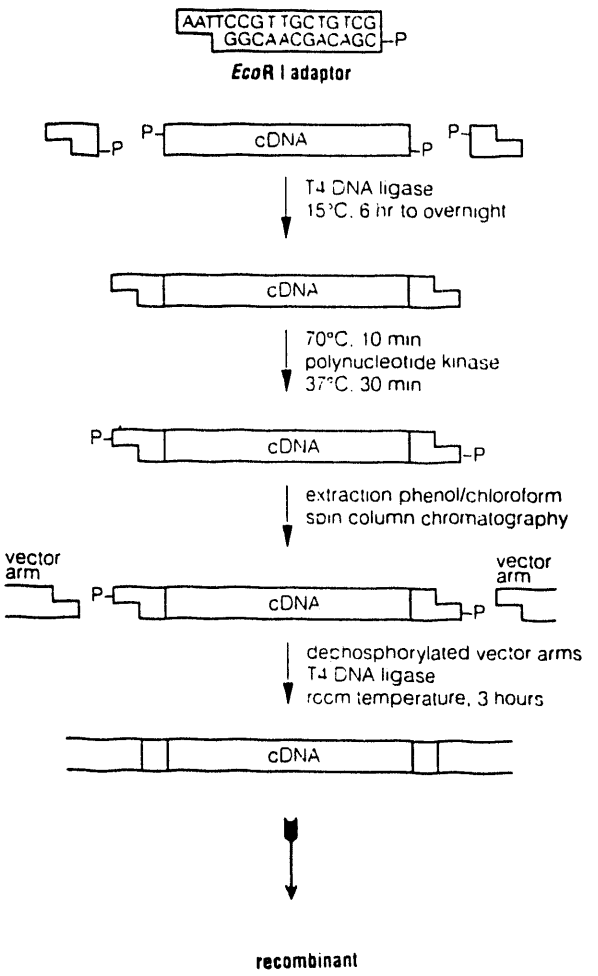


Figure 7 - Cloning Scheme #2
A. CDNA Synthesis



B. Ligation/Recombinants

The synthesis of cDNA involved **first strand synthesis** with MLV reverse transcriptase and **second strand synthesis** with DNA polI/RNaseA and then ligated into lambda gtll arms (i.e., constructing phage library). Material from the library was used to infect selective bacterial strains and plaques were probed with specific antibodies for cellulases and ligninases.

Additionally, the PPO 'model system' has provided the basis for our study of the lignocellulosic enzymes. Also, **immunoprecipitation** has been included in our standard protocol(s) to render increased detection and activity of PPO as well as lignocellulases in batch cultures. Figure 8 displays samples before and after immunoprecipitation.

Immunoprecipitation:

A protein-specific antibody may permit quantitative isolation of the protein of interest by immunoprecipitation (Lerner and Steitz, 1979). This procedure consists of three steps:

1. The specific antibody was added to the cell extract (e.g., CA8000/pBR322, CA8000/pCV1) from 6 day and 13 day C. versicolor filtrates.
2. Protein A-Sephrose beads were added to the antibody-cell extract mixture to bind all immunoglobulin molecules (Fc portion) in the precipitation mix.
3. The pellets were centrifuged and washed thoroughly several times to remove the unprecipitated material.

After the final wash, the pellets were resuspended in 100 ul of 2X SDS sample buffer at pH 6.8. The samples were prepared for SDS Urea Pollyacrylamide Gel Electrophoresis (Figure 8).

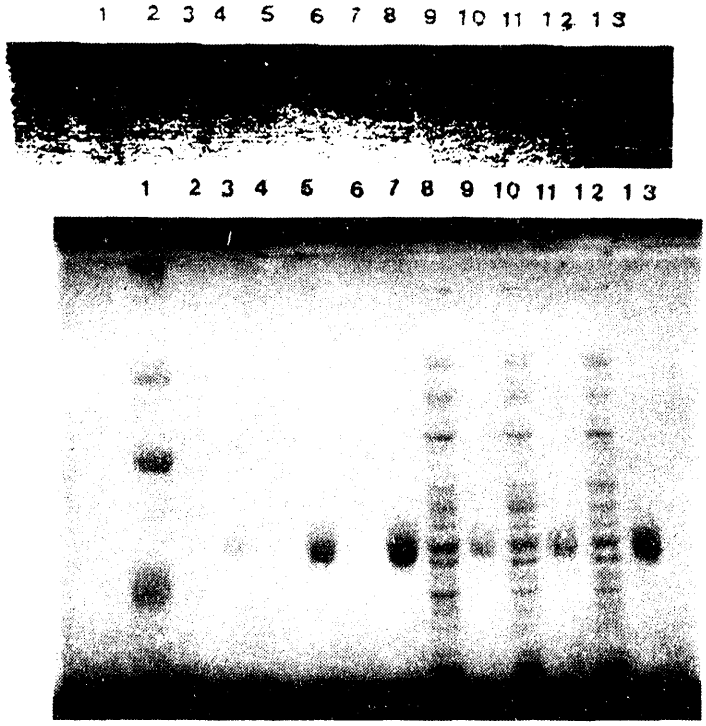


Figure 8 - Lanes: (1) HMW marker; (2) Tyrosinase; (3) Ab + Tyrosinase (Precipitate); (4) 6-Day filtrate; (5) 6-Day filtrate - Ab (Precipitate); (6) 13-Day filtrate; (7) 13-Day filtrate + Ab (Precipitate); (8) CA8000 Extract; (9) CA8000 + Ab (Precipitate); (10) CA8000/pBR322 Extract; (11) CA8000/pBR322 Extract + Ab (Precipitate); (12) CA8000/pCV1 Extract; (13) CA8000/pCV1 Extract + Ab (Precipitate)

21. **Project Title:** Separations by Reversible Chemical Association

Principal Investigator: C. J. King

Project Site: University of California at Berkeley
Lawrence Berkeley Laboratory and
Department of Chemical Engineering

Description:

Investigate the application of reversible chemical complexation as a means of separation of polar organic compounds from dilute aqueous solutions. Solutes of interest include oxygenated compounds produced by fermentation, such as carboxylic acids, alcohols, and amino acids. These approaches can substantially reduce energy requirements, and minimize the wastes, of conventional chemical separations (distillation, evaporation, conventional solvent extractions) in industry.

General objectives of the project include the measurement of phase equilibria and the rates at which equilibria are established; establish guidelines for the selection of complexation agents; develop suitable methods of process integration (into other unit operations), and to define the most promising applications of the method.

Specific objectives include the identification of regeneration methods for organic acids extracted from solutions; identification of process approaches for the extraction of organic acids at pH above the pKa of the acid; identify suitable extraction agents for glycol and other polyfunctional alcohols.

1993 Accomplishments:

Recovery of Carboxylic Acids

We completed our research on measurement and interpretation of equilibria for uptake of lactic and succinic acids by basic adsorbents and extractants, including for pH > pK_a of the acid. We tested our adsorbents with a real fermentation broth furnished by Brian Davison of ORNL, determined with this broth and synthetic solutions the competitive uptakes of phosphate and sulfate, and devised appropriate processing methodology for dealing with broths containing such competing anions. Finally, we investigated reversible esterification with *n*-butanol as a means of recovery of lactic acid from aqueous trimethylamine solutions that have been used to regenerate extractants and sorbents strongly enough basic to take up carboxylic acids effectively at pH > pK_a of the acid.

Water-Enhanced Solvation

We also completed research on the extent to which co-dissolved water increases solvation of various solutes in electron-donor solvents, notably ketones and esters. The conclusion is that there is very substantial enhancement of solvation for carboxylic acids, but the effect is much less for alcohols, glycols and phenols.

Recovery of -OH-Bearing Compounds

We investigated reversible reaction with an aldehyde as a means for recovering diols from dilute aqueous solutions. We measured chemical equilibria and rates, as well as vapor-liquid equilibria, for formation of dioxolanes by reaction of formaldehyde and acetaldehyde with 1,2-propanediol. Rates are somewhat slow, but are accelerated with sufficient amounts of catalysts (acidic polymeric resins). It is difficult to

strip excess formaldehyde from solution, and, in the case of aldehyde, the equilibrium for the forward reaction is such that the volatility of the dioxolane product over solution is rather low.

1994 Plans:

Recovery of -OH-Bearing Compounds

We intend to extend and formulate conclusions from our research on recovery of glycols from dilute aqueous solutions by means of reversible reaction with aldehydes to form dioxolanes. We are presently investigating the possibility of extracting or adsorbing, as well as stripping, the product dioxolane from aqueous solution.

In additional research directed toward recovery of multi-OH compounds, we shall investigate ways of implementing complexation with organoboronates on an industrial scale, including mounting the boronate functionality on solid sorbents. Means of regeneration will also be explored, probably involving swinging pH to convert from anionic boronate to un-ionized boronic acid. One possibility is saturation with CO_2 to lower pH, followed by stripping the CO_2 to raise pH.

Finally, we have noted that activated carbons have significant capacity for glycols and sugars. This must reflect chemical interactions with surface functional groups, since glycols and sugars adsorb negatively at inert surfaces. We shall explore this effect further, seeking better carbons, as well as polymeric sorbents that deploy the functional groups that are effective on carbon surfaces.

Recovery of Carboxylic Acids

Following our work on extraction and adsorption at pH > pK_a, we will seek leachants that effectively remove carboxylic acids from amine extractants and sorbents at high pH. We will determine the equilibria for back extraction of acetic acid from amine extractants into basic aqueous calcium/magnesium solutions, as would be used for manufacture of the benign road de-icer, CMA (calcium magnesium acetate) by fermentation coupled with extraction. We will also determine equilibria for extraction and adsorption of multiple acids with amine extractants and adsorbents. Finally, we will examine selectivities between carboxylic acids and sugars, starch fragments, etc., with an eye to identifying those separation methods that most effectively remove carboxylic acids without simultaneous uptake of substrate.

Annual Technical Summary Report:

Separations of carboxylic acids, alcohols and glycols from aqueous solution are critically important for economical production of these chemicals from biomass by fermentation, for recovery from aqueous waste streams, and for production by petrochemical routes. Separations using reversible chemical complexation with reactive extractants or adsorbents are effective for these needs, because these agents are selective and give high capacities for dilute solutes.

In earlier years we defined equilibria and methods of regeneration for recovery of carboxylic acids by means of amine extractants and related basic adsorbents. We also examined phenol- and boronate-based extractants for recovery of alcohols and glycols.

Recovery of Carboxylic Acids: We completed our research on equilibria for uptake of lactic and acetic acids by basic extractants and adsorbents, including recovery from aqueous solutions at pH > pK_a of the carboxylic acid. We have established that several amine-based extractants and adsorbents have high capacities for recovering these carboxylic acids from aqueous solutions at pH as much as two pH units above the pK_a of the acid, as is typical of attractive industrial fermentation conditions. These extractants

and sorbents can be effectively regenerated with aqueous solutions of trimethylamine (TMA), which in turn can be regenerated by thermal dissociation of the resultant trimethylammonium carboxylates to form product carboxylic acid and recycle TMA.

Working with a real fermentation broth and synthetic solutions, we have determined the equilibria for competitive uptake of sulfate, phosphate, lactate and succinate by amine-based extractants and adsorbents. Uptake of sulfate and phosphate is much less for the extractants than for the adsorbents. Sulfate, phosphate and other strong-acid anions can be dealt with through the use of a mixed-bed ion exchanger before adsorption or extraction in an external loop.

Thermal dissociation of trimethylammonium carboxylates gives good recoveries of those carboxylic acids (e. g., succinic, fumaric) that can precipitate from aqueous solution. Recovery of lactic acid is more difficult. We have measured equilibria and kinetics for recovery of lactic acid from trimethylammonium lactate by esterification with *n*-butanol to form volatile *n*-butyl lactate, which can be distilled and then hydrolyzed to form lactic acid and recycle alcohol.

Water-Enhanced Solvation. We completed research identifying the degree to which solvation of carboxylic acids, alcohols, glycols and phenols in ketone and ester solvents is increased by the presence of small amounts of co-dissolved water. Relatively small amounts of water increase solvation of carboxylic acids by large amounts. Activity coefficients decrease by factors of 2 to 3, 6 to 8, and 7 to 10 due to the presence of water for mono-, di- and tri-carboxylic acids, respectively. Activity coefficients decreased by a factor of about 1.5 for ethanol and 1,2-propanediol as solutes in the presence of water. Water-enhanced solvation of phenols is small, when existent. The strong effect of water on solvation of carboxylic acids has already been shown to be useful for regeneration of ketone and ester extracts of carboxylic acids, and should also be useful for solvent leaching of sorbents laden with carboxylic acids.

Recovery of -OH-Bearing Compounds. We prepared a paper covering our work on the use of ion-pair extraction with an organoboronate for recovery of 1,2-propanediol, fructose, sorbitol and glycerol from water. It has been accepted for publication.

We also initiated research on recovery of diols by reversible formation of dioxolanes through reaction with aldehydes. Dioxolanes are far less polar than glycols and are therefore much more readily stripped from aqueous solution. The recovered dioxolane is then converted back to product glycol and aldehyde, either directly by hydrolysis or indirectly through methanolysis. In these reactions it is important to devise effective means of forcing the equilibria in the desired directions, such as by carrying out the reactions in distillation columns with high hold-up and appropriately placed feeds.

We measured chemical equilibria and rates for formation of dioxolanes by reactions of formaldehyde and acetaldehyde with 1,2-propanediol. We also measured vapor-liquid and liquid-liquid equilibria in these systems. Rates are somewhat slow, but are accelerated with sufficient amounts of catalysts (acidic polymeric resins).

The concentration of dioxolane in chemical equilibrium with a solution of 1% to 2% 1,2-propanediol in water and a stoichiometric concentration of formaldehyde is so low that stripping the dioxolane out of the solution is economically not feasible. Excess formaldehyde would be very difficult to remove from aqueous solution.

The reaction product of propylene glycol and acetaldehyde is 2,4-dimethyl-1,3-dioxolane. The chemical equilibrium constant for the forward reaction was measured at various temperatures and ranged from a value of 18 at 25°C to a value of 8 at 80°C. A kinetic experiment at 60°C showed considerably faster kinetics than for reaction with formaldehyde at 90°C. However, the forward equilibrium constant does

not give a high conversion to the dioxolane in the presence of large amounts of water, i. e., for dilute solutions.

We are investigating extraction of product dioxolanes, so as to see if the formation of dioxolanes can be made more favorable in that way.

22. Project Title: Metabolic Engineering

Principal Investigator: J. E. Bailey

Project Site: California Institute of Technology

Description:

Application of recombinant DNA methods to restructure metabolic networks can improve the production of metabolite and protein products by altering the pathway distributions and rates. Recruitment of heterogeneous proteins enables the extension of existing pathways to obtain new chemical products, alter post-translational protein processing, and degrade recalcitrant wastes. Although some of the experimental and mathematical tools required for metabolite engineering are available, complex cellular responses to genetic perturbations can complicate predictive design. This project seeks to expand the technology for using directed genetic alterations, to improve cellular-base biocatalysis. The project involves mathematical analysis of metabolic reaction networks and regulation, and experimental validation of proposed metabolic processes.

At the same time, the project has a very applied end use, which is to solve the problem of growth of aerobic cell cultures, especially at the high cell-density environments, with maintenance of dissolved oxygen concentrations above the growth limits. With the bacterial hemoglobin expressed intra-cellularly, it is possible for cells to utilize the oxygen more efficiently.

1993 Accomplishments:

Several experiments were completed to elucidate the manner in which expression of *Vitreoscilla* hemoglobin (VHb) improves growth and productivity of oxygen-limited *Escherichia coli* and other cultured microorganisms and higher cells. The accomplishment of these experiments required establishment of a unique nuclear magnetic resonance-bioreactor facility with the capability of accomplishing phosphorous-31 NMR measurements of intracellular concentrations in aerated, growing bacterial cultures. Also, a new method for determination of cytochrome levels in intact cells based upon deconvoluted dual-wavelength absorption spectroscopy was established. The results of these measurements showed that the presence of hemoglobin in *Escherichia coli* enhances respiratory functions and associated energetic activities and rates in oxygen-limited *E. coli*. These data provide a basis for interpretation of the effects of *Vitreoscilla* hemoglobin expression in industrial aerobic organisms and for guiding future applications of this technology. These experiments demonstrated that the presence of hemoglobin results in a more highly energized membrane, higher ATP production rates and also alters the level of oxygen-regulated protein expression.

1994 Planned Activities:

This subproject terminates September 30, 1993.

Annual Technical Summary Report:

Two important experiments to investigate VHb physiological effects in *E. coli* have been completed. First, measurements have been made of the stoichiometry of proton extrusion out of the cell relative to oxygen reduced. The methodology outlined in the proposal has been refined. Substantial effort was required to identify cultivation conditions and experimental protocols which gave reproducible absolute values for proton pumping stoichiometry, although under every experimental condition the VHb-expressing strains consistently exhibited a larger H^+/O ratio than the control strains. In the final experiments, *E. coli* JM101:pRED2 (this is the original hemoglobin expression vector) and JM101:pUC9 were grown in batch cultivations in a bench-top fermentor in LB medium with agitation conditions adjusted to provide oxygen-limited cultures in early exponential phase. Samples harvested during late exponential phase were analyzed following the protocol of Kawahara *et al. Agricul. Biol. Chem.* **52**, 1979 (1988). These experiments, which have been reproduced, demonstrate that the number of protons translocated outside the cell per oxygen consumed (H^+/O) was 3.0 +/- 0.1 for the VHb expressing strain and 2.0 +/- 0.1 for the control strain. This higher proton translocation efficiency is consistent with earlier suggestions, based only upon overall metabolic stoichiometry, that the presence of VHb enhances the efficiency of respiratory function.

Based upon the data and the reported higher efficiency of proton pumping of the low-affinity terminal oxidase cytochrome relative to that of the higher oxygen-affinity terminal oxidase cytochrome d, experiments were conducted to evaluate the influence of VHb expression in two different respiratory mutants. The VHb expression vector used in these experiments was plasmid PMSV1 which was constructed from the parental plasmid PMS421, a low copy plasmid chosen to minimize possibilities of plasmid-host interaction that would complicate interpretation of the data. PMSV1 was constructed by inserting the 2.2 kb *HindIII* fragment from pRED2. These plasmids were introduced into *E. coli* ECL933 (F(*cyo-lac*), *bla*$^+$, *cyo*$^+$, *cyd*$^+$), ECL936 (F(*cyo-lac*), *bla*$^+$, *cyd*$^+$, *Dcyo::kan*), and ECL937 (F(*cyo-lac*), *bla*$^+$, *cyo*$^+$, *Dcyd::kan*); these three strains are wild-type, missing cytochrome o, and missing cytochrome d, respectively. Shake-flask experiments with these strains provide interesting preliminary results. In particular, expression of VHb in these strains (confirmed by Western blot analysis) has no detectable effect on growth trajectories of the wild-type or the cytochrome o mutant cells. However, in strain ECL937, which lacks cytochrome d activity and must use only the low-affinity, higher proton pumping efficiency terminal oxidase cytochrome o, expression of hemoglobin results in more rapid growth than the corresponding control. This suggests a mechanism in which hemoglobin or oxygenated hemoglobin is in some fashion interacting positively to enhance the function of cytochrome o while VHb has small effect on strains which utilize only cytochrome d.

Each of the strains ECL933, ECL936, and ECL937 was transformed with either pMSV1 or pMS421 and grown in a batch bioreactor at 37 degrees C, pH controlled at 7.0, and constant aeration such that the dissolved oxygen level falls below 2% of air saturation within 2 hours following inoculation. Exhaust gas carbon dioxide and oxygen content were analyzed by an infrared spectrophotometer and a paramagnetic analyzer respectively.

The growth trajectories for all of the strains investigated were quite similar with the exception of ECL937:pMSV1. This construct, which expresses hemoglobin in a cytochrome d- deficient genetic background obtained a final optical density approximately 25% higher (OD_{600} = 2.5) compared to the final density of the other strains. These specific growth rates for ECL937:pMS421 and ECL937:pMSV1 were 0.37 h^{-1} and 0.48 h^{-1}, respectively.

Significant differences were also seen in these experiments in oxygen uptake rates and respiratory quotient values. The specific oxygen uptake rates OUR (in mmol/L/H) were approximately 25% higher in VHb-expressing transformants of ECL936 and ECL937 compared to the OUR values for the plasmid-control

strains ECL936:pMS421 and ECL937:pMS421. Additionally, the respiratory quotient (RQ; the ratio of CO_2 produced to oxygen consumed), was calculated for these cultivations. Strain ECL937:pMSV1 exhibited an RQ value at the end of the cultivation which was about two-fold higher than the RQ for the control strain ECL937:pMS421. The effect of VHb was not significant on the RQ patterns for ECL936 and ECL933. These results are qualitatively consistent with the shake-flask experiments in the sense that VHb had the greatest effect on the mutant obliged to employ cytochrome o as its sole terminal oxidase.

In order to assess the influence of high-level VHb expression on proton pumping stoichiometry in the ECL mutants, it was necessary to construct a new set of control and VHb expression vectors. Specifically, the antibiotic resistance marker for this marker had to be changed to that for spectinomycin because of the existence of a variety of other antibiotic resistance markers in the genome of the mutants we intend to study. These constructions have been completed, the mutants have been transformed, and initial proton pumping stoichiometry measurements have been accomplished. The estimated H^+/O ratios are changed due to VHb in the parental host containing both cytochromes but, as expected, less affected in mutants lacking either cytochrome d or cytochrome o.

The capability of accomplishing nuclear magnetic resonance (NMR) spectroscopy measurements of growing *Escherichia coli* cultures was applied to study the energetics of *Vitreoscilla* hemoglobin-expressing *E. coli*. Also, batch fermentations of a set of terminal oxidase mutants of *E. coli* with and without VHb expression were conducted and analyzed.

Fed-batch cultivations of wild-type *E. coli* strains MG1655 and an isogenic hemoglobin-expressing strain designated GRO21 were conducted. Cultivations were done using low-phosphate medium and feeding protocols specifically developed to enable on-line phosphorous-31 NMR spectra to be acquired on a sample of growing cells circulated from the bioreactor into the NMR sample chamber and back to the bioreactor under aerated conditions as described in the previous progress report. According to these measurements, the nucleoside triphosphate pool content as well as the inorganic phosphate levels in the cell, the cytoplasmic pH, and UDPG contents were identical for MG1655 and GRO21 cultures. However, these results are significant since the specific growth rate for GRO21 was 0.064 h^{-1} compared to a specific growth rate or 0.038 h^{-1} for MG1655. Since the net rate of ATP synthesis must be equal to the rate of ATP dilution by growth which is the product of the specific growth rate and the intracellular ATP concentration, these results together indicate that the hemoglobin-expressing strain exhibits a net rate of ATP synthesis which is more than 65% higher than the wild-type strain.

Employing an NMR technique called saturation transfer, a more direct estimate can be obtained of the rate of ATP synthesis. In order to implement this saturation-transfer technique on the spectrometer employed in this research, a substantial amount of hardware modification and new software development was necessary. These have been accomplished, but, in order to provide necessary sensitivity and acquisition times, the saturation transfer experiments were not done in the on-line, growing-cell configuration but instead in the more traditional dense cell pellet, batch glucose utilization configuration.

Results of saturation transfer experiments, following normalization by the dry cell density of the sample, indicated, averaged over six experiments for each strain, an ATP synthesis rate which was approximately 30% greater for VHb-expressing GRO21 compared to wild-type strain MG1655.

Previous publications on saturation transfer measurements of *E. coli* have emphasized the importance of conducting control experiments to verify that the ATP synthesis flux measured by saturation transfer is based upon activity of the membrane-bound ATPase and not from glycolysis. Following suggestions by previous investigators who studied other *E. coli* strains, the saturation transfer experiments were repeated employing the specific inhibitor DCCD for ATPase and iodoacetic acid for the glycolytic enzyme GAPDH. Addition of DCCD caused disappearance of the observed transfer in both GRO21 and MG1655

while addition of iodoacetate gave results identical to those with untreated cells. These experiments confirm that the observed differences in ATP synthesis flux can be attributed to different activities of the membrane-bound ATPase in each of the strains studied here. This result is qualitatively consistent with other earlier findings in fed-batch cultivations that greater protein synthesis occurred in VHb-expressing strains than in isogenic strains although the specific oxygen uptake rate was identical for the two strains previously studied.

The current mechanistic model for the action of VHb in *E. coli* is based upon hypothesized enhancement by VHb of cytochrome o activity in *E. coli*. Interpretation of previous experiments has been limited by absence of direct experimental information on the relative quantities of cytochrome o and cytochrome d under the cultivation conditions and in the wild-type and mutant strains employed in our experiments. In order to address this question, a new assay has been established which provides simultaneous estimates of levels of cytochrome o, cytochrome d, and VHb in whole cells. Establishing this assay required modification of a dual-wavelength spectrofluorometer and implementation of a kalman filter computer algorithm (a modified least squares method) for deconvolution of the acquired spectra. Such deconvolution is necessary due to overlap in the signals for cytochrome o, cytochrome d and VHb. Based upon this method, we have obtained cytochrome assays consistent with literature values for wild-type cells. The presence of VHb has no significant effect on cytochrome d levels in either ECL933 or ECL937. However, cytochrome o levels are 2- to 7-fold higher in VHb-expressing transformants (by pMSV1) than in controls (containing pMS421). Because cytochrome o is more highly expressed at higher culture oxygen levels in this regime of conditions, this result is consistent with our "increased effective dissolved oxygen" hypothesis.

4.4.1 Chemical Processes

23. Project Title: Electroreduction of Lipids.

Principal Investigator: P. Pintauro

Project Site: Tulane University

Description:

A novel method of hydrogenating polyunsaturated fatty acids found in vegetable oils to monounsaturated acids low in the trans isomer product is being explored. The selectivity of the electrochemical route is superior to that of heterogeneous catalysts at high temperature, since it allows the production of the nutritionally preferred cis (oleic acid) isomer, which is not the preferred thermodynamic product at higher temperatures (the trans isomer is more stable). The electrochemical selectivity could be extended to other classes of reductions in which the stereoselectivity is necessary.

1993 Accomplishments:

Final oil hydrogenation experiments were carried out in the radial flow Raney nickel powder reactor to determine the inter-relationship between current density, electrolyte oil content, and reactor performance. In order to predict soybean oil hydrogenation, a mathematical computer model was written. This model will also predict current efficiencies as a function of applied current and electrolyte oil content. A complete oil electro-hydrogenation plant flow sheet has been finalized and calculations for some preliminary designs have been made.

Annual Technical Summary Report:

A final set of oil hydrogenation experiments were carried out in the radial flow Raney nickel powder reactor to determine the inter-relationship between current density, electrolyte oil content, and reactor performance. Soybean oil hydrogenation experiments were performed at current densities ranging from 10 mA/cm^2 and oil contents of between 10 w/v% and 45 w/v%. The reaction medium consisted of a two-phase solution of soybean oil in a water/t-butanol solvent containing tetraethylammonium p-toluenesulfonate as the supporting electrolyte. In each experiment the following were determined: (1) the final fatty acid profile and iodine value, (2) the hydrogenation current efficiency, (3) the % total trans isomers, and (4) the % free fatty acids. Optimum reactor performance (86% current efficiency) was achieved at 20 mA/cm^2 with an electrolyte containing 20 w/v% soybean oil.

A mathematical computer model was written to predict soybean oil hydrogenation current efficiencies as a function of applied current and electrolyte oil content. The analysis employed porous electrode theory and phenomenological reaction rate expressions for hydrogen evolution and oil hydrogenation on Raney nickel powder. Three parameters in the model (the exchange current density for the production of adsorbed hydrogen, a rate constant for H_2 evolution, and a reaction order constant in the hydrogen evolution rate expression) were found by force-fitting the model's current efficiency predictions to 15 experimental soybean oil current efficiencies (where the applied current and soybean oil content of the electrolyte differed). With the optimized values of the kinetic parameters the model was able to accurately reproduce the 15 experimental current efficiencies with an average error of 4.1% (and a maximum error of 11%). In a techno-economic analysis of the electrocatalytic oil hydrogenation process, the model will be used to determine an oil's final iodine value (extent of hydrogenation), the optimum thickness of the Raney nickel bed cathode, and the anode/cathode voltage drop for a large-scale oil hydrogenation reactor.

The flow sheet for a complete oil electro-hydrogenation plant has been finalized and some preliminary design calculations have been made. The flow sheet includes the electrochemical reactors and the separation equipment required to remove oil product from the solvent/supporting electrolyte downstream from the reactor. Based on experimental data for a single anode/cathode element radial flow reactor, the total size and number of multiple element shell-and-tube reactors for a 5 MM lb/year soybean oil hydrogenation plant were determined. For a reactor operating at 20mA/cm^2 and 80% current efficiency, a total of four shell-and-tube reactors are needed. Each cylindrical reactor is 200 cm in length, 80 cm in diameter and contains 400 anode/cathode tubular elements.

24. **Project Title:** Product Recovery in Supercritical Solvents. (Project continuing through carry-over funds from FY 1991)

Principal Investigator: M. Antal

Project Site: University of Hawaii

Description:

The National Science Foundation has supported considerable research in the Renewable Resources Research Laboratory (R^3L) of the University of Hawaii concerning the conversion of simple sugars and fermentation products into high value chemicals in near- and supercritical water. Our work has emphasized the production of furfural from xylose, HMF from sucrose, fructose, and glucose, ethene (and PVC) from ethanol, and acrylic acid from lactic acid. This approach has the following benefits:

1) Reactions of organic compounds in water with density $\rho > 0.3$ g/mL and temperatures above about 200°C usually involve heterolytic mechanisms that can be catalyzed to produce high yields of relatively few products.

2) These reactions are often fast, achieving equilibrium in one minute or less.

3) The products of these reactions usually command a significantly higher value than the reactants.

4) Distillation of the reactant is not necessarily required since the reaction chemistry requires water as a media to achieve the desired selectivity.

5) Products of the reaction may phase separate from the water after exiting the reactor, thus facilitating separation.

6) Products of the reaction are available at high pressure.

The Biocatalysis program of DOE became interested in this research because of its potential economic impact and its ability to save energy by avoiding distillation and providing products at high pressures without high compression costs. During the first phase of the DOE sponsored activity we surveyed the possibilities of producing high value products from a wide variety of fermentation products. Many promising lines of research were identified, which could keep my laboratory busy for the next decade or more. The following sections summarize our 1992 accomplishments (giving emphasis to "big picture" issues such as the extent to which a given conversion chemistry demonstrates all the benefits discussed in the preceding paragraph), 1993 plans, industry involvement, technical highlights, and project output.

1993 Accomplishments:

Identified conditions which result in a high conversion of citric acid to itaconic acid with a selectivity approaching 100%.

Identified conditions which provide an 80% (or more) yield of methacrylic acid from itaconic acid with 100% conversion of the feed. Hydroxy isobutyric acid is a major byproduct, which is easily converted to methacrylic acid. The combined yield of methacrylic acid and hydroxy isobutyric acid from itaconic acid exceeds 90% with 100% conversion of the feed.

The NMR studies at Princeton are almost complete, and confirm our view of the mechanism. Our new supercritical flow reactors are becoming operational, and we expect to verify the itaconic yields.

Annual Technical Summary Report:

This is a manuscript submitted to a peer review journal that gives a complete summary description of work performed under this contract.

Formation of Methacrylic Acid from Citric and Itaconic Acids in Near- and Supercritical Water

Citric acid rapidly reacts in hot (250°), compressed (34.5 MPa) liquid water to form itaconic and citraconic acids with a selectivity approaching 100%. At higher temperatures (360°C), in the absence and presence of NaOH, itaconic acid decarboxylates to form methacrylic acid. The yield of methacrylic acid depends on the temperature, pH, and buffer strength of the medium, reaching a maximum of about 80% (by mole) of the itaconic acid feed. Conditions which favor the production of methacrylic acid also lead to the formation of its hydration product: hydroxy isobutyric acid. Under optimum conditions the combined yield of methacrylic acid and hydroxy isobutyric acid from itaconic acid exceeds 90%. Results are consistent with well established dehydration and decarboxylation mechanisms. The condition of the reactor wall appears to influence the yields by catalyzing parasitic fragmentation reactions.

Introduction

Between 200 and 400 million pounds of citric acid are produced annually in the USA by fermentation of molasses and other sugars using the microorganism *Aspergillus niger* (Leeper et al.). A lesser quantity of itaconic acid is manufactured by a similar technology using *Aspergillus terreus* (Leeper et al.). The recovery of citric acid from its fermentation broth via calcium salt precipitation is a costly, highly complex, sophisticated operation (Bouchard and Merrit). Itaconic acid is separated from its broth by acidification, clarification and other purifications, followed by concentration, crystallization and collection of the acid (Tate). Thus these two acids depict the principal problem facing all fermentations: how to recover the product from its broth inexpensively? One attack on this problem is to employ further the aqueous broth as a medium for the transformation of the fermentation product into a higher value petrochemical feedstock. If this transformation were to be accomplished in a reactive distillation tower (Doherty and Buzad), recovery costs could be reduced dramatically. Earlier work in this laboratory examined the utility of near- and supercritical water as a reaction medium for the acid-catalyzed conversion of ethanol to ethene (Antal, et al., Xu et al., Xu et al.), 1-propanol and 2-propanol to propene (Ramayya et al., Narayan and Antal, Narayan and Antal), xylose to 2-furaldehyde (Antal et al.), glucose and fructose to 5-(hydroxymethyl)-2-furaldehyde (Antal et al.), and the dehydration of lactic acid to acrylic acid (Mok, Antal, and Jones). For almost all of these renewable substrates, conditions were identified which result in a favorable yield of the desired product. Moreover, in all cases the possibility exists of avoiding a costly recovery step by employing reactive distillation with water as the reaction medium of choice. The goal of this work is to evaluate the utility of near- and supercritical water as a medium for

the conversion of citric and itaconic acids to methacrylic acid. By avoiding costly separations, we hope to realize an economical method for producing a valuable petrochemical from a renewable feedstock. Nevertheless, the loss of two molecules of carbon dioxide and one molecule of water from citric acid results in a 55% weight loss during the conversion process. Consequently, if the process is to be economical, the reaction chemistry must be quite specific.

Because citric acid possesses one hydroxyl and three carboxyl groups, it reacts at elevated temperatures to form a plethora of reactive products that can further participate in a large number of secondary reactions. Acids with a tertiary hydroxyl in the α position are known to decompose to a ketone upon heating (Roberts), thus citric acid (in the melt phase) decomposes to acetone dicarboxylic acid which further decomposes to acetone (Bouchard and Merritt). On the other hand, β-hydroxy acids usually dehydrate to an α-β unsaturated acid (sometimes with the loss of carbon dioxide) at elevated temperatures (Hurd); hence cis/trans-aconitic acid is a major unstable intermediate product of citric acid decomposition in its melt phase. Above 180°C aconitic acid decomposes to form itaconic and citraconic anhydrides (Bruce), which revert to their acid counterparts in the presence of water (Bouchard and Merritt).

Prior research (Mok, Antal, and Jones) concerning the dehydration of lactic acid in supercritical water led us to anticipate gains in selectivity if the citric acid reactions were carried out in water. The work on lactic acid suggested the facile formation by intramolecular catalysis of an unstable β-lactone intermediate from β-hydroxy acids, which rearranges at elevated temperatures to form an unsaturated acid. Since the hydroxyl group of citric acid is β to two carboxyl groups, we expected it to quickly react in water at elevated temperatures and form cis/trans-aconitic acid (see Figure 1). Furthermore, the literature (Arnold, Elmer and Dodson) offers a general mechanism for the decarboxylation of unsaturated acids, which led us to anticipate the facile conversion of aconitic acid to itaconic acid (see Figure 2), and its subsequent decarboxylation to methacrylic acid. Because of this prior work we felt optimistic about the possibility of identifying conditions which would lead to the selective formation of methacrylic acid from either citric or itaconic acids. Nevertheless, the earlier work in aqueous solutions also revealed competing, parasitic pathways which could decrease yields of methacrylic acid at every step of the process. For example, in the lactic acid study (Mok, Antal, and Jones) acid-catalyzed dehydration/decarbonylation pathway was found which (in this case) could transform citric acid into acetoacetic acid. Acetoacetic acid readily decomposes into carbon dioxide and acetone at low temperatures (Merck). Moreover, equilibration of the propene dicarboxylic acids is known to be fast in water solutions at elevated temperatures (Linstead and Mann; Sakai), thus itaconic acid may rearrange to citraconic and mesaconic acids (see Figure 1) more rapidly than it decarboxylates to form methacrylic acid. There is no reason to expect either of these two acids to decarboxylate readily and form methacrylic acid. Also, itaconic acid can add water across the double bond, forming citramalic acid. Finally, methacrylic acid is unstable at elevated temperatures. Like itaconic acid, methacrylic acid can also add water across its double bond, forming hydroxy isobutyric acid. Methacrylic acid can also decompose by decarboxylation to form propene, which can attack remaining methacrylic acid and form its propyl ester (Bauer). Clearly, even in water the selective conversion of citric and itaconic acids to methacrylic acid is a challenging problem in chemical reaction engineering.

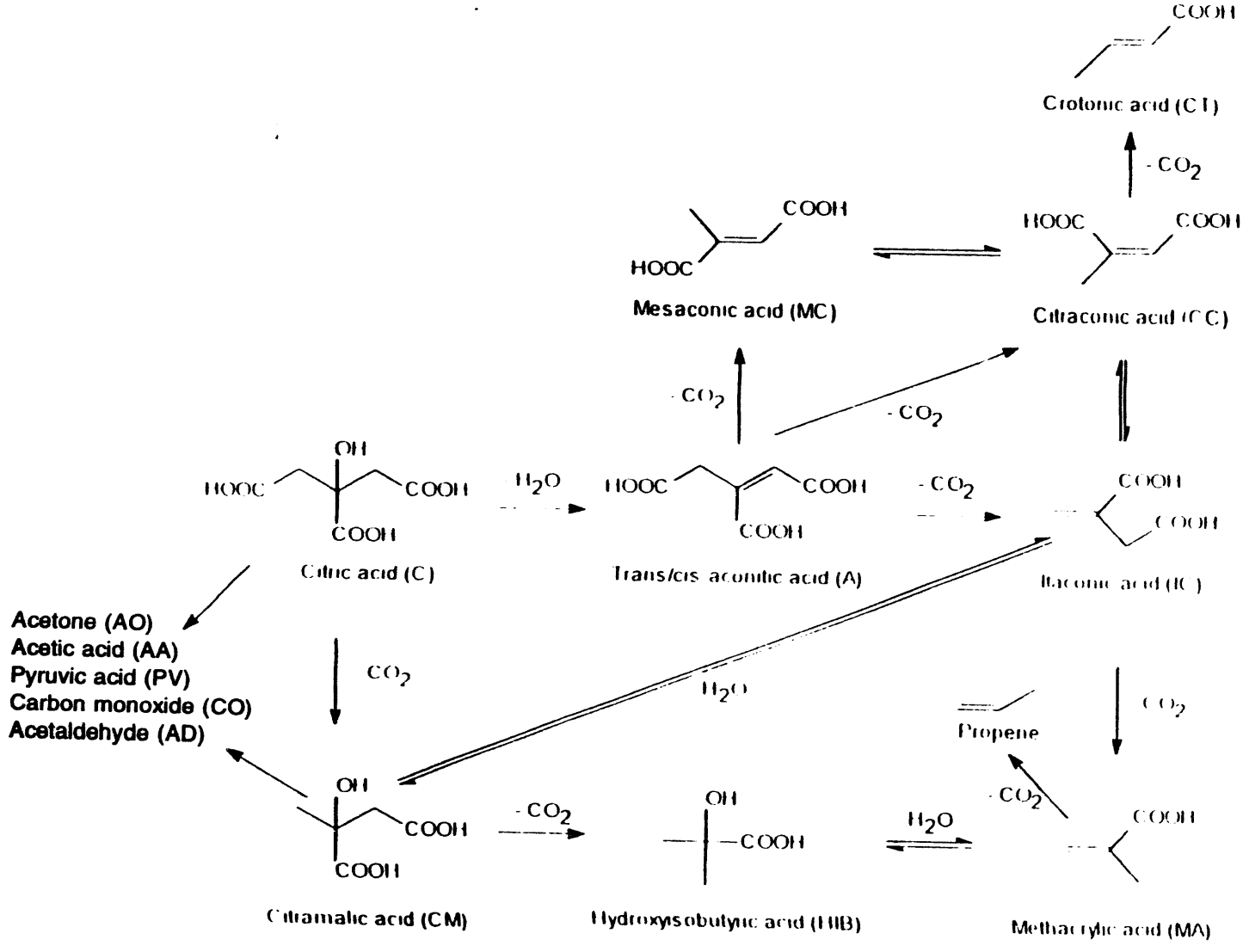
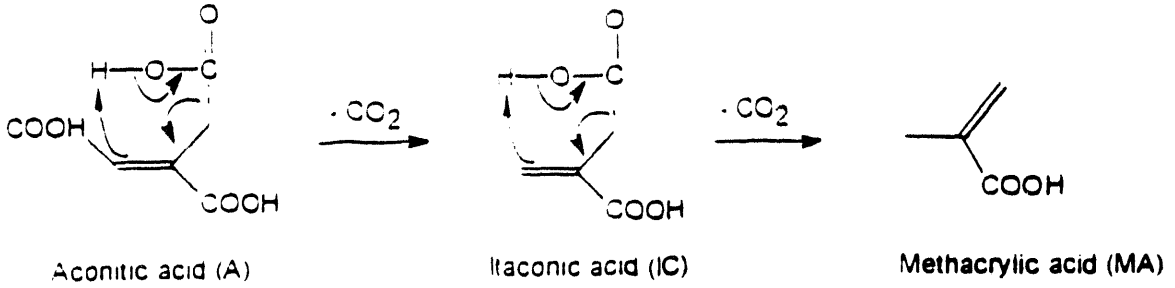


Figure 1 - Important pathways for decomposition of citric acid in water



Aconitic acid (A) Itaconic acid (IC) **Methacrylic acid (MA)**

Figure 2 - Expected mechnism of methacrylic acid fromation from aconitic acid (following Arnold, Elmer and Dodson. 1950)

Apparatus and Experimental Procedures

The principles underlying the design of the two plug flow reactors employed in this work have been described in earlier publications (Cutler, Antal, and Jones; Ramayya and Antal, Xu, DeAlmeida, and Antal). These two reactors realize residence times ranging from about 1 to 10 s, and 20 to over 100 s at temperatures as high as 400°C and pressures up to 34.5 MPa. Details concerning the operation of these reactors were presented in earlier publications (Antal, et al., Ramayya, et al., Antal, et al.). Samples of the liquid effluent from either reactor were analyzed by HPLC using a 300 x 7.8 mm Biorad Aminex HPX-87H ion exclusion column operated at room temperature with a flow of 0.6 ml/min of 0.01 M trifluoroacetic acid solution. Products were detected by a Waters Series R400 RI detector in series with a Hewlett Packard Model 1040A UV-Vis diode array detector. Gaseous products were analyzed using a Hewlett-Packard Model 5890 Series II GC equipped with a TCD connected in series with a FID, and an Alltech Carbosphere stainless steel packed column (80/100 mesh, 6 ft x 1/8 in). The carrier was an 8% hydrogen in helium mixture flowing at 30 ml/min. The GC furnace was programmed to hold the column at 35°C for 4.2 min, followed by a 15°C/min ramp to 227°C and a 35°C/min ramp to 350°C, which was held for 9.5 min. Daily syringe injections of one to three certified gas standards were used for calibration.

Liquid products were identified by a comparison of their retention times and UV-Vis spectra with those of known standards. These standards were obtained from Sigma Chemical Co. or Aldrich Chemical Co. in the highest available purity (usually 98% or better). No significant impurities in the standards were detected by HPLC analysis. Duplicate injections of at least two samples from the reactor at each reaction condition were subject to analysis.

Results and Discussion

Tables I, II, and III display the results of experiments involving citric acid, and miscellaneous other reactants. Product yields are expressed as absolute mol % (100 x mole of product/mole of reactant fed). In almost all cases, the reactions were conducted at 34.5 MPa (P_r=1.56) and temperatures ranging from 220° to 400°C. The critical temperature of water is 374°C; consequently reactions conducted below this temperature involved hot compressed liquid water as the solvent; whereas above this temperature the water was supercritical. The high pressure ensured that over the entire temperature range the density of the water was sufficiently high to sustain liquid phase, heterolytic reactions and minimize the role of gas phase, free radical reactions in product formation.

Experiments 1-4 in Table I illustrate the effect of temperature on the reaction chemistry. Below 250°C decarboxylation of citric acid occurs slowly with high selectivity. In experiment 2 the only liquid products detected were itaconic and citraconic acids, with a trace of mesaconic acid. The excellent carbon balance and the stoichiometric agreement of the carbon dioxide yield with the sum of the yields of the three

propene dicarboxylic acids are consistent with the fact that no parasitic fragmentation byproducts (such as acetone and acetic acid) were observed in this experiment. Unfortunately, fifteen months later this auspicious result was not reproduced. Parasitic byproducts carbon monoxide, acetic acid, and pyruvic acid appear in experiment 83. Other results (see below) strongly suggest that activation of the reactor wall is responsible for catalyzing these parasitic reactions. Above 250°C parasitic reactions begin to evidence themselves in a declining carbon balance, the appearance of unwanted byproducts (acetone and acetic acid), and a growing discrepancy between the carbon dioxide yield and the formation of itaconic, citraconic, and mesaconic acids. At 280°C the addition of base NaOH increases the reaction rate but not the sum of the yields of the propene dicarboxylic acids. However experiments 6-8 reveal a trend that results in improved selectivity and reaction rate by 320°C as a consequence of the presence of NaOH. Moreover, the desired product methacrylic acid begins to appear in significant yields. Nevertheless, the conditions recorded for experiment 2 appear optimal for the conversion of citric acid to itaconic, citraconic, and mesaconic acids.

Since it is not difficult to obtain a quantitative conversion of citric acid to the propene dicarboxylic acids (primarily itaconic acid), the focus of this paper shifts to the reaction chemistry of these acids in hot compressed and supercritical water. The first question of interest is the rate of isomerization of itaconic acid relative to its rate of decarboxylation. A comparison of experiments 24 with 32, and 27 with 33 (under "Effect of Reactant" in Table 2) show that the rate of interconversion between itaconic and citraconic acids is very high relative to the rates of all other reactions. Almost identical products are obtained independent of which acid is used as reactant. Consequently, these two acids should be regarded to be a single reactant as far as discussions of reaction specificity are concerned.

The second question of interest concerns the acidity of itaconic acid in liquid water at high temperatures. According to Le Chatelier's Principle it is expected that increasing temperature will affect the dissociation equilibrium according to the sign of ΔH_{dissoc}. Thus sulfuric acid (whose dissociation is strongly exothermic) becomes a weak acid in supercritical water (Quist et al., Narayan and Antal); whereas *tert*-butanol becomes a relatively strong acid in compressed liquid water at 250°C (Xu and Antal). A qualitative test of the ability of a putative acid to dissociate in liquid water at temperatures above about 300°C is to examine the stability of 2-propanol in its presence. At elevated temperatures very low concentrations of Arrhenius and Bronsted acids serve as effective catalysts for the quick dehydration of 2-propanol to propene. In the absence of acid, 2-propanol is stable at these conditions. An experiment involving the dehydration of 1.0 M 2-propanol in the presence of 0.1 M itaconic acid at 360°C, 34.5 MPa and 27 s residence time resulted in a 70% conversion of the alcohol to propene (which was the only significant gaseous product). At 375°C a similar conversion of 2-propanol can be effected by 0.0005 M sulfuric acid after 1.5 s (Antal et al.). Thus itaconic acid is weak relative to sulfuric acid at these conditions; neve theless this result shows that its dissociation must be considered in any examination of its reaction chemistry in water at elevated temperatures.

Another important question concerns the stability of methacrylic acid in water at elevated temperatures. Experiment 40 in Table 3 shows that methacrylic acid decomposes under relatively severe conditions to form propene, crotonic acid, α-hydroxyisobutyric acid and acetone. If we suppose that the difference between the carbon dioxide and propene yields is propene which reacted with remaining methacrylic acid to form an undetected ester, then the carbon balance for the experiment approaches unity. Obviously, great care must be taken in the choice of reaction conditions to avoid the degradation of methacrylic acid into unwanted byproducts which are capable of attacking the parent.

Among others, experiments 20-29 in Table 2 show that decarboxylation of itaconic acid is fast above 350°C. Unfortunately, the appearance of parasitic reaction byproducts (such as acetic acid, pyruvic acid, acetone, and acetaldehyde) and methacrylic acid degradation products (propene), signal a loss of selectivity at these temperatures. To improve the carbon balance and to gain insight into the parasitic reaction

chemistry, we engaged in a search for undetected and/or unidentified products. Only one, small, unidentified HPLC peak was found, which did not absorb strongly in the UV. We prepared methyl esters of product samples from the reactor and authentic standards for all identified, significant products and injected these esters into the GC-MSD. No large, unidentified peaks were detected. One small, unidentified peak had an m/z value of 144, which could be the methyl ester of paraconic acid (see Figure 3). One explanation for the lower carbon balance in these experiments involves possible reactions of methacrylic acid with the itaconic/citraconic acid reactants and/or their other decomposition products. Experiments 44-47 in Table 3 were designed to test this hypothesis. A comparison of the results of experiment 44 with 50, and experiment 47 with 48 indicates complete recovery of the methacrylic acid reactant, combined with additional methacrylic acid formed from the itaconic acid reactant. Evidently methacrylic acid is not attacked by the product "soup" that results from the decomposition of itaconic acid.

Anticipating that the pH and buffer strength (reactant concentration) of the medium would affect the reaction chemistry, we sought to improve selectivity by systemically varying these at temperatures where the rate of decarboxylation is relatively high. Listed in Table 2 under "Effect of pH and reactant concentration" are blocks of data organized on the basis of increasing temperature. Within each block data are organized on the basis of increasing pH. Short residence time experiments conducted using the capillary tube reactor are grouped separately. As expected (Mok, Antal, and Jones), experiments 71, 20, and 25 show that decreasing pH favors the parasitic decarbonylation pathway that results in acetone formation. To verify that the effect of added NaOH in experiment 25 is due to a change in pH, and not the nature of the cation or the general presence of ions, we executed experiments with KOH and NaCl as additives. Results using KOH (see experiment 30) were effectively identical to those with NaOH; whereas those with NaCl (experiment 31) were quite similar to those with pure water (see experiment 20, which was conducted at a slightly higher temperature). From these results we concluded that the cation exerts little influence on the reaction: pH is a controlling variable.

The mechanism for decarboxylation of unsaturated acids given in Figure 2 indicates the key role played by the double bond and the β-carboxylic hydrogen in the loss of carbon dioxide. The importance of the double bond is illustrated by the results of experiments 41 and 42, which show that methylsuccinic acid (MS) (the saturated analog of itaconic acid, see Figure 3) does not decarboxylate at reaction conditions. Experiment 51 provides evidence for the role of the β-carboxylic hydrogen in decarboxylation: at high pH only isomerization occurs (no decarboxylation is observed). Moreover, the absence of vinylacetic acid (see Figure 3) as a product in all our experiments indicates that only the β-carboxyl group in itaconic acid leaves as carbon dioxide. The low yields of crotonic acid are also consistent with our expectations: the rate of decarboxylation of citraconic and mesaconic acids to crotonic acid is slow because these two propene dicarboxylic acids lack a double bond β to their carboxyl groups. The indicated mechanism also helps us to anticipate how an increase in pH (due to a decrease in the acid to base ratio) will affect the rate of decarboxylation of itaconic acid. The carboxylic acid group which is α to the double bond in itaconic acid is expected to be the more acidic since it is closer to the electron withdrawing double bond; consequently a negligible amount of the carboxylic acid group β to the double bond should be ionized as long as the pH of the buffer is lower than the pKa of the second dissociation constant at reaction temperature and pressure. As the double bond becomes more electron rich due to the loss of a proton from the α carboxyl, it is more likely to form an intra-molecular bond with the hydrogen on the β carboxyl, which initiates decarboxylation. When the pH of the solution exceeds the pKa of the second dissociation constant, then the rate of decarboxylation will decrease as there are fewer β carboxyl hydrogens available to initiate decarboxylation. Thus the mechanism displayed in Figure 2 leads us to anticipate a relatively narrow range of reactant itaconic acid to base ratios that favor the desired decarboxylation.

Because neither the pKa_1 nor the pKa_2 of itaconic acid in near-critical water is known, it is not possible to speak quantitatively of the pH of a near-critical buffer composed of itaconic acid and base NaOH. The

situation is exasperated by the fact that the pH changes as itaconic decomposes to methacrylic acid, whose pKa is also unknown at reaction conditions. Consequently, at present we must be content with phenomenological observations. Experiments 34, 63, 64, 65, 78, 79, and 80 show that high yields (approaching 80%) of methacrylic acid can be obtained at low reactant concentrations (0.05 to 0.1 M) with reactant to base ratios between 5:1 and 10:1 after about 25 s residence time at 360°C. Higher concentrations of itaconic acid with the same reactant to base ratio result in lower yields of methacrylic acid (see experiments 72 and 73). Similarly, higher reactant concentrations with a higher acid to base ratio also result in lower yields (experiments 35 and 25). An increase in temperature to 375°C at the favored conditions (experiment 75) evidences a slight decrease in methacrylic acid yield, and variations of reactant concentration and acid to base ratio (experiments 74, 76, and 77) at this temperature do not improve the yield.

Reasoning that secondary reactions could be responsible for destroying the desired methacrylic acid product at long residence times, we conducted experiments with a capillary tube flow reactor that offers very short residence times (<10 s) at these conditions. Instead of the anticipated improvement in methacrylic acid formation, we observed a rise in the yields of parasitic byproducts acetic acid, pyruvic acid, and acetone, and a decrease in the methacrylic acid yield (see experiments 54, 81, 82, 63, and 34). This difference in the performance of the two reactors has been observed in earlier work involving the formation of 2-furaldehyde from xylose at low acid concentrations (Antal et al.), and studies of the decomposition of glucose and fructose in water at 250°C. In spite of many attempts to passivate the wall of this reactor relative to reactants that are sensitive to base-catalyzed decompositions, some catalytic activity still remains. Moreover, the series of experiments 63, 78, and 86 indicates that even the larger, annular flow reactor became depassivated over time. The steady decline over time in the yield of methacrylic acid at the "optimal condition" was accompanied by a steady rise in the acetic acid and pyruvic acid yields. These results suggest that further gains in selectivity could be realized in a reactor fabricated from material that lacked this type of activity.

Table 1 Results of Citric Acid Experiments

Exp #	Reactant	Catalyst	Temp / °C	Res. time/s	Conversion %	CO2	CO	C3H6	MA	IC	CC	MC	CT	AO	AA	AD	PY	HIB	Cbal	
Effect of temperature																				
1	C 0.5 M	nil	220	95	20	12	0	0	0	7	2	0	0	0	0	0	0	0	89	3/20/92 L 7
2	C 0.5 M	nil	250	97	37	40	0	0	0	30	9	1	0	0	0	0	0	0	102	2/22/92/L 16
83	C 0.5 M	nil	250	106	64	56	3	0	0	35	12	1	0	0	2	0	2	0	87	5/17/93 s1,s3
3	C 0.5 M	nil	280	43	75	72	2	0	0	41	19	3	0	2	3	0	3	0	95	11/27/92 N2,N4
4	C 0.5 M	nil	320	41	100	130	7	0	7	26	19	14	0	6	4	0	2	0	88	11/27/92 N16,N18
6	C 0.5 M	10 mM NaOH	280	43	86	89	3	0	1	46	23	4	0	0	3	0	2	0	87	11/27/92 N6,8,10
7	C 0.5 M	10 mM NaOH	300	38	100	103	5	2	4	42	24	10	0	5	4	0	2	0	92	12/28/92 d1-d5
8	C 0.5 M	10 mM NaOH	320	44	100	121	6	0	6	35	25	16	0	6	6	0	3	0	96	11/27/92 n12,n14
84	C 0.05 M	10 mM NaOH	250	108	69	75	1	0	0	56	16	2	0	1	2	0	2	0	108	5/17/93 s4,s5
78	C 0.05 M	10 mM NaOH	375	25	100	177	7	1	56	1	0	0	3	12	13	7	7	8	95	5/4/93 s11,s12
Effect of pH																				
70	C 0.5 M	10 mM H2SO4	280	41	79	74	2	0	0	36	19	3	0	2	4	0	2	0	85	4/23/93 s1,s2
3	C 0.5 M	nil	280	4	75	72	2	0	0	41	19	3	0	2	3	0	3	0	95	11/27/92 N2,N4
6	C 0.5 M	10 mM NaOH	280	43	86	89	3	0	1	46	23	4	0	0	3	0	2	0	87	11/27/92 N6,8,10
63	C 0.1 M	2 mM NaOH	280	43	92	82	4	0	1	50	21	4	0	3	3	0	3	0	88	4/13/93 m3,m1
65	C 0.01 M	0.2 mM NaOH	280	43	100	88	2	0	3	50	21	5	0	6	0	0	2	0	87	4/13/93 l1,l2
62	C 0.1 M	10 mM NaOH	280	43	99	91	4	0	3	45	22	6	0	4	3	0	2	0	83	4/13/93 s3,s4
64	C 0.01M	1 mM NaOH	280	43	100	100	4	0	6	38	20	8	0	4	0	0	2	0	80	4/13/93 n3,n4
4	C 0.5 M	nil	320	41	100	130	7	0	7	26	19	14	0	6	4	0	2	0	88	11/27/92 N16,N18
8	C 0.5 M	10 mM NaOH	320	44	100	121	6	0	6	35	25	16	0	6	6	0	3	0	96	11/27/92 n12,n14
60	C 0.1 M	10 mM NaOH	375	3.2	100	144	0	0	23	11	16	7	2	6	11	0	6	3	81	4/8/93 s13
61	C 0.01 M	2 mM NaOH	375	3.2	100	153	0	0	34	0	4	0	0	13	38	0	15	4	80	4/8/93s19,20
52	C 0.1 M	50 mM NaOH	375	1.1	100	127	0	0	26	10	26	27	0	9	6	0	7	1	83	3/24/93 s7-8
53	C 0.01 M	50 mM NaOH	375	1.1	100	0	0	0	0	5	4	10	0	0	68	0	83	0	81	3/24/93 s10,12,13
Effect of residence time																				
14	C 0.5 M	10 mM NaOH	300	38	100	103	5	2	4	42	24	10	0	5	4	0	2	0	92	12/28/92 d2-d5
15	C 0.5 M	10 mM NaOH	300	51	100	103	5	4	5	39	22	11	0	5	4	0	2	0	89	12/28/92 c2-c5
16	C 0.5 M	10 mM NaOH	300	94	100	109	4	4	10	27	15	14	0	5	4	0	2	2	80	12/28/92 b2-b5
17	C 0.5 M	10 mM NaOH	300	138	100	120	4	0	12	19	10	13	0	4	4	0	1	3	71	12/28/92 a2-a5

Table 2. Results of Itaconic Acid Experiments

Exp #	Reactant	Catalyst	Temp/°C	Res. time/s	Conversion/%	CO2	CO	C3H6	MA	IC	CC	MC	CT	AO	AA	AD	PY	HIB	Cbal	Sample
Effect of temperature																				
20	IC 0.5 M	nil	360	64	83	47	4	2	20	18	11	10	1	4	2	4	1	2	76	10/27/92 N4, N6
21	IC 0.5 M	nil	375	62	90	66	6	2	26	13	10	8	2	5	4	6	1	3	80	10/27/92 N8, N10
22	IC 0.5 M	nil	385	56	91	67	5	2	26	12	9	6	2	6	3	7	0	3	76	10/27/92 N20,N22
23	IC 0.5 M	nil	400	54	92	67	6	2	30	10	8	4	4	6	5	10	0	3	78	10/27/92 N30,N28
24	IC 0.5 M	10 mM NaOH	350	71	93	78	0	2	34	7	7	3	1	2	3	2	1	4	66	12/03/92 A1-A4
25	IC 0.5 M	10 mM NaOH	360	61	96	86	4	1	38	4	4	3	1	3	3	2	1	4	66	12/03/92 B1-B4
26	IC 0.5 M	10 mM NaOH	365	61	98	93	4	2	42	2	2	2	1	3	3	2	0	5	72	12/03/92 C1-C4
27	IC 0.5 M	10 mM NaOH	370	58	99	100	4	1	44	1	1	1	2	3	3	3	0	5	70	12/03/92 D1-D4
28	IC 0.5 M	10 mM NaOH	375	55	99	111	6	4	46	1	1	1	2	4	3	0	0	4	78	11/17/92 K7-K10
29	IC 0.5 M	10 mM NaOH	400	49	100	110	4	7	45	0	0	0	2	6	3	1	0	3	71	11/17/92 K13-K16
55	IC 0.01 M	10 mM NaOH	360	3.5	98	53	0	0	53	2	7	0	0	0	27	0	25	3	89	4/7/93 n1,n2
49	IC 0.01 M	10 mM NaOH	375	2.1	97	56	0	0	59	3	12	10	0	0	11	0	13	5	89	3/22/93 s8-10
56	IC 0.01 M	2 mM NaOH	360	3.5	96	63	1	0	33	4	9	5	0	0	33	0	17	5	83	4/7/93 m2,m3
58	IC 0.01 M	2 mM NaOH	375	3.2	97	55	0	0	40	3	6	3	0	0	32	0	16	5	81	4/8/93 s3,s4
65	IC 0.10 M	20 mM NaOH	360	27	99	?	?	?	75	1	0	0	2	?	3	1	1	?	?	4/20/93 s16,s17
74	IC 0.1 M	20 mM NaOH	375	21	99	103	3	1	67	1	0	0	2	4	4	2	2	8	89	4/28/93 s3,s4
85	IC 0.05 M	10 mM NaOH	320	95	96	80	1	1	53	4	1	3	2	1	22	0	8	10	89	5/17/93 s8,s9
63	IC 0.05 M	10 mM NaOH	360	25	99	?	?	?	81	1	0	0	2	?	5	0	3	10	?	4/20/93 s7,s8
75	IC 0.05 M	10 mM NaOH	375	21	99	96	3	1	70	1	0	0	2	5	6	5	5	9	87	4/28/93 m1,m2
Effect of reactant																				
24	IC 0.5 M	10 mM NaOH	350	71	93	78	0	2	34	7	7	3	1	2	3	2	1	4	66	12/03/92 A1-A4
32	CC 0.5 M	10mM NaOH	350	67	93	72	2	3	33	7	6	5	1	3	3	2	1	4	68	12/23/92 B2-B5
27	IC 0.5 M	10 mM NaOH	370	58	99	100	4	1	44	1	1	1	2	3	3	3	0	5	70	12/03/92 D1 D4
33	CC 0.5 M	10 mM NaOH	370	58	99	86	3	8	44	1	1	1	2	3	3	2	0	4	69	12/23/92 D1 D4
Effect of residence time																				
36	IC 0.5 M	10 mM NaOH	350	36	84	44	2	0	24	16	12	12	1	2	2	0	0	3	72	1/7/93 s26,s30
37	IC 0.5 M	10 mM NaOH	350	47	87	53	3	0	28	12	10	10	0	2	2	1	1	3	72	1/7/93 s22,s24
24	IC 0.5 M	10 mM NaOH	350	71	93	78	0	2	34	7	7	3	1	2	3	2	1	4	66	12/03/92 A1-A4
38	IC 0.5 M	10 mM NaOH	350	93	95	80	4	0	48	5	3	4	0	4	2	1	1	4	71	1/7/93 s10,s16
39	IC 0.5 M	10 mM NaOH	350	130	98	93	4	0	47	2	2	2	1	4	2	2	1	5	73	1/7/93 s2,s6
54	IC 0.05 M	10 mM NaOH	360	3.5	93	29	0	0	31	7	19	11	0	0	8	0	5	4	77	4/7/93 s1,s2
81	IC 0.05 M	10 mM NaOH	360	5.1	92	56	0	0	50	8	11	6	1	3	9	0	5	6	91	5/7/93 s3,s4,s5
82	IC 0.05 M	10 mM NaOH	360	9.2	98	79	0	0	66	2	2	1	2	4	11	0	7	8	92	5/7/93 s1,s2
63	IC 0.05 M	10 mM NaOH	360	25	99	?	?	?	81	1	0	0	2	?	5	0	3	10	?	4/20/93 s7,s8
34	IC 0.05 M	10 mM NaOH	360	62	100	100	3	3	72	0	0	0	0	0	7	0	6	9	93	2/4/93 s17,s19
49	IC 0.01 M	10 mMNaOH	375	2.1	97	56	0	0	59	3	12	10	0	0	11	0	13	5	89	3/22/93 s8-10
77	IC 0.01 M	10 mM NaOH	375	20	100	100	0	3	65	0	0	0	0	0	14	0	7	11	91	4/28/93 l1,l2

Table 2. (continued)

Exp #	Reactant	Catalyst	Temp/°C	Res time/s	Conversion/%	CO2	CO	C3H6	MA	IC	CC	MC	CT	AO	AA	AD	PY	HB	Cbal	Sample
Effect of pH and reactant concentrations																				
31	IC 0.5 M	10 mM HNO3	350	70	80	42	2	0	20	20	14	14	0	4	3	2	0	2	79	1/26/93 s8,s11
24	IC 0.5 M	10 mM NaOH	350	71	93	78	0	2	34	7	7	3	1	2	3	2	1	4	66	12/03/92 A1-A4
30	IC 0.5 M	10 mM KOH	350	66	93	67	3	4	32	7	6	5	1	2	3	1	1	3	66	12/23/92 A1 A4
71	IC 0.5 M	10 mM H2SO4	360	60	80	39	11	0	14	20	19	13	0	10	3	0	0	0	81	04/23/m1,m2
20	IC 0.5 M	nil	360	64	83	47	4	1	20	18	11	10	1	4	2	4	1	2	76	10/27/92 N4, N6
25	IC 0.5 M	10 mM NaOH	360	61	96	86	4	1	38	4	4	3	1	3	3	2	1	4	66	12/03/92 B1-B4
35	IC 0.25 M	10 mM NaOH	360	61	99	96	4	2	60	1	1	1	2	3	6	4	2	15	91	2/4/93 s11,s12
64	IC 0.05 M	5 mM NaOH	360	27	98	?	?	?	74	2	0	0	2	?	8	0	3	7	?	4/20/93 s11,s14
80	IC 0.05 M	5 mM NaOH	360	25	98	82	0	1	60	2	2	1	3	3	12	8	4	7	91	5/4/93 s14,s15
73	IC 0.5 M	100 mM NaOH	360	25	99	102	3	1	57	1	0	0	1	4	2	0	1	7	79	4/23/93 l1,l2
72	IC 0.2 M	40 mM NaOH	360	25	99	105	3	1	67	1	0	0	1	5	2	0	2	9	88	4/23/93m1,m2
65	IC 0.10 M	20 mM NaOH	360	27	99	?	?	?	75	1	0	0	2	?	3	1	1	7	?	4/20/93 s16,s17
79	IC 0.10 M	20 mM NaOH	360	25	99	86	4	1	72	1	0	0	2	4	4	2	3	10	95	5/4/93 s6,s8
63	IC 0.05 M	10 mM NaOH	360	25	99	?	?	?	81	1	0	0	2	?	5	0	3	10	?	4/20/93 s7,s8
78	IC 0.05 M	10 mM NaOH	360	25	99	89	0	1	72	1	1	1	0	4	7	4	4	10	97	5/4/93 s1,s3
86	IC 0.05 M	10 mM NaOH	360	25	98	92	5	2	63	2	0	0	2	2	11	0	5	9	90	5/17/93 s11,s12
14	IC 0.05 M	10 mM NaOH	360	62	100	100	3	3	72	0	0	0	0	0	7	0	6	9	93	2/4/93 S17 S19
57	IC 0.01 M	1 mM NaOH	360	35	94	51	1	0	20	6	11	6	0	0	32	0	14	4	75	4/7/93 l1,l2
55	IC 0.05 M	10 mM NaOH	360	35	98	53	0	0	53	2	7	0	0	0	27	0	25	3	89	4/7/93 m1,m2
56	IC 0.01 M	2 mM NaOH	360	35	96	63	1	0	33	4	9	5	0	0	33	0	17	5	83	4/7/93 m2,m3
87	IC 0.5 M	10 mM HNO3	370	58	85	68	3	0	28	15	13	10	1	6	3	2	0	3	81	1/26/93 s13,s17
27	IC 0.5 M	10 mM NaOH	370	58	99	100	4	1	44	1	1	1	2	3	3	3	0	5	70	12/03/92 D1-D4
88	IC 0.5 M	10 mM KOH	370	58	99	93	4	6	46	1	1	1	2	4	3	3	0	4	70	12/23/92 C1-C4
74	IC 0.1 M	20 mM NaOH	375	21	99	103	3	1	67	1	0	0	2	4	4	2	2	8	89	4/28/93 s3,s4
75	IC 0.05 M	10 mM NaOH	375	21	99	96	3	1	70	1	0	0	2	5	6	5	5	9	97	4/28/93 m1,m2
76	IC 0.05 M	25 mM NaOH	375	21	99	98	2	2	69	1	0	0	1	4	6	2	3	12	95	4/28/93 n1,n2
77	IC 0.01 M	10 mM NaOH	375	20	100	100	0	3	65	0	0	0	0	0	14	0	7	11	91	4/28/93 l1,l2
48	IC 0.1 M	10 mM NaOH	375	2.1	80	20	0	0	19	20	33	16	0	0	3	0	1	0	88	3/22/93 s3,s5
59	IC 0.01 M	1 mM NaOH	375	3.2	94	52	0	0	28	6	8	4	0	0	33	0	11	4	75	4/8/93 s8,s9
58	IC 0.01 M	2 mM NaOH	375	3.2	97	55	0	0	40	3	6	3	0	0	32	0	16	5	81	4/8/93 s3,s4
50	IC 0.1 M	50 mM NaOH	375	1.1	89	36	0	0	36	11	28	10	0	0	2	0	2	0	87	3/24/93 s17,s18
49	IC 0.01 M	10 mMNaOH	375	2.1	97	56	0	0	59	3	12	10	0	0	11	0	13	5	89	3/22/93 s8-10
51	IC 0.01 M	50 mM NaOH	375	1.1	75	0	0	0	0	24	18	52	0	0	6	0	3	0	97	3/24/93 s20,21,23

Table 3. Other results

Exp #	Reactant	Catalyst	Temp/°C	Res times	Conversion/%	CO2	CO	C3H6	MA	IC	CC	MC	CT	AO	AA	AD	PY	HIB	Cbal	Sample
40	MA 0 5 M	10mM NaOH	370	58	23	6	2	2	77	0	0	0	2	2	0	0	0	5	87	2/11/93s1,s2
41	MS 0 1 M	nil	350	67	4	0	0	0	0	0	0	0	0	0	0	0	0	0	97	1/26/93 s1,s2
42	MS 0 1 M	10 mM NaOH	350	67	2	0	0	0	0	0	0	0	0	0	0	0	0	0	98	1/26/93 s5,s6
43 *	0 5 M IC+0 12 M MA	10 mM NaOH	370	62	98	99	5	2	64	2	2	1	2	4	3	1	0	5	78	2/11/93 S6,S8
44 *	0 1 M IC+0 1 M MA	50 mM NaOH	375	1	91	82	0	0	146	9	20	7	0	0	3	0	1	6	99	3/4/93 s10,s9
45 *,**	0 1 M IC+0 1 M MA	50 mM NaOH	375	1	89	34	0	0	137	10	24	9	0	0	0	0	2	6	95	3/4/93 s19,s20
46 *,**	0 1 M IC+0 1 M MA	10 mM NaOH	375	1	80	17	0	0	101	20	30	11	0	0	3	0	1	2	85	3/4/93 s14 s15
47 *	0 1 M IC+0 1 M MA	10 mM NaOH	375	1	80	21	0	0	118	20	31	14	0	0	3	0	1	4	95	3/4/93 s2 s3
89	A 0 025 mM	1mM H2SO4	250	90	100	102	0	0	0	62	20	5	0	0	0	0	0	0	96	4/18/92 L22

* Yields are based on reactant Itaconic acid, whereas Cbal is based on both reactants

** Conducted at pressure 4000 psi instead of normal 9000 psi

4.5 Process Control and Systems Analysis

25. Project Title: Bioengineering Simulation Technology (BEST) Development.

Principal Investigators: R. Wooley, G. Phillipidis, R. Landucci

Project Site: National Renewable Energy Laboratory

Description:

The development of the necessary mathematical algorithms to simulate bioprocesses, which is carried out in part through a subcontract, to encode the algorithms into commercially available ASPEN/SP-386. Once developed and validated, the bioprocess simulator can be used to rapidly and accurately determine the technical and economic feasibility of existing and proposed bioprocesses, or evaluate the impact of proposed bioprocess improvements and R&D accomplishments. These analyses can be done on a consistent basis to allow comparison of alternative processes and the assessment of progress from different R&D projects. It can also be used to establish the most cost effective research goals and, thereby, minimize the time to commercialization and maximize the impact of the research investment.

1994 Planned Activities:

The BEST project team will use the results of the ASPEN+(BPS) evaluation to assess the need for additions and modifications to BPS to satisfy NREL simulation needs. An assessment will be made of the possibility of using the unit operations added to ASPEN/SP in negotiation with ASPEN Technology for modifications or enhancements to ASPEN+. Possibly an exchange of ASPEN/SP developed BEST code for additional programming by ASPEN Technology.

This assessment will need to be delayed until the end of the first quarter 1994.

Annual Technical Summary Report:

Delivery of unit operation models developed jointly between NREL and Simulation Sciences (SimSci) have been further delayed due to the turn-over of key personnel at SimSci. The additional batch and CSTR unit operations with rigorous mixing and aeration and heat transfer calculations will hopefully be delivered in the 1st quarter '94, baring any further problems at SimSci. Delivery of these additions along with all source code and documentation will complete the current contract with Simulation Sciences for bio processing unit operations additions to the chemical simulator, ASPEN/SP.

An ASPEN+ and ASPEN BPS (Bioprocess Simulator) training class is scheduled for the second week of November and will serve as the kick-off of an evaluation of the bioprocessing capabilities of this commercially available software. The evaluation will compare this software to the needs of NREL bioprocessing engineering and to the software developed by Simulation Sciences under the BEST project.

26. **Project Title:** A Biological and Chemical Process Integration and Assessment Computer Model: BCPI.

Principal Investigator: J. D. Ingham

Project Site: Jet Propulsion Laboratory

Description:

The objective is to develop a computer model (BCPI) that can be used to determine the energy requirements and economics of biological and chemical processes for the production of industrial chemicals. The goal of this component of the JPL Biocatalysis Project is to support the DOE Advanced Industrial Concepts Division Catalysis/Biocatalysis Program, by contributing to the technology base for chemical and biocatalyzed processes for large-scale production of industrial chemicals from renewable resources.

There are several commercial computer-aided design (CAD) programs, such as ASPEN[9,10,11], that can be used for detailed chemical process assessments; however, their primary purpose is to develop detailed process designs for specific chemical plants. Except for recent work on the BEST simulator (based on ASPEN) at NREL, CAD programs have not been satisfactorily adapted for bioprocess assessments. In any case, operational CAD programs are not normally available at moderate cost, are not very user-friendly, and require special computer capabilities; such as a 386 computer with 6-16 MB of expanded memory. In addition, as CAD simulators have been improved, computer requirements have been extended to accommodate larger databases and increased complexity, and this trend is expected to continue. BCPI software is being developed to minimize or circumvent these factors that tend to limit applications for rapid, reliable process assessments.

A preliminary version of the BCPI computer model was used to estimate fermentation costs for ethanol, and for comparisons for an ethyl acetate process with an ASPEN simulator[12]. A series of process reaction types (for example, specific fermentation processes and chemical processes such as hydrogenation, dehydration, esterification) are included in the BCPI model for selection from menu lists, and the selected type is modified as appropriate for an assessment. For a specific reaction of each type, default values for numerous parameters and variables, such as stoichiometric factors, properties, rates, concentrations, materials costs, and economic values, are displayed and can be changed during the assessment run, or in the program, to create a new assessment. Mass and energy balances are provided and costs are computed on the basis of a factored estimate. A large number of known processes will be included and verified to ensure that cost estimate protocols are reliable. After any base-case estimate, each additional assessment (where numerous process variations can be incorporated) may be completed within a few minutes. Since

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9 Peters, M.S., Timmerhaus, K.D. (1980), Plant Design and Economics for Chemical Engineers, McGraw-Hill, New York, NY.

10 Douglas, J.M. (1988), Conceptual Design of Chemical Processes, McGraw-Hill, New York, NY.

11 N.K. Rohatgi and J.D. Ingham, "Conversion of Bioprocess Ethanol to Industrial Chemical Products: Applications of Process Models for Energy-Economic Assessments", Thirteenth Symposium on Biotechnology, Solar Energy Research Institute, Golden, CO, June 6, 1991. Published in Applied Biochemistry and Biotechnology, **34/35**, 515-526 (1992).

12 Ibid.

only the subprograms for each specific assessment are put into the computer memory (and are later automatically deleted), expanded memory or other special computer capabilities are not needed. The current version consists of a series of interactive menu-driven subprograms that run consecutively. Process-specific subprograms are automatically preselected when the process name is selected from the first menu. The completed program will include a user-accessible library of equipment types that are automatically scaled according to process flow rates, with appropriate cost factors for materials, year of construction, and specific process requirements. Procedures will be provided to incorporate alternative feedstock and conversion options and to link and integrate a series of conversion steps that can be selected from the library of subprograms. The purpose of the model is to provide rapid, reliable assessments of new process concepts.

1993 Accomplishments:

- Completed BCP1 model improvements to include the capability to display each process unit with clearly defined process streams, and completed modifications needed to assess processes where two or three consecutive biochemical or chemical conversions are required.

- Preparation of a report to provide an overview of the US chemical processes industries (CPI). The results should be useful in determining which process research areas should result in the most significant improvements in the utilization of renewable resources and in increased process energy efficiency and conservation.

- Completion of an energy-economic assessment of a specific bioprocess for the production of citric acid to be used as a feedstock for conversion to methyl methacrylate.

- Completion of a final report on the prior Catalysis and Biocatalysis Program for the period 1980-1991.

1994 Planned Activities:

The BCP1 model database subprograms will be extended for the technical and economic assessment of a large number of specific biological and chemical processes. In addition, more than twenty specific separation or conversion processes of direct interest to the BCTR Program will be assessed to determine potential energy and economic benefits. Results of model calculations will be verified by comparison with information available for operational or previously described industrial operations, to confirm the reliability of model results. A survey of industrial companies will be completed. The purpose of the survey effort is to obtain comments about the model, develop mutual interests and cooperation, and improve model validation and industrial applications.

Annual Technical Summary Report:

In the planning and implementation of research to improve energy efficiency, it is often necessary to determine if new proposed advances in biochemical or chemical technology would be expected to result in decreased energy consumption and potential cost advantages in the production of industrial chemicals. A computer model (designated BCP1) is being developed to assess proposed process concepts and anticipated energy-economic benefits.

The objective is to develop the BCP1 model and use it to determine the energy requirements and economics of biological and chemical processes and process improvements for the production of industrial chemicals. The goal is to support the DOE Advanced Industrial Concepts Division Catalysis/Biocatalysis

Program, by contributing to the technology base for chemical and biocatalyzed processes for large-scale production of industrial chemicals from renewable resources. Advantages of the BCP1 model (in comparison with alternative models) are that it can be used with any IBM-type computer, with no expanded or extended memory, is totally user-friendly, and can be readily adapted for multiple assessments of many types of biological or chemical processes. There is no limit to the database or subprogram size because the entire model can be operated from any number of disks and drives. The reason for this advantage is that only one subprogram is processed and in the computer memory at a time, with the values of variables passed from one subprogram to the next. It is more convenient (but not really necessary) to use a computer that operates at higher speed, e.g., 16 MHz, because of the minor time delays required to consecutively load each subprogram. Eventually, if the application is so extensive that more than one diskette is required, the model display will provide prompts to insert the next disk, or a hard disk can be used to provide uninterrupted program operations.

Commercial computer-aided design (CAD) programs, such as ASPEN, can be used for chemical process assessments; however, their primary purpose is to develop detailed process designs for specific chemical plants. To complete a study estimate of plant costs with moderate predictive accuracy, can cost thousands of dollars. Except for recent work on the BEST simulator (based on ASPEN) at NREL, CAD programs have not been satisfactorily adapted for bioprocess assessments. In any case, operational CAD programs are not normally available at moderate cost, are not very user-friendly, and require special computer capabilities. A 386 computer with 6-16 MB of expanded memory is normally a minimal requirement. In addition, as CAD simulators have been improved, computer requirements have been extended to accommodate larger databases and increased complexity, and this trend is expected to continue. BCP1 software is being developed to minimize or circumvent these factors that tend to limit applications for rapid, reliable energy and economic assessments of various process concepts, particularly for timely research applications.

A preliminary version of the BCP1 computer model was used previously to estimate fermentation costs for ethanol, and for a comparative study of an ethyl acetate process. A series of process reaction types (for example, specific fermentation processes and chemical processes such as hydrogenation, dehydration, esterification) are included in the BCP1 model for selection from menu lists, and the selected type is modified as appropriate for an assessment. For a specific reaction of each type, default values for numerous parameters and variables, such as stoichiometric factors, properties, rates, concentrations, materials costs, and economic values, are displayed and can be changed during the assessment run, or in the program, to create a new assessment. Mass and some energy balances are provided, and costs are computed on the basis of a factored estimate. A large number of known processes will be included and verified to ensure that cost estimate protocols are reliable. After any base-case estimate, each additional assessment (where numerous process variations can be incorporated) may be completed within a few minutes. Since only the subprograms for each specific assessment are put into the computer memory (and are later automatically deleted), expanded memory or other special computer capabilities are not needed. The current version consists of a series of interactive menu-driven subprograms that run consecutively. Process-specific subprograms are automatically preselected when the process name is selected from the first menu. The completed program will include subprograms that contain information on equipment types that may be automatically scaled according to process flow rates, with appropriate cost factors for materials, year of construction, and specific process requirements. Procedures will be provided to incorporate alternative feedstock and conversion options and to link and integrate a series of conversion steps that can be selected from the library of subprograms. The purpose of the model is to provide rapid, reliable assessments of new processes to evaluate potential energy or economic advantages.

Recent accomplishments (1993)

For some estimates it is desirable to include two (or more) sequential conversion steps in an overall process, as in the conversion of starch to sugar, which is then converted to ethanol, or another fermentation product. Program modifications have been completed to provide assessments of sequential processes of this type. For a process involving three conversion steps, five summary reports are produced: three for each independent conversion, one to integrate and combine the first and second steps, and the final summary where all three conversion steps are combined. Also, a series of subprograms has recently been developed to allow the user to include and display increased process detail as needed. These modifications can show each unit process separately, including operational features and process stream compositions. These features of the model have been included for several sample processes. At the present time, specific process applications and expansion of the database subprograms is being emphasized instead of further model development. The purpose is to increase the number of process types that can be assessed, and to use the current model for the evaluation of several proposed process advancements.

An energy-economic assessment of a specific bioprocess for the production of citric acid to be used as a feedstock for conversion to methyl methacrylate was completed using the BCP1 model for Prof. M.J. Antal, Jr (Hawaii Natural Energy Institute, University of Hawaii). In the proposed process, citric acid as a dilute fermentation stream would be dehydrated directly to methacrylate in supercritical water. These assessments resulted in a selling price estimate of .84 $/lb and energy consumption of 24.2 btu/lb for citric acid (anhydrous). For an aqueous solution of citric acid (180 g/l) to be used as feedstock for conversion to methacrylate, the corresponding estimates were: 0.29 $/lb and 5.4 btu/lb. The price and energy for the dilute solution are 35% and 22%, respectively, relative to pure citric acid. The actual cost for production is less because selling and associated costs would not apply to the intermediate citric acid, when the final product is methacrylic acid.

Other work included preparation of a report to provide an overview of the US chemical processes industries (CPI). Processes were selected from those for the fifty highest volume chemicals produced in the US in 1991.

Each summary description consists of four parts:

(i) Description of the market
(ii) Process description
(iii) Energy intensity
(iv) Environmental profile

This information is to provide assistance for project planning and implementation, in support of the objectives of the BCTR Program. The results should be useful in determining which process research areas should result in the most significant improvements in the utilization of renewable resources and in increased process energy efficiency and conservation. Also a final report on the previous Catalysis and Biocatalysis Program for the period 1980-1991 was prepared and published.

27. **Project Title:** Program Support to BCTR Computer Aided Catalyst Design Program

Principal Investigator: A. L. Tonkovich

Project Site: Battelle Pacific Northwest Laboratory

Description:

The purpose of this task is to assist DOE-CE/OIT/AICD in gathering information in support of the Catalystsis by Design (CACD) program. The emphasis is placed on characterizing the types of catalysts currently under investigation, and then assessing the potential impact of these catalysts on the production of the top 50 chemicals.

1993 Accomplishments

The project was initiated in mid-FY93. To date, we have conducted interviews with the CACD principal investigators to characterize the types of catalysts under investigation and how they are being studied (i.e., what assumptions and applicable uses). Research is also underway to characterize the limitations in the efficient production of the top 50 chemicals. Those chemicals that are manufactured via a catalyst identified in the CACD study are candidates for potential production energy savings rising from an improved catalyst performance.

1994 Planned Activities

Because of the late start on this project, it is anticipated that activities begun in FY93 will continue into the first quarter of FY94. It is further anticipated that carryover funding from FY93 will be sufficient to complete these activities. No direct follow-on activities are being proposed in FY94 at this time.

In a related arena, PNL has been working with the AIChE's Center for Waste Reduction Technologies (CWRT) to develop a scope of work for a planned CWRT RFP on chemical reaction engineering. The RFP will be used to solicit industry and academic research proposals dealing with waste reduction through improved reaction selectivity and yield. BCTR potentially has the opportunity to participate in this research through cost-sharing, either directly to CWRT or via PNL, which is a full member of CWRT.

Annual Technical Summary Report

New effort.

28. **Project Title:** BCTR Program Planning and Support

Principal Investigators: J. Young and L. Fassbender

Project Site: Battelle Pacific Northwest Laboratory

Description:

Pacific Northwest Laboratory (PNL) supports the DOE Advanced Industrial Concepts Division (AICD) in systems analysis for the Biological and Chemical Technology Research (BCTR) Program. Meeting the

diverse requirements of BCTR requires systems analysis activities to target the industry R&D needs with the potential for greatest impact.

1993 Accomplishments

The project was initiated in mid-FY93. To date, we have conducted interviews with the CACD principal investigators to characterize the types of catalysts under investigation and how they are being studied (i.e., what assumptions and applicable uses). Research is also underway to characterize the limitations in the efficient production of the top 50 chemicals. Those chemicals that are manufactured via a catalyst identified in the CACD study are candidates for potential production energy savings rising from an improved catalyst performance

1994 Planned Activities

BCTR Program Planning and Support

The specific activities to be conducted jointly by PNL, JPL, and NREL are as follows: Energy savings opportunities will identified for targeting better ways to produce high volume chemicals. Environmental impacts will be evaluated for existing and new production technologies based on a qualitative review of the process flows. Improved chemical and alternative biological routes to commodity chemicals via biomass, wastes, fossil feedstocks, carbon dioxide, or synthesis gas will be studied. This includes evaluation of alternative chemical intermediates that can be catalytically converted to or substitute for large volume organic chemicals and evaluation of the use of improved process technology such as biological, electrochemical, advanced separations, and reaction engineering.

Statistics will be provided for existing or alternative routes on capital cost, the generation of affluents, energy use, and other important factors affecting future market penetration. The results will include identification of strategic opportunities for improved or alternative chemicals production routes based on relative savings in energy use, effluent generation, and capital costs over conventional technology. The goal will be a set of recommended R&D targets to obtain significant energy savings by 2030 with reduced environmental impact and increased economic competitiveness. It is anticipated that the greatest amount of energy can be saved by improving production technology for the top 50 bulk commodity chemicals, although other process industries are possible targets as well.

The purpose of this task is to assist DOE-CE/OIT/AICD in gathering information in support of the Computer Aided Catalyst Design (CACD) program. The emphasis is placed on characterizing the types of catalysts currently under investigation, and then assessing the potential impact of these catalysts on the production of the top 50 chemicals.

Annual Technical Summary Report

None. Program support.

5.0 PROGRAM OUTPUT

5.1 Publications in Professional Journals, Books and Symposium Chapters, and DOE Technical Publications (by institution).

Allied Signal, Inc.

H. Zemel, J. F. Quinn and M. B Koch: On the Role of Water, Support, and Organic Solvent in the Operation of an Immobilized Enzyme Bioreactor. In "Opportunities with Industrial Enzymes", R. Heinemann and B. Wolnak, edit., p.66, (1992).

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L. Yang, T. J. Raeker and A. E. DePristo, "Surface Segregation in Bimetallic Clusters: Predictions using a Molecular Dynamics/Monte-Carlo Corrected Effective Medium Theory" Surf. Sci., 290, 195-205, (1993).

B. C. Khanra and T. S. King, "Studies of the Segregation-Dependence of Proton Knight Shifts in Ru-Cu Bimetallics," J. Phys. Chem., 97, 4164-4166, (1993).

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J. Tahir-Kheli and W. A. Goddard III, "Spinons and Holons in 1-D Three Band Hubbard Models for High T_c Superconductors," (in press).

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5.2 Patent Awards and Filings

California Institute of Technology

W. A. Goddard III, Cell Multipole Method (CMM), filed, (August, 1993).

National Renewable Energy Laboratory (NOT fully funded by BCTR, but global patent info may have some BCTR input)

P. F. Weaver and P. C. Maness, "Photoconversion of Gasified Organic Materials into Biologically Degradable Plastics," U.S. patent allowed, March 1993, global patent application filed.

5.3 Special Awards and Presentations

California Institute of Technology

"Enzyme Engineering for Unusual Environments: Biocatalysts for the Chemical Industry," First Biennial Lilly Biocatalysis Symposium, Eli Lilly, Indianapolis, Indiana, (April 16, 1993).

Pacific Northwest Laboratory

A. C. Hess, M. I. McCarthy, J. C. White, J. A. Anchell, J. B. Nicholas, M. R. and Thompson, "Ion Exchange and Acid Catalysis in Clays and Zeolites," Chemical Design Automation News, invited contribution, (August 1, 1993).

University of California at Berkeley

C. J. King, "Recovery of Carboxylic Acids with Functionalized Sorbents and Extractants," invited presentation at Gordon Research Conference on Reactive Polymers, Ion-Exchangers and Adsorbents, Newport RI, (August 25, 1993).

C. J. King, "Advances in Separation Techniques: Recovery of Polar Organics from Aqueous Solution," invited Plenary Lecture, 11th Intl. Congress of Chemical Engineering, Chemical Equipment Design and Automation (CHISA'93), Prague, Czech Republic, (August 30, 1993).

Clarence G. Gerhold Award in Separations Technology (First Recipient), American Institute of Chemical Engineers, (November 1992).

Centennial Medallion (one of 200 given), American Society for Engineering Education, (June 1993).

Dr. Klibanov was honored by the 1993 Arthur C. Cope Scholar Award from the American Chemical Society.

Dr. Klibanov was elected to the National Academy of Engineering for "Research in Enzyme and Protein Technology and Contributions to the Field of Biocatalysis in Nonaqueous Solvents."

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