Priorities in Biotechnology Research for International Development: Proceedings of a Workshop (1982)

Pages
273

Size
5 x 9

ISBN
0309328373

Board on Science and Technology for International Development; Office of International Affairs; National Research Council

PRIORITIES IN BIOTECHNOLOGY RESEARCH FOR INTERNATIONAL DEVELOPMENT

PROCEEDINGS OF A WORKSHOP

Washington, D.C. and
Berkeley Springs, West Virginia
July 26-30, 1982

Board on Science and Technology
for International Development
Office of International Affairs
National Research Council

NATIONAL ACADEMY PRESS
Washington, D.C. 1982

NOTICE: The project that is the subject of this report was approved by the Governing Board of the National Research Council, whose members are drawn from the councils of the National Academy of Sciences, the National Academy of Engineering, and the Institute of Medicine. The members of the committee responsible for the report were chosen for their special competences and with regard for appropriate balance.

This report has been reviewed by a group other than the authors according to procedures approved by a Report Review Committee consisting of members of the National Academy of Sciences, the National Academy of Engineering, and the Institute of Medicine.

The National Research Council was established by the National Academy of Sciences in 1916 to associate the broad community of science and technology with the Academy's purposes of furthering knowledge and of advising the federal government. The Council operates in accordance with general policies determined by the Academy under the authority of its congressional charter of 1863, which establishes the Academy as a private, nonprofit, self-governing membership corporation. The Council has become the principal operating agency of both the National Academy of Sciences and the National Academy of Engineering in the conduct of their services to the government, the public, and the scientific and engineering communities. It is administered jointly by both Academies and the Institute of Medicine. The National Academy of Engineering and the Institute of Medicine were established in 1964 and 1970, respectively, under the charter of the National Academy of Sciences.

These proceedings have been prepared by the Board on Science and Technology for International Development, Office of International Affairs, National Research Council, for the Bureau for Science and Technology, U.S. Agency for International Development, Washington, D.C., under Grant No. DAN-5538-G-SS-1023-00, Letter of Agreement No. 19.

Copies available from:

Board on Science and Technology
for International Development
National Research Council
2101 Constitution Avenue, N.W.
Washington, DC 20418 USA

NRC Advisory Panel

ROBERT H. BURRIS, Department of Biochemistry, University of Wisconsin, Madison, Wisconsin, USA, Chairman

JERRY J. CALLIS, U.S. Department of Agriculture, Plum Island Animal Disease Center, Greenport, New York, USA

WILLIAM C. DAVIS, Department of Veterinary Microbiology and Pathology, Washington State University, Pullman, Washington, USA

STANLEY FALKOW, Department of Medical Microbiology, Stanford University School of Medicine, Stanford, California, USA

ELMER L. GADEN, Department of Chemical Engineering, University of Virginia, Charlottesville, Virginia, USA

RALPH W.F. HARDY, Central Research and Development Department, DuPont Experimental Station, Wilmington, Delaware, USA

WILLIAM R. PRITCHARD, School of Veterinary Medicine, University of California, Davis, California, USA

JAMES SHEPARD, Department of Plant Pathology, Kansas State University, Manhattan, Kansas, USA

H. GARRISON WILKES, Department of Biology, University of Massachusetts, Boston, Massachusetts, USA

Speakers and Contributors

MARTIN ALEXANDER, Laboratory of Soil Microbiology, Department of Agronomy, Cornell University, Ithaca, New York

DAVID BALTIMORE, Nobel Laureate, Director, Whitehead Institute; Professor, Department of Biology, Massachusetts Institute of Technology

NYLE C. BRADY, Senior Assistant Administrator, Bureau for Science and Technology, U.S. Agency for International Development

HENRY R. BUNGAY, Department of Chemical and Environmental Engineering, Rensselaer Polytechnic Institute, Troy, New York

G. B. COLLINS, Agronomy Department, University of Kentucky, Lexington, Kentucky

WILLIAM C. DAVIS, Travis C. McGuire, and Lance E. Perryman, Department of Veterinary Microbiology and Pathology, College of Veterinary Medicine, Washington State University, Pullman, Washington

LOWELL LEWIS, Agriculture and University Services, Agricultural Experiment Station, University of California, Berkeley, California

M. PETER MCPHERSON, Administrator, U.S. Agency for International Development

M. S. SWAMINATHAN, Director General, International Rice Research Institute, Los Baños, Laguna, Philippines

KENNETH WARREN, Director for Health Sciences, Rockefeller Foundation, New York, New York

Preface

Recent research in cell biology, molecular genetics, recombinant DNA, and related fields appears to be laying the groundwork for important technological developments. The research areas range from those requiring very complex and expensive laboratory techniques and equipment to those that are within the capacity of relatively modest laboratories.

The resulting technologies are likely to have a major impact on a variety of economic activities involving food, chemistry, pharmaceuticals, and energy and environment. There are also likely to be significant industrial applications through new products and processes. Through direct participation, or through the impact of work done elsewhere, developing countries will feel the effect of these new developments in science and technology over the coming years.

In early 1982, the Bureau for Science and Technology of the U.S. Agency for International Development (AID), recognizing that important developments are taking place in biotechnology research, asked the Board on Science and Technology for International Development (BOSTID) of the National Research Council to convene a workshop as early as possible in 1982 to assess the implications of the "new biology" for its programs and to recommend priorities for support in biotechnological fields related to international development. This workshop was held July 26-30, 1982, in two stages.

For the first stage, an open meeting was convened July 26-27 at the National Academy of Sciences in Washington, D.C., to address the state of the art of the new biology. Overviews of developments in the fields of agriculture, health, and energy were presented, followed by panel discussions that included both U.S. and Third World participants. This session was attended by 175 workshop participants, observers, and members of the news media.

The second stage of the workshop, held July 27-30 at Coolfont Conference Center in Berkeley Springs, West Virginia, drew together 75 invited participants and observers in small working groups. The results of these deliberations, which take the form of recommended priorities for programs that might be undertaken by AID and other donor agencies in support of biotechnology activities related to agriculture, health, and energy and international development, are presented in these proceedings.

Participants in the opening session of this workshop were welcomed by Walter A. Rosenblith, foreign secretary of the National Academy of Sciences, and workshop chairman Robert H. Burris, Department of Biochemistry, University of Wisconsin. In addition, they heard brief remarks by M. Peter McPherson, administrator of the Agency for International Development, and Nyle C. Brady, senior assistant administrator, Bureau for Science and Technology, Agency for International Development. Academy President Frank Press opened the second day of the workshop.

Keynote addresses were given by Nobel Laureate David Baltimore, director of the Whitehead Institute and professor of biology at the Massachusetts Institute of Technology, on "Priorities in Biotechnology"; and M. S. Swaminathan, director general of the International Rice Research Institute, Los Baños, Philippines, who spoke on "Perspectives in Biotechnology Research from the Point of View of Third World Agriculture."

Contents

Summary of Recommendations and Conclusions

VACCINES

Infections are a major cause of human and animal morbidity and mortality in developing countries. Although poverty, chronic undernutrition, illiteracy, lack of medical care, and contaminated water and food all contribute to the spread of infectious organisms, immunization remains one of the most economical means of preventing specific diseases. Overall, vaccines offer immense benefit for comparatively low cost, a primary consideration for decision makers in developing countries.

This working group used three criteria to determine which animal and human diseases should receive priority for new or additional vaccine research and development funded by the U.S. Agency for International Development (AID) and other bilateral and multilateral donors. A fourth criterion was added which was specific either to human or animal diseases. The criteria considered for each disease are:

- Current vaccine status, based upon the availability of an effective, inexpensive, safe vaccine
- Feasibility of biotechnological approaches to developing a better, cheaper, and safer candidate vaccine within the next 5 years
- Current funding for vaccine research and development--whether available or likely to be available in sufficient amounts from sources other than AID (for example, World Health Organization, National Institutes of Health, pharmaceutical companies). This criterion was considered in relation to the relative importance of the disease in developing countries.
- Public health significance [for human diseases] as measured by incidence; prevalence; morbidity; mortality; or severity
- Economic losses [for animal diseases] in terms of livestock production or decrease in availability of animal protein.

The following sections identify eight human and four animal diseases as well as one zoonotic disease (affecting both human and animals), which have been selected and recommended as high priority for new funding.

Human Diseases

Rabies (Human). Rabies is a fatal disease that continues to be a serious problem in some developing countries. Although vaccination is part of the post-exposure treatment, current vaccines have undesirable, dangerous side effects. Research in this area should be directed toward:

- Development of a better, cheaper, and safer vaccine for pre- and post-exposure treatment. In the meantime, AID and other donors should encourage production of rabies vaccine in developing countries.
- Improvement in production techniques of current vaccine strains to lower costs
- Continued use of genetic engineering to clone the viral glycoprotein and test it as a vaccine
- Organic synthesis of peptides for vaccine development.

Dengue. Dengue fever and associated hemorrhagic syndromes are caused by four closely related viruses (designated dengue serotypes 1, 2, 3, and 4) that are transmitted chiefly by Aedes aegypti mosquitoes. The disease is highly endemic in tropical Asia and certain Pacific islands and endemic in Central and South America and the Caribbean region. An effective vaccine would have to confer immunity to all four virus serotypes. Research in this area should be directed toward:

- Development of vaccine based upon viral surface glycoproteins using molecular cloning.

Japanese Encephalitis. Large outbreaks of Japanese encephalitis have occurred in Southeast Asia, as well as epidemics during the last decade in Thailand, Burma, and India. Research in this area should be directed toward:

- Identification, characterization, and production by biotechnological techniques of the protective antigen of the Japanese encephalitis virus
- Studies of the efficacy of this glycoprotein product as a human vaccine.

Bacterial Respiratory Diseases. The primary bacterial respiratory diseases in man are caused by Streptococcus pneumoniae, Hemophilus influenzae Type B, and Bordetella pertussis. These bacteria are a major cause of morbidity worldwide in children under 5 years of age. Research in this area should be directed toward:

- Improvement of existing vaccines for pneumonia and pertussis by standard vaccine methods and the use of biotechnology
- Development of a vaccine for H. influenzae Type B using biotechnology; there are specific needs in the areas of immunopotentiation and carrier-hapten presentation.

Bacterial Enteric Diseases. These are a major cause of childhood mortality in large areas of the world where rehydration therapy is not provided promptly. Research in this area should be directed toward:

- Improvement of experimental vaccines for Campylobacter jejuni, Escherichia coli, Salmonella, Shigella, and Vibrio cholerae by standard methods.
- Development of defined, lethal deletion mutants of pathogenic enterobacteria. Such mutants serve as excellent immunogens because they do not need to be inactivated and yet are innocuous because they cannot replicate.
- Development of bioengineered protein vaccines as has been done for enterotoxigenic E. coli diseases of neonatal pigs and calves.

Chlamydial Infections. A widespread public health problem in many developing countries, chlamydial infections are the leading cause of blindness (trachoma). Chlamydia spp. resemble bacteria but reproduce only within living animal cells. Infection is spread by flies, through contact with ocular discharges, or through sexual contact. Adults may develop urethritis or conjunctival scarring, while babies can develop pneumonia or severe conjunctivitis.

Chemotherapy is available to treat individual cases but is cumbersome and expensive to administer. Post-infection immunity has been shown in humans, and some investigators now believe that immunity is cell-mediated. No vaccine is available, although a monoclonal antibody diagnostic test for Chlamydia trachomatis may soon be prepared commercially. Research in this area should be directed toward:

- Basic identification and characterization of candidate immunogenic antigens of Chlamydia trachomatis and C. psitticae
- Production of candidate protective antigens using biotechnological approaches.

Malaria. Malaria is the most important infectious disease in the world today. No vaccine is currently available. Research in this area should be directed toward:

- Identification, characterization, and molecular cloning of Plasmodium falciparum gamete, erythrocyte, and sporozoite antigens, which are candidate protective immunogens, using biotechnological methods
- Basic research on P. malarie, P. ovale, and P. vivax for identification of specific antigens.

Leishmaniasis. Kala-azar (visceral leishmaniasis) and cutaneous and mucocutaneous leishmaniasis are sandfly-transmitted protozoan diseases with grossly underestimated public health importance in South America, Africa, and the Middle East. Research in this area should be directed toward:

- Identification and cloning of antigens of Leishmania varieties.

In addition, the working group recommended that:

- A targeted research effort aimed at vaccine development utilizing biotechnology be funded for each of the above high-priority diseases. Such a research effort would involve: (1) identification and characterization of immunogenic antigens, (2) synthesis and production of such antigens through biotechnology, and (3) formulation of a candidate vaccine suitable for testing.

- This targeted research be conducted in collaboration with scientists from developing countries. A major advantage of biotechnology and genetic engineering is that they do not require highly sophisticated laboratory equipment; for the most part, well-equipped, clinical laboratory facilities will suffice. However, such an effort will require an interdisciplinary team, reliable laboratory reagents, and appropriate animal facilities for testing. Few developing countries possess the capability to produce promising laboratory antigens in quantities sufficient for early clinical testing and subsequent mass production. In addition, reliable quality control for vaccine production is lacking in many parts of the world.

- Training be an integral part of the collaborative research effort. The key requirement for research and development of

new vaccines is trained, experienced scientists. Both long-term graduate work for developing country scientists in developed country universities and short-term training in any appropriate site are equally important for the transfer of knowledge and techniques.

Animal Diseases

Neonatal Diarrhea. This disease complex affects swine and cattle and is caused by bacterial as well as viral agents. Research in this area should be directed toward:

- Development of vaccines for enterobacterial and viral diarrhea agents. Enterobacterial agents should be emphasized given the current research support for viral diarrheas. Biotechnological approaches promise valuable new immunogens in each of these categories and concomitant new antigens for use in diagnostic tests.

Bacterial Respiratory Disease. This disease complex is the single most important cause of morbidity, mortality, and economic loss in all livestock in the developed and developing world. Research in this area should be directed toward:

- Identification and characterization of the significant immunogenic antigens of Pasteurella hemolytica, P. multocida, and Bordetella bronchiseptica
- Production of such immunogens by bio- or organic synthesis.

African Swine Fever (ASF). ASF is endemic in several African countries, Spain, and Portugal, and results in severe economic losses. Research in this area should be directed toward:

- Vaccine development using biotechnology. It is conceivable that vaccines can be developed, but additional information is required on the immunology of the disease, the biology and molecular properties of the virus, and its etiology.

Hemotropic Diseases of Animals (Specifically Babesiosis and Anaplasmosis). Hemotropic diseases of livestock are major impediments to the economic production of meat, milk, and fiber in many parts of the developing world. Research in this area should be directed toward:

- Isolation, characterization, and production of protective antigens using biotechnological approaches.

Zoonotic Disease

Tuberculosis (Mycobacteria). Tuberculosis (TB) is highly prevalent worldwide. The major economic impact of this disease on livestock production is compounded by its zoonotic implications. Research on this disease should be directed toward:

- Development of improved diagnostic tools to distinguish in humans BCG (Bacille Calmette Guérin) vaccine reactions from those resulting from infection
- Improved production of TB-specific antigens, including purification of PPD (purified protein derivative)
- Assessment of the efficacy of BCG vaccine currently in use
- Development of an effective vaccine, including bio- or organic synthesis of the immunogen, that can be used in areas of high incidence (especially in animals) in developing countries. Such a vaccine is desirable but not considered a near-term possibility.

ANIMAL PRODUCTION

Worldwide, animal products contribute over 56 million tons of edible protein and over 1 billion megacalories of energy annually. With its high biological value, this protein is equivalent to more than 50 percent of the protein produced from all cereals, yet the proportion of research funds currently going to animal production is less than 15 percent of the total for agriculture. The role of biotechnology in animal production is largely one of supplying tools to assist animal producers with breeding, health, and nutrition.

This working group identified the following research goals within four broad areas: genetics, reproduction, nutrition, and health.

Genetics

Manipulation of genetic material and its exchange among countries have become important means of upgrading animal production worldwide. Genetic research goals deserving the highest priority include:

- Demonstration of germ plasm transfer (embryos and semen) without risk of transmitting disease
- Identification and transfer of germ plasm associated with resistance to disease and health problems
- Determination of genetic markers to identify or increase superior germ plasm from indigenous species. Using monoclonal antibody technology will speed up the process of genetic selection.

- Identification of genetic factors related to production and, particularly, to puberty, morbidity, and proliferance
- Storage (using cryopreservation and similar techniques) and classification of germ plasm on a worldwide species basis. Germ plasm banks representing exotic and endangered species should be established as soon as possible in the different ecological zones.

Reproduction

The following represent immediate research needs in the area of animal reproduction:

- Use of ova transfer as a tool for improving livestock production within the shortest time possible and for controlling disease. Aspects of this research are cryopreservation and transportation, twinning and single birth, superovulation, maturation of oocytes in vitro, oocyte fusion and sexing, cloning, unisex parentage, and interspecies transfer.
- Reduction of embryo wastage in superovulating species
- Development and use of alternate methods for semen preservation and handling in artificial insemination as well as study of seminal physiology, fertility, and aspects of disease control through artificial insemination
- Use of monoclonal antibodies for improving disease diagnostic techniques, vaccine production, ova transfer, and semen and embryo sexing
- Study of testicular hypertrophy
- Improvement of reproductive management in developing countries.

Nutrition

Improving the nutrition of indigenous animals in developing countries is of great importance. Because many developing countries import animal feed, methods for greater utilization of local biomass and by-products for animal feed must be developed. It would thus be useful to integrate the research efforts on animal production with those of agronomists, range scientists, irrigation specialists, soil scientists, and economists.

Important research areas in nutrition include:

- Natural range management in tropical areas

- Methods to identify chemical inhibitors in plants to permit more efficient use of indigenous fodder species
- New or more effective uses of local biomass and feedstuff residues such as aquatic weeds, cereal stalks, and grain milling by-products and ensilage (particularly by-products of biomass energy projects such as the leaves and stems of trees and shrubs, as well as stillage and fermentation residues). The entire plant must be considered for human and animal use. Plant breeding programs must consider the effects of a plant's lignin, silica, and tannin content on its value as an animal feed and an energy source.
- Methods of evaluating plant cell usability by different species
- Feeding strategies for local areas. Through knowledge of feeding behavior of animals, the best complementarity of animal and plant ecosystems for most efficient use can be identified as well as constraints within ecosystems such as chemical inhibitors that affect utilization of plants by animals.
- Needs for a balanced diet to improve overall nutritional efficiency. Projects in this area are well suited to a multidisciplinary approach.

Health

Animal losses from disease are a major concern of livestock producers in developing countries. Based on world priorities, specific diseases that merit additional research include:

- Parasitic diseases: babesiosis and anaplasmosis, theileriosis (East Coast fever), and trypanosomiasis (number one priority in Africa)
- Intestinal parasites, including flukes
- Viral diseases: African swine fever, foot-and-mouth disease, goat catarrhal fever, Gumboro disease in poultry, hog cholera, rabies, Rift Valley fever, and rinderpest
- Bacterial diseases: brucellosis, buffalo neonatal polyarthritis (important in Asia), contagious bovine pleuropneumonia, leptospirosis, mastitis, streptothricosis (important in West Africa), and tuberculosis.

Two general recommendations were also made:

- It is recommended that AID and other donors give priority in support to:

-- Verification of the ability to transfer embryos and semen between geographic regions without risk of transmitting disease
-- Establishment of embryo replication capabilities in developing countries
-- Use of genetic markers for animal production using monoclonal antibody techniques.

- Because few research institutions in developing countries have begun to use new biotechnological procedures, it is recommended that a careful process of identifying the most useful and productive technologies and related manpower and training needs for local requirements be undertaken. This will involve:

  -- Workshops on priorities and implementation strategies
  -- Participation in conferences by prospective researchers and trainees
  -- Technician training through short courses
  -- Longer term research manpower development at the graduate level.

MONOCLONAL ANTIBODIES

Antibodies, protein molecules produced by certain cells in the body, are a basic constituent of animal and human disease-fighting immune systems. In 1975, a new era in immunology was launched with the discovery of the hybridoma technique, a method for creating pure and uniform antibodies against a specific target. Application of this technology has provided a breakthrough in the methods available for analysis of the antigenic composition of microorganisms, for rapid diagnosis, and to aid in the development of vaccines. The technology affords an opportunity to make important advances in understanding the diseases of humans, animals, and plants with greater specificity, speed, and at reduced cost. The technique itself is relatively simple and straightforward to use and can be readily developed and made available for use in developing countries.

Human Health

Many diseases still lack adequate or effective diagnostic reagents, resulting in continued high morbidity and mortality among populations. Some of these diseases are found worldwide while others are specific to developing countries.

- It is therefore recommended that means be provided to accelerate and expand the capability of developing country institutions to prepare or use monoclonal antibodies as diagnostic reagents. Priority should be given to developing antibodies for use in diagnosis or detection of the following diseases or pathogens:

  -- Bacterial: Hemophilus, Mycobacterium tuberculosis, Pneumococcus, Streptococcus, E. coli, Salmonella, Shigella, Campylobacter jejuni
  -- Metazoan: cysticercosis/hydatidosis
  -- Protozoan: ascariasis, amebiasis, echinococcosis, filariasis, malaria, schistosomiasis, toxoplasmosis
  -- Viral: equine encephalitis, German measles, hepatitis, hepatitis B virus, herpes II, influenza, rabies.

Among all the medically important viruses, hepatitis B virus (HBV) can be singled out as one of the most important to mankind. In many developing countries of Asia and Africa, this virus is present in up to 60 percent of the population and may lead to the development of viral hepatitis and hepatocellular carcinoma. Thus control of this disease is a goal of many developing countries.

- It is therefore recommended that high priority be given to a feasibility study of the establishment of two monoclonal antibody centers, one in Asia and one in Africa. As one of their major goals, these centers should seek to produce monoclonal antibodies against the HBV antigens for use in study of the epidemiology of HBV infection. Once developed, these centers should have the capability of producing monoclonal antibodies to other disease-causing organisms and should serve as centers for maintenance and distribution of hybridoma cell lines and diagnostic antibody reagents. The centers also should serve as sites for wet laboratory training courses.

Animal Health

As with human disease, diagnostic reagents do not exist for many animal diseases, or, if available, are inadequate for accurately identifying infectious agents responsible for disease outbreaks.

- It is recommended that a program involving a network of institutions be developed to prepare antibodies for use as diagnostic tests and epidemiological studies. Priority should be given to making antibodies used in the diagnosis or detection of the following diseases or pathogens:

-- Bacterial: Brucella, E. coli (causing neonatal diarrhea), Mycobacterium tuberculosis, Salmonella
-- Metazoan: cysticercosis/hydatidosis, fascioliasis, schistosomiasis
-- Protozoan: babesiosis (bovine), coccidiosis (avian, ruminants)
-- Rickettsial: anaplasmosis (bovine)
-- Viral: African swine fever, atrophic rhinitis, bluetongue (bovine, caprine, ovine), bovine paralytic rabies, bovine viral diarrhea, caprine arthritis, encephalitis, equine infectious anemia, foot-and-mouth disease (bovine, swine), infectious bovine rhinotracheitis, Marek's disease (avian), Newcastle disease (avian), pleuropneumonia.

- Because few typing reagents exist for the development of selective breeding for increased resistance to disease, it is recommended that a full set of monoclonal antibodies be made for each functional polymorphic, antigenically distinguishable gene product on white blood cells. This includes gene products of the major histocompatibility gene complex, gene products that define T and B cell differentiation antigens, and gene products that define antigens common to both T and B cells.

Plant Health

High-quality diagnostic antisera capable of identifying a wide variety of viral, bacterial, or fungal pathogens are not currently available for many of the plant diseases that affect the world's important food and fiber crops. Furthermore, the epidemiology of many of these agents has not been investigated. Failure to diagnose these pathogens properly in important food crops leads to significant decreases in agricultural productivity. Many of these diseases are found worldwide and result in significant crop losses.

- It is therefore recommended that monoclonal antibody technology be utilized to produce antibodies for use in diagnostic and epidemiological studies of important viral and bacterial plant diseases. Priorities should be given to developing antibodies to the following disease agents:

-- Rice: rice dwarf, rice grassy stunt, rice ragged stunt, rice tungro
-- Maize: maize chlorotic leaf spot, maize rough dwarf, maize streak

-- Cassava: cassava mosaic virus
-- Citrus: citrus tristeza virus, Spiroplasma citri, Xanthomonas citri
-- Potato: potato viruses M, S, X, and Y; potato leaf roll
-- Fruit trees: Prunus necrotic ring spot, apple mosaic, prune dwarf, tobacco streak, tomato ring spot, tobacco ring spot viruses.

Other Recommendations

- It is recommended that AID or another technical assistance agency establish international working teams to ascertain the level at which each of the interested developing countries is prepared to use monoclonal antibody technology for diagnostic purposes.

It would be useful to employ the "Request for Proposal" (RFP) system commonly used by the National Institutes of Health in each of the subject countries as a means of identifying centers with appropriate facilities and the desire to exploit this new technology.

- To identify and assess needs for implementing individual programs that incorporate training, laboratory "set-up," and performance, it is recommended that AID or other donors establish planning grants, perhaps in reference to the RFPs. The donors would be responsible for determining the source and availability of existing diagnostic reagents or those that will soon be available. Priorities for support should be (1) diagnostics, (2) reagents for genetic and epidemiological studies, and (3) therapeutics.

ENERGY

Energy derived from biomass already contributes significantly to civilization. Biomass, in the form of firewood, agricultural residues, and animal dung, is the principal energy source in many countries, and even highly developed nations use wood extensively to heat homes and generate power.

This working group has identified biotechnologies with substantial potential for use in developing countries, keeping in mind institutional constraints, environmental problems, and needs for trained manpower that may be encountered. Specific recommendations have been made both for short-range approaches to exploiting biomass resources and for more complicated technologies that may reach fruition more slowly. All these recommendations emphasize a

systematic development of any biomass-related program, with careful economic assessment at each step.

Selecting and Analyzing the Applications of Biotechnology to Energy Production

- It is recommended that modeling techniques be used to evaluate options for biotechnology in developing countries. Such techniques could include engineering and economic analyses and energy material balances, as well as an analysis of the macroeconomic aspects such as effects on food production and food prices and even balance of payments.

- It is recommended that technologies or processes that generate multiple products from biomass and increase rural employment or improve village self-sufficiency be emphasized. Processes that yield both fuel and food or feed should receive priority over those with single outputs.

- It is recommended that simple, inexpensive diagnostic techniques be developed for monitoring biological conversion. These might include the use of monoclonal antibodies to identify viable organisms in a bioreactor such as a biogas unit or an alcohol fermenter.

Improved Utilization of Wood and Other Lignocellulosic Materials

- It is recommended that the conversion of lignocellulosic biomass into fuels and chemicals be studied at the pilot project level, preferably as a collaborative effort among organizations with experience in this area.

Utilization of Polysaccharide (Principally Starchy) Materials

It is recommended that:

- Improved bioreactor designs be developed for important fermentation processes such as ethanol and fuel gas production--for example, columns or systems using immobilized microorganisms and continuous feed. The potential for lowering capital requirements and production costs is significant.

- Advanced separation and purification processes be applied to biomass processing, which could mean increased energy efficiency over that obtained using traditional processes such as distillation. Application of these processes could also result in reduced energy consumption and increased availability of by-products with fuel value such as bagasse.

- Improved saccharification processes be applied to the conversion of starchy and inulin-rich biomass into ethanol. Examples of these processes are: (1) low-temperature saccharification, (2) simultaneous saccharification and fermentation, and (3) introduction of genes for amylase production.

- Environmental control technology for bioprocessing of wastes be improved, emphasizing processes that coproduce fuel gas, organic chemicals, and other saleable compounds. For example, improvements in anaerobic digestion of stillage could lower capital requirements, reduce pollution problems, and generate revenues from sale of coproducts, as well as lower the costs of producing ethanol, microbial protein, and other products.

- Suitable methods for biomass storage be devised to decrease the significant preprocessing losses found in current field practice for many crops such as cassava. New or improved methods could reduce feedstock cost, improve processing efficiency, and minimize transportation losses of biomass.

Microbiological Research and Development

- It is recommended that an international network be established to screen potential species for fuel and chemical production, and that a committee be established to elaborate a more complete screening inventory as well as a set of guidelines for executing the screening and processing of the results.

- Because most microbiological processes are single-species systems, it is recommended that mixed culture systems and their interactions be studied; for example, anaerobic holocellulose hydrolyzers, acid formers, and methanogens; thermophilic lignocellulosic biodegraders; and nitrogen-fixing algal and bacterial systems.

By-products, Coproduction, and Diesel Fuel Substitutes, Including Environmental Control Technology

- It is recommended that a more balanced approach be taken to fuel production, emphasizing the development of processes for the production of possible diesel fuel substitutes, such as alkanes, butanol, and vegetable oils. Biomass sources for diesel fuel production may include woody biomass from which turpentine is recovered, oilseeds from which fuels may be extracted, selected aquatic biomass, and oil-producing microorganisms. Integral to this emphasis on by-product utilization are effective means for environmental control.

Institutional Interaction and Training

- To stimulate the use of biotechnology for energy production in developing countries, it is recommended that adequate curricula be developed for in-country training and that overseas degree and nondegree training continue.

BIOLOGICAL NITROGEN FIXATION

Nitrogen is frequently the limiting nutrient in agricultural productivity. Although 78 percent of the earth's atmosphere is composed of nitrogen gas (N_2), plants are unable to use it in this form. The nitrogen must first be "fixed," that is, combined with other elements such as hydrogen, carbon, or oxygen before it can be assimilated by higher plants.

The ability to fix gaseous nitrogen is restricted to certain prokaryotes (cyanobacteria, actinomycetes, bacteria) that contain the enzyme nitrogenase. While a number of these organisms fix atmospheric nitrogen in the free-living state, some form symbiotic associations with higher plants. Examples of the latter include the legume-*Rhizobium*, nonlegume-actinorhiza (*Alnus*, *Casuarina*), and nonlegume-cyanobacteria (*Azolla*) symbioses.

Based on a discussion of various research opportunities in biological nitrogen fixation, this working group identified a number of goals as warranting support by AID and other donors. Recommended areas for priority are listed below; indicated in parenthesis is an estimated time scale for a well-organized research effort to achieve significant results.

- It is recommended that AID and other donors give the highest priority to support of research designed to optimize nitrogen fixation by (1) cyanobacteria and the Azolla system in

association with rice (3-10 years), and (2) the legume-Rhizobium symbiosis. The latter requires developing stress-tolerant strains of Rhizobium spp. (5-10 years), selecting legumes suitable for local conditions (5+ years), and developing techniques for producing effective strains of Rhizobium spp. that do not persist in the soil long enough to compete with more desirable rhizobia added later (5 years).

- It is recommended that AID and other donors also recognize the importance of research directed toward the following goals:

  -- Development of the trait in legumes that allows them to use fixed nitrogen simultaneously with soil nitrogen (time scale for productive research: 10-20 years)
  -- Development of improved technology for production and use of legume inoculants (3-5 years)
  -- Development of fast-growing, nitrogen-fixing trees (10+ years)
  -- Evaluation of grass species and associated bacteria for their ability to promote nitrogen fixation and accumulation of dry matter (10-15 years).

PLANT CELL AND TISSUE CULTURE

Research efforts in plant cell and tissue culture have increased dramatically worldwide in recent years including efforts in developing nations.

Plant propagation using shoot tip culture is the most advanced area in plant cell and tissue culture and is currently being used to propagate elite genotypes of several plant species in a number of countries. Propagation using plant tissue culture can have a direct effect on crop improvement by more rapid and efficient propagation of certain species, and it can have an indirect effect by elimination of disease in other species. This technology is uniquely suited to solving certain problems in agriculture and forestry and was highlighted by the working group as the area most likely to make a near-term contribution to economic development in many developing countries.

This working group identified eight research areas as prospects for near-term, mid-term, and long-term applications of plant cell and tissue culture techniques, respectively:

- Near-term (many new applications possible now or within 5 years)
  -- Clonal propagation

-- Disease elimination
-- Germ plasm exchange
-- Gene transfer by wide hybridization

- Mid-term (some applications to plant improvement possible within the next 5-10 years)
  -- Variant selection (including somaclonal selection)
  -- Production of haploids

- Long-term (plant improvement applications unlikely for at least 10-15 years)
  -- Secondary natural products
  -- Molecular genetic engineering in plants.

The group made a conscious decision not to recommend research on specific plants, feeling that such judgment can only be made locally by people most familiar with the problems and needs. It was felt more appropriate to recommend guidelines to be used in evaluating proposals for applying plant cell and tissue culture to particular problems.

- It is recommended that development assistance agencies evaluating research proposals in the area of plant cell and tissue culture:

  -- Give highest priority to proposals that include provisions ensuring that the products of tissue culture technology reach farmers and consumers. This generally requires programs for testing and evaluating new products and capabilities for commercial seed or stock production. Proposals that will result in immediate application should be given special attention.

  -- Give high priority to proposals on well-defined plant improvement problems. For a given problem, proposals to use the most feasible tissue culture methods should be given preference over those proposing to use methods that need more development. Conversely, low priority should be given to proposals to learn techniques using model plants such as tobacco; often such projects have never progressed from tobacco to other important plants.

  -- Give high priority to proposals that will permit the near-term extension of plant cell and tissue culture techniques to plant improvement problems using any or all of the following: clonal propagation, disease elimination, germ plasm exchange, or gene transfer by wide hybridization using embryo culture. The research

goals of such proposals will include the development of procedures for efficient clonal propagation and regeneration of elite genotypes, preferably by adaptation of known procedures for the species. When procedures for the species have not been developed, careful judgments should first be made based on success with related species regarding the likelihood of near-term success.

-- Require that proposals to begin work in plant cell and tissue culture include a thorough evaluation of the minimal infrastructure requirements and specify a plan for removing constraints. Granting agencies should be prepared to provide funds for infrastructure needs when necessary.

- It is also recommended that:

  -- Efforts be made to provide training for personnel from developing countries in laboratories of scientists willing to engage in long-term collaboration. To the maximum extent possible, opportunities for training in-country by visiting scientists are preferable to training abroad.

  -- Consideration be given to establishing regular scientific exchanges at all levels for developing country scientists engaged in plant cell and tissue culture research. Regional, problem-oriented workshops and newsletters directed toward particular applications of the technology should be given special attention.

  -- Private sector involvement in the application of plant cell and tissue culture to problems in developing countries be encouraged. Private firms may be able to provide short-term training and could be contracted to do research and development work on problems of special importance to developing countries.

  -- Efforts to establish tissue culture laboratories begin small, with preference given to institutional settings that are amenable to the eventual development of strong interdisciplinary teams of plant scientists.

Introduction

Genetic selection, manipulation, and alteration have been integral features of agriculture and food processing for centuries, and the rate of change of these techniques has been understandably slow. In the past 30 years, however, powerful new technologies based on research in molecular genetics have greatly increased possibilities for manipulating the inherited characteristics of plants, animals, and microorganisms. Recent scientific discoveries in immunology, recombinant DNA, and cell culture—and the potential they hold for significant advances in agriculture, health, and energy--have impressed scientists, world leaders, investors, and the public in general. Media coverage, devoted to such topics as interferon and the meteoric rise of genetic engineering firms, has created expectations of miracle drugs, new crops, and new technologies such as techniques for transferring nitrogen-fixing genes to nonleguminous plants (for example, rice and corn), or replacing abnormal genes with normal ones. Work is progressing on these and other topics, but at a more measured pace than the public has been led to believe.

Developing countries have also been caught up in the euphoria surrounding the so-called "biotechnology revolution." The basic development goals of most Third World countries include increasing food production, improving public health, and making available increased amounts of energy for domestic and industrial purposes. Compared with many traditional development programs, the anticipated results from biotechnology appear to be cost-effective and within the time frame and general manpower requirements of most developing countries.

While some commercial applications of biotechnology, including genetic engineering, monoclonal antibodies, and new plant and animal technologies, are now emerging from current research, many basic research issues remain. Also, since most of the research conducted thus far has been in developed countries and on problems of interest to them, problems relating to developing countries require special emphasis.

OBJECTIVES OF THIS WORKSHOP

In response to worldwide interest in the potential of biotechnology, and at the request of the U.S. Agency for International Development (AID), the Board on Science and Technology for International Development (BOSTID) convened a workshop in Washington, D.C., and Berkeley Springs, West Virginia, July 26-30, 1982. Scientists and policy-level planners from both the United States and a large number of developing countries were brought together to review the state of the art in their respective disciplines and to recommend priorities in biotechnology research for international programs in agriculture, health, and energy.

Biotechnology encompasses many areas, for example, enzyme technologies, microbial applications, immobilized cells and enzymes, cell culture, genetic engineering, immunology, and biomass technologies. However, the time frame for application to developing countries, logistical considerations, and appropriateness for AID's and other donors' development objectives limited the topics for consideration to the following:

- Vaccines
- Animal production
- Monoclonal antibodies
- Energy
- Biological nitrogen fixation
- Plant cell and tissue culture.

Results of research in these six topics have potential for wide application. Within varying degrees, breakthroughs may occur in these research topics during the next 5-10 years.

ORGANIZATION OF THE WORKSHOP

An advisory panel appointed by the National Research Council assisted BOSTID in the selection of topics and identification of U.S. and Third World participants. Approximately 60 scientists (35 from 20 developing countries and 25 from the United States—see Appendix) took part in the workshop.

Plenary sessions open to the public were held the first two days of the workshop in Washington, D.C. Major addresses were given at the opening session by Nobel Laureate David Baltimore, Massachusetts Institute of Technology, and M. S. Swaminathan, director general, International Rice Research Institute, the Philippines (see Part One). Lowell Lewis, University of California, Berkeley, presented an overview of biotechnology's role in agriculture, which was followed by a panel discussion by agricultural experts from

developing countries (see Appendix for list of panel members). Kenneth Warren, Rockefeller Foundation, and Henry R. Bungay, Rensselaer Polytechnic Institute, presented similar overviews for health and energy, respectively, which were also followed by panel discussions.

In addition to the overviews by Lewis, Bungay, and Warren, BOSTID commissioned the following background papers: William C. Davis and colleagues, monoclonal antibodies; Martin Alexander, biological nitrogen fixation; and G. B. Collins, plant cell and tissue culture (see Part Three).

Working groups (see Appendix), formed to address the six research topics, then met in closed sessions at Coolfont Conference Center in Berkeley Springs, West Virginia, for three days. During the final half-day plenary session, the working groups presented their summary recommendations.

Generally, the working groups first identified problems common to developing countries that might be solved by the technology in question. They were then asked to determine the priorities for research, including specific examples where possible, the likely time scale for applications, requirements for special equipment or facilities, the implications for manpower and training, the level of effort required, and the possible costs. In determining priorities, the groups considered research topics that are not receiving very much support and generally gave lower priority to topics for which support is high (for example, development of a malaria vaccine). Working groups were also asked to consider possible institutional arrangements for undertaking the suggested research. The reports of the working groups appear in Part Two.

Two evening sessions of the workshop were devoted to crosscutting issues, and the Time/Life film, "Life: Patent Pending," was shown. Iver P. Cooper, an attorney in Washington, D.C., discussed some of the legal aspects of biotechnology, and Thomas C. O'Brien, National Bureau of Standards (NBS), presented NBS goals for anticipating the standards and measurements required by the biotechnology industry. Christian Orrego, Massachusetts Institute of Technology, addressed the working groups on the institutional aspects of biotechnology, with emphasis on communications, training, and collaborative research.

PART ONE

Opening Remarks and Keynote Addresses

Opening Remarks

M. PETER McPHERSON

On behalf of the Agency for International Development, it is my great pleasure to welcome you to this workshop.

Biotechnology holds enormous promise for helping to fulfill some of humanity's most fundamental needs--from increasing food and energy supplies to improving health care. However, theoretical potential is one thing; translating that potential into practice is another. It is my hope that this important workshop can help to bridge this gap.

Clearly, the challenge of applying innovative scientific approaches to developing country problems will require the talents of many individuals and institutions. Therefore, the Agency for International Development has asked the National Academy of Sciences to convene this workshop as a means of reaching out to the scientific community at large, both in the United States and abroad. I believe this collaborative undertaking serves two essential and closely related purposes.

First, such efforts are indispensable for assuring that the research agenda reflects developing country needs and is not confined to academically interesting problems or problems with the highest monetary impact for the industrialized nations. Unless the emerging research agenda is broadened to include issues of particular concern to the developing world, technology transfer and capacity building will become little more than slogans without substance. Consequently, AID--and the other donor agencies represented here--are looking to this workshop for help in defining appropriate avenues for the development and application of biotechnologies overseas.

Second, by combining our forces through undertakings like this, the international scientific and development communities can help lay the foundations for genuine self-reliance throughout the developing world. By this, I do not mean self-sufficiency--for what nation can be self-sufficient in the twentieth century? Nor do I mean the kind of self-reliance that enables a nation to survive from day to day, but at a level that approaches mere subsistence. Rather, I mean self-reliance at the highest level of productivity that is permitted

by the current state of knowledge. This kind of self-reliance rests, in turn, on having strong scientific capabilities within each nation, able to modify and adapt new or improved technologies to local conditions.

I can assure you that AID is fully committed to making this goal a reality. Today's workshop represents one important facet of our commitment. I wish you well in your own efforts over the next few days to link innovative biotechnical approaches to developing country problems. We shall await with interest your findings and recommendations.

Opening Remarks

NYLE C. BRADY

I am honored to join you for this conference, which represents the growing cooperation between the U.S. Agency for International Development and the National Academy of Sciences. It also reflects, in turn, the strengthening of AID's ties to the broader U.S. and overseas scientific community.

AID'S RECORD OF SUPPORT FOR SCIENCE AND TECHNOLOGY

AID's emphasis on science and technology for development goes back many years. In the agency's earliest days, under the Point IV Program, technical assistance was the focal point of the U.S. foreign aid effort. The development of effective means of family planning has been supported in large measure by AID, together with the fight against smallpox, malaria, and other infectious diseases. AID has also contributed significantly to development of the oral rehydration method for controlling diarrheal diseases, perhaps the major killer of children under age five in developing nations.

AID was a major contributor to the development and adoption of the high-yielding wheat and rice varieties--known as "Green Revolution." This support has taken several forms. AID has continued to contribute to the international agricultural research centers, providing 25 percent of the centers' operating expenses each year.

In addition, AID has provided catalytic support for the kind of agricultural research and institution building required at the national level for this new technology to take hold. In post-independence India, for example, AID financing and leadership was instrumental in helping to marshall a 20-year institution-building effort that led to the establishment of nine new agricultural universities. These new institutions integrated research, teaching, and extension and have been widely accredited with underpinning India's phenomenal agricultural advances.

CURRENT AID EFFORTS TO STRENGTHEN
THE ROLE OF SCIENCE AND TECHNOLOGY

Within this historical context, and in light of today's urgent need, the administrator of AID is endeavoring to establish an even stronger and more central role for science and technology within AID's overall assistance efforts.

Significant internal changes have been made that include the establishment of sector councils in the technical areas of priority: agriculture, nutrition, health, population, energy and natural resources, human resources and engineering. The sector councils concentrate on ways to improve the agency's technical competence and program quality in each area.

Four agency directorates have been established to work with the councils and provide a focus for the agency's work. The directorates are headed by internationally recognized leaders: Jack Robins, former dean of the College of Agriculture at Washington State University, directs Food and Agriculture; Jack Vanderryn, a well-trained scientist, who was formerly with the Department of Energy, heads Energy and Natural Resources; Ruth Zagorin, an experienced social scientist and administrator who served with the International Development Research Centre in Canada and then with the U.S. Department of Agriculture leads the Human Resources Directorate; and Jarrett Clinton, a former AID officer who was quite successful in Indonesia and was, most recently, at the Population Council, presides over Health and Population. These individuals not only have line authority over activities within the Science and Technology Bureau, but also agency-wide responsibilities to help improve the role of science and technology more generally.

Under Mr. McPherson's leadership, AID is also strengthening its relationships with the scientific community at large. First, several new initiatives have been taken under the authority of Title XII of the Foreign Assistance Act to augment the role of U.S. universities in AID's development assistance efforts. Memoranda of Understanding are being developed to formalize relationships with universities that are willing to commit themselves to long-term relations with AID. A Joint Career Corps is being planned for university staff who opt to spend one-third of their professional time working with AID, and a Joint Venture mechanism to make more effective use of the resources of small U.S. universities is being explored.

Second, we are continuing to strengthen our 15-year relationship with the National Academy of Sciences, during which various NAS commissions have conducted a wide variety of studies in developing countries on behalf of AID. The Board on Science and Technology for International Development is currently playing the lead role on behalf of the Academy and is seeking to identify innovative technologies of potential economic value for developing nations. BOSTID will also

establish networks of developing country institutions to explore and improve those technologies.

These steps and organizational changes within AID are being taken with one overriding objective in mind: to move science and technology to a higher level within the agency's own priorities and to give AID's technical staff a larger role in the agency's activities.

In this process, we hope to make the fullest possible use of scientists from the developing countries as well as from the United States. Moreover, all conferences cosponsored by AID and the Academy will have two components: a "show and tell" aspect for sharing of new knowledge; and a "so what" component, to discuss what AID and others should be doing that we are not already with regard to the issues in question.

THIS CONFERENCE

This conference is an excellent example of this kind of cooperative endeavor. New developments in cell biology, molecular genetics, recombinant DNA, and related fields offer extraordinary promise for opening new vistas in the fields of health, energy, food, and agriculture. However, much of the work that is now under way is still in the realm of basic research or is oriented primarily toward issues that concern the highly industrialized economies. AID is concerned about the role of these biotechnologies in the development process, and we believe the Academy is particularly well qualified to provide us with recommendations of opportunities, strategies, and policies for possible program initiatives.

More specifically, we want to address the questions: To what extent should the developing countries be involved in biotechnology research themselves, and in which areas? Is this the right time to encourage the developing countries to set up their own programs in particular areas? And, if such programs are advisable to one degree or another, what if anything should AID be doing, within our limited resources, to facilitate developing country research along the recommended lines?

This, then, is the agenda for this conference. The agency is delighted to participate in this important event, together with the National Academy of Sciences and the distinguished guests from many countries of the developing world.

Keynote Address

Priorities in Biotechnology

DAVID BALTIMORE

Over the next few decades biology will likely have an impact similar to that made by electronics over the last few decades. Recent advances in molecular biology, immunology, and cell culture promise to bring biology out of the laboratory and into society with both the excitement and complications that this implies.

The new capability is called "biotechnology," yet biotechnology is not new. Biology has been harnessed for years to produce wines, beers, vinegar, foods, solvents, ethanol as an energy source, and drugs. What is new about the "new" biotechnology is the style of approach and methodologies now available.

The "old" biotechnology chiefly manipulated microorganisms or plant foods, and selection was the major force behind production of desired traits. Occasionally, mutagenesis increased our ability to select, but there was very little possible in the way of directed alteration. The new biotechnology uses knowledge about the interior of cells to direct and manipulate the products they make. Although we are still essentially manipulating elements that were provided by nature, we are now taking the pieces out of cells, manipulating them, reinserting them, and thus heightening the capabilities of those cells to produce what we desire. It is important to remember that the amount of manipulation involved is as small as possible and that the major materials--DNA sequences in many cases--are provided by nature and are the products of millions of years of evolution. This is, of course, mainly recombinant DNA technology.

Major advances have also been made in hybridoma research which is harnessing immunology in new and surprising ways, in cell culture which is making cells of both plants and animals available as direct sources of materials in protoplast fusion techniques, and in a variety of other methods. Thus the new biotechnology is becoming broader in scope and involves the input of an enormously wide range of technological capabilities that come out of laboratory science.

Despite the fact that biotechnology is a very high technology in terms of being at the forefront of basic sciences, it is a very

appropriate technology for solving the problems of people at all levels of development. The capabilities of molecular biology go well beyond the kinds of synthetic methods connected with recombinant DNA techniques. Molecular biology has developed a range of powerful analytic tools that give the modern biologist the power to unravel the most complex of biologic systems. We can, for instance, in simple cases decode all of the information of a virus and understand its intricate details at levels that were not imaginable 10 years ago. We can attack the more complicated systems of larger parasites and, building on knowledge of the natural history of parasitic organisms, use powerful techniques of molecular biology to determine the life cycle of the organism, its pathologies, as well as the key events that are targets for therapy. In genetic disease, a worldwide problem, we can begin to determine the nature of gene malfunctions and the underlying cause of genetic disease, thus enabling us to direct therapies. More important, we are developing powerful new screening methods to identify carriers of specific genes on the basis of genetic linkage rather than by direct analysis of the lesion. We can now attack the molecular basis of cancer in ways that were not imaginable a few years ago, and we can now understand developmental anomalies as we begin to unravel our understanding of biological development. None of these tasks is easy, and they all require large numbers of highly skilled people working over long periods of time. Nevertheless, these investigations are now possible, whereas not many years ago they were not worth talking about.

RECOMBINANT DNA RESEARCH

More familiar to us in any discussion of the application of molecular biology to societal problems are the synthetic capabilities that result from recombinant DNA methods. By extracting genes, hooking them up appropriately, and inserting them into microorganisms, we can produce new proteins. I believe it will some day be possible to extract the gene for almost any protein and to insert that gene in a microorganism, resulting in production on whatever scale we desire. Although there are technical problems in doing this and each protein seems to present its own set of problems, these problems are solvable in theory and are being solved in practice. The host microorganisms used are either bacteria, generally *Escherichia coli*, or yeast cells. However, other systems such as *Bacillus subtilis* and mammalian cells are being used.

The development of vectors to bring DNA into mammalian cells and to achieve production in cell culture will be an increasingly valuable technology, partly because as we learn more about the nutrition of cells, the growth of cells will become cheaper and the production of mammalian proteins in mammalian cells may be a feasible route.

It is certainly feasible today for pharmaceutically active compounds and may be for other kinds of materials in the future. Materials made in this way are familiar to all of us: insulin; bovine growth hormone; chymosin or rennin to help in the manufacture of cheese; urokinase, which shows important potential for the treatment of vascular disease; interferon, which may or may not be important for a variety of things. Most exciting, however, is the production of crucial surface proteins of certain viruses that continue to plague our lives--in developing countries, hepatitis B; in many areas of the world, foot-and-mouth disease virus; in all parts of the world, influenza virus and rabies, a rarely seen scourge but one of the most terrifying. The major antigens for all of these viruses have been cloned and expressed in bacteria. The utility of these in the development of vaccines is being investigated, and there is great hope. However, in no case do any of these things or in fact any material resulting from recombinant DNA research represent a product of commercial success.

The utility of recombinant DNA methods is heightened by the ability to use either the purified products made from cells (cells can express these products at high enough levels that purification is relatively easy) or whole cells, which in certain cases--specifically in industrial processes--represent a feasible way of taking advantage of the ability to direct the capabilities of cells.

In this regard, the power of having specific antibodies is something we are only beginning to realize, and the major power that we may have in the short run will be diagnostic. The ability to diagnose specific cancers has been widely discussed, but the ability to develop immunoassays for pesticides, toxins, viruses and other microbes, and parasites may develop in the short run. It is not so much that hybridomas are giving us a new kind of antibody that was not there before; rather, the technologies that are being developed around the availability of a constant source of antibody are allowing us to use that antibody to test and diagnose very low levels of materials. More speculative is the use of monoclonal antibodies in therapy, largely for cancer. For this, there appears to be much less hope than has been suggested in the popular press.

APPLICATIONS TO DEVELOPMENT

Although in recent years much of the application of biotechnology has focused on the needs of the developed world, the most urgent needs are clearly in the developing world. The companies that jumped into biotechnology have paid some attention to the areas in which there are obviously high profits to be made, such as hepatitis B or foot-and-mouth disease, and we are beginning to see research laboratories pay

more attention to the parasitic diseases, viruses, and even the cancers, that specifically afflict the less-developed world. However, at the moment this work is driven more by a profit motive than by anything else. It is exciting to see that the Agency for International Development is trying to change that orientation to one of need rather than of profit.

A number of research possibilities relate directly to the needs of developing countries--particularly, modifying plants to increase resistance to disease, to tolerate unfavorable growing conditions, to fix nitrogen more efficiently, and to provide a better balanced protein--yet almost all of the biotechnology is found in the developed world.

What would it take for less-developed countries to have the necessary capability to utilize the new technologies, and thereby to set their own priorities and to solve their own problems? The answers to this question appear to fall into three categories: education, infrastructure, and support.

To explain these categories, it is perhaps best to first consider how the new biotechnology came into being: it was an outgrowth of ordinary research by exceedingly well-trained and well-educated people working in a system that believes in supporting investigator-initiated research. However, that training and the ability to self-define problems were coupled to an infrastructure of supply companies that provided specialized equipment such as centrifuges; enzymes, which are the heart of the technology; fine chemicals; laboratory gadgetry, some of which is crucial to research; specialized chromatography media; and so on. This infrastructure is possibly the most crucial part of the system. Education can be obtained as well as support, but if the infrastructure is lacking, these technologies become very difficult to use. Infrastructure includes the provision of a constant source of electricity for cooling as well as for continuing experiments, and often for the air conditioning necessary for the growth of specific organisms.

The development of modern biotechnology, it should be realized, was in a sense serendipitous. Thousands of researchers followed their own noses without preconceptions. Their major finds were enzymes. I was fortunate enough to find reverse transcriptase, an enzyme central to most of the methodologies for turning microorganisms into protein factories. However, this discovery resulted from studying the life cycle of a tumor virus that had no relation whatsoever to biotechnology. I am not arguing that the Agency for International Development should fund all biomedical research; it certainly cannot. Rather, it is important to see the spirit in which this kind of field moves forward.

Education

Working in the new biotechnology, therefore, even in its most practical mode, requires almost the same elements needed for its discovery, including highly educated people. Approximately 30 years is needed, starting with early nurturing through a long period of apprenticeship and graduate school and postdoctoral work, to develop a researcher fully capable of handling modern biotechnology. The apprenticeship, the crucial part of the educational program, must be carried out in up-to-date laboratories to get a "hands-on" understanding of where the difficulties are. Those of us who train people in modern biology know that the most important experience is acquired when things go wrong. One must be in a situation to see how many different things can go wrong and how you get around those things to learn how to solve problems. One of the saddest occurrences--and this can happen even in the United States where the density of science is so high--is when someone who is inexperienced and working in a relatively isolated area spends six months making the same mistake because he does not know it is a mistake.

Infrastructure

Biotechnology research is continually dependent on infrastructure. No group can make all of its own enzymes, for example; it must depend on commercial sources simply because the time and energy required to make them is beyond the capability of any one institution. The hardest requirement to meet in a developing country will probably be the acquisition of materials from abroad. For example, enzymes are very unstable and must be delivered in a frozen state usually within 48 hours of shipping. Unless they are kept cold through their life, they die. A continual supply of electricity is thus an absolute necessity. It is also important that such reagents are not held up in customs, and that the developing country laboratory have available the foreign exchange necessary for their purchase.

Support

The third area--support--has two sides: equipped, functional laboratory facilities and ongoing, high-level support to maintain continuity and to encourage scientists to return after their inevitable time spent abroad. The part of support that involves money is, of course, crucial, but with the availability of funds from various international sources, money is probably not the limiting factor in the development of biotechnology.

Is it realistic to expect developing countries to make the necessary commitments to training, infrastructure, and support? For most countries it is probably not realistic because it is too expensive, and it inevitably involves a long-term political commitment. Therefore, given the expense and difficulty, is it necessary for developing countries to have this technology? The answer is clearly yes. It is not prudent to maintain a continual dependence on the developed world where much of the current work is profit-oriented--not a solid base on which to build solutions to local problems.

The alternative to either having a national capability or being dependent on the developed world is international cooperation among developing countries, and this is a difficult prescription that incurs both political and logistical problems. However, the establishment of regional laboratories, supported cooperatively and with the help of international agencies, is crucial if the developing world is to participate fully in the new biotechnology. This tradition exists in agriculture, and it could exist in molecular biology with the appropriate thought and will. Such laboratories could act as training centers in addition to undertaking projects applicable to the region in which they are located. Although such institutions would focus on diseases and on local agricultural needs, it is important that they also contribute to the base of science on which the field depends. That sense of involvement in the international community of scientists makes an institution attractive to work for. Such a cooperative venture might first be attempted in South America where there are some efforts to move in that direction.

Although the extent of commitment necessary to be a full participant in the biotechnological revolution is emphasized here, it is important to remember that biology does have a way of becoming simpler, easier, and cheaper as it matures. It is reasonable, therefore, to hold out the hope that the future for biotechnology will not be, as in physics, bigger machines and more money; rather, it will be--based on the history of the field--smaller machines and less money but more intelligence.

One example is vaccine production. Historically, vaccine production has been done in animals, in eggs, or in tissue culture. In the last 5 years, however, there have been attempts to develop subunit vaccines, and the cloning projects described earlier for certain viruses are an example of that. Clearly, pure protein vaccines are safer, easier to transport, and more stable than intact virus vaccines, even killed virus, and it is hoped that recombinant DNA methods will provide ways of making protein antigens in bacteria and yeast as has now been done on a pilot scale. Nevertheless, several groups of researchers, working mainly on the West Coast, have discovered a shortcut to vaccine production that will make it even

cheaper, stabler, and more precise: to make short peptides chemically that mimic the antigenic sites on proteins.

This is an old idea; work of this kind first was conducted in the 1960s and sporadically since then, but most previous successes apparently never penetrated the consciousness of the scientific community. Newspaper accounts of recent successes, however, show that at least in a laboratory setting peptides that correspond to antigenic determinants on foot-and-mouth disease virus, hepatitis virus, influenza virus, and probably others can be made. Whether they will provide the simple, widely applicable route to making vaccines against specific viruses has yet to be shown, but it certainly avoids the most complex step of the biotechnological process--the high-level expression of gene products in microorganisms. If it is only necessary to isolate a genome, sequence it, pull out the appropriate region, and make a peptide chemically, resulting in an antigen that can protect an animal against the virus, we will see vaccines available for essentially all viruses in the world in the next few years--however, remember the "if" at the beginning of that sentence. It will then be possible to go directly from a nucleic acid sequence to a protective antigen.

CONCLUSION

Does biotechnology then really relate to the important needs of the developing world? On the one hand, other recent revolutions, such as that in microelectronics of which we are now in the middle, appear to have had relatively little impact on the developing world because they do not go to the heart of the basic necessities of life. Biotechnology, on the other hand, relates closely to these necessities because biology is the science of life and can contribute to better health, to larger food supplies in highly specific ways that are tailored to local circumstances, and to energy needs. Although the application of biotechnology requires a highly developed educational system, a complex infrastructure, and strong, stable support, it is still relatively cheap on the scale of international science and is remarkably adaptable to local circumstances. Biotechnology is likely to make specific contributions in the areas of vaccines; a deeper understanding of the disease process; food supplements, possibly specific amino acids to supplement deficiencies in plant proteins; better crop plants in which those needs could be engineered; better animal health, which is, of course, a great hope; and with luck, cheaper energy.

Finally, no such discussion is complete without some mention of whether biotechnology poses any special safety hazards. Since the first discovery of DNA methods, debate has raged about the possible unique hazards associated with this methodology. In spite of the most

careful scrutiny, no problems have arisen, and it is safe to say that none are expected. It is certainly important to remain vigilant in using this new kind of technology--in using any new technology--because these are still early times. Our experience, although reassuring, is limited, and there are new possibilities and methodologies coming along all the time. However, none of these poses in any theoretical way--or in any obvious way--a hazard. In fact, for highly pathogenic microorganisms it is clearly a much safer way of working. For example, the Lassa fever virus found in Africa is so contagious and so deadly to work with that almost no progress has been made on it since its isolation, except for a little work in the high containment laboratories at the Centers for Disease Control. If the genome of this virus could be cloned and removed from the laboratory and worked on without fear, we might then understand why this virus is one of the very rare lethal viruses of man.

Experience with the foot-and-mouth disease virus is instructive in this regard. Only in the last few years were we able to remove this virus from an island in the United States where the amount and style of work to be done was limited by the local circumstances. The ability to remove it as a DNA molecule has opened up a range of experimentation that was never before possible. The same thing has obviously happened in many other countries where the cloning of the foot-and-mouth disease virus has greatly expanded the ability to work with and understand the virus.

SUMMARY

We are in the decades of biology; many new capabilities are with us and many more are to come. If the developing world is to reap the benefits of this technology, it probably needs local capabilities, the acquisition of which requires tremendous commitment. One possibility for accomplishing this is the pooling of resources on a regional basis to establish laboratories that can attack regional problems. Over this week of intensive study, this workshop will probably come to a clear understanding of just which areas are ripe for investigation and will consider the institutional framework that will best serve the needs of the developing world. We still expect new breakthroughs and new capabilities to come from the research laboratory, and it is important that these be available to benefit the whole world.

Keynote Address

Perspectives in Biotechnology Research from the Point of View of Third World Agriculture

M. S. SWAMINATHAN

INTRODUCTION

Biotechnology encompasses many facets of the management and manipulation of the biological system. In 1981, the European Federation of Biotechnology defined this branch of science as "the integrated use of biochemistry, microbiology and chemical engineering in order to achieve the technological application of the capacities of microbes and culture cells." A recent offshoot of biotechnology research is genetic engineering, which involves gene splicing and recombinant DNA cloning.

There has been considerable public and political interest in this field of technology because of reported breakthroughs possibly of great significance to life on earth. Some examples of these are: the development of new nitrogen-fixing plants, single-cell edible protein, new crops resistant to pests, bacteria for use in waste recycling and pollution control, and petrochemical substitutes; the use of gene therapy to correct monogenic diseases such as sickle-cell anemia; the use of genetic screening for the isolation of genes responsible for birth defects; the microbial production of human insulin; the production of interferon and growth hormones; and a general increase in the knowledge base of immunological processes including cancer (Stewman and Lincoln 1981).

It is widely believed that biotechnology will probably have its most important impact on agriculture and health. Bollinger (1980) has called tissue culture "the botanical equivalent of the laser, in that there are more potential applications than originally conceived." The vast panorama of possibilities for the use of tissue culture techniques in agriculture, animal husbandry, aquaculture, and forestry is just unfolding.

Fears have also been expressed about the potential dangers inherent in some fields of biotechnology research such as genetic engineering. In addition, doubts have been voiced about the wisdom of allocating too many resources to new technologies, often at the cost

of intensifying work that uses already successful and well-tested techniques (Sprague et al. 1980). In addition to considerations of safety and strategy, another concern is the growing secrecy attached to biotechnology research because of commercial considerations.

Developing countries are naturally attracted to the potential applications of biotechnology research to alleviating hunger, providing energy, and improving quality of life. Naturally, the precise priorities of developing countries vary widely. The National Institute of Biotechnology and Applied Microbiology in the Philippines, for example, has accorded priority to research on biofuels, nitrogen fixation, food fermentation, plant hydrocarbons, antibiotics, vaccines, microbial insecticides, and biomass production. The National Biotechnology Board, established by the government of India, has chosen genetic engineering, photosynthesis, tissue culture, enzyme engineering, alcohol fermentation, and immunotechnology as areas of immediate interest.

Nearly every developing country has plans or programs for harnessing the tools of biotechnology for national development. It is, therefore, important that all countries realistically understand the problems and potentials associated with biotechnology research. This conference is a very important milestone in this respect. Since food is the first among the hierarchical needs of man, priorities in biotechnology research as related to agriculture are discussed here.

AGRICULTURAL SCENARIO AND BALANCE SHEET

Current world food stocks are satisfactory because of the good harvests of the last few years in North America as well as in most countries of Asia (Figure 1). The situation of global food security, however, is far from satisfactory, since fluctuations in production caused by aberrations in weather, pest epidemics, and unfavorable public policies could be considerable. Both the Food and Agriculture Organization of the United Nations (FAO) and the World Food Council have been emphasizing the need for greater interest in the development of enduring national, regional, and global food security systems. The present satisfactory level of global food reserves thus should be regarded only as a breathing spell during which greater efforts for elevating and stabilizing food production should be launched.

How has the world as a whole been able to keep food production slightly above the rate of population growth? New technologies leading to the development of high-yielding and management-responsive varieties of wheat, rice, and other major food crops have been important factors. A major agricultural asset of many developing countries is the vast, untapped production reservoir arising from the prevailing gap between potential and actual yields even at current levels of technology. An important agricultural liability is the

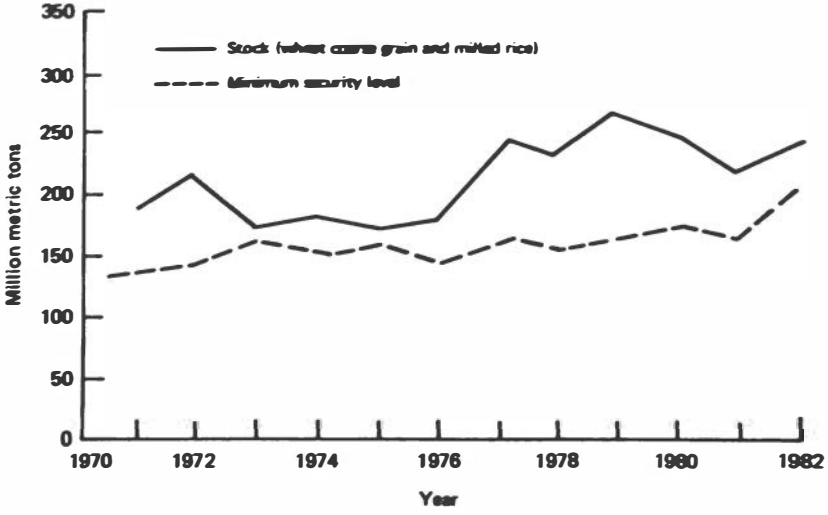


FIGURE 1 World carryover stocks and minimum security levels of cereals (including China and the USSR): 1970-1982.

shrinking availability of land for crop production. Farms in many countries in Southeast Asia are getting increasingly smaller, and in most of the states of India, the average size of a farm holding is less than 1 hectare (ha).

Enhanced food production must, therefore, result largely from improved productivity and more intense cropping; this is where high-yield technology is of particular relevance. For example, in the Philippines an additional 3.2 million ha would have been needed to achieve the 1980 rice production level had there been no yield improvement since 1960 (Table 1). In India, over 34 million ha of additional land would have been needed to produce the quantities of rice and wheat harvested in 1979 but for the productivity advances made since the mid-1960s (Table 2).

In spite of this impressive progress, the vulnerability of world food production systems, particularly to weather conditions, is very great because of the following factors:

- Much of the surplus food grains available to supply other countries on commercial or concessional terms are produced in

TABLE 1 Rice production, area, and yield in the Philippines: 1960, 1970, 1980[a].

Year	Production of rough rice (thousand tons)	Area (thousand ha)	Yield (t/ha)
1960	3,705	3,198	1.16
1970	5,343	3,112	1.72
1980	7,723	3,459	2.23

[a]About 3.2 million ha more would have been needed to achieve the 1980 production level at the 1960 yield level.

SOURCE: Bureau of Agricultural Economics, Philippine Ministry of Agriculture.

TABLE 2 Impact of new technology on yield and land requirements in India.

	Year	Area (thousand ha)	Yield (t/ha)	Production (thousand tons)	Area (thousand ha)
Rice	1966-1970	36,360	0.98	35,770	—
	1979	40,480	1.33	53,770	14,331
Wheat	1961-1966	13,191	0.83	10,950	—
	1979	22,560	1.57	35,510	20,222

SOURCE: Ministry of Agriculture, Government of India.

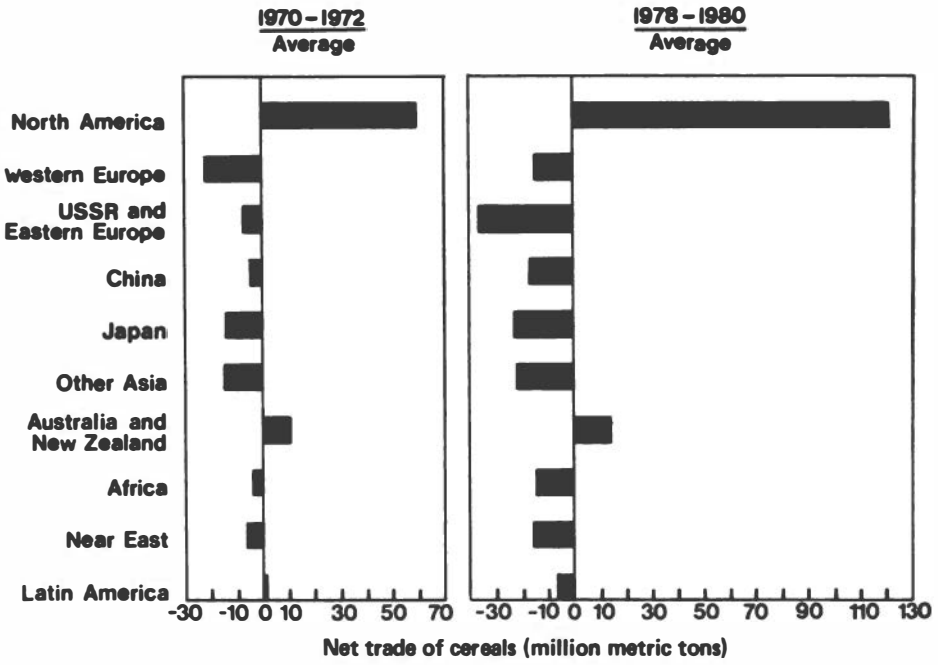


FIGURE 2 Net trade of cereal grains (million tons): 1970-1972 and 1978-1980.

a few countries such as the United States, Canada, Australia, and New Zealand (Figure 2).

- About 16 crops and 7 farm animals provide the bulk of human food needs. Consequently, if the production of one of the major crops such as wheat or rice is adversely affected over large geographic areas, there could be acute food scarcity.
- The escalating cost of energy and the consequent rise in the cost of food production lead to a slow diffusion of new technology, which is based to a great extent on purchased inputs. When the cost of production is high, the risk involved is also greater. The cost, risk, and return of

farming determines the extent to which a farm family will invest both money and effort. Even in Africa, where per capita food production declined during the 1970s, there are examples of a sharp increase in production when farmers were given a remunerative price. This is particularly important in many developing countries where the arable area per agricultural worker is declining.

Small, fragmented holdings create problems in efficient management. Further, serious problems of soil erosion and land degradation arising from indiscriminate deforestation are encountered in many developing countries. According to FAO, 0.6 percent of the existing forest cover disappears every year in Asia, which is equivalent to 1.8 million ha every year or 3.5 ha per minute. On a global basis, 21 ha of tropical forest are disappearing every minute.

A recent study by a committee established by the government of India to assess fuelwood supplies and needs found that, in contrast to the present requirement of 133 million tons (t)* of fuelwood per year, current efforts can provide only 39 million tons. The committee therefore concluded that at least 1.5 million ha of land should be planted annually with fast-growing fuelwood species (Swaminathan 1982). Scientific use of land thus becomes exceedingly important if the food, fuel, fodder, fiber, and other needs of the population are to be met adequately.

The FAO study (1979), Agriculture: Toward 2000, provides a wealth of valuable statistics. For example, for 90 developing countries as a group, the net cereals deficit of 36 million tons in 1978-1979 will be doubled by 1990 and doubled again by 2000. In other words, 18 years from now the gap between demand and supply in these countries will be 144 million tons. Sixty percent of total cereals was consumed as feed in developed countries, and the demand for animal products tends to grow with increased income. FAO has further projected that by the year 2000, 7 percent of the population of developing countries will remain undernourished. Can we integrate appropriate segments of emerging technologies with traditional ones to find speedy and effective solutions to the problems of undernutrition and malnutrition?

AGRICULTURAL PRODUCTION STATEGIES

The major pathways of improved productivity will have to be increased yields and more intensive cropping. Multiple cropping is possible in the tropics and subtropics because of the abundance of

*Metric tons are used throughout this report.

TABLE 3 Increase in areas under irrigation (million ha): 1961-1965 to 1977 (annual average).

	1961-1965	1977	Percent increase
Africa	5.8	7.8	13.4
Asia	100.0	128.8	28.8
South America	4.9	6.5	32.6
All developing countries	110.0	144.9	31.7
All developed countries	39.0	52.9	35.6
World	149.0	198.0	32.9

SOURCE: Data taken from FAO.

sunshine throughout the year. The major constraint, however, is water availability, and most developing countries are making major investments in bringing more land under irrigation (Table 3).

More nutrients are required for both higher productivity and more intensive cropping. Many of the soils in Southeast Asia and Latin America suffer from mineral stress, and thus plant nutrition, soil and plant health care, as well as irrigation require simultaneous attention. At the same time, steps must be taken to expand the area under cultivation through the reclamation of soils affected by factors retarding healthy crop growth. For example, in South and Southeast Asia about 86 million ha can be made more productive provided that problems of salinity, alkalinity, and other adverse soil conditions are rectified (Table 4). Fortunately, it is possible to breed varieties tolerant to some of the adverse soil factors (Table 5).

Developing countries face the dilemma of having to improve both production and consumption simultaneously. This will be possible under current economic conditions only if production costs are kept as low as possible so that the output price is reasonable to a majority of consumers. The alternative is to subsidize either input, output, or both. Most developing countries cannot bear the financial burden involved in extensive farmer and consumer subsidies. Since energy in

TABLE 4 Extent of problems affecting rice lands of South and Southeast Asia.

Soil problem	Area (million ha)
Salinity	53.2
Alkalinity	7.0
Acid sulfate soil condition	5.3
Peat soil condition	21.0
TOTAL	86.5

SOURCE: International Rice Research Institute, Los Baños, Philippines.

TABLE 5 Performance of rice varieties/lines tolerant of problem soils.

Soil problem	Variety or line	Yield (t/ha)
Salinity	IR50, IR5657-44	3.6
Alkalinity	IR36, IR4595-4	3.6
Strong acidity	IR42, IR4683-54	4.2
Peat	IR42, IR8192-31	3.1
Aluminum toxicity	IR43, IR6115-1	3.8
Phosphorus deficiency	IR52, IR8192-200	4.4
Zinc deficiency	IR36, IR8192-31	2.9
Iron deficiency	IR36, IR52	2.8

SOURCE: IRRI (1981).

the form of fertilizer, irrigation, tillage, and postharvest technology operations is an important component in the cost of production, fossil fuel requirements must be reduced. Thus the challenge to those in charge of technology development is to bring about continuous improvement in the productivity of major farming systems per unit of land, water, time, and energy without detriment to the long-term productive potential of the soil. Can biotechnology help in this task? Taking rice as an example, the following illustrates the scope for integrating recent advances in biotechnology with the techniques already in use.

APPLICATION OF BIOTECHNOLOGY TO RICE PRODUCTION

In its study, Agriculture: Toward 2000, FAO (1979) has projected the need for the production of an additional 300 million tons of rice between 1974-1976 and the end of the century. The rate of growth in world demand for rice during the 1980s is expected to be on the order of 2.9 percent a year. In contrast to the other cereals, the demand for rice would remain overwhelmingly for direct use as human food. If past trends in demand and production continue, the developing countries' gross import requirement for rice would rise from 8.3 million tons of paddy in 1974-1976 to 33 million tons in 2000. Through expanded irrigation and improved productivity, it should be possible for developing countries to meet 200 million tons of the additional demand through greater home production (Table 6). This will require an optimum blend of technology, services, and public policies.

Several years of breeding and selection work are needed to incorporate useful genes from a suitable donor strain into a commercially popular variety. The breeding of the rice variety IR36, which now occupies over 10 million ha in Asia, took about 7 years. Seed production on a scale necessary to cover large areas takes another 2-3 years. Thus about 10 years is needed from the time a cross is made until it makes a widespread impact, even when two or three crops can be grown in a year. Using such techniques as "rapid generation advance," some strains of rice will produce four crops a year.

Another recent example of the time taken for transferring a desirable gene from one genetic background to another is the work on high-lysine corn. The opaque-2 gene discovered at Purdue University in the early 1960s has been associated with several undesirable traits. It is only after 12 years of patient research that scientists at the International Maize and Wheat Research Center in Mexico (CIMMYT) have been able to combine the high-lysine character with other desirable traits.

TABLE 6 Area, yield, and production of rice (paddy) in developing countries: 1974-1976 and 2000.

Land and water class	Harvested area (million ha)		Yield (t/ha)		Production (million tons)	
	1974-1976	2000	1974-1976	2000	1974-1976	2000
Rainfed	62.6	57.8	1.5	2.1	95.4	123.8
Irrigated	36.5	64.8	2.7	4.1	97.6	264.9
Total	99.1	122.6	1.9	3.2	193.0	388.7

SOURCE: FAO (1979).

Thus, in the 1980s, improved food production will depend largely on material already in the plant breeders' assembly line. Research and development establishments should be bridging the gap between potential and actual yields in small farmers' fields by helping to eliminate the constraints responsible for this gap. Any new research program planned and initiated now will be relevant largely to increasing and stabilizing food production in the 1990s. It is essential, therefore, that in agricultural research and development planning, both the short- and long-term goals and needs are given appropriate and concurrent attention.

In the following sections, improved yield and nutrient supplies for rice are used as examples of research goals for which the application of tissue culture and genetic engineering techniques could be of great value.

Raising the Ceiling of Yields

The potential and actual experimental rice yields under two levels of incident solar radiation representing the wet and dry seasons in monsoon Asia are indicated in Figure 3. The term "favorable conditions" refers to the conditions under which diseases, insects, water, fertilizers, and cultural practices do not limit rice growth and yield. The data suggest that further increases of rice yield by 2.2 t/ha for the wet season and 3.5 t/ha for the dry season

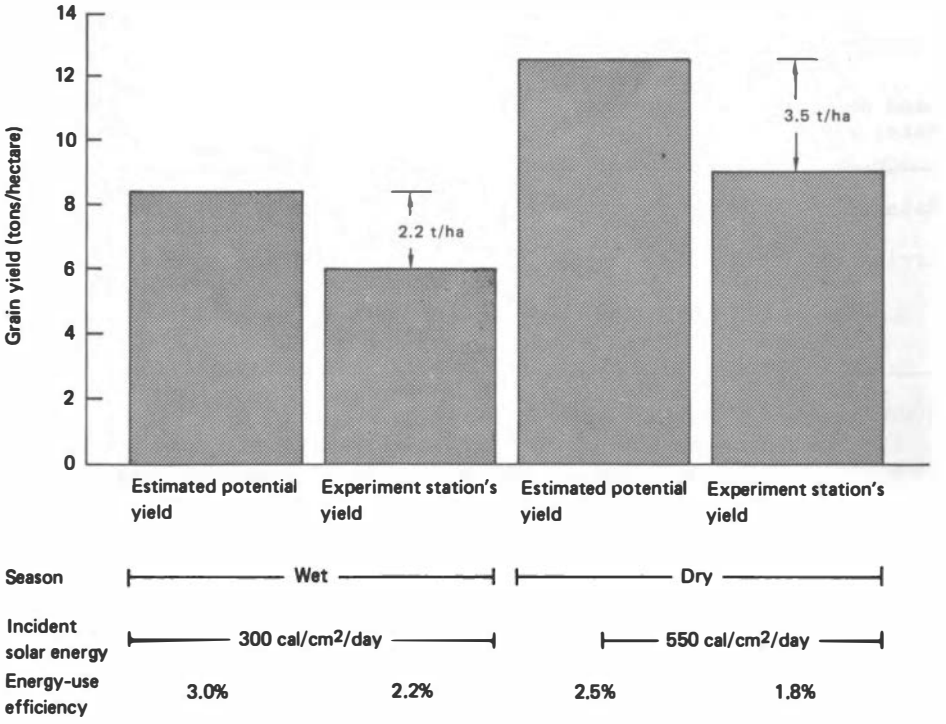


FIGURE 3 Potential under favorable conditions and actual experimental rice yields during the wet and dry seasons in monsoon Asia.

would be realistic targets. The following assumptions underlie this conclusion:

- Estimated potential yields were calculated assuming that the amount of incident solar energy during the grain filling period and efficiency of energy use by a crop ultimately determine the yield.
- A yield of 6 t/ha for the wet season and 9 t/ha for the dry season were chosen for the experiment station. These yields can be easily produced with a good variety and appropriate

management. Yields higher than these have been reported, but they were obtained under exceptionally favorable conditions or by using "a special treatment" such as carbon dioxide enrichment before or after heading.

- The 300 and 550 calories (cal) per square centimeter (cm^2) per day represent levels of incident solar energy during the ripening period of the rice crop in monsoon Asia during the wet and dry seasons, respectively. In Australia and the state of California, much higher levels of solar energy, that is, 600-700 $cal/cm^2/day$, are available.
- A 3.0 percent energy-use efficiency is given for the wet season and 2.5 percent for the dry season. Energy-use efficiency is expected to decrease as the level of incident solar energy increases. But these high values were obtained for the vegetative growth stage. During the ripening period, senescence is unavoidable and thus the energy-use efficiency during that period is inevitably lower than during the vegetative stage. Differences in energy-use efficiency between the estimated potential yields and the experiment station's yields are between 0.7 and 0.8 percent.

Bridging the Gap Between Actual and Potential Yields

A number of physiological factors may contribute to reducing the gap between actual and potential yields:

- Increasing biomass production
  - -- Fast leaf area development
  - -- Low maintenance respiration
  - -- Low photorespiration
- Increasing a crop's sink size
  - -- Large number of spikelets per shoot
  - -- Large grain size
  - -- Greater partition of assimilates into spikelet formation
  - -- Increase in the harvest index (up to 0.6)
- Assuring better grain filling
  - -- Slow senescence
  - -- Maintenance of a healthy root system
- Increasing lodging resistance
  - -- Stiff culm
  - -- Slow senescence
  - -- Maintenance of a healthy root system

In the past, slower initial growth was correlated with higher yields because the old varieties were tall and susceptible to lodging. With short-statured and lodging-resistant varieties, fast

initial growth is one requirement for maximum crop photosynthesis. In theory, the land should be covered with leaves as early as possible, and the leaves should be erect to increase photosynthesis.

Dark respiration is divided into two components, growth respiration and maintenance respiration. Growth respiration cannot be changed unless biochemical pathways are modified. Maintenance respiration is used for protein turnover and active ion transport. It is possible to look for a variety with a lower maintenance respiration. The total elimination of photorespiration in rice may increase net photosynthesis by 50 percent. Reduced maintenance respiration and photorespiration should increase leaf photosynthesis.

A small sink size generally limits rice yields under favorable conditions. To increase the sink size, the number of spikelets per shoot and grain size must be increased simultaneously. This is now being attempted by the Plant Physiology Department of the International Rice Research Institute (IRRI). In a physiological sense, greater partitioning of the assimilates into spikelet formation before heading will result in a larger sink size. Young spikelets compete for assimilates with flag leaf and culm; this process is probably under hormonal control. In terms of morphology, short culm and small flag leaf favor greater partitioning of assimilates into spikelets.

Increased sink size should also be coupled with an increased harvest index. Modern *indica* varieties have a harvest index of about 0.5; this could be increased to 0.6.

Slow senescence assures active photosynthesis during the grain filling period and contributes to increased resistance to lodging. Leaf sheath contributes to the breaking strength of the shoot by 30-60 percent.

Maintenance of a healthy root system is also important, in addition to water and nutrient uptake, for (1) production of cytokinin, a plant hormone responsible for senescence; and (2) better anchorage of the crop stand to prevent root lodging.

Stiff culm must be considered in addition to short plant stature, and the increased importance of resistance to lodging can be clearly illustrated as follows. With a harvest index of 0.5, 8.2 tons of straw supports 8.2 tons of grain (estimated potential yield), for a total biomass of 16.4 tons. When the harvest index is increased to 0.6, 5.5 tons of straw must support 8.2 tons of grain. Thus the straw must be much stiffer even though the total biomass production is less (5.5 + 8.2 = 13.7 tons). Special attention should be given to this characteristic since recent varieties such as IR36, IR42, and IR50 have less resistance to lodging than IR8.

The Role of Biotechnology Research

The possible applications of biotechnology to rice improvement are indicated below. Although the basic techniques were developed in the past, the application of these techniques to crop improvement is a new avenue of research.

- Cell and tissue culture
  - -- Induction and selection of useful mutants at the cellular level (salt tolerance, aluminum toxicity tolerance, high-lysine and high-protein content, low photorespiration, disease resistance, low oxygen tolerance)
  - -- Embryo culture (intra- and interspecific hybridization)
  - -- Anther and pollen culture (reducing breeding time)
  - -- Protoplast fusion (interspecific and intergeneric hybridization, hybrid rice improvement, Azolla improvement)
- Genetic engineering (incorporation of nitrogen fixation genes)

Modification of single cells is the end of research for microorganisms, but it is just the first step for higher plants. Thus further advances in cell and tissue culture techniques are indispensable if genetic engineering is to be useful for the improvement of higher plants such as rice. In higher plants, cells must be modified, multiplied, and regenerated into the whole plant. Further, the modified traits must be manifested in the whole plant and retained for succeeding generations. In cell and tissue culture, two problems require intensive research:

- Understanding mechanisms of genotypic differences in plant regeneration and developing methods to overcome such differences.
- Developing methods to regenerate plants from long-term cultured callus and cell suspension.

Without solving these two problems, the usefulness of cell and tissue culture, and thus genetic engineering, may remain confined to a very narrow range of materials and problems.

Plant cell and tissue culture can be put to a number of good uses immediately. Among the various techniques in cell and tissue culture, induction and selection of useful mutants at the cellular level is probably the most promising approach to rice improvement. At IRRI, work on salt tolerance and aluminum toxicity tolerance is now under way, and work on high-lysine and high-protein rice will soon be initiated.

Low photorespiration has been studied at several laboratories, and presently, tissue culture is being used to reduce the photorespiration rate of tobacco plants. Photorespiration inhibitors are also being studied intensively at several laboratories.

Tissue culture has also been used to increase the lysine content of rice, but results indicate that there has been a greater increase in the protein and only a slight increase in the lysine content of the grains.

Disease resistance can be increased by tissue culture when particular toxins are directly involved in the disease development. Application of tissue culture to sheath blight resistance will soon be initiated at IRRI.

Embryo culture has been used to achieve interspecific hybridization at IRRI, although no results have been obtained thus far. Because of the simplicity of the technique, embryo culture may be useful in some instances where post-fertilization abnormalities impair embryo development.

Anther culture may not be useful in rice breeding unless further advances are made in plant regeneration and pollen culture. In China, several rice varieties were produced by anther culture, but they were japonica varieties. Plant regeneration problems hamper usefulness of anther culture in most indica varieties.

Other applications of tissue culture techniques include the standardization of methods for breeding perennial plants like coconut, rubber, and fast-growing fuelwood trees. Coconut, for example, is afflicted by diseases of unknown etiology such as cadang-cadang in the Philippines and root wilt in India. However, healthy palms with a high yield potential can be found growing in the midst of severely infested coconut palms. Because coconut is an obligatorily cross-pollinated plant, it is difficult to fix desirable genotypes. Vegetative propagation of disease-resistant, high-yielding palms through tissue culture may be of particular value here.

Biological Nitrogen Fixation

The incorporation of nitrogen fixation genes into rice by means of genetic engineering is the most ambitious subject. At least 17 genes are involved in the nitrogen fixation system, but it is still not known whether or not manipulation of such a large number of genes is possible. In view of the widespread interest in this field, problems relating to nitrogen fixation are reviewed separately here in some detail.

Wetland rice has been grown without nitrogen fertilizer for centuries. Long-term fertility experiments in both temperate and tropical regions indicate that about 50 kilograms (kg) of nitrogen (N_2) per hectare are absorbed by every crop of rice in non-nitrogen

plots. Most of this amount is compensated for by biological nitrogen fixation.

Wetlands are quite well suited to biological nitrogen fixation as the floodwater provides a suitable home for phototrophic, nitrogen-fixing organisms, and the anaerobic soil is a suitable site for anaerobic nitrogen fixation. The wetland rice root provides another site for nitrogen fixation by supporting nitrogen "rhizobacteria" (bacteria that live in and around the root), but dryland rice has little ability to do this.

The major nitrogen-fixing agents in wetland rice soils are (1) free-living, blue-green algae (BGA); (2) blue-green algae symbiotic with the water fern, Azolla; (3) heterotrophic, nitrogen-fixing bacteria associated mainly with the roots of rice; (4) heterotrophic, nitrogen-fixing bacteria growing on plant residues; and (5) rhizobia in symbiosis with aquatic legumes.

Blue-green Algae. Inoculation of BGA is widely used in Egypt, India, and Burma, and the preparation of inoculum is well developed as a small-scale village biotechnology. Because little is known about the relationship between inoculation and grain yield, studies on the availability of algal nitrogen and the ecology of blue-green algae have been initiated at IRRI, which has published an extensive survey of the literature on blue-green algae in rice soils (Roger and Kulasooriya 1980). Field experiments there show that rice yields resulting from inoculated algae are on an average 16 percent higher than yields based on uninoculated algae (Table 7).

Although the preparation and transport of inoculum is easier than for Azolla, the establishment of inoculated BGA is rather sporadic. Little is known about the conditions leading to successful inoculation and about the factors that govern the survival of the blue-green

TABLE 7 Average effects of algae inoculation on rice yields in field experiments.

	Control grain yield (kg/ha)	Increased grain yield (kg/ha) resulting from algae inoculation	Percent increase over control
Mean	3016	485	16.1
Number of data	30		87

SOURCE: Roger and Kulasooriya (1980).

algae. The existence of indigenous BGA and algae predators and mineral deficiencies in the soil would limit the establishment of inoculated BGA.

BGA is still not a familiar tool for gene engineering, since only a limited number of strains are free from bacteria, and no vector of gene information has been found. Some urgent requirements in this area are:

- Strains should be collected and selected for environmental adaptation as well as for nitrogen fixation.
- Information is needed on how to encourage the growth of desirable organisms and discourage the growth of undesirable indigenous algae and predators.
- Low-cost sources of a controlling predator such as neem (Azadirachta indica) seed should be sought.
- A possible gene transfer suitable to BGA should be sought; protoplast fusion would be one of the techniques.

Azolla-Anabaena Symbiosis. The potential of Azolla as a nitrogen-fixing, green manure crop suitable to rice culture has been recently recognized by many researchers, agricultural administrators, and farmers. Azolla pinnata can fix nitrogen at the maximum rate of about 3 kg/ha daily and A. filiculoides at the rate of 10 kg/ha daily.

Azolla requires high levels of phosphorus in the soil, a relatively mild temperature (preferably less than 30°C), good water control, and the control of insects that cause serious damage, particularly in the tropics. Areas that meet these requirements only have limited rainfed tropical rice culture.

Azolla must be maintained throughout the year as a vegetatively growing plant and transferred and inoculated in the fresh state. Azolla forms sporocarps (male and female organs), which can be used for germ plasm maintenance and propagation, but the conditions necessary to induce these artificially are not known, and artificial mating has been thus far unsuccessful. Crossing Azolla species by asexual recombination could prompt an improvement in strains, and protoplast fusion may be of value for this. This technique could also be tried between Azolla and Anabaena (Figure 4).

Azolla has been used in China and northern Vietnam for centuries, and recently trials to use it as a green manure crop were conducted in other Asian countries. The International Network of Soil Fertility and Fertilizer Evaluation for Rice (INSFFER), organized by IRRI, has examined the effect of Azolla green manuring. It confirmed that one crop of Azolla gave an increase in rice yield equivalent to that obtained by the application of 30 kg N_2/ha of urea (Table 8).

In the southern Philippines, the Azolla technique was successfully introduced over several thousand ha, and farmers reported

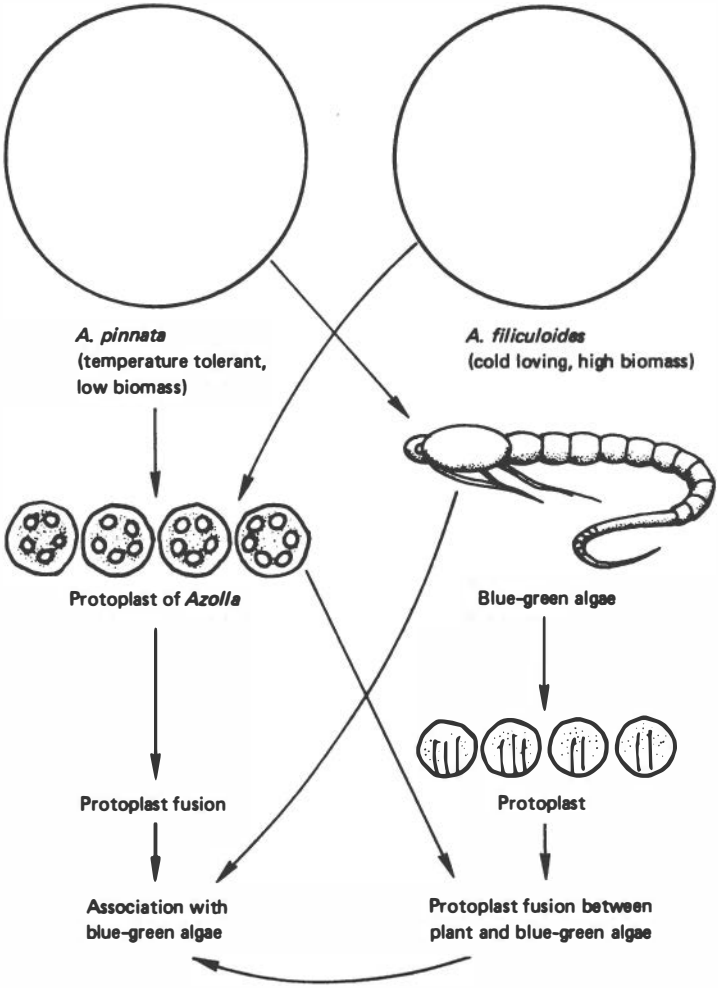


FIGURE 4 Potential application of protoplast fusion to Azolla.

TABLE 8 The effect of *Azolla* incorporation as compared with chemical nitrogen fertilizer.

	Grain yield in t/ha (index)	
	1979	1980
No nitrogen	2.6 (100)	3.2 (100)
30 kg N_2/ha urea split	3.2 (122)	3.8 (123)
60 kg N_2/ha urea split	3.7 (141)	4.2 (139)
Azolla grown before transplanting	3.2 (122)	4.0 (123)
Azolla after transplanting and incorporated	3.1 (118)	3.9 (123)
Azolla after transplanting but not incorporated	3.1 (119)	4.0 (123)
30 kg N_2/ha + *Azolla* before transplanting	3.7 (143)	4.4 (140)
30 kg N_2/ha + *Azolla* after transplanting	3.5 (134)	4.4 (135)
Azolla before and after transplanting	3.6 (139)	4.2 (137)
Number of sites	13	19

Source: INSFFER.

that two crops of *Azolla* (before transplanting and after harvest of rice) could replace chemical nitrogen fertilizer. IRRI maintains *Azolla* collections that include six species and screens strains suitable for the use in the tropics.

Because of its high protein content, *Azolla* is also a potential source of animal and fish nutrition and can be used by farmers with small land holdings.

Nitrogen Fixation Associated With Wetland Rice. Since the discovery of a sensitive assay for detecting nitrogen fixation through acetylene reduction, many scientists have reported that several non-nodulating plants, particularly gramineous plants, fix nitrogen in association with bacteria living in and around the root. To a small extent, C_4 grasses in the tropics and wetland plants (including rice) show higher activities than others.

Direct evidence of nitrogen fixation using $^{15}N_2$ has been obtained for wetland rice, and Digitaria and Paspalum. In wetland rice soils, about 5-10 kg N_2/ha may be fixed by the heterotrophic bacteria associated with rice. When various groups of nitrogen-fixing bacteria were isolated from the stem and roots of wetland rice, it was found that, at most, nitrogen-fixing bacteria occupied 90 percent of the heterotrophic bacteria isolated from the root.

Inoculation of bacteria isolated from roots (Azospirillum) to plants was sometimes positive in its impact on growth and nitrogen fixation, but the results were quite variable. Differences among cultivars in their ability to support nitrogen fixation in the root zone have been reported. Screening of varieties more capable of stimulating nitrogen fixation in the root zone is one way to increase fixation by this mechanism.

Despite many reports of the potential of nitrogen fixation in the root zone of non-nodulating plants, convincing data that pinpoint the importance of this mechanism are very limited. Further, despite many reports of the presence of Azospirillum and its inoculation, convincing data establishing the actual involvement and dominance of this bacteria in nitrogen fixation are scarce. Too little is also known of its physiology for it to be used effectively, and of the plant characteristics needed to support it more efficiently.

Sesbania and Aeschynomene are leguminous plants that can grow in an aquatic environment, and Sesbania rostrata and Aeschynomene indica form stem nodules. Because of these nodules, nitrogen fixation by S. rostrata is less inhibited by nitrogen from fertilizer than ordinary root nodule-bearing leguminous plants. Sesbania sesban is widely used in the tropics for intercropping among rice.

Because of its height, Sesbania competes with rice plants at its later growth stage, and is, therefore, grown at a late stage of the first crop of rice. It is allowed to grow further after harvest of the rice crop and is used as fertilizer for the second crop, a practice common in Vietnam. Collection of Sesbania species and strains is needed to exploit this possibility.

Comprehensive, Integrated Use of Biological Nitrogen Fixation in Rice Culture. None of the nitrogen-fixing systems mentioned thus far can be the sole source of nitrogen in rice cultivation. Combinations of possible sources should be used based on a clear understanding of the relative importance of each nitrogen-fixing agent under different

environmental, cultural, and social conditions. Azolla, for example, is more suitable for double cropping of rice in irrigated areas than in rainfed areas. In contrast, the BGA technique can be easily adopted in rainfed areas. Continuous flooding is needed for the production of large quantities of biomass by indigenous BGA. In such cases, inoculation of every crop would not be effective because of well-established indigenous BGA biomass.

Nitrogen fixation by BGA and heterotrophic bacteria could coexist because their ecological niches are different. BGA is active mainly in early stages of rice growth, while nitrogen fixation associated with root bacteria is active during the reproductive stage.

The final goal of the technology of nitrogen fixation is, of course, to minimize dependence on chemical nitrogen fertilizer through the use of biofertilizers. IRRI scientists believe that the continuation and strengthening of currently operating systems of interaction between nitrogen-fixing bacteria and rice roots is the most feasible way to increase the nitrogen fixation associated with rice. In view of the fact that the living root is inhabited with 10^7-10^9 cells of bacteria per gram dry root, the introduction of nitrogen-fixing genes by genetic engineering techniques is worth trying. Most of the heterotrophs isolated from rice roots can fix nitrogen, although their ability to fix nitrogen is 10 times lower than that of the most active nitrogen-fixing bacteria. If the nitrogen-fixing activity of these "rhizobacteria" of rice could be increased, and if they were able to excrete NH_4 to outside cells (derepression), progress may be expected.

Another encouraging feature is that most of these "rhizobacteria" can utilize the hydrogen gas produced in a paddy field from organic matter. If the ability of these bacteria to use hydrogen as an energy source could be improved, wetland rice root would be quite unique as a recycler of energy wasted as hydrogen. IRRI is willing to cooperate in such research with scientists and institutions interested in increasing rice yields without an associated increase in the consumption of mineral fertilizers.

Protoplast Fusion

The University of Nottingham and IRRI are undertaking a joint project to utilize the scope offered by protoplast fusion in the following areas:

- Hybrid rice production. The People's Republic of China is cultivating nearly 6 million ha of hybrid rice, based on cytoplasmic male sterile lines identified in varieties adapted to temperate conditions. Male sterile lines and restorers should also be developed for varieties

characterized by adaptation to tropical conditions as well as by good combining ability. In addition, the scope for heterosis in root growth and early seedling vigor must be studied, possibly using the tools of somatic and sexual genetics. It will be necessary to use anther culture for production of haploids to maintain the chromosome number at the diploid level (Figure 5).

- Protoplast fusion and hybrid cell regeneration will be used in interspecific hybridization in Azolla. Attempts will also be made to bring about fusion between the cells of Azolla and Anabaena.

Genetic Engineering

There has been an impressive increase in the amount of published material on the application of genetic engineering research to crop improvement. A conference convened in 1980 by the Rockefeller Foundation on "Genetic Engineering for Crop Improvement" resulted in an excellent account of possibilities and problems (Rachie and Lyman 1981). Meetings organized by the U.N. Industrial Development Organization for developing a project on the establishment of an International Centre for Genetic Engineering and Biotechnology have also provided valuable information, and this author (Swaminathan 1981) has reviewed the potential value of integrating emerging and current techniques of plant breeding.

It is clear from a survey of the published literature that knowledge is lacking about the structure and organization of genetic information in plants, as well as about the processes related to gene regulation and expression. A somatic cell population directly isolated from the plant represents a valuable source of genetic variation of interesting, practical consequences. If a better understanding is gained of the biochemical processes that control cell differentiation and the development of organs with specialized functions in whole plants, cellular totipotency (the ability to grow whole plants from single cells) could be put to many uses.

An area of research that needs urgent attention is the development of fast-growing fuelwood trees. All aspects of research related to "energy agriculture" deserve the highest priority if further damage to soil health as a result of deforestation and soil erosion is to be arrested in the tropics and subtropics. For example, could annual varieties of bamboo be developed by crossing bamboo with sugarcane and other appropriate crops? Leguminous shrubs and trees provide considerable opportunities for improvement. Now that an International Research Institute for the Winged Bean (Psophocarpus tetragonolobus) has been established in Sri Lanka, it should be possible to initiate a research program designed to develop erect and self-standing strains of winged bean.

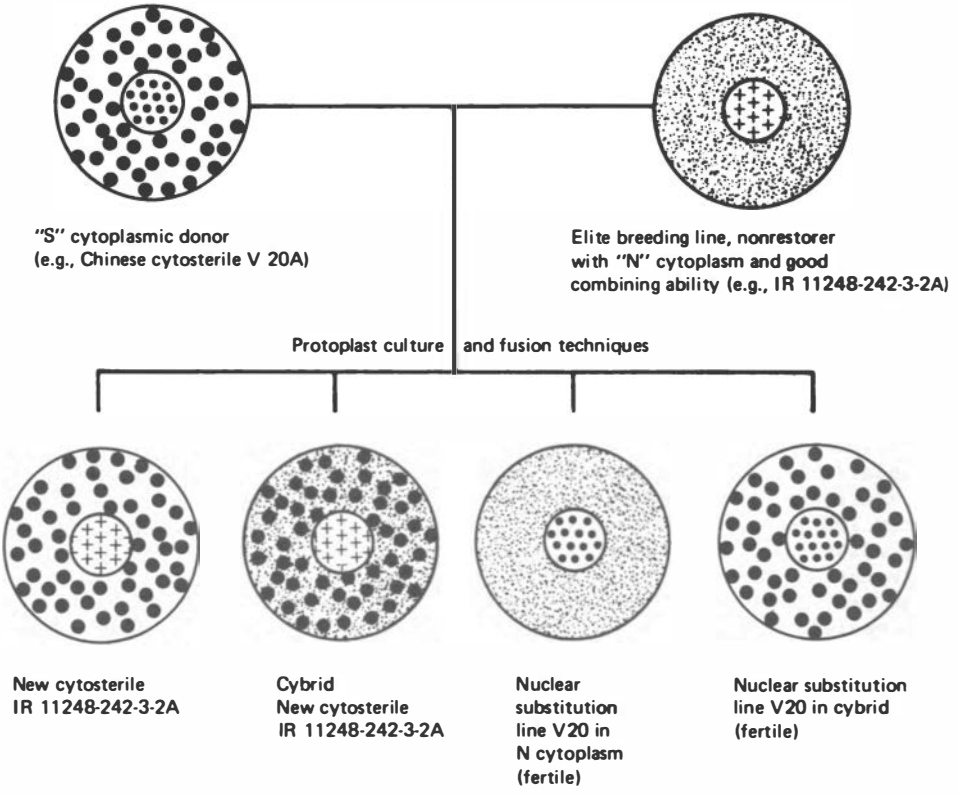


FIGURE 5 Use of protoplast culture and fusion techniques to develop new cytoplasmic male sterile lines in rice.

In the choice of problems, it is obvious that those that defy solution through already available techniques should be selected. Solving a problem rather than worshipping a tool should be the goal. This does not mean that speculative and imaginative research by gifted scientists, which may or may not yield the results sought, should not be supported. In the interest of generations yet to be born, imaginative research should always receive support without a prior judgment about its ultimate value.

NEEDS OF DEVELOPING COUNTRIES

Developing countries are naturally concerned about the needs to avoid technological obsolescence and to profit from the most recent advances in technology. While science helps to advance the frontiers of knowledge and hence is of value regardless of the source of the new knowledge, for technology to be effective in advancing production it must be compatible with specific ecological, socioeconomic, and sociocultural factors. Technologies that can help purchase time and facilitate quantum jumps in production and prosperity are of particular interest to developing countries. Biotechnology has raised considerable hopes in this respect.

For Third World countries to gain both a realistic appreciation of the usefulness of the new techniques and practical benefits from them, they must receive assistance in the following areas.

Choice of Research Priorities and Implementation of Programs

To avoid later disappointments, careful thought should be given to the choice of research fields where new tools will help to accelerate progress and solve problems that have so far defied solution. This is particularly important if research goals have applied objectives. The following institutional structures may be helpful in this respect:

- International and regional brain banks. Banks such as the International Bank for Reconstruction and Development (World Bank) and the Asian Development Bank were established chiefly to render financial support to worthwhile development projects. Thought must now be given to the organization of "brain banks" that would supply objective and up-to-date advice on technology transfer. This objective has become particularly urgent in the context of the growing commercialization of skills and know-how and the enlarging secrecy around the knowledge base of discovery. To be successful, "brain banks" obviously need the support and guidance of leading scientists and technologists who are not only authorities in their respective fields of specialization but are also humanists.
- Institutional cooperation. Appropriate institutions in developing and developed countries can enter into cooperative arrangements to maximize the benefits of their complementary strengths. This will be very helpful to purchase time and sharpen priorities. The Board for International Food and Agricultural Development of the U.S. Agency for International Development is an important program in this field.

- Investigator-initiated tie-up. In addition to interinstitutional collaboration, there should be scope for individual scientists in developed and developing countries to collaborate on projects of mutual interest. Simplified administrative procedures and appropriate financial support mechanisms are needed to foster such collaboration.
- Periodic seminars and workshops. The organization of seminars and workshops in developing countries under the joint auspices of the science academies of developed and developing countries will be helpful to reviewing progress, clarifying issues, and determining priorities. Periodically, seminars should also be held for policy makers and political leaders to familiarize them with the state of the art in biotechnology and to help them make investment decisions based on a scientific understanding of the likely returns.

Research Resources

The major research resources needed for organizing effective national programs are: (1) trained manpower, (2) chemicals and equipment, and (3) information and literature. Assistance in these areas will be of value for strengthening national research programs. In manpower development, priority should be accorded to filling critical gaps in internal competence. Training and help will also be needed in the design of laboratories with adequate safety measures. Where possible, assistance should be given in the manufacture of enzymes, radiochemicals, etc., within the country. The information network could help spread knowledge about the scientific aspects, legal aspects (patent regulations, plant breeders' rights, etc.), and commercial aspects of biotechnology applications. For imparting training and for the dissemination of information, institutions like INTSOY (International Soybean Program) could be promoted.

International Centers and Programs

The United Nations and other organizations are presently considering several ideas including the establishment of international and regional centers. The immediate establishment of a modified version of the TOKTEN scheme (Transfer of Know-how Through Expatriate Nationals) sponsored by the U.N. Development Program, will be useful. A review of experience gained under the TOKTEN scheme, made at a workshop held in Islamabad, Pakistan, in January 1982, has resulted in several useful recommendations. The TOKTEN scheme itself needs to be broad-based in some selected fields like biotechnology so as to include not only expatriate nationals but also other appropriate experts.

In December 1982, the opportunities now available for the appropriate integration of emerging and traditional technologies in such fields as biotechnology, microelectronics, computer sciences, and satellite imagery and communication will be discussed at a workshop to be held at IRRI under the sponsorship of the U.N. Advisory Committee on Science and Technology for Development. It is through the planned and purposeful integration of the old and the new that we can sow the seeds for sustainable development.

REFERENCES

Bollinger, W. H. (1980) Sustaining renewable resources: Techniques from applied botany. In: Techniques from Applied Botany. Academic Press, New York, New York, USA.

FAO (Food and Agriculture Organization of the United Nations) (1979) Agriculture: Toward 2000. FAO, Rome, Italy.

IRRI (International Rice Research Institute) (1981) Annual Report. IRRI, Los Baños, Philippines.

Rachie, K. O. and J. M. Lyman (1981) Genetic Engineering for Crop Improvement. Proceedings of a conference organized by the Rockefeller Foundation, May 12-15, 1980, New York, New York, USA.

Roger, P. A. and S. A. Kulasooriya (1980) Blue-green Algae and Rice. International Rice Research Institute, Los Baños, Philippines.

Sprague, G. F., D. E. Alexander, and J. W. Dudley (1980) Plant breeding and genetic engineering: a perspective. Bioscience 30:17-21.

Stewman, S. and D. Lincoln (1981) Recombinant DNA breakthroughs in agriculture, industry and medicine. Futures 13:128-140.

Swaminathan, M. S. (1981) Plant Breeding in Preparation for the 21st Century. Meghmad Saha Medal Lecture, Indian National Science Academy, New Delhi.

Swaminathan, M. S. (1982) Report of the Fuelwood Study Committee of the Planning Commission. Government of India, New Delhi.

PART TWO
Reports of the Working Groups

Vaccines

INTRODUCTION

Infections are a major cause of human and animal morbidity and mortality in developing countries. Up to 40 percent of the infants in many communities die before reaching 5 years of age, usually directly or indirectly from infection. Bacterial, viral, and parasitic pathogens take heavy tolls in human suffering and subject livestock owners to economic loss and uncertainty.

Some of these pathogens infect both humans and animals. In many developing countries animals live in close association with humans. Animals and fowl provide reservoirs for viruses and other pathogenic organisms that either infect man directly or become infective for humans by means of genetic recombination mechanisms. Advances in molecular biology have provided new tools to detect variation in human and animal viruses and, thus, possibly elucidate obscure relationships.

Conventional means of controlling major communicable diseases include chemotherapy, vector control, vaccination (against specific organisms), health education, and slaughter of infected livestock. For those diseases without a satisfactory vaccine, control becomes very expensive and is particularly difficult for countries with few resources. Vector control requires continuous importation of pesticides; drugs for parasitic diseases are often toxic and ineffective, and reinfection may occur at any time; education about personal protective measures induces little compliance; and livestock slaughter can mean economic ruin for small farmers.

Although poverty, chronic undernutrition, illiteracy, lack of medical care, and contaminated water and food all contribute to the spread of infectious organisms, immunization remains one of the most economical means of preventing specific diseases. An effective vaccine can produce long-lasting immunity against certain diseases, in some cases a lifetime. A small number of doses is usually required for protection. Hospitals or health clinics are not prerequisites for

a successful vaccination campaign in remote rural areas, although a health or veterinary infrastructure facilitates access by the population and improves immunization coverage. Overall, vaccines offer immense benefit for comparatively low cost, a primary consideration for decision makers in developing countries.

Vaccines for the prevention of human and animal diseases are produced by any one of several conventional methodologies that have become rather static with regard to efficacy, safety, stability, and cost. The very good vaccines such as those for the human diseases—yellow fever and measles--or the animal diseases--rinderpest and hog cholera--have been in use for more than 10 years and have helped greatly reduce the incidence of these diseases in many areas of the world. However, some vaccines of questionable efficacy are also still in use because of the reduction in research that occurs once an effective vaccine is in use.

Current vaccines are attenuated organism vaccines such as modified-live virus measles vaccine, killed whole organism vaccines such as pertussis vaccine, inactivated toxins such as diphtheria toxoid, and subunits or "split products" derived from agents such as influenza virus. Problems of impotency, instability, reactogenicity (adverse side effects), and actual transmission of disease (reversion of attenuated organisms to wild type and inadequate inactivation of organisms) are continuing problems.

Contributions of Biotechnology to Vaccine Development

The problems and shortcomings just outlined continually alert vaccine researchers to the need for better products. Technical breakthroughs are applied quickly and with great effect. For example, when the cell culture technique was developed 30 years ago, polio vaccine development, testing, and worldwide distribution occurred with great rapidity. The same is likely to happen as breakthroughs in biotechnology yield new vaccine products.

Research strategies for development of better, cheaper, and safer vaccines are now being developed using the new "biotechnology." Through the use of monoclonal antibodies and recombinant DNA technology (see the working group report on monoclonal antibodies), it is now possible to define and produce immunogenic components much more quickly. Genetically engineered vaccines should be more stable and safer than conventional vaccines, as only a single or small number of components is needed rather than the entire infectious organism. Actual vaccine manufacture in many cases should be cheaper because standard fermentation techniques can be used as opposed to the costly and sophisticated tissue culture techniques currently required (Bachrach 1981).

Specific research approaches for development of new vaccines include:

- Recombinant DNA technology and its application to large-scale antigen production
- Antigenic peptide production by solid-phase synthesis methods
- Related development of adjuvants, immunoenhancers, and novel delivery systems (liposomes, slow-release methods, etc.).

Each of these research elements is crucial for the development of efficacious, inexpensive protein immunogens. Similarly, for the development of new, live attenuated organism vaccines, the use of proscribed mutations of pathogenic microorganisms represents a novel approach to obtaining safe, potent vaccines (Henderson 1981).

NEW FUNDING PRIORITIES IN TARGETED VACCINE DEVELOPMENT USING BIOTECHNOLOGY

For which diseases does biotechnology offer the hope of vaccine development? This working group began by listing 30 human and 19 animals diseases of importance in developing countries (see the working group report on animal production for additional discussion of animal diseases). Three criteria were then used to help classify each disease as high, medium, or low priority for new or additional vaccine research and development funded by the Agency for International Development (AID) and other bilateral and multilateral donors. A fourth criterion was added which was specific either to human or animal diseases. Tables 1 and 2 show the results of these deliberations. Criteria considered for each disease are:

- Current vaccine status, based upon the availability of an effective, inexpensive, safe vaccine
- Feasibility of biotechnological approaches to developing a better, cheaper, and safer candidate vaccine within the next 5 years
- Current funding for vaccine research and development--whether available or likely to be available in sufficient amounts from sources other than AID (for example, World Health Organization, National Institutes of Health, pharmaceutical companies). This criterion was considered in relation to the relative importance of the disease in developing countries.
- Public health significance [for human diseases] as measured by incidence; prevalence; morbidity; mortality; or severity
- Economic losses [for animal diseases] in terms of livestock production or decrease in availability of animal protein.

TABLE 1 Assessment of new funding priorities in vaccine development: human diseases.[a]

Disease	Public health significance	Current vaccine status	Feasibility of using biotechnology in vaccine development	Current funding	Priority for new funding
Bacterial respiratory diseases	High	Fair	Good	Low	High
Bacterial enteric diseases	High	Low protection	Good	Medium	High
Dengue	High	None	Good	Medium	High
Japanese encephalitis	High	Poor	Good	Low	High
Rabies	High	Variable	Good	Low	High
Chlamydial infections	High	None	BRN	Low	High
Malaria	High	None	Good	Medium	High
Leishmaniasis	Medium	None	BRN	Low	High
Tuberculosis	High	Questionable	Medium	Low	High
Bacterial meningitis	High	Good	BRN	Low	Medium
Respiratory syncytial virus	High	None	BRN	Fair	Medium
Hemorrhagic fevers	High	None	BRN	Fair	Medium
Chagas disease	Medium	None	BRN	Fair	Medium

TABLE 1 (Continued)

Disease	Public health significance	Current vaccine status	Feasibility of using biotechnology in vaccine development	Current funding	Priority for new funding
Diphtheria-pertussis-tetanus	High	Good	NA	NA	Low
Gonorrhea	High	None	BRN	Fair	Low
Leprosy	High	Low	Medium	High	Low
Typhoid	Medium	Fair	BRN	Fair	Low
Hepatitis A	High	None	Medium	Low	Medium
Hepatitis B	High	Good	Good	High	Low
Herpes	Medium	None	Medium	High	Low
Measles	High	Excellent	NA	NA	Low
Mumps	Medium	Good	NA	Low	Low
Polio	High	Good	Good	High	Low
Rotaviruses	High	None	Low	High	Low
Rubella	Medium	Good	NA	High	Low
Yellow fever[b]	High	Good	NA	Low	Low
Typhus	High	Low	BRN	Low	Low
Filariasis	High	None	BRN	Medium	Low
Schistosomiasis	High	None	BRN	Good	Low

TABLE 1 (continued)

Disease	Public health significance	Current vaccine status	Feasibility of using biotechnology in vaccine development	Current funding	Priority for new funding
African trypanosomiasis	Medium	None	BRN	Fair	Low
Rift Valley fever	SEE ANIMAL SECTION				

[a]Abbreviations: BRN--basic research needed; NA--not applicable.
[b]See Pan American Health Organization (1981).

TABLE 2 Assessment of priorities in vaccine development: animal diseases.[a]

Disease	Economic losses in developing countries	Current vaccine status	Feasibility of using biotechnology in vaccine development	Current funding	Priority for new funding
Tuberculosis	High	Questionable	Medium	Low	High
Neonatal diarrhea	High	Poor	High	Medium	High
Bacterial respiratory disease	High	Poor	BRN	Medium	High
African swine fever	High	None	High	Low	High
Hemotropic diseases	High	Poor	BRN	Low	High
Rabies	High	Variable	High	Medium	Medium
Rift Valley fever	High	Good	High	Medium	Medium
Newcastle disease	High	Poor in LDCs	Variable	Low	Medium
Foot-and-mouth disease	High	Medium	High	High	Low

TABLE 2 (continued)

Disease	Economic losses in developing countries	Current vaccine status	Feasibility of using biotechnology in vaccine development	Current funding	Priority for new funding
Rinderpest	High	Excellent	NA	Low	Low
Vesicular stomatitis virus	Low	None	High	Low	Low
Bluetongue	Low	None	High	Low	Low
Hog cholera	High	Good	Good	Low	Low
African horse sickness	Medium	Medium	High	Low	Low
Equine encephalitis	High	Good	Good	Medium	Low
Malignant catarrhal fever	Low	None	Medium	Low	Low
Pulmonary adenomatosis	Medium	None	Low	Low	Low
Pseudorabies	Medium	Poor	Medium	Low	Low
Brucellosis	High	Medium	BRN	Low	Low

[a]Abbreviations: BRN--basic research needed; NA--not applicable.

Based on the above criteria, the working group rated the relative importance of each disease for new funding by AID or other donors.

The following sections identify eight human and four animal diseases as well as one zoonotic disease (affecting both humans and animals), which have been selected and recommended as high priority for new funding (see Table 3). This section should be read with the following in mind:

- A targeted research effort aimed at vaccine development utilizing biotechnology should be funded for each high-priority disease. Such a research effort would involve: (1) identification and characterization of immunogenic antigens, (2) synthesis and production of such antigens through biotechnology, and (3) formulation of a candidate vaccine suitable for testing.
- This targeted research should be conducted in collaboration with scientists from developing countries. A major advantage of biotechnology and genetic engineering is that they do not require highly sophisticated laboratory equipment; for the most part, well-equipped, clinical laboratory facilities will suffice. However, such an effort will require an interdisciplinary team, reliable laboratory reagents, and appropriate animal facilities for testing. Few developing countries possess the capability to produce promising laboratory antigens in quantities sufficient for early clinical testing and subsequent mass production. In addition, reliable quality control for vaccine production is lacking in many parts of the world.
- Training should be an integral part of the collaborative research effort. The key requirement for research and development of new vaccines is trained, experienced scientists. Both long-term graduate work by developing country scientists in developed country universities and short-term training in any appropriate site are equally important for the transfer of knowledge and techniques.

Human Diseases

Rabies (Human). Rabies is a fatal disease that continues to be a serious problem in some developing countries. Although vaccination is part of the post-exposure treatment, current vaccines have undesirable, dangerous side effects. The Merieux Institute has developed an effective vaccine, but its high cost limits its widespread use. Improvement of this vaccine is an ideal project for developing countries with good production laboratories. Protein G has been cloned, and prospects for a vaccine by the genetic engineering route are therefore high.

TABLE 3 Disease problems of high priority for vaccine development.

Disease	Years required to develop candidate vaccine[a]
HUMAN	
Rabies	2-5
Dengue	5
Japanese encephalitis	5
Bacterial respiratory diseases	Greater than 5
Bacterial enteric diseases	Greater than 5
Chlamydial infections	Less than 5
Malaria	2-5
Leishmaniasis	Less than 5
ANIMAL	
Neonatal diarrhea (bacterial and viral)	5
Bacterial respiratory disease (bacterial and viral)	Less than 5
African swine fever (ASF)	Greater than 5
Hemotropic diseases[b]	5
ZOONOTIC (Human and Animal)	
Tuberculosis	Greater than 5

[a]These figures are estimates based on the assumption that adequate funding is available from AID or other sources.
[b]See the later section, "Hemotropic Diseases of Animals (Specifically Babesiosis and Anaplasmosis)."

RESEARCH OBJECTIVES:

- Development of a better, cheaper, and safer vaccine for pre- and post-exposure treatment. In the meantime, AID and other donors should encourage production of rabies vaccine in developing countries.
- Improvement in production techniques of current vaccine strains to lower costs.
- Continued use of genetic engineering to clone the viral glycoprotein and test it as a vaccine.
- Organic synthesis of peptides for vaccine development.

Dengue. Dengue fever and associated hemorrhagic syndromes are caused by four closely related viruses (designated dengue serotypes 1, 2, 3, and 4) that are transmitted by Aedes aegypti mosquitoes. The disease is highly endemic in tropical Asia and certain Pacific islands and endemic in Central and South America and the Caribbean region. Dengue hemorrhagic fever (DHF) and DHF with shock syndrome have resulted in high mortality rates among children in tropical Asia. Recently, DHF appeared in Cuba.

No vaccine is available for this disease, and current efforts are not, to the working group's knowledge, utilizing biotechnology to develop one. An effective vaccine would have to confer immunity to all four virus serotypes. It is therefore suggested that AID and other donors increase support to institutions undertaking dengue fever research.

RESEARCH OBJECTIVE:

- Development of vaccine based upon viral surface glycoproteins using molecular cloning.

Japanese Encephalitis. Large outbreaks of Japanese encephalitis have occurred in Southeast Asia, as well as epidemics during the last decade in Thailand, Burma, and India. This disease is usually found in rural and semiurban areas. The vaccines presently available do not offer complete protection and are costly. A simple glycoprotein present on the virus surface is considered the main immunogenic component of the agent. Direct cloning and expression of this viral gene seems possible, but no efforts known to the working group are being made at the moment to utilize such biotechnological approaches.

RESEARCH OBJECTIVES:

- Identification, characterization, and production by biotechnological techniques of the protective antigen of the Japanese encephalitis virus
- Studies of the efficacy of this glycoprotein product as a human vaccine.

Bacterial Respiratory Diseases. The primary bacterial respiratory diseases in man are caused by Streptococcus pneumoniae, Hemophilus influenzae Type B, and Bordetella pertussis. These bacteria are a major cause of morbidity worldwide in children under 5 years of age.

The widely used and efficacious killed whole cell vaccine for pertussis is effective in infants. However, it is significantly reactogenic, frequently causing fever, prolonged crying, and, in extremely rare cases, neurologic sequelae and death. Pertussis has

been dropped from the general pediatric vaccination program in England and some European countries primarily because of its reactogenicity.

The existing efficacious vaccine for pneumonia is compounded of capsular polysaccharides from the major serotypes. This vaccine is recommended primarily for older persons and those who have repeated respiratory infections. The immunity induced by this vaccine is of limited duration.

No effective vaccine is currently available for Hemophilus influenzae. Capsular polysaccharide has been shown to protect against the disease; however, the polysaccharide is weakly immunogenic in children under 2 years of age, the age of peak incidence. H. influenzae is a major cause of acquired mental retardation.

RESEARCH OBJECTIVES:

- Improvement of existing vaccines for pneumonia and pertussis by standard vaccine methods and the use of biotechnology.
- Development of a vaccine for H. influenzae Type B using biotechnology; there are specific needs in the areas of immunopotentiation and carrier-hapten presentation.

Bacterial Enteric Diseases. These are a major cause of childhood mortality in large areas of the world where rehydration therapy is not promptly provided. The major causative agents are Shigella, Salmonella, Escherichia coli, Campylobacter jejuni, and Vibrio cholerae. Experimental vaccines have been obtained for the first three microorganisms, although the protective agents are not known. Basic research is needed to identify the relevant antigens so that effective vaccines can be obtained. It is recommended that AID increase support for diarrheal disease centers in developing countries in order to enhance their capabilities in biotechnology-related research. One example of such a center is the International Center for Diarrheal Disease Research in Bangladesh.

RESEARCH OBJECTIVES:

- Improvement of experimental vaccines for E. coli, Salmonella, Shigella, and V. cholerae by standard methods.
- Development of defined, lethal deletion mutants of pathogenic enterobacteria. Such mutants serve as excellent immunogens because they do not need to be inactivated and yet are innocuous because they cannot replicate.
- Development of bioengineered protein vaccines as has been done for enterotoxigenic E. coli diseases of neonatal pigs and calves.

Chlamydial Infections. A widespread public health problem in many developing countries, chlamydial infections are the leading cause of blindness (trachoma). Chlamydia spp. resemble bacteria but reproduce only within living animal cells. Infection is spread by flies, through contact with ocular discharges, or through sexual contact. Adults may develop urethritis or conjunctival scarring, while babies can develop pneumonia or severe conjunctivitis.

Chemotherapy is available to treat individual cases but is cumbersome and expensive to administer. Post-infection immunity has been shown in humans, and some investigators now believe that immunity is cell-mediated. No vaccine is available, although a monoclonal antibody diagnostic test for Chlamydia trachomatis may soon be prepared commercially.

RESEARCH OBJECTIVES:

- Basic identification and characterization of candidate immunogenic antigens of Chlamydia trachomatis and C. psitticae
- Production of candidate protective antigens using biotechnological approaches.

Malaria. Malaria is the most important infectious disease in the world today. There are an estimated 150 million cases per year and a very high mortality rate for children (1 million deaths in Africa alone per year). Chemotherapy is available, but the emergence of drug-resistant mutants of the parasite poses a major health threat, especially in Southeast Asia and South America. In addition, vector control is thwarted by the increasing prevalence of insecticide-resistant mosquitos.

No vaccine is currently available for use against malaria. Design of a vaccine must take into account that the malaria parasite has three specific stages in its life cycle--sporozoites, erythrocytic forms, and gametes--in which different, specific antigens are being expressed. Thus a vaccine against a particular stage, for example, sporozoite, will not provide immunity against the other stages. In addition, there is mounting evidence of the existence of both species- and strain-specific antigens in malaria.

It is recommended that AID and other donors fund research directed toward the following specific and important areas of malaria vaccine development that are currently not supported.

RESEARCH OBJECTIVES:

- Identification, characterization, and molecular cloning of Plasmodium falciparum gamete, erythrocyte, and sporozoite

antigens, which are candidate protective immunogens, using biotechnological methods
- Basic research on P. malarie, P. ovale, and P. vivax for identification of specific antigens.

Leishmaniasis. Kala-azar (visceral leishmaniasis) and cutaneous and mucocutaneous leishmaniasis are sandfly-transmitted protozoan diseases with grossly underestimated public health importance in South America, Africa, and the Middle East. Mucocutaneous leishmaniasis is an increasing problem in newly colonized forested areas. There is evidence of natural immunity, and development of a vaccine is being attempted by conventional methodologies. Chemotherapeutic agents may be very toxic, and it is difficult to maintain residual insecticides by periodic application.

RESEARCH OBJECTIVE:

- Identification and cloning of antigens of Leishmania varieties.

Animal Diseases

Neonatal Diarrhea. This disease complex affects swine and cattle and is caused by bacterial as well as viral agents. The disease induced by bacteria displays high prevalence globally and is generally caused by enterotoxigenic strains of Escherichia coli. Monoclonal antibodies that bind to the pili of the enterotoxigenic strains of E. coli are being licensed for use in Canada. Genetic engineering has already been used to produce protective antigens. In fact, a vaccine against swine and calf diarrhea has been developed in this manner and is commercially available in Europe.

RESEARCH OBJECTIVE:

- Development of vaccines for enterobacterial and viral diarrhea agents. Enterobacterial agents should be emphasized given the current research support for viral diarrheas. Biotechnological approaches promise valuable new immunogens in each of these categories and concomitant new antigens for use in diagnostic tests.

Bacterial Respiratory Disease. This disease complex, which has an obscure etiology involving viral, bacterial, and environmental stress factors, is the single most important cause of morbidity, mortality, and economic loss in all livestock in the developed and developing world. Fatal respiratory disease usually involves

overwhelming Pasteurella hemolytica, P. multocida, or Bordetella bronchiseptica infection. Conventional immunoprophylactic approaches and antibiotic treatment regimens are ineffective, and in many settings research groups are searching for new ideas. Clearly, novel approaches based upon genetically engineered species of Pasteurella immunogens are worthy of investigation.

RESEARCH OBJECTIVES:

- Identification and characterization of the significant immunogenic antigens of P. hemolytica, P. multocida, and Bordetella bronchiseptica
- Production of such immunogens by bio- or organic synthesis.

African Swine Fever (ASF). ASF is endemic in several African countries, Spain, and Portugal, and results in severe economic losses. In 1978, ASF spread to the Dominican Republic, Haiti, and Brazil, but it has now been eradicated from the Dominican Republic, and similar efforts are under way in Haiti. The highly virulent strain of the virus, which causes practically 100 percent mortality among domestic pigs, is perpetuated in three species of African wild pigs, which develop an asymptomatic infection. A less virulent strain of ASF virus results in lower (20-30 percent) mortality and is capable of inducing chronic infection.

As no vaccine is available for this infection, slaughter of all pigs found in infected areas is the only measure for elimination of the disease. However, double fencing of domestic pigs in Kenya is greatly reducing the spread of the disease from wild pigs such as warthogs. Soft ticks have been implicated as vectors and reservoirs of the virus, and their possible role in persistence of the virus in infected areas deserves further investigation.

RESEARCH OBJECTIVE:

- Vaccine development using biotechnology. It is conceivable that vaccines can be developed, but additional information is required on the immunology of the disease, the biology and molecular properties of the virus, and its etiology.

Hemotropic Diseases of Animals (Specifically Babesiosis and Anaplasmosis). Hemotropic diseases of livestock are major impediments to the economic production of meat, milk, and fiber in many parts of the developing world. Major efforts are being made to control trypanosomiasis and theileriosis (East Coast fever), but tick-borne babesiosis and anaplasmosis (variety of vectors) are of great importance and, although poorly understood, are the subject of little research. Beginnings have been made in the culture of protozoan

Babesia spp. but not of rickettsial Anaplasma. An isolated glycoprotein of Babesia spp. has tentatively been identified as the immunogenic agent.

These organisms are clearly good candidates for the biotechnological approach to antigen identification and synthesis—a similar recommendation was made by an AID/Rockefeller Foundation Task Force in May 1982 (Rockefeller Foundation 1982). Development of vaccines for these diseases could lead to major improvements in livestock production in many parts of the world.

RESEARCH OBJECTIVE:

- Isolation, characterization, and production of protective antigen using biotechnological approaches.

Zoonotic Disease

Tuberculosis (Mycobacteria). Tuberculosis (TB) is highly prevalent worldwide. The major economic impact of this disease on livestock production is compounded by its zoonotic implications. Diseased cattle can transmit tuberculosis to man by way of milk and through direct exposure, and this most commonly occurs among herdsmen and nomadic populations in developing countries.

Currently available diagnostic tools using protein derivatives appear to be adequate to detect infected animals in developing countries where the prevalence of the disease is high. In countries where prevalence is low, however, more specific and reliable diagnostic tests are needed. The alternatives available for control in livestock are herd testing and slaughter, identification and segregation of cattle, and pasteurization of milk.

Research on improving a vaccine for animal TB has direct as well as indirect applicability to human tuberculosis. Control of animal tuberculosis has been shown to reduce the disease reservoir for humans. One possible approach to vaccine production may be the identification of the immunogenic components of the organism and their synthesis by organic recombinant DNA techniques once they have been identified.

RESEARCH OBJECTIVES:

- Development of improved diagnostic tools to distinguish in humans BCG (Bacille Calmette Guérin) vaccine reactions from those resulting from infection
- Assessment of the efficacy of currently used BCG vaccine

- Improved production of TB-specific antigens, including purification of PPD (purified protein derivative)
- Development of an effective vaccine, including bio- or organic synthesis of the immunogen, that can be used in areas of high incidence (especially in animals) in developing countries. Such a vaccine is desirable but not considered a near-term possibility.

GENERAL ISSUES IN THE DEVELOPMENT AND MANUFACTURE OF VACCINES

Manpower and Training

In recent years, there has been a tendency toward greater exclusivity regarding patenting of the crucial elements of important biologicals. As a result, free exchange of and access to information is not easily available. Developing countries in South America, Africa, and Asia, however, are more aware of the importance of prophylaxis in public health and are developing a reasonably competent cadre of scientists capable of assimilating new biotechnologies and putting them to good use in their own countries. AID and other donors can be effective agencies for facilitating and catalyzing the transfer of such technologies through the support of (1) secondment of scientific manpower from developed countries, (2) well-designed training programs, and (3) career development awards to identify qualified personnel from developing countries.

Commercial Vaccine Production in Developing Countries

Several new approaches to the commercial production of vaccines have resulted from biotechnology research. Recombinant DNA technologies are being used to produce bacterial clones and synthesize protein antigens for use as vaccines. One example of this approach is the cloning in E. coli of a protective antigen for foot-and-mouth disease. Commercial production of such vaccines involves conventional fermentation technology currently in use in developing countries. Since only a small portion of the infectious organism's DNA is encoded in the bacteria, special containment facilities are not required to produce vaccines against highly virulent diseases. Additional advantages should include a high degree of stability, consistency of the product, a high yield of vaccine antigen, and low overall production costs.

Several potential problems are, however, associated with this approach. For example, a vaccine could become contaminated with bacterial products other than the desired antigen, resulting in

allergic sensitization of the recipient or other undesirable effects. The antigens produced may be too specific or restricted to provide protection against all forms of the disease. Particularly in the case of some viral diseases where cell-mediated immunity is the protective mechanism, highly specific antigenic components may not be effective. Bacteria are unable to glycosylate proteins, and glycosylation may be necessary for appropriate immunologic recognition of certain antigens. Thus alternatives to bacteria such as yeast must be used where glycosylation is important. However, they may lack the mechanism for assembling and processing complete antigens. Commercial production of vaccines by large-scale yeast culture could be easily accomplished by existing technology.

Another approach to production of vaccines is genetic engineering of mammalian cell lines, which may be particularly necessary for some viral antigens. Production systems using these cell lines would require closely monitored tissue culture processes. Potential hazards include viral infection of the culture system—or identification of latent viruses in the cell line--and the possibility of inducing autoimmunity by cell antigens copurified with the vaccine antigen. Commercial production of this type would be difficult to implement in some developing countries.

Organic synthesis of protein antigens for use as vaccines is potentially advantageous for vaccine production and can be accomplished without large, costly production facilities or equipment. Synthetic vaccines would be amenable to highly effective quality control. The development of such vaccines requires that the structure of the protective antigen be well understood and the amino acid sequence known. Extensive immunologic manipulation of such peptides will be necessary to confer sufficient immunogenicity to them. These manipulations may include carrier-hapten preparation, incorporation in liposomes, or the use of adjuvants.

Technology Transfer

Some developing countries may decide to manufacture new vaccines locally rather than import them. Genetically engineered cell lines for vaccine production may be acquired through royalties to or licensure from the inventors. In such cases, the licensure agreement should include training of production and quality control personnel by the inventing institute or corporation. Such agreements could also include continuing product support by both parties. A second possibility would be the establishment in developing countries of facilities owned by the inventor. An alternate arrangement would be for developing countries to purchase the antigen by means of a supply contract. The purchaser could thus avoid most of the capital investment costs while retaining control of dosage, vaccine

formulation, and method of administration (for example, jet gun, adjuvant system).

Several developing countries are preparing to invest in production facilities for new vaccines. In such instances, industrial scale-up will be required (for example, microcarrier fermentation, immunochromatography). It would also be useful to promote a program of collaboration and training between biologic product manufacturers in developed and developing countries. This might be possible through such organizations as the Pharmaceutical Manufacturers Association in the United States and the State Pharmaceutical Corporation in India, or between individual companies (Institute of Medicine 1979).

Vaccine Testing and Clinical Trials

While there is no question that the testing and field trials of vaccines designed to prevent important tropical diseases must be conducted in the affected countries, it is also important that full information and protocols of laboratory and animal testing be provided to the appropriate health authorities of the country where the clinical trials are to be held. Furthermore, it would be useful to involve host country scientists in the initial stages of laboratory work to the fullest extent possible so that human trials are conducted collaboratively. Additional planning, coordination, data collection, and proper evaluation are required. Trials should, as far as possible, be monitored by a team consisting of experts from the host country, the donor, and preferably a third disinterested party (Institute of Medicine 1980).

Methods of Administering Vaccines

Better methods of administering vaccines are required in developing countries for reasons of cost, safety, and efficacy. Sufficient attention has not been given to newer and more effective methods of administration, for example, through pressure nozzles or skin applications. In many public health disease immunization programs, the potential of the oral route has to be more fully exploited; in many cases it may be worthwhile to design oral vaccines for this purpose.

REFERENCES

Bachrach, H. L. (1981) Genetic Engineering in Plants and Animals. Paper prepared for a U.S. Department of Agriculture Workshop on Emerging Technologies, 1981, Chicago, Illinois, USA.

Henderson, W. (1981) The Potential of Genetic Manipulation for the Improvement of Vaccines Against Animal Diseases in Developing Countries. U.N. Industrial Development Organization, Vienna, Austria.

Institute of Medicine (1979) Pharmaceutical Innovation and the Needs of Developing Countries. National Academy of Sciences, Washington, D.C., USA.

Institute of Medicine (1980) U.S. Participation in Clinical Research in Developing Countries. National Academy of Sciences, Washington, D.C., USA.

Pan American Health Organization (1981) Meeting on Modernization of Yellow Fever Vaccine Production Techniques. January 12-14, 1981, Washington, D.C., USA.

Rockefeller Foundation (1982) The Major Hemotropic Diseases of Food-Producing Animals. Study sponsored by the U.S. Agency for International Development and the Rockefeller Foundation for the Joint Research Committee of the Board for International Food and Agricultural Development, Washington, D.C., USA.

Animal Production

INTRODUCTION

Animals not only produce high-quality protein to supplement large quantities of moderate-quality, economically produced staples in the human diet. They also provide traction for agriculture and other needs; manure for fertilizer, solid fuel, and biogas; and serve as a means of capital generation, insurance against risk, and a mode of exchange. Worldwide, animal products contribute over 56 million tons of edible protein and over 1 billion megacalories of energy annually. With its high biological value, this protein is equivalent to more than 50 percent of the protein produced from all cereals, yet the proportion of research funds currently going to animal production is less than 15 percent of the total for agriculture.

The role of biotechnology in animal production is largely one of supplying tools to assist animal producers with breeding, health, and nutrition, but it is important not to overwhelm producers with this technology. Activities in this area should, therefore, include support of research on monoclonal antibody production, embryo splitting, cloning and sexing of gametes, and genetic engineering, as well as better utilization and implementation of already established techniques in management schemes and educational delivery programs.

Integrated research is also necessary. Human health research should be developed, as far as possible, in conjunction with that involving animal health, since many disease problems are related to the close contact of people and animals, and research on crop production should be integrated with that on animal production. As new, more productive crops are developed, there is a need to consider the entire plant with respect to human and animal use and digestibility as well as energy production (see the working group report on plant cell and tissue culture).

Finally, although man and animals may compete for space and foodstuff, they can also complement one another. Approximately 65 percent of the earth's land is not suited for farming or human

occupancy; better ways must be found to utilize this space for animal production.

RECOMMENDED RESEARCH ON ANIMAL PRODUCTION

Genetics

Manipulation of genetic material and its exchange among countries have become important means of upgrading animal production worldwide. Animal products should be selected for their compatibility with a developing country's environment and socioeconomic needs. Advances in biotechnology can reduce the risk of disease in transfer and can greatly speed the process of selection.

Biotechnological research areas that should be investigated in connection with the male animal include studies linking genes and disease resistance, the use of monoclonal antibodies for male sexing (identifying male offspring at fertilization), and the use of performance factors and adaptation to environment as measures for genetic evaluation. For the female, *in vitro* fertilization, oocyte culture, embryo sexing, and embryo replication are areas of possible investigation.

Using biotechnological techniques, it should be possible to develop research projects that greatly accelerate evaluation of methods to improve animal production. Production of identical siblings could eliminate genetic variables in applied research programs, greatly reducing the number of animals required per project and thus expenses.

Genetic research goals deserving the highest priority include:

- Demonstration of germ plasm transfer (embryos and semen) without risk of transmitting disease
- Identification and transfer of germ plasm associated with resistance to disease and health problems
- Determination of genetic markers to identify or increase superior germ plasm from indigenous species. Using monoclonal antibody technology will speed up the process of genetic selection.
- Identification of genetic factors related to production and, particularly, to puberty, morbidity, and proliferance
- Storage (using cryopreservation and similar techniques) and classification of germ plasm on a worldwide species basis. Germ plasm banks representing exotic and endangered species should be established as soon as possible in the different ecological zones.

Reproduction

For males, production, collection, processing, and use of spermatozoa are topics of interest to many researchers, who are also concerned about seminal quality, preservation, and handling, including alternate storage methods to freezing. The factors that affect the mobility of spermatozoa--those that inhibit as well as stimulate or initiate mobility--must also be studied, as well as the epididymal physiology and maturation of the spermatozoa. Investigations of disease spread in semen and sexing semen are also needed. An important related research topic, of course, is the fertility of various species in the tropics.

Other areas of possible genetic research include an evaluation of the physiological and psychological aspects of sexual behavior in indigenous breeds and a study of pheromones, chemical substances produced by an animal that stimulate one or more behavioral responses from other individuals of the same species. The factors that stimulate testicular hypertrophy must also be identified (unilateral castration stimulates hypertrophy and semen production in the remaining testicle). Finally, andrologic profiles of animals in the tropics in relation to behavior, mobility, and fertility must be compiled.

Regarding females, spread of animal disease can now be avoided by means of embryo transfer in which germ plasm is transported via embryo from one area to another, although this remains to be confirmed. Embryo transfer from one species to another, which is likely to be achieved in the near future, is also a means of establishing species in regions where they did not previously exist. Causes of embryo wastage in the female is a further area of concern, as well as cryopreservation of embryos in association with existing artificial insemination facilities. Further, the methods available for early diagnosis of pregnancy--RIA (radioimmunoassay), ELISA (enzyme-linked, immunosorbent assay), and ultrasound--need further study.

A number of aspects of the transfer of livestock among geographical zones must be examined more thoroughly including:

- Breed differences
- Economic feasibility of environmental manipulation to enhance reproductive efficiency
- Nutritional requirements
- Effect of stress on organs--thyroid, anterior pituitary, adrenal glands--in relation to reproductive behavior
- Heat detection and silent estrus
- Puberty changes in relation to nutrition.

Projects on reproduction might also investigate new techniques as well as improve established practices. Educational programs for those

in the livestock industry may be necessary to implement and improve existing techniques.

Applying Biotechnology to Reproduction. Applications of biotechnological research will be most relevant to mammalian germ plasm manipulations that improve animal production. Some of these manipulations are direct and immediately available, while others are more indirect and hold promise for the future.

A number of manipulations are currently being practiced. Artificial insemination extends the use of available male gametes and permits a superior animal to sire thousands of offspring. Superovulation produces larger numbers of female gametes for fertilization and, in combination with embryo transfer, extends the reproductive potential of the female and accelerates herd development. Cryopreservation suspends the life processes of spermatozoa, ova, and embryos, thus permitting "banking" for crosses within a time frame that would otherwise be impossible. Finally, twinning, by embryo splitting or half blastomere development, produces two genetically identical individuals (this technique is being extended to produce multiple siblings). All of these manipulations are not practiced successfully on all species; they have been largely applied only to cattle and horses in the United States. All merit improved efficiency and ease of performance and reduced cost. Given that they are already proven, these techniques rate high priority for continued support.

For the immediate future (within 5 years), it is highly probable that biotechnology will produce a number of breakthroughs that will improve animal reproduction.

Sex regulation will identify the sex of a preimplantation-stage embryo or separate X- from Y-bearing sperm cells before fertilization. This work is being undertaken using the H-Y antigen as a criterion.

Maturation of oocytes in vitro enables the harvest of hundreds of female gametes from a single ovary and, combined with fertilization in vitro, can "rescue" problem animals or species from reproductive obscurity. This has been achieved in rodents and primates, but only sporadic success has so far been reported for livestock species.

Oocyte fusion involves two female ova "mutually fertilizing" each other without benefit of a male gamete. This permits female-female crosses and ensures that the resulting offspring will be homozygous females. Blastocyst-stage embryos have already been obtained this way using Sendai virus, but current studies are focusing on the use of polyethylene glycol and square-wave electrical fields as ways of inducing fusion.

Cloning produces multiple copies of genetically identical animals. This has been done in mice by nuclear transplantation, but other methods hold promise for easier production of more copies.

Fusion of embryonic cells to ova whose nuclei have been destroyed by an ultraviolet light beam is probably the most promising of these. When ways are found to use somatic cell nuclei (which has not yet been accomplished), an infinite number of copies of an already proven animal can be made.

In unisex parentage, embryos are produced from the genome of only one parent or from two male gametes. With this method, one pronucleus of a newly fertilized ovum is destroyed by an ultraviolet light beam, and the other is permitted to duplicate before the cell cleaves, thus restoring diploidy. This is done with cytochalasin B which blocks cytokinesis but permits karyokinesis to proceed. Depending on which pronucleus is destroyed, the embryo will be of only paternal or maternal origin but will always be female and 100 percent homozygous. The same technique can also be used to selectively destroy the female pronucleus in an ovum where the cell surface had been treated to permit polyspermy, making a male-male cross possible.

Interspecies embryo transfer, where the embryo of one species is transferred to a surrogate mother of another species, can extend and conserve the limited germ plasm of exotic and endangered species whose genomes may be important for the future. This technique could also be used to eliminate vertical transmission of species-specific diseases. To date, successful term pregnancies have been achieved by this method in members of the horse, cattle, and rodent families. (Pregnancy was initiated in the cat and mustelid families but did not go to term.)

Within 10 years, in addition to continued improvement of all the techniques noted, at least four major technological advances involving manipulation of mammalian germ plasm are foreseen:

- Rapid selection for genetic traits using monoclonal antibodies for identification of specific gene carriers
- Gene transplantation to introduce a particular trait into an animal or to correct a genetic defect
- Customized animals produced from transferred nuclei selected from cells of the desired phenotype
- Ovary transplantation to rescue the germ plasm of sterile superior females.

To summarize, the following represent immediate research needs in the area of animal reproduction:

- Use of ova transfer as a tool for improving livestock production within the shortest time possible and for controlling disease. Aspects of this research are cryopreservation and transportation, twinning and single birth, superovulation, maturation of oocytes in vitro, oocyte fusion and sexing, cloning, unisex parentage, and interspecies transfer.

- Reduction of embryo wastage in superovulating species
- Development and use of alternate methods for semen preservation and handling in artificial insemination as well as study of seminal physiology, fertility, and aspects of disease control through artificial insemination
- Use of monoclonal antibodies for improving disease diagnostic techniques, vaccine production, ova transfer, and semen and embryo sexing
- Study of testicular hypertrophy
- Improvement of reproductive management in developing countries.

Nutrition

Improving the nutrition of indigenous animals in developing countries is of great importance. Because many developing countries import animal feed, methods for greater utilization of local biomass and by-products for animal feed must be developed. It would thus be useful to integrate the research efforts on animal production with those of agronomists, range scientists, irrigation specialists, soil scientists, and economists.

Important research areas in nutrition include:

- Natural range management in tropical areas
- Methods to identify chemical inhibitors in plants to permit more efficient use of indigenous fodder species
- New or more effective uses of local biomass and feedstuff residues such as aquatic weeds, cereal stalks, and grain milling by-products and ensilage (particularly by-products of biomass energy projects such as the leaves and stems of trees and shrubs, as well as stillage and fermentation residues). The entire plant must be considered for human and animal use. Plant breeding programs must consider the effects of a plant's lignin, silica, and tannin content on its value as an animal feed and an energy source.
- Methods of evaluating plant cell usability by different species
- Feeding strategies for local areas. Through knowledge of feeding behavior of animals the best complementarity of animal and plant ecosystems for most efficient use can be identified, as well as constraints within ecosystems such as chemical inhibitors that affect utilization of plants by animals.
- Needs for a balanced diet to improve overall nutritional efficiency. Projects in this area are well suited to a multidisciplinary approach.

Health

Animal losses from disease are a major concern of livestock producers in developing countries. Needs for research on specific disease problems vary among developing countries and within certain areas of a country. Some general research topics, however, that warrant attention include development and refinement of better diagnostic techniques through monoclonal antibodies (see the working group report on this subject); development of better preventive measures, including improved vaccines and expansion of vaccine production to susceptible diseases (see the working group report on vaccines); and review of the effectiveness of some eradication programs. Based on world priorities, specific diseases that merit additional research include:

- Parasitic diseases: babesiosis and anaplasmosis, theileriosis (East Coast fever), and trypanosomiasis (number one priority in Africa)
- Intestinal parasites, including flukes
- Viral diseases: African swine fever, foot-and-mouth disease, goat catarrhal fever, Gumboro disease in poultry, hog cholera, rabies, Rift Valley fever, and rinderpest
- Bacterial diseases: brucellosis, buffalo neonatal polyarthritis (important in Asia), contagious bovine pleuropneumonia, leptospirosis, mastitis, streptothricosis (important in West Africa), and tuberculosis.

Control of genetic disease may be aided by genetic selection, gene manipulation and engineering, or both. Specific areas could include mastitis and tick resistance and development of an improved immune response.

Work in biotechnology has until now largely focused on cattle, but the role of other types of animals, both domestic (swine, poultry, sheep and goats, buffalo and camels) and exotic (deer and antelope, capybara, etc.) merit attention to determine any significant applications of the new biotechnology. These species contribute to the total food supply and complement biomass utilization in ways that might be exploited to advantage.

Biotechnology may also have important applications to both diagnosis of and resistance to toxicological agents in the environment, particularly those resulting from use of fertilizers, herbicides, and pesticides in agriculture. Furthermore, biotechnological applications to breeding and health programs may provide important opportunities for improving the overall productivity of farming systems, providing alternative management possibilities, and solving stress-related problems. Management-associated diseases include: mineral deficiencies and toxicities, metabolism/nutrition-mediated diseases, stress-mediated diseases, and neonatal enteritis.

In the area of postharvest technology and marketing of animal products, improved systems of delivering and storing animal products for consumers are critical for developing countries and are likely to have important biotechnology implications.

In all these areas, improvements in infrastructure and application of existing technology are likely to be more urgent than modern biotechnology. However, the prospective biotechnological advances that could be applied are likely to make the impact of infrastructural changes more attractive and potentially more effective in the long run in increasing animal production.

GENERAL RECOMMENDATIONS

At the turn of the century, plagues such as rinderpest constituted the most important single threat to the livestock industry. Over time, however, through vaccination and improved management practices, most of the devastating diseases have been for the most part controlled. Although infectious diseases in livestock are still important, genetic, reproductive, and nutritional problems have assumed greater importance in overall livestock production in developing countries. Thus research on these problems should have greater priority, especially as there appears to be evolving, through monoclonal antibody technology, better diagnostic capabilities and safer and more effective vaccines. However, in view of the objectives of this workshop--to identify research opportunities in biotechnology of the highest use to developing countries--it was concluded that direct applications of biotechnology to genetic and reproductive needs are more important than indirect applications in nutrition and health. Nevertheless, the latter aspects remain of utmost importance if the full benefits of biotechnological techniques are to be gained.

Most significant advances in breeding can be achieved through the technology now available by increasing the gene frequency of high-performance stock in small numbers of selected animals through embryo replication. Using this technique, it is possible to generate in one year herds of producing animals that would have taken a hundred years with traditional breeding programs. However, transfer of this technology and high-performance genes to developing countries requires the ability to transfer embryos and semen without risk of disease transmission. Although such transfer remains to be demonstrated, there is no technical barrier to accomplishing this in the near future. Another aspect of biotechnology related to these advances is the ability to use monoclonal antibodies to identify the presence or absence of specific genes responsible for productive traits and the presence or susceptibility of the animal to disease. This capability is likely to become established within the near future with limited investment in research funding.

- It is therefore recommended that the U.S. Agency for International Development (AID) and other donors give priority in support to:

  -- Verification of the ability to transfer embryos and semen between geographic regions without risk of transmitting disease
  -- Establishment of embryo replication capabilities in developing countries
  -- Use of genetic markers for animal production using monoclonal antibody techniques.

No special requirements are associated with the application of biotechnology to improving animal production. Institutions with facilities for such routine procedures as storing semen for artificial insemination can accommodate the new technologies.

- Because few research institutions in developing countries have begun to use new biotechnological procedures, it is recommended that a careful process of identifying the most useful and productive technologies and related manpower and training needs for local requirements be undertaken. This will involve:

  -- Workshops on priorities and implementation strategies
  -- Participation in conferences by prospective researchers and trainees
  -- Technician training through short courses
  -- Longer term research manpower development at the graduate level.

Technical training to acquire the technology through short courses is readily available. Graduate-level opportunities at advanced centers in the United States, Europe, and Japan are likely to be more difficult in terms of cost, access to limited positions because of competition with nationals, and the problem of relevance of highly specialized, narrow research topics to developing country needs. Strengthening of graduate training and research capabilities in developing country centers should be emphasized.

Monoclonal Antibodies

INTRODUCTION

Antibodies, protein molecules produced by certain cells in the body, are a basic constituent of animal and human disease-fighting immune systems.* When the immune system detects a foreign substance or antigen in the body, it stimulates cells that make a diverse number of antibodies against the antigen. The antibody-producing cells recognize the physiochemical characteristics (electric charge, pattern, or shape) of the particular antigen and produce an antibody that binds specifically to the antigen, thus effectively neutralizing and destroying it.

The standard method of obtaining antibodies to protect against a specific disease antigen is to inject an animal (or human) with the antigen for which an immune response is desired. The immune system then responds by producing a variety of antibodies, each specific to a different part of the injected antigen molecule. Blood serum removed from the animal contains this antibody mixture.

Obtaining antibodies from immunized animals is, however, a slow and tedious operation, and it is very difficult to isolate a specific antibody. At the end of several extraction and purification steps, the antibodies are usually only weakly specific, available in small amounts, and of relatively low activity. Moreover, attempts to culture antibody-secreting cells have failed, since such cells do not survive long enough nor produce sufficient antibodies in culture to become worthwhile sources of antibodies.

In 1975, a new era in immunology was launched with the discovery of the hybridoma technique, a method for creating pure and uniform

*For a separate description of this technology and the state of the art, see "Biomedical and Biological Application of Monoclonal Antibody Technology in Developing Countries," by William C. Davis and colleagues, in Part Three.

antibodies against a specific target. Scientists learned how to fuse myeloma (cancer) cells with antibody-producing cells from an immunized donor. The hybrid cell or "hybridoma" resulting from this fusion has the ability to multiply rapidly and indefinitely in culture and to produce an antibody of predetermined specificity, known as a "monoclonal antibody." This new hybridoma technology for production of standardized reagents (antibodies) of a given class, specificity, and affinity has provided scientists with a tool that permits the analysis of virtually any antigenic molecule. Such reagents can be made in virtually unlimited amounts whenever needed, thus making them readily available to all investigators or diagnostic laboratories.

Application of this technology has provided a breakthrough in the methods available for analysis of the antigenic composition of microorganisms, for rapid diagnosis, and to aid in the development of vaccines. The technology affords an opportunity to make important advances in understanding the diseases of humans, animals, and plants with greater specificity, speed, and at reduced cost. The technique is relatively simple and straightforward to use and can be readily developed and made available for use in developing countries. This can be accomplished through cooperation between institutions in developed and developing countries.

POTENTIAL APPLICATIONS IN DEVELOPING COUNTRIES

Scientists are now in a period of transition from the use of conventional serological methods to the use of monoclonal antibodies. Such a transition requires replacement of conventional antibody reagents that must be constantly reproduced and standardized as supplies become exhausted, as well as development of reagents that provide greater specificity and thereby higher resolution than previously obtainable with complex antisera. The range of activity in this field is immense and cannot be fully described in this report. However, several activities are described here merely to indicate the scope of the areas that may relate to the needs of developing countries.

Monoclonal antibodies have been made against a spectrum of antigenic components, including hormones, drugs, serum components, white and red blood cells, bacteria, viruses, fungi, single and multicellular parasites, and natural toxins derived from plants and microorganisms. They have also proven of value in delineating the composition of viral antigens and in virus epidemiology. Antibodies generated against animal viruses such as rabies, influenza, parainfluenza, herpes measles, SV-40, reovirus, and tumor viruses have made it possible to identify previously unrecognized substrains that complicated diagnosis and the development of effective vaccines. Similar use in plants has led to the delineation of virus substrains

involved in the pathogenesis of major crop diseases such as those induced by fruit tree viruses (Prunus necrotic ring spot, apple mosaic, and tobacco streak) and potato viruses (M, S, X, and Y; potato leaf roll). Production of monoclonal antibodies against surface antigens of parasites at various stages in their life cycle has proven useful in dissecting the complex antigenic structure of composite membrane molecules and identifying molecules for use in diagnosis and the development of effective vaccines. For malaria, trials are planned for the near future to test the potential of membrane antigens as vaccines. For trypanosomes, studies have begun to show promise, and in the mouse model evidence has just been obtained that cross protection can indeed be obtained between related but antigenically dissimilar variants.

These examples emphasize the utility of monoclonal antibodies in addressing some of the difficult problems faced by developing countries. The following sections describe some specific areas in which the use of monoclonal antibody technology will prove of exceptional value.

Human Health

The development of routine diagnostic reagents for use in diagnostic laboratories exemplifies effective use of monoclonal antibody technology. Although there is little difference in the amount of time needed to develop diagnostic reagents by conventional procedures and to develop identical reagents using monoclonal antibody technology, the advantage of the new technology is that antibodies only have to be generated once. The specificity obtained is exact and readily standardized. The resulting savings in time and money has been recognized, and commercial enterprises are rapidly turning to the new technology for the routine production of serological reagents. Monoclonal antibodies have already been made against a number of compounds of potential value in diagnostic procedures, including hormones, bacteria, viruses, parasites, white and red blood cells, and serum proteins.

The reagents generated thus far would be equally useful in diagnosing diseases in developing countries and would greatly facilitate and improve the existing diagnostic capability. Although effective reagents are still not available for many diseases (see Table 1), for some, such as malaria and schistosomiasis, considerable progress has been made (see the working group report on vaccines). It would be useful to devise a mechanism that would accelerate and expand the capability of institutions to prepare the needed diagnostic reagents. Once this technology is established and monoclonal antibodies are made to the prevalent, serious disease-causing organisms, these antibodies could provide the means of identifying the

TABLE 1 Infectious diseases occurring in humans.

Disease	Requires improved diagnostic capability	Disease control possible[a]	Requires vaccine or better vaccine
BACTERIAL			
Brucellosis	X		
Colibacillosis	X	X	
Leprosy	X		X
Leptospirosis	X	X	
Pseudomonoiasis	X		X
Salmonellosis	X	X	X
Syphilis	X		X
Tuberculosis	X	X	X
Venereal gonococcus	X		X
CHLAMYDIAL			
Trachoma	X	X	X
METAZOAN			
Ascariasis	X	X	X
Cysticercosis/hydatidosis	X	X	X
Filariasis	X	X	X
Schistosomiasis	X	X	X
PROTOZOAN			
Amebiasis	X	X	X
Giardiasis		X	X
Malaria	X	X	X
Toxoplasmosis	X	X	X
VIRAL			
Equine encephalitis	X		
Hepatitis	X	X	X
Herpes II venereal	X		X
Influenza	X	X	X
German measles	X		X
Rabies		X	X

[a]By vaccination, treatment, or by limiting spread.

antigenic constituents of the pathogenic organisms that can be used for the development of effective vaccines. Malaria is an example of how monoclonal antibodies can be used for such purposes. The cell membrane antigens identified may prove useful in development of an effective vaccine.

Monoclonal antibodies also offer potential for the development of immunotherapeutic reagents. Although the technology is not well advanced in this area, it may be possible to develop reagents that could protect against such agents as snake venoms, plant- and microorganism-derived toxins, and rabies. For a single use, monoclonal antibodies derived from mouse-mouse hybridomas would prove effective. For continued use, however, antibodies would have to be derived from human-human hybridomas. The technology for developing such reagents is, nevertheless, in its infancy and can only be considered as a potential application that would be extremely useful as an additional therapeutic tool.

Success in generating hybridomas that make antibodies of high specificity and affinity against reproductive tract hormones (human chorionic gonadotrophin, progesterone) opens the possibility of their use in both diagnosis and therapy. An abundant source of antibodies of consistent characteristics would be helpful in devising low-cost methods of detecting pregnancy at an early stage that use nonradioactive, isotopic immunoassays. Stability and reliability of these kits at temperatures prevalent in tropical countries would stimulate their widespread use in several parts of the world where such facilities are not available. Mechanisms should be devised to make these developments more immediately useful, as well as model systems for evolving similar assays for diseases of pregnancy.

Food Production

Monoclonal antibody technology is not limited to problems of human health; it will prove equally valuable in improving food production in developing countries.

Animal Health. Significant animal losses from disease occur in developed countries, and losses in developing countries are even more severe. For cattle, it is estimated that some $4 billion per year is lost from disease in the United States alone. This is, in part, attributable to a lack of knowledge about the immune function in food-producing animals, a lack of adequate diagnostic reagents to identify infectious agents responsible for disease outbreaks, and a lack of information essential for improving methods for breeding for disease resistance. As with humans, monoclonal antibody technology has provided the means of developing a set of diagnostic reagents, and the value of this technological breakthrough cannot be overemphasized. The

spectrum of diseases in food animals the world over is quite large, and effective diagnostic reagents are only available for a few. The development of standard diagnostic reference reagents for international use would be of great value. A number of the important diseases are listed in Table 2.

A multinational program is needed to address effectively the problems of food animal disease, since in many instances diseases are not restricted to a single continent. For example, anaplasmosis is a persistent disease caused by a rickettsial organism. Because wild ruminants serve as a reservoir, there is no effective way to eradicate the disease, and no effective vaccine has been developed. A better method is needed to identify infected animals, and greater efforts must be expended on producing a vaccine. Wild animals also serve as a reservoir for and prevent eradication of bluetongue virus. Here, however, disease control is complicated by the fact that many subtypes--the current estimate is 21--of the virus exist, and vaccination against one type does not effectively protect against infection by other types. Bluetongue is also one of the diseases that prevents the shipment of sperm and embryos to developing countries to aid in upgrading native breeds. There is an urgent need to accelerate research on this virus, both to develop typing reagents and to produce a vaccine (see working group reports on vaccines and animal production).

Where a disease is more localized and important to food production--for example, salmonellosis and colibacillosis--research programs utilizing monoclonal antibody technology to address specific needs are required. Although such diseases exist worldwide, strain variation at the local level accounts for regional problems, which necessitate development of diagnostic reagents to identify the strains of bacteria causing the local disease. At both the national and international levels, monoclonal antibodies are needed to facilitate the identification of antigens in pathogenic organisms that can be used to develop effective vaccines.

Monoclonal antibody technology also provides a much-needed mechanism for improving methods of selective breeding. Conventional methods of producing typing reagents have provided a limited but important view of the antigenic polymorphic determinants present on white blood cells, which can be used to define gene systems for selective breeding. Monoclonal antibody technology has shown that the potential is much larger and that it can serve as a mechanism for identifying not only gene systems that influence susceptibility to infectious diseases but also those that control the inheritance of desirable performance traits.

An international collaborative program that includes developing countries is needed to develop monoclonal antibody typing reagents for use in selective breeding studies. The compilation of genetic profiles both of animals that might be used to upbreed native animals

TABLE 2 Infectious diseases occurring in animals.

Disease	Requires improved diagnostic capability	Disease control possible[a]	Requires vaccine or better vaccine
BACTERIAL			
Atrophic rhinitis (swine)	X		
Brucellosis (bovine)	X	X	
Colibacillosis (swine, poultry)		X	
Leptospirosis (bovine, swine)	X	X	
Pleuropneumonia (swine)			X
Salmonellosis (swine, poultry)		X	
Tuberculosis (bovine)	X	X	X
METAZOAN			
Ascariasis		X	X
Cysticercosis/hydatidosis		X	X
Fascioliasis	X	X	X
Schistosomiasis		X	X
PROTOZOAN			
Babesiosis (bovine)	X	X	X
Coccidiosis (avian, ruminants)		X	X
Theileriosis		X	X
Trypanosomiasis	X	X	X
RICKETTSIAL			
Anaplasmosis (bovine)	X		X
VIRAL			
African swine fever	X		X
Aujeszky's disease (swine)		X	
Bluetongue (ruminants)	X		X
Bovine paralytic rabies	X	X	X

TABLE 2 (continued)

Disease	Requires improved diagnostic capability	Disease control possible[a]	Requires vaccine better vaccine
Bovine viral diarrhea		X	X
Equine infectious anemia	X		X
Foot-and-mouth disease (swine, bovine)		X	X
Hog cholera		X	
Infectious bovine rhinotracheitis (bovine)	X	X	X
Low production syndrome (poultry)	X	X	X
Marek's disease (poultry)		X	X
Newcastle disease (poultry)		X	
Rinderpest (bovine)	X		X

[a]By vaccination, treatment, or by limiting spread.

and of native animals is also needed. Native animals, although resistant to disease, are often of poor quality and cannot be used over a short time period to improve breed performance. In contrast, the exotic breeds that can provide the gene pool for improving the quality of native stock do not possess the genes that contribute to resistance to endemic disease. The latter thus prevents the direct introduction of improved breeds and necessitates upbreeding through crossbreeding.

A typing system is needed that permits the identification and retention of desirable traits. Monoclonal antibody technology has afforded the first major breakthrough to achieve this objective. With precise typing and the use of ova transplantation, selective breeding could be greatly accelerated. This technology would be of equal value in improving both large and small food animals.

Plant Health. The food chain for animals and humans is based on plant agriculture. Thus any improvements to plant health and crop production by reducing or controlling outbreaks of plant pathogens will have a direct impact on animal and human nutrition. Large numbers of viral, fungal, and bacterial pathogens constantly threaten

entire food crops, and the use of genetically uniform plants to maximize production has increased the risk of such calamities. International quarantine programs have been established to prevent the spread of major pathogens via infested or infected seed or plant parts or produce, and serology has played an important role in such programs.

The persistent difficulty, until now, has been the development of adequate quantities of standardized immune reagents for worldwide use. An international program is needed to address the problems associated with producing standardized immunological reagents for disease diagnosis and epidemiology. Monoclonal antibody technology provides a means of meeting these goals, and with a joint commitment from developed and developing countries rapid progress can be made. Developed countries can provide the input for diagnostic reagents for pathogens common to both developed and developing countries. Some developing countries, however, need to address immediately pathogens that are contributing to economic losses.

Continuing programs that use serological techniques for potatoes, fruit, and cereal crops illustrate the need for immune reagents for research and diagnostic tests for certification (see Table 3). For example, potatoes are propagated vegetatively to ensure crop uniformity. Certification of pathogen-free seed pieces for cultivation requires testing for such organisms as Erwinia carotovora var. atroseptica (the causative agent of blackleg of potato), potato leaf roll virus, and potato viruses M, S, X, and Y. It has been difficult, however, to develop reagents capable of distinguishing between strain variants, and this capability is often necessary to determine whether a disease outbreak occurred with a variant of the pathogen characteristic of the area where the seed stock was put into production or with a variant characterisic of the region where the seed pieces were propagated. Monoclonal antibody technology offers a solution to these problems and provides a means for epidemiological surveys of pathogenic strain variants.

Ilarviruses present similar problems in fruit trees. Prunus necrotic ring spot, prune dwarf, and apple mosaic viruses cause serious diseases in cherry, peach, plum, and apple trees. The failure of these viruses to elicit a strong immune response hinders the production of high-quality serological reagents capable of detecting them in certified propagation stock or in field outbreaks. Early work with monoclonal antibodies has shown that these problems can be overcome.

Several viruses cause major difficulties in citrus production. For example, citrus tristeza is nearly ubiquitous, and certain strains of this virus can destroy entire orchards. Control measures are to remove infected orchards. Although mild strains of this virus cause little or no disease and can protect against infection by the severe form, conventional sera cannot distinguish between these strains. It is hoped that monoclonal antibodies may resolve these problems.

TABLE 3 Infectious diseases occurring in plants.

Disease	Requires improved diagnostic capability[a]	Work in progress[b]
POTATO		
Bacterial		
Erwinia carotovora var. *atroseptica*		X
Pseudomonas solanacerum		X
Viral		
Andean potato latent		X
Potato leaf roll		X
Potato virus M		X
Potato virus S		X
Potato virus X		X
Potato virus Y		X
Tobacco mosaic		X
CITRUS (bacterial and spiroplasma)		
Citrus tristeza virus	X	X
Spiroplasma citri	X	X
Xanthomonas citri	X	X
FRUIT TREES (peach, plum, cherry, almond, apricot, apple)		
Viral		
Apple mosaic		X
Prune dwarf	X	X
Prunus necrotic ring spot		X
Tobacco ring spot	X	X
Tobacco streak	X	X
Tomato ring spot	X	X
COCONUT		
Cadang-cadang	X	
Lethal yellowing	X	
CASSAVA		
Cassava mosaic virus	X	X
SUGARCANE		
Ratoon stunt virus	X	X
Sugarcane mosaic virus	X	X

TABLE 3 (continued)

Disease	Requires improved diagnostic capability[a]	Work in progress[b]
WHEAT, BARLEY, GRAINS		
Viral		
Barley stripe mosaic	X	
Barley yellow dwarf	X	
RICE		
Viral		
Rice dwarf	X	
Rice grassy stunt	X	
Rice ragged stunt	X	
Rice tungro	X	
MAIZE		
Viral		
Maize chlorotic leaf spot	X	
Maize rough dwarf	X	
Maize streak (Africa)	X	

[a]Relationships of pathogen host vector and environment not clearly understood.
[b]Can be controlled or reduced in severity.

Proper diagnostic capabilities will also increase the efficiency of crop production and utilization. For example, potatoes infected with potato leaf roll virus should be consumed within a month or two of harvest, since prolonged storage may result in development of an internal net necrosis, rendering the tubers useless except for animal feed. Barley yellow dwarf virus symptoms in infected wheat, barley, and oats may resemble nitrogen deficiency. Proper virus diagnosis can prevent both needless crop spoilage and application of expensive nitrogen fertilizer when it is not needed.

One primary area in which monoclonal antibodies will affect plant production is through the development of highly specific antibodies to different strains of plant pathogens. With this technique it will now be possible to detect and differentiate strains of viral, bacterial,

and fungal pathogens that have not been recognized, opening up a new era of epidemiological research on plants. For example, there are presently more than 100 proposed members of the potato virus Y group of plant viruses, many of which are probably not unique. Antibodies to only a handful of these viruses have been produced, and many of these antisera are of such low quality that they are of limited value in strain differentiation. Production of monoclonal antibodies to a number of carefully selected members of this virus group from different crop species and geographical areas of the world would provide a basis for determining the true number of viruses in this group and a means of determining to what geographical area and to which crop species certain strains may be restricted. Specific virus-vector-host relations could also be elucidated using this technology.

Recent research reports on strains of Prunus necrotic ring spot, apple mosaic, and tobacco streak viruses, which infect a number of fruit, forest, vegetable, and ornamental crops, demonstrate the presence of strains of these viruses that were previously unrecognized. Epidemiological research programs are already in progress to investigate the distribution of these virus strains in commercial fruit orchards in the United States, Canada, New Zealand, Australia, England, Western Europe, and South Africa.

In a similar program with Xanthomonas campestris, the causal agent of a serious disease in Brassica spp. (cabbage), epidemiological surveys in Hawaii have yielded valuable information on the number and distribution of strains of this bacterium.

RECOMMENDATIONS FOR USE OF MONOCLONAL ANTIBODIES

Human Health

Many diseases still lack adequate or effective diagnostic reagents, resulting in continued high morbidity and mortality among populations. Some of these diseases are found worldwide, while others are specific to developing countries.

- It is therefore recommended that means be provided to accelerate and expand the capability of developing country institutions to prepare or use monoclonal antibodies as diagnostic reagents. Priority should be given to developing antibodies for use in diagnosis or detection of the following diseases or pathogens:

  -- Bacterial: Hemophilus, Mycobacterium tuberculosis, Pneumococcus, Streptococcus, E. coli, Salmonella, Shigella, Campylobacter jejuni

-- Metazoan: cysticercosis/hydatidosis
-- Protozoan: ascariasis, amebiasis, echinococcosis, filariasis, malaria, schistosomiasis, toxoplasmosis
-- Viral: equine encephalitis, German measles, hepatitis, hepatitis B virus, herpes II, influenza, rabies.

Such monoclonal antibodies should be developed in institutions that need diagnostic reagents.

Among all the medically important viruses, hepatitis B virus (HBV) can be singled out as one of the most important to mankind. In many developing countries of Asia and Africa, this virus is present in up to 60 percent of the population and may lead to the development of viral hepatitis and hepatocellular carcinoma. Thus control of this disease is a goal of many developing countries. Epidemiological studies are urgently needed to define the distribution of the virus. The antigenic structures such as HB_s, HB_a, and HB_e, which are reportedly related to the infection of hepatitis B virus, are very complex. The presence of these antigens in human serum is strongly linked to HBV infection.

Through the use of monoclonal antibody technology, it will now be possible to identify these antigenic components clearly. Furthermore, through the joint efforts of AID and developing countries, the epidemiology of this viral disease is being worked out, and it may be possible to eradicate this disease in the future. The results of such an effort may also have far-reaching effects on the occurrence of human hepatocellular carcinoma, which has been implicated in HBV infection.

- It is therefore recommended that high priority be given to a feasibility study of the establishment of two monoclonal antibody centers, one in Asia and one in Africa. As one of their major goals, these centers should seek to produce monoclonal antibodies against the HBV antigens for use in study of the epidemiology of HBV infection. Once developed, these centers should have the capability of producing monoclonal antibodies to other disease-causing organisms and should serve as centers for maintenance and distribution of hybridoma cell lines and diagnostic antibody reagents. The centers also should serve as sites for wet laboratory training courses.

Animal Health

As for human disease, diagnostic reagents do not exist for many animal diseases, or, if available, are inadequate for accurately identifying infectious agents responsible for disease outbreaks.

- It is recommended that a program involving a network of institutions be developed to prepare antibodies for use as diagnostic tests and epidemiological studies. Priority should be given to making antibodies used in the diagnosis or detection of the following diseases or pathogens:

  -- Bacterial: Brucella, E. coli (causing neonatal diarrhea), Mycobacterium tuberculosis, Salmonella
  -- Metazoan: cysticercosis/hydatidosis, fascioliasis, schistosomiasis
  -- Protozoan: babesiosis (bovine), coccidiosis (avian, ruminants)
  -- Rickettsial: anaplasmosis (bovine)
  -- Viral: African swine fever, atrophic rhinitis, bluetongue (bovine, caprine, ovine), bovine paralytic rabies, bovine viral diarrhea, caprine arthritis, encephalitis, equine infectious anemia, foot-and-mouth disease (bovine, swine), infectious bovine rhinotracheitis, Marek's disease (avian), Newcastle disease (avian), pleuropneumonia

- Because few typing reagents exist for the development of selective breeding for increased resistance to disease, it is recommended that a full set of monoclonal antibodies be made for each functional polymorphic, antigenically distinguishable gene product on white blood cells. This includes gene products of the major histocompatibility gene complex, gene products that define T and B cell differentiation antigens, and gene products that define antigens common to both T and B cells.

This undertaking should center around international cooperation. Activities currently involving the production or use of such antibodies to develop selective breeding programs are under way at the following universities: University of Edinburgh, Scotland; John Curtin School of Medical Research, Australia; and University of California at Davis, Michigan State University, University of Wisconsin, Washington State University, United States. The International Laboratory for Research on Animal Diseases (ILRAD), Africa, should develop a program in this area.

Plant Health

High-quality diagnostic antisera capable of identifying a wide variety of viral, bacterial, or fungal pathogens are not currently available for many of the plant diseases that affect the world's

important food and fiber crops. Furthermore, the epidemiology of many of these agents has not been investigated. Failure to diagnose these pathogens properly in important food crops leads to significant decreases in agricultural productivity. Many of these diseases are found worldwide and result in significant crop losses.

- It is therefore recommended that monoclonal antibody technology be utilized to produce antibodies for use in diagnostic and epidemiological studies of important viral and bacterial plant diseases. Priorities should be given to developing antibodies to the following disease agents:

  -- Rice: rice dwarf, rice grassy stunt, rice ragged stunt, rice tungro
  -- Maize: maize chlorotic leaf spot, maize rough dwarf, maize streak
  -- Cassava: cassava mosaic virus
  -- Citrus: citrus tristeza virus, Spiroplasma citri, Xanthomonas citri
  -- Potato: potato viruses M, S, X, and Y; potato leaf roll
  -- Fruit trees: Prunus necrotic ring spot, apple mosaic, prune dwarf, tobacco streak, tomato ring spot, tobacco ring spot viruses.

Several national and international agricultural research institutes could serve as a network to provide trained plant scientists in immunology, plant pathology, plant breeding, and entomology. Monoclonal antibody production centers could most easily be established in research institutes that have animal or human cell culture facilities nearby. Close cooperation between plant scientists and veterinary scientists working with monoclonal antibodies would be advantageous.

Other Recommendations

- It is recommended that the Agency for International Development (AID) or another technical assistance agency establish international working teams to ascertain the level at which each of the interested developing countries is prepared to use monoclonal antibody technology for diagnostic purposes.

It would be useful to employ the "Request for Proposal" (RFP) system commonly used by the National Institutes of Health in each of the subject countries as a means of identifying centers with appropriate facilities and the desire to exploit this new technology.

RFPs would include size and scope of the diagnostic population (human, plant, or animal), endemic diseases that might be subject to the application of monoclonal antibody techniques, description of the facilities and the kinds of equipment available, serologic tests that are presently performed, curriculum vitae of the principal investigator emphasizing relevant experience as well as those of all junior personnel, and finally, a detailed description of the level of participation. Laboratories should participate in any such program by using both monoclonal antibodies available from established commercial sources (licensed products) as well as antibodies produced by their laboratories. AID or another donor would provide for consultative services and on-site assistance to laboratories.

At the next level, individual centers should be aided, where sufficient need and infrastructure exist, in developing facilities and manpower to produce their own diagnostic antibodies. Members of the working teams might provide consultative assistance. The principal initial need, however, is to train personnel in laboratories in either developed or developing countries so that they can "take the technology home" with them. AID or other donors would support visits to developing countries by experts in this field to help with any problems encountered, planning, and execution once each laboratory begins producing some standard monoclonal antibodies.

- To identify and assess needs for implementing individual programs that incorporate training, laboratory "set-up," and performance, it is recommended that AID or other donors establish planning grants, perhaps in reference to the RFPs. The donors would be responsible for determining the source and availability of existing diagnostic reagents or those that will soon be available. Priorities for support should be (1) diagnostics, (2) reagents for genetic and epidemiological studies, and (3) therapeutics.

It is anticipated that future support of some aspects of this technology will be tailored to the needs of each developing country. These may include (1) application of monoclonal technology for the isolation of appropriate antigens for vaccine development (both human and animal), (2) antibodies to detect selective genetic markers to assist in the selective breeding of valuable livestock, (3) therapeutic antibodies for the treatment of human diseases in endemic populations, and (4) application of monoclonal technology to diagnosis and epidemiology of plant pathogens.

Energy

INTRODUCTION

Energy derived from biomass already contributes significantly to civilization. Biomass, in the form of firewood, agricultural residues, and animal dung, is the principal energy source in many countries, and even highly developed nations use wood extensively to heat homes and generate power.* Brazil is leading the way in developing alcohol fuels to supplement or replace gasoline, while the United States, Canada, and other countries have more modest alcohol fuels programs based on various biomass feedstocks.

In planning biotechnology programs, the diversity of needs and resources among developing countries should be recognized. Brazil's combination of land, climate, and scientific talent is not typical, and, because of differences in one or more of these areas, many developing countries will explore other avenues for applying biotechnology to their energy problems. The more significant benefits of biotechnology for the energy systems of developing countries will be longer term--those associated with increased biomass production--although shorter term gains will arise from biomass conversion. With the introduction of highly productive plant species and efficient conversion technologies, biomass can become a major source of fuels and chemicals, thereby reducing dependence on fossil feedstocks.

This working group has identified biotechnologies with substantial potential for use in developing countries, keeping in mind institutional constraints, environmental problems, and needs for trained manpower that may be encountered. Specific recommendations have been made both for short-range approaches to exploiting biomass resources and for more complicated technologies that may reach

*See Part Three for a separate background paper prepared on this subject, "Overview of New Biomass Industries," by Henry R. Bungay.

fruition more slowly. All these recommendations emphasize a systematic development of any biomass-related program, with careful economic assessment at each step. In addition to recent advances in biotechnology that may be valuable to developing countries, some pioneering research topics have been suggested.

RECOMMENDATIONS

Selecting and Analyzing the Applications of Biotechnology to Energy Production

Any selection of biotechnologies for conversion of biomass to energy should take into account alternative technologies (such as thermoconversion processes), the resources and constraints of a developing country, as well as the possible coproduction of food, feed, fiber, and other products. A useful strategy, particularly in developing countries, might emphasize processes that initially lead to saleable products on a small-scale basis but have sufficient flexibility to enable production of commodities and energy such as ethanol at a later time. In the United States, for example, hydrolysis processes convert lignocellulosics into sugar syrups that may be sold directly or fermented to ethanol and microbial protein. This strategy is aimed at a rapid introduction of biomass products into the marketplace.

- It is recommended that modeling techniques be used to evaluate options for biotechnology in developing countries. Such techniques could include engineering and economic analyses and energy material balances, as well as an analysis of the macroeconomic aspects such as effects on food production and food prices and even balance of payments. These analyses should be initiated prior to any major experimental or developmental work and maintained throughout the course of a project to update the economic data.

- It is recommended that technologies or processes that generate multiple products from biomass and increase rural employment or improve village self-sufficiency be emphasized. Processes that yield both fuel and food or feed should receive priority over those with single outputs. Processes that increase rural employment might include growing lignin-degrading fungi on wood to provide edible mushrooms and a more digestible cellulose fraction that could serve as a feed for ruminants.

- It is recommended that simple, inexpensive diagnostic techniques be developed for monitoring biological

conversion. These might include the use of monoclonal antibodies to identify viable organisms in a bioreactor such as a biogas unit or an alcohol fermenter.

Improved Utilization of Wood
and Other Lignocellulosic Materials

- It is recommended that the conversion of lignocellulosic biomass into fuels and chemicals be studied at the pilot project level, preferably as a collaborative effort among organizations with experience in this area. Special attention should be given to identifying the more promising local lignocellulosic crops and the economy of the various technological options, for example, biomass densification, use of bagasse from sugarcane as a substrate for cellulose hydrolysis, alternatives to costly and energy-intensive aerobic systems for producing enzymes, processes for using the mixed sugars from hemicellulose. Also, more efficient use of firewood warrants special attention.

In the short term, the use of densified biomass should be evaluated, along with alternative uses of firewood. Pulverized biomass and agricultural wastes such as sawdust and rice hulls can be compacted into pellets or briquettes by heat and pressure. The increased combustion efficiency realizable with a more uniform product more than repays the monetary and energy investments in the densification process, and storage stability and resistance to moisture are greatly improved. The capital cost of the processing equipment is not unreasonable.

In the mid-term, the use of bagasse from sugarcane as a substrate for cellulose hydrolysis should be emphasized. Such an emphasis may mean improved ethanol fermentation economics and energetics and the availability of a sizable new source of energy, chemicals, food, feed, and fibrous products. Because bagasse constitutes a large weight fraction of sugarcane, the additional fermentable sugar obtained from hydrolysis of bagasse would greatly increase the amount of ethanol derived from a crop of cane. Each of the various promising technologies for hydrolysis should be tested and evaluated.

In the longer term, alternatives to costly, energy-intensive aerobic systems that are now used for producing cellulase enzymes should be explored. Examples included:

- Developing biological pretreatment systems or biological pulping for lignocellulosic biomass by employing basidiomycetes or other filamentous fungi

- Studying product distribution in the anaerobic conversion of whole cellulose into organic acids
- Studying the extent of lignin physical modifications and chemical breakage and their relation to anaerobic degradation
- Studying the action of the cellulase-hemicellulase complex of anaerobic bacteria.

Another topic for longer term research might be the processes for utilizing the mixed sugars from hemicellulose. These sugars are not fermented by the normal yeasts that produce ethanol, but other organisms can convert xylose and other pentoses to such products as acetone/butanol or 2,3-butanediol. Certain yeasts (Pachysolen tannophilus), some bacteria (for example, Clostridia), and new strains obtained by recombinant DNA techniques can ferment pentoses to ethanol. Another potentially attractive route for pentose utilization is chemical conversion to furfural. Each of these alternatives should be evaluated.

Utilization of Polysaccharide (Principally Starchy) Materials

It is recommended that:

- Improved bioreactor designs be developed for important fermentation processes such as ethanol and fuel gas production--for example, columns or systems using immobilized microorganisms and continuous feed. The potential for lowering capital requirements and production costs is significant.

- Advanced separation and purification processes be applied to biomass processing, which could mean increased energy efficiency over that obtained using traditional processes such as distillation. Application of these processes could also result in reduced energy consumption and increased availability of by-products with fuel value such as bagasse.

- Improved saccharification processes be applied to the conversion of starchy and inulin-rich biomass into ethanol. Examples of these processes are: (1) low-temperature saccharification, (2) simultaneous saccharification and fermentation, and (3) introduction of genes for amylase production. Application of these processes could mean more economic utilization of cassava, babassu, Jerusalem artichoke, and other polysaccharide-rich materials, and near-term availability of ethanol in countries with limited production of sugar crops.

- Environmental control technology for bioprocessing of wastes be improved, emphasizing processes that coproduce fuel gas, organic chemicals, and other saleable compounds. For example, improvements in anaerobic digestion of stillage could lower capital requirements, reduce pollution problems, and generate revenues from sale of coproducts, as well as lower the costs of producing ethanol, microbial protein, and other products.

- Suitable methods for biomass storage be devised to decrease the significant preprocessing losses found in current field practice for many crops such as cassava. New or improved methods could reduce feedstock cost, improve processing efficiency, and minimize transportation losses of biomass.

Microbiological Research and Development

From the biological point of view, the production of fuels and chemicals from biomass is still in an early stage of development and could profit from the screening of a large number of species of plants, algae, fungi, and bacteria for their potential. Any such screening effort must be global in scope, since the organisms of potential interest are very unlikely to be encountered only in the natural ecosystems of developed countries.

- It is recommended that an international network be established to screen potential species for fuel and chemical production, and that a committee be established to elaborate a more complete screening inventory as well as a set of guidelines for executing the screening and processing of the results. Screening should include the following properties:

  -- Native trees or shrubs that are highly productive on marginal, saline, or otherwise impaired soils
  -- Microorganisms that produce extracellular enzymes highly insensitive to temperature and pH variations (cellulases, amylases, etc.)
  -- Plants and microorganisms that produce hydrocarbons or similar fatty molecules in high yields
  -- Marine algae with high growth rates for protein production in seawater lagoons
  -- Microorganisms that produce novel insecticides and pesticides
  -- Fermentative microorganisms highly resistant to end-product inhibition.

- Because most microbiological processes are single-species systems, it is recommended that mixed culture systems and their interactions be studied: for example, anaerobic holocellulose hydrolyzers, acid formers, and methanogens; thermophilic lignocellulosic biodegraders; and nitrogen-fixing algal and bacterial systems.

By-products, Coproduction, and Diesel Fuel Substitutes, Including Environmental Control Technology

Diesel fuel is increasingly the preferred liquid fuel in developing countries. Thus conversion of biomass to ethanol and methane may not be of primary interest.

- It is recommended that a more balanced approach be taken to fuel production, emphasizing the development of processes for the production of possible diesel fuel substitutes, such as alkanes, butanol, and vegetable oils. Biomass sources for diesel fuel production may include woody biomass from which turpentine is recovered, oilseeds from which fuels may be extracted, selected aquatic biomass, and oil-producing microorganisms. Integral to this emphasis on by-product utilization are effective means for environmental control.

Production of diesel fuel from biomass may be achieved by both short-term and longer term development. For example, in the short term, available vegetable oils could be evaluated for their use as tractor fuel; longer term development should be implemented to increase yields and to produce oil more suitable as a fuel.

The use of biomass might be more readily accepted if adapted to immediate needs. Thus, for example, production of vegetable oil for use as both a food and diesel fuel could be introduced rapidly. Similarly, techniques for increasing turpentine yields from woody biomass could be implemented immediately. Mid-term developments might include improvements in the acetone/butanol process with the research goal of increased butanol tolerance and an increased ratio of butanol to acetone. A project with longer term developments might be the use of high-yield aquatic biomass.

Institutional Interaction and Training

- To stimulate the use of biotechnology for energy production in developing countries, it is recommended that adequate curricula be developed for in-country training and that overseas degree and nondegree training continue. Training at

local energy research and development facilities with their human and material resources, both inside and outside of the universities, should be optimized. The flow of scientific and technical information on energy within each developing country and exchanges with the regional and global research and development system should be freed from impediments. Finally, national and international interaction between research institutes undertaking study of energy issues should be stimulated.

Curricula development for undergraduate training in biotechnology and related areas should take into account the importance of such fields as biochemical engineering, molecular biology, microbiology, and biochemistry without sacrificing traditional areas of importance such as plant taxonomy and ecology.

Graduate training in biotechnology and related areas at the masters and doctorate levels should be joint efforts of universities, research institutes, and industry. Some international funds should support the participation in such programs of visiting teachers from both developed and developing countries.

Intensive advanced training courses in areas of biotechnology of particular relevance to energy research and development in a given country or region should be offered on a regional or international basis with the participation of visiting specialists.

Interactions between university departments and research institutes dedicated to energy research and development in biotechnological areas on the national, regional, and international levels should be stimulated. These interactions may take many forms such as:

- Joint research projects run by research and development institutions in different developing countries and pertaining to a geographical region, accompanied by periodic review meetings, workshops, and the like
- Long-term bilateral collaboration in teaching and research between an institution in a developed country and one in a developing country
- Active participation of developing country institutions in international networks for study of specific problems in biotechnological research and development
- Linkage of institutions in developing countries to data bank systems and computerized, satellite-mediated systems for information exchange
- Exchange of scientists and engineers among research institutions working in the same field and related programs.

Biological Nitrogen Fixation

Nitrogen is frequently the limiting nutrient in agricultural productivity. Although 78 percent of the earth's atmosphere is composed of nitrogen gas (N_2), plants are unable to use it in this form. The nitrogen must first be "fixed," that is, combined with other elements such as hydrogen, carbon, or oxygen before it can be assimilated by higher plants.*

The ability to fix gaseous nitrogen is restricted to certain prokaryotes (cyanobacteria, actinomycetes, bacteria) that contain the enzyme nitrogenase. While a number of these organisms fix atmospheric nitrogen in the free-living state, some form symbiotic associations with higher plants. Examples of the latter include the legume-*Rhizobium*, nonlegume-actinorhiza (*Alnus*, *Casuarina*), and nonlegume-cyanobacteria (*Azolla*) symbioses. In the symbiotic association, the plant provides both a specialized environment for the prokaryote and a source of energy and receives fixed nitrogen in return. The legume-*Rhizobium* symbioses include soybean and alfalfa and are of unquestioned agricultural importance. The *Azolla*-*Anabaena* symbioses have documented potential as an alternative source of nitrogen for rice. Nonlegume-actinorhiza associations, such as *Casuarina*, offer potential for the production of lumber, fuel, and paper pulp.

*See Part Three for a separate background paper prepared on this subject, "Research to Enhance Biological Nitrogen Fixation: Misplaced Emphasis?" by Martin Alexander. Although the author was a member of the working group, the opinions expressed in his paper were not evaluated by the group and should not be interpreted as part of their deliberations.

ORGANISMIC ASPECTS OF BIOLOGICAL NITROGEN FIXATION (BNF)

Legumes and Rhizobia

Since only a relatively small percentage of the world's known legumes has been considered seriously as crop plants, it is important to seek those that are useful both as food crops and as a source of raw materials. Even though a legume might be unacceptable as a food, it may contain constituents that could be used as is or modified to products of high value such as drugs. The soybean is an excellent example of a crop that has in recent decades risen rapidly to prominence in the world's agricultural economy. Its success is based not only on its direct use as an oil and protein food for humans, but also on its secondary uses such as a high-protein animal feed. A multi-use crop has great advantages and should be considered in selecting unexploited legumes as possible new crops.

A number of legumes currently cultivated may have potential for expanded use, resulting in increased production of food or raw materials for other uses. These plants include: soybean, winged bean, common bean, chickpea, cowpea, green gram, black gram, pigeon pea, mung bean, lentil, pea, broad bean, lima bean, Sesbania, Acacia, and some of the Medicago spp. (burr and barrel medic annuals, alfalfa). In addition, a number of forage legumes such as Desmodium and Centrosema can be useful. Special products such as guar gum and gum arabic from legumes may have additional uses.

Tree legumes that fix nitrogen such as Leucaena should be examined in detail and a search made for additional useful trees of this type. Study of this group of plants has lagged because they have been of little economic importance. They can, however, be used profitably in certain regions. Although lists have been made of potentially useful legumes, it would be helpful to reexamine these and to set priorities for testing them in particular areas.

Plant Breeding and Genetic Engineering. Selection of legumes should be based on how well they adapt to local conditions. Since many factors are involved--for example, temperature, salinity, mineral stresses, pH--classical plant breeding approaches may be augmented eventually by modern genetic tools. For example, delay of senescence could allow a longer period for nitrogen fixation. While this might be achieved by the use of plant growth substances, a more useful solution would be to change the genetic nature of the plant by breeding for the desired traits. This is now being tested in soybeans, and preliminary results suggest that such plants may have equivalent seed yield, with additional nitrogen and organic matter in the leaves for recycling as residues to the soil. This is particularly useful in areas where a crop of soybeans is followed immediately by a planting of winter cereal.

Although classical techniques could be applied more effectively to the leguminous nitrogen-fixing systems than they now are, new approaches, including recombinant DNA, should not be neglected. Since nitrogen fixation in nodulated legumes is not consistently good, strains of *Rhizobium* should be selected or engineered to give maximal response under field conditions.

Problems related to host plant specificity and strain variation have been recognized for half a century. The differences between effective and ineffective strains are often quite subtle, and the factors responsible for this need clarification. Biotechnology and genetic manipulation will undoubtedly play a major role in unraveling the basis of these differences.

Recent studies have shown very promising potential for the transfer of desirable characteristics by way of plasmids. A number of symbiotic properties can be transferred on plasmids among *Rhizobium leguminosarum* strains, resulting in dramatic improvements in their characteristics. However, a present drawback is a lack of markers for effectiveness and efficiency of nitrogen fixation. Although there are well-controlled tests indicating a positive correlation between active nitrogen fixation and the possession of an uptake hydrogenase (hup), hup^+ and hup^- strains are scattered over the entire spectrum of effectiveness in *Rhizobium*. In the absence of any positive correlation between rhizobia in the free-living state and their effectiveness in association with a legume, the only clear-cut means of determining effectiveness is testing them under controlled conditions in the greenhouse and then in the field. However, the demonstration that a particular strain of rhizobia is effective in the greenhouse does not mean that this will also be the case under field conditions.

Plasmid technology also might be used to introduce other quite different desirable properties such as the ability to produce antibiotics capable of controlling organisms normally detrimental to the nitrogen-fixing organism and thus promoting *Rhizobium* growth by making it more competitive in the rhizosphere. It is also possible that plasmid technology may provide a means of establishing desirable nitrogen-fixing characteristics in other soil microorganisms.

Although the news media often suggest that genetic engineers are on the verge of transferring nitrogen-fixing (*nif*) genes to nonlegumes, this goal will probably not be achieved in the immediate future. Transfer of these genes to closely associated organisms is not particularly difficult--they were transferred to *E. coli* some years ago to produce a functional nitrogen-fixing system. However, while these genes have been transferred into yeast, they were not functional (that is, expressed). Although the transfer of *nif* genes into corn may be somewhat more difficult than into yeast, the transfer itself should be achievable.

As with yeast, transfer of the nif genes will probably be the easiest part of the process. Engineering the capabilities for the system to operate under low partial pressures of oxygen, to provide the energy needed for nitrogen fixation, and to integrate it generally into the system in a way that the genes will be expressed will likely be more difficult. Because of these difficulties, the transfer of nitrogen-fixing genes--a high-risk, long-range project--should not be considered a primary goal of developing countries at this time. However, it is important that work continue toward this goal.

Soil Amendments. Proper plant growth is basic to the exploitation of any plant-bacterial symbioses. In some cases, the use of proper soil amendments has allowed legumes to thrive on soils that, in their native state, were poorly adapted to the growth of legumes. Notable examples are in Brazil and Indonesia, where adequate applications of lime and phosphate to the soils resulted in a condition that supported excellent yields of soybeans. The mobilization of phosphorus is often enhanced by the presence of the proper mycorrhizae in the soil. Vesicular-arbuscular mycorrhizae appear to enhance phosphorus uptake in nitrogen-fixing plants. Although soil amendments can often cure deficiencies, it may be more advantageous in some instances to select or tailor plants to the soil type or climatic conditions.

Questions continue to be raised about the usefulness of supplying fertilizer nitrogen to leguminous plants. In the major soybean production areas of the United States, for example, that practice is not recommended. In some areas of less productive soils where the plants are under stress in early growth, the addition of small amounts of nitrogen to soybean plants may be useful. However, this practice should be examined carefully in all areas to prevent the addition of costly nitrogen fertilizers where they will yield no net economic gain for the farmer. Careful control of this factor could lead to a substantial reduction in the use of nitrogen fertilizer worldwide. Some of the other commonly used legumes fix little nitrogen and must be supplied high levels of combined nitrogen to assure an adequate crop. Recent plant genetic studies with Phaseolus (beans) produced genotypes that obtain an increased fraction of their nitrogen from the atmosphere. Similar improvements in other crops are entirely feasible.

Effective Rhizobium Inoculants. These are a critical requirement for the successful growth of legumes. It is important to produce legume inoculants locally, and this should be possible in developing countries. Their importation in most cases should be discouraged because the organisms may not be well adapted to the environment. If organisms are selected for conditions peculiar to a particular area and inoculants are prepared with proper quality control by responsible companies, the chances for acceptance and effective use are much

improved. Modification of the classical technology for growing the organisms may simplify production of inoculants in isolated areas: rather than using a highly concentrated broth of the inoculant for mixing with presterilized peat, the usual inoculant carrier, the broth can be diluted before addition. Using this technology, small manufacturing plants should be able to cope with large-scale production of inoculants. Although there is some question about the availability of peat in some developing countries, it is believed that it can be obtained in most countries. It can be sterilized in polypropylene bags by autoclaving, followed by introduction of the organisms.

In the same vein, other sources of nutrients for the growth of rhizobia should be explored. Sucrose has been used traditionally, but some cheaper sources of energy may be substituted. Even more costly is the yeast extract that is usually furnished as a source of nitrogen and growth substance for the rhizobia. Perhaps a cheaper nutrient, such as corn steep liquor, could be substituted without reducing the growth rate of the organisms.

Although some advocate single strain cultures of the bacteria in the inoculant, the use of mixed cultures to ensure effective inoculation over a broader spectrum of cultivars still seems preferred. Moreover, while preinoculated seed have seldom proven successful in the past, studies of inoculum survival on the seed coat should continue as preinoculated seed are a convenient vehicle for distributing root nodule bacteria. Research should also continue on other methods of inoculation.

Rhizobium inoculants are often subject to various stresses both prior to planting and in the field. Often in the tropics, exposure of the inoculum to high temperatures in storage results in inactivation. A suggested solution to this problem is use of a temperature-sensitive color spot on the bag of inoculant that changes color if the bag is exposed to a high temperature. In advising the customer of the inoculant's condition, such a device could motivate distributors to treat cultures with care. It is, however, also desirable to seek strains that tolerate higher temperatures without sacrificing nitrogen-fixing effectiveness. Genetic selection and other techniques could be applied to this problem.

The association between the leguminous plant and Rhizobium must be fitted to the field situation. The demonstration that a particular strain of rhizobia is effective in the greenhouse does not necessarily mean that it will perform in the same manner in the field where it may be subjected to a variety of stresses and may compete directly with indigenous strains of rhizobia for nodulation of the legume in question. Rhizobia must be selected or engineered to meet the existing field conditions.

There is currently much emphasis on developing rhizobia that withstand a variety of stresses before and during the infection,

including drying, temperature, pH, predation, salinity, and competition with other organisms. For the immediate future, it is more expedient to select for resistance to multiple stresses by screening local hardy strains than by using modern genetic methods to introduce resistance. Existing recombinant DNA technology may, however, play an important role in improving the nitrogen-fixing capability of strains tolerant to specific stresses. In this regard, it is important to keep in mind that the effectiveness of the nitrogen-fixing symbiosis must be maintained; there is no point in having a stress-resistant Rhizobium if it cannot effectively nodulate the host plant and fix atmospheric nitrogen. Recombinant DNA and associated technologies may also provide appreciable insight into the molecular basis for stress resistance by comparing rhizobia that are resistant to a specific stress or stresses with those that are not. In the more distant future, modern methods of molecular genetics may well be directly applicable to the introduction of multiple characters responsible for stress tolerance in Rhizobium.

It may be expecting too much to achieve dramatic increases in rates of nitrogen fixation by either selection or construction of elite rhizobia. Increases in the range of 5-15 percent are, however, certainly possible and could be of great importance if achieved for widely grown crops such as soybeans.

Because competition among bacterial strains in the soil is an overriding problem in soybeans and certain other legumes, it has been suggested that serious consideration be given to developing bacteria that do not persist in the soil. Although persistence means that inoculation is not needed each year, it may at times be undesirable if, for example, the benefits of superior strains produced by genetic methods are not realized because inocula from previous years prevent their effective use. Under such conditions, a method of destroying indigenous rhizobia by activating preexisting genetic lesions may be desirable. Alternatively, using strains that die each year should be considered. These techniques could prevent transferring a recognized problem in developed countries to new legume-producing areas.

Agronomic Practices. Approaches to optimizing agricultural productivity are influenced by economic factors. The advent of high-yielding crops adapted to intensive fertilization has diminished the agronomic practice of intercropping. With the rising costs of nitrogenous fertilizer, it may be desirable to reexamine the potential of intercropping between legumes and nonlegumes. It may also be desirable to expand utilization of multiple cropping systems and to introduce an additional, rapidly growing crop in periods between crops.

Rice

Sources of nitrogen for lowland rice and other flooded crops include the Azolla-Anabaena associations, free-living cyanobacteria (blue-green algae), and loose associations with free-living, nitrogen-fixing organisms.

Azolla-Anabaena azollae Associations. Azolla is a genus of small freshwater floating ferns whose members contain the nitrogen-fixing cyanobacteria, Anabaena azollae, as a symbiont in their dorsal leaf lobes. These associations can provide nitrogen for a rice crop, and because millions of people depend upon rice as their primary food, the use of Azolla as an alternative source of nitrogen for rice warrants attention. Moreover, it appears that Azolla may add other desirable materials to a rice crop.

The Azolla-Anabaena azollae association is a highly productive and novel symbiosis. Those species or strains most efficient in the use of phosphorus, tolerant of extremes in temperature, and resistant to pests should be selected through screening procedures. Maximal manipulation and benefits of the association will be obtained through control of sporulation. Spores should prove useful for breeding programs as a means of preserving germ plasm, and work has started on their potential use in inoculation.

Paddy fields are presently inoculated with a few hundred kilograms to several tons of wet Azolla per hectare, and selected strains capable of more rapid multiplication would decrease these amounts. If sporulation can be controlled at will, spores could be used directly as inoculum, or as a means of producing vegetative material for inoculum.

The search must continue for strains of Azolla that withstand high and low temperatures, and additional research is required on the effects of herbicides and herbicide residues on Azolla growth, as well as disease resistance, particularly to fungal diseases. Four of the six known Azolla species thrive over a rather wide pH range of from 4 to 9.

There is some evidence that Azolla strains vary in their demand for phosphate, and improvement will be possible in this area. In addition, Azolla can be preloaded with sufficient phosphate to carry it through several generations. Slow-release forms of phosphate, perhaps from floating forms of fertilizer, should be considered. As the Azolla-Anabaena azollae decomposes, it releases the fertilizer that it has assimilated, and thus both nitrogen and phosphorus and perhaps potassium become available to the rice.

Although the genetic diversity of Azolla is just beginning to be demonstrated, one can select from existing strains, and the future may see the development of new varieties or hybrids that will be particularly active in specific environments. Simple methods for

preservation of germ plasm and maintenance of the sporophyte (asexual reproductive cycle) should be developed so that the farmer can carry inoculum over from one year to the next with minimal loss.

The application of Azolla is not practical in the culture of deep-water rice, which constitutes about 30 percent of the rice production in Thailand and Bangladesh. However, the Azolla system has distinct advantages over other systems in that it is available and manageable. Further, the heterocyst frequency and nitrogen-fixing capability of the Azolla endophyte are greater than those in free-living cyanobacteria, and nitrogen fixation in Azolla is not markedly suppressed by combined nitrogen in the aqueous environment. The extremely high levels of nitrogen fixation--several hundred kilograms (kg) of nitrogen per hectare per year--reported for Azolla are based on multiple growth cycles of the association over an entire year and are not pertinent to normal cultural practice. More realistic expectations might be 50-75 kg of nitrogen per hectare per crop, which could be achieved with an active growth period of about 6 weeks.

Free-Living Cyanobacteria. Although cyanobacteria normally exhibit a high capacity for nitrogen fixation when tested under ideal conditions, they often fix little nitrogen under field conditions. Obviously, a number of characteristics of the organisms as well as ecological factors may limit fixation, but proper selection and genetic modification of cyanobacteria may improve their performance and result in inocula of consistent quality. Although rice has been grown for centuries without appreciable fertilization, rice culture is usually limited to one crop a year, and yields under these conditions have been quite low. Experiments at the International Rice Research Institute (IRRI) have demonstrated fixation by cyanobacteria of 30-50 kg of nitrogen per hectare per year, but this yield is erratic and inadequate to support high production of rice. Many variables, including predation, need study and control before cyanobacteria that fix high levels of nitrogen in the laboratory will benefit rice by providing significantly high levels of combined nitrogen under field conditions.

Heterotrophic Organisms. Although associative fixation in rice may make some contribution to yields, it is estimated to be substantially less than that made by cyanobacteria and the Azolla association. Fixation has generally been reported in the range of 3-7 kg of nitrogen per hectare per year. Because variations in fixation may depend upon the varieties of rice used, the latter should be screened carefully to identify those that establish the best association with bacteria. There is some difficulty in distinguishing the fixed nitrogen contributed by cyanobacteria, Azospirillum spp., clostridia, and other organisms that fix nitrogen under conditions

suitable for rice culture. Studies of paddy varieties may be the most productive approach to improving associative fixations.

Related to this, the exact changes that occur during the process of puddling rice fields are not well defined. Weeds are plowed under, and after they have undergone extensive decomposition, the rice is planted at a time judged empirically suitable by the vigor of generation of gas from plant residues. These processes need to be better defined.

Grass Associations

Many grasses grown under nonaquatic conditions may stimulate limited biological nitrogen fixation in their root zones by providing carbon compounds to heterotrophic, nitrogen-fixing bacteria. Such associative nitrogen fixation--characteristically studied as an association between grasses and *Azospirillum* spp.--contributes considerably less nitrogen than does fixation by leguminous plants and *Rhizobium*. Although of less importance quantitatively, the system warrants further investigation because of its potential contribution to cereals that constitute the food base for a large percentage of the world's population. A wide variety of organisms should be isolated and tested against many cereal cultivars to find a highly effective association between the two agents. The grass or the bacteria may need genetic modification to improve the association. Development of a good associative fixation for wheat, corn, and rice could have a major impact on food production for human consumption.

Certain salt-tolerant grasses grow for long periods under difficult soil conditions with the addition of virtually no fertilizer. For example, with adequate water *Diplachne fusca* (Kollar grass) grows at least 8 months of the year in Pakistan and furnishes adequate forage for cattle. The sources of fixed nitrogen appear to be an algal mat that develops on the soil surface and free-living organisms that develop below the soil surface and contribute additional nitrogen. Productivity may be as high as 80 tons of green matter per hectare per year. The grass in association with cyanobacteria and associative fixers can be the base of a profitable agricultural operation.

Actinorhizal Associations

Many families of nonleguminous plants form nitrogen-fixing symbioses with actinomycetes of the genus *Frankia*. Woody species forming such actinorhizal associations may offer agricultural and forest husbandry opportunities analogous to those projected for woody legumes nodulated by *Rhizobium*.

The nitrogen-fixing nonlegume Purshia tridentata already provides forage over large areas of rangeland, and other genera are harvested for wood. Casuarina, for example, grows at a sufficiently rapid rate in Thailand that it can be harvested for construction lumber within 5 years of planting. Trimmings from these trees are used for fuel, and the trees themselves may be a potential source of paper pulp, although this use has not been tested. Casuarina is a vigorous nitrogen fixer and is well nodulated in areas where the plant has existed for some time. When it is introduced into new areas, nodulation is poor. To date, the actinomycete that produces nodules has not been isolated in pure culture. Attempts should continue to isolate the endosymbiont so that it can be used as an inoculant. Casuarina is quite tolerant to salt and to water stress, but as there appears to be considerable variation in tolerance, strains of the trees should be selected for desirable properties.

RECOMMENDED RESEARCH ON BIOLOGICAL NITROGEN FIXATION

Based on a discussion of various research opportunities in biological nitrogen fixation, this working group identified a number of goals as warranting support by the U.S. Agency for International Development (AID) and other donors. Recommended areas for priority are listed below; other programs not assigned priority are covered in this report. Indicated in parenthesis is an estimated time scale for a well-organized research effort to achieve significant results.

- It is recommended that AID and other donors give the highest priority to support of research designed to optimize nitrogen fixation by (1) cyanobacteria and the Azolla system in association with rice (3-10 years), and (2) the legume-Rhizobium symbiosis. The latter requires developing stress-tolerant strains of Rhizobium spp. (5-10 years), selecting legumes suitable for local conditions (5+ years), and developing techniques for producing effective strains of Rhizobium spp. that do not persist in the soil long enough to compete with more desirable rhizobia added later (5 years).

Work on Azolla could be carried out most effectively at international and developing country national research institutions, supplemented by studies located at laboratories in the United States. Work on stress-tolerant strains of Rhizobium and selection of legumes would best be conducted at local and national laboratories within specific countries, with field studies at the local level. Development of the characteristics that provide nonpersistent rhizobia should be carried out at established laboratories well acquainted with modern genetic manipulations, which may again involve laboratories in the United States.

- It is recommended that AID and other donors also recognize the importance of research directed toward the following goals:

  -- Development of the trait in legumes that allows them to use fixed nitrogen simultaneously with soil nitrogen (time scale for productive research: 10-20 years)
  -- Development of improved technology for production and use of legume inoculants (3-5 years)
  -- Development of fast-growing, nitrogen-fixing trees (10+ years)
  -- Evaluation of grass species and associated bacteria for their ability to promote nitrogen fixation and accumulation of dry matter (10-15 years).

Studies of legumes that use fixed nitrogen and soil nitrogen simultaneously can be performed at national laboratories in developing countries. As the work will in all likelihood involve the use of ^{15}N as a tracer, it may be necessary to send samples for analysis to the United States or elsewhere. Work on development of the technology needed to produce inocula could also be done at selected national laboratories. It would also be desirable to continue the innovative and developmental programs of the Project for Nitrogen Fixation by Tropical Agricultural Legumes (NifTAL) at the University of Hawaii and of other comparable institutions. Studies on fast-growing trees can be made at national laboratories, especially those interested in forest technology. The International Center for Research on Agroforestry may be a particularly good coordinator for such work. Finally, investigations dealing with nitrogen fixation by organisms associated with grasses may be done most effectively at international laboratories such as the International Center for Research in the Semi-Arid Tropics (ICRISAT) or in other institutions where the necessary scientists are available.

PERSONNEL DEVELOPMENT

It is generally accepted that any successful program to develop biological nitrogen fixation worldwide requires a greater emphasis on training of personnel. Two approaches to training are usually taken. First, short-term training programs of a week or so are used in a number of countries to inform investigators in the country and to present techniques that can be mastered in a relatively short time. Such programs have filled an important niche. The second approach is longer term training in active research centers such as doctoral and postdoctoral training in universities and research institutions; an investment in either form of training by AID would be fully justified.

Successful short-term refresher courses have been held in Brazil and Africa at the Microbiological Resource Centers (MIRCENs). One program funded by the British has supported a senior investigator from England in Indonesia for 4 years, while at the same time supporting the training in England of 10 people from Indonesia, who returned to their country to continue their research. A similar plan is under way to train 100 young people from Pakistan in agriculture. Difficulty experienced in placing individuals abroad might be alleviated by participation of an international organization such as the Food and Agriculture Organization (FAO) of the United Nations. All programs, in all countries, of course, are suffering from the rapidly increasing cost of education.

The institutional arrangements for training investigators in biological nitrogen fixation are apparently inadequate. Efforts by NifTAL and other groups to furnish short courses have successfully extended Rhizobium technology to some areas. However, to establish a sustained program in a country, it is important that individuals be sent to centers of activity in nitrogen fixation where they will have an opportunity to study in depth before returning to their own country. It would also be useful to send senior investigators abroad to aid in interpreting local problems and suggesting possible solutions. Training deserves high priority in any program.

A substantial percentage of available funds would be well spent on training programs that extend over a number of years and concentrate on problems that occur in developing countries. Development of bilateral linkages will prove particularly useful for training scientists from developing countries and will confer a measure of continuity on research programs.

Dissemination of information in specialized areas is important for stimulating and maintaining personnel development. The Rhizobium newsletter that originated in Australia has been terminated, and its loss severs a link among nitrogen-fixing researchers in a large number of developing countries. Consequently, there is an urgent need to explore other means of maintaining and broadening communications among workers on biological nitrogen fixation.

Plant Cell and Tissue Culture

INTRODUCTION

During the past decade, the public imagination has been fired by advances in plant cell biology and molecular genetics.* Indeed, knowledge of genetically engineered plant cells like the "pomato" and the "sunbean" has leaked out of the laboratory. These and other important achievements in plant cell and molecular biology come at a time when the world urgently needs to find ecologically sound, energy-efficient ways of substantially increasing the productivity of agricultural and forest lands while bringing marginal land into production to meet growing worldwide demands for food, feed, fuel, and fiber.

Plant cell and tissue culture is, in simplest terms, a technique that uses single plant cells, unorganized groups of cells (callus), or organized cell masses (tissue). In an ideal system (presently possible with a relatively small number of species), cells are manipulated in a series of chemical and physical environments, which cause them first to multiply (cloning) and later to regenerate whole plants that survive transplantation to the greenhouse or the field. Research to extend the use of this system to varieties indigenous to developing countries could have immediate potential for application.

Research efforts in plant cell and tissue culture have increased dramatically worldwide in recent years including efforts in developing nations. Sixty-nine countries are now represented in the International Association for Plant Tissue Culture (up from 48 in 1978), including 40 nations in Africa, Asia, and Latin America. However, much of the new technology still is not being routinely applied.

---

*See Part Three for a separate background paper prepared on this subject, "Plant Cell and Tissue Culture: An Overview," by G. B. Collins.

Plant propagation using shoot tip culture is the most advanced area in plant cell and tissue culture and is currently being used to propagate elite genotypes of several plant species in a number of countries. Propagation using plant tissue culture can have a direct effect on crop improvement by more rapid and efficient propagation of certain species, and it can have an indirect effect by elimination of disease in other species. This technology is uniquely suited to solving certain problems in agriculture and forestry and was highlighted by the working group as the area most likely to make a near-term contribution to economic development in many developing countries.

The large numbers of cells produced in the laboratory using tissue culture techniques provide plant scientists with an opportunity to study the effects of chemical or physical stresses on the cell population. A great deal of spontaneous variation occurs as cells multiply in culture, and exposure to radiation and certain chemicals can increase the degree of variant formation. From the resulting cultures of normal and variant cells, it is sometimes possible to isolate cell lines having unique characteristics (salt tolerance, for example).

The selection of unique cell lines followed by whole plant regeneration is a valuable laboratory technique. Although practical limitations to plant improvement exist, variant selection using tissue culture has a reasonable potential for making important contributions within the next 10-15 years. On the other hand, techniques that manipulate and utilize only portions of cells (protoplasts, for example) and fragments of genetic material (cloned genes, for example) are still in the developmental stages, and very few, if any, practical applications to plant improvement are likely in the next decade using this high-technology aspect of plant cell and tissue culture.

REVIEW OF RESEARCH AND APPLICATIONS USING PLANT CELL AND TISSUE CULTURE TECHNIQUES

This working group identified eight research areas as prospects for near-term, mid-term, and long-term applications of plant cell and tissue culture techniques, respectively:

- Near-term (many new applications possible now or within 5 years)
  - -- Clonal propagation
  - -- Disease elimination
  - -- Germ plasm exchange
  - -- Gene transfer by wide hybridization

- Mid-term (some applications to plant improvement possible within 5-10 years)
  - -- Variant selection (including somaclonal selection)
  - -- Production of haploids

- Long-term (plant improvement applications unlikely for at least 10-15 years)
  - -- Secondary natural products
  - -- Molecular genetic engineering in plants.

Since plant cell and tissue culture techniques are comparatively new and evolving rapidly, efforts to utilize this technology to solve practical problems can also be expected to generate additional fundamental knowledge and thus help expand the scientific base of plant biology. This will contribute in important ways to the self-reliance and scientific capabilities of the countries where the technology is used.

The advantages of each research area as well as possible pitfalls are discussed below.

Clonal Propagation

An immediate opportunity exists to apply the established techniques of clonal propagation to a wide variety of plants including forest, horticultural, and agronomic species. These techniques have special applications to plants currently propagated asexually or others with long generation times such as trees. In reforestation efforts and for species being examined for fuelwood or other uses, these propagation techniques provide a means of producing large quantities of selected or elite propagules (cuttings, seeds, or spores) for evaluation, and they can often overcome many propagation and breeding difficulties that prohibit effective reforestation. In certain breeding programs, this aspect of plant tissue culture can be used both to increase the supply of limited plant material and to reduce the time required to introduce selected genetic traits. For example, it would be advantageous to propagate F_1 hybrids of some perennial crops such as cocoa and coffee.

When generalizing about any new technology, especially one as broadly applicable as plant cell and tissue culture, there are always limitations. In most situations, the methods of *in vitro* multiplication must be standardized for each species and perhaps for varietal or cultivar differences within species as well. However, one of the primary opportunities represented by clonal propagation technology is the introduction of plant tissue culture approaches into plant improvement programs in developing countries. In the near term, this will introduce developing country scientists to the various

techniques and managerial necessities of modern plant cell and tissue culture, thereby smoothing the way for the eventual addition of more research-oriented activities using the basic technology.

Disease Elimination

Tissue culture techniques have been used widely to provide pathogen-free plants, a need that is widespread for all major crops and ornamental plants. Disease-free plants facilitate the international exchange of germ plasm. Moreover, the yield and quality of many clonally reproduced crops are often significantly increased by elimination of disease.

The technique for producing disease-free plants is neither difficult nor does it require expensive laboratory equipment. It may be convenient to combine applied goals with basic research during the tissue culture phases of disease elimination. Thus disease elimination is a productive area for research support.

The two groups of plant pathogens most frequently encountered are microorganisms such as bacteria and fungi which attack plant tissues on exposed surfaces or cause systemic infections, and viruses and viroids which reside within the plant cell. The first category of pathogens--surface-borne bacteria and fungi--are often eliminated by the routine process of surface sterilization of the explant (living tissue) before placing it in culture. Plants subsequently derived from uncontaminated cultures will be free of these pathogens. Virus-free plants may be obtained by meristem culture and shoot tip graftings. Viruses occur less frequently in meristematic cells and often are not transmitted to progeny plants derived from the meristem in culture.

It is essential that any facility producing virus-free plants also be capable of testing the derived plants to be certain that they are virus free (virus indexing). Plants certified to be free of one virus may or may not be free of others. Virus indexing may be accomplished with biological assays (by exposing highly susceptible plants to sap or tissue from the tissue culture-derived plant) or with serological or new monoclonal antibody techniques.

Germ Plasm Exchange

Germ plasm resources are important to any plant improvement effort. Many opportunities exist for locating novel genetic traits in wild, semidomesticated, or indigenous cultivars and land races of all horticultural, forest, or agronomic species. Plant cell and tissue culture allows the transfer of plant materials in a disease-free condition for utilization in improvement programs worldwide. Such

transfers encourage the initiation of additional plant tissue culture activities on a wider range of plant species.

Many of the issues relating to germ plasm resources are not directly related to the scientific or technological approaches under discussion at this workshop. These issues are either political in nature or are being addressed by other organizations such as the international agricultural research centers funded by the Consultative Group on International Agricultural Research (CGIAR) or the International Board for Plant Genetic Resources (Rome).

Finally, it was observed that, in addition to relying on germ plasm centers, the biological, bibliographic, and human resources of botanical gardens, particularly those in developing countries, could be exploited, especially for vegetatively propagated plants.

Gene Transfer by Wide Hybridization

Traditionally, embryo culture utilizing tissue culture methods has been used to rescue hybrid embryos after wide crosses. Practiced for more than 40 years, this method is still appropriate in certain situations. Somatic hybridization using plant tissue culture methods is now feasible in certain species and may extend the limits of hybridization beyond those obtained using embryo culture.

Embryo Culture. Following certain wide sexual crosses, hybrid embryos can be rescued from the maternal environment where they ordinarily abort in the early stages of development. This is accomplished by aseptic removal and culture on an appropriate medium that allows for the development of intact and fertile plants. This technique overcomes the problem of cross sterility due to improper endosperm formation and subsequent embryo abortion after wide crosses.

Hybrid embryo rescue is the first step in the transference of derived traits from one plant species to another. Other steps include backcrossing the hybrid to the desired cultivated type while selecting for the desired trait from the donor species. This technique has been used in many crops such as barley, cotton, tomato, and beans and requires close interaction among cytologists, biochemists, and geneticists, as well as plant breeders. It can be applied where the problems are well defined (for example, combining disease resistance with high yield) and where a breeding program is well established.

It is also possible to produce totally new plant forms (for example, Triticale and Raphanobrassica) by wide hybridization while retaining the complete genomes of both parents. Historically, however, these have been long-term projects because of hybrid sterility problems.

Hybridization by Somatic Cell Fusion. The advances achieved during the 1970s in the inter- and intrageneric fusion of protoplasts

to produce somatic hybrids were both exciting and promising. Protoplast fusion appeared to provide unlimited flexibility in producing exotic new plants. However, the pace of development has slowed substantially in recent years. The main examples of success in producing somatic hybrids have been with species in the family Solanaceae (potato, tobacco, and petunias).

Somatic hybridization using protoplasts is a high technology research area that requires well-developed tissue culture techniques and genetic methods for selection. Further development of this field appears to depend on solving several technical limitations, including the inability to regenerate plants from cultured protoplasts for most species. Another serious limitation at present is the absence of suitable genetic markers required for the reliable isolation of fusion products in vitro and for the identification and analysis of true hybrid plants. A substantial research effort will be required to overcome these limitations.

The advent of routine protoplast culture methods and sensitive genetic markers to identify somatic hybrids will undoubtedly accelerate development of this field dramatically. Even so, somatic hybrids are not likely to provide productive new crops in the near or mid-term. Other wide hybrids, for example, Triticale and Raphanobrassica, required long periods for development before they were acceptable as crops. The stabilization and refinement of wide hybrids as crops can be expected to require substantial commitments to conventional genetic and plant breeding programs. In the long term, however, somatic hybrids appear very promising to generate an exciting array of new plant types, some of which may be very useful as new sources of food, fiber, pharmaceuticals, latex, and oil.

Variant Selection (Including Somaclonal Selection)

There are now several reports of new variants obtained by plant cell and tissue culture techniques. Regeneration has been recorded in 103 species (63 via organogenesis and 40 via somatic embryogenesis) of which 46 species are classified as of agronomic importance. The new variants have morphological, physiological, qualitative, and quantitative characteristics that show chromosomal, nuclear, and cytoplasmic differences from the original plants. The perceived advantage of this technique for crop improvement is the possibility of selecting novel genotypes, thus saving field space and time. Variant selection can be expanded through protoplast and cell suspension cultures, with or without the use of physical or chemical mutagens. This approach could allow research institutions in developing nations to work toward the release of new varieties within ongoing breeding programs. Of course, plant regeneration frequencies must be high and screening techniques very well defined. To make an effective and

rapid impact on breeding programs, the selected traits must be genetically transmitted and expressed throughout successive generations.

A limitation to this approach is that some important agronomic and forestry species have not yet been regenerated (for example, all of the large-seeded legumes, including soybeans, beans, peas). Furthermore, it is sometimes difficult to design screening methods for cellular variants that will be expressed as whole plant variants. Because at this time there is no known product of somatic cell selection in an important food, forage, or fiber crop that has been used in crop improvement, the perceived advantages of the method have not yet been realized.

Production of Haploids

Production of haploids by anther (part of the plant flower that produces pollen) culture has been used successfully for a growing number of species. Haploids are cells or organisms that contain only one representative from each of the pairs of homologous chromosomes found in the normal diploid cell. The most common use of haploids is to produce instantly homozygous lines by chromosome doubling of haploids (several other research applications of haploids are cited in Part Three in "Plant Cell and Tissue Culture: An Overview").

The advantages of anther-derived haploids are: (1) large numbers of haploids can be produced efficiently, and (2) complete inbreds can be obtained in one step by chromosome doubling. Anther-derived haploids have been used in the cultivar synthesis of tobacco in several countries and in rice, wheat, and corn in China. Moreover, it is currently possible to obtain barley haploids using a haploid embryo rescue technique after an interspecific cross.

A number of limitations exist to the application of tissue culture approaches to haploid production. First, large numbers of anther-derived haploids are not the rule, and culture techniques have not been described for many important crops. In corn, for example, haploids are produced at a comparatively low frequency and require labor-intensive procedures. Second, the haploid embryo rescue method is specific to barley. It should be noted, finally, that homozygous lines for use in crop improvement can be obtained in all of the above-mentioned crops and most other crops by conventional breeding methods within 4-5 years.

Secondary Natural Products

In spite of impressive advances in the area of synthetic organic chemistry, we continue to depend on plants for some of the vital

chemicals that can be derived from them, for example, the important insecticidal pyrethrins isolated from chrysanthemums. Plant tissue, organ, and cell cultures from a large number of medicinal plants have been established, and it is now possible to grow plant cells in suspension cultures at the 100-200 liter scale. Controlled biosynthesis using such plant cell cultures would have several advantages: (1) desired compounds could be produced under controlled conditions independent of climatic or agronomic conditions and at potentially reduced cost; (2) products from plants of tropical, temperate, or mountainous regions could be produced at one location; and (3) products from rare and endangered species could be made available.

Some fundamental problems still exist that have a direct bearing on the successful exploitation of plant cell, tissue, and organ cultures for biosynthesis of valuable chemical compounds. To date, the recovery of high value products from in vitro systems has not been economically feasible. A major drawback has been the need for continuous cell selection since plant cells have a tendency to change in culture (in contrast to microorganisms). Further research into the mechanisms of variant cell formation in plant tissue culture will be needed to develop techniques for maintaining stable cell lines.

Molecular Genetic Engineering in Plants

This is an emerging field whose potential for transferring genes from one plant to another is long range. However, plant genes are good candidates for recombinant DNA insertion into microorganisms. Once inserted the cloned DNA can be used for research purposes and for vat chemical production of specialty chemicals. Pharmaceuticals and diagnostic products can be expected in 10-15 years.

The transfer of cloned genes between plants for crop improvement must be viewed as a long-term prospect. The problems involve identification of specific genes and gene groups and their incorporation and expression in appropriate tissues of the multicellular plant. Research in this field requires a well-developed infrastructure in many research areas including plant cell and tissue culture, especially protoplast, microbial genetics, molecular biology, and recombinant DNA technology. It will also require reciprocal and continuing access to expertise from one or more traditional botanical research areas such as plant biochemistry, physiology, genetics, pathology, and breeding.

Genetic engineering technology has not developed to the point where it has been demonstrated that foreign genes introduced into the genome of plant cells can be stably incorporated, expressed, and inherited by the whole plant. It will require many years of research and a substantial investment to develop this technology. Investments

in well-established, reliable technologies should not be diverted in any country while awaiting development of reliable molecular genetic engineering of crop plants. However, shorter term opportunities to utilize other novel techniques of molecular biology could result in analytical approaches that assist conventional genetic and plant breeding programs.

ISSUES RELATED TO PLANT CELL AND TISSUE CULTURE

Education and Training

Trained personnel is the most important component of any successful plant cell and tissue culture program. Training should include a solid background in the life sciences followed by graduate-level work in botany, microbiology, biochemistry, or molecular biology. In addition to learning the specialized techniques used in plant cell and tissue culture work, the ideal training program will include exposure to agronomy, genetics, and plant breeding. For trained plant scientists, intensive 6-month programs in operating tissue culture laboratories can be very useful.

As much as possible, in-country training is preferable to training abroad. The possibility of using a unique agricultural or silvicultural problem to attract a small team of younger plant scientists to a developing country for a period of up to 4 years of concentrated effort should be explored. The team's research could be used to provide training for host country scientists who would then continue the work of the laboratory at the end of the visiting team's stay. The setting for an effort of this kind might be a university, a research institution, or a private firm.

Infrastructure

A successful plant cell and tissue culture program will require equipment, chemicals, and library and technical resources not commonly available in developing countries. A thorough evaluation of the minimal requirements for success and a plan to deal with local constraints must be part of any serious proposal to engage in a new effort.

Ready access to small quantities of relatively inexpensive specialty enzymes and chemicals is a key barrier to tissue culture work in most developing countries. Tissue culturists must overcome this independently using ad hoc arrangements. It was suggested that an effort be made to establish a formal mechanism to resolve, in a general way, such small-scale foreign exchange and importation problems. Support for a central "enzyme bank" of some kind was expressed by the developing country scientists.

Research Collaboration

Experience with successful plant cell and tissue culture programs in India, Brazil, and China has demonstrated the importance of ongoing research collaboration with comparable groups in the United States, Europe, or Japan. Collaborations have generally been long term with bilateral funding and have included elements of equal partnership with benefits to all parties. The successful programs are regularly monitored for progress and appropriateness of objectives. Research collaboration often results from training programs.

Communications

One of the most difficult problems facing developing country scientists is an almost total lack of information about the latest developments in their research area. This is particularly true in a field progressing as rapidly as plant cell and tissue culture. Support is needed for:

- Expanded local libraries by means of subscription to a larger number of journals and abstracts, or installation of microcomputer terminals connected to libraries and data bases in developed countries
- Subscriptions to newsletters of professional societies
- Regular participation in workshops and meetings, particularly of a regional nature, where plant scientists can exchange ideas.

Private Sector Involvement

It may be possible to establish self-supporting plant cell and tissue culture laboratories in developing countries by taking advantage of high value species such as floricultural, horticultural, or medicinal plants. In addition, opportunities exist for involving the private sector in the United States and other developed countries. The unique scientific and managerial talents that are found in the private sector can be used in training, for example. Contacts made in this way would facilitate establishment of joint ventures. In general, whenever collaborative research is possible between various private and public organizations, it should be encouraged.

Development of Research Centers

Experience in industrialized and developing countries alike has shown that successful cell and tissue culture programs generally start small (1-3 professionals) and grow rapidly with the additional involvement of plant scientists trained in physiology, biochemistry, genetics, or molecular biology. Efforts, therefore, should be made to establish small tissue culture laboratories in an institutional environment that will facilitate future growth including the development of interdisciplinary approaches by teams of cooperating scientists.

The rate of growth in new programs can be dramatic. In Brazil, for example, the number of research groups using plant cell and tissue culture grew from zero to over 20 in a decade. Over half of these groups can be traced to the stimulus of a single U.S. scientist who became deeply involved through training and research collaboration beginning in 1971. Transfer of the technology to other countries has followed a pattern characterized by long-term relationships between individual scientists. Extension to other areas of the world will benefit from programs prepared to provide continuous support over a period of 10 years or more.

RECOMMENDATIONS

This working group made a conscious decision not to recommend research on specific plants, feeling that such judgments can only be made locally by people most familiar with the problems and needs. It was felt more appropriate to recommend guidelines to be used in evaluating proposals for applying plant cell and tissue culture to particular problems.

- It is recommended that development assistance agencies evaluating research proposals in the area of plant cell and tissue culture:

  -- Give highest priority to proposals that include provisions ensuring that the products of tissue culture technology reach farmers and consumers. This generally requires programs for testing and evaluating new products and capabilities for commercial seed or stock production. Proposals that will result in immediate application should be given special attention.
  -- Give high priority to proposals on well-defined plant improvement problems. For a given problem, proposals to use the most feasible tissue culture methods should be given preference over those proposing to use methods that

need more development. Conversely, low priority should be given to proposals to learn techniques using model plants such as tobacco; often such projects have never progressed from tobacco to other important plants.

-- Give high priority to proposals that will permit the near-term extension of plant cell and tissue culture techniques to plant improvement problems using any or all of the following: clonal propagation, disease elimination, germ plasm exchange, or gene transfer by wide hybridization using embryo culture. The research goals of such proposals will include the development of procedures for efficient clonal propagation and regeneration of elite genotypes, preferably by adaptation of known procedures for the species. When procedures for the species have not been developed, careful judgments should first be made, based on results with related species, regarding the likelihood of near-term success. In general, important opportunities exist with many tropical species, especially those that propagate asexually and those that have long generation times, such as trees. In this regard, plant tissue culture propagation appears particularly suited to problems concerning reforestation, fuelwood supplies, and the development of tree crops.

-- Require that proposals to begin work in plant cell and tissue culture include a thorough evaluation of the minimal infrastructure requirements and specify a plan for removing constraints. Granting agencies should be prepared to provide funds for infrastructure needs when necessary.

- It is also recommended that:

  -- Efforts be made to provide training for personnel from developing countries in laboratories of scientists willing to engage in long-term collaboration. To the maximum extent possible, opportunities for training in-country by visiting scientists are preferable to training abroad.

  -- Consideration be given to establishing regular scientific exchanges at all levels for developing country scientists engaged in plant cell and tissue culture research. Regional, problem-oriented workshops and newsletters directed toward particular applications of the technology should be given special attention.

  -- Private sector involvement in the application of plant cell and tissue culture to problems in developing

countries be encouraged. Private firms may be able to provide short-term training and could be contracted to do research and development work on problems of special importance to developing countries.

-- Efforts to establish tissue culture laboratories begin small, with preference given to institutional settings that are amenable to the eventual development of strong interdisciplinary teams of plant scientists.

PART THREE
Background Papers

Agriculture Overview

LOWELL LEWIS

INTRODUCTION

Although the world has made significant gains in total food production, the problems of chronic malnutrition and hunger persist in many nations. Enough grain is produced presently to provide every man, woman, and child with some 3,000 calories a day, more than the average level of consumption in the United States. Increases in world food production are largely due to the "Green Revolution," which introduced high-yielding varieties of wheat, rice, and other grains and improved agronomic practices. In many countries, production increased three- to fourfold. It is estimated that more than 50 million hectares have been planted with high-yielding varieties, and that the increases in production can meet the food needs of over 300 million people.

According to C. P. Timmer of the Harvard Business School (personal communication, 1982), highly skewed rural incomes and lack of adequate purchasing power among consumers and food producers are creating widespread hunger and stagnating food production. For these reasons, many development agencies are shifting their emphasis from direct relief to attempts to reform the agricultural and social patterns within developing countries. One way to address poverty is to increase agricultural productivity--the actual units produced per units input.

World food production must double in the next 40-80 years, depending on the rate of population growth. In 1975, when the world population was 4 billion, 3.3 billion tons of food were produced. If population grows at an annual rate of 2 percent, the 8 billion people of the year 2015 will require 6.6 billion tons of food. Two-thirds to three-fourths of this increase must come from intensified production on existing land and the rest from bringing new, marginal lands into production. Both tasks will require new and appropriate technology.

The elements of the "Green Revolution," along with improved plant varieties, were chemical fertilizers, irrigation, and mechanization--fossil fuel-intensive and technology-intensive solutions. In the future, agricultural productivity must be increased with different inputs. The next generation of agricultural research must focus on removing obstacles to animal food production such as widespread disease, breeding plant varieties to withstand environmental stress and resist pests, and improving photosynthetic efficiency, nutrient and water uptake, and biological nitrogen fixation.

ANIMAL PRODUCTIVITY

Diseases and parasites severely constrain animal productivity in many developing countries, particularly in the tropics, and they account for the annual loss of more than 50 million cattle and buffalo and 100 million sheep and goats worldwide. The cost of human attempts to control such diseases is staggering. One Rockefeller Foundation report (Muriithi 1978) noted that tick-borne diseases in Africa and South America are controlled by dipping millions of cattle weekly.

In spite of the impact of tropical animal diseases, developing countries have 60 percent of the world's animals. However, these animals produce only 20 percent of the world's animal products. The opportunities to augment such production are manifold, especially in light of increasing demand. The Food and Agriculture Organization of the United Nations (FAO) has estimated that the effective demand for animal products in developing countries will rise 100 percent from 1976 to 1990.

Three areas of biotechnology could strengthen animal productivity in developing countries by several magnitudes: the use of vaccines and the use of monoclonal antibodies in the control of disease, and the techniques of embryo transfer.

Vaccines

Vaccines are the most effective and least expensive means of controlling infectious disease. Today, new techniques of genetic engineering are greatly enhancing a vaccine's effectiveness. One example is the new vaccine against foot-and-mouth disease, an important cattle disease found in South America, Asia, and Africa. This disease is a major threat to the livestock industry, and its spread in the United States would cost billions of dollars.

In the past, foot-and-mouth vaccines had the intended effect of stimulating the animal's immune response, but they fell short of the desired effect because the vaccine viruses had to be weakened or killed to prevent actual transmission of the disease. Scientists at

the U.S. Department of Agriculture's laboratory at Plum Island, New York, have now produced a superior vaccine. They used genetic engineering to remove the portion of the virus that makes it pathogenic; the remaining virus evokes a much stronger immune response without transmitting the disease.

Promising research is also under way to develop a new, more effective vaccine against bluetongue virus, which attacks cattle, sheep, and goats. This virus destroys embryos. If cows are infected late in gestation, it causes severe neurologic defects in the offspring. In the adult animal, the virus impairs health and weight gain.

Some 20 varieties of bluetongue virus exist, and they are difficult to distinguish. Unfortunately, scientists have failed to develop a good vaccine because they do not know which strains are most important. Since there is no effective way to test animal tissues as they are being transported between countries, this virus has stopped international shipment of embryos and sperm, thus preventing a number of West African countries from introducing superior breeding stock from other countries. A solution to this problem lies in new techniques associated with monoclonal antibodies, which offer enhanced identification and manipulation of specific disease strains.

For the last 100 years, scientists have had only limited success in developing vaccines against parasitic diseases. Parasites inhibit the immune response of the host and produce so many different antigens that the host cannot develop specific antibodies to attack the parasite. Vaccines are not, therefore, available for some of the most prevalent diseases of our time, including malaria.

Two parasitic diseases believed to be responsible for the death of 3 million head of cattle a year are theileriosis, or East Coast fever, and trypanosomiasis, known as sleeping sickness when it occurs in man. These diseases inhibit the use of cattle, and even human settlement, on 7 million square kilometers of land, which could probably support an additional 120 million head of cattle. If just 10 percent of this number were available each year, an additional 120,000 tons of meat could be produced annually, as well as additional milk.

Theileriosis is found in many parts of Africa, the Middle East, India, and Pakistan. In eastern Africa, mortality of 90-95 percent is expected if susceptible cattle are introduced into an infested area without precautions.

Trypanosomiasis inhibits the use of cattle throughout the entire central portion of Africa south of the Sahara and in parts of South America. Symptoms include anemia, retarded growth, poor production, and incapacity to work. This disease is transmitted by the tsetse fly, which prevents grazing, cultivation, and human settlement in many areas. Although it elicits an immune response, this parasite survives in animals by changing its cell coat and so its antigenic structure;

thus an animal's immune response cannot overtake it. One variant may be eliminated, but only in time for another to emerge and proliferate. Scientists hope ultimately to define some part of the molecule that does not change with each cell coat and elicit an immune response to that component of the molecule to obtain more effective immunity.

Monoclonal Antibodies

Since 1975, scientists have been able to use antibodies in new, powerful ways to diagnose and manipulate the agents of disease. In the past, scientists employed the natural immune defenses of humans and animals to produce vaccines and antibody sera. Vaccines consisted of weakened or destroyed disease agents that stimulated the immune response, while therapeutic sera consisted of antibodies themselves. Scientists also used antibodies as a diagnostic tool for distinguishing strains of disease. Such diagnostic tools, called reagents, were obtained by selecting antibodies that attacked a certain type of pathogen; however, this could not be done with precision. The body produces extremely diverse antibodies when invaded by a foreign substance—several hundred to a thousand different types. Because it is almost impossible to separate them, conventional antisera contained mixtures of antibodies.

Scientists have known that the full resolution power of antibodies is available only if one antibody-producing cell is selected and grown in culture. The single cell's progeny, or clone, is then a source of large amounts of identical antibody against a single antigenic determinant. However, antibody-secreting cells cannot be maintained in a culture medium.

In the last 6-8 years, scientists have devised a solution to this problem. They can now immortalize antibody-producing cells by fusing one with a tissue culture adapted tumor cell, that is, a cell of a similar lineage but one that has become malignant and thus has gained immortality. The result is a plentiful source of antibody that is up to a thousand times more specific than before because it is made from identical cells, all cloned from a single antibody-producing cell. Scientists are now able to select and mass produce the antibody of interest for diagnosis of disease, dissection of a given pathogenic organism, and identification of the component most useful in production of a vaccine.

One of the most important immediate uses of monoclonal antibodies is for more precise identification of a disease. Once it is known which subtypes of a virus or bacteria are causing the disease, it is possible to use existing vaccines or develop additional ones to fit the disease. Monoclonal antibodies are also important for the study of genetic susceptibility to disease. Studies in mice and humans

indicate that certain diseases can be recognized by genetic markers, which can in turn be recognized by antibodies. The highly specific monoclonal antibody will enable scientists to obtain good genetic profiles of animals, particularly breeding stock, and identify those carrying the most desirable traits, such as resistance to disease, milk production in dairy herds, and weight gain in cattle.

While monoclonal antibodies promise breakthroughs in the control of animal and human disease, the development of vaccines for some of the most complex parasitic diseases will not come quickly. For example, the International Laboratory for Research on Animal Diseases (ILRAD) in Nairobi has learned an enormous amount about the biology of trypanosomiasis, but this new information has only confirmed how difficult it will be to produce a vaccine for this disease. In spite of early optimism, the development of such a vaccine in the next decade is not certain.

On the optimistic side, scientists investigating malaria now believe they can develop a vaccine for this devastating disease within the next 5-10 years. This disease currently infects 300 million people and results in the death of 1 million African children every year.

Embryo Transfer

Embryo transfer, an innovation of the last decade and now a $20 million industry in the United States, could have a stunning impact on animal food production in developing countries if its initial cost and requirements for technical sophistication do not prove prohibitive.

The term "embryo transfer" now applies to a sequence of steps that begins with the stimulation of multiple egg production at the time of ovulation. Artificial insemination is followed by embryo recovery, usually providing 10-15 embryos from a single cow, which are stored under laboratory conditions. The embryos are eventually transferred from the genetic mother to host or surrogate mothers.

The major use of embryo transfer is to increase the reproductive rate of valuable cows. Normally only one embryo per year is obtained from a cow. Now a genetically superior donor, through its recipient cows, can produce 50-60 superior calves in a single year. In addition, embryo transfer accelerates the introduction of new genetic material into indigenous cattle; with a herd of recipient cows, it requires one generation. The conventional method of artificial insemination requires three generations before the offspring are seven-eighths the genetic makeup of the new breed.

Because embryos can be frozen for international shipment, a developing country could import genetically superior embryos and make rapid progress in upgrading their cattle. This method also reduces

disease problems that accompany the introduction of superior breeding cattle to a foreign country.

Upgrading cattle stock could double or triple animal food production. Milk production could jump from 4,000-5,000 pounds (lbs) per year per cow to 12,000 lbs. The weight of beef cattle at 1 year could rise from 500 lbs per animal to 800-900 lbs, with an accompanying improvement in quality of meat.

According to a recent Science article (Seidel 1981):

> Embryo transfer could permit the introduction of cattle to many areas of the world where it is now prohibited because imported mature animals die or are severely harmed by infection with local disease. When embryos are transferred to indigenous recipients, the young receive appropriate immunity through the first colostrum, or milk, and the developing immune system of the calf becomes specifically attuned to the disease organisms in the environment.

Although embryo transfer appears to produce high gains relative to the required investment, its use by developing countries depends on a high initial capital outlay and the existence of a technological infrastructure. The cost of 10-15 embryos is $2,000, but the largest cost of the procedure is maintaining recipient cows. Seidel notes that "techniques will continue to be simplified and efficacy will increase, especially for freezing embryos; consequently costs will decline drastically. It will probably be decades, however, before embryo transfer will be used on a scale comparable to artificial insemination."

PLANT PRODUCTIVITY

Plant Cell and Tissue Culture

Tissue culture, an innovation of the last two decades, could be directly applied to increasing food production in developing countries with dramatic results. This technology produces genetic duplicates of superior plants, each grown from bits of tissue that are taken from a single parent plant and placed in a test tube. This represents the fastest method of cloning, up to a million times faster than traditional methods, and scientists have perfected test-tube

environments for hundreds of plants, ranging from carrots and tomatoes to fir trees and giant sequoias. The duplicates produced are of uniformly high quality, free of disease, and more vigorous than plants grown from seeds.

Developing countries can use this technology in several ways. Breeders can clone the best-adapted, superior plant varieties to produce enough duplicates for large-scale production. In the United States, it is normal for a commercial nursery to produce 300,000 such clones in a month, quickly paying off the $50,000 capital outlay for facilities. Tissue culture methods can also be used to reestablish such varieties in a disease-free state. Many varieties in the existing gene pool are riddled with pathogens--viruses, bacteria, and fungi. If breeders begin with clean material, they can increase productivity of a variety from 30 to 300 percent.

Another use of tissue culture is to select naturally occurring mutants among somatic cells--one in every 10,000 to 1 million cells in the plant body is a naturally occurring mutant. Mutants can be selected for tolerance to disease, saline soils, drought, heat, or cold. By exposing cell cultures to a disease toxin, for instance, scientists can quickly identify the one that survives and can regenerate a new population of plants from it. In potatoes, this technique allows production of new varieties within a year.

Breeders also use cultures of the anthers, which contain pollen, to produce haploids, cells with half the normal number of chromosomes. These can be used to telescope the normal time it takes to produce new varieties. Scientists in the Far East have successfully used this technique to produce new varieties of wheat, rice, tobacco, and other crops in about one-third the time it takes to produce varieties by traditional methods.

By placing plant embryos in tissue culture, plant breeders can rescue rare hybrids--offspring that combine desirable traits of two species. Many potentially valuable hybrids are aborted as embryos because of incompatibility in their environment, such as a deficiency or toxic element in the fruit. Using tissue culture techniques, scientists can remove the embryo relatively early and rear it in the test tube where it grows free of all stresses. This technique has been used for 50 years in the United States. Orchid breeders have developed hybrids involving 5-70 different genera, let alone species, an unheard of feat before tissue culture.

Plant breeders can assist nature through test-tube pollination, a technique used when normal pollination of the flower does not result in fertilization. Researchers excise the ovules, the parts of the flower that contain the eggs, and directly apply the pollen to produce fertilization. In this way, seeds that would not occur in nature are obtained for breeding purposes.

Protoplast fusion, a form of tissue culture that enables breeders to make wide crosses between genera, probably will not have any

practical use in developing countries for at least 10 years. Protoplasts--somatic cells from which the walls have been removed--are taken from two parent plants and fused into a single walled cell in an artificial medium.

Protoplast fusion has been used for fusion of a potato cell and a tomato cell, producing an exotic hybrid that is sterile; it produces flowers but no seeds. Its usefulness lies in its expanded gene pool, which is now available to the plant breeder. However, scientists will have to engineer chromosome eliminations to reduce the large number of unnecessary genes that interfere with the use of this hybrid for breeding purposes. The present usefulness of protoplast fusion is to provide new germ plasm for an existing crop.

Nitrogen Fixation

Nitrogen is essential for plant growth, and in most of the world it is the major limiting factor in crop production. Since man-made nitrogen fertilizer is prohibitively expensive in many developing countries, crops capable of fixing their own nitrogen have acquired urgent importance in the developing world.

The most efficient of these crops, the legumes, form a symbiotic relationship with the soil bacteria Rhizobium, encapsulating them in root nodules. These bacteria manufacture the nitrogen-fixing enzyme nitrogenase, which provides two-thirds of all the nitrogen fixed on the plant--about 200 million tons annually.

Researchers are now searching for super strains of Rhizobium by exploring their activity with different plant varieties under different conditions of acidity, temperature, and soil nutrient level. Some procedures developed at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) have increased the rate of nitrogen fixation in legumes 300-500 percent. These results should reach the farmer's fields in the 1980s. More immediately, legume-based cropping systems could be widely used to build soil in developing countries through sequential cropping, interplanting, and winter ground cover.

In Brazil, soybeans are now a major source of foreign income, although there was no production of any consequence 10 years ago. Their success can be attributed to the good yields achievable without appreciable fertilizer. Cowpeas, another legume crop, are being cultivated in the African Sahel to build up residual nitrogen supplies and to provide a popular food crop as well.

A number of factors limit nitrogen fixation in the field, although these symbiotic systems show dramatic potential in the laboratory. For example, indigenous rhizobia that do not fix nitrogen may compete with the added Rhizobium and take over positions on the roots where there could be beneficial activity. Furthermore, rhizobia

inoculated onto seeds often do not survive various farming practices. For instance, the inoculated seeds may be stored for some time before planting, often at high temperatures, or they may be planted in dry, hot soil.

A very promising system for nitrogen fixation in connection with rice cultivation is blue-green algae. When rice is grown underwater, coexisting organisms such as blue-green algae, and possibly bacteria, fix nitrogen. There is now evidence that these algae fix enough nitrogen to sustain the crop without additional inputs of nitrogen--and have done so for hundreds of years. Insufficient nitrogen exists, however, for high yields; this can be reversed by inoculating the field with more algae or bacteria.

While scientists are using genetic engineering to search for more efficient nitrogen-fixing organisms, research is also needed on the reasons that the presently used organisms have limited efficiency. Algae transported to laboratories fix more nitrogen than they do in the field. Probable limiting factors are the presence of phosphorus or iron in the water, competition with the non-nitrogen-fixing algae that are present, and predation by small animals. Once these limitations are confirmed, they can be overcome or bypassed.

Farmers in the Far East are using a remarkable form of nitrogen fixation that exists in some flooded rice fields. The wild fern Azolla contains an algae Anabaena in the hollows of its leaves; together they fix 300-500 lbs of nitrogen per acre per year. This can be compared to clover and alfalfa, which fix 100-200 lbs per acre per year. The Chinese and Vietnamese have used this wild fern in rice cultivation as a green manure crop, and it may be useful in other parts of Asia, Africa, and Latin America.

While work on biological nitrogen fixation of legumes will likely be expanded in the 1980s, it is much less likely that scientists in this decade will incorporate a nitrogen-fixing system into cereal grains, although this prospect has received much speculation. Even if this were accomplished, plant energy would be required to fix the nitrogen, resulting in about 20 percent lower yields.

LIMITATIONS TO AGRICULTURAL PRODUCTIVITY

Socioeconomic Factors

The major factor limiting agricultural production in developing nations is the lack of a monetary incentive; in many countries the prices received for agricultural products are simply too low to reward the farmer for his efforts. Governments tend to keep these prices low for at least two reasons. First, if prices are allowed to increase so that the farmer receives a fair price, the cost of food becomes prohibitively high, possibly resulting in civil unrest. Second, if

the government subsidizes the price of food so that the farmer receives a fair price and the consumer pays the low price, the country soon runs out of money.

Sometimes government policies create a system of incentives that render scientific achievements undesirable. For example, it has been noted in some countries that farmers are reluctant to use high-yielding dwarf varieties of grain because, under present conditions, straw yield is more valuable than grain. In one case a government has put a ceiling on the price of wheat that is substantially below the world market price, and has mandated compulsory deliveries of a major portion of the crop. The government then uses this portion to subsidize the price of bread to the urban poor. However, while the price of grain is kept artificially low, the price of meat is not controlled and has become very high. Consequently, the price of animal feed is high, making the parts of a grain crop that can be fed to animals very valuable. Growers can produce twice as much straw from the traditional varieties as they can from the high-yielding dwarf varieties.

The risk factor also limits agricultural productivity. Developing country farmers who have lived on the edge of starvation and economic disaster for centuries may not risk a change in their production system, even though they understand that a change would be profitable in the long run.

Another obstacle to productivity is poor marketing. Too often farmers have been encouraged to plant a commodity only to find that there is no market for it, or no way to transport it to market. Many countries lack reliable transportation systems and a food processing and marketing infrastructure.

Finally, the extension of agricultural techniques is often not suited to the farmer's needs, and fails to take into account a valuable source of information--already existing cultural and agricultural practices. Many extension needs could be met by an effective cooperative extension service similar to that existing in the United States. Although developing countries may have a titular cooperative extension, it does not necessarily serve the production needs of farmers.

Differences in Nutritional Needs of Developing Countries

Biochemical differences in populations may play a decisive role in the transfer of agriculture-related technology to developing countries. Not all populations of the world have the same nutritional requirements. For instance, much of the world's adult population cannot digest the lactose in milk. The only exceptions are the Northern Europeans and their descendants in America and three African tribes: Fulani of West Africa, Tutsi of Middle Africa, and Masai of

Kenya and Tanzania. Thus the quantities of milk the United States has provided to developing nations for several decades have been indigestible to most of the recipients. If they drank it in any quantity, much of its protein value was lost in the resulting diarrhea. In the United States, 70 percent of blacks are intolerant of lactose (see Kretchmer 1972).

In spite of digestion problems, milk has provided needed calories, and under no circumstances should milk shipments be discontinued without providing a substitute. Many dairy products such as yogurt and pressed cheeses are digestible to non-milk drinkers. Alternatively, milk could be treated with a microbially produced lactase to convert lactose into a digestible sugar form of glucose and fructose.

Patent Laws

Recent patent laws and a related U.S. Supreme Court decision could have a chilling effect on collaborative research between U.S. and developing country scientists. The Plant Variety Protection Act of 1970, with its 1980 amendments, extends patent rights to breeders of sexually produced plants and seed growers. The act applies to seeds, transplants, and more than 350 plant species, including tomatoes, carrots, cucumbers, okra, celery, and peppers. It also extends the period of patent protection for plants from 17 to 18 years to conform with the provisions of the International Convention for the Protection of New Varieties of Plants.

Such patent laws present a problem to developing nations because patented seeds are more expensive, and royalties can place a heavy burden on farmers. There was a 150 percent rise in the total cost of seeds and plants used by American farmers between 1972 and 1977.

Although a nation's patent laws are not necessarily binding internationally, technologically advanced countries are taking new measures to protect the products of expensive genetic research. For their part, developing countries are also concerned about genetic material from their countries used in foreign breeding programs. Should selection become available that uses that material in the breeding line, the developing country must then pay a royalty to purchase the new variety.

Two countries may enter into a testing agreement for scientific exchange of germ plasm. Under these agreements, research organizations in developing nations can use the resulting plant varieties for their own scientific investigations. However, most of these research organizations are not equipped to handle commercial distribution. If, for example, the University of California developed a plant variety with commercial application in a developing country, it would probably enter into a commercial agreement with a firm in

that country, presuming there was protection under the patent laws of that nation.

One problem for developing countries is that their valuable natural resource, the untapped germ plasm of wild plants, is not patentable. For example, a promising new plant discovered in a developing country could be sent via a collaborative program to a U.S. scientist, but that country would receive no benefit from the eventual breeding triumph. Existing patent laws apply only to plant varieties that have been manipulated by man or discovered as sports or mutations of cultivated plants.

A related U.S. Supreme Court decision extended patent rights to the products of genetic engineering. In June 1980, the Supreme Court ruled that new forms of life created in the laboratory are eligible for patents under current patent law. The case, Diamond v. Chakrabarty, concerned a scientist for the General Electric Company who developed and patented a microorganism that degrades crude oil into carbon dioxide and protein.

CONCLUSION

Biotechnology is essential to solving the food production problems of the developing world. Its success, however, depends on realistic applications in the context of sociocultural, economic, and political limitations in each country. As David Saxon, president of the University of California, has said, it is becoming urgent that both scientists and the general public be able to distinguish the credible from the incredible and the science from the pseudoscience if we wish to grasp tangible benefits from the abstract and infinite possibilities of the scientific world.

REFERENCES

Kretchmer, N. (1972) Lactose and lactase. Scientific American. October:131-138.

Muriithi, I. E. (1978) The impact and implications of theileriosis and trypanosomiasis on livestock production in Africa. Inauguration Symposium on Current Trends in Immunology and Genetics and their Implications for Parasitic Diseases. International Laboratory for Research on Animal Diseases and the Rockefeller Foundation.

Seidel, G. E., Jr. (1981) Superovulation and embryo transfer in cattle. Science. Vol. 211.

Overview of New Biomass Industries

HENRY R. BUNGAY

INTRODUCTION

The oil-importing nations are fighting for economic survival because fuel costs are rapidly draining their financial resources. Worldwide demand for petroleum has dropped because of a surprisingly effective conservation effort based on automobiles with greatly improved mileage and on more sensible use of fuels for homes, buildings, and factories. Without such conservation in the more developed nations, petroleum prices would continue to escalate, leading to greater distress of many nations. Alternatives must be examined, nevertheless, and the use of biomass as a feedstock for the fuel and chemical industries has attractive prospects. In the beginning, biomass industries should have opportunities for healthy profits as small factors in large markets keyed to expensive fossil feedstocks. The prices of products made from biomass must be established to meet competition.

Other technologies for harnessing the sun's energy have monumental engineering problems. Photovoltaic systems are expensive to construct and require ongoing maintenance and backup or storage for times when the sun is not shining. Mounting these systems in space and beaming microwave energy continuously to the earth seems far too expensive, especially when the microwave intensity may be roughly the same as that of sunlight. The ingrained spending habits of space programs may preclude any faint hope of profit. Boilers powered by focused sunlight, wind energy, generation of electricity using thermal differences in the oceans, wave energy, and other means of direct or indirect harnessing of solar energy could make significant contributions when conditions and logistics are favorable.

Photosynthesis is a natural way of harnessing energy from the sun, and the resulting biomass has some very attractive features: no required backup system as the crop is a form of storage; good reactivity, especially for sugary or starchy materials; stable prices for cellulosic materials because many different plants are roughly

equivalent so none need have an exclusive market; and well-established technology for the agricultural practices. Disadvantages are: wide distribution compared to the concentrated deposits of coal, oil, and gas; high moisture content to add mass and lower fuel value; and some of the most promising conversion technologies still need research and development.

Although there is abundant biomass worldwide, many countries with dry climates have severe shortages of trees and other vegetation. The rate of growth of biomass is several times the rate of fossil fuel consumption. However, growth of a standing crop depends on plants dying and decaying to provide nutrients for new growth. Soil fertility may decline rapidly if man intercepts growth and does not return nutrients to the land such as organic matter, nitrogen compounds, and phosphate. Biomass production can be increased dramatically by using fast-growing species and sound management, but there are maximum growth rates and efficiency of capturing sunlight. There are also few areas of the world where water is so abundant that its cost can be discounted. Nevertheless, sufficient resources exist in various regions, and a shift to large biomass industries is getting under way. Farms of the future may produce food and fiber and energy feedstocks.

Biomass fuels would have less merit if their percentage contribution to total energy usage declined because consumption of other sources of energy continued to soar. However, all sources of energy have limitations, and none can keep up with an insatiable demand. It is likely that energy costs will soon be so high that nations will approach an energy ceiling under which biomass can become a significant contributor.

Today's energy business collects fossil gas, coal, or oil and subjects them to minimal clean-up and processing to produce relatively inexpensive fuels. A competing biomass industry should not rely on sophisticated processing to obtain products little different from the feedstocks of today's energy industries. Biomass conversion can achieve commercial success much sooner if it is focused on more valuable, high-quality fuels and chemicals rather than on cheap substitutes for gas and heating oil.

Biomass factories are limited in size because of critical feedstock logistics. The cost of hauling feedstock is significant, and a reasonable plant size might be based on distances suitable for loading, hauling, and unloading by normal work shifts. Two round-trips per truck per day would be feasible. A factory processing 1,000 tons of feedstock per day (dry basis) should be large enough to realize economies of scale while drawing feedstock from a 50-80 kilometer (km) radius to keep hauling costs within reason. Per unit of product, it costs several times as much to build a small plant as a large plant, giving the latter a distinct advantage. Locating factories near the source of biomass would be an economic boon to rural areas.

Thus for the establishment of biomass industries, land must be affordable, and there must be suitable soil and climate for biomass cultivation. Future fertilizer and water costs are unpredictable; biological nitrogen fixation and the recycling of phosphates and water could stabilize the costs for biomass plantations. It is difficult to estimate real costs given the available subsidies, depletion allowances, tax incentives, supports in the form of government highways, and the like, but biomass industries will resemble agribusiness with variations due to weather and market interplays. The pattern of costs should be steady except for these perturbations.

BIOMASS FEEDSTOCKS

Terrestrial Plants

New multi-purpose crops that supply food, fiber, and chemicals may revolutionize agriculture, but existing biomass can lead the way. Many nations have enough trees to supply a large bioconversion program, and most agricultural residues are put to poor use. Residues are usually left on the ground to rot, both to provide some erosion control and to revert some organic matter back to the soil. As these residues are diverted to bioconversion, new methods of soil management must be devised.

Some of the new crops being studied have exciting prospects. For example, mimosa is a woody legume that derives much of its nitrogen from the air. Several plants with reasonably high productivity can thrive in alkaline soils, and some tolerate quite brackish water. This would allow some presently underutilized soils near saline waters to become important for cultivating biomass.

Aquatic Plants

Water areas can be much less expensive than land. Although fresh waters are usually part of reservoir, shipping, or recreational systems, there may be opportunities for growing biomass. Clearly, streams naturally choked by water hyacinth might be dedicated to biomass production. Lakes, streams, marshes, or freshwater impoundments can be used to grow many different plants, but floating species offer advantages for easy harvesting. Hydrilla, duckweed, and various algae show some promise, and water hyacinth is very productive in hot climates. Of the rooted plants, cattails are particularly attractive because of wide geographic range and high productivity.

Several aquatic plants can convert atmospheric nitrogen, thus eliminating or greatly reducing the need for chemical fertilizer. In India, nitrogen-fixing algae are grown in very shallow ponds, allowed

to dry, and used as fertilizer. Use of the water fern Azolla, which has internal symbiotic algae, is also common in Asia. Azolla is an efficient fixer of atmospheric nitrogen and produces excellent fertilizer for rice fields. It may serve as a good food or as a feedstock for fuels or chemicals.

Marine plants have high maximum growth rates, and while the open oceans offer vast growing areas, the ocean surfaces are unfortunately too low in nutrients. There are schemes to anchor giant kelp in the oceans and to use wave action to pump nutrient-laden water from the depths. Proponents of kelp have promulgated wildly optimistic yield estimates and have minimized the problems of kelp nutrition and of economical processing. A floating marine plant with much more potential is sargassum. It grows in relatively shallow waters that hold nutritious particulate matter that would settle in the open oceans, and it could possibly be harvested by wind action. Some algae excrete potentially valuable oils or waxes when they are nitrogen deficient, but rates are poor. Genetic modifications might result in strains with good productivity.

Plants Containing Sugars or Starch

Land plants that produce sugars are well known (sugarcane, maple trees, sweet sorghum, sugar beets). Although used mostly as food, sugars are also a source of chemicals. Starchy plants such as corn, potatoes, and cassava are important foods. In the United States, there is sufficient corn grown to allocate some portion to fermentation to produce ethanol. The economics of corn alcohol, however, would be rather unattractive without governmental incentives.

Plant Exudates

Guayule is considered a promising alternative to the rubber produced on tree plantations. Many plants contain latex or suspensions of steroids, waxes, or hydrocarbons. Most such plants are found in arid regions and tend to have low growth rates; modern genetics may, however, transform them into miniature chemical factories. Plants that produce hydrocarbons or chemicals directly have an exciting future--but for rubber and high value organic compounds, not for cheap fuels.

The exudates from coniferous trees such as pine seem to have been overlooked as potential major contributors for replacing petrochemicals. Applying the herbicide paraquat to pine trees increases resin and turpentine yields severalfold, even in excess of the typically optimistic estimates for exudates from arid land plants on a per hectare basis. If wood and pine chemicals are valuable

coproducts, these could be quite profitable operations. The existing industry, gum naval stores, has declined because of obsolete technology and high labor costs, and new methods would be important in exploiting the greatly increased yields of exudates (Layman 1982).

"Energy Cane"

Until very recently, only the sucrose in sugarcane attracted commercial interest, and strains were chosen for the highest yield at the lowest cost. Many other strains that produced excessive leaves or stalks were shelved or discarded. It is still the practice in many countries to burn the leaves prior to cutting, thus creating air pollution and a hazard if sudden high winds spread the flames and devastate adjacent fields. After extracting the sugar, the residue (bagasse) is discarded at some sugar refineries, but most now use it to replace expensive boiler fuels.

An exciting program in Puerto Rico is growing "energy cane" using sugarcane varieties that produce roughly the same amount of sugar as do customary varieties but with about twice as much total biomass (Alexander 1982). Such a crop could provide a reasonable sugar yield and boiler fuel far in excess of the needs of the sugar refinery. This additional biomass is satisfactory as fuel for generation of electric power, although it must be dried and sized for handling and burning. Puerto Rico's economy has suffered greatly from increases in the price of oil, so lower cost fuel from energy cane is very attractive.

THERMOCHEMICAL CONVERSION

Woody plants provide most of the energy in rural regions of many nations. One survey of firewood plants has found that a shift to different species could increase their availability in many areas (National Research Council 1980). Today, the technology for burning wood ranges from an open fire to modern, fluidized-bed combustion of sized and dried particles. Even highly developed nations derive significant benefit from wood for heating homes and generating power; biomass is low in sulfur and requires less pollution control equipment than coal. Less-developed nations must balance the greater efficiencies of central power stations burning wood against the costs of distribution systems and power line losses.

Thermochemical conversion of biomass to fuels and chemicals copies technologies used for coal. Biomass is widely distributed and cannot achieve economies of scale anywhere near those of very large coal conversion factories, but it is quite reactive and may be less costly to process. Practically any carbonaceous feedstock will suffice for thermochemical conversion.

The options are gasification, pyrolysis, and liquefaction. Pyrolysis of biomass was important commercially until displaced by less costly conversions of petroleum. Liquefaction of biomass requires high temperature and high pressure, and thus a large investment and high operating costs. Neither of these thermochemical conversions can compete with gasification at present (Rogers et al. 1981).

Much more research has been conducted on conversion of coal than on biomass, and experiences with large-scale gasification such as the SASOL process in South Africa must be evaluated (Heylin 1979). This process produces synthesis gas--a mixture of carbon monoxide and hydrogen--which can be used to make many different chemicals. Methanol is the leading candidate for a fuel product, and ammonia made with nitrogen from the air and hydrogen from synthesis gas would fit into a biomass cycle as fertilizer. Natural gas is the preferred feedstock in the United States for synthesis gas--although coal is cheaper--because of its ease of handling and the clean-up problems presented by coal. Biomass would have to be relatively inexpensive to become the feedstock of choice because it is bulky, widely distributed, and costly to collect.

The numerous designs developed for biomass-based gasifiers have largely been directed toward small units. Apparently engineers recognize the scale required for an economic operation and tend to think of gasification of biomass as an auxiliary operation for disposal of surplus material. For example, several designs are aimed at generation of low Btu (British thermal unit) gas from waste piles for local use. Large gasifiers for coal can serve as future models for biomass; small gasifiers are not yet a particularly attractive investment.

Typical schemes for gasification are shown in Figure 1. Countercurrent flow provides heat economy in that the hot gas warms the incoming feedstock. The very rough economic analysis for combustible gas shown in Figure 2 also indicates the effects of plant size. Although 1,000 tons per day is considered a very large size for pulp manufacture, this is relatively small in Figure 2. Large scale also implies considerable risk, and private companies are reluctant to embark on such a major expenditure without government help or loan guarantees. Even the South African ventures are unattractive in the present financial climate, and their production of liquid fuels was motivated mainly by the need to be more independent of oil suppliers with hostile political views.

Figure 2 shows that a feedstock cost of about $30 per dry ton (t) as used in other economic portions of this paper would require a selling price of $8-9 per million Btu of product gas. Because this value is significantly above the present value of natural gas, gasification is not now commercially viable for biomass, especially for small installations. Special circumstances such as availability

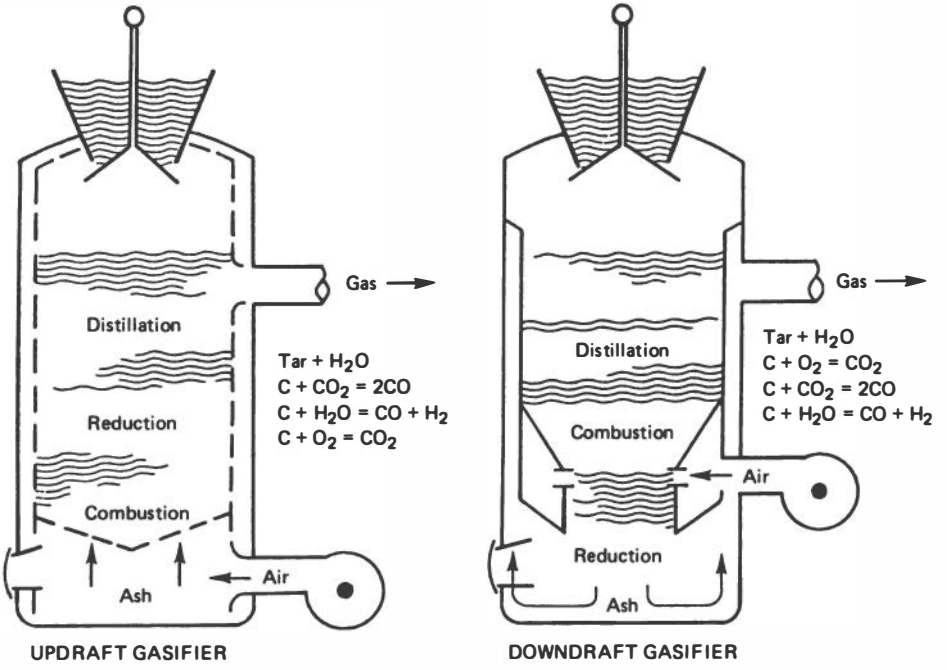


FIGURE 1 Schemes of the updraft and downdraft gasifiers.

of very low-cost feedstock could allow profitable operation, but competing processes might do better.

ANAEROBIC DIGESTION

Conversion by anaerobic digestion seems very attractive because the process requires simple equipment and little control. However, there has been widespread dissatisfaction with operating anaerobic digestion of sewage sludges because the microbial cultures are easily upset. Anaerobic digestion for fuel gas is not likely to be so crude and simple if reliability must be assured. Furthermore, the product

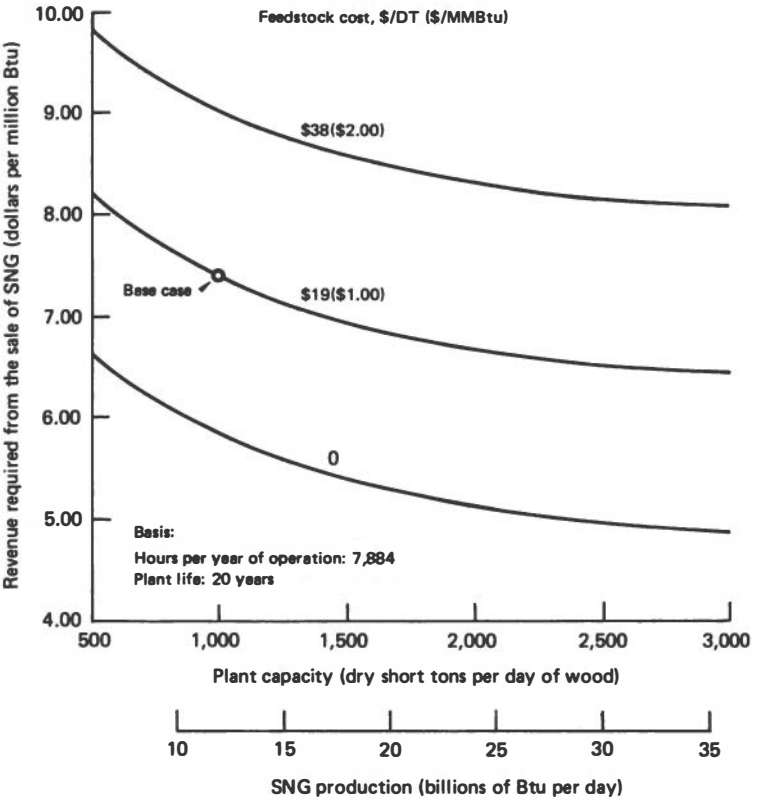


FIGURE 2 Production of SNG (synthetic natural gas) from wood by direct gasification and combined shift/methanation: effect of plant size on revenue required from the sale of SNG by a regulated producer. Source: Kohan et al. (1979).

gas is roughly two-thirds methane and one-third carbon dioxide that must be removed to bring the heating value up to that of pipeline gas. This requires investment in a relatively sophisticated purification process.

Several countries have sponsored major programs for generation of methane gas with moderate heating value from the small-scale digestion of manures or organic wastes. China reports fair success, but efforts in India have a less favorable evaluation. Individual farmers often have insufficient manure or capital, or both, to warrant constructing a digester, so several must band together. Fair distribution of gas and of the sludge used as fertilizer is difficult. In any event, some installations have been abandoned, while others appear to work reasonably well.

The prospects for anaerobic digestion are improving because of new reactor designs such as those using beds packed with organisms. Reportedly, methane of 90 percent purity has been obtained from digesters packed with immobilized organisms, thus eliminating the gas purification steps. Dilute feed streams were used. The pretreatments that aid digestion are much the same as those used for hydrolysis processes which yield more valuable products. Thus digestion to products other than methane seems sensible. Heavily loaded digesters tend to accumulate organic acids, inhibiting the production of methane. Such short-chain acids have reasonable value, but isolating them from crude mixtures could be expensive.

The most modern arrangement for digestion of biomass is the two-stage, thermophilic digester shown in Figure 3. Warm digesters can be relatively small because digestion rates are fast, but some of the methane must be used to heat the digester. Although the packed-bed digester may soon reach commercial practice, construction details and mass transfer problems need more study.

A rough economic analysis of digestion is given in Table 1. Untreated lignocellulosic materials tend to give conversion yields of about 36 percent of the organic matter (McFarlane and Pfeffer 1981). While pretreatment can increase this to about 75 percent, chemicals, heat, labor, and equipment add significant costs. It seems that the economics hinge on the cost of feedstock for anaerobic digestion, and costly feedstocks preclude the chance of profit until gas prices become much higher. Manures may be considered free, but there are competing uses such as fertilization and other processes.

Scale-up is less of a problem for anaerobic digestion than for thermochemical processes. For anaerobic digestion using reactors large in size, more modules could be added to scale up to any desired production rate, and the economies of scale would be relatively modest.

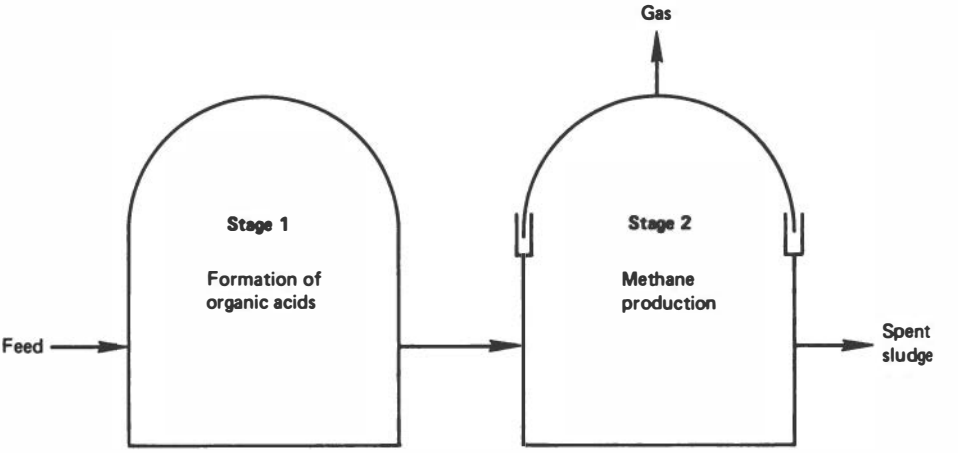


FIGURE 3 Two-stage anaerobic digestion.

ALCOHOL FROM SUGARS

The alcohol program in Brazil is based mainly on sugarcane, although some installations use other feedstocks. Juices, extracts, or molasses are fermented to ethanol by rather conventional means, but there have been several interesting improvements such as shortening the batch fermentation time to less than 10 hours. Process economics are not very good, but the benefits to the country have been sizable in terms of sparing the high costs of petroleum imports. Had the Brazilian sugar earmarked for ethanol been sold instead in the international sugar markets, sugar prices would have been quite dismal rather than just severely depressed. Some other nations have more modest alcohol programs and have learned much from Brazilian experiences.

Fuel alcohol made by fermentation using corn has also been marketed in the United States. When corn prices are depressed, fuel alcohol offers an alternative use; however, government assistance in various forms has been needed to encourage the production of fuel alcohol. Distillers' dried grains (the term applied to evaporated bottom liquor from the distillation step) is sold as cattle feed, which represents significant additional income. A massive fuel

TABLE 1 Economics of anaerobic digestion to methane.[a]

Percent conversion	Required price for product gas	
	$/million Btu[b]	$/gigajoule
30	39	38
40	24	23
60	13	13

[a]This table is based on the following assumptions: (1) lignocellulosic feedstock is priced at $27.50/dry metric ton; (2) the cost of purifying methane is not considered; (3) plant size is 454 tons of feedstock per day; and (4) a 15 percent yearly return on investment is obtained.

[b]The present price of natural gas has passed $6 per million Btu.

SOURCE: Based on Jones (1980).

alcohol effort based on corn would overwhelm the markets for this by-product and make profits unlikely.

CELLULOSE HYDROLYSIS

Hydrolysis of hemicellulose to mono- and oligo-saccharides is easily accomplished with either acids or enzymes. Native cellulose is much more resistant to hydrolysis because of its highly ordered crystalline structure and the physical barrier of lignin that surrounds the cellulose fibers. Some of the most striking advances made in programs supported by the U.S. Department of Energy have been various pretreatments that render cellulose amenable to easy hydrolysis.

The approximate composition of lignocellulosic materials is: cellulose, 25-50 percent; hemicellulose, 20-35 percent; and lignin, 18-35 percent. Each major component should be converted to products to maximize income and to minimize waste treatment costs.

Acid Hydrolysis

Acid hydrolysis of wood is an old technology, and projects conducted during World War II led to the Madison process, which optimized time, temperature, and acid strength. While this process is not economical in the United States, other countries, particularly the USSR, have many plants for hydrolyzing wood to sugars. A few plants produce alcohol by fermenting the sugar, but single-cell protein for animal feed is the most common product. Furfural is sometimes derived from the pentose fraction of the hemicellulose.

Acid hydrolysis leads to a sequence of reactions. Hydrolysis is approximately a thousand times faster for hemicellulose than for cellulose. The sugars from each are degraded by acid to resins, polymers, and furfural derivatives. Reaction conditions are thus set for a compromise between hydrolysis and degradation such that the final mixture contains unreacted biomass, unwanted products, and the desired sugars. Since the sugars from hemicellulose are formed early, there is time for considerable degradation, leading to major losses. Using sulfuric acid, the maximum yield of fermentable sugars is about 55 percent by weight of starting cellulose. Superior performance is obtained with other acids, and methods are being developed for more economical recovery of these expensive acids.

Hemicellulose can be removed by hydrolysis with dilute acid with very little effect on the cellulose. Bacteria, molds, and yeast produce some ethanol from these sugars, which are predominantly xylose, but their poor tolerance of ethanol prevents its accumulation. Alcohol-tolerant yeast now used commercially cannot ferment xylose. An enzyme can be added to form xylulose from xylose. Common yeast can then be used to ferment xylose to ethanol with excellent yields. This enzyme is identical to glucose isomerase, which is relatively inexpensive because of its widespread use in converting glucose to high-fructose syrups for commercial sweeteners. A serious drawback of this process is the need to recycle unreacted xylose back from the fermentation step to the enzyme in order to approach again the concentrations of the equilibrium mixture. New mutants have isomerase activity and thus need no supplemental enzyme, but tolerance to ethanol still needs improvement. Furthermore, organisms such as *Pachysolen tannophilus*, which have the inherent ability to ferment xylose, may be improved enough to merit commercial consideration. Utilizing hemicellulose to produce additional ethanol will mean a 50-70 percent improvement in productivity in factories using biomass.

Enzymatic Hydrolysis

The rate of enzymatic hydrolysis of cellulose is very slow unless the feedstock is pretreated. Fortunately, some effective new

pretreatments have been developed that result in yields of over 90 percent of the theoretical amount of glucose. Thus as the costs of enzymes continue to drop dramatically, enzymatic hydrolysis is likely to become highly profitable. Enzyme fermentation has been so improved that there is no need for an enzyme purification step, and filtered broth can be diluted and added directly to the hydrolysis reactor.

The molds that produce cellulase have been studied intensively at the U.S. Army Natick Laboratories, and these efforts, in addition to those by other groups (particularly at Rutgers University), have created excellent strains. A modification of the Natick process, developed at the University of California, Berkeley, has contributed engineering improvements and alternative treatments.

The Gulf Oil Chemicals Company has terminated research and development on its process and has donated rights to the University of Arkansas. In this process, feedback inhibition by sugars of the enzymes does not occur because ethanol formation proceeds simultaneously with saccharification. Municipal solid wastes will be used, and credits are taken for waste disposal when estimating costs. There seems to be some difficulty in reducing the feedstock to a size suitable for bioconversion because in the design of one factory, ball milling accounts for over one-third of the capital cost. Natick workers have shown that two-roll wet milling is effective with waste paper and less expensive than milling or grinding. Residues from the Arkansas experiments have been fed to animals, but it is unlikely that regulatory agencies will approve meat from these animals for human consumption.

The success of biomass refineries depends upon converting all of the major components to valuable products. Despite vast markets for ethanol, sales of ethanol alone will probably not result in a profitable operation. Inability to find buyers for the large amounts of lignin produced could limit the construction of new refineries. Because lignin burns well, it could defray most of the factory fuel costs. Furthermore, the availability of large amounts of relatively inexpensive lignin should be a stimulus to finding more valuable uses.

Direct Bioconversion to Ethanol

The use of expensive enzymes to hydrolyze cellulose to glucose for fermentation could become obsolete before its first commercial tests if a project at the Massachusetts Institute of Technology (MIT) succeeds. MIT researchers are seeking direct fermentation of lignocellulose with anaerobic organisms that first hydrolyze to sugars and then ferment the sugars to ethanol (Avgerinos and Wang 1980). This process is relatively inexpensive compared to producing cellulase

enzymes with a highly agitated aerobic fermentation because little mixing and no aeration are employed. Coarsely shredded biomass (corn stover seems best at present) is brought in contact with an organism that hydrolyzes both the cellulose and hemicellulose but ferments only the resulting glucose. A second organism is added later to ferment sugars from hemicellulose to ethanol. Unreacted feedstock is dried and burned for power. Because the process is simple and there is little investment in treating the feedstock, profits are possible with a low conversion to products. If conversion of cellulose is too low, the factory may produce too much excess power, and the marketing of such a product mix would be complicated.

MIT has developed several microbial mutants that produce mostly ethanol and few by-products when grown on purified cellulose. However, actual feedstocks do not work as well, and too much acetic acid is produced. Further improvement of the culture should solve this problem.

Lignin-Oriented Processes

For some years, there has been interest in removing lignin from wood fibers by extraction with an organic solvent. With this method the lignin is not damaged and could command a premium price as a substitute for phenol in phenol-formaldehyde plastics or for various formulations such as adhesives and oil well drilling muds. If lignin and products from hemicellulose are used to defray costs, solvent pulping may compete with traditional pulping methods. The problem, however, is efficient recovery of expensive solvents, which is complicated by absorption to the cellulose fraction.

Two groups have developed processes for solvent pulping that emphasize lignin recovery. Each yields several products and uses a mild treatment so that the lignin fractions are relatively unaffected and remain reactive. Such lignin is much superior to the damaged, substituted material that results from paper pulping by the conventional Kraft and sulphite processes.

The Biological Energy Corporation process—a collaborative effort among the General Electric Company, University of Pennsylvania, Hanneman Medical Center, and Lehigh University—uses alcoholic solvents with acid or alkali to extract lignin from wood chips while simultaneously hydrolyzing the hemicellulose to soluble mixed sugars (see Figure 4). Lignin precipitates as the extract is evaporated to a sugar syrup, and the vapors go to solvent recovery. The solids from the wood chip extraction are cellulose with desirable properties for use in paper. Commercial success, however, hinges on solvent recovery, and it is difficult to erase the last traces of solvent from the cellulose.

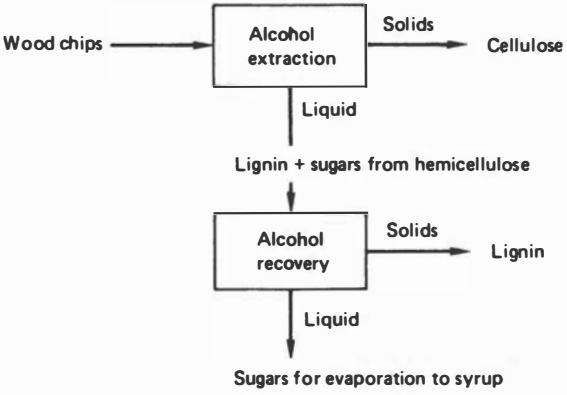
The Battelle-Geneva Corporation pulping process differs primarily in that it uses phenol rather than alcohol (see Figure 4). Hot water and phenol are miscible and dissolve lignin while hydrolyzing the hemicellulose. Upon cooling, a water-rich layer containing the sugars and a phenol-rich layer containing the lignin are formed. Again, a key economic factor is recovery, and phenol is more difficult to recover than common alcohols. Economic information released by Battelle-Geneva is shown in Table 2. Plant cost is roughly the same as for other processes for refining lignocellulosic materials.

Steam explosion of wood chips to make a filler for construction materials such as "Masonite" is well known. In this process, the cellular structure of wood is impregnated with steam and thus disintegrates when the steam is suddenly expanded. However, because cellulosic fibers are shortened and weakened, there is little prospect of manufacturing good paper products by steam explosion. A Canadian company, Stake Technology, utilizes a continuous process using a mechanical screw plus steam to explode wood chips. The product is more digestible than wood by cattle and could be fractionated to lignin and to hemicellulose sugars and cellulose.

Another Canadian company, Iotech, uses steam explosion as a pretreatment for cellulose hydrolysis and optimizes the time and temperatures in a gun for batch explosion of wood chips (see Figure 4). The cost is the same as or less than that for the size reduction steps of other schemes proposed for wood hydrolysis. Thus pretreatment accomplishes multiple purposes: cellulose is weakened for easier enzymatic hydrolysis, hemicellulose is hydrolyzed and partially destroyed, and lignin is melted and more easily removed.

The choice between organic solvent pulping of wood and steam explosion will depend on the value of the products. Paper pulp commands a relatively high price compared to glucose from hydrolyzing the cellulose, and this may justify the cost of solvent treatment. The lignin from the Iotech process is easily recovered by extraction with dilute alkali. As yet there are no claims for superiority by either of the groups extracting lignin with solvents or the Iotech Corporation. If cellulose in the Iotech process can be tailored to uses that rival the value of paper pulp, this all-aqueous technology should be highly profitable. About 7 percent of the exploded wood goes to the production of cellulase enzymes. Cellulase broth is added directly to the rest of the wood to achieve better than 85 percent of the theoretical glucose from cellulose. Hemicellulose hydrolyzes readily during steam explosion and is washed out as a solution to be evaporated to a syrup for feeding cattle. Solid residue from enzymatic hydrolysis is rich in lignin that can be recovered in quite pure form by extraction with dilute alkali, or the residue can be sold directly for adhesive applications such as binding of plywood or chipboard where some inert fillers are desirable in the resin.

BIOLOGICAL ENERGY CORPORATION



BATTELLE/GENEVA

IOTECH

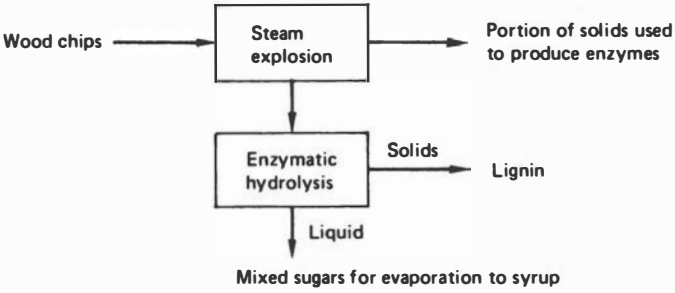


FIGURE 4 Three biomass refining process.

TABLE 2 Economics of biomass refining: Battelle-Geneva and Iotech processes.

BATTELLE-GENEVA PROCESS[a]		
Plant size:	700 t/day wood[b]	
Capital investment:	$34 million	
Feedstock cost:	$7.5 million/yr	
Labor, materials, chemicals	$14 million/yr	
Product value:		
Cellulose pulp @ $350/t	$35 million/yr	
Pentose syrup @$.08/lb	$ 7 million/yr	
Lignin @ $.15/lb[c]	$15 million/yr	
IOTECH PROCESS[d]		
Plant size:	1,000 t/day wood	
Capital investment:	$70 million	
Projected production costs:	Syrup cost per kg (dry)	Lignin cost per kg
Raw materials	$0.021	$0.046
Energy	.021	.026
Maintenance	.013	.015
Depreciation	.013	.015
Overhead	.009	.018
Direct labor	.003	.011
Apportioned unit cost	$0.080	$0.131
Probable selling price (per kg)	$0.170	$0.440

[a]Derived from information in Krieger (1982).
[b]Needed to produce 100,000 t/yr cellulose pulp.
[c]Lignin goes to phenol and oil.
[d]Data provided by the Iotech Corporation and taken from Bungay (1981).

Tests of the Iotech process on a 1,000-liter scale at the Gulf pilot plant in Kansas achieved about 90 percent of the theoretical glucose from cellulose, and fermentation to ethanol performed well. Recipes for wood binders using the lignin have received favorable evaluations by potential customers. Finally, the first Iotech factory may be built in New York State. Because the initial products will be syrups and lignin, capital costs will be kept down and plant start-up will be easier. Fermentation products such as ethanol are projects for the future. Economics furnished by the Iotech Corporation are shown in Table 2.

Another advance that will lower the cost of enzymes is recycling. Of the dozen or so components of cellulase, several bind strongly to cellulose. These can be recovered easily from the hydrolysate by adsorption on cellulosic material which can then be blended with the solids fed to hydrolysis. A fraction of the enzymes on the solid residue from hydrolysis can be desorbed by simple means (Sinitsyn et al. 1982), and the extract can be used to supplement the fresh enzyme. Beta-glucosidase, a key component that splits cellobiose to glucose, is not very stable and cannot be recovered by any method based on adsorption on cellulose. Low-cost beta-glucosidase can, however, be obtained by a separate fermentation. There are already indications that about 50 percent of the cellulose can be recycled, and the impact on costs will be dramatic.

Another possible product is the leafy residue resulting if trees are harvested while green. Protein content is about 23 percent, and similar material is sold in Europe as cattle feed. However, when entire trees are collected, soil fertility will decline quickly unless fertilizers are used.

ENERGY BALANCES

Energy balances and energy efficiency of processes have been disputed at great length with little regard for the more important question of economics. It is, of course, senseless to produce ethanol as a gasoline substitute if it takes as much or more gasoline to produce the ethanol. When the energy needed to pump irrigation water, manufacture fertilizer and farm machinery, fuel both agriculture and the conversion factories, and meet all other requirements is added up, it is difficult to show a positive energy balance. However, for a residue such as corn stover, the main crop has already paid for many of these expenditures. Less valuable tree species require little energy investment because the harvesting machinery is usually in place for the principal trees. Nevertheless, a plantation maintained exclusively for biomass must be very energy conscious. The ideal would be a multi-purpose crop with food or fiber products of high value to defray costs.

Processes with energy savings have already been quite successful. For example, older distillation technology for ethanol required more heat energy than the heat of combustion of the product. Today, good engineering with heat exchangers and vapor recompression to power subsequent distillation stages can save two-thirds of the distillation energy, but with a marked increase in equipment costs. Substituting other procedures for all or for part of distillation can be highly cost effective. Vapor phase drying of ethanol reduces the energy for recovery to about 20 percent of the heat of combustion of the product ethanol. Another attractive option is to purify the ethanol partially by distillation and to extract into gasoline to obtain gasohol directly.

CONCLUSION

In a developed nation with solid markets for organic chemicals, biomass refining will be highly profitable. Other countries may find it more expedient to burn biomass as an easily implementable solution to finding substitutes for imported oil. Gasification of biomass and anaerobic digestion to methane have inherent limitations that require cheap feedstocks, and other circumstances must be favorable to justify an investment. New digester designs may improve prospects for methane, but new designs for fermenters to aid biomass refining are also appearing.

There are good prospects that the private sector will commercialize processes for biomass fuels and chemicals with little need for government help. Nevertheless, governments must be sympathetic and realize that competing industries have many kinds of direct and indirect subsidies. Biomass should be given a chance to compete fairly without prejudice.

REFERENCES

Alexander, A. G. (1982) Second Generation Energy Cane: Concepts, Costs, and Benefits. Paper presented at Symposium on Fuels and Feedstocks from Tropical Biomass II, April 1982, San Juan, Puerto Rico.

Avgerinos, G. C., and D. I. C. Wang (1980) Direct microbiological conversion of cellulose to ethanol. Annual Reports on Fermentation Processes 4:165.

Bungay, H. R. (1981) The Iotech Process. Paper presented at the U.S. Alcohol Fuels Symposium, October 1981, Washington, D.C., USA.

Heylin, M. (1979) South Africa commits to oil-from-coal process. Chemical and Engineering News. September 17, p. 13.

Jones, J. L. (1980) Methane production via bioconversion: a technoeconomic evaluation. Chemical Engineering Progress 76:58.

Kohan, S. M., P. M. Barkhordar, and F. A. Schooley (1979) Mission Analysis for the Federal Fuels from Biomass Program. Available from the U.S. National Technical Information Service, Accession No. SAN-0115-T3.

Krieger, J. (1982) Process yields marketable biomass fractions. Chemical and Engineering News. May 31, p. 38.

Layman, P. (1982) Naval stores markets on hold during recession. Chemical and Engineering News. March 22, p. 30.

McFarlane, P. N., and J. T. Pfeffer (1981) Biological Conversion of Biomass to Methane. Available from the U.S. National Technical Information Service, Accession No. SERI/TR-98357-1.

National Research Council (1980) Firewood Crops: Shrub and Tree Species for Energy Production. National Academy of Sciences, Washington, D.C., USA.

Rogers, D. Z., D. J. Wilhelm, and D. S. Ross (1981) Feasibility Study on New Processes for Fuel From Biomass. Available from the U.S. National Technical Information Service, Accession No. DOE/ET/23023-T4.

Sinitsyn, A. P., H. R. Bungay, and L. S. Clesceri (1982) Enzyme Management in the Iotech Process. Paper presented at the American Chemical Society national meeting, Kansas City, Kansas, USA.

Biomedical and Biological Application of Monoclonal Antibody Technology in Developing Countries

WILLIAM C. DAVIS, TRAVIS C. McGUIRE, and LANCE E. PERRYMAN

INTRODUCTION

No single discovery has had a greater impact on the development of medicine and biology than the finding that exposure to a pathogen or other foreign antigenic substance elicits the formation of a set of unique serum glycoproteins (antibodies) that react specifically with the antigen. This simple observation at the turn of the century led to the development of serological techniques for such varied purposes as the identification of pathogenic organisms and blood typing to distinguish heritable differences in blood group antigens.

The subsequent recognition that these antisera actually contain antibodies with many specific reactivities revealed a much greater potential for the use of serological techniques in biological analysis. Through refinements in immunization procedures and sequential absorption, it was found that certain antisera could distinguish multiple fine differences in the antigenic composition of whole organisms as well as differences in complex molecules of biological or synthetic origin. This finding, in turn, led to a wide range of applications of serological techniques in biology and medicine. The only limitations to the use of serum as a source of antibody were (1) the need to constantly reproduce and standardize reagents where there was a continuing requirement for a specific reagent, and (2) the inability to control the quantitative composition of the antibodies synthesized. The latter limitation restricted the full potential of antibodies as a bioanalytical tool.

The most recent turn in this saga of events has been the finding that it is possible to immortalize individual, antibody-producing cells (single clones) by hybridization with tissue culture-adapted plasma tumor cells. This development has launched a new era of exceptional dimension and potential. The ability to construct hybrids that produce antibodies of a given class, specificity, and affinity has provided scientists with a tool that permits the analysis of

virtually any antigenic molecule. Since the initial discovery using mice and plasma tumor cell lines derived from mice, useful plasma tumor cell lines have been identified in rats and humans. This finding has expanded the potential of making monoclonal antibodies in man as well as in rodents.

This discovery is so recent that its potential has only begun to be realized. The findings that have already emerged from application of the technology, however, have revealed the tip of the iceberg. For example, the use of monoclonal antibodies to study rabies has revealed unsuspected diversity in the antigenic composition of the causative virus and the reason that existing vaccines have failed to provide protection in all parts of the world. The implication of the finding is that many of the disease-causing organisms for humans, animals, and plants may possess similar diversity not recognized by conventional serological diagnostic reagents. Application of the technology to the study of malaria and trypanosomiasis has provided the first major breakthrough in analysis of their antigenic composition and clues as to how effective vaccines can be made to reduce the incidence of these devastating diseases. Application of the technology to study of the cells that regulate the quality and expression of the immune response has allowed investigation of the immune response in humans and animals with precision heretofore only possible with inbred strains of animals.

Conventional methods of serology are now being supplanted with those using monoclonal antibodies. Thus the impact of the technology is only beginning to be felt even in developed countries. The implications are clear, however, for emerging nations. Immediate benefits will accrue with the augmentation of existing methods of diagnosing disease and the development of vaccines. Moreover, this technology affords an opportunity to make major advances in human and animal medicine over a shorter time at greatly reduced cost. It also provides a mechanism for improving specific food production industries.

This report indicates where immediate and future benefits will accrue from monoclonal antibody technology and provides information on the requirements for setting up a laboratory and producing monoclonal antibodies.

APPLICATIONS OF MONOCLONAL ANTIBODY TECHNOLOGY

Medical

Control of a disease can be exercised at various stages during its development. For example, diagnosis of an infection is followed by either treatment of the disease, isolation of the infected individual in order to limit spread of the infection to susceptible individuals or groups, or both. Infections are usually prevented by

means of vaccination, which increases the number of immune individuals and thereby decreases the spread of infection. Other methods can also be used; in the case of vector-borne diseases, the vector can be eliminated.

The medical uses of monoclonal antibodies can best be demonstrated in the areas of diagnosis, treatment, and prevention of disease. Obviously, a variety of other uses can be envisioned.

Diagnosis. Detection of the presence of a pathogen, chemical, drug, or metabolite usually depends on either its presence or the presence of a specifically associated substance. To diagnose infectious diseases, it is often necessary to demonstrate the presence of the causative organism or a specific immune response to the causative organism. For many infectious diseases, it is possible to demonstrate the causative organism throughout most of the course of the infection; examples include venereal diseases caused by gonococcal and chlamydial organisms. In this case, an antibody-based test specifically identifies the various organisms associated with a particular venereal disease and provides means for diagnosis. Monoclonal antibodies are particularly useful for such tests because they can be formulated to recognize unique antigens on particular organisms. They can also be made to recognize only one type of organism and not cross-react with closely related organisms, allowing a very specific diagnosis.

Monoclonal antibodies have an advantage over polyclonal antisera (conventional antisera prepared in animals) in that they react with only one determinant and molecules related to that determinant. Restricted reactivity allows for precise screening to find those monoclonal antibodies unique to the organism of interest. In addition, once a suitable monoclonal antibody has been identified, it can be reproduced indefinitely, providing a standardized reagent. In contrast, polyclonal antisera usually require extensive, laborious absorptions to remove cross-reactions and to obtain specific reactivity. Requirements for batches of antisera from different animals and absorptions make standardization of polyclonal antisera difficult and sometimes impossible. The example of the use of monoclonal antibodies to diagnose venereal disease can be applied to any disease or group of diseases where the causative agent is antigenically distinct.

For many infectious diseases, detectable organisms are present in the lesion, blood, or secretions for only a few days in the initial phase of the disease. Detection during such a relatively short phase of the process is not a suitable basis for a diagnostic test. In many persistent infections, the organisms are present in too low a number or are complexed with the host antibody, thereby preventing detection. In most situations in which organism detection is not a feasible basis for diagnosis, measurement of an antibody response to

the causative organism forms the basis for the diagnostic test. Such tests require the use of the organism or antigens derived from the organism to react with serum or secretions to assay for the presence of an antibody.

Monoclonal antibody technology has proven useful in improving tests for measuring antibody response. One improvement is use of monoclonal antibody to isolate the antigen to be used in the test. This is accomplished by making a monoclonal antibody to the specific organism or to an antigen of the organism. The antibody is then made in large quantities, isolated by routine procedures, and attached to agarose beads by a cyanogen bromide coupling procedure. The antibody-coated beads are then reacted with a heterogeneous mixture containing the antigen of interest, washed thoroughly, and the antigen is eluted from the matrix. The purified antigen is then used to determine the presence or absence of antibody in the infected host. An isolated antigen eliminates cross-reactions with antibodies to other organisms that occur and confuse interpretation of results when crude antigens are used for detection of antibody.

A second improvement in tests for antibody in the host can be made with monoclonal antibodies that bypass the need for large quantities of antigen derived from the organism of interest. In this case, a monoclonal antibody is made to an antigen in or on the same organism for which antibody is also made in the infected host. The monoclonal antibody is then used as antigen to make a monoclonal anti-idiotypic antibody, which reacts with a part of the binding site on the starting monoclonal antibody. Under certain circumstances, reaction of the monoclonal antibody to organism-derived antigen and the monoclonal anti-idiotypic antibody can be inhibited by the organism. Such a reaction can be used to search for the organism during isolation procedures and in tissues and fluids. In addition, if the monoclonal antibody and antibodies made by the host react with the same determinant on the organism, the reaction of the monoclonal antibody, to either the organismal antigen or the monoclonal anti-idotypic antibody, will be inhibited by antibodies present in the serum or fluids of the infected individuals. In the latter case, antibodies in the infected individual would react with the monoclonal anti-idiotypic antibodies and block their reaction with the monoclonal antibody to the organism. This type of test requires that some of the host antibodies and the monoclonal antibody to an organismal antigen recognize the same antigenic determinant and that the monoclonal anti-idiotype antibody recognize the binding site on both the host antibodies and the monoclonal antibody. If these conditions can be met, then a test for host antibodies to an organismal antigen can be made without the laborious task of preparing antigen from the organism. This type of test will prove useful for diagnosis of parasitic, viral, bacterial, and fungal diseases where defined antigens are unavailable.

In noninfectious diseases such as those of the endocrine system, a variety of monoclonal antibodies are already in use to measure concentrations of hormones in body fluids. Similar assay systems can be made for any chemical, drug, body metabolite, or other substance to which a monoclonal antibody can be made. Perhaps the best example is the measurement of embryonic antigens that are expressed in the case of intestinal adenocarcinomas. The application of monoclonal antibodies to the diagnosis of a variety of hormonal, neoplastic, metabolic, infectious, and other diseases will certainly increase as the repertoire of monoclonal antibodies increases.

Treatment. For monoclonal antibodies to be most effective, it may be necessary to produce those of the same type as the species into which they are injected for treatment. For example, mouse antibodies to isotypic determinants on the IgM of neoplastic B lymphocytes of people are useful as a treatment until the individual being treated makes antibodies (particularly anti-idiotype) to the mouse antibodies. Thereafter, any further injection of monoclonal antibodies of mouse origin is quickly removed. Furthermore, the antigen-antibody complexes formed might result in serum sickness.

The preferred treatment is with monoclonal antibodies of human origin made from a fusion of human lymphocytes with a mouse or human plasma tumor cell. Human cell lines that fuse with human lymphocytes have been developed for use in making antibodies for treatment of people, but this relatively new development has not been established clinically. The same rationale is true when using monoclonal antibodies in the treatment of animal disease.

It should be kept in mind that monoclonal antibodies made from fusions in the mouse system can be used in humans and other animals only on a single or very short-term basis. For example, mouse monoclonal antibodies could be used on a one-time emergency basis for treatment or protection after exposure to tetanus, botulism, snake venoms, rabies, herpes B, or Marburg viruses. Monoclonal antibodies could also be used to treat several serious viral infections for which adequate vaccines are not currently available.

In certain animal diseases such as diarrhea of calves caused by E. coli infection, monoclonal antibodies to the pili of the organism might be given orally to protect against the disease. This would be a particularly effective therapy if monoclonal antibodies of bovine cow origin, IgA type mixed with secretory piece, were used; large quantities of antibody to the antigenic determinants that give a protective effect could be administered.

The many other possibilities for monoclonal antibody treatment of important animal diseases may be of special interest to developing countries attempting to improve animal production.

Prevention (Vaccination). The best example of disease prevention using monoclonal antibodies is vaccine production. Monoclonal

antibodies can be used to identify and isolate antigens from all types of organisms causing infectious disease, and the antigens can then be used as vaccines.

Vaccines are not yet available for a variety of infectious diseases, particularly for complex protozoan organisms such as malaria and trypanosomes. Malarial parasites have elaborate life cycles and, therefore, are difficult to study. However, evidence suggests that effective immunity might be obtained if antibodies could be induced to antigens present on the developmental forms of the parasite. For instance, when monoclonal antibodies to malaria sporozoites were given to mice prior to infection, subsequent infection was prevented. Thus in a single experiment a determinant on the difficult-to-isolate sporozoite stage of the malarial organism was identified, and the solution to a major disease was brought closer. With the monoclonal antibody available, it will now be possible to isolate the molecule carrying the determinant by affinity chromatography and to test its capability to induce protective antibodies when injected as a potential vaccine.

Similar approaches can be used to find antigens on other infectious organisms that will induce protective immune responses. This procedure is by no means limited to single-cell parasites; it extends to all types of pathogens and may be of exceptional value with complex parasites. Monoclonal antibody technology might well prove to be the technological breakthrough needed to help with multicellular parasites as well.

In addition to using monoclonal antibodies to identify and characterize antigens that can be used for vaccination, the process can be carried one step further. Monoclonal antibodies can also be used to develop synthetic vaccines as well as antigens for diagnostic assays. For example, if the antigenic determinant of interest is on a protein, the protein can be digested into small polypeptides and the polypeptide reacting with the monoclonal antibody isolated by affinity chromatography. The amino acid sequence of the polypeptide can then be determined. Assuming the number of amino acids composing the critical determinant is from 7 to 20, the polypeptide can be synthesized and coupled to a suitable carrier molecule, injected into test animals, and evaluated for its ability to induce a protective immune response. Antigens that fail to elicit immunity when present in the organism can be engineered so that they become more immunogenic and, as a result, more effective vaccines.

One novel application of monoclonal antibodies in deriving a vaccine is the use of monoclonal anti-idiotypic antibodies as antigen. For example, clonal anti-idiotypic antibodies made against monoclonal antibody to variant surface glycoprotein of an African trypanosome were used to induce antibodies in mice that would neutralize the trypanosome. These antibodies were then used to induce the formation of an anti-idiotypic antibody.

How do anti-idiotypic antibodies when injected into animals induce the production of antibodies that recognize the same antigen used to make the original monoclonal antibody? The binding site on the monoclonal antibody that recognizes the antigen has unique determinants (idiotypes) that are recognized by the monoclonal anti-idiotypic antibody. The idiotype is involved in binding to the natural antigen. The recognition of the idiotype by an anti-idiotypic antibody requires a similar interaction. In this instance, however, the binding site of the anti-idiotypic antibody mimics the architectural configuration of the natural antigen so accurately that the anti-idiotypic antibody can elicit a response comparable to that of the natural antigen, and this obviates the need for antigen in the vaccine. It is apparent that not all anti-idiotypic antibodies can be used as antigen, since not all of the antibodies possess regions of the binding site that correctly mimic the antigen.

Health and Nutrition

In recent years, the relationship of nutrition to immunological functions has been more clearly defined. Deficiencies in protein, total calories, amino acids, vitamins, minerals, and fatty acids can severely depress the immune system. The examples presented here will demonstrate the profound effect that malnutrition can have on resistance to disease, the differential effects that malnutrition can have on the subcomponents of the immune system, and the role that monoclonal antibodies can play in the delineation of the immune defects that may result.

Protein-calorie malnutrition results in increased susceptibility to infections and fatal diseases. In cases of severe deficiency, both B lymphocyte and T lymphocyte subpopulations are affected, resulting in a decline in antibody production and cell-mediated immunity, respectively. At a less severe level, a differential effect is observed. The B lymphocyte function declines proportionally to dietary restriction, but the T lymphocyte activity is unaffected (or in some instances enhanced) until dietary restriction becomes severe.

Zinc is essential for the preservation of normal immune responsiveness. Zinc deprivation in man, cattle, and rodents leads to dermatitis, diarrhea, and increased incidence of infections. More has been learned of zinc's role in the activities of lymphocytes from the study of humans with acrodermatitis enteropathica, of cattle with lethal trait A46, and of rodents experimentally fed zinc-deficient diets. Zinc is a weak mitogen, able to stimulate DNA synthesis in lymphocytes. It has also been shown to be a polyclonal activator of B lymphocytes, resulting in increased synthesis and secretion of antibodies. The greatest effect of zinc appears to be on the thymus and the T lymphocytes which differentiate and mature in the thymus.

In zinc deficiency, thymic atrophy occurs rapidly, leading to a marked decrease in the absolute numbers of T lymphocytes and a consequent decrease in helper T lymphocytes (T_H) as well as cytotoxic T lymphocytes (T_C). Natural killer cell activity also declines.

Since dietary deficiencies influence the immune system, and since lymphocyte subpopulations are affected to different degrees, diagnosis and study of the immune defects in malnutrition would be enhanced by the existence of reagents specific for each lymphocyte subpopulation. Such studies are equally important for both developed and developing countries. These reagents can be more readily produced by cell hybridization techniques than by conventional alloimmunization or xenoimmunization. In man and rodents, B lymphocytes, monocytes, natural killer cells, helper cells (T_H), cytotoxic cells (T_C), and suppressor T cells (T_S) can be identified and quantitated using specific monoclonal antibodies. Similar reagents are required for domesticated animals to facilitate analysis of immune status and to improve food production through reduction of infectious diseases.

Monitoring Drug Use and the Presence of Toxic Substances

Antibody-dependent immunoassays have been developed to evaluate drug usage. Examples include: the quantification of exogenous steroid levels to evaluate endocrine organ activity, hormone replacement therapy, or production of hormones by neoplastic cells; the quantification of therapeutic compounds in blood, tissues, or excretions to determine whether adequate dosages are employed; and the detection of illegal drugs or toxic compounds in human blood.

Although many of these assays are performed with antibodies obtained by conventional means, the use of monoclonal antibodies has several advantages. The precision of the antibody allows differentiation of closely related compounds. Since hybridoma cells can be grown in ascitic fluid, virtually unlimited quantities of monoclonal antibodies can be produced. This facilitates standardization of assays since a given antibody with defined specificity and constant binding affinity can be produced and made available for an indefinite period. The use of such reagents by multiple laboratories would reduce interlaboratory variability of test results and thereby allow more reliable comparisons of data.

Monitoring the Immune System

The activity of the immune system depends on complex regulatory interactions among macrophages, B lymphocytes, and subpopulations of T lymphocytes--T_H (helper), T_C (cytotoxic), T_S (suppressor),

T_D (delayed type hypersensitivity), etc. Absence or impaired function of one or more of these cell subpopulations generally results in a complex pathology, often with increased susceptibility to infections. More subtle perturbations of reactivity or numbers may also have serious pathological consequences.

Diagnosis and investigation of immunologically mediated disorders have been enhanced by the limited availability of monoclonal antibodies that bind specifically to defined lymphocyte surface antigens. Some bind to cell membrane glycoproteins that are only present at certain stages of lymphocyte maturation, while others bind to all mature T lymphocytes and some only to specific T cell subset differentiation antigens. Using these reagents, it is possible to define the exact step at which lymphocyte maturation is inhibited in various immunodeficiency diseases. Similarly, these reagents are useful in monitoring the return of functional lymphocyte subpopulations following transplantation of hematopoietic stem cells in the immunodeficient patient. The availability of monoclonal antibodies specific for T_4 and T_8 antigens in humans has allowed determination of the normal ratios of these two surface antigens; an alteration in the ratio may occur in certain diseases. For example, in naturally occurring immunoinflammatory diseases--such as hemolytic anemia, systemic lupus erythematosus, inflammatory bowel disease, hyper-IgE syndrome, ectopic eczema, and multiple sclerosis--there is a significant reduction in T_8 cells which allows T_4 cells to provide continuous help to B lymphocytes in a less regulated manner. In other diseases such as lepromatous leprosy, antigen-specific T_8 cells are stimulated and increased in number. This may account for the anergy observed in this and similar diseases.

Monoclonal antibodies have also been useful in diagnosing T-cell malignant diseases. For example, malignant T lymphocytes from patients with the Sezary syndrome bear the T_4 phenotype and may provide help to B lymphocytes involved in immunoglobulin synthesis. This syndrome has been treated with a polyclonal anti-T cell reagent (ATGAM^R) with some success.

The production of monoclonal antibodies to human and domestic animal lymphocyte subpopulations is advantageous as it facilitates diagnosis, investigation, and, ultimately, therapy of lymphocytic malignancies, immunodeficiencies, and immunoinflammatory diseases. It may also permit the development of more effective methods of inducing immunity to infectious organisms.

Plant Research

Monoclonal antibody technology is playing a growing role in plant research and management. Many diseases affect the production of plant food crops for human and animal consumption as well as the development

and maintenance of pathogen-free seeds and plants. An exceptionally large number of viral, fungal, and bacterial pathogens constantly threaten entire food crops, and the use of genetically uniform plants to maximize production has increased the risk of such threats. To minimize the occurrence of widespread plant disease, national and international programs have been established to identify major pathogens and prevent their spread through shipment of contaminated seeds, plant stocks, or crops. Elaborate quarantine and certification programs have been established for many plants, and serology has played an important role in such programs. The persistent difficulty, until now, has been the development of adequate quantities of standardized immune reagents for worldwide use.

Ongoing programs with potatoes, fruit, and cereals illustrate the need for immune reagents for use in research, diagnostic tests, and certification. For example, potatoes are propagated through seed pieces, derived from whole potatoes, to provide pathogen-free seed pieces for cultivation and uniformity of crop. Certification requires testing for the presence of such organisms as Erwinia carotovora var. atroseptica (the causative agent of blackleg of potato), potato leaf roll virus, potato virus X, and potato virus Y. The first two organisms cause devastating losses, while the latter two cause reduced yields. Serological techniques are the main means of detecting such pathogens; however, it has been difficult to develop reagents with sufficiently narrow reactivity to distinguish between strain variants. On a number of occasions it has been essential to determine whether disease outbreaks resulted from variants of the pathogen characteristic of the region where the seed stalk was propagated or characteristic of the region where it was put into production. Monoclonal antibody technology augments the precision of existing antisera and presents a solution to the problem. It also provides a means of conducting epidemiological surveys of farm land before introducing a given food plant.

The development of biological control measures to reduce disease in potatoes has added another dimension to the utility of monoclonal antibodies. Recent research has revealed that the fungus causing Verticillium wilt can be potentially controlled by the presence of specific strains of the bacterium Pseudomonas fluorescence, which may produce an antibiotic or other growth-inhibiting factor. However, this bacterium can only be used effectively if reagents are available with sufficient specificity to distinguish the beneficial subtype from the myriad types present in the field. Monoclonal antibodies present a way to make such reagents.

Fruit trees, which are afflicted by numerous viruses, present another interesting problem. Citrus tristeza virus, in particular, has been a problem since the development of citrus fruit industries; certain strains of this virus can destroy entire orchards. The primary approach to control has been removal and destruction of the

infected orchards. Some years ago in Brazil, a severe outbreak led to the destruction of over a million citrus trees.

No effective method of testing for this virus has been developed. This problem is compounded by the fact that a number of variant forms of the virus exist in the field, with some causing severe disease while others cause little or no disease. The latter types do, in fact, protect against infection by those causing severe disease. The decision to destroy an orchard depends upon which form is present. Available serologic reagents made by conventional procedures fail to distinguish one form from the other. Monoclonal antibodies provide a means of resolving this problem.

Ilarviruses also cause major disease problems in fruit trees. Prunus necrotic ring spot and prune dwarf viruses cause disease in cherry trees; apple mosaic and tulare apple mosaic viruses cause disease in apple trees. Serological assays have been used to aid in the selection of virus-free stalk for distribution, diagnosis of field outbreaks, and certification of plants being introduced into new areas. Conventional reagents have been useful, but they are difficult to maintain in sufficient supplies to meet commercial needs worldwide. The failure of these viruses to elicit a strong immune response has added to the problem and has limited the development of reagents capable of detecting the many forms of the virus. Early programs with monoclonal antibodies have already shown that the difficulties can be surmounted.

Similar problems exist in the cereal grains; however, less use has been made of serological assays to identify and control the pathogens, in part because reagents made by conventional procedures lack sufficient specificity. Although the reagents made were often used to distinguish a virus type, they could not be used to distinguish strain variants. The utility of monoclonal antibodies for resolving the problem is apparent. Field diagnosis of barley yellow dwarf virus and barley stripe mosaic virus, for example, would allow detection of strain variants responsible for disease outbreaks as well as determination of whether the disease was seed borne or the result of field contamination.

Production of Food Animals

The utility of selective breeding to improve production of food animals has been recognized and practiced for many years. Because of its importance, considerable emphasis has been placed on applying new technologies to aid in the manipulation and control of reproduction and growth cycles to maximize the full genetic potential of food animals. Experience has revealed that selective breeding is no simple task, especially when the methods for identifying multiple desirable genetic traits are limited. Examples of such traits in beef cattle

are high fertility, twinning, heavy weight at weaning, food conversion efficiency, carcass quality, and resistance to disease.

Application of knowledge gained from breeding laboratory animals and insects has required the development of techniques to select for desirable traits based on the measurement of heritability of traits rather than techniques based on direct identification of genes or gene complexes. Heritability is defined as the percentage of the difference among animals in performance traits passed on from parents to offspring; for example, weight gain in pasture might be 30 percent higher in animals carrying the selected trait. The improvement, if indeed a heritable trait, is expected to be passed on and to be cumulative in subsequent generations.

This has proven to be the case for a number of selected traits that are under polygenic control. Artificial insemination and ova transplantation have augmented the utility of this method of selective breeding. In developed countries, this technology has helped modernize livestock and poultry breeding by accelerating the introduction and distribution of desired traits in a population. Examples of the success of these techniques are the 50 percent increase in milk production by dairy cows over the past 30 years and the development of high-yield breeds of beef cattle and strains of chickens and turkeys.

The benefits of applied genetics are only now exerting an effect on food production. However, the results point to a need to expand and improve the techniques for selective breeding. For example, with long-lived food animals such as cattle where advances are essential, little progress has been made in analyzing the genetic traits regulating the immune response and susceptibility to a variety of diseases. In the United States alone, an estimated annual loss of $4.5 billion is attributable to disease. Although evidence exists that resistance is under genetic control, none of the parameters of measurement used provide a way to identify the traits involved.

The magnitude of the problem is exemplified by the fact that none of the high-performance breeds developed in Europe or America can be introduced into developing countries in Africa or South America where food shortages are expected to become acute. These breeds are highly susceptible to endemic diseases, particularly trypanosomiasis and anaplasmosis. Interbreeding with native animals to offset susceptibility has shown that multiple genetic factors are involved in resistance and that intense selective breeding programs are needed to develop resistant breeds with high performance traits. Examination of native stocks of both cattle and other species (such as the water buffalo) has revealed the potential of germ plasm resources as well as the need for improved methods of genetic selection to improve breed performance. Other domestic food animals present the same type of problem.

The advent of monoclonal antibody technology has provided an important tool for improving selective breeding programs both in terms of analysis of the immune response to infectious agents and delineation of a library of genetic traits that can be used to help identify and concurrently select animals for disease resistance and the desired performance traits. Extensive studies in mice, chickens, and humans have revealed that a number of genes and gene complexes code for antigenic molecules present on white and red blood cells. Some genes code for differentiation antigens that define sub-populations of white blood cells; others code for antigens common to all white blood cells. The differentiation antigens have proven quite useful, as mentioned previously, in elucidating the function of the immune system. Others have proven useful as genetic markers to identify important genetic traits through linkage analysis.

One especially relevant finding has been the association between the inheritance of histocompatibility genes and susceptibility to certain types of disease. Studies in humans have revealed a striking association between the transmittance of the gene coding for the human major histocompatibility gene complex antigen, B27, and the occurrence of a serious joint disease, ankylosing spondylitis. Ninety-five percent of the patients with the disease are positive for the antigen.

Other less striking examples have shown similar associations. In each instance, the findings have indicated that genes, not readily identifiable alone, may be associated the transmittance of another identifiable trait. For food animal research, studies indicate that similar associations exist in animals and that genetically and antigenically distinct membrane molecules can serve as markers to trace the patterns of inheritance of linked genes that influence the expression of disease. Studies also point to the possibility of identifying genes that control the expression of desirable traits. Monoclonal antibodies will play a pivotal role in such studies because they provide the precision needed to identify the array of gene products and the means of developing standardized typing reagents.

Of special interest is the fact that the technology and computer programs developed for selective breeding of cattle can be extended to other species. In each case, the information gained and the battery of monoclonal antibody typing reagents can be used in emerging countries where programs need to be implemented to accelerate the upbreeding of food animals.

REQUIREMENTS AND PROCEDURES FOR IMPLEMENTING MONOCLONAL ANTIBODY TECHNOLOGY

Monoclonal antibody technology can be readily applied in a number of areas, and in many instances as a direct result of ongoing programs in developed countries. A considerable effort is now under way to

make monoclonal antibodies that can be used in the study of health and nutrition, in the monitoring of drug use, in the monitoring and analysis of the immune system, in the study of food plants and their pathogens, and in the development of methods to identify disease-resistant stocks of food animals for selective breeding. However, how and when will such apparently sophisticated technology be made available for use in emerging nations?

Well-trained technical staff and adequately equipped laboratories are needed to produce monoclonal antibodies effectively, and this places an obvious constraint on use of this technology. The initial cost outlay for effective implementation of this technology appears quite large. However, comparison with the costs of continued use of conventional procedures for preparing immune reagents for medical and biological purposes with their attendant limitations quickly dispels this notion. The following section outlines the requirements for setting up a laboratory and producing monoclonal antibodies. In most situations, the best use of such facilities will occur where the laboratory is centrally located and associated with other major laboratories.

Training

The production of monoclonal antibodies requires knowledge of basic tissue culture techniques and familiarity with the principles and procedures of immunology. Furthermore, experience with gel diffusion, complement fixation, immunofluorescence, radioimmunoassay, and enzyme-linked immunosorbent assays is also beneficial, as well as experience in the breeding, rearing, and handling of large numbers of mice under disease-free conditions.

Specifically, the investigator should be experienced in sterile techniques, and the preparation of tissue culture media and sterile glassware. Throughout the production of monoclonal antibodies, cells must be evaluated microscopically to determine viability and rate of growth. Experience with establishing and maintaining cells in vitro is also necessary. Finally, an essential part of the production of monoclonal antibodies involves identifying and characterizing antibodies obtained following fusion of myeloma cells with spleen cells obtained from immunized mice. The assay systems used will depend on the nature of the antigen and the intended use of the monoclonal antibody (some assay systems are described later).

Realistic Time Frame for Implementation

From the time that an adequately equipped laboratory is established, it may take as long as a year before the first usable

monoclonal antibodies are obtained. During this period, three major tasks must be undertaken.

First, stable clones must be obtained. Approximately 4 months of continuous laboratory work are required, beginning with immunization of mice and adaptation of the parent myeloma cell lines to growing in a new laboratory, through fusion of cells, evaluation of resulting hybridomas, and finally establishment of stable clones producing monoclonal antibodies. And the cell lines obtained require constant attention. It is, therefore, essential that investigators are able to devote the time and effort required for successful completion of the procedure.

The second major task is development of assay systems to evaluate cell culture supernatants efficiently and accurately for the desired antibody activity. The importance of reliable assay systems cannot be overemphasized, nor can the necessity for development and validation of the assay system before cell fusion is done. There may not be enough time to resolve the technical difficulties of an assay system after cell hybrids begin to grow. Because the investigator has only a few days to test supernatants from hybrids and thus decide which to retain or discard, a reliable assay must be available when needed.

The third task is full characterization of the monoclonal antibody once obtained, including tests for specificity and affinity of antigen binding, definition of the immunoglobulin isotype produced by the clone, and characterization of the secondary activities of the antibody (precipitation, complement fixation, etc.). Once the laboratory is experienced in fusion procedures and a complete battery of assay systems is established, additional monoclonal antibodies may be obtained and characterized within 4 months.

Laboratory Facilities

Laboratory Space. The central production laboratory should include a minimum of 61 square meters of work space, hot and cold running water, an adequate electrical supply to support several pieces of electrical equipment, and a temperature control system to keep the ambient temperature below 31°C. Temperature regulation in the laboratory is necessary to maintain cell culture incubators at the proper temperature for growth and maintenance of cells. Alternatively, the temperature of the incubators must be controlled. Space for breeding and rearing of mice is also required.

Equipment. The central production laboratory must be equipped with the following:

- Humidified, temperature-controlled incubator with a CO_2-in-air atmosphere. For a hot climate, the incubator's

temperature must be controlled for low as well as high temperatures.

- Tissue culture sterile hood
- Inverted microscope, preferably a phase microscope with 4X, 10X, and 40X objectives
- Standard microscope
- Temperature-controlled water bath
- Centrifuges: 3,000-rpm (revolutions per minute) table-top centrifuge; 3,000-6,000 rpm refrigerated centrifuge
- Autoclave
- Sterile filter and reservoir (5-10 liter)
- Freezer to -70°C
- Carbon dioxide tanks, nitrogen tanks (liquid and gas phase tanks)
- pH meter
- Balance
- Liquid nitrogen storage containers
- Still for production of double distilled, deionized water or a system to distill by filtration through resins.

Depending upon the nature of assays used, additional equipment may be useful: an ELISA (enzyme-linked immunosorbent assay) reader, fluorescent microscope, gamma scintillation counter, spectrophotometer, and fluorescent-activated flow cytometer.

Supplies and Preparation. The following supplies are required for the efficient production of monoclonal antibodies:

- Tissue culture multi-well plates with covers (sterile)
  - -- 96-well flat bottom plates, about 0.28 cm^2 (square centimeters) per well
  - -- 24-well flat bottom plates, about 2.0 cm^2 per well
  - -- 6-well flat bottom plates about 17 cm^2 per well.
- Tissue culture flasks (sterile)--25 cm^2, 75 cm^2, and 150 cm^2
- Sterile screw-cap test tubes, pipettes, beakers, flasks; 1-, 5-, 10-, 20-, 50-ml (milliliter) syringes; 16-, 18-, 25-gauge needles
- Polyethylene glycol (PEG) of molecular weight (MW) between 1,000 and 4,000
  - -- 41.6 percent PEG/15 percent DMSO (dimethylsulfoxide): Autoclave 50 g (grams) PEG to liquefy and sterilize. When it has cooled to about 60°C, add 70 ml of serum-free Dulbecco's Minimal Essential Medium (DMEM) containing DMSO (55 ml DMEM + 15 ml DMSO).
  - -- 25 percent PEG: Autoclave 50 g PEG to liquid. When it has cooled to about 60°C, add 150 ml serum-free DMEM. Both PEG solutions may be stored for up to 2 weeks at 4°C.

-- 50 percent PEG: Autoclave and then aliquot 1 ml in sterile 4-dram vials.

- Selection medium
  - -- HAT (50x). Dissolve the following in 99 ml of double distilled water containing 0.01 M (mole) sodium hydroxide (NaOH): 6.8 mg (milligrams) hypoxanthine, 0.95 mg aminopterin, and 19.5 mg thymidine. Filter, sterilize, and add 1 ml 1 M HEPES to give a final concentration of 10 mM (millimolar) HEPES.
  - -- HT (50x). Prepare as for HAT (50x) but omit the aminopterin. HAT (50x) and HT (50x) can be stored for 1-2 months at 4°C.
  - -- Final HAT medium. Add 1 ml buffered HAT (50x) to 49 ml DMEM containing antibiotics, 10 mM HEPES, 20 percent heat-inactivated fetal calf serum, and 5 x 10^{-5} M 2-mercaptoethanol (2-ME).
  - -- Culture medium. This is DMEM containing antibiotics, 10 mM HEPES, 15 percent fetal calf serum, and 5 x 10^{-5} M 2-mercaptoethanol.
  - -- Comments. Fetal calf serum lots may vary in their ability to support growth of cells. However, use of 2-ME in medium minimizes the effect and the need to test lots of serum. DMEM containing antibiotics, buffer, and fetal calf serum can be stored for 2 weeks at 4°C, but 2-ME must be diluted and added to the medium on the day it is to be used for fusion. Otherwise, it can be added at the time of preparation. Glutamine should be added after 2 weeks of storage.

Animal Space and Management. A continuous supply of BALB/c mice is required at all stages of production of monoclonal antibodies including immunization, donation of thymuses for use as feeder cells, and production of antibody-containing ascitic fluid. Mice should be reared in a separate room from other species. Adequate temperature control and ventilation is needed to reduce the probability and prevalence of infectious diseases.

Breeding cages must contain one female and one male for maintaining lineage by brother-sister mating and two females and one male for use in preparation of hybridomas. A simple cage card system is used for recording the dates of the breeding as well as birthdates of each litter produced. Mice are weaned at 3 weeks of age, separated by sex into new cages, and identified by a card indicating birthdate. Mice 4-6 weeks of age are ideal thymus donors for feeder cells. Mice intended for spleen cell donors can be immunized beginning at 3 weeks of age. For most immunization protocols, such mice will be 6-9 weeks old by the time immunization is complete and will be large enough to yield an adequate number of spleen cells.

Substantial numbers of mice are used in the production of ascitic fluid. These mice should be adults to maximize the volume of fluid produced. Mice are injected intraperitoneally with 0.5 ml of filter-sterilized pristane (2-, 6-, 10-, 14-tetramethylpentadecane). Seven to 60 days later, the mice are injected with 10^7 viable hybridoma cells and are examined daily starting 5 days after injection. Ascites appears from 6 to 20 days following injection. To expedite ascites production on a timely basis, several cages of mice should be pristane primed and available at all times.

Production of Monoclonal Antibodies

Since the initial discovery that stable antibody-producing hybrids can be formed, the techniques of fusion and processing cell cultures have been greatly simplified. It is now possible to obtain consistently large numbers of hybrids and select the lines producing the antibody of choice.

The critical component in the production of hybridomas is the preparation of cells for fusion. Where rodents are used as a source of cells for fusion, immunization protocols must be devised that optimize the proliferative response to antigen in the spleen. Plasmablasts appear to be more suitable as fusion partners than mature plasma cells. For many antigens it is sufficient to immunize once, and then 2 or more weeks later boost with a single intravenous injection of antigen 72 hours before collection of the spleen. For others it may be important to hyperimmunize and use an adjuvant. Where *in vitro* techniques are employed, as when attempting to use human cells to produce human monoclonal antibodies, no clear guidelines can be given. Both culture conditions and the antigen must be worked with to obtain the desired proliferative response.

The fusion and culture of hybridomas is quite straightforward. Figure 1 outlines one effective approach to producing and processing many hybrids on a regular basis with a limited staff. Although detailed for use with spleen cells from immune mice, the scheme is equally useful when cultures of *in vitro* immunized cells are used as a source of plasmablasts.

Phase I: Fusion and Primary Culture of Hybridomas.

1 Boost mice with an intravenous injection of antigen 72 hours before use. On the day of fusion, collect the spleen and make a free cell suspension by injecting and flushing the spleen with sterile serum-free medium. Centrifuge the cells and resuspend in lysing solution. When sterile water (H_2O) is used, suspend the cell in 1 ml of H_2O and immediately dilute in 20 ml of medium. Any delay will result in the

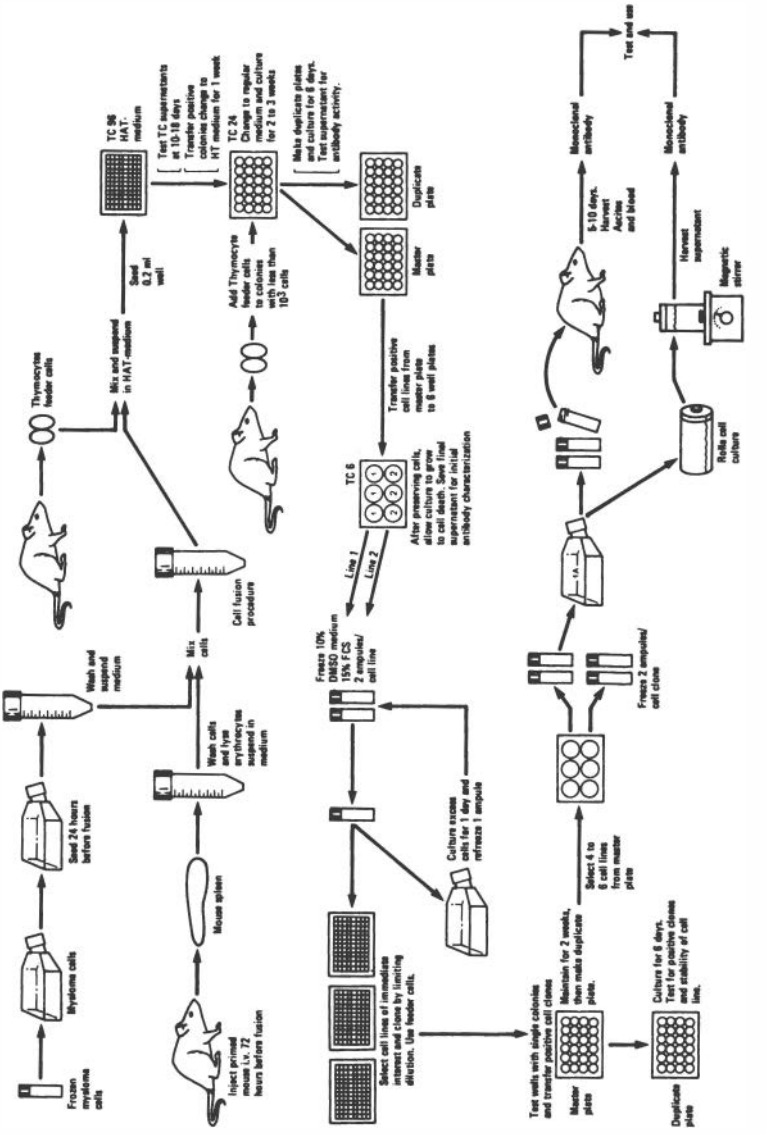


FIGURE 1 Production of monoclonal antibodies.

loss of plasmablasts and a consequent reduction in antibody-producing hybrids.

2 Maintain myeloma cells in cell culture and seed into fresh flasks and medium 16-24 hours before fusion to ensure they are in the early log phase of growth at the time of fusion. At the time of use, collect the cells, centrifuge, and resuspend in serum-free medium.

3 Count both myeloma and spleen cells and then mix in the appropriate ratio. Depending on the properties of the tumor cell, the ratio of spleen to tumor cells may vary from 5:1 to 2:1. For NS-1 and SP2 cells, a good ratio is 5:1. For X63 Ag8.653, a ratio of 2.5:1 yields better results.

4 Following mixing, the cells are centrifuged into a loose pellet by spinning at 1,000 rpm for 10-15 minutes. Remove the supernatant and overlay the pellet with 1 ml of PEG. For 3 minutes, mix the PEG into the pellet. In so doing break up the pellet into uniform small clumps.

This step can be performed with several types of PEG 1,000-4,000 MW at a concentration of 45-50 percent in medium or 41.6 percent in the presence of DMSO. Equal success is achieved whether fusing cells at room temperature or at 37°C. The variable, of course, is whether an excessive number of plasmablasts are lost during the lysis of erythrocytes.

5 Following fusion dilute the cells in 30 ml of serum-free medium, with the first 10 ml medium being added and mixed at 1 ml per minute. Slow dilution reduces the risk of osmotic disruption of the fused cells.

Centrifuge the cells and resuspend in complete medium containing HAT and then dispense into 96-well tissue culture plates, 10 plates for each 10^8 splenocytes used in the fusion. Add 10^6 thymocytes to each well to serve as feeder cells. The latter step has proven critical for optimizing that outgrowth of newly formed hybrids. Feeder cells and 2-ME in the medium exert a synergistic effect.

6 In carbon dioxide incubators with high humidity, incubate the cultures for 3-4 days between changes of culture medium. With rapidly growing cultures, replace half the medium with fresh HAT medium every fourth day.

7 Identify and mark the wells containing hybrid colonies on day 9 or 10 and then allow the colonies to grow to 500 or more

cells. In rapidly growing cultures, supernatants can be collected and assayed for antibody activity by day 12 to 14. Where appropriate, collect and test the supernatants from the largest colonies first; test the remainder 2-4 days later.

8 After each test for antibody, transfer positive cell lines to 24-well plates. Add 3-5 x 10^6 feeder cells to each well to promote rapid cell growth. Maintain cells in static culture for a minimum of 2 weeks by removing 50-75 percent of the hybrid cells every 2-3 days. This maneuver selects for stable, rapidly growing, antibody-producing hybrids. Slow growing hybrids and hybrids that cease to synthesize antibody are eliminated. The culture should be maintained in a HAT medium for 14 days from the time of fusion, 7 days in HT medium, and then continued in regular culture medium.

9 After 2 weeks, make duplicate cultures and allow the cells to overgrow and die. Collect the supernatants and assay for the presence of antibody. Take cultures producing antibodies of the desired specificity from the master plate and expand into 6-well plates (2-3 wells for each cell line). Harvest the cells twice for preservation in liquid nitrogen (4-10 x 10^6 cells per ampule in 1 ml of fresh medium containing 10 percent DMSO). Replenish the cultures with fresh medium and allow the cells to overgrow and die. Collect final supernatant for further analysis. The 6-well plates are essential for minimizing labor and time during this phase of hybridoma production.

Phase II: Cloning and Preservation of Hybrids.

1 Following preliminary selection of hybrids, screen the final supernatants in detail to identify antibodies of immediate interest. Take the parent cell lines from the freezer and clone by limiting dilution. When viability is good, clone the cell lines immediately. Take the remainder of the excess cells and culture them in a T75 flask for 1-2 days. As soon as enough cells are present, harvest the cells and freeze one ampule to replace the ampule used for cloning. The culture should not be allowed to proliferate more than necessary to avoid change in composition of the cell line at this early stage of processing. As in the initial phase, use feeder cells to promote growth.

2 At 6-8 days mark wells containing a single colony. At 12-14 days assay supernatants from the marked wells. Transfer 24-48 positive cultures to 24-well plates and maintain in

static culture as described previously. Make up duplicate culture plates at 14 days and test 7 days later.

3 After noting which cloned lines are stable (that is, all cloned lines are positive), expand 4-6 clones of each cell line into 6-well plates for cell preservation and production of antibody. Record which lines are stable (that is, all transferred cultures are positive) and which are unstable. Reclone unstable lines or mark for recloning when used for antibody production.

4 Preserve 4-6 clones from each cell line in liquid nitrogen.

Phase III: Production of Antibody.

1 To produce antibody, culture cloned cell lines in vitro or grow in ascites form in mice.

For in vitro culture, take cells from the freezer and grow to confluency in a T75 flask. Transfer to a rollacell bottle and grow to a higher cell density. Add fresh medium as required. Where desired, increase the cell density further by introducing a sterile magnetic stirring bar into the culture bottle and placing it upright on a magnetic stirrer. The cells can be grown to cell death or maintained by removal of excess cells and replacement of spent antibody-rich medium.

For in vivo production of antibody, prepare additional ampules of frozen cells, 10^7 cells per ampule. As needed, thaw the ampules of cells, wash once to remove the DMSO, then inject the cells intraperitoneally into BALB/c mice pretreated with pristane. Collect ascites and test for antibody activity and then freeze until needed. The use of frozen cells from culture for ascites production avoids the problem of having to adapt and stabilize each new line to growth in vivo.

Methods for Assaying Monoclonal Antibodies

General Considerations. There are two major problems in selecting an assay system for screening antibody activity in supernatants from hybridomas. One problem is lack of a test that can be done easily and in one day. Often it is necessary to evaluate for the presence of antibody activity in 1,000 supernatants. When large numbers of supernatants are to be tested, the assay system should have relatively few steps and an endpoint that can be quickly measured.

The second major problem is selection of an antibody assay that provides the maximum amount of information in relation to the type of antibody(ies) desired from the fusion. For instance, if the goal is to make monoclonal antibodies that are cytotoxic for B lymphocytes, the initial screening assay should be a cytotoxicity test. Such an assay would eliminate, in the first round, all antibodies incapable of mediating cytotoxicity. In subsequent assays, the tests should permit assessment of antibody isotype, specificity, and affinity. High-affinity antibodies might be sought for use in diagnostic assays and low-affinity antibodies for affinity column purification. Thus, selection of an assay system is based primarily on what kind of antibody is being sought and its intended use, and, therefore, considerable time and thought must be given to selection of the assay systems. Proper selection can greatly reduce the workload and ensure that the desired monoclonal antibodies are identified at a minimum cost. Details of the following tests can be found elsewhere. This discussion will focus on the advantages and disadvantages of each test.

Enzyme-linked Immunosorbent Assay (ELISA). When ELISA procedures are used to detect antibody, the antigen is usually attached nonspecifically to the wells of a plastic microtiter plate. The supernatants from cultures are introduced and incubated with the antigen, and the plates are washed. A reagent is then added to assay for the presence of antibody. Several reagents can be used and two will be described.

One reagent is rabbit antiserum to mouse immunoglobulins, which has been conjugated with either alkaline phosphatase or horseradish peroxidase. After incubation, the unattached enzyme-antibody conjugate is washed away, and an appropriate substrate is added. The suitable enzyme substrates are those that change color in the presence of enzyme. Therefore, if mouse antibody is present in a supernatant, the enzyme-conjugated, rabbit antimouse immunoglobulin would bind. In the presence of substrate, a color change occurs. If no mouse antibody is present in the supernatant, no color change occurs. For the ELISA test, enzyme-conjugated, rabbit antimouse immunoglobulin can be used to detect the various classes and subclasses of mouse immunoglobulins that occur in the supernatants (such as IgG_1, IgG_{2a}, IgG_{2b}, IgG_3, IgA, and IgM). Here, the antimouse immunoglobulin reagents must have broad specificity. If monoclonal antibodies occurring in a single subclass, such as IgG_1, are desired, the specificity of the antimouse immunoglobulin reagent should be restricted to mouse IgG_1.

The second reagent to consider is enzyme conjugated to purified staphylococcal protein A. The protein A binds to the Fc portion of some immunoglobulins. In the mouse, protein A usually binds to the subclasses of monoclonal antibodies, IgG_{2a}, IgG_{2b}, and IgG_3, but it can bind to the IgG_1 subclass and on occasion IgM. Selecting

monoclonal antibodies that bind protein A has advantages when purification of the monoclonal antibody is considered. The supernatant or ascitic fluid containing the antibody can be passed over a protein A affinity column for rapid purification. The bound monoclonal antibody can be eluted by lowering the pH. Mouse antibodies that do not bind protein A can be detected by adding a rabbit antimouse antiserum to the wells after incubation with mouse monoclonal antibody. This step is followed by the enzyme-protein A conjugate and then the substrate. Rabbit IgG, which comprises most of the antibodies to mouse immunoglobulins, readily binds to protein A. In some instances, the addition of the rabbit antimouse immunoglobulin reagent provides an increase in sensitivity.

Primary advantages of the ELISA procedures are the ease and rapidity with which the test can be run with several hundred samples. In addition, if the enzyme substrate reaction is controlled properly, the test can be read visually without elaborate equipment. When visual reading is used, the test is very inexpensive and can be adapted to almost any laboratory.

The disadvantage of the ELISA test is that appropriate concentrations of antigen must be attached to the plastic plate. In some instances where antigens are, for example, in serum or other high-protein media, it is necessary to enrich the antigen to obtain binding at the desired concentrations. Also, the conjugation of enzyme to reagent must be done to provide proper ratios for optimal color development and specific binding.

Radioimmunoassay (RIA). To detect the presence of mouse monoclonal antibodies by radioimmunoassay, the antigen of interest is usually bound to plastic microtiter plates as in the ELISA test. Supernatants, possibly containing mouse monoclonal antibodies, are incubated with antigen-coated wells and washed and incubated with either ^{125}I-rabbit IgG antibodies to mouse immunoglobulins or ^{125}I-protein A. As in the ELISA test, rabbit antimouse antiserum can be added after the addition of supernatants and before the addition of ^{125}I-protein A both to increase the sensitivity of the assay and to identify mouse immunoglobulin classes and subclasses that do not bind to protein A. The ^{125}I is coupled to the anti-immunoglobulin antibodies or the protein A by several different procedures that result in a covalent bond.

The RIA test can be performed as easily and quickly as the ELISA test except that counting the wells for the presence of ^{125}I is slower. It provides a very quantitative measure of binding which is useful in many research applications.

The major disadvantages of the radioimmunoassay are the requirements for a gamma ray counter and a constant supply of ^{125}I, which makes it relatively expensive. Further, a facility to handle radioisotopes without endangering personnel is needed.

Immunofluorescence. In some cases, it is necessary to assay for monoclonal antibodies that bind to antigens on tissue sections and on the surface of living cells. An appropriate assay under these conditions is a fluorescent-tagged, rabbit IgG antimouse immunoglobulin or fluorescent-tagged protein A. The substrate, whether fixed or unfixed, is incubated with mouse antibody-containing supernatants, washed and then incubated with the fluorescent-tagged reagent, and washed and examined with a fluorescent microscope. As with ELISA or radioimmunoassay, a rabbit antimouse immunoglobulin reagent can be inserted after the supernatant and before the fluorescent-tagged protein A to increase sensitivity and to detect nonprotein A binding mouse antibodies.

An immunofluorescent assay usually takes more time to read, often more time to perform, and requires a good fluorescent microscope. Fluorescent microscopy is usually selected for a specific purpose such as assaying for internal cellular antigens that cannot be readily isolated and bound to plastic. It is also useful for detecting antibodies that bind to the surface antigens on live organisms or cells.

Cytotoxicity. When antibodies are needed for use in a cytotoxicity test, then that test should be used for screening. The supernatants are incubated with live cells or organisms, washed, and a complement source is added. The cells or organisms are evaluated for cell death by visual observation after the addition of vital dyes such as Eosin or Trypan blue. The most difficult aspects of cytotoxicity testing with mouse monoclonal antibodies are the source of complement and the identification of a suitable target cell. In general, rabbit serum works best as a complement source, and 25 percent or less of the antibodies can be detected with guinea pig complement. Background activity is a problem with all heterologous complements. The density of the antigen on the cell surface must be sufficiently high to facilitate antibody complement interaction. When the density is too low or the cell of interest is in low concentration, interesting antibodies may go undetected.

This test requires a phase-inverted microscope and is generally performed using special microplates that can be easily and quickly scored. It should be kept in mind that not all classes and subclasses of mouse antibodies bind complement and cause cytotoxicity. However, cytotoxicity of a live cell or organism establishes that the antigen being recognized is on the surface of the cell organism. In the case of pathogens, the demonstration of an antigen on the surface by monoclonal antibody may be an important first step for isolating an antigen for possible use in the development of a vaccine.

Flow Cytometry. A valuable analytical tool for both analyzing and separating cells is the fluorescence-activated cell sorter

(FACS). Cells or organisms to be separated or analyzed are usually incubated with a mouse monoclonal antibody that binds to the surface, washed, and then incubated with fluorescein-conjugated, rabbit IgG antimouse immunoglobulin and washed again. During sorting, the labeled cells flow through a laser beam and trigger a separation device. In this way, cells or organisms in heterogeneous populations can be isolated for further experimentation. In the analyzer mode, the cells can be categorized by size, intensity of fluorescence, and number of cells with differing amounts of fluorescence.

The FACS is very expensive and should be considered only for large centralized research laboratories in developing countries that have ready access to service representatives. However, such instrumentation adds an important dimension to the use of monoclonal antibodies and must be considered a vital component of a well-equipped laboratory. (A new mercury-arc lamp-based analyzer recently introduced on the market may prove of exceptional value, especially in developing countries with needs in clinical diagnostics.) For example, a FACS can be used to develop a set of monoclonal antibodies of the same specificity but of different isotypes. Many of the hybridomas produced are capable of undergoing a switch in the synthesis of antibody isotype without change of specificity. Thus in any culture there can be a predominant isotypic form of antibody (e.g., IgM) being produced by the majority of cells in culture and another isotype being formed by a switch variant (e.g., IgG_{2a}). The FACS permits the selection and isolation of those cells producing the variant. Where there is a need for the development of an antibody for therapeutic or diagnostic use, this permits the use of existing cell lines producing antibody of the wrong isotype, but correct specificity, to be used in the development of the needed cell line.

The FACS can also be used to isolate rare antigenic variants of parasites for analysis and propagation. In diseases such as trypanosomiasis, the use of the FACS in conjunction with monoclonal antibodies is perhaps the only way that much progress can be made in defining the full spectrum of antigenic variants.

Cost Estimate

The cost of furnishing supplies and equipment for a monoclonal antibody laboratory depends on the number of people employed, the sophistication of equipment utilized, and the preexisting availability of required equipment and supplies. Assuming all required equipment and supplies would have to be purchased for the laboratory, the following list is an estimate of initial costs; labor costs and the laboratory facility itself are not included.

Item	Estimated Cost (U.S. dollars)
CO_2 incubator	$3,000-5,000
Tissue culture sterile hood	3,000-8,000
Inverted microscope	1,000-8,000
Standard microscope	1,000-3,000
Water bath	300
Centrifuge	2,000-8,000
Autoclave	1,500-15,000
pH meter	400-1,000
Balance	1,200
Liquid nitrogen storage containers	3,000-6,000
Water distillation system	6,000-8,000
Mouse cages	3,000
Purchase of 50 mice for breeding stock	200
Maintain 150 mice for 12 months @ $.035 per day per mouse	1,890
Tissue culture flasks and plates	4,000
Tissue culture medium, buffers, antibiotics	2,000
Fetal calf serum	2,000
Plates for assay of antibodies	2,000
Additional glassware (beakers, bottles, pipettes)	1,000
TOTAL ESTIMATED COST	$41,000 - 75,000

Estimated costs for optional equipment are: fluorescent microscope, $1,500; gamma scintillation counter, $15,000-25,000; and flow cytometer, $125,000-250,000. Total estimated cost with added equipment, $180,000-353,000.

The estimate for tissue culture supplies will vary according to the pace of activity in the laboratory. It takes $1,000 worth of supplies, on the average, to carry the clones of hybridomas from a single fusion through to the first stage of culture and preservation. It takes approximately $300 per cell line to complete cloning and standardization.

SUGGESTED READING

Galfre, G. and C. Milstein (1981) Preparation of monoclonal antibodies: strategies and procedures. Methods in Enzymology 73:3-46.

Grant, T. J., S. Moreira, and A. A. Sahbe (1960) Report on general aspects of tristeza and stem pitting in citrus varieties in Sao Paulo, Brazil. State Horticultural Society 73:12.

Hämmerling, G. J., U. Hämmerling, and J. F. Kearney, eds. (1981) Monoclonal Antibodies and T-Cell Hybridomas: Perspectives and Technical Advance. Elsevier/North-Holland Biomedical Press, New York, New York, USA.

Hooker, W. J., ed. (1981) Compendium of Potato Diseases. American Phytopathological Society, St. Paul, Minnesota, USA.

Kenneth, H. F., T. J. McKearn, and K. B. Bechtol (1980) Monoclonal Antibodies: A New Dimension in Biological Analysis. Plenum Press, New York, New York, USA.

Kung, P. C., M. A. Talle, M. De Maria, N. Ziminski, R. Look, J. Sifter, and G. Goldstein (1981) Creating a useful panel of anti-T cell monoclonal antibodies. International Journal of Immunopharmacology 3:175.

Milstein, C. (1980) Monoclonal antibodies. Scientific. October.

Milstein, C. and E. Lennol (1980) The use of monoclonal antibody techniques in the study of developing cell surfaces. Current Topics in Developmental Biology 14:1.

National Institutes of Health (1981) New Initiatives in Immunology. NIAID Study Group Report, National Institutes of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland, USA.

Oi, V. T. and L. A. Herzenberg (1980) Immunoglobulin-producing hybrid cell lines. Pp. 351-372, in: Selected Methods in Cellular Immunology, edited by B. B. Mishell and S. M. Shügi. W. H. Freeman and Co., San Francisco, USA.

Potocnjak, P., N. Yoshida, R. S. Nussenzweig, and V. Nussenzweig (1980) Monovalent fragments F(ab) of monoclonal antibodies to a sporozoite surface antigen (Pb44) protect mice against malarial infection. Journal of Experimental Medicine 151:1504.

Reinherz, E. L. and S. F. Schlossman (1981) The characterization and function of human immunoregulatory T lymphocytes subsets. Immunology Today 2:69.

Sacks, D. L., K. M. Esser, and A. Sher (1982) Immunization of mice against African trypanosomiasis using anti-idiotypic antibodies. Journal of Experimental Medicine 155:1108.

Smith, K. M. (1972) A Textbook of Plant Virus Diseases. Academic Press, New York, New York, USA.

Shows, T. B., and A. Y. Sakaguchi (1980) Gene transfer and gene mapping in mammalian cells in culture. In Vitro 16:55.

de St Groth, F., S. Scheidegger, and D. Scheidegger (1980) Production of monoclonal antibodies: strategy and tactics. Journal of Immunological Methods 35:1.

U.S. Congress (1981) Impact of Applied Genetics: Micro-organisms, Plants, and Animals. Office of Technology Assessment, U.S. Congress. U.S. Government Printing Office, Washington, D.C., USA.

Wadi, J. A. (1982) Biological Control of Verticillium Dahline on Potato. Ph.D. thesis, Washington State University, Pullman, Washington, USA.

Wiese, M. V., ed. (1977) Compendium of Wheat Diseases. American Phytopathological Society, St. Paul, Minnesota, USA.

Wiktor, T. J., and H. Koprowski (1980) Antigenic variants of rabies virus. Journal of Experimental Medicine 152:99.

World Health Organization (1980) Report of the Fourth Meeting of the Scientific Working Group on the Immunology of Malaria. World Health Organization TDR/IMMAL-SWG(4)/80.3, Geneva, Switzerland.

Research to Enhance Nitrogen Fixation: Misplaced Emphasis?

MARTIN ALEXANDER

INTRODUCTION

Nitrogen fixation in agricultural land is rarely limited by a lack of highly active nitrogen-fixing microorganisms. Although this point is often stressed by those concerned with the practical aspects of nitrogen fixation, it seems to be overlooked as a result of the enormous amount of exciting research being undertaken on the biochemistry and genetics of nitrogen-fixing organisms.

If this view is correct, it follows logically that a significant imbalance exists between basic studies of nitrogen fixation by these microorganisms and the investigations needed to explain why so little nitrogen is fixed in nature. The enormous progress made in recent years in understanding the biochemistry and genetics of nitrogen-fixing microorganisms often leads only to improved organisms when no improvement is apparently needed. Conversely, little attention is being given to overcoming the stresses that prevent the available nitrogen-fixing microorganisms from doing what laboratory and greenhouse studies suggest they ought to do. If the reason for extensive research interest is, in fact, promoting nitrogen fixation for food production and not merely enlarging the scientific literature per se, it is incumbent to assess what are the real major research needs.

No attempt is made here to evaluate the priorities for research on higher plants because the author does not have the competence to comment on this field. The enormous amount of interesting research on nitrogen fixation in the rhizosphere of cereals and pasture grasses will also not be considered, because, in contrast with the microbiological systems discussed below, it is not clear that sufficient energy is available under natural conditions to sustain appreciable nitrogen fixation by microorganisms residing on root surfaces or their immediate environs, especially when they are competing with the ecologically adapted, resident microflora. Similarly, attention will not be given to the nodulated nonlegumes

because their role in agricultural systems is largely uncertain, and because there are significant microbiological obstacles to their practical exploitation in the near future.

RHIZOBIUM INOCULUM

Highly active, nitrogen-fixing strains of *Rhizobium* often fail to survive during storage of the inocula or following their application to seeds. Although poor survival of the rhizobia results in little nitrogen being fixed under field conditions, almost no research is being done to develop strains that are resistant to the stresses.

Excellent strains are now available for use as inocula by farmers, and quality control in many of the inoculum-producing facilities is quite good. Nevertheless, the bacteria fail to survive in sufficient number in the carriers. For example, when the inoculum is stored under practical conditions, the moisture level may be too low or too high, both of which deleteriously affect the survival of rhizobia. Moreover, their survival is markedly affected by restricted aeration in the inoculum carrier (Roughley 1968). Similarly, inocula in the temperate zone are often exposed to low or high temperatures, and many of the inocula prepared for use in the tropics are stored or are inadvertently placed in sites that reach excessively high temperatures, jeopardizing survival of the organism. Thus the numbers of rhizobia fall rapidly when the temperature reaches 45°C, and a thousandfold-order decline can occur in a short period at 55°C (Wilson and Trang 1980).

It is common knowledge among American, European, and Australian agronomists and microbiologists who have worked in the tropics that the excellent inocula prepared in the United States, Europe, or Australia are often worthless because of the poor storage conditions in tropical regions. Nevertheless, few programs are under way to isolate strains that are tolerant of such stresses or to create new genotypes having traits that prevent the destruction of those bacteria in inoculum preparations that are extremely effective in symbiotic nitrogen fixation.

Should a highly effective strain survive transport in large numbers, the populations of these bacteria often fail, nevertheless, once they are applied to seeds. Although emphatic recommendations can be made about how to apply the inoculum to the seed and store it thereafter, if these recommendations are heeded only occasionally, the value of the rhizobia is only partially obtained. For example, the farmer may mix inoculum with seed in an enclosed shed prior to going out into the field, and may then store the seed for short or long periods, at moderate or high temperatures, and in the dark or in sunlight. It would be far more appropriate to have bacteria able to endure the actual stresses in farm practice.

The death of root nodule bacteria applied to and dried on seed can be dramatic. Even under ideal conditions and with only a few hours elapsing between the time the seeds are inoculated and they are sown, the decline may be appreciable. In some instances, as much as a thousandfold fall in rhizobial numbers occurs during the first 24 hours, and even a hundredfold decline of Rhizobium trifolii, for example, may take place within 1 hour after application of the bacteria to the seed (Salema et al. 1982). Appreciable declines in numbers of R. japonicum take place within 12 hours of their application to soybean seeds (Iswaran 1971). Exposing the inoculated seed to 3 or more hours of sunlight often leads to poor inoculation results (Alexander and Chamblee 1965). Nevertheless, light-tolerant mutants of other species have been isolated, and light tolerance of bacteria can be modified by growth conditions so that genetic or cultural manipulation can be used to protect bacteria against solar inactivation (see Kunisawa and Stanier 1958).

A number of materials have been devised for application to seeds to reduce loss of viability. For example, pelleting of seeds of subterranean clover or barrel medic has resulted in longer survival of the rhizobia prior to their introduction into dry soil (Goss and Shipton 1965). For reasons unknown, however, pelleting does not always improve the survival of the root nodule bacteria (Herridge and Roughley 1974). In some instances, so many viable cells are lost that, independent of the seed coating material, the only way of ensuring sufficient rhizobia on seeds for adequate nodulation involves the use of extremely large numbers of these bacteria (Davidson and Reuszer 1978), and these cell densities are often not attainable by the procedures used to prepare inoculants in developing countries. Some novel approaches have been attempted in recent years to overcome the problem of poor survival on seeds. One example is the entrapment of the bacteria in a polyacrylamide gel for the inoculation of soybeans (Dommergues et al. 1979).

Rhizobium strains differ in their capacity to survive the stresses on inoculated seeds. For example, strains of R. trifolii differ in their rates of decline on inoculated seed, some strains showing good and others poor survival (Philpotts 1977). Strains of the rhizobia fixing nitrogen in cowpeas vary enormously in their susceptibility to drying, the cell densities of some falling to levels less than 1 percent of the original population, others enduring drying for 11 days with 50 percent of the original inoculum retaining viability (Osa-Afiana and Alexander 1982b). Such information has not been effectively used in research designed to improve the nitrogen-fixing capacity of rhizobia in the field. Differences among Rhizobium strains could be exploited in future research designed to overcome or minimize the deleterious effects that prevent already excellent nitrogen-fixing rhizobia from performing as predicted in greenhouse or laboratory tests.

Seeds of several economically important legumes contain inhibitors that suppress the bacteria nodulating the developing root system. Such inhibitors have been found in seeds of subterranean clover, white clover (Thompson 1960, Hale and Mathers 1977), soybeans, and mung beans (Jain and Rewari 1976). It appears that part of the toxicity can be removed by washing or soaking the seeds prior to sowing, but, given the difficulties in convincing farmers merely to inoculate, it is not likely that they can be convinced to wash seeds free of, from their viewpoint, an unknown inhibitor of a possibly unimportant germ. It is not difficult to obtain mutants of Rhizobium resistant to inhibitors—a procedure used to obtain strains immune to antibacterial chemicals applied for seed protection (Odeyemi and Alexander 1977a)--and antibiotic-resistant mutants have also been obtained for ecological and genetic research. Thus a feasible means of overcoming the effect of seed toxins is the development of toxin-resistant rhizobia, an approach not yet attempted.

ESTABLISHMENT OF RHIZOBIUM

The ideal strain for inoculation should have, in addition to the capacity for nitrogen fixation, the ability to persist and to grow in soil or around plant roots. These abilities to survive and grow are especially important for rhizobia used with legumes that must be replanted each year. Species of Rhizobium vary in their ability to colonize plant roots; for example, R. lupini grows faster and reaches larger populations than R. trifolii (Chatel and Parker 1973a). Furthermore, strains of a single species differ in their ability to colonize the roots of higher plants (Chatel and Greenwood 1973a). Nevertheless, little attention has been given to developing strains that are better colonists or are better able to become established in the rhizosphere or in the soil than the commonly used rhizobia. For that reason, many of the highly effective strains of root nodule bacteria are often of little or no value under natural conditions.

Indeed, the available evidence, which is admittedly scant, indicates that the highly effective strains currently important in agriculture fail to grow under most natural soil conditions. Brief periods of proliferation may follow the moistening of dry soil or a freezing-drying cycle, but the extent of such growth is slight and its duration is short. Moreover, the natural organic substrates in soil and those that enter in the form of root remains or residues of above-ground portions of plants fail to support appreciable replication. Under natural conditions, the only apparent means for these organisms to increase significantly is associated with excretions from plant roots (J. Pena-Cabriales and M. Alexander, Cornell University, unpublished data). Thus existing strains of these bacteria are not well adapted for growth in the underground habitat,

and practical benefit might be derived from research designed to increase their potential for such replication. Further, although highly active nitrogen-fixing rhizobia fail to replicate under most soil conditions, no attention is being given to developing or identifying strains that are able to grow in soil in the absence of plant roots.

Many Rhizobium strains active in nitrogen fixation are unable to become established when in "competition" with indigenous strains of Rhizobium having low effectiveness. Studies of this "competition" pointedly illustrate the current overemphasis on research on the nitrogen-fixing capacity to the almost complete exclusion of research on the ecologically significant traits of these bacteria. The numerous studies conducted of competition among Rhizobium strains for the nodulation of leguminous plants generally agree that this is a major practical problem and frequently results in the highly active nitrogen fixer being almost worthless in practice. In some instances, as with R. japonicum, some of the inoculum strains fail to form an appreciable percentage of nodules, regardless of the rate or technique of inoculation, because of the poor "competitiveness" of the organism (Boonkerd et al. 1978). In other studies of R. japonicum, about 5 percent of the nodules was formed by bacteria introduced with the inoculum (Johnson et al. 1965). Similarly, field studies with R. leguminosarum showed that the frequency of nodulation by test strains ranged from about 5 to 13 percent (Strivastava et al. 1980). Poor competitiveness can sometimes be overcome by using very large numbers of the rhizobia in the inoculant, thereby increasing the number of nodules derived from the inoculum organism (Amarger 1974). However, it is often not possible to produce inoculants that have enough bacteria to overwhelm the more competitive rhizobia in the soil.

It is generally agreed that the selected rhizobia should be not only infective and effective in nitrogen fixation but also able to compete successfully with indigenous rhizobia. However, the physiological traits associated with "competitiveness" remain to be worked out. The desired strains are obtained by screening them under greenhouse conditions, yet with these presumed "competitive" strains, field conditions often radically alter a bacterium's potential competitiveness. Thus some strains are good competitors with other strains of Rhizobium at low temperatures but fare poorly at high temperatures (Roughley et al. 1980). Furthermore, a Rhizobium strain that can successfully compete with other strains in the presence of one variety of legume may be wholly unsuccessful or fare poorly in the root zone of other varieties of the same plant species. Finally, the particular soil into which the plant is introduced can also modify the presumed competitive abilities (Roughley et al. 1976).

Successful establishment and extensive nodulation by introduced Rhizobium are also greatly influenced by their very poor movement through soil. Very few cells of R. trifolii, for example, are able to

move even 3 centimeters from their point of introduction (Hamdi 1971), and insignificant movement occurs even in the presence of the developing root system, the boring activities of earthworms, and high rates of water infiltration. It is not clear how research can result in greater movement and mobility of these bacteria through the soil matrix, since movement is affected by particle size and the amount of water applied (Hamdi 1974). Size of soil particles, however, is not a variable that can be controlled in nature, and although the amount of water can be increased, the economics of irrigation preclude such practices if the sole purpose is to increase the vertical movement of beneficial bacteria.

SURVIVAL OF *RHIZOBIUM* IN SOIL

Highly effective rhizobia, following their introduction into soil with seeds, often do not survive in significant numbers. Nevertheless, little attention is being given to this problem or to the use of strains of rhizobia that are able to persist in sufficient numbers to ensure nodulation of succeeding generations of legumes.

The problem of survival is clearly illustrated by Australian studies of the nodulation of subterranean clover by *R. trifolii*. In a typical study, while all of the nodules in the first year were formed by the inoculum strain, in the second year nearly all were formed by strains not in the original inoculum (Roughley et al. 1976). In other studies of the same bacterium-legume association, 80 percent of the nodules in the first year were derived from the introduced bacteria. However, none of the nodules appearing at the end of the succeeding season were derived from the added bacterium, and all of the plants bore nodules generated by strains that were not as effective in the symbiosis as the originally introduced microorganism (Bergersen 1970).

Poor survival is also evident in laboratory studies of certain soils, even those at pH values near neutrality. In one investigation, for example, the four test strains of *R. japonicum* fared poorly, and three of the four declined to fewer than 10,000 per gram in 6 weeks or less (Vidor and Miller 1980). Nevertheless, some rhizobia maintain their viability for appreciable periods of time in other soils that are moist and are not under temperature or pH stress (Danso and Alexander 1974).

In the 1950s and early 1960s, considerable attention was directed to the poor survival of rhizobia in Western Australia which resulted from a problem known as "second-year clover mortality." In the problem area, clover sown in certain soils became established and nodulated in the first year, but subsequent nodulation failed to occur and the plant died in the second year. Plant mortality was attributed to the inability of the root nodule bacterium to survive from the first season to the second, an apparent consequence of the high soil

temperatures and extreme desiccation prevailing in these sandy soils (Marshall et al. 1963).

The problem of poor survival is not intractable because strains vary considerably in their susceptibility under natural conditions. Without considering for the moment the identity of the particular stresses that result in the loss of viability, it is evident that some strains of R. trifolii persist poorly in the soils of Australia, whereas other strains endure in large numbers (Gibson et al. 1976). Similarly, the steady-state populations of strains of the same species may be quite different at varying periods after the sowing of inoculated seeds (Chatel and Greenwood 1973b).

As a rule, the ability to endure in large numbers is thought to be a beneficial attribute of Rhizobium used for seed inoculation. Persistence is not, however, a desirable trait for indigenous rhizobia which frequently--with their endurance of various stresses--make it difficult to introduce new effective strains to replace partially effective indigenous organisms.

BIOLOGICAL STRESSES ON RHIZOBIUM

Although Rhizobium strains active in nitrogen fixation appear to compete poorly with indigenous microorganisms and are particularly susceptible to predation, especially during the period the root system is actively developing and nodulation should occur, research designed to overcome these problems is almost nonexistent.

Evidence of the influence of indigenous microorganisms on root nodule bacteria comes from several sources. In an early report on the failure of inoculation of subterranean clover in certain soils of Australia, the indigenous microbial community was blamed for preventing colonization of the rhizosphere by the added nitrogen-fixing inoculum strain (Hely et al. 1957). Similarly, in areas of New Zealand it was observed that establishment of white clover was poor, even when lime, fertilizers, or micronutrients were added to the soil. Establishment was successful, however, if the indigenous microbial community was reduced by the application of antimicrobial agents. These agents presumably reduced the activity of components of the indigenous community, thereby permitting the Rhizobium to colonize the rhizosphere and bring about nodulation (Beggs 1961, 1964). In tests of subterranean clover (Harris 1953) and white clover (Anderson 1957) under more defined conditions, it was observed that nodulation by effective R. trifolii was reduced, delayed, or prevented by fungi or other bacteria. Because the microorganisms responsible for these deleterious changes did not usually produce antibiotics acting against the rhizobia in culture, the harmful effects were attributed to competition.

In recent studies, it was observed that rhizobia fail to grow in soil even if a carbon source they can use is added in reasonable amounts. If enormous concentrations of the carbon source were added, however, proliferation occurred. Evidently, the indigenous populations are better able than the rhizobia to make use of low concentrations of the organic compound, but at high concentrations sufficient quantities remain for the slow-growing and poorly competitive root nodule bacteria. Moreover, if antibiotics toxic to many native soil bacteria but not to rhizobia are added to the soil, thereby suppressing the potential competitors, the rhizobia are able to proliferate (J. Pena-Cabriales and M. Alexander, Cornell University, unpublished data).

Competition for limited supplies of nutrients is probably an extremely important interaction among natural microbial communities. Soils are almost invariably deficient in readily available carbon compounds. Because members of the genus Rhizobium and members of other genera of nitrogen-fixing bacteria grow slowly, they are not presumably effective competitors in soil. Probably more than is presently realized, competition restricts the activity of nitrogen-fixing organisms, both free-living and those that, like Rhizobium, must compete in the rhizosphere prior to invading the host plant.

Means of overcoming or minimizing the impact of competition have not attracted widespread attention. One possible approach is to apply inhibitors to the seed (or possibly to the foliage for chemicals that are translocated downward) in the hope that these chemicals suppress the competing organisms but not the nitrogen-fixing species. It is not difficult to obtain such chemicals for seed treatment since many of the commercially important fungicides, including those designed for treatment of legume seeds, inhibit many bacteria, and the rhizobia can be made resistant to these fungicides. Such an approach has been used with species of Rhizobium and with several leguminous species. The chemicals presumably act, at least in part, by inhibiting the indigenous bacteria, thus allowing the introduced root nodule bacterium to proliferate with minimal competition (Odeyemi and Alexander 1976).

Biologically formed toxins can influence the establishment of rhizobia on roots. For example, extracts of soils supporting clovers that nodulated poorly were toxic to R. trifolii growing in laboratory media, and it has been postulated that such inhibitors restrict the development of rhizobia in nature (Chatel and Parker 1972). If, in fact, soil toxins affect the colonizing ability of these microorganisms, it should not be difficult to add the capacity to resist soil toxins to the traits of effective rhizobia.

Predation seems to be a significant factor in reducing the populations of rhizobia and other bacteria that at times are abundantly present in soil. When artificially high numbers of

Rhizobium are added to soil, their population size is drastically diminished. This decline is paralleled by the proliferation of indigenous protozoa, which apparently feed on the large numbers of rhizobia and reduce their density appreciably without eliminating them (Danso et al. 1975). The high rhizobial densities needed to trigger protozoan predation are not common in natural soils. However, as inoculated seeds imbibe water and begin to develop roots, the Rhizobium begins to grow. As the potential prey for protozoa becomes more numerous, the predators begin to feed, thereby restricting the size of the Rhizobium population.

Studies of beans and R. phaseoli suggest that the protozoa are a major factor in the restricted growth of root nodule bacteria. One study found that the suppression of protozoa with thiram resulted in enhanced colonization of beans by R. phaseoli (Ramirez and Alexander 1980). In subsequent studies, it was noted that the abrupt decline in population of R. phaseoli around germinating seeds and their developing root systems was delayed if the seed was treated with chemicals that inhibited the protozoa. Inhibition of protozoa by the fungicide, thiram, was accompanied by a high initial rate of nodulation by the test Rhizobium, a yield increase, and a greater amount of nitrogen fixed by beans (Lennox and Alexander 1981).

Research designed to develop strains that are more active in nitrogen fixation is thus of little utility until a means of overcoming protozoan attack is developed. One such means is the use of chemicals that inhibit protozoa but have no effect on either the host plant or the rhizobia, and research in this direction has already been initiated (Ramirez and Alexander 1980, Lennox and Alexander 1981). It is possible that Rhizobium strains less attractive to predators might be developed, but this approach is not likely because of the diversity of protozoa and the wide range of bacteria they can consume. Nevertheless, bacterial capsules or other surface structures may reduce the rate of predation to such an extent that the rhizobia are able to colonize the rhizosphere more extensively.

ABIOTIC STRESSES ON RHIZOBIUM

Soil Acidity

Soil acidity is a major reason for the absence of nodulation and the failure of Rhizobium to colonize legume roots or to survive in soil. Yet few of the presently available, highly effective strains of Rhizobium are able to tolerate low pH soils, and means of overcoming the problem are not being sought.

Much of the world's arable land--including a high percentage of that in tropical areas--is acidic, and this, agronomists agree, is a major constraint to legume cultivation in these regions. Moreover,

ample evidence exists that the survival, growth, and effectiveness of many of the most active strains of Rhizobium are influenced deleteriously at low pH.

Although many legumes are acid-sensitive, even when growing on fixed nitrogen, many grow well under acid conditions; for example, serradella grows at pH 4.0, and white clover develops readily at pH 5.0 (Mulder et al. 1966). Even for a single legume species such as alfalfa (Jo et al. 1980), tolerances to soil acidity vary among varieties, and plant breeders are endeavoring to develop varieties able to cope with this significant stress. It has been observed for alfalfa (Jo et al. 1980) and peas (Mulder et al. 1966) that nodulation is more markedly affected at low pH than root development or plant growth. Apparently, some phase of the infection process induced by the bacteria is inhibited at low pH, although root development and bacterial growth do not show this high sensitivity (Evans et al. 1980). In other instances, as with Medicago truncatula, the absence of nodulation in acid soils may result from the inability of R. meliloti to survive or grow (Robinson and Loneragan 1970). Nodulation failures owing to poor survival of rhizobia in acid soils are particularly likely when inoculation is not practiced every year, a common occurrence even in developed countries. Indeed, frequent inoculation of legumes growing in acid soils was recommended nearly 60 years ago (Bryan 1923).

If nodules are not formed at low pH values, the plant may develop using fixed nitrogen, but the farmer does not get the special benefit he expects from legumes. Serradella and alfalfa, for example, continue to grow at the expense of fixed nitrogen even when the low pH prevents nitrogen fixation (Mulder et al. 1966).

Some species of Rhizobium do not multiply in culture even at moderate acidities; R. meliloti, for example, often does not grow below pH 5.3. Other species have greater tolerance, however. Although the pH range for survival is expected to be wider than that for replication, many of the active nitrogen fixers fail to survive in sterile soil at pH 5.2 (where suppression is not a result of some harmful microorganism) (Lowendorf et al. 1981). Nevertheless, strains of a single species of Rhizobium vary in their pH sensitivity, whether sensitivity is assessed by growth in culture, survival in sterile soil, or nodulation of host plants (Lowendorf et al. 1981, Munns et al. 1979). Such strains may be useful as legume inoculants because they nodulate at pH values at which other strains do not (Mulder et al. 1966, Munns et al. 1979) or because they can survive longer in acid soils (Lowendorf et al. 1981).

Acid soils also frequently contain levels of aluminum, manganese, or iron that may be injurious to nodulation or growth of rhizobia. From the viewpoint of developing resistance in the nitrogen-fixing symbiosis, only aluminum has received attention. Rhizobium strains that vary in their ability to tolerate aluminum in culture can be

selected (Keyser and Munns 1979), and differences in sensitivity are also evident in the behavior of such strains in soil (P. G. Hartel, A. M. Whelan, and M. Alexander, Cornell University, unpublished data). Nevertheless, the aluminum stress in nature appears to affect the host plants and not the rhizobia (Munns et al. 1981; P. G. Hartel, A. M. Whelan, and M. Alexander, Cornell University, unpublished data), and thus efforts to overcome this stress should be directed to the plant.

Drought

The drying of soil has a devastating effect on many rhizobia, and the populations of highly effective strains are reduced catastrophically. Nevertheless, the biology of drought tolerance in these rhizobia and its exploitation are rarely investigated.

Nearly all areas of the world are subject to rain-free periods of sufficient duration to result in extensive drying of soil, and the rhizobia that must survive in these soils to bring about nitrogen fixation in the succeeding crop may thereby become too few in number to cause extensive nodulation. With many strains of all species of Rhizobium, a single exposure of the soil to drying reduces the viable population by 99 percent or more, and several cycles of soil wetting followed by drying, a common occurrence in nature, reduce the population still further (Pena-Cabriales and Alexander 1979). With some strains of the bacteria that infect Lotus corniculatus, the decline in soil kept dry for several months--again, a common occurrence in many tropical regions--may be 10,000-fold (Foulds 1971).

The conditions under which water is lost from the soil affects the extent of the decline (Bushby and Marshall 1977). Without question, prolonged drought or even a short period of drying is a major stress on Rhizobium, yet little information exists on the soil properties affecting the reduction in population size and on microbial and other factors that are related to differences among strains and soils (Bushby and Marshall 1977), although the type and amount of clay are of great importance (Osa-Afiana and Alexander 1982a). From observations that nearly half of the viable cells of some strains of cowpea rhizobia survive one drying cycle whereas more than 99 percent of the cells of other strains die under identical circumstances (Osa-Afiana and Alexander 1982b), it appears that means can be devised to obtain cultures not seriously affected by the drying of soil.

High Temperatures

High temperatures harm the survival and colonizing ability of effective rhizobia and nodulation and nitrogen fixation by legumes.

Nevertheless, a significant research program to obtain temperature-resistant rhizobia or Rhizobium-legume associations has not been initiated.

High soil temperatures may occur at seeding time, during plant growth, and following harvest. The period following harvest should not be ignored as survival of the bacteria at that time is important. In tropical and subtropical regions as well as in the southeastern United States, the temperature near the soil surface is often above 40°C and sometimes may reach 60°C. Rhizobia are subject to these temperatures following harvest and before sowing, after planting into hot, dry soil (as sometimes occurs in Australia), or both. Some strains such as R. trifolii and strains nodulating Lotus pedunculatus (Brockwell and Philips 1970, Chatel and Parker 1973b) survive very poorly under these conditions. The death of R. trifolii in the fields of Western Australia is responsible for the poor growth of subterranean clover in the second year after planting; the rhizobia fail to survive in appreciable numbers in the hot, dry soil at the end of the first season (Chatel and Parker 1973b). This problem is not as great in soils of heavy texture and is prevented to some degree by certain clays (Marshall 1964). The moist soils are more sensitive to heat than dry soils (Wilkens 1967), and marked declines are evident even at 36°C (Danso and Alexander 1974).

Rhizobia must grow to colonize the rhizosphere and cause nodulation, and during the growth period their sensitivity to temperature is important. The optimum temperatures for growth in culture vary among strains and species, and values of 27-39°C have been noted. The maximum temperatures are generally 35-39°C, but proliferation may take place up to 42°C (Allison and Minor 1940, Bowen and Kennedy 1959, Munevar and Wollum 1981b). Differences in growth and colonizing abilities probably explain why some strains are more active in nodulating soybeans at low temperatures and others are more active at high temperatures (Weber and Miller 1972). Studies of Vicia atropurpurea and Medicago tribuloides confirm that certain rhizobia form more nodules at low temperatures, while others produce more at high temperatures (Pate 1961). Thus super nitrogen fixers could be easily displaced in nature by less effective strains simply because of their temperature responses. Furthermore, some strains of R. leguminosarum fail to induce nodulation at 30°C even though they and their host, peas, grow at that temperature (Frings 1976). In addition, temperature affects the relative activity of the rhizobia; a bacterium that is highly effective at one temperature is less active at a different temperature (Munevar and Wollum 1981a). For these reasons, greater nitrogen gains probably can be achieved by improvements in the heat resistance of the symbiosis.

Rhizobium strains or species vary greatly in their susceptibility to high temperatures. R. meliloti survives better than R. trifolii on seed lying for long periods in hot, dry soil (Brockwell and Philips

1970); R. lupini and R. japonicum are less susceptible than R. trifolii when in dry soil at elevated temperatures (Chatel and Parker 1973b, Marshall 1964). Rhizobium strains that nodulate and fix nitrogen similarly on Cicer arietinum at low temperatures exhibit differences in nodulation and nitrogen-fixing activity at 30°C (Dart et al. 1976). Thus it should be possible to obtain or devise rhizobia able to survive, grow, or fix nitrogen in high temperature regions.

Salinity and Alkalinity

Nodulation and survival of effective rhizobia are affected deleteriously in saline and alkaline soils. Considerable attention has been given to the effects of salts on rhizobia growth in culture but not to finding means of overcoming poor bacterial survival or the sensitivity of nodulation.

Many soils rich in salt and with high pH values are often considered undesirable for legumes. For example, berseem clover, guar, cowpeas, and lentils nodulate poorly in the highly saline-alkali soils of India, and peas may be wholly devoid of nodules (Bhardwaj 1974). Studies performed to establish the effect of salts on the growth of rhizobia in culture have found that high salt levels are usually required to inhibit growth of the bacteria (Ethiraj et al. 1972, Mendez-Castro and Alexander 1976, Steinborn and Roughley 1975). Comparisons of the sensitivities of microorganisms and plants, moreover, show that bacteria can proliferate at salt levels that prohibit growth of the host (Bhardwaj 1975). However, one cannot assume that, because replication of the bacteria is not seriously affected, there are no problems related to the symbiosis. First, rhizobia do not survive in some of these soils (Bhardwaj 1975, Pant and Iswaran 1970), and it is not certain whether it is possible to exploit the differences among Rhizobium strains in salt tolerance either for growth or the ability of some rhizobia to "acclimate" or mutate to even higher levels of salt tolerance, as has been shown several times (Ethiraj et al. 1972, Mendez-Castro and Alexander 1976, Steinborn and Roughley 1975). Second, nodulation is more sensitive to these stresses than root development, at least for soybeans (Bernstein and Ogata 1966), and alkali- and salt-induced delays in nodulation of berseem clover and lentils may result in reduced yields (Bhardwaj 1975).

Fungicides

Many fungicides reduce and some abolish nodulation by strains of Rhizobium used in inoculants, prompting the development of several ways to minimize the problem.

The seeds of many legumes are attacked by a variety of plant pathogens, especially fungi, sometimes greatly reducing yields. In countries in which farmers can afford to purchase fungicides, the chemicals have been widely used, even after it was realized that they are harmful to Rhizobium. Seed-applied fungicides are not specific for plant pathogens or even fungi, and many kill rhizobia and render the added inoculant useless. Thus, for example, the number of R. phaseoli added to bean seeds declines when the fungicides thiram, captan, or PCNB are applied to seeds (Graham et al. 1980). Furthermore, some of the best seed-protecting chemicals also reduce nodulation by R. phaseoli (Graham et al. 1980), R. japonicum, and R. leguminosarum (Stovold and Evans 1980). Such decreases in nodulation of the peanut lead to reduced nitrogen fixation and pod yield (Chendrayan and Prasad 1976).

Several methods have been proposed or are used to minimize the impact of these fungicides. Because the chemicals vary greatly in their effect on rhizobia (Curley and Burton 1975, Diatloff 1970b), a simple approach is to use those seed protectants that have the least deleterious effects. Unfortunately, these are not always the most desirable chemicals for control of the pathogens. A second approach involves covering the fungicide-treated seeds with a coating material; poly (vinyl acetate) has been used for this purpose (Diatloff 1970a). A third approach obtains fungicide-resistant mutants derived from effective rhizobia and applies them to the seeds together with the chemicals (Odeyemi and Alexander 1977b). Each procedure is more useful in permitting nodulation or increasing yields than the conventional practices used by farmers. Nevertheless, because of wide-ranging farming practices and the continual introduction of new fungicides and different rhizobia, fungicide toxicity remains an issue.

LIMITATIONS ON NITROGEN FIXATION BY BLUE-GREEN ALGAE (CYANOBACTERIA)

The ability of farmers in Asia to grow lowland rice for millenia with no input of fixed nitrogen has been attributed to the activity of blue-green algae (cyanobacteria). Although insufficient nitrogen is fixed for high yields of rice, little attention has been given to defining why so little nitrogen is fixed in paddy fields under natural conditions.

The blue-green algae naturally present in paddy fields or added by inoculation rarely fix nitrogen to the extent that is assumed to be their potential. Studies of the ecology of these algae have revealed that several factors limit their nitrogen-fixing activity. Insufficient phosphorus is often found in the water phase above the soil, and insufficient iron may limit algal development in waters that are at high pH or become alkaline during photosynthesis. In the

absence of adequate levels of these elements, excellent nitrogen fixers can do very little, while additions of these nutrients stimulate growth and nitrogen fixation. Most blue-green algae grow poorly below pH 6.0, where they become inactive, are displaced by green algae, or both in waters of even slight acidity. Thus, nitrogen fixation and development of blue-green algae are correlated with soil pH (Roger and Reynaud 1979, Wilson and Alexander 1979). Competition with algae that fix little or no nitrogen may also reduce the nitrogen gain potentially brought about by the photosynthetic prokaryotes (Wilson et al. 1979). These organisms are also quite susceptible to grazing by invertebrates that sometimes flourish in the water, and ostracods (Grant and Alexander 1981, Wilson et al. 1980) and daphnids (Roger and Reynaud 1979) may proliferate at their expense.

Each one of these limitations can probably be overcome. The phosphorus (Roger and Reynaud 1979) and iron (Ryther and Kramer 1961) required for optimal algal growth vary appreciably so that inoculant strains with a low nutrient demand could be developed. (Recommending the addition of phosphorus to the water is not an attractive option for poor farmers.) The acid limitation may be overcome by using or developing inoculants of acid-tolerant, blue-green algae. The occurrence of such algae in some moderately acidic soils suggests that this is not a far-fetched option. Competition from other algae may be reduced by using herbicides, many of which are algicidal, together with an inoculum consisting of a herbicide-resistant cyanobacterium. This has been done in laboratory studies of paddy soils treated with simetryne and a variant of Aulosira spp. that was resistant to this herbicide (Wilson et al. 1979). Because the invertebrate grazers and not the nitrogen fixers are suppressed by insecticides (Osa-Afiana and Alexander 1981), they represent a feasible means of increasing algal activity. However, the cost of herbicides and insecticides may be too high for most rice farmers in developing countries, and a more attractive approach might involve finding algae that are better competitors or are less susceptible to predation. The literature of limnology and oceanography indicates that predator-resistant algae are reasonably common in aquatic environments.

Some of the ecological factors that govern nitrogen fixation by blue-green algae also limit the growth and activity of Azolla. Phosphorus is a major limiting nutrient, and its addition to paddy water enhances Azolla development and nitrogen fixation (Singh 1977, Talley et al. 1981, Watanabe et al. 1981). Moreover, some Azolla populations may be more efficient than others in using phosphorus (Talley et al. 1981). Iron also sometimes restricts the growth and activity of the fern symbiosis (Talley et al. 1981), and Azolla, like algae, fails to develop if the pH of paddy soils is low (Singh 1977). In addition, the fern, like the free-living algae, is subject to attack by pests, but this can be controlled by insecticides (Singh 1979). Temperature also greatly affects growth; Azolla

mexicana flourishes at warmer temperatures, while A. filiculoides grows well at cooler temperatures (Rains and Talley 1979).

CONCLUSION

From the standpoint of the microbial contribution to symbiotic nitrogen fixation and to nitrogen fixation by blue-green algae in paddy fields, the factors limiting nitrogen fixation are rarely the lack of highly effective strains of Rhizobium or of blue-green algae potentially active in fixation. The limiting factors are nearly always the absence of strains adapted to ecologically significant stresses in soil or in paddy fields. Basic and applied research that is designed to increase food production, whether in the long or short run, should seek to define these stresses and find means to overcome them.

REFERENCES

Alexander, C. W., and D. S. Chamblee (1965) Effect of sunlight and drying on the inoculation of legumes with Rhizobium species. Agronomy Journal 57:550-553.

Allison, F. E., and F. W. Minor (1940) The effect of temperature on the growth rates of rhizobia. Journal of Bacteriology 39:365-371.

Amarger, N. (1974) Compétition pour la formation des nodosités sur la féverole entre souche de Rhizobium leguminosarum apportées par inoculation et souche indigènes. Comptes Rendus Academic Sciences, Ser. D. 279:527-530.

Anderson, J. K. (1957) The effect of soil microorganisms on the plant-rhizobia association. Phyton 8:59-73.

Beggs, J. P. (1961) Soil sterilant aid to clover establishment indicates anti-nodulation factor in soils. New Zealand Journal of Agricultural Research 103:325.

Beggs, J. P. (1964) Growth inhibitor in the soil. New Zealand Journal of Agricultural Research 108:529-535.

Bergersen, F. J. (1970) Some Australian studies relating to the long-term effects of the inoculation of legume seeds. Plant Soil 32:727-736.

Bernstein, L., and G. Ogata (1966) Effects of salinity on nodulation, nitrogen fixation, and growth of soybeans and alfalfa. Agronomy Journal 58:201-203.

Bhardwaj, K. K. R. (1974) Growth and symbiotic effectiveness of indigenous Rhizobium species of a saline-alkaline soil. Proceedings, Indian National Science Academy 40:540-543.

Bhardwaj, K. K. R. (1975) Survival and symbiotic characteristics of Rhizobium in saline-alkali soils. Plant Soil 43:377-385.

Boonkerd, N., D. F. Weber, and D. F. Bezdicek (1978) Influence of Rhizobium japonicum strains and inoculation methods on soybeans grown in rhizobia-populated soil. Agronomy Journal 70:547-549.

Bowen, G. D., and M. M. Kennedy (1959) Effect of high soil temperatures on Rhizobium spp. Queensland Journal of Agricultural Science 16:177-197.

Brockwell, J., and L. J. Philips (1970) Studies on seed pelleting as an aid to legume seed inoculation. 3. Survival of rhizobium applied to seed in hot, dry soil. Australian Journal of Experimental Agriculture and Animal Husbandry 10:739-744.

Bryan, O. C. (1923) Effect of acid soils on nodule-forming bacteria. Soil Science 15:37-40.

Bushby, H. V. A., and K. C. Marshall (1977) Some factors affecting the survival of root-nodule bacteria on desiccation. Soil Biology and Biochemistry 9:143-147.

Chatel, D. L., and R. M. Greenwood (1973a) Differences between strains of Rhizobium trifolii in ability to colonize soil and plant roots in the absence of their specific host plants. Soil Biology and Biochemistry 5:809-813.

Chatel, D. L., and R. M. Greenwood (1973b) The colonization of host-root and soil by rhizobia. II. Strain differences in the species Rhizobium trifolii. Soil Biology and Biochemistry 5:433-440.

Chatel, D. L., and C. A. Parker (1972) Inhibition of rhizobia by toxic soil-water extracts. Soil Biology and Biochemistry 4:289-294.

Chatel, D. L., and C. A. Parker (1973a) The colonization of host-root and soil by rhizobia. I. Species and strain differences in the field. Soil Biology and Biochemistry 5:425-432.

Chatel, D. L., and C. A. Parker (1973b) Survival of field-grown rhizobia over the dry summer period in Western Australia. Soil Biology and Biochemistry 5:415-423.

Chendrayan, K., and N. N. Prasad (1976) Effect of seed treatment with Tillex and Ceresan on Rhizobium groundnut symbiosis. Madras Agricultural Journal 63:520-522.

Curley, R. L., and J. C. Burton (1975) Compatibility of Rhizobium japonicum with chemical seed protectants. Agronomy Journal 67:807-808.

Danso, S. K. A., and M. Alexander (1974) Survival of the two strains of Rhizobium in soil. Soil Science Society of America, Proceedings 38:86-89.

Danso, S. K. A., S. O. Keya, and M. Alexander (1975) Protozoa and the decline of Rhizobium populations added to soil. Canadian Journal of Microbiology 21:884-895.

Dart, P. J., J. Day, R. Islam, and J. Dobereiner (1976) Symbiosis in tropical grain legumes: some effects of temperature and the

composition of the rooting medium. Pp. 361-384 in: P. S. Nutman, ed. Symbiotic Nitrogen Fixation in Plants. Cambridge University Press, London, England.

Davidson, F., and H. W. Reuszer (1978) Persistence of Rhizobium japonicum on the soybean seed coat under controlled temperature and humidity. Applied and Environmental Microbiology 35:94-96.

Diatloff, A. (1970a) Overcoming fungicide toxicity to rhizobia by insulating with a poly (vinyl acetate) layer. Journal of the Australian Institute of Agricultural Science 36:293-294.

Diatloff, A. (1970b) The effects of some pesticides on root nodule bacteria and subsequent nodulation. Australian Journal of Experimental Agriculture and Animal Husbandry 10:562-567.

Dommergues, Y. R., H. G. Diem, and C. Divies (1979) Polyacrylamide-entrapped Rhizobium as an inoculant for legumes. Applied and Environmental Microbiology 37:779-781.

Ethiraj, S., H. R. Sharma, and S. R. Vyas (1972) Salt tolerance of rhizobia. Indian Journal of Microbiology 12:87-92.

Evans, L. S., F. K. Lewin, and F. A. Vella (1980) Effect of nutrient medium pH on symbiotic nitrogen fixation by Rhizobium leguminosarum and Pisum sativum. Plant Soil 56:71-80.

Foulds, W. (1971) Effect of drought on three species of Rhizobium. Plant Soil 35:665-667.

Frings, J. F. J. (1976) The rhizobium-pea symbiosis as affected by high temperatures. Mededelingenblad Landbouwhogeschool Wageningen 76/77.

Gibson, A. H., R. A. Date, J. A. Ireland, and J. Brockwell (1976) A comparison of competitiveness and persistence amongst five strains of Rhizobium trifolii. Soil Biology and Biochemistry 8:395-401.

Goss, O. M., and W. A. Shipton (1965) Nodulation of legumes on new light land. I. Survival of rhizobia on inoculated pelleted seed held for varying periods before sowing in dry soil. Journal of Agriculture of West Australia 6:616-621.

Graham, P. H. J., G. Ocampo, L. D. Ruiz, and A. Duque (1980) Survival of Rhizobium phaseoli in contact with seed protectants. Agronomy Journal 72:625-627.

Grant, I. F., and M. Alexander (1981) Grazing of blue-green algae (cyanobacteria) in flooded soils by Cypris sp. (Ostracoda). Soil Science Society of America Journal 45:773-776.

Hale, C. N., and D. J. Mathers (1977) Toxicity of white clover seed diffusate and its effect on the survival of Rhizobium trifolii. New Zealand Journal of Agricultural Research 20:69-73.

Hamdi, Y. A. (1971) Soil-water tension and the movement of rhizobia. Soil Biology and Biochemistry 3:121-126.

Hamdi, Y. A. (1974) Vertical movement of rhizobia in soil. Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene, Abteilung II. 129:373-377.

Harris, J. R. (1953) Influence of rhizosphere micro-organisms on the virulence of Rhizobium trifolii. Nature 172:507-508.

Hely, F. W., F. J. Bergersen, and J. Brockwell (1957) Microbial antagonism in the rhizosphere as a factor in the failure of inoculation of subterranean clover. Australian Journal of Agricultural Research 8:24-44.

Herridge, D. F., and R. J. Roughley (1974) Survival of some slow-growing Rhizobium on inoculated legume seed. Plant Soil 40:441-444.

Iswaran, V. (1971) Survival of Rhizobium japonicum on soybean seeds. Israel Journal of Agricultural Research 21:79-80.

Jain, M. K., and R. B. Rewari (1976) Studies on seed coat toxicity to rhizobia of urid (Phaseolus mungo), mung (Phaseolus aureus), and soybean (Glycine max). Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene, Abteilung II. 131:163-169.

Jo, J., S. Yoshida, and R. Kayama (1980) Acidity tolerance and symbiotic nitrogen fixation capacity of some varieties of alfalfa. Nippon Socchi Gakkaishi 26:174-178. (In Japanese.)

Johnson, H. W., U. M. Means, and C. R. Weber (1965) Competition for nodule sites between strains of Rhizobium japonicum applied as inoculum and strains in the soil. Agronomy Journal 57:179-185.

Keyser, H. H., and D. N. Munns (1979) Tolerance of rhizobia to acidity, aluminum, and phosphate. Soil Science Society of America Journal 43:519-523.

Kunisawa, R., and R. Y. Stanier (1958) Studies on the role of carotenoid pigments in a chemoheterotrophic bacterium. Corynebacterium poinsettiae. Archiv für Mikrobiologie 31:146-156.

Lennox, L. B., and M. Alexander (1981) Fungicide enhancement of nitrogen fixation and colonization of Phaseolus vulgaris by Rhizobium phaseoli. Applied and Environmental Microbiology 41:404-411.

Lowendorf, H. S., A. M. Baya, and M. Alexander (1981) Survival of Rhizobium in acid soils. Applied and Environmental Microbiology 42:951-957.

Marshall, K. C. (1964) Survival of root nodule bacteria in dry soils exposed to high temperatures. Australian Journal of Agricultural Research 15:273-281.

Marshall, K. C., M. J. Mulcahy, and M. S. Chowdhury (1963) Second-year clover mortality in Western Australia—a microbiological problem. Journal of the Australian Institute of Agricultural Science 29:160-164.

Mendez-Castro, F. A., and M. Alexander (1976) Acclimation of Rhizobium to salts, increasing temperature and acidity. Revista Latinoamericana de Microbiologia 18:155-158.

Mulder, E. G., T. A. Lie, K. Dilz, and A. Houwers (1966) Effect of pH on symbiotic nitrogen fixation of some leguminous plants.

Symposium of the Ninth International Congress of Microbiology, Moscow, USSR, pp. 133-151.

Munevar, F., and A. G. Wollum (1981a) Effect of high root temperature and Rhizobium strain on nodulation, nitrogen fixation, and growth of soybeans. Soil Science Society of America Journal 45:1113-1120.

Munevar, F., and A. G. Wollum (1981b) Growth of Rhizobium japonicum strains at temperatures above 27°C. Applied Environmental Microbiology 42:272-276.

Munns, D. N., J. S. Hohenberg, T. L. Righetti, and D. Lauter (1981) Soil acidity tolerance of symbiotic and nitrogen-fertilized soybeans. Agronomy Journal 73:407-410.

Munns, D. N., H. H. Keyser, V. W. Fogle, J. S. Hohenberg, T. L. Righetti, D. Lauter, M. G. Zaroug, K. L. Clarkin, and K. W. Whiteacre (1979) Tolerance of soil acidity in symbioses of mung bean with rhizobia. Agronomy Journal 71:256-260.

Odeyemi, O., and M. Alexander (1977a) Resistance of Rhizobium strains to phygon, spergon, and thiram. Applied and Environmental Microbiology 33:784-790.

Odeyemi, O., and M. Alexander (1977b) Use of fungicide-resistant rhizobia for legume inoculation. Soil Biology and Biochemistry 9:247-251.

Osa-Afiana, L. O., and M. Alexander (1981) Factors affecting predation by a microcrustacean (Cypris sp.) on nitrogen-fixing blue-green algae. Soil Biology and Biochemistry 13:27-32.

Osa-Afiana, L. O., and M. Alexander (1982a) Clays and the survival of Rhizobium in soil during desiccation. Soil Science Society of America Journal 46:285-288.

Osa-Afiana, L. O., and M. Alexander (1982b) Differences among cowpea rhizobia in tolerance to high temperature and desiccation in soil. Applied and Environmental Microbiology 43:435-439.

Pant, S. D., and V. Iswaran (1970) Survival of groundnut Rhizobium in Indian soils. Mysore Journal of Agricultural Science 4:19-25.

Pate, J. S. (1961) Temperature characteristics of bacterial variation in legume symbiosis. Nature 192:637-639.

Pena-Cabriales, J., and M. Alexander (1979) Survival of Rhizobium in soils undergoing drying. Soil Science Society of America Journal 43:962-966.

Philpotts, H. (1977) Survival of Rhizobium trifolii strains on inoculated seed held at 35°C. Australian Journal of Experimental Agriculture and Animal Husbandry 17:995-997.

Rains, D. W., and S. W. Talley (1979) Use of azolla in North America. Pp. 419-431 in: Nitrogen and Rice. International Rice Research Institute, Los Baños, Philippines.

Ramirez, C., and M. Alexander (1980) Evidence suggesting protozoan predation on Rhizobium associated with germinating seeds and in the rhizosphere of beans (Phaseolus vulgaris L.). Applied and Environmental Microbiology 40:492-499.

Robinson, A. D., and J. F. Loneragan (1970) Nodulation and growth of Medicago truncatula in acid soil. I. Effect of calcium carbonate and inoculation level on the nodulation of Medicago truncatula on a moderately acid soil. Australian Journal of Agricultural Research 21:427-434.

Roger, P. A., and P. A. Reynaud (1979) Ecology of blue-green algae in paddy fields. Pp. 287-309 in: Nitrogen and Rice. International Rice Research Institute, Los Baños, Philippines.

Roughley, R. J. (1968) Some factors influencing the growth and survival of root nodule bacteria in peat culture. Journal of Applied Bacteriology 31:259-268.

Roughley, R. J., W. M. Blowers, and D. F. Herridge (1976) Nodulation of Trifolium subterraneum by introduced rhizobia in competition with naturalized strains. Soil Biology and Biochemistry 8:403-407.

Roughley, R. J., E. S. P. Bromfield, E. L. Pulver, and J. M. Day (1980) Competition between species of Rhizobium for nodulation of Glycine max. Soil Biology and Biochemistry 12:467-470.

Ryther, J. H., and D. D. Kramer (1961) Relative iron requirement of some coastal and offshore plankton algae. Ecology 42:444-446.

Salema, M. P., C. A. Parker, D. K. Kidby, and D. L. Chatel (1982) Death of rhizobia on inoculated seed. Soil Biology and Biochemistry 14:13-14.

Singh, P. K. (1977) Multiplication and utilization of the fern "Azolla" containing nitrogen-fixing algal symbiont as green manure in rice cultivation. Riso 26:125-137.

Singh, P. K. (1979) Use of azolla in rice production in India. Pp. 407-418 in: Nitrogen and Rice. International Rice Research Institute, Los Baños, Philippines.

Steinborn, J., and R. J. Roughley (1975) Toxicity of sodium and chloride ions to Rhizobium spp. in broth and peat culture. Journal of Applied Bacteriology 39:133-138.

Stovold, G. E., and J. Evans (1980) Fungicide seed dressings: their effects on emergence of soybean and nodulation of pea and soybean. Australian Journal of Experimental Agriculture and Animal Husbandry 20:497-503.

Strivastava, J. S., B. D. Singh, B. Dhar, V. P. Singh, R. B. Singh, and R. M. Singh (1980) Use of streptomycin resistance and phage sensitivity as markers in competition studies with Rhizobium leguminosarum. Indian Journal of Experimental Biology 18:1171-1173.

Talley, S. W., E. Lim, and D. W. Rains (1981) Application of Azolla in crop production. Pp. 363-384 in: J. M. Lyons, et al., eds. Genetic Engineering of Symbiotic Nitrogen Fixation and Conservation of Fixed Nitrogen. Plenum Press, New York, New York, USA.

Thompson, J. A. (1960) Inhibition of nodule bacteria by an antibiotic from legume seed coats. Nature 187:619-620.

Vidor, C., and R. H. Miller (1980) Relative saprophytic competence of Rhizobium japonicum strains as determined by the quantitative fluorescent antibody technique (FA). Soil Biology and Biochemistry 12:483-487.

Watanabe, I., N. S. Berja, and D. C. Del Rosario (1981) Growth of Azolla in paddy fields as affected by phosphorus fertilizer. Soil Science and Plant Nutrition (Tokyo) 26:301-307.

Weber, D. F., and V. L. Miller (1972) Effect of soil temperature on Rhizobium japonicum serogroup distribution in soybean nodules. Agronomy Journal 54:796-798.

Wilkins, J. (1967) The effects of high temperatures on certain root-nodule bacteria. Australian Journal of Agricultural Research 18:299-304.

Wilson, D. O., and K. M. Trang (1980) Effect of storage temperature and enumeration methods on Rhizobium spp. numbers in peat inoculants. Tropical Agriculture 57:233-238.

Wilson, J. T., and M. Alexander (1979) Effect of soil nutrient status and pH on nitrogen-fixing algae in flooded soils. Soil Science Society of America Journal 43:936-939.

Wilson, J. T., S. Greene, and M. Alexander (1979) Effect of interactions among algae on nitrogen fixation by blue-green algae (cyanobacteria) in flooded soils. Applied and Environmental Microbiology 38:916-921.

Wilson, J. T., S. Greene, and M. Alexander (1980) Effect of microcrustaceans on blue-green algae in flooded soils. Soil Biology and Biochemistry 12:237-240.

Plant Cell and Tissue Culture: An Overview

G. B. COLLINS

INTRODUCTION

The need for new and innovative research methods that will lead to increased agricultural productivity cannot be stressed too strongly. Genetically superior crop varieties and efficient production practices have enabled farmers in the United States and in other developed countries to increase production steadily for the past several decades, and they are equally important to increased agricultural output in developing countries.

The application of conventional plant breeding methods and performance testing for manipulating and evaluating useful genetic variation will probably continue to be the primary approach leading to development of superior plant varieties. However, much excitement and potential have been provided by the rapid development of plant cell and tissue culture methods, recombinant DNA technology, and additional molecular approaches to crop improvement (see Marx 1982, Goodin 1982).

Plant cell and tissue culture has already contributed significantly to crop improvement and offers great potential for the future. Manipulations of genetic material using plant cell and tissue culture provide input to plant breeding efforts at two levels. The first is to make available to breeders materials such as genetic and cytogenetic variants, haploids, wide-cross hybrids, disease-free stocks, and large numbers of propagules. The second and possibly the most important long-range contribution of plant cell and tissue culture methods is to provide the bridge between molecular genetic engineering techniques and plant breeding. Most schemes envisioned thus far for gene, chromosome, and organelle transfer, which utilize a wide variety of tested and untested systems for delivering the donated material, require a cultured cell or tissue of the recipient plant species. Thus plant cell and tissue culture methods and their applications deserve to be fully assessed for crop improvement purposes.

Economic plant species represent a diverse assortment of biological types including annuals and perennials, herbaceous and woody species, moncots and dicots, self- and cross-pollinated groups, those that are vegetatively propagated, and apomictic forms. The reproductive cycles of these plants vary widely, and they are multicellular, highly differentiated organisms that cover a massive range of sizes and morphological forms. Plant cell and tissue culture provides a system for potentially reducing any plant species to the single-cell state. Such cells can be grown and manipulated under aseptic conditions in the laboratory where as many as 5 to 500 million cells or cell aggregates may be contained in a single culture flask. Assuming that single cells can be selected and ultimately regenerated into whole plants, the efficiency in sheer number of experimental units is striking, especially when contrasted to whole plants numbering only in the thousands per acre of land. In addition to being able to grow plant cells as single units, it is now possible to produce protoplasts by enzymatically removing the cell wall. Many manipulations can be carried out at the protoplast level and for a number of species the cultural requirements have been defined so that an adult plant can be regenerated from a single protoplast or fused protoplasts from two different species. Such *in vitro* manipulations of protoplasts, cells, tissues, and organs of plants for the purpose of generating or facilitating the production of useful improvements in agricultural crop species will be addressed here.

HISTORY OF PLANT CELL AND TISSUE CULTURE

The earliest well-documented effort to use plant tissue culture was recorded at the turn of the century by Haberlandt (1902) when he attempted to regenerate plants from single cells. Very little progress, however, was recorded until the 1930s. Three key studies include White's (1939) establishment of an artificial nutrient medium for the successful culture of the *Nicotiana glauca* x *N. langsdorfii* hybrid and the successful definition of the requirements for the long-term culture of carrots reported in the same year by Gautheret (1939) and Nobécourt (1939).

Although many pioneering studies on plant cell and tissue culture could be cited, only a few will be mentioned here. The importance of auxin to cytokinin ratios (growth regulators) for achieving different forms of plant tissue growth in culture, including morphogenesis, was provided by Skoog and Miller (1957). The discovery of somatic embryogenesis in carrots and the elaboration of this plant regeneration process by Steward (1958) still serves as a model system today. Bergmann (1960) pioneered single-cell cloning of plant cells. The culture medium devised by Murashige and Skoog (1962) for tobacco has served as the starting point for the development of refined growth

media for dozens of plant species in last two decades of active research on plant tissue culture. The initial work by Cocking (1960) on plant protoplast isolation and culture has been of comparable significance to the development of protoplast technology. Bourgin and Nitsch (1967) attracted the attention of the scientific community with the production of haploid plants by anther culture in Nicotiana. Carlson et al. (1972) set the stage for an extensive research effort on plant protoplasts when they successfully produced a somatic cell hybrid by fusing protoplasts of two Nicotiana species. Finally, timely reviews of plant cell and tissue culture are available as edited volumes by Street (1977), Reinert and Bajaj (1977), Vasil and Vasil (1980), Conger (1981b), and Thorpe (1981).

TISSUE CULTURE TERMINOLOGY AND BASIC TECHNIQUES

Methods

The term "plant tissue culture" has been rather generally applied in the recent literature to all forms of plant cultures grown in vitro, ranging from undifferentiated single-unit protoplasts to complex multicellular and highly organized organ cultures. The most frequently encountered types of plant tissue cultures include callus, cell suspension, organ, meristem tip, and protoplast:

- Callus culture. Masses of unorganized cell clusters grow on the surface of an agar solidified nutrient growth medium. Initial explant tissue sources commonly used include embryonic, seedling, mature vegetative plant parts, or reproductive tissues such as ovules. Tissue is ordinarily subcultured at 2-4 week intervals.
- Cell suspension culture. Plant cells and cell aggregates grow in liquid growth medium with agitation for aeration and to break up cell clusters. Initiating tissue for the culture is either callus or wounded vegetative tissues as described for callus initiation. Subculturing routinely takes place at 3.5- or 7-day intervals by dilution into fresh culture medium.
- Organ culture. Plant organs such as roots, shoots, embryos, anthers, ovaries, and ovules are introduced into culture on an agar solidified growth medium or often with a solid support and a wick contact to a liquid medium. Subculture is normally at intervals of 2-4 weeks, followed by transfer to media with a different nutritional and hormonal composition to obtain a specific growth response.
- Meristem tip culture. Shoot meristem tips of varying size, depending on plant species and experimental objective, are

inoculated into culture on an agar solidified medium. Either single complete plants or proliferation of large numbers of shoot buds may be generated by controlling media composition and culture conditions. The final stage of culture generally involves transfer of plants to a root-inducing medium.

- Protoplast culture. Protoplasts are most frequently isolated from leaf mesophyll tissues, root tissue, or cell suspension cultures by enzymatic digestion, followed by clean-up and concentration steps. Protoplasts are cultured in liquid medium to perform manipulations and subsequently to achieve cell wall regeneration and cell division, followed by transfer to agar medium for callus proliferation and plant regeneration.

All these cultures are grown in temperature- and light-controlled facilities. Growth media components include major and minor inorganic nutrient salts, organic components including vitamins and amino acids, sucrose as the typical carbon energy source, and phytohormones. Frequently, complex organic additives such as coconut water, casein hydrolysate, and yeast extract are incorporated in the growth medium, presumably to compensate for undefined nutritional or hormonal requirements.

The facilities and equipment needed for carrying out plant cell and tissue culture research are rather modest. Major items of equipment include an analytical balance, autoclave, dry sterilizing oven, pH meter, glass distilled water source, laminar flow hood, microscopes (dissecting, inverted, and compound binocular), gyratory shaker, table-top centrifuge, incubator, and refrigerator. Chemicals, glassware, and routine laboratory supplies are also required.

Limitations

In Vitro Methods. To use plant cell and tissue culture methods to improve a given crop species, it is necessary to have efficient *in vitro* methods for that crop, including, ideally, all of the cultures listed above. Unfortunately, this is not the case for very many plant species outside of the Solanaceae. A recently published monograph edited by Conger (1981a) reviews the status of *in vitro* methodologies for agricultural crop groups including ornamentals, fruits, vegetables, agronomic crops, and trees and reveals that all types of *in vitro* cultures are possible for only a few species.

A basic problem encountered in achieving *in vitro* culture capability with all plant species, especially with genotypes of agricultural importance, is the general lack of quantitative information on the physiology, biochemistry, and developmental aspects

of plant cells in culture. Successful culturing of most species has been achieved using empirical approaches that involve manipulation of tissue explants from a restricted number of genotypes on media varying in nutritional and hormonal components. The specific roles and interactions of growth regulators, including auxins, cytokinins, abscisic acid, and gibberellins, are poorly understood.

Although in vitro systems are well developed for tobacco, potato, petunia, carrot, and a few other plant species, the information gathered in the process of achieving successful cultures for these plants is not readily transferrable to other important crop plants. This does not imply, however, that little progress has been made in placing plants into in vitro culture in the relatively short period of concentrated research. Callus, suspension, and meristem tip cultures are, in fact, now possible for hundreds of plant species (Conger 1981b). It is not necessary to achieve all types of in vitro culture capability to make use of one or more available cultural systems for the species. For example, in vitro clonal propagation has been used extensively for many years for ornamental species using meristem tips or other vegetative tissue explants.

Regeneration of Complete Plants. Another major problem is the inability to regenerate complete plants from single cells and protoplasts. To be agriculturally useful, transformed cells, selected variant cells, heterokaryon fusion products, and haploid microspores must be capable of regeneration into a complete plant and ultimately production of vegetative progeny or seed that express the desired trait. The plant regeneration problem has been particularly difficult in such important groups as the grasses, cereals, and woody species. However, plant regeneration has been recently recorded for difficult species such as corn via somatic embryogenesis from scutellar callus (Lu et al. 1982), and plants have been regenerated from protoplasts of orange, mandarin, grapefruit, lemon, and sour orange (Vardi et al. 1982). In the case of corn and the citrus species cited above, embryonic explant tissue sources were necessary to achieve regeneration. Restrictions on plant regeneration from cell cultures often include explant source (embryonic or juvenile), genotype of explant donor, and limited period for which the culture can be maintained before regenerative potential is lost.

Gamborg and Shyluk (1981) list 45 crop plants for which plants have been regenerated from tissue cultures, including 25 agronomic species, 11 tree species, and 9 vegetable species. Few of these examples involved regeneration from the single-cell level.

Evans et al. (1981) address the question of plant regeneration in a different manner. They have classified successful reports of plant regeneration on the basis of whether plant recovery was by organogenesis or somatic embryogenesis, and further noted explant source, media, and hormone concentrations used. In their survey, they

found 26 crop and 14 noncrop species capable of plant regeneration via somatic embryogenesis, with vegetable and tree species dominating the crop list. Twenty grass and cereal crop species and hybrids have reportedly regenerated plants via an organogenic pathway. Important crop species in the group include Indian grass, fescue, sugarcane, barley, millet, wheat, oats, rice, sorghum, and corn. An additional 43 crop species excluding the Gramineae (grasses) have been reported capable of regenerating plants either directly or indirectly through an intermediate callus stage via the mechanism of organogenesis. The status of additional types of tissue and cell cultures for crop plants such as anther, protoplast, meristem tip, ovule/ovary, and embryo will be provided as specific areas of application are discussed.

Cytogenetic and genetic variability have been repeatedly documented in plant cell and tissue cultures. Chaleff (1980) offers examples for several plant species of chromosomal and genetic changes in cultured cells and in plants regenerated from them, and, of course, numerous examples also exist of the regeneration of cytologically normal plants from tissue cultures. In general, less variability is encountered in plants regenerated from short-term cultures, in cultures where regeneration is by somatic embryogenesis versus organogenesis, and from organized shoot meristem cultures. Aberrations in regenerated plants usually take the form of morphological alterations, albinism, and sterility. Variability generated or perpetuated in plant cell cultures is not necessarily bad if the deleterious effects can be avoided or managed, and if useful variations can be selected and utilized as has been so aptly demonstrated in the case of sugarcane and potato improvement.

A major proportion of the realized and anticipated applications of plant cell culture techniques to crop improvement involves the recovery of a gene, hybrid, haploid, or otherwise altered plant. This, however, represents only the first step in a traditional breeding procedure for the crop. More often than not, several years will be required to establish the new trait in an acceptable genetic background and to evaluate its performance and its possible effects on the agronomic, chemical, and quality traits of the crop. Furthermore, cell culture methods are not applicable to different crop species with equal success. Rather, each species or crop must be considered individually regarding its biology (sexual versus vegetatively propagated, annual versus perennial, self-pollinated versus cross-pollinated, diploid versus polyploid) and its cultural capability *in vitro*. The *in vitro* method chosen is then considered as a tool for use with the appropriate breeding procedure.

SPECIFIC APPLICATIONS OF PLANT CELL AND TISSUE CULTURE IN CROP IMPROVEMENT

Plant cell and tissue culture methods can be applied in the following areas:

- Clonal propagation
- Elimination of disease
- Long-term storage of germ plasm and germ plasm exchange
- Wide hybridization and gene transfer
- Production of haploids and homozygous breeding lines
- Generation of genetic variation and variant selection
- Genetic engineering of economic plant species
- Basic physiological/developmental genetic studies.

These areas are listed somewhat arbitrarily and serve only to categorize research for discussion. Some overlap between areas is obvious, and additional or alternative divisions and subdivisions of research areas are possible. Even additional applications of tissue and cell culture can be envisioned.

Clonal Propagation

In vitro methods have been used extensively for asexual propagation of crop species, and the procedures have become well developed and very important commercially (see Conger 1981b, Constantin et al. 1981, Tomes et al. 1982). Reasons for choosing an *in vitro* system for clonal propagation include ease and efficiency of increase, maintenance of heterozygosity, and sexual sterility or incompatibility problems. Production of sufficient numbers of plants of a unique genotype for evaluation and further development is the typical objective of clonal propagation, especially in cases where seed production is not possible. It also has useful applications in breeding and basic research studies.

Clonal propagation is accomplished by multiplication of axillary shoots, adventitious shoot production, or somatic embryogenesis (Murashige 1978). *In vitro* clonal propagation procedures have been applied rather extensively to ornamentals, vegetables, and fruit crops. Orchids were the first plant to be propagated commercially utilizing *in vitro* methods. According to Hughes (1981), tissue culture techniques have been used to propagate species from more than 40 families exclusive of the widely propagated Orchidaceae. Skirvin (1981) suggests that all fruit crops could be grown in culture provided patience and resources were not limiting. Bottino (1981) notes that at least 24 vegetable crops are being propagated using *in vitro* techniques. These methods are being used on a large scale

for nursery potato production, and several other examples of research and commercial application are suggested for vegetable crops such as asparagus, cauliflower, and cassava.

Clonal propagation has not been used to any extent for commercial purposes in agronomic crops. In fact, as Conger (1981a) suggests, most agronomic crops are seed propagated, and in vitro clonal manipulations are largely restricted to the research needs of maintaining specific genotypes or proliferating a rare sterile, or sexually incompatible, genotype. Mott (1981) is optimistic about the future potential for woody species where special problems of plant size and generation time place a high premium on the development of in vitro time-saving techniques. Several woody fruit tree species--Malus, Prunus, Pyrus, Vitis, Rubus, Ribes, Actinidia, Vaccinium, and Morus--have already been successfully induced to produce multiple shoots and to root in vitro (Lane 1982). Practically all of the woody species work has been reported in the past 6-10 years.

A new dimension and role for in vitro progagation can be envisioned if the efficient production of somatic embryos can be established in more species of economic plants. This would provide large numbers of propagules characterized with a low frequency of aberrations for use in conjunction with fluid drilling technology. This method is more likely to be feasible with high-income crops such as vegetables, although application to other crops is possible, especially where disease, space, and seasonal transplant problems may be severe.

The extensive list of plant species for which buds, shoots, embryoids, or plants have been produced in vitro is indicative of the interest in and potential of clonal propagation in crops, even in view of cautions that have been expressed (Vasil and Vasil 1980).

Elimination of Disease

One of the most important applications of tissue culture to date has been the use of meristem tip culture to eliminate viruses from infected plants. This involves the aseptic inoculation of the meristematic dome and one or more leaf primordia into culture on a simple nutrient agar. Quak (1977) and Walkey (1978) have outlined the basic procedure and its application to economic plant species to eliminate viruses. Both reviews list species studied and discuss the efficiency of including a heat treatment to improve the success rate for eliminating viruses.

The major application of the meristem tip culture method is in vegetatively propagated species where parental clones have become infected by a virus. Meristem tip culture has at least two advantages over other types of tissue culture for eliminating viruses: (1) the

integrity of the original plant or clone is maintained with minimal genetic variation, and (2) the methods developed for meristem tip culture are relatively straightforward and can be used directly or with slight modification for clonal progagation.

Scientists disagree as to how the meristem tip culture eliminates a virus. The host species, the virus, and growth conditions are considerations. In many instances, the virus may reside in the meristem tip so that one or more of the methods for virus indexing must be applied to the regenerated plants.

Although the use of meristem tip culture for virus elimination is cited as the most widely used and significant contribution of tissue culture to crop improvement (Conger 1981b, Daub and Carlson 1981), the plants obtained should be evaluated for yield and quality. Walkey (1978) cites examples of changes in flowering, fruiting, fruit color, and cold unit requirements to break dormancy in meristem tip culture-regenerated plants.

Although meristem tip culture and other tissue culture methods for disease elimination will be largely applied to vegetable and ornamental species, these techniques have some utility in eliminating viruses from the elite germ plasm and clonal stock of outcrossing species of field crops.

Long-Term Storage of Germ Plasm and Germ Plasm Exchange

Plant breeders and other plant scientists face the difficult problem of maintaining germ plasm for future unpredictable needs that require genetic variability for variety development. The need to preserve all potentially valuable germ plasm is balanced by the time, labor, and space required to maintain germ plasm for a large number of economic plant species. Germ plasm maintenance is an especially critical problem for vegetatively propagated species because of the labor and expense required and the fact that field increase involves exposure to climatic uncertainties, pests, and identification errors.

Maintenance of species propagated by seed is also not without problems, including seed viability, seed-borne pathogens, and maintenance of heterozygous species.

The increasing exchange of germ plasm among scientists as well as the interstate and intercountry exchange of commercial germ plasm also poses serious phytosanitary problems. The introduction of a pest through seed or vegetative plant parts represents a substantial threat to the agriculture in the region of entry.

Tissue culture methods have been evaluated and are currently being used for the storage and preservation of crop germ plasm; tissue and cell cultures are being used increasingly as the forms in which germ plasm is exchanged between countries. The use of meristem tip culture for maintaining germ plasm and as an exchange form is a

logical extension of the research methods developed to use this same type of tissue culture system in rapid clonal propagation and for eliminating viruses and pathogens from plant germ plasm (see Kartha 1981a, 1981b, 1982; Withers 1980).

Tissue cultures can be stored using either a slowed growth rate method where cultures may be maintained for a few years during which periodic transfer to fresh media may be required or an extremely low-temperature storage method (cryopreservation) in which metabolic activities are arrested and storage may be extended to many years with culture viability retained. Storage of coffee germ plasm has been possible for 2 years using meristem culture (Kartha 1982), and this method offers the greatest potential for storage and maintenance of several important woody species of plants.

Storage of germ plasm in the form of tissue cultures utilizing cryopreservation techniques and temperatures in the -196°C range offers great promise for maintaining germ plasm of crop plants. Research in this area is not well advanced, however, and numerous problems remain to be resolved. Cell or tissue viability, genetic integrity, and ability to regenerate plants at the end of the storage period are prerequisites to the successful application of the technique. Tissue condition, tissue preconditioning, cryoprotectants, and methods of freezing and thawing are methodological considerations still under investigation. Current research has centered on the use of meristems for long-term storage because of the high incidence of genetic variability in plants regenerated from long-term callus and suspension cultures coupled with the relatively few species in which single-cell regeneration can be accomplished. Somatic embryos may eventually represent a useful form of cultured plant tissue for long-term storage with acceptable genetic fidelity in the recovered plant.

Short-term storage and long-term maintenance of plant germ plasm using meristem tip culture is presently possible for a large number of plant species (Kartha 1981b). Although many details have yet to be worked out for efficient, long-term storage of germ plasm that satisfies all the requirements described, the demonstration by Kartha (1982) that 75 percent of a test group of strawberry meristems regenerated into mature plants after 80 weeks of storage at -196°C indicates the potential of the method. Successful low-temperature culture of peas, potatoes, carrots, Trifolium, and chickpea has also been reported.

Wide Hybridization and Gene Transfer

Hybridization between wild relative species and cultivated crop species has been an experimental objective of researchers attempting to improve crops for centuries. Usually the wild species is viewed as

a source of genetic variation for disease or insect resistance, environmental stress tolerance, chemical traits, or agronomic traits, or simply as a source of genetic variation for broadening the germ plasm base. The importance, methods, and uses of interspecific and intergeneric hybrids in crop plants have been reviewed by Hadley and Openshaw (1980).

Barriers to sexual hybridization between species are numerous and may be classified as either pre-fertilization or post-fertilization types. The various mechanisms of intra- and interspecific incompatibility are too numerous and complex to describe in this report. Although it is possible to develop methods for overcoming sexual incompatibility without knowing precisely the barrier to hybridization, selection of the most suitable method is facilitated by knowing the mechanism of incompatibility and the site of failure.

Hybridization with a related species would be a valuable contribution to improved breeding of many crops. Tissue culture-assisted sexual hybridization and somatic cell fusion can facilitate successful hybridization in cases where normal sexual procedures fail. The methods available in tissue culture-assisted wide hybridization include in vitro pollination and fertilization, fertilized ovule or ovary culture, and hybrid embryo culture (Rangaswamy 1977; Zenkteler 1980; Yeung et al. 1981; Steward 1981; Raghavan 1980).

Improvement in the tissue culture media and culturing techniques for specific crop species has stimulated research utilizing fertilized ovule/embryo culture in many economic crops such as cotton, soybean, clover, barley, and tomato. Work with Nicotiana using fertilized ovule culture (Reed and Collins 1978) and with Trifolium using embryo rescue (Phillips et al. 1982) are examples of the efficacy of these techniques in facilitating wide crossing between cultivated species and their wild relatives. The in vitro pollination and fertilization method is most useful where pre-fertilization barriers exist, and embryo culture is useful for rescue of failing post-fertilization zygotes.

The second in vitro method for production of wide hybrids between species is by fusion of somatic cells or protoplasts from the two species with subsequent regeneration of an intact hybrid plant. A vast amount of literature is available on protoplasts, protoplast fusion, and techniques related to somatic cell fusion. Keller et al. (1982) document this technique and progress made in its application in hybrid production.

Genomic hybrids, cytoplasmic hybrids, and partial hybrids with some chromosomes eliminated represent the types of combinations possible by somatic cell fusion. The ability to combine and transfer nuclear and cytoplasmic genomes is of immense interest and potential importance in crop improvement. The short-term expectations, however, have generally been far too optimistic. A few examples of the reasons

for the over-optimism include: lack of plant regeneration capacity from fused heterokaryons, instability/incompatibility of nuclear and cytoplasmic genomes in the same cell, sterility of hybrids, polyploid chromosome number of hybrids unless haploid somatic cells are available for fusion, lack of expression of donor species traits in the hybrid, and technical problems related to returning the raw hybrid to a commercially acceptable form.

Although the long-term potential for crop plant hybridization via somatic cell fusion is real, the immediate applications appear limited. The methodologies required for protoplast isolation, culture, fusion, and plant regeneration are available for only a few crop species, and even here they are incomplete. Once the techniques are available, somatic cell hybrids will likely have very limited and specific applications to transfer of nuclear genes and cytoplasmic traits not achievable by other methods. Transfer of cytoplasmic male sterility, organelle genome manipulation, cytoplasmic mixing, and basic studies on heterosis, gene expression, chromosome elimination, organelle fusion, and recombination are possible areas for exploitation. A hybrid between two species obtained by normal sexual crossing or by any of the tissue culture methods described is only the first step in a long and tedious plant breeding program. Normal breeding techniques must be used to develop a commercially acceptable cultivar in which the contributions (nuclear, cytoplasmic, or unique combinations of the two) of the donor species are incorporated into an acceptable commercial background genotype and finally evaluated for performance. The introgression of exotic genetic material is generally disruptive to a species, and a genotype that can accommodate the integration of foreign genetic material must be established.

Useful nuclear and cytoplasmic traits have become established through natural selection in wild plant species. Differences of extreme practical value also distinguish crop plants (legumes versus nonlegumes, C_3 versus C_4 plants). Hybridization by either sexual or parasexual methods will undoubtedly make the greatest contribution to crop improvement when the genomes of the donor and crop species are closely homologous, except for a restricted number of useful donor genome traits. In cases where important nuclear and cytoplasmic traits are present in genetically divergent species, approaches that facilitate the transfer of only the fraction of the genome required for expression of the trait(s) are preferable. *In vitro* pollination/fertilization and ovule/embryo culture should be exploited for hybridization of crop species with closely related but sexually separated species. Further refinements in the methods for protoplast manipulation will provide somatic cell fusion hybridization capability for some crop species. Unique cytoplasmic and cytoplasmic/nuclear combinations between related species offer the greatest immediate contribution from somatic cell fusion.

Production of Haploids and Homozygous Breeding Lines

The efficient production of haploid vegetative plants from immature pollen grains through *in vitro* cultures of anthers has progressed rapidly since 1967 when the technique was presented (Bourgin and Nitsch 1967). The basic anther culture procedure has been modified for different plant species, and, although haploid plants can be produced in a large number of species, their production is not a routine operation for many crop plants (Collins and Genovesi 1982, Maheshwari et al. 1980). Recent notable anther culture improvements and successful attempts in producing haploid plants have been made in such economic species as wheat, corn, *Brassica*, fescue, rubber tree, tomato, poplar, rye, potato, triticale, and grapes (Collins and Genovesi 1982).

Haploids and haploid derivatives are generally used as sources of haploid cells and tissues and plants for selection experiments, for production of homozygous lines and generation of aneuploid stocks, and in genetic analyses. Specific applications include scaling down from higher to lower ploidy levels, production of pure lines for hybrid production, identification and recovery of male sterile types, and rapid pure-line fixation for species such as trees with long sexual cycles. The time saved in achieving homozygosity is offset by the loss of generations ordinarily used in a breeding program for selection and evaluation.

Haploids from a wide range of genotypes can provide three basic types of material. The hemizygous genetic condition in plants makes efficient selection possible since recessive alleles are not masked by dominant alleles as is the case in diploid heterozygous material. Haploid vegetative plant tissue can be used to isolate protoplasts from species to be hybridized by somatic cell fusion. Hybrids produced by fusion of haploid cells result in diploid hybrids rather than polyploid hybrids obtained from diploid somatic cell fusions. Finally, doubling the number of chromosomes of haploid cells or plants results in homozygous true-breeding lines. The homozygous lines may be used as inbred lines for hybrid production, to fix gene combinations at the conclusion of a backcrossing scheme, or to stabilize other unique germ plasm in homozygous form.

A second method involving *in vitro* procedures used for haploid production is the so-called Bulbosum method of Kasha and Kao (1970). This method involves the interspecific hybridization of cultivated barley (*Hordeum vulgare*) with *H. bulbosum* and the subsequent preferential elimination of the *H. bulbosum* chromosomes, resulting in barley haploids. The haploids survive with the aid of *in vitro* hybrid embryo culture. Kasha and Reinbergs (1980) have detailed the successful development within 5 years of a new barley variety utilizing the Bulbosum method. Advantages noted for the haploid

breeding method included speed, ease of selection, and a unique opportunity to study gene action and the effects of environment. Performance and stability of haploids and doubled haploids were judged satisfactory.

At least one additional *in vitro* method is available to produce haploid plants. Jensen has used ovule culture to produce haploids of barley (personal communication with C. J. Jensen, Riso National Laboratory, Roskilde, Denmark). This method is very similar to the well-known one of wide crossing in which chromosomes of one species are eliminated such as with the Bulbosum method, or the unfertilized egg cell simply develops into a viable haploid seed.

Haploids and doubled haploids are potentially very useful materials for crop breeding and variety development as well as in numerous supporting areas such as mutant selection, hybridization via protoplast fusion, genetic analysis, and the production of cytogenetic stocks. Several cultivars and breeding lines developed utilizing haploid breeding procedures have been released for barley, rice, and tobacco. Extensive programs are under way for wheat, corn, and several woody species.

A major problem associated with the use of haploid breeding methods is the lack of efficient haploid production in large numbers from diverse genotypes of most crop species, with the exception of *Nicotiana*, *Petunia*, and certain genotypes of rice. In the small grains, albino haploid plantlet production limits the usefulness of the anther culture procedure.

Haploids and doubled haploid lines have not been extensively evaluated agronomically in many crop species. No problems have been encountered with burley tobacco, but the situation is different with flue-cured tobacco. For burley tobacco, unlike flue-cured, doubled haploids are similar in performance to their respective inbred cultivar parents (Deaton et al. 1982). Significant vigor reduction has been observed in doubled haploid lines of flue-cured tobacco (Arcia et al. 1978). Results from additional species and more extensive testing will be required to determine the extent of the problem encountered with the haploid and doubled haploid condition.

Generation of Genetic Variation and Variant Selection

In the past decade, two potentially significant approaches to manipulation of genetic variation have been employed utilizing tissue culture methods. The first system uses different forms of tissue culture, such as callus, cell suspension, and protoplasts, in conjunction with a positive selection scheme to isolate useful genetic variants. In some studies, the plant tissues or cells have been mutagenized prior to applying a selective agent or condition. In

general, it has been possible to select a wide range of variants from plant tissue cultures in a number of different species.

The second type of genetic variation is that which is spontaneously generated by or associated with various tissue culture methods (Larkin and Scowcroft 1981). Cytological variation has been frequently found to be characteristic of callus and suspension cultures of plant cells, especially of older cultures such as those having undergone several subculture passages in vitro.

Genic mutations are rare and most often recessive events which makes reliance on conventional diploid genetics and breeding methods inefficient for mutant recovery. The capability to grow millions of haploid cells in a culture flask provides a decided advantage in terms of number of selectable units, hemizygous condition, and a system for introducing a screening or selecting agent. For such a system to be useful in crop improvement, the variant cell must be capable of regeneration into a plant; the variant must be genetically determined versus an epigenetic event; and the variant gene must be expressed at the whole plant level as well as being transmitted stably to successive sexual generations.

According to the literature (Chaleff 1980, Tomes et al. 1982, Vasil 1980, Thorpe 1981), a large number of mutants have been selected from tissue culture, and a few of these have been characterized as stable genetic variants at the whole plant level. Disease resistance, herbicide tolerance, stress tolerance (heavy metals, salt, drought, chilling), and various biochemical variants (amino acid overproduction, antibiotic resistance, nitrate reductase negative, and photorespiration) are examples of the most sought-after mutant traits using tissue culture methods. Examples of successful in vitro mutant recovery include selection of cells from a susceptible genotype and eventual recovery of plants with resistance to southern corn leaf blight (Gengenbach et al. 1977); selection of wildfire bacterial disease resistance in tobacco (Carlson 1973); lysine plus threonine resistance in corn (Hibberd and Green 1982); ethionine-resistant alfalfa (Reisch et al. 1981); and 2,4-D resistant trefoil (Swanson and Tomes 1980).

There are very few cases of variant selection in which plants have been regenerated and both the genetic basis and inheritance pattern of the trait have been established; and even fewer cases where the performance of the derived mutant line has been evaluated for all agronomic and chemical attributes important to the crop. Ethionine-resistant alfalfa plants exhibit considerable variation in physical traits (Reisch and Bingham 1981), and sterility also appears to be a problem (personal communication with E. T. Bingham, University of Wisconsin, Madison). Thorough evaluation of tissue culture-derived plants is necessary to determine their usefulness in crop improvement.

Although the information about spontaneous variability arising in plants regenerated from tissue culture is too preliminary to generalize for all crop plants, the reported variation for plant morphology, chromosome number, sterility, and vigor is striking (Larkin and Scowcroft 1981). If useful variation can be identified and utilized as reported for sugarcane (Heinz et al. 1977) and potato (Shepard et al. 1980), tissue culture variation may be considered advantageous. However, if useful selected or transferred variants are overwhelmed by negative variants originating in culture, special effort will be required to reduce the variation or to use only certain types of cultures.

Spontaneously recovered somaclonal or protoclonal variants and selected variants (with or without mutagenesis) from tissue and cell culture systems are excellent sources of plant variation. These may be useful if variant cells can be regenerated into plants where the selected or useful genes are expressed, stable, and transmitted to progeny plants in subsequent generations. The large number of variants isolated to date is encouraging, although only a few variants have been fully characterized and thus can be considered for potential use in crop improvement.

In assessing the utility of variant selection at the cellular level, a few additional considerations are necessary. First, the correlation of *in vitro* cellular response and *in vivo* whole plant response is not well established. Some genes of interest may not be expressed *in vitro* and other genes expressed in response to a selective agent or regime *in vitro* may not be expressed in the intact, highly differentiated plant. Culture conditions and type of tissue culture system utilized may have to be manipulated to optimize gene expression and selection.

Second, it is difficult to select numerous important agronomic traits in culture. This results from difficulties in imposing a selective regime and in obtaining and recognizing the desired response. Drought tolerance, yield, maturity, insect resistance, plant architecture, and lodging resistance are examples of important traits for which *in vitro* selection and expression at the whole plant level require innovative approaches for success. In general, complex traits that are under quantitative genetic control do not permit simple selection and fixation unless extremely large numbers of experimental units are evaluated.

The variation encountered in tissue culture-derived plants and the depressed agronomic performance of these materials is a final important consideration. The selected variant plant or its progeny must be thoroughly evaluated. It will probably also be necessary to incorporate the selected gene(s) into several genetic backgrounds to achieve optimum expression of the selected trait and maximum performance of the crop species. These requirements mean that isolation of the variant is only the first step in the

breeding/evaluation procedure, much like the starting point from a sexual cross or selection from within an established population in a conventional breeding program.

Genetic Engineering of Economic Plant Species

The excitement generated about the potential for plant genetic engineering ultimately rests on the achievement of gene, chromosome, and organelle transfer from a donor source into agriculturally important, economic species of plants. Although plant molecular genetics is progressing rapidly, there is a series of critical steps en route to the final step of "engineering" a gene into a new crop cultivar. Some current research areas in higher plant molecular genetics include gene isolation, gene sequencing, gene cloning, development of gene vectors, direct gene transfer and uptake, chromosome micromanipulation, gene integration, control of gene expression, gene stability and transmission, and organelle transfer. The time frame for successful execution of genetic engineering of improved crop plants is likely to be measured in decades rather than years. Many of the sought-after traits for genetic engineering in crops are complex, polygenically controlled processes such as increased growth rate and yield, improved photosynthetic efficiency, improved biological nitrogen fixation, improved pest resistance, and resistance to environmental stresses.

The research effort in genetic engineering is large and intensive in both the private and public sectors of the United States and of several other countries. Although plant research lags far behind the prokaryote and animal research areas, the importance of contributions made by genetic engineering to crop improvement is substantial. The ultimate product of genetic engineering is an improved cultivar or breeding line. The role of the plant breeder is to provide different germ plasm backgrounds for gene, chromosome, or organelle transfer; to evaluate the whole plant expression of the engineered trait and overall species performance as influenced by the engineered trait; and finally, to release a new cultivar or germ plasm source.

Plant cells and tissues growing in vitro are visualized as the bridge between molecular genetics and new crop cultivars ultimately developed by plant breeders. Plant protoplasts with restrictive cell walls removed are considered to be the most logical and efficient recipient structures for the introduction of foreign genes and organelles. Use of protoplasts for genetic engineering makes plant regeneration from transformed protoplasts a necessity, just as plant regeneration is required for mutant selection using protoplasts or suspension cultured cells.

It is beyond the scope of this report to provide a status report on research progress in higher plant molecular genetics. Several

recent reviews elucidate the goals of and approaches to genetic engineering of economic plant species, including a report on a Rockefeller Foundation conference on genetic engineering for crop improvement (Rachie and Lyman 1981), chapters on organelle uptake and introduction of purified DNA by Chaleff (1980), and reviews by Flavell (1981) and Cocking et al. (1981). Plant protoplasts are envisioned as the key to the successful delivery and incorporation of genes and organelles through the use of genetic engineering methods.

Manipulation of one or a few nuclear or organelle genes with necessary promoter control sequences via molecular genetic methods is of utmost importance in contrast to either sexual or parasexual genetic techniques where entire genomes involving hundreds or thousands of genes are transferred to the recipient species. The elimination of undesirable genetic material and stable incorporation of the useful gene is a time-consuming and difficult breeding sequence following introduction of an entire genome.

The direct application of molecular genetics to the development of improved cultivars and economic plant species germ plasm is not feasible presently. However, plant cell and tissue culture methods are viewed as essential to future research aimed at introducing genes, chromosomes, and organelles into plants. Protoplasts, egg cells, and immature pollen grains in culture are the most promising recipient structures for such transformation experiments. The capability to culture single plant cells and protoplasts from economic plant species and to regenerate plants from them is prerequisite to such genetic engineering approaches, and a research effort is required in this area.

Basic Physiological, Developmental, and Genetic Studies

Plant cell and tissue culture research can provide major indirect contributions to crop improvement by generating knowledge about the growth, development, physiology, and genetics of cells in culture with some correlations to whole plant relationships. Examples include dissection of biochemical pathways involved in determination of important plant products and processes, biochemical and genetic control of organogenesis and embryogenesis, enzyme regulation, host-pathogen interactions, membrane permeability, selection and characterization of auxotrophic mutants for use as selectable markers in transformation experiments, and control of gene expression in plants. Of course, many more biochemical, physiological, developmental, and genetic questions can be addressed through the use of large numbers of cells in culture on defined growth medium under precisely controlled culture environments. The specific experiments must be designed by scientists from various disciplines seeking information related to individual plant species and problems peculiar to the species.

RESEARCH NEEDS AND PRIORITIES

Plant cell and tissue culture technologies currently available are enabling plant scientists to make major contributions to crop improvement, and an expanded role for these technologies is envisioned for the future. Areas that could make the most significant contributions, especially in developing countries, include clonal propagation, disease elimination, haploid production, germ plasm exchange, wide hybridization, and mutant selection.

The most important research need is to develop efficient procedures for whole plant regeneration from protoplasts and single cells of economic plant species, and this capability must be extended to a wide range of genotypes, to variant protoplasts or cells in culture, and to long-term cultures. An understanding of the genetic and developmental control of the regeneration process and totipotency is needed to transfer conditions for successful manipulations with one species to other species. Breeding responsive genotypes is a logical approach to achieving efficient plant regeneration systems. Because regeneration of whole, genetically stable plants underlies all types of plant cell and tissue culture-mediated approaches to plant improvement, research on this problem is of the highest priority.

Although development of all types of tissue culture techniques for every plant species cannot be justified, efficient methods must be available for each *in vitro* manipulation attempted. The capability to initiate cultures, satisfy nutritional requirements, control organizational responses, and impose appropriate selective conditions must be available.

Another important research need is to develop a strategy for a total improvement program for a specific crop species. It will then be possible to establish plant cell and tissue culture as an adjunct tool for breeding and other production/management research programs for the crop. Such a program might initially follow these steps:

- Identify the crop to be improved.
- Identify and characterize the breeding and improvement objectives for the crop.
- Establish the specific problems encountered for which alternate methods such as one or more plant cell and tissue culture techniques can be applied.
- If an *in vitro* approach is feasible, determine the status of the specific tissue culture method.
- Develop the appropriate tissue culture methods if unavailable to include plant regeneraton.

Research involving tissue culture must be pursued collaboratively with the plant breeder or other plant scientist who is seeking to establish the improved crop species in a production system. Other scientists

may be brought into the collaboration such as physiologists, microbiologists, biochemists, and molecular biologists as specific expertise is required. Additional personnel, such as production agronomists and extension workers, may be required to help evaluate and test the crop plant materials.

Improved crop varieties and production systems are key to improved agricultural productivity in practically every agricultural system. In developing countries, where equipment, fertilizer, technology, and research-extension capabilities may be limiting, improved varieties and production practices probably represent the least expensive improvement within the shortest time in crop agriculture.

REFERENCES

Arcia, M. A., E. A. Wernsman, and L. G. Burk (1978) Performance of anther-derived dihaploids and their conventionally inbred parents as lines, F_1 hybrids, and in F_2 generations. Crop Science 18:413-418.

Bergmann, L. (1960) Growth and division of single cells of higher plants in vitro. Journal of General Physiology 43:841-851.

Bottino, P. J. (1981) Vegetable crops. Pp. 141-164 in: Cloning Agricultural Plants Via In Vitro Techniques, edited by B. V. Conger. CRC Press, Boca Raton, Florida, USA.

Bourgin, J. P., and J. P. Nitsch (1967) Obtention de Nicotiana haploides à partir d'étamines cultivées in vitro. Annales de Physiologie Végétale 9:377-382.

Carlson, P. S. (1973) Methionine sulfoximime resistant mutants of tobacco. Science 180:1366-1368.

Carlson, P. S., H. H. Smith, and R. D. Dearing (1972) Parasexual inter-specific plant hybridization. Proceedings, National Academy Sciences 69:2292-2294.

Chaleff, R. S. (1980) Genetics of Higher Plants: Applications of Cell Culture. Cambridge University Press, Cambridge, England.

Cocking, E. C. (1960) A method for the isolation of plant protoplasts and vacuoles. Nature 187:927-929.

Cocking, E. C., M. R. Davey, D. Pental, and J. B. Power (1981) Aspects of plant genetic manipulation. Nature 293:265-270.

Collins, G. B., and A. D. Genovesi (1982) Anther culture and its application to crop improvement. Pp. 1-24 in: Application of Plant Cell and Tissue Culture to Agriculture and Industry, edited by D. T. Tomes, B. E. Ellis, P. M. Harney, K. J. Kasha, and R. L. Peterson. University of Guelph Press, Guelph, Ontario, Canada.

Conger, B. V. (1981a) Agronomic crops. Pp. 165-215 in: Cloning Agricultural Plants Via In Vitro Techniques, edited by B. V. Conger. CRC Press, Boca Raton, Florida, USA.

Conger, B. V., ed. (1981b) Cloning Agricultural Plants Via In Vitro Techniques. CRC Press, Boca Raton, Florida, USA.

Constantin, M. J., R. R. Henke, K. W. Hughes, and B. V. Conger (1981) Propagation of Higher Plants Through Tissue Culture: Emerging Technologies and Strategies. Pergamon Press, Oxford, England.

Daub, M., and P. S. Carlson (1981) Technologies and strategies in plant cell and tissue culture--new approaches to old problems. Environmental and Experimental Botany 21:269-275.

Deaton, W. R., P. D. Legg, and G. B. Collins (1982) A comparison of burley tobacco doubled-haploid lines with their source inbred cultivars. Theoretical and Applied Genetics 62:69-74.

Evans, D. A., W. R. Sharp, and C. E. Flick (1981) Growth and behavior of cell cultures: embryogenesis and organogenesis. In: Plant Tissue Culture: Methods and Applications in Agriculture, edited by T. A. Thorpe. Academic Press, New York, New York, USA.

Flavell, R. B. (1981) The analysis of plant genes and chromosomes by using DNA cloned in bacteria. Philosophical Transactions of the Royal Society of London, Series B: Biological Sciences 292:579-588.

Gamborg, O. L., and J. P. Shyluk (1981) Nutrition, media and characteristics of plant cell and tissue cultures. Pp. 21-44 in: Plant Tissue Culture: Methods and Applications in Agriculture, edited by T. A. Thorpe. Academic Press, New York, New York, USA.

Gautheret, R. J. (1939) Sur la possibilité de réaliser la culture indéfinie de tissu de tubercules de carotte. Comptes rendus Hebdomadaires des Séances de l'Académie des Sciences 208:118-121.

Gengenbach, B. G., C. E. Green, and C. M. Donovan (1977) Inheritance of selected pathotoxin resistance in maize plants regenerated from cell cultures. Proceedings, National Academy of Sciences 74:5113-5117.

Goodin, P. (1982) Plasmids: New key to genetic engineering. Agricultural Research. 30(12):8-11.

Haberlandt, G. (1902) Kultureversuche mit isolierten Pflanzellen. Akademie der Wissenschaften (Vienna, Austria) 111:69-92.

Hadley, H. H., and S. J. Openshaw (1980) Interspecific and intergeneric hybridization. Pp. 133-159 in: Hybridization of Crop Plants, edited by W. R. Fehr and H. H. Hadley. American Society of Agronomy, Madison, Wisconsin, USA.

Heinz, D. J., M. Krishnamutthi, L. G. Nickell, and A. Maretzki (1977) Cell, tissue and organ culture in sugarcane improvement. Pp. 3-17 in: Applied and Fundamental Aspects of Plant Cell, Tissue and Organ Culture, edited by J. Reinert and Y. P. S. Bajaj. Springer-Verlag, Berlin, Germany, and New York.

Hibberd, K. A., and C. E. Green (1982) Inheritance and expression of lysine plus threonine resistance selected in maize tissue cultures. Proceedings, National Academy of Sciences 79:559-563.

Hughes, K. W. (1981) Ornamental species. Pp. 5-50 in: Cloning Agricultural Plants Via In Vitro Techniques, edited by B. V. Conger. CRC Press, Boca Raton, Florida, USA.

Kartha, K. K. (1981a) Tissue culture for virus elimination and germplasm preservation. Pp. 123-147 in: Genetic Engineering for Crop Improvement, edited by K. O. Rachie and J. M. Lyman. Rockfeller Foundation, New York, New York, USA.

Kartha, K. K. (1981b) Meristem culture and cryopreservation--methods and applications. Pp. 181-211 in: Plant Tissue Culture: Methods and Applications in Agriculture, edited by T. A. Thorpe. Academic Press, New York, New York, USA.

Kartha, K. K. (1982) Cryopreservation of germplasm using meristem and tissue culture. Pp. 139-161 in: Application of Plant Cell and Tissue Culture to Agriculture and Industry, edited by D. T. Tomes, B. E. Ellis, P. M. Harney, K. J. Kasha, and R. L. Peterson. University of Guelph Press, Guelph, Ontario, Canada.

Kasha, K. J., and K. N. Kao (1970) High frequency of haploid production in barley (Hordeum vulgare L.). Nature 225:874-876.

Kasha, K. J., and F. Reinbergs (1980) Achievements with haploids in barley research and breeding. Pp. 215-230 in: Fourth John Innes Symposium and Second International Haploid Conference, edited by D. R. Davies and D. A. Hopwood. John Innes Charity, Norwich, England.

Keller, W. A., G. Setterfield, G. Douglas, S. Gleddie, and C. Nakamura (1982) Production, characterization, and utilization of somatic hybrids of higher plants. Pp. 81-114 in: Application of Plant Cell and Tissue Culture to Agriculture and Industry, edited by D. T. Tomes, B. E. Ellis, P. M. Harney, K. J. Kasha and R. L. Peterson. University of Guelph Press, Guelph, Ontario, Canada.

Lane, W. D. (1982) Tissue culture and in vitro propagation of deciduous fruit and nut species. Pp. 163-186 in: Application of Plant Cell and Tissue Culture to Agriculture and Industry, edited by D. T. Tomes, B. E. Ellis, P. M. Harney, K. J. Kasha, and R. L. Peterson. University of Guelph Press, Guelph, Ontario, Canada.

Larkin, P. J., and W. R. Scowcroft (1981) Somaclonal variation--a novel source of variability from cell cultures for plant improvement. Theoretical and Applied Genetics 60:197-214.

Lu, C., I. K. Vasil, and P. Ozias-Akins (1982) Somatic embryogenesis in Zea mays L. Theoretical and Applied Genetics 62:109-112.

Maheshwari, S. C., A. K. Tyagi, and K. Malhatra (1980) Induction of haploidy from pollen grains in angiosperms--the current status. Theoretical and Applied Genetics 58:193-206.

Marx, J. L. (1982) Plant biotechnology briefing. Science 216:1306-1307.

Murashige, T. (1978) Principles of rapid propagation. Pp. 14-24 in: Propagation of Higher Plants Through Tissue Culture: A

Bridge Between Research and Application, edited by K. W. Hughes, R. Henke, and M. Constantin. Technical Information Center, U.S. Department of Energy, Oak Ridge, Tennessee, USA.

Murashige, T., and F. Skoog (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum 15:473-497.

Nobécourt, P. (1939) Sur la perénnite et l'augmentation de volume des cultures des tissus végétaux. Comptes Rendus des Séances de la Société de Biologie et de Ses Filiales 130:1270-1271.

Phillips, G. C., G. B. Collins, and N. L. Taylor (1982) Interspecific hybridization of red clover (Trifolium pratense L.) with T. sarosiense Hazsl. using in vitro embryo rescue. Theoretical and Applied Genetics 62:17-24.

Quak, F. (1977) Meristem culture and virus free plants. Pp. 598-615 in: Applied and Fundamental Aspects of Plant Cell, Tissue and Organ Culture, edited by J. Reinert and Y. P. S. Bajaj. Springer-Verlag, Berlin, Germany.

Rachie, K. O., and J. M. Lyman, eds. (1981) Genetic Engineering for Crop Improvement. Rockefeller Foundation, New York, New York, USA.

Raghavan, V. (1980) Embryo culture. International Review of Cytology, Supplement 11B 21:209-240.

Rangaswamy, N. S. (1977) Applications of in vitro pollination and in vitro fertilization. Pp. 412-425 in: Applied and Fundamental Aspects of Plant Cell, Tissue and Organ Culture, edited by J. Reinert and Y. P. S. Bajaj. Springer-Verlag, Berlin, Germany.

Reed, S. M., and G. B. Collins (1978) Interspecific hybrids in Nicotiana through in vitro culture of fertilized ovules. Journal of Heredity 69:311-315.

Reinert, J., and Y. P. S. Bajaj, eds. (1977) Plant Cell, Tissue and Organ Culture. Springer-Verlag, Berlin, Germany.

Reisch, B., and E. T. Bingham (1981) Plants from ethionine resistant alfalfa tissue cultures: variation in growth and morphological characteristics. Crop Science 21:783-788.

Reisch, B., H. Duke, and E. T. Bingham (1981) Selection and characterization of ethionine-resistant alfalfa (Medicago sativa L.) cell lines. Theoretical and Applied Genetics 59:89-94.

Shepard, J. F., D. Bidney, and E. Shakin (1980) Potato protoplasts in crop improvement. Science 208:17-24.

Skirvin, R. M. (1981) Fruit crops. Pp. 51-139 in: Cloning Agricultural Plants Via In Vitro Techniques, edited by B. V. Conger. CRC Press, Boca Raton, Florida, USA.

Skoog, F., and C. O. Miller (1957) Chemical regulation of growth and organ formation in plant tissues cultured in vitro. 11th Symposium Society Experimental Biology 11:118-131.

Steward, F. C. (1958) Growth and development of cultivated cells, III. Interpretations of the growth from free cell to carrot plant. American Journal of Botany 45:709-713.

Steward, Mc. D. (1981) In vitro fertilization and embryo rescue. Environmental and Experimental Botany 21:301-315.

Street, H. E. (1977) Plant Tissue and Cell Culture, 2nd Edition, Blackwell Scientific Publications, London, England.

Swanson, E. B., and D. T. Tomes (1980) Plant regeneration from cell culture of Lotus corniculatus and the selection and characterization of 2,4-D tolerant cell lines. Canadian Journal of Botany 58:1205-1209.

Thorpe, T. A. (1981) Plant Tissue Culture: Methods and Application in Agriculture. Academic Press, New York, New York, USA.

Tomes, D. T., B. E. Ellis, P. M. Harney, K. J. Kasha, and R. L. Peterson (1982) Application of Plant Cell and Tissue Culture to Agriculture and Industry. University of Guelph Press, Guelph, Ontario, Canada.

Vardi, A., P. Spiegel-Roy, and E. Galun (1982) Plant regeneration from citrus protoplasts: variability in methodological requirements among cultivars and species. Theoretical and Applied Genetics 62:171-176.

Vasil, I. K. (1980) Perspective in plant cell and tissue culture. International Review of Cytology, Supplements 11A and 11B. Academic Press, New York, New York, USA.

Vasil, I. K., and V. Vasil (1980) Clonal propagation. International Review of Cytology, Supplement 11A. Academic Press, New York, New York, USA.

Walkey, D. G. A. (1978) In vitro methods for virus elimination. Pp. 245-254 in: Frontiers of Plant Tissue Culture, edited by T. A. Thorpe. University of Calgary, Calgary, Canada.

White, P. R. (1939) Potentially unlimited growth of excised plant callus in an artificial nutrient. American Journal of Botany 26:59-64.

Withers, L. A. (1980) Preservation of germplasm. International Review of Cytology, Supplement 11B. Academic Press, New York, New York, USA.

Yeung, E. C., T. A. Thorpe, and C. J. Jensen (1981) In vitro fertilization and embryo culture. Pp. 253-271 in: Plant Tissue Culture: Methods and Applications in Agriculture, edited by T. A. Thorpe. Academic Press, New York, New York, USA.

Zenkteler, M. (1980) Intraovarian and in vitro pollination. International Review of Cytology, Supplement 11B. Academic Press, New York, New York, USA.

APPENDIX
List of Participants

NATIONAL RESEARCH COUNCIL

ROBERT H. BURRIS, Department of Biochemistry, University of Wisconsin, Madison, Wisconsin, USA, Chairman
NAWAL AFIFI, Department of Clinical Pathology, Faculty of Medicine, University of Cairo, Cairo, Egypt
MARTIN ALEXANDER, Department of Agronomy, Cornell University, Ithaca, New York, USA
IDRISU ALHAJI, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria, Nigeria
POONSOOK ATTHASAMPUNNA, Industrial Research Division, Thailand Institute of Scientific and Technological Research, Bangkok, Thailand
DAVID BALTIMORE, Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts, USA
EDWIN BINGHAM, Department of Agronomy, University of Wisconsin, Madison, Wisconsin, USA
FRANCISCO BOLIVAR, Departmento de Tecnologia Biologia, Instituto de Investigaciones Biomédicas, Universidad Nacional Autonoma de Mexico, Mexico City, Mexico
W. HUGH BOLLINGER, Native Plants, Inc., Salt Lake City, Utah, USA
NANTAKORN BOONKERD, Department of Agriculture, Bangkok, Thailand
JORGE BOSHELL, Instituto Nacional de Salud, Bogota, Colombia
HENRY R. BUNGAY, III, Department of Chemical and Environmental Engineering, Rensselaer Polytechnic Institute, Troy, New York, USA
JERRY J. CALLIS, U.S. Department of Agriculture, Plum Island Animal Disease Center, Greenport, New York, USA
MOHINDRA S. CHADHA, Bio-Organic Division, Bhabha Atomic Research Centre, Bombay, India

CHUNGMING CHANG, Department of Microbiology and Immunology, Yang-Ming Medical College, Taipei, Taiwan
IVER P. COOPER, Antonelli, Terry & Wands, Washington, D.C., USA
OTTO JESU CROCOMO, Centro de Biotecnologia Agricola (CEBTEC), Fundacao de Estudios Agrarios Luiz de Queiroz, Piracicaba, Brazil
JOSEPH DANIEL, Department of Zoology, University of Tennessee, Knoxville, Tennessee, USA
WILLIAM C. DAVIS, Department of Veterinary Microbiology and Pathology, Washington State University, Pullman, Washington, USA
JARDIM FREIRE, Department of Soils, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil
SAMSON GOMBE, Reproductive Biology Unit, University of Nairobi, Nairobi, Kenya
LANCE GORDON, Connaught Laboratories, Inc., Swiftwater, Pennsylvania, USA
C. EDWARD GREEN, Department of Agronomy and Plant Genetics, Institute of Agriculture, University of Minnesota, St. Paul, Minnesota, USA
IRA GREEN, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland, USA
EDWARD HALK, American Type Culture Collection, Rockville, Maryland, USA
MOHAMED HALLATOU, Institut Agronomique et Vétérinaire, Hassan II, Agadir, Morocco
HU HAN, Institute of Genetics, Academia Sinica, Beijing, China
ADOLFO HUBE, Center for Artificial Insemination and Embryo Transplants, Faculty of Veterinary Sciences, Universidad Austral de Chile, Valdivia, Chile
KAREN HUGHES, Department of Botany, University of Tennessee, Knoxville, Tennessee, USA
S. O. KEYA, Department of Soil Science, University of Nairobi, Nairobi, Kenya
AKLILU LEMMA, Centre for Science and Technology for Development, United Nations, New York, New York, USA
LOWELL LEWIS, Agriculture and University Services, University of California, Berkeley, California, USA
MARIO MARTELLE, Instituto de Investigaciones Pecuarias, Mexico City, Mexico
ROBERT E. MCDOWELL, JR., Department of Animal Science, Cornell University, Ithaca, New York, USA
KAMINI MENDIS, Department of Parasitology, University of Colombo, Colombo, Sri Lanka
K. K. G. MENON, Hindustan Lever Research Center, Bombay, India
RALPH L. MOTT, Department of Botany, North Carolina State University, Raleigh, North Carolina, USA
FREDERICK A. MURPHY, College of Veterinary Medicine and Biomedical Sciences, Colorado State University, Ft. Collins, Colorado, USA
TOM O'BRIAN, National Bureau of Standards, U.S. Department of Commerce, Washington, D.C., USA

CHRISTIAN ORREGO, Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts, USA
W. G. PADOLINA, National Institutes of Biotechnology and Applied Microbiology, University of the Philippines at Los Banos, Laguna, Philippines
CARLOS PALACIOS, Instituto de Investigaciones Veterinarias, Maracay, Venezuela
GERALD PETERS, C. F. Kettering Research Laboratory, Yellow Springs, Ohio, USA
DONALD PHILLIPS, Department of Agronomy, University of California, Davis, California, USA
FRANCISCO PINHEIRO, Pan American Health Organization, Washington, D.C., USA
RODOLFO QUINTERO, Instituto de Investigaciones Biomédicas, Universidad Nacional Autonoma de Mexico, Mexico, City, Mexico
CARLOS ROLZ, Instituto Centroamericano de Investigacion y Tecnologia Industrial, Guatemala City, Guatemala
G. R. SANDHU, National Agriculture Research Centre, Islamabad, Pakistan
DIDIN SASTRAPRADJA, Indonesian Institute of Science, Jakarta, Indonesia
SETIJATI SASTRAPRADJA, National Biological Institute, Bogor, Indonesia
CHARLES D. SCOTT, Oak Ridge National Laboratories, Oak Ridge, Tennessee, USA
MARO RAN-IR SONDAHL, Department of Genetics, Instituto Agronomico de Campinas, Sao Paulo, Brazil
M. S. SWAMINATHAN, International Rice Research Institute, Los Banos, Philippines
G. P. TALWAR, National Institute of Immunology, New Delhi, India
NICOLAU VAN UDEN, Department of Microbiology, Gulbenkian Institute of Science, Lisbon, Portugal
JAMES VOSS, Department of Clinical Sciences, College of Veterinary Medicine and Biomedical Sciences, Colorado State University, Ft. Collins, Colorado, USA
KENNETH WARREN, Rockefeller Foundation, New York, New York, USA
WILLIAM J. WECHTER, Hypersensitive Diseases Research, Upjohn Company, Kalamazoo, Michigan, USA
H. GARRISON WILKES, Department of Biology, University of Massachusetts, Boston, Massachusetts, USA
DYANN WIRTH, School of Public Health, Harvard University, Boston, Massachusetts, USA
DONALD L. WISE, Dynatech Corporation, Cambridge, Massachusetts, USA
VICTOR YANG, Promon Engenharia, S.A., Sao Paulo, Brazil
OSKAR ZABORSKY, National Science Foundation, Washington, D.C., USA

WORKING GROUPS

Vaccines

Jerry J. Callis, Chairman
Francisco Pinheiro, Co-chairman
Dyann Wirth, Rapporteur
Jorge Boshell
Lance Gordon
Kamini Mendis
K. K. G. Menon
Frederick A. Murphy
Christian Orrego
Karen Bell, BOSTID Staff
Alfred Buck, AID Observer

Animal Production

Samson Gombe, Chairman
Robert E. McDowell, Jr., Co-chairman
James L. Voss, Rapporteur
Idrisu Alhaji
Joseph C. Daniel
Adolfo Hube
Carlos Palacios
Michael G. C. McDonald Dow, BOSTID Staff
Douglas Butchart, AID Observer

Monoclonal Antibodies

William C. Davis, Chairman
Chungming Chang, Co-chairman
Edward Halk, Rapporteur
Nawal Afifi
Ira Green
Mario Martelle
W. G. Padolina
G. P. Talwar
William J. Wechter
Maryalice Risdon, BOSTID Staff
James Erickson, AID Observer

Energy

Victor Yang, Chairman
Henry R. Bungay, III, Co-chairman
Donald L. Wise, Rapporteur
Poonsook Atthasampunna
Rodolfo Quintero
Carlos Rolz
Didin Sastrapradja
Charles D. Scott
Nicolau Van Uden
Oskar Zaborsky
Griffin Shay, BOSTID Staff
Paul Weatherly, AID Observer

Biological Nitrogen Fixation

Robert H. Burris, Chairman
G. R. Sandhu, Co-chairman
Gerald Peters, Rapporteur
Martin Alexander
Francisco Bolivar
Nantakorn Boonkerd
Jardim Freire
S. O. Keya
Donald Phillips
Setijati Sastrapradja
Michael Greene, BOSTID Staff
Lloyd Frederick, AID Observer

Plant Cell and Tissue Culture

Edwin T. Bingham, Chairman
Hu Han, Co-chairman
W. Hugh Bollinger, Rapporteur
Mohindra S. Chadha
Otto Jesu Crocomo
C. Edward Green
Mohamed Hallatou
Karen W. Hughes
Ralph L. Mott
Maro Ran-ir Sondahl
H. Garrison Wilkes
David Mog, BOSTID Staff
Robert Morris, AID Observer
Donald Plucknett, Observer
Gary Toenniessen, Observer

PANEL LIST

Agriculture Panel

M. S. Chadha
W. G. Padolina
Carlos Palacios
G. R. Sandhu
Nantakam Boonkerd

Health Panel

Nawal Afifi
Mario Martelle
G. P. Talwar

Energy Panel

Poonsook Atthasampunna
Carlos Rolz
Nicolau Van Uden
Victor Yang

BOSTID STAFF

DENNIS WOOD, Professional Associate, Workshop Coordinator
KAREN BELL, Professional Associate
SABRA BISSETTE, Proceedings Editor
BRENDA BUCHBINDER, Staff Assistant
MICHAEL G. C. MCDONALD DOW, Deputy Director
MICHAEL GREENE, Professional Associate
CHERYL HAILEY, Administrative Secretary
JOHN HURLEY, Director
ELIZABETH MCGAFFEY, Administrative Secretary
MELVALEE MITCHELL, Assistant Text Processing Coordinator
DAVID MOG, Professional Associate
LOLA MORALES, Administrative Secretary
MARYALICE RISDON, Staff Assistant
GRIFFIN SHAY, Professional Associate
SHERRY SNYDER, Reports Editor
WYETHA TURNEY, Text Processing Coordinator

U.S. AGENCY FOR INTERNATIONAL DEVELOPMENT OBSERVERS

IRVING M. ASHER, Office of the Science Advisor
ALFRED BUCK, Office of Health, Bureau for Science and Technology
DOUGLAS BUTCHART, Office of Agriculture, Bureau for Science and Technology
JOHN DALY, Office of the Science Advisor
JAMES ERICKSON, Office of Health, Bureau for Science and Technology
CURTIS FARRAR, Bureau for Science and Technology
LLOYD FREDERICK, Office of Agriculture, Bureau for Science and Technology
ROBERT MORRIS, Office of Agriculture, Bureau for Science and Technology
PAUL WEATHERLY, Office of Energy, Bureau for Science and Technology

OTHER INVITED OBSERVERS

DONALD PLUCKNETT, CGIAR Secretariat, World Bank, Washington, D.C.,
USAGARY TOENNIESSEN, Rockefeller Foundation, New York, New York, USA